Application of Fluorescence Spectroscopy for Monitoring Microbial Contamination of Drinking Water

Thesis submitted to The Hebrew University of Jerusalem, Robert H. Smith Faculty of Agriculture, Food and Environment for the M.Sc in Biochemistry and Food Sciences

By Kwanele Siyabonga Simelane

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Declaration

This study was conducted under the supervision of,

Professor Shlomo Sela: Agricultural Research Organization (ARO), Volcani Center, Institute of Postharvest and Food Science, Department of Food Quality and Safety.

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Professor Shlomo Sela

(Supervisor)

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Kwanele Siyabonga Simelane

(Student)

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Abbreviations

EEM- Excitation-emission matrix

Ex/Em- Excitation/Emission

HPC- Heterotrophic plate count

CFUs- Colony forming units

EPA- Environmental Protection Agency

CWS- Contamination warning system

3D- Three dimensional

ASC- Action Script

PARAFAC- Parallel factor analysis

DOC- Dissolved organic carbon

NOM- Natural organic matter

UF- Ultra-filtration

NF- Nano-filtration

mg/L- Milligrams per liter.

GRW- Grand River water

SORS- Second order Raleigh scattering

FORS- First order Raleigh scattering

α- Primary peak

β- Secondary peak

δ- Protein-like peak in the

E. coli- Escherichia coli

B. subtilis- Bacillus subtilis

P. aeruginosa- Pseudomonas aeruginosa

ARO- Agricultural Research Organization

ml- milliliter

g- gram

µm- micrometer

µl– micro liter

µg– microgram

AO- Acridine orange

ppb- Part per billion

ppm- Part per million

T- number of bacteria per ml

N- Average number of bacteria per microscopic image

A- Active surface area of the filter (mm^2)

a- Area of the microscopic image (mm^2)

V- Volume of sample filtered

R^2- coefficient of determination (a measure of the strength and direction of the linear relationship between two variables)

SD- standard deviation

SEM- standard error of the mean.

T_c - concentration of tryptophan in one cell;

S_r - slope for the regression of fluorescence upon the cell number (per ml);

S_t - slope of the regression of fluorescence by tryptophan concentration (in µg per L).

Abstract

The heterotrophic plate count (HPC) and fecal coliform determination presently used for microbial quality evaluation in drinking water are time- and labor-consuming. When the microbial results are known, the water may have been consumed. Therefore, the current microbial tests of drinking water quality are not effective for protecting consumers' health. Operators of water treatment systems need to know the water quality in near real-time in order to respond. Contamination of the water system, whether intentional, accidental, or due to an inadequate disinfectant residuals needs to be detected rapidly. The purpose of this study was therefore to determine if an optical measurement, i.e. excitation-emission matrix (EEM) technique can be used to detect, quantify and distinguish bacterial contaminants in drinking water to ensure consumer safety. If proven effective, this technique has the potential to be further developed for online monitoring of drinking water quality. Fluorescence intensity of three laboratory-grown bacterial species (*E. coli, Bacillus subtilis* and *Pseudomonas aeruginosa*) was determined between an excitation wavelength range 210-590 nm and emission wavelength range 220-600 nm. The EEM of the three bacterial species revealed two excitation/emission peaks at 225/(334-338) and 280/(332-338) nm. Linear regression analysis showed a significant correlation ($p < 0.01$) between number of bacteria and fluorescence emission intensity at all excitation wavelengths with R^2 ranging from 0.80 to 0.99. Since, all three bacterial species displays identical fluorescence spectra, this method cannot distinguish between different bacterial species in drinking water. Fluorescence measurements could detect the presence of at least 10^4 CFU/ml in tap water.

EEM analysis of natural water-borne bacteria revealed only a single fluorescence peak localized at excitation/emission wavelengths of 210/368-370 nm. Significant correlation ($R^2 = 0.84$; $p > 0.01$) was also identified between HPC and the fluorescence intensity of tap water at this excitation/emission wavelength.

Overall, this study suggests that fluorescence intensity measurement at specific EEM region is effective in monitoring bacterial contamination of drinking water. Thus, EEM has the potential to be applied as a rapid technique for online monitoring of drinking water quality.

Application of Fluorescence Spectroscopy for Monitoring Microbial Contamination of Drinking Water

1. Background

1.1 Water microbial quality and its significance

World Health Organization (WHO) considers that drinking water should be suitable for human consumption and for all usual domestic purposes including personal hygiene. Diverse regulatory agencies adopt similar definitions (Bartram et al., 2003). Drinking water should therefore be suitable for consumption, washing/showering and domestic food preparation. In human health terms, exposure to water and its constituents can occur through ingestion, contact and aerosol inhalation. Piped drinking-water supplies typically involve source abstraction, treatment and distribution. The latter may include ancillary devices at domestic or institutional levels, such as softeners, activated carbon treatment, vending machines, dispensers, etc. It should also include those obtained from non-piped sources, such as from springs and community wells, in bottles and as ice. Drinking water should be controlled to ensure the quality and safety of water in order to protect the health of the consumers. Contaminants including high level of total bacteria, and pathogenic microorganisms may appear in drinking water as a result of failure in the well-field, water treatment system or the distribution network (Nygard et al., 2007; Henderson et al., 2009; Richardson et al., 2009).

The typical microbial contamination incident is often linked to one or several system malfunctions in some cases combined with environmental factors, such as flooding (Stedmon et al, 2011). Once pathogenic organisms have entered the drinking water system they are generally unable to proliferate rapidly, and thus they will be overcome due to rapid washout. Thus microbial contamination incidents are characterized as being stochastic (random), short-termed and difficult to detect (Stedmon et al., 2011). Fecal material is generally considered to be among the most severe types of contamination (Richardson et al., 2009). Feces may contain a vast number of microbial pathogens that can have adverse effect on human health even at very low concentrations (Stedmon et al., 2011). Therefore, control of faecal contamination in drinking-water systems and sources, is of primary importance. Faecal indicator bacteria, such as *E. coli* are the current parameter of choice in monitoring faecal pollution (Bartram et al., 2003). Drinking water supplies in rural

areas are more prone to human waste contamination due to poor or even lack of hygienic practices and contamination monitoring system (Richardson et al., 2009).

There exists a need for better and fast monitoring of water systems given that current laboratory-based methods, which include heterotrophic plate count (HPC) and detection of *E. coli* are too slow to develop operational response and do not provide a level of public health protection in real-time (Storey et al., 2011). There is a clear need to be able to rapidly detect and respond to instances of accidental or deliberate contamination, due to the potentially severe consequences to human health. Detecting water contamination in real-time is the most optimal way to ensure an appropriate and timely response (Storey et al., 2011).

Online monitoring of water quality parameters, transmitting data in real-time to the utility's supervisory control and data acquisition system can serve the dual purpose of early detection of contamination and can save utility operators hours of sampling and testing time that would otherwise be required to collect even a fraction of the same data (EPA, 2005). Therefore, this will ensure public safety because contamination will be detected timely and the water will be prevented from being distributed to the public.

The EPA suggested that a successful drinking water contamination warning system (CWS) should be developed that encompasses monitoring technologies and detection strategies, combined with enhanced public health surveillance to collect, integrate, analyze, and communicate information to provide a timely warning of potential water contamination incidents and initiate response actions to minimize public health and economic impacts (EPA, 2005). The success of a CWS depends on its ability to effectively integrate these components and analyze the resulting information in a timely manner to inform response actions that can substantially reduce the potential consequences of a contamination incident (EPA, 2005).

1.2 Fluorescence spectroscopy and its potential use for detecting the presence of various types of organic matter in water

Fluorescence spectroscopy involves measuring the intensity of the light emitted by molecules and ions after being excited by a light of shorter wavelength. The increase (red shift) of the wavelength of the emitted light as compared with that of the excitation is a fundamental property of the fluorescence (Lackowicz, 1999). It reflects the fact that an optically excited electronic system explores a partial non-optic loss of

energy prior to the following light emission. Fluorescence spectra can be measured by *(i)* applying an excitation at a single wavelength and scanning emission wavelengths (emission spectra), *(ii)* scanning excitation wavelengths and measuring emission at a single wavelength (excitation spectra), *(iii)* scanning both excitation and emission wavelengths but maintaining a constant difference between them (synchronous spectra), *(iv)* scanning excitation wavelengths and measuring an emission spectrum for each excitation (excitation-emission matrix-EEM or the 3D fluorescence map or the contour of the total luminescence).

Fluorescence spectroscopy offers rapid and consistent analyses of water purity with high instrumental sensitivity. This is due to the fact that pure water is not fluorescent *per se*, and the observed fluorescence results from the presence of (mostly) organic impurities including both natural organic matter (NOM) and organic matter related to or affected by anthropogenic activity.

Obviously, EEMs covering all the combinations of excitation and emission wavelengths provide maximal information about fluorescing substances as compared with other above-mentioned fluorescence-measuring approaches. Hence, the fluorescence EEM method, especially in combination with the powerful chemometric techniques, such as parallel factor analysis (PARAFAC) (see Andersen and Bro, 2003; Stedmon and Markager, 2005; Borisover et al., 2009 and references therein) provides a basis for capturing changes in the fluorescence spectra of water due to the presence of various organic fluorophores. Compared to other available techniques of organic matter characterization, this method is highly sensitive to the presence of humic-like and protein-like materials (Hudson et al., 2007; Peiris et al., 2009). The fluorescence EEM analysis method was extensively used for characterizing different types of water including; rivers, lakes, and recycled water (Saadi et al., 2006; Hudson et al., 2007; Henderson et al, 2009; Borisover et a., 2009; Borisover et al., 2011 and multiple references therein). An example of EEM is shown in Fig 1 below.

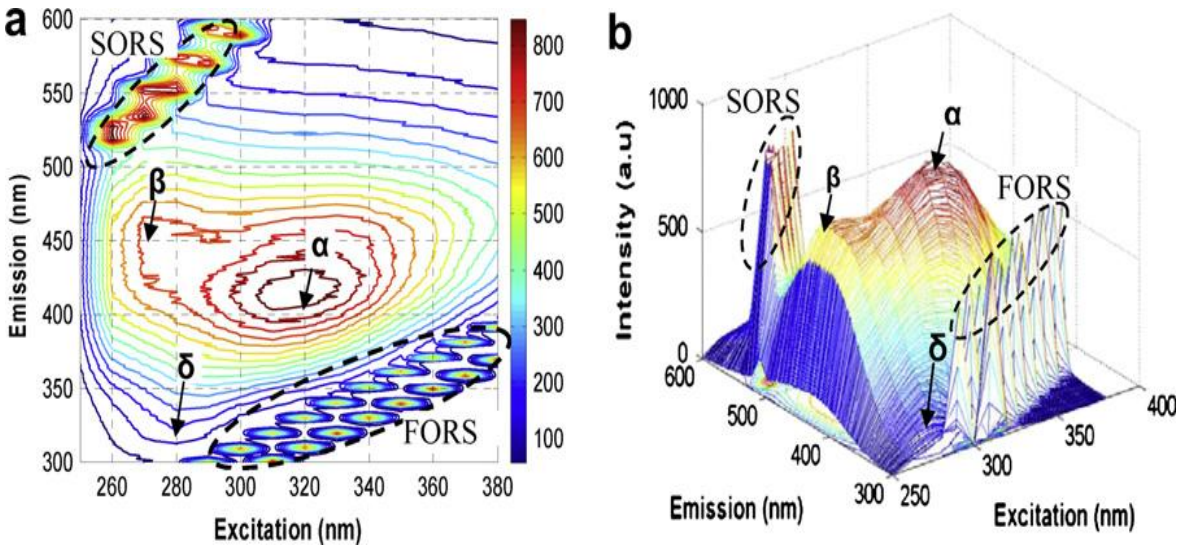

Fig. 1. Typical fluorescence features seen in the (a) fluorescence EEM for Grand River water (GRW) and (b) 3D view of the same EEM. First order Raleigh scattering (FORS) and Second order Raleigh scattering (SORS) regions are indicated using dashed-lines. The fluorescence EEM of GRW water (i.e. raw water) shows a primary peak (α) at Ex/Em 320 nm/415 nm and secondary peak (β) at Ex/Em 270 nm/460 nm that correspond to humic substances. The proteinaceous matter-related peak in the (δ) region is not clearly visible due to the very low concentration levels of the protein-like substances present in GRW (Reprinted from Peiris *et al.*, 2010).

1.3 Use of fluorescence spectroscopy for monitoring treatment of drinking water and its quality

Organic matter fluorescence was suggested as an early warning system for detection of contamination events in drinking water derived from groundwater systems (Stedmon et al., 2011). Further, in Saxony, Central East Germany, EEM fluorescence and ultra-high-resolution mass spectrometry have been utilized in monitoring levels of organic carbon in freshwaters (Herzsprung et al., 2012). Increased levels of organic carbon in freshwaters pose serious challenges for processing and the commercial supply of drinking water. Organic material in water can cause aesthetic problems such as an unpleasant taste, odor, and color. DOM does not pose a health risk itself but may become transformed into potentially harmful disinfection byproducts (DBPs) when subjected to raw water treatment with reactive species such as free chlorine, ozone, chloramines,or chlorine dioxide (Herzsprung et al., 2012; Hertkorn et al., 2008). For these reasons, dissolved organic carbon (DOC; frequently used as an indicator for

DOM) causes increased flocculation costs to remove the unwanted organic byproducts. Optimizing the processing of drinking water requires substantial research among engineers and water chemists directed at characterization and removal of humic substances from raw waters (Herzsprung et al., 2012). The potential of fluorescence spectroscopy technique in monitoring sewage contamination of drinking water as well as formation of disinfection byproducts has been also explored by Hua et al. (2007) and Henderson et al. (2009).

With the increased use of membranes in drinking water treatment, fouling – particularly the hydraulically irreversible type – remains the main operating issue that hinders performance and increases operational costs (Peldszus et al., 2011). The main challenge in assessing fouling potential of feed water is to accurately detect and quantify feed water constituents responsible for membrane fouling. Thus the utilization of fluorescence EEMs has made it possible to detect protein-like substances, humic substances, fulvic acid and particulate/colloidal matter with high sensitivity in surface water. The application of principal component analysis to fluorescence EEMs allowed estimation of the impact of surface water constituents on reversible and irreversible membrane fouling in drinking water treatment plant (Peldszus et al., 2011).

Fluorescence EEMs analysis method has also been used for characterization of NOM and the associated fouling events in ultra-filtration (UF) and nano-filtration (NF) based drinking water treatment processes (Peiris et al., 2009). The ability of this approach to characterize water NOM with a wide range of DOC concentrations (i.e., from 8.0 DOC-mg/L for raw water to 0.4 DOC-mg/L for NF permeate) without pre-dilution or pre-concentrations steps has also been demonstrated (Peiris et al., 2008).

1.4 Problem statement and hypothesis

The current routine methods used to measure microbial quality of drinking water are based on a daily water sampling followed by filtration of a specific water volume and cultivation on specific agar media to detect the presence of total bacteria (heterotrophic plate counts) and indicator bacteria (e.g. coliforms), typically associated with the presence of fecal material (Tallon et al., 2005). The present microbial techniques are time-consuming because they might take for at least twenty four hours (time for plating and incubating the plates for colony count) (Stedmon et al., 2011). Additionally, the grab sampling approach only allows for a marginal

amount of the distributed water to be analyzed. Given the stochastic nature of microbial contamination, the chance of actually catching a contamination and the time take to complete the microbiological tests, the chance of actually catching the contamination incident before it reaches the consumers is zero (Stedmon et al., 2011). Further, the plating approach of bacteria enumeration only allows culturable bacteria to be enumerated in the water sample, yet usually culturable bacteria account for about 0.01% of the total bacteria in drinking water; hence the actual quantity of bacteria which might affect water quality cannot be determined through plating (Bartram et al., 2003). Drinking water distribution systems provide a stressful environment for bacteria since they are low in nutrients (Stedmon et al., 2011). Stressed bacteria may exist also in viable but not culturable form (Oliver, 2005), thus it is possible that bacteria including waterborne pathogens may not be able to cultivate under standard laboratory conditions.

Determination of the EEM fluorescence is a highly sensitive technique that allows the detection of certain fluorescing amino-acids, i.e., tryptophan, tyrosine, and phenylalanine, which serve as the basic components of proteins. Therefore, measuring EEMs could be useful also for detecting intact or degraded microorganisms in drinking water. This idea is supported by an early observation that the fluorescence emission response measured at a certain pair of the excitation/emission wavelengths (i.e., of 230 and 330 nm, respectively) was sensitive to the type and class of microorganisms (Determann et al. 1998). The relationship between tryptophan-like fluorescence and microbial activity was demonstrated also by examining increases in protein-derived fluorescence during algal exponential growth phase (Stedmon and Markager, 2005).

Although many applications of fluorescence spectroscopy, especially those using EEM, were suggested to monitor the quality of natural surface water and ground water (Hudson et al., 2007; Borisover et al., 2009; Stedmon et al., 2011; Herzsprung et al., 2012; Matilainen et al., 2011), as well as to characterize the presence of NOM, humic and protein-like substances, to the best of our knowledge, none of these applications directly addressed possible correlation between the microbial load of drinking water and EEM fluorescence. We hypothesized that measuring fluorescence can also be effective mean in characterizing the microbial quality of drinking water and hence could be utilized as additional tool for real-time monitoring of drinking water quality.

2. Objectives

The general objective of the study was to test if fluorescence spectroscopy can be utilized to detect microbial quality of drinking water.

The specific objectives were:

1. Detect the presence of bacteria in drinking water using EEMs of fluorescence.
2. Assess the sensitivity of detection.
3. Determine measurements specificity and fluorescence background in tap water.
4. Examine the ability of fluorescence EEMs to distinguish between different bacterial species.

3. Materials and methods

3.1 Fluorescence measurements general description

Fluorescence spectra of all aquatic samples were measured with an RF-5301PC spectrofluorometer (Shimadzu, Japan) equipped with 150-W Xenon lamp (Ushio Inc., Japan) as described by Borisover et al. (2009, 2011). The cuvette used was the standard quartz cell (Hellma, USA; Suprasil, with the 200-2,500nm spectral range) having a path length of 10mm and the chamber volume of 3,500 μL. Anti-stat polyshield delicate task wipers (Kimtech, USA) were used to wipe the cuvette before it could be inserted into the cuvette holder of the spectrofluorometer. Excitation–emission matrices (EEMs) were generated at 23±2°C. Fluorescence emission spectra between 220 and 600 nm were collected at 2-nm increments, with excitation wavelengths ranging from 210 to 590 nm at 5-nm increments. Scanning rate per map was about 9 minutes and the excitation and emission optical slits were at 10nm. Optical density at 210nm was checked in every sample before fluorescence measurement using UV-Visible spectrophotometer, Genesys 10UV Scanning, (Thermo Scientific; Cat. 335906-02; Madison WI 53711; USA) to ensure it is sufficiently low to prevent an inner filter effect. Instrument stability was controlled by measuring the intensity of the Raman peak of water at excitation wavelength at 275 nm and emission wavelength at 305nm and pH was maintained around 7.0(±1). Further, the Action Script (ASC) file containing the measured fluorescence emission data was converted with Microsoft Excel and Surfer 7.0 software, to a fluorescence map (an example is shown in Fig. 2).

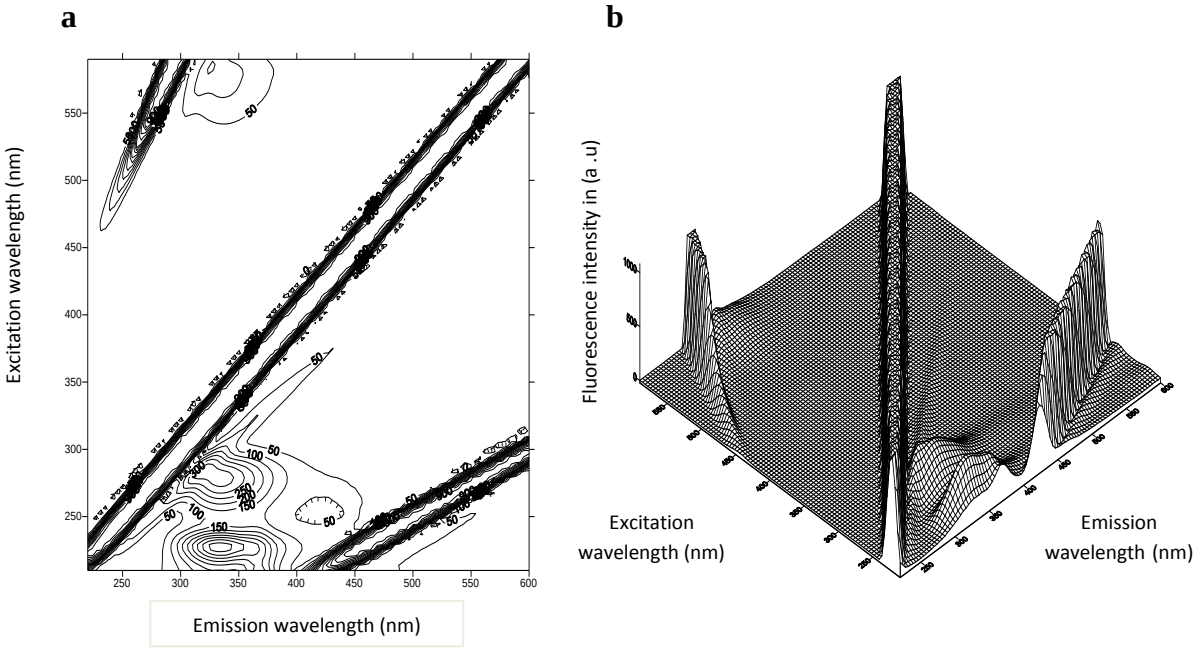

Fig. 2. Contour (top view) fluorescence map (a) and 3-D fluorescence map (b) generated by a suspension of 10^6 *E. coli* colony forming units per ml of filtered tap water.

3.2 Water Sampling

Tap drinking water from the water distribution system in the Institute of Soil, Water and Environmental Sciences building (The Volcani Center, ARO) was sampled by first allowing the water to flow for three minutes before collected into three sterile glass 1 liter bottles. 800 ml of the sampled tap water was filled into each bottle. When indicated, the collected water was filtered through a 0.2 µm syringe-driven filter unit (Millipore, Ireland). During the filtration, the first 10 ml of filtered water was discarded since it may contain fluorescing substances released from the filter. For weekly monitoring of fluorescence of tap water, 10 ml was extracted, after thorough mixing, from each bottle of 800 ml sampled water into three sterile 20 ml glass vials; the rest was utilized for total bacterial count and heterotrophic count. The fluorescence of triplicated water samples was measured as described in 3.1.

3.3 Heterotrophic plate counts of drinking-water

Heterotrophic plate count of drinking-water was carried out essentially as described before by (Allen et al., 2004; Reasoner, 2004). Breafly, 5, 10, 50 and 100 ml of tap water were passed through 0.45 µm pore size membrane filters fitted on Millipore

Filtration System apparatus (Millipore, Israel). Membrane filters with retained bacteria were placed into R2A agar (Cat. 218261, Bacto, Australia) in 50mm Petri dish plates (Miniplast Ein-Shemer, Israel) and incubated at 24^0C for 7 days. The number of colony forming units (CFUs) was counted and used for calculation of bacterial concentration.

3.4 Bacterial Strain

3.4.1 Laboratory-grown bacteria

The following bacterial strains were used in this study, *E. coli ATCC25922, Bacillus subtilis 3610 and Pseudomonas aeruginosa ATCC 27853*.

3.4.2 Bacteria and growth conditions

Bacteria were transferred from pure cultures kept at -20 ^{0}C (with 20% glycerol solution) for *E. coli* and *P. aeruginosa* and at -80 ^{0}C for *B. subtilis*, were grown on LB agar medium (Diffco; Becton, and Company Sparks, USA) and incubated at 37 ^{0}C for 24hs. Several well isolated colonies were harvested, suspended in a 10 ml volume of LB broth and incubated at 37 ^{0}C with shaking for 24 hs to prepare a stock. The optical density of the stock was measured at 600 nm using the Cell Density Meter; Model CO8000 (TAMAR, Cambridge, UK). A tenfold dilution of 100 µl was performed for plate count to determine the total number of CFUs in the stock.

3.4.3 Bacteria for fluorescence measurement

For fluorescence measurement, the LB broth medium was removed by centrifugation for 10 minutes at 5000 rpm and at 25 ^{0}C. The cells were washed three times with 20 ml of 0.2 µm filtered tap water and centrifuged as described above. Finally, the pellet was suspended in 10ml volume of filtered tap water. Double serial dilution was carried out up to the ratio (1:4096). In brief, twelve 20 ml disposable scintillation glass vials with screw caps (Kimble Chase, China) were washed with distilled water and then rinsed with filtered tap water. The vials were filled with each 5 ml of filtered or unfiltered tap water, (as indicated), under the safety hood, using 5 ml pipette. Serial dilution of 1:1 (5 ml stock bacterial suspension: 5 ml filtered or unfiltered tap water) was prepared starting from the stock of bacteria being studied. The fluorescence

measurements of the diluted bacterial suspensions were carried out as described in section 3.1.

3.4.4 Isolation of natural water-borne bacteria

Water-borne bacteria from local drinking water distribution system were sampled directly from the distribution system in the Institute of Soil, Water and Environmental Sciences building (The Volcani Center, ARO). Bacteria were separated from the water by filtration of 1000 ml of tap water through a 0.45 µm filter membrane (Millipore, Israel). In order to prepare a 10 ml stock solution, bacteria retained on the membrane were released from the filter by vortexing at maximum intensity (Labnet international, USA) with 10 ml of filtered tap water, which exhibited negligible fluorescence at the wavelengths of interest. A serial 10 folds dilution of the suspended bacteria was conducted in order to count the total number of bacteria by epifluorescence microscopy (see section 3.6).

3.5 Preparation of L- tryptophan solution

A 100 ppm stock solution was prepared by dissolving 1 mg of L-tryptophan powder (T8941-25G, Sigma-Aldrich, USA) into 10 ml of filtered low-fluorescence tap water by shaking. Visually, tryptophan was fully dissolved in water which is expected due to its high aqueous solubility at room temperature (11.4 g/L at 25 °C). Yet, to guarantee the lack of any traces of solid residuals in solution, the stock solution was filtered through a 0.22 µm syringe-driven filter unit. The tryptophan stock solution was double diluted until 0.3 ppb. Fluorescence of L-tryptophan was measured from the lowest 0.3 ppb, to the highest (9.6ppb) concentration as described in the section 3.1.

3.6 Enumeration of total number of bacteria in tap water by microscopy

For direct enumeration of total number of bacterial cells in water, protocols given by Boulos et al. (1999) and based on the study by Hobbie et al. (1977) were used.

A 900 µl of a well-mixed water sample was transferred into a clean glass tube and mixed again to ensure the bacteria cells are suspended evenly. About 100 µl of 25% glutaraldehyde solution (filtered with 0.22 µm membrane) was added into the tube. The tube was thoroughly mixed and incubated at room temperature for 1 h. Black polycarbonate membrane filters (polycarbonate 0.22 µm pore-size; 25 mm diameter;

Nucleopore, USA) were placed into the filtration apparatus. Then, the polycarbonate membrane filters were wetted by passing 10 ml of 0.22 µm-filtered double distilled water. The water samples with final glutaraldehyde concentration of 2.5% were filtered through the wetted polycarbonate membrane filters and then covered with 1 ml of 0.01% Acridine Orange (AO) for 2 minutes to stain the retained bacteria. The filter was rinsed with 10 ml of filtered double distilled water and the filters were placed on a glass slide between two drops of low fluorescence immersion oil and covered with cleaned cover slips. Specimens were viewed under epifluorescence microscope (Olympus, U-V1xC, 1K39362, and Tokyo, Japan), set 470/20-nm excitation filter, and a 525/50 nm barrier filter. At least 10 snapshots microscopic images (with calibration bar) for each sample were taken, as in the example shown in Fig. 3. To calculate the number of bacteria per ml of sample, the following formula was used:

$T = N\,(A/a)/\,V$; T= total number of bacteria per ml; N= Average number of bacteria per microscopic image; A=Active surface area of the filter (mm^2); a= Area of the microscopic image (mm^2); V=Volume of sample filtered (ml). 10 snapshots for each filter was calculated and presented as mean T and standard error of the mean (SEM).

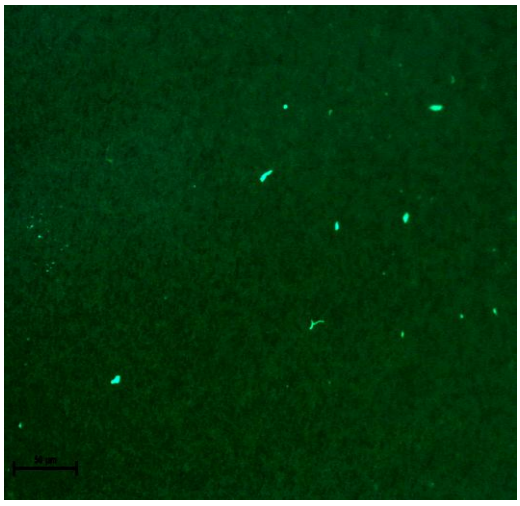

Fig.3. Snapshot of fluorescence microscope image taken during total count of bacteria in tap water.

4. Results

4.1 Fluorescence maps of different bacteria

In order to examine if fluorescence can be utilized to detect bacteria in tap water, it is necessary to determine which excitation/emission combinations should be used. EEMs of fluorescence were obtained for different lab-grown bacteria suspended in filtered tap water at a similar cell concentration and also for natural water-borne bacteria (Fig. 4, 5, 6, 7).

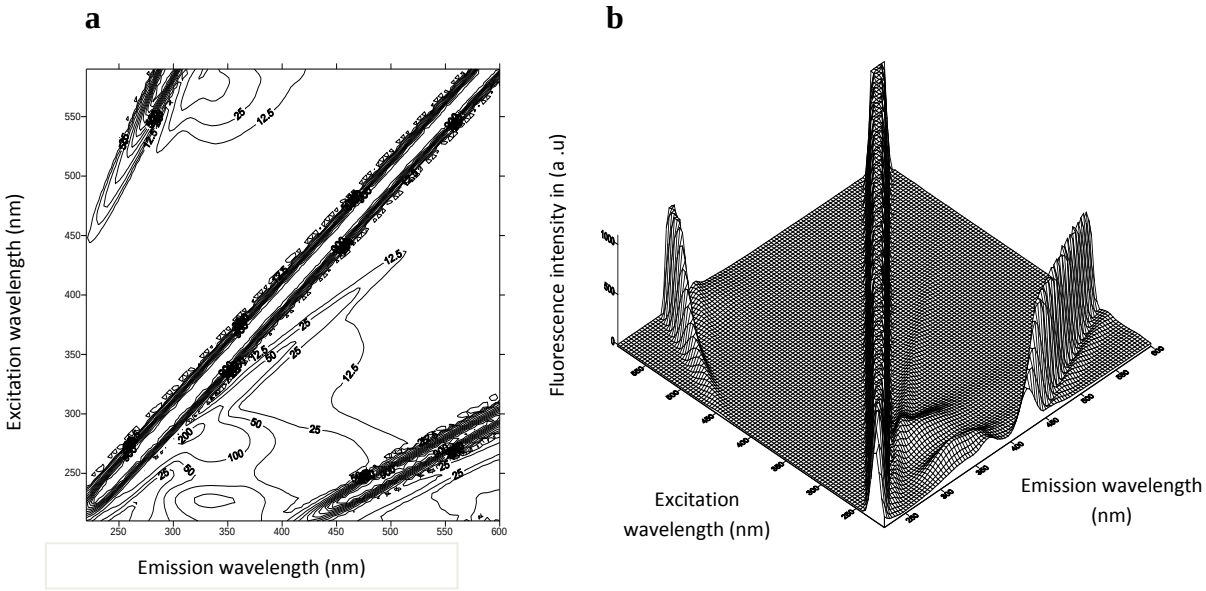

Fig. 4. A representative contour (top view) fluorescence map (a) and 3-D fluorescence map (b) generated by 10^5, *E. coli* CFU per ml of filtered tap water.

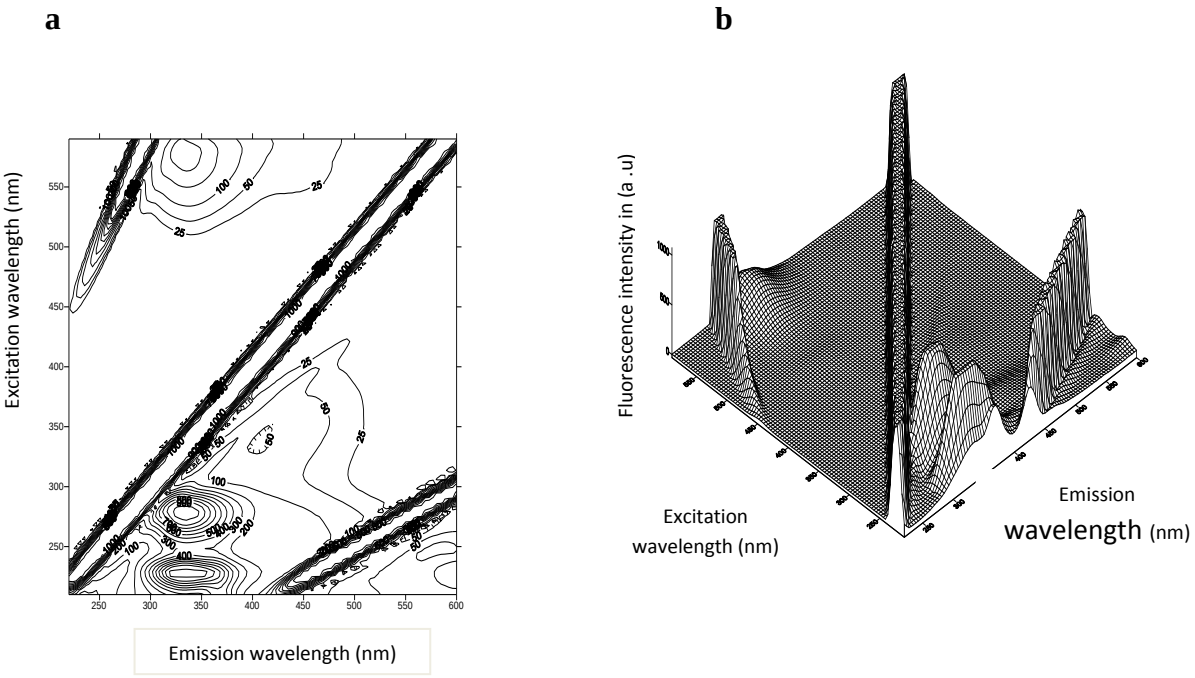

Fig. 5. A representative contour (top view) fluorescence map (a) and 3-D fluorescence map (b) generated by 10^5, *B. subtilis* CFU per ml of filtered tap water.

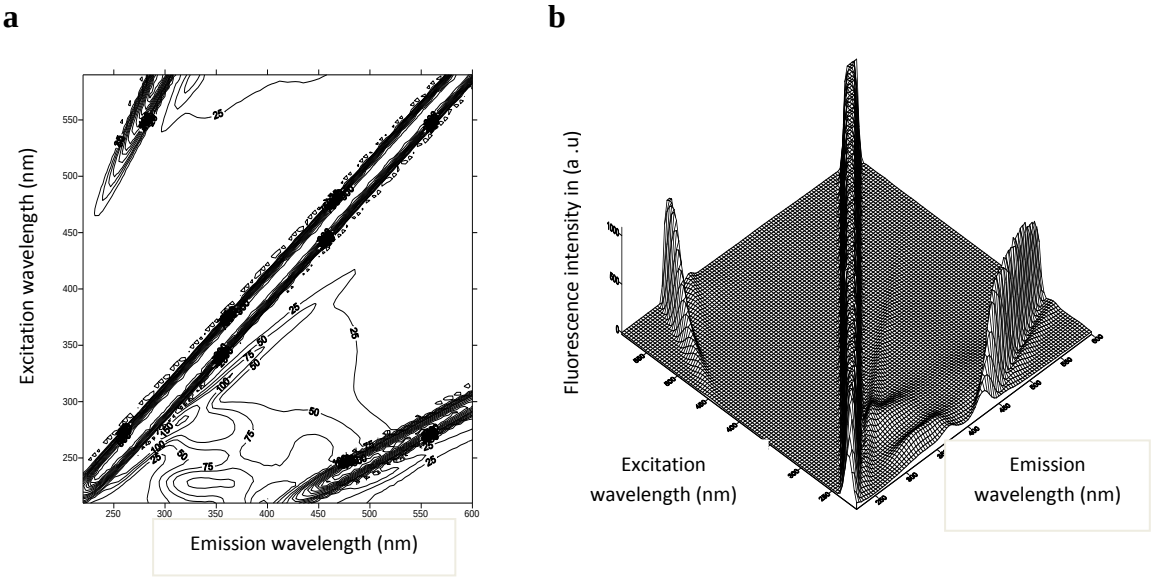

Fig. 6. A representative contour (top view) fluorescence map (a) and 3-D fluorescence map (b) generated from 10^5, *P. aeruginosa* CFU per ml of filtered tap water.

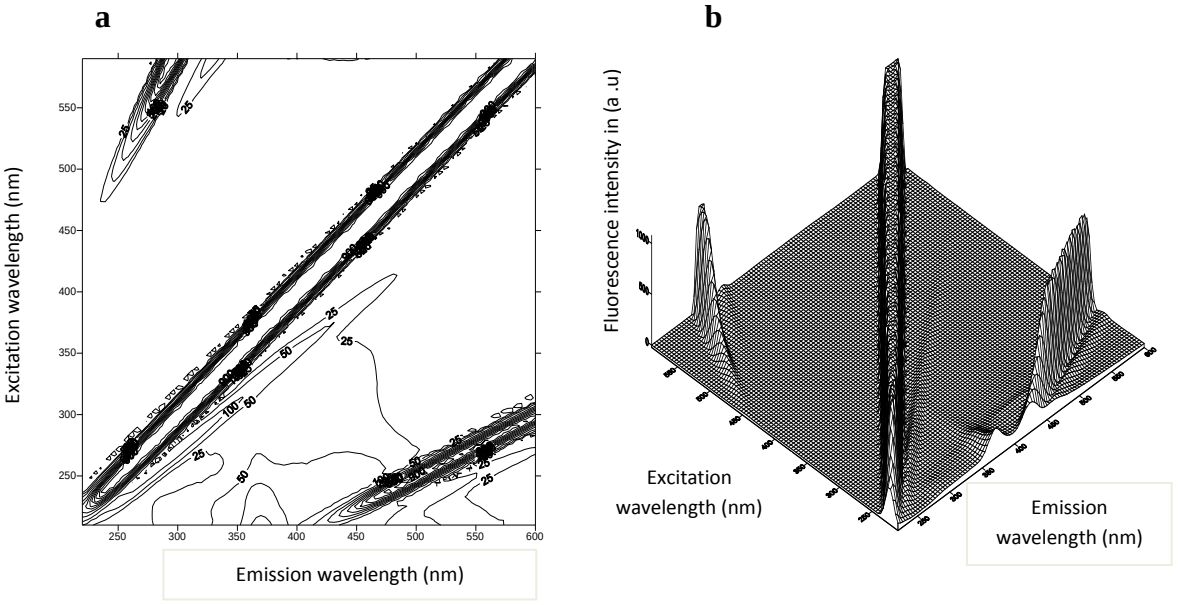

Fig. 7. A representative contour (top view) fluorescence map (a) and 3-D fluorescence map (b) generated by 10^5 cells of natural water-borne bacteria per ml of filtered tap water.

Table 1, summarizes the fluorescence intensity of the different bacteria at the three observed EEM locations where peaks fluorescence were observed.

Bacteria	**Fluorescence intensity in arbitrary units (Excitation/Emission wavelengths nm)**			**CFUs/ml**
E. coli	172.2 (210/368)	242.8 (225/336)	181.2 (280/366)	9.4 x 10^5
B. subtilis	584.7 (210/368)	1015.7 (225/336)	1013.7 (280/336)	8.6 x 10^5
P. aeruginosa	135.7 (210/370)	166 (225/336)	141 (280/332-38)	9.4 x 10^5
Natural water-borne bacteria	315 (210/370)	184 (225/370)	48 (280/344)	4.5 x 10^5

The fluorescence maps demonstrate the presence of two major peaks in laboratory-grown bacteria 225/336, and 280/(332-338 nm) (Fig. 4, 5, 6), while water-borne bacteria have a distinct peak located at 210/(368-370) nm. Among the laboratory grown bacteria, *B. subtilis* has a much pronounced peak at excitation/emission 225/336 nm compared to *E. coli, and P. aereginosa*.

4.2 The effect of bacteria concentration on fluorescence

4.2.1 *E. coli*, *B. subtilis* and *P. aeruginosa*

To test the fluorescence response to bacterial concentration, suspension of increasing concentration of three different bacteria species, *E. coli*, *P. aerugiosa and B. subtilis* were prepared and the emission intensities were recorded in three different excitation/emission wavelength regions, where major peaks were identified. The data is presented in Table 3-5.

Table 2. Mean fluorescence intensity of different *E. coli* concentrations in filtered tap water

Sample (dilution factor)	**Mean fluorescence emission intensities[a] arbitrary units at different combinations of excitation/emission wavelengths nm[b] (+/-SD)**			**Mean E. coli CFU/ml (+/-SD)**
	210/368-370	**225/334-336**	**280/332-336**	
d5 (1:32)	576 (54)	1016[c] (0)	1016c (0)	4×10^6 (3.5×10^5)
d6 (1:64)	232 (67)	465 (34)	374 (29)	2×10^6 (1.8×10^5)
d7 (1:128)	131 (14)	120 (19)	81(3)	1.2×10^6 (1.7×10^5)
d8 (1:256)	87 (5)	87 (46)	31(2)	5×10^5 (4×10^4)
d9 (1:512)	31 (13)	26 (10)	8 (1)	2.7×10^5 (4.6×10^4)
d10 (1:1024)	43 (2)	18 (4)	7 (1)	1.4×10^5 (2.3×10^4)
d11 (1:2048)	32 (8)	12 (1)	6 (3)	6.9×10^4 (6.9×10^4)
d12 (1: 4096)	21 (4)	9 (1)	4 (1)	3.4×10^4 (3.4×10^3)
Blank[d]	13 (2)	6 (1)	2 (1)	0

[a]Fluorescence intensities at the excitation/emission wavelength pair of interest in three replicated experiments.

[b]The range of emission wavelengths (where applicable) represents the interval in which the wavelength of maximal emission varied among the samples.

[c]Emission intensity is over the scale of the spectrofluorometer.

[d] Filtered tap water without bacteria.

Table: 3. Mean fluorescence intensity of different *B. subtilis* concentrations in filtered tap water

Sample (dilution factor)	**Mean fluorescence emission intensities[a] arbitrary units at different combinations of excitation/emission wavelength nm[b] (+/-SD)**			**Mean *B. subtilis* CFU/ml (+/-SD)**
	210/368-370	**225/334-338**	**280/332-336**	
d6 (1:64)	553 (45)	975 (57)	936 (38)	7.5×10^5 (1.5×10^5)
d7 (1:128)	254 (26)	497 (91)	438 (49)	3.8×10^5 (7.7×10^4)
d8 (1:256)	125 (10)	205 (33)	168 (29)	1.9×10^5 (3.9×10^4)
d9 (1:512)	66 (4)	68 (11)	54 (10)	4.5×10^5 (4.9×10^4)
d10 (1:1024)	46 (1)	29 (1)	19 (0)	4.7×10^4 (9.7×10^3)
d11 (1:2048)	37 (4)	24 (1)	15 (0)	2.3×10^4 (4.8×10^3)
d12 (1: 4096)	34 (3)	24 (5)	14 (1)	1.2×10^4 (2.4×10^3)
Blank[c]	25 (1)	15 (1)	11 (2)	0

[a]Fluorescence intensities at the excitation/emission wavelength pair of interest in three replicated experiments.

[b]The range of emission wavelengths (where applicable) represents the interval in which the wavelength of maximal emission varied among the samples.

[c] Filtered tap water without bacteria.

Table: 4. Mean fluorescence intensity of different *P. aeruginosa* concentrations in filtered tap water

Sample (dilution factor)	**Mean fluorescence emission intensities[a] (abrtitrary units) at different combinations of excitation/emission wavelength nm[b] (+/-SD)**			**Mean *P. aeruginosa* CFU/ml (+/-SD)**
	210/368-370	**225/334-336**	**280/334-338**	
d7 (1:128)	477 (19)	993 (32)	847 (63)	3.8×10^6 (4.7×10^5)
d8 (1:256)	257 (17)	415 (11)	347 (5)	2×10^6 (1.7×10^5)
d9 (1:512)	131 (6)	145 (30)	130 (16)	8.8×10^5 (8.3×10^4)
d10 (1:1024)	80 (2)	74 (190)	56 (2)	4.7×10^5 (5.9×10^4)
d11 (1:2048)	60 (18)	58 (29)	53 (24)	2.3×10^5 (2.9×10^4)
d12 (1: 4096)	43 (6)	56 (8)	50 (1)	1.2×10^5 (1.5×10^4)
Blank[c]	29 (0)	36 (0)	47(0)	(0)

[a]Fluorescence intensities at the excitation/emission wavelength pair of interest in three replicated experiments.
[b]The range of emission wavelengths (where applicable) represents the interval in which the wavelength of maximal emission varied among the samples.
[c]Filtered tap water without bacteria.

The data obtained for each bacterial strain (Table 2-4) were used for linear regression analysis. The regression lines for the three pairs of excitation/emission wavelength of interest are presented below (Fig. 8-16).

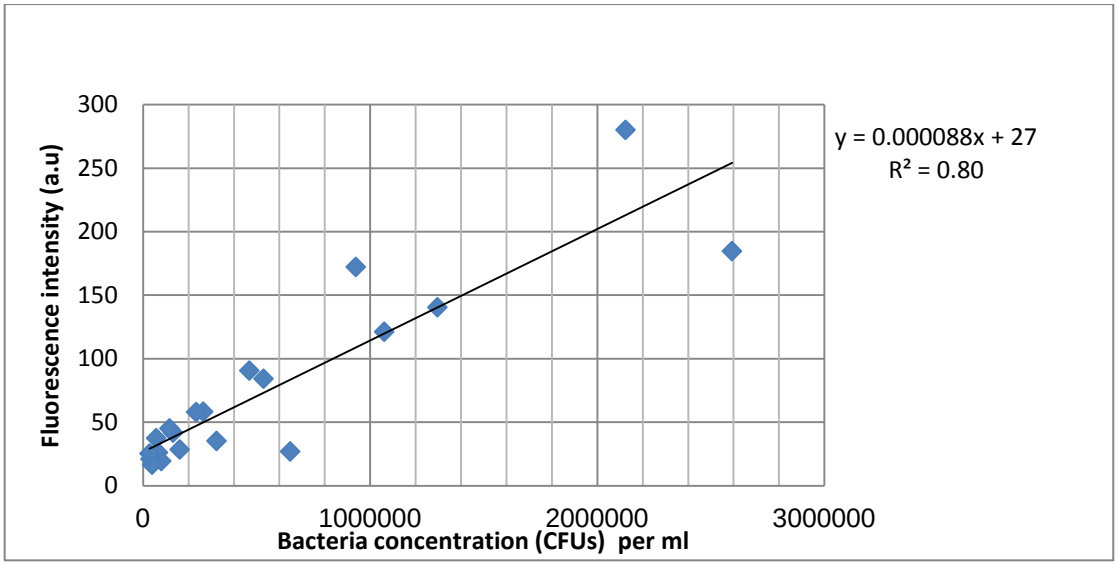

Fig. 8. Fluorescence intensity (in arbitrary units, a.u) at excitation/emission wavelengths of 210/(368-370) nm plotted against concentration of *E. coli* suspended in filtered tap water. The direct line represents the linear regression.

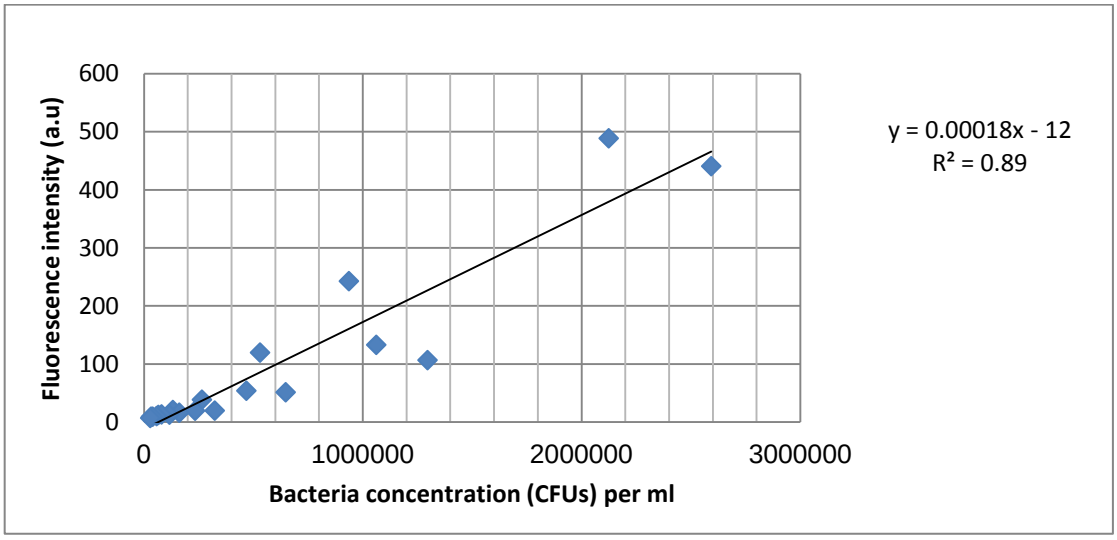

Fig. 9. Fluorescence intensity (in arbitrary units, a.u) at excitation/emission wavelengths of 225/(334-336) nm plotted against concentration of E. coli suspended in filtered tap water. The direct line represents the linear regression.

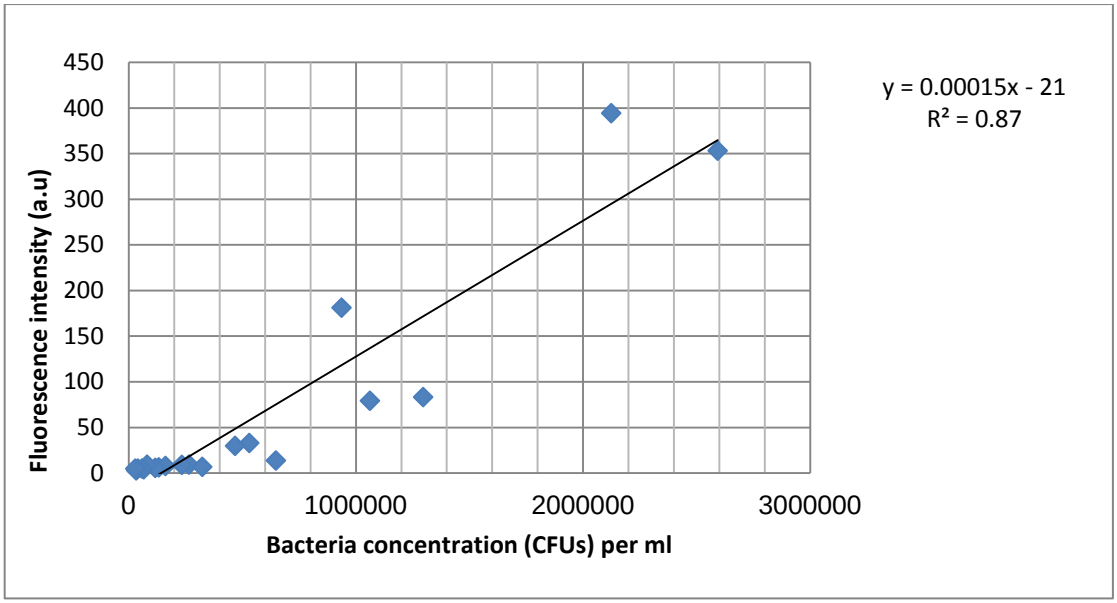

Fig. 10. .Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 280/(332-336) nm plotted against concentration of *E. coli* suspended in filtered tap water. The direct line represents the linear regression.

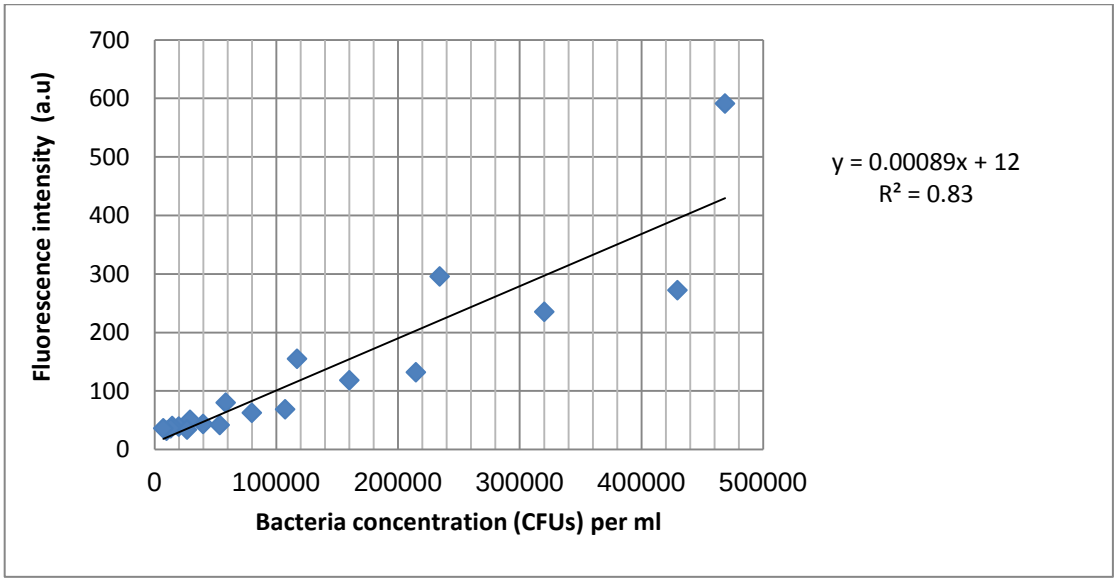

Fig. 11. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 210/(368-370) nm plotted against concentration of *B. subtilis* suspended in filtered tap water. The direct line represents the linear regression.

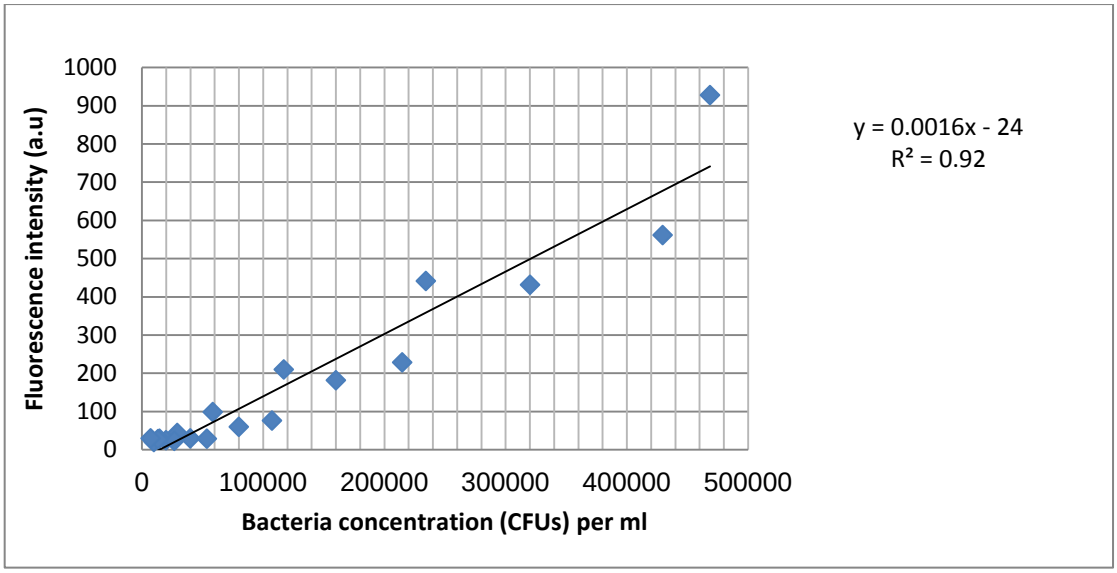

Fig. 12. Fluorescence intensity at excitation/emission wavelengths of 225/(334-338) nm plotted against concentration of *B. subtilis* suspended in filtered tap water. The direct line represents the linear regression.

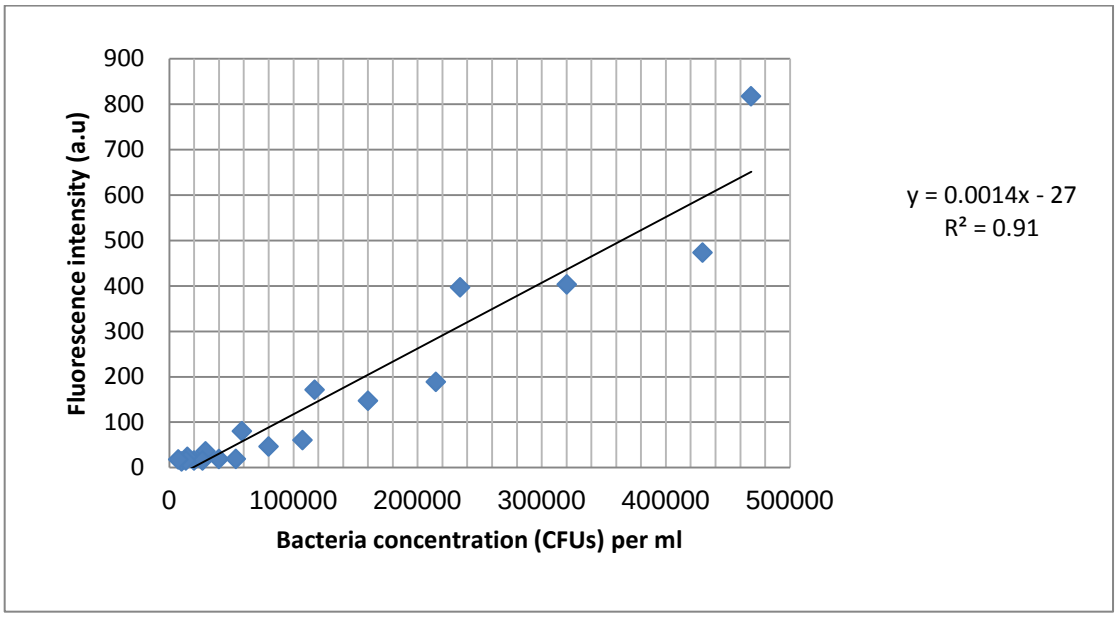

Fig. 13. Fluorescence intensity (in arbitrary units, a.u) at excitation/emission wavelengths of 280/(332-336) nm plotted against concentration of *B. subtilis* suspended in filtered tap water. The direct line represents the linear regression.

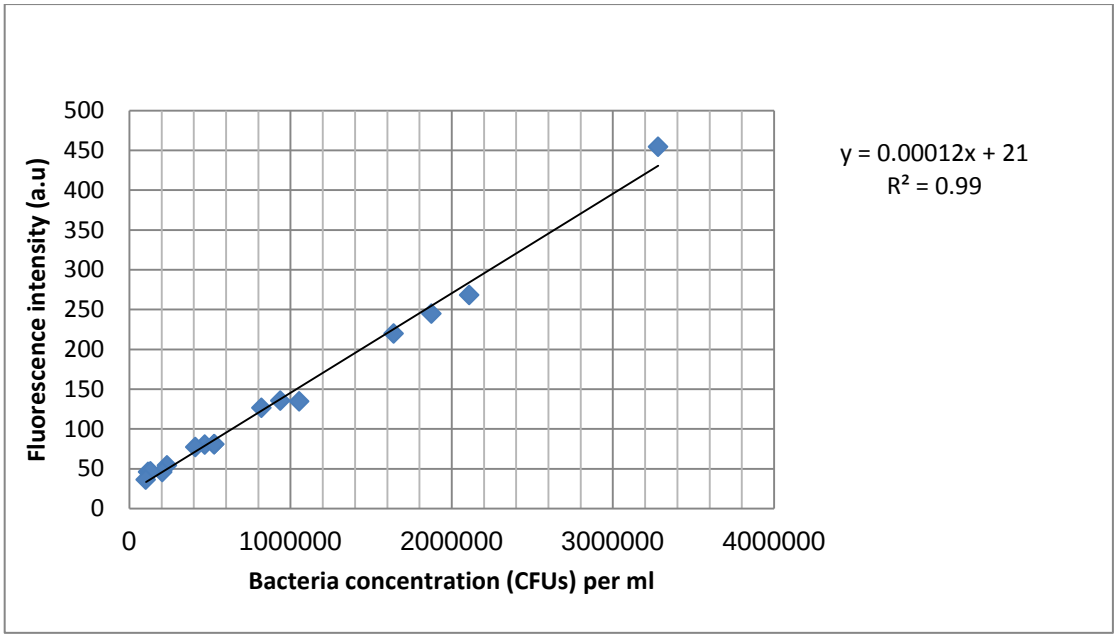

Fig. 14. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 210/(368-372) nm plotted against concentration of *P. aeruginosa* suspended in filtered tap water. The direct line represents the linear regression.

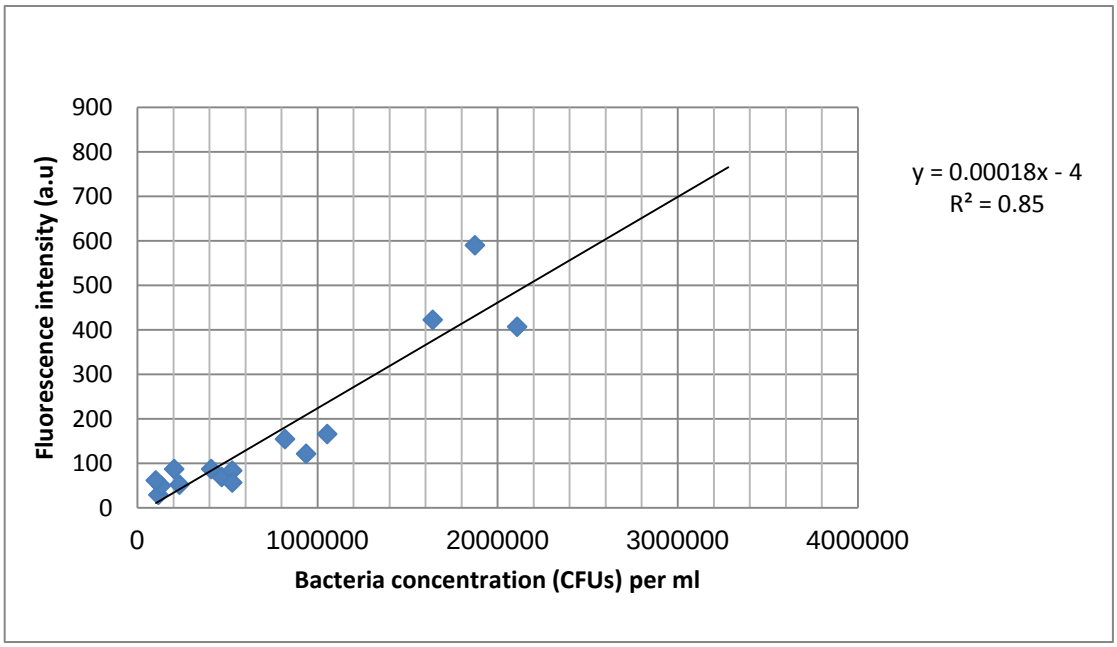

Fig. 15. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 225/(334-336) nm plotted against concentration of *P. aeruginosa* suspended in filtered tap water. The direct line represents the linear regression.

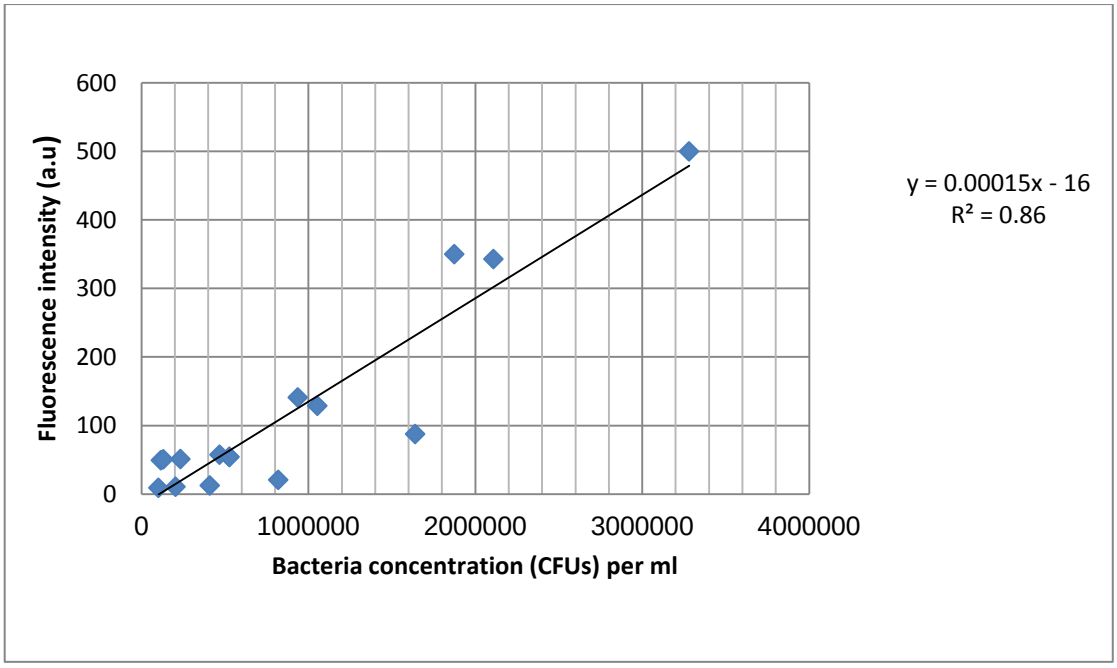

Fig. 16. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 280/(334-338) nm plotted against concentration of *P. aeruginosa* suspended in filtered tap water. The direct line represents the linear regression.

In the three laboratory grown-bacteria studied, fluorescence emission has a linear correlation to concentration at all three tested excitation/emission peaks.

4.2.2 Natural water-borne bacteria

Since the three bacterial species were single cultures laboratory strains, fluorescence emission intensities (a.u.) were also measured for natural water-borne bacteria which composed of mixed populations. Fluorescence emission intensities were tested for increasing bacterial concentrations, in three independent dilution series. The emission intensities recorded at the three different excitation/emission wavelengths pairs are shown in Table 5.

Table: 5. Mean fluorescence intensities of natural water-borne bacteria at different concentrations in pre-filtered tap water: at the excitation/emission wavelength pairs of interest in three replicated experiments.

Sample (dilution factor)	**Mean fluorescence emission intensities[a] (arbitrary units) at different combinations of excitation/emission wavelength nm[b] (+/-SD)**			**Mean cell number per ml (+/-SEM)**
	210/368-370	**225/368-372**	**280/438-444**	
d0	537 (103)	308 (80)	71 (11)	4.2×10^5 (3.8×10^4)
d1 (1:2)	317 (8)	194 (17)	57 (7)	2.1×10^5 (1.9×10^4)
d2 (1:4)	161 (16)	123 (9)	45 (4)	1×10^5 (9.5×10^3)
d3 (1:8)	103 (10)	83 (6)	43 (3)	5.2×10^4 (4.8×10^3)
d4 (1:16)	64 (1)	59 (5)	41 (3)	2.6×10^4 (2.4×10^3)
d5 (1:32)	45 (3)	46 (2)	40 (3)	1.3×10^4 (1.2×10^3)
d6 (1:64)	39 (4)	46 (8)	40 (4)	6.6×10^3 (5.9×10^2)
d7 (1:128)	32 (0)	38 (0)	39 (4)	3.3×10^3 (3×10^2)
d8 (1:256)	31 (1)	37 (0)	40 (4)	1.6×10^3 (1.5×10^2)
d9 (1:512)	31 (2)	38 (0)	39 (3)	8.2×10^2 (74)
d10 (1:1024)	31 (2)	37 (0)	38 (2)	4.1×10^2 (37)
Blank[c]	28 (1)	33 (0)	37 (1)	0

[a]Fluorescence intensities at the excitation/emission wavelength pair of interest in three replicated experiments.
[b]The range of emission wavelengths (where applicable) represents the interval in which the wavelength of maximal emission varied among the samples.
[c] Filtered tap water without bacteria.

By combining all data obtained in the replicated experiments (Table 6), the linear regressions of fluorescence intensity upon the concentration of natural water-borne bacteria were tested for the three pairs of excitation/emission wavelengths of interest (Fig. 17, 18, 19).

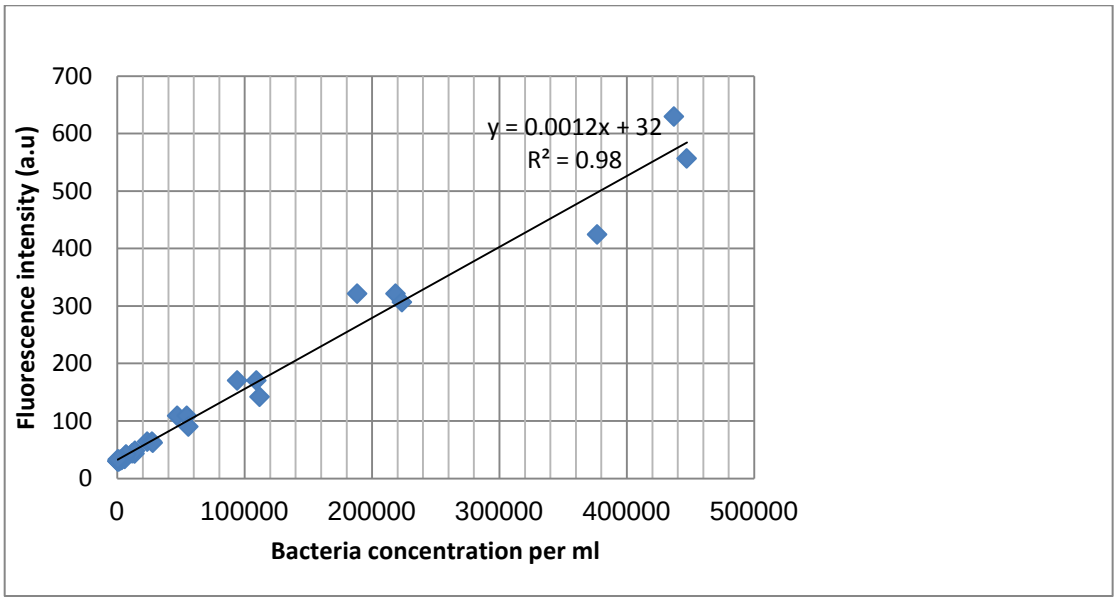

Fig. 17. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 210/(368-370) nm plotted against concentration of natural water-borne bacteria, suspended in filtered tap water. The direct line represents the linear regression.

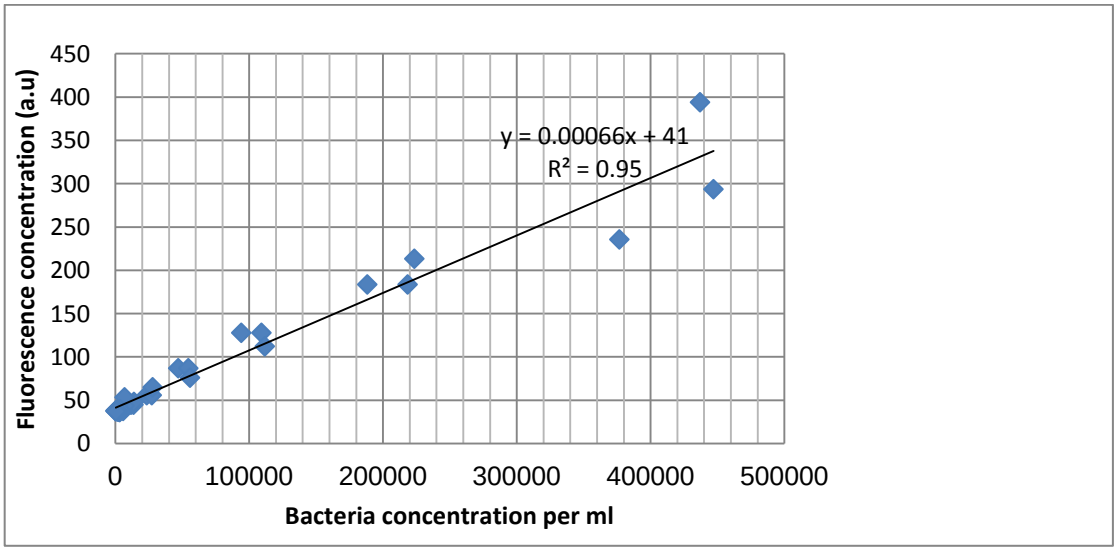

Fig. 18. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 225/(368-372) nm plotted against concentration of natural water-borne bacteria suspended in filtered tap water. The direct line represents the linear regression.

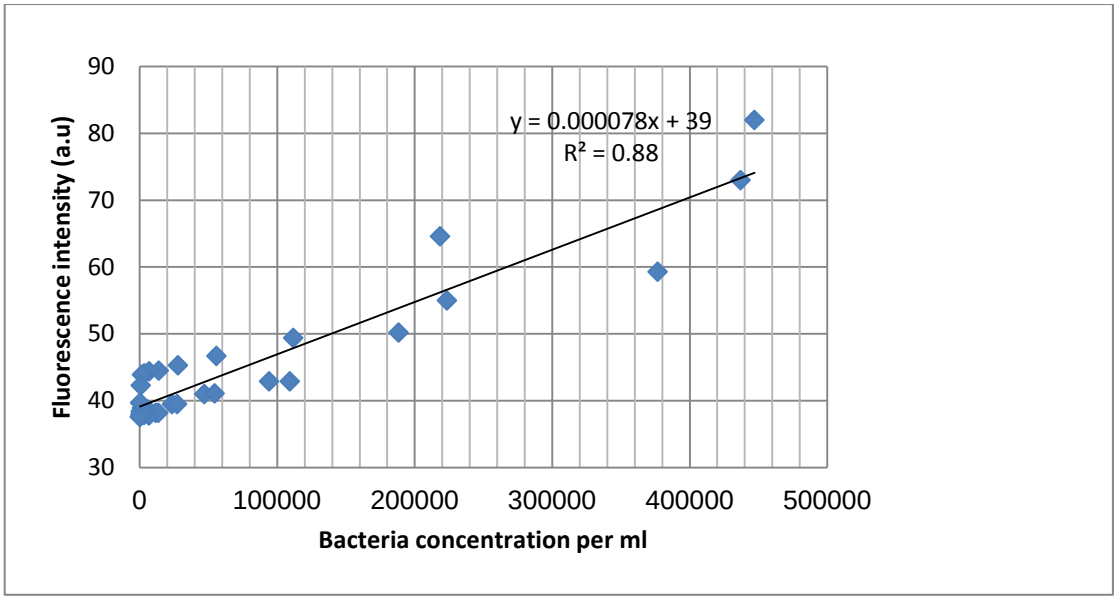

Fig. 19. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 280/(438-444) nm plotted against concentration of natural water-borne bacteria suspended in filtered tap water. The direct line represents the linear regression.

Natural water-borne bacteria fluorescence emission has a linear correlation to concentration at all three tested excitation/emission peaks (Fig. 17-19). It is clear that fluorescence emission intensity is more sensitive to bacteria concentration at 210/368-370 peak.

4.3 The fluorescence of L- tryptophan solution

After identifying fluorophores in water containing different bacteria (i.e., *E. coli*, *B. subtilis*, *P. aeruginosa*, and natural water-borne bacteria), we wanted to know which of the identified peaks corresponds to the presence of tryptophan, since, we know from Cantor et al. (1980) that tryptophan is the major aromatic amino acid that contributes to fluorescence of bacteria. The intensities of fluorescence emission of L-tryptophan dissolved in filtered tap water were measured at various L-tryptophan concentrations in two independent experiments (Table 6). The typical contour and 3D fluorescence maps of L-tryptophan solution in filtered tap water are shown in Fig. 20.

Table 6. L-tryptophan dissolved at different concentrations in pre-filtered tap water: fluorescence intensities at the excitation/emission wavelength pairs of interest in duplicated experiments.

	Mean luorescence emission intensities at different combinations of excitation/emission wavelength nm[a] (+/-SD)		
Sample (ppb)	**210/368-370**	**220/354-356**	**275/354-356**
9.6	563 (49)	1016[b] (0)	1016[b] (0)
4.8	223 (3)	438 (3)	419 (5)
2.4	67 (3)	111 (35)	82 (28)
1.2	25 (5)	19 (1)	8 (1)
0.6	20 (3)	19 (1)	6 (0)
0.3	19 (1)	13 (1)	5 (1)
Blank[c]	17 (2)	11(1)	5 (1)

[a]The range of emission wavelengths (where applicable) represents the interval in which the wavelength of maximal emission varied among the samples.

[b]Emission intensity is over the scale of the spectrofluorometer.

[c]Filtered tap water without bacteria.

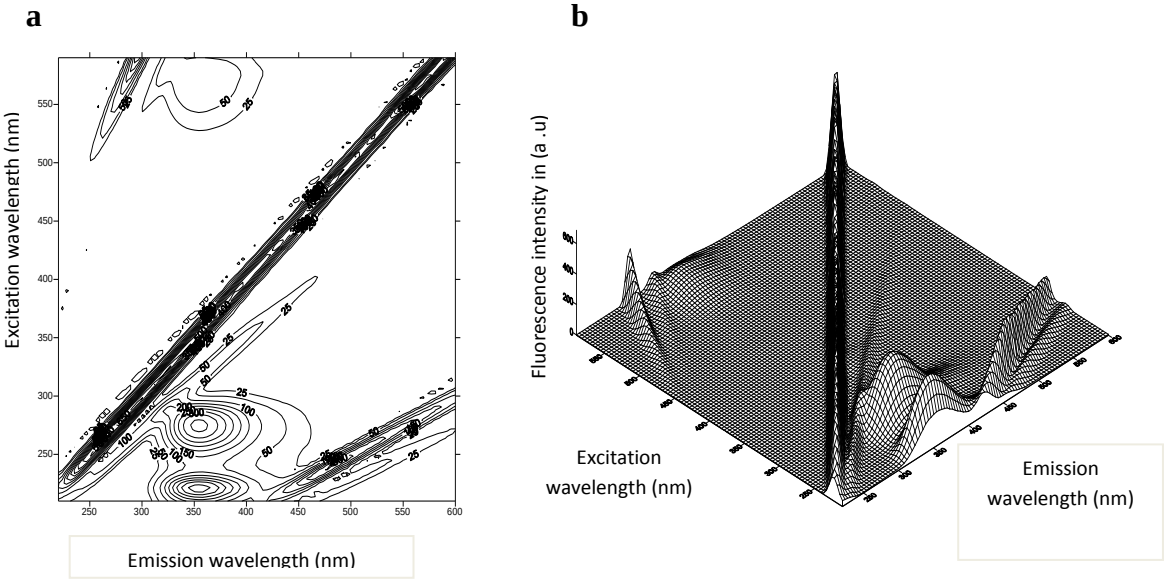

Fig. 20. Contour (top view) fluorescence map (a) and 3-D fluorescence maps (b) of the 4.8ppb L-tryptophan solution in filtered tap water.

Tryptophan displays two major peaks at excitation/emission 220/354-356 and 275/354-356.

A linear regression analysis of fluorescence intensity upon the concentration of L-tryptophan in duplicate experiments was performed for three pairs of excitation/emission wavelengths of interest (Fig. 21, 22, 23).

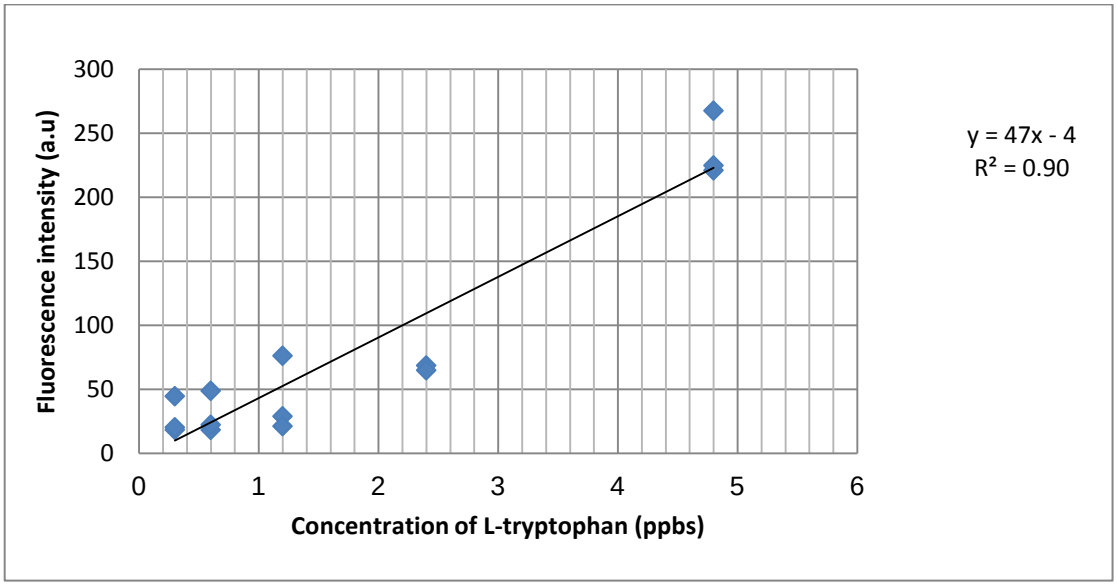

Fig. 21. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 210/(368-370) nm plotted against concentration of L-tryptophan dissolved in filtered tap water. The direct line represents the linear regression.

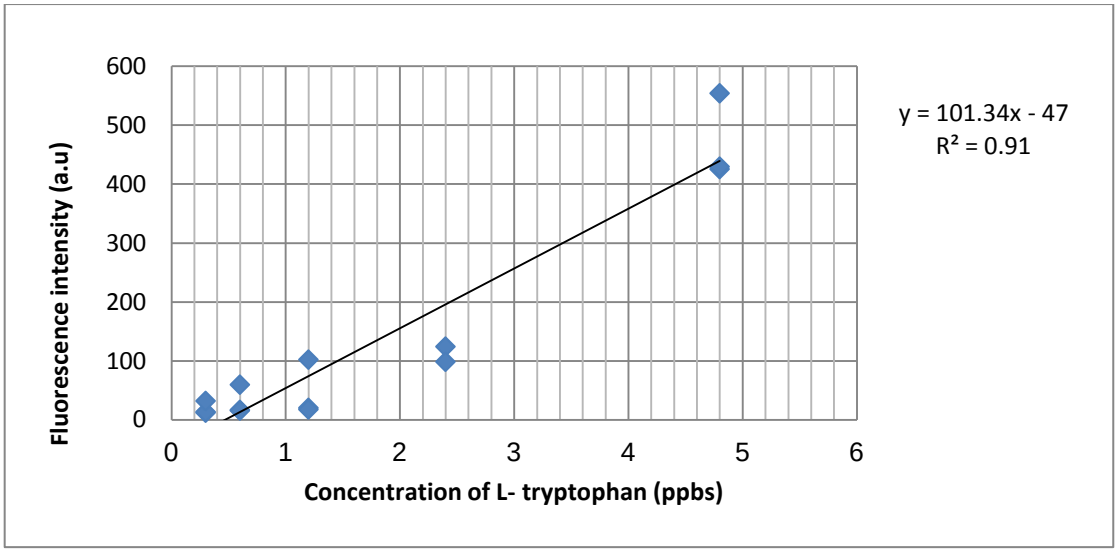

Fig. 22. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 220/(354-356) nm plotted against concentration of L-tryptophan dissolved in filtered tap water. The direct line represents the linear regression.

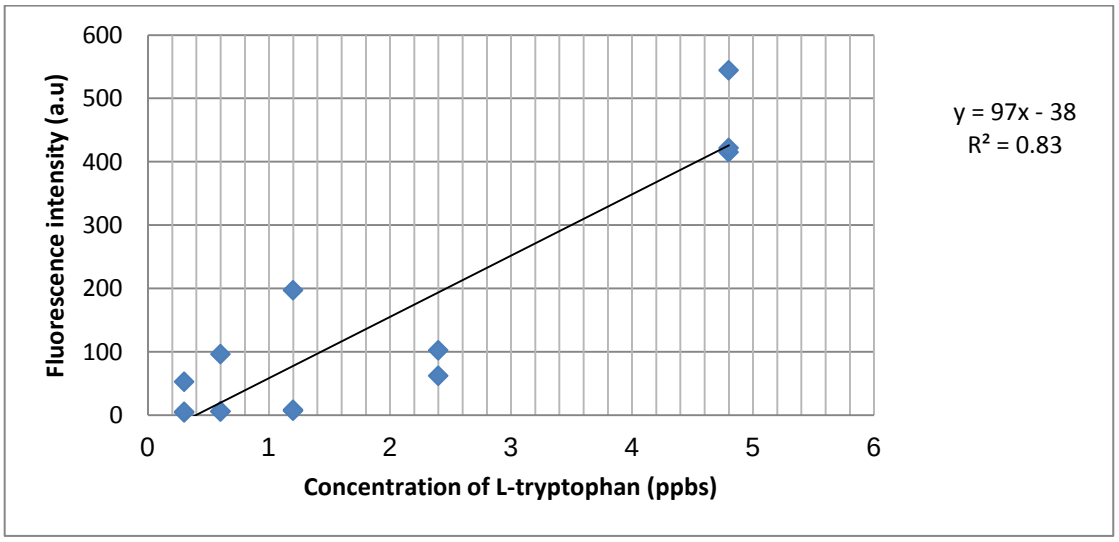

Fig. 23. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 275/(354-356) nm plotted against concentration of L-tryptophan dissolved in filtered tap water. The direct line represents the linear regression.

L-tryptophan fluorescence emission has a linear correlation to concentration at all three tested excitation/emission peaks. The fluorescence intensity is more sensitive to L-tryptophan concentration at 220/(354-356) nm.

4.4 Fluorescence intensity monitoring of natural tap water

4.4.1 HPC

The number of culturable waterborne bacteria (HPC) in drinking water was determined weekly, between the months of January 29 to May 28 2013. Base on the data presented in Fig. 17, 18 and 19, the fluorescence characterized by excitation at 210 nm and emission range at 368-370nm demonstrated the strongest sensitivity to the concentration of natural water-borne bacteria. In order to examine if fluorescence intensity can be used for measuring the total number of waterborne bacteria in drinking water, weekly monitoring of tap water was performed between the months of December 23 2012 to July 10 2013. The fluorescence emission intensity at excitation/emission wavelength of 210/368-370 nm was determined as well as HPC and the data are shown in Fig. 24.

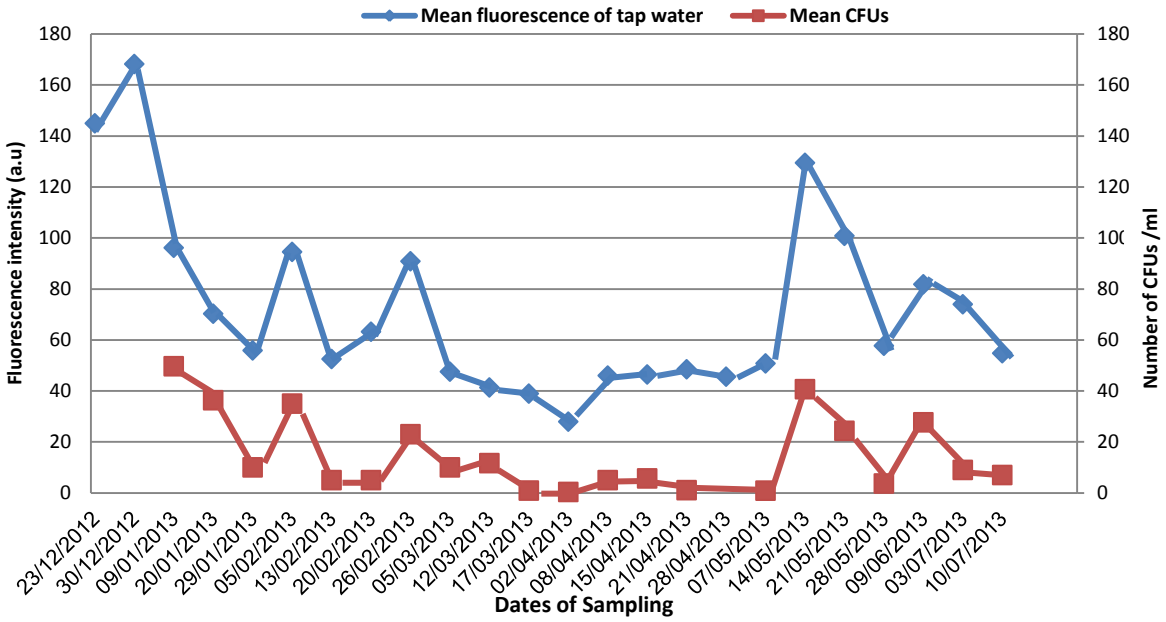

Fig. 24. The weekly monitoring of tap water fluorescence (in arbitrary units) at Excitation/emission wavelength 210/(368-70) nm and the heterotrophic plate counts (per ml) of tap water. HPC data monitoring started with a delay; on the third week of the fluorescence monitoring. Scale for both y-axes is the same.

Linear regression analysis was performed (Fig. 25), and showed a strong linear correlation between the fluorescence intensity and the number of bacterial CFU.

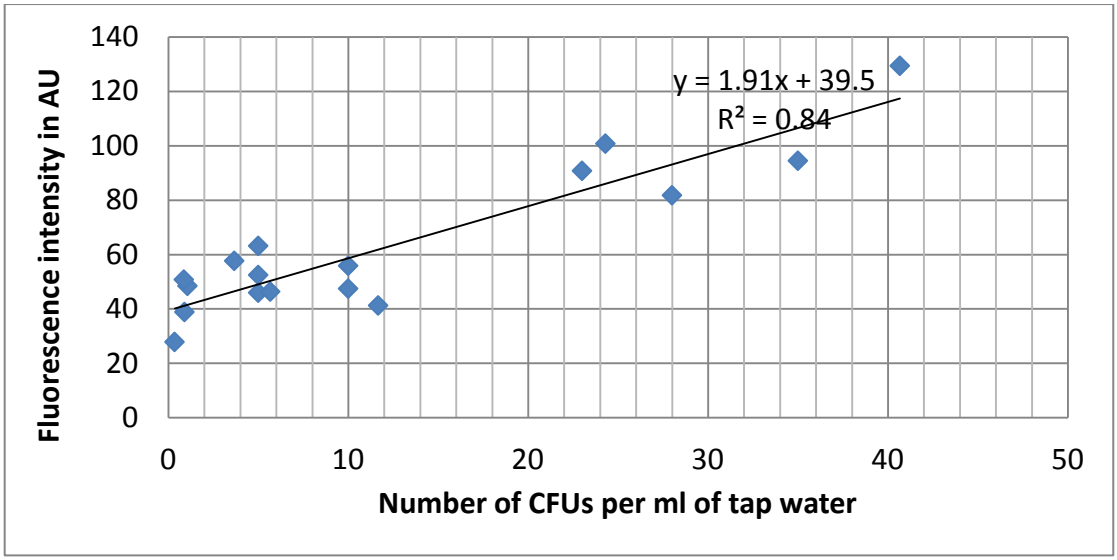

Fig. 25. Fluorescence intensity (in arbitrary units) at excitation/emission wavelengths of 210/368-370 nm plotted against CFU per milliliter of tap water. The data were accumulated during 24 weeks monitoring in the Institute of Soil, Water and Environmental Science building (The Volcani Center). The direct line represents the linear regression.

4.4.2 Total number of bacteria

In parallel to HPC determination, the total number of waterborne bacteria in drinking water was determined weekly by microscopic count. The fluorescence emission intensity at excitation/emission wavelength of 210/368-370 nm as well as the total counts are presented in Fig. 26. Both fluorescence intensity and bacterial counts display similar dynamics.

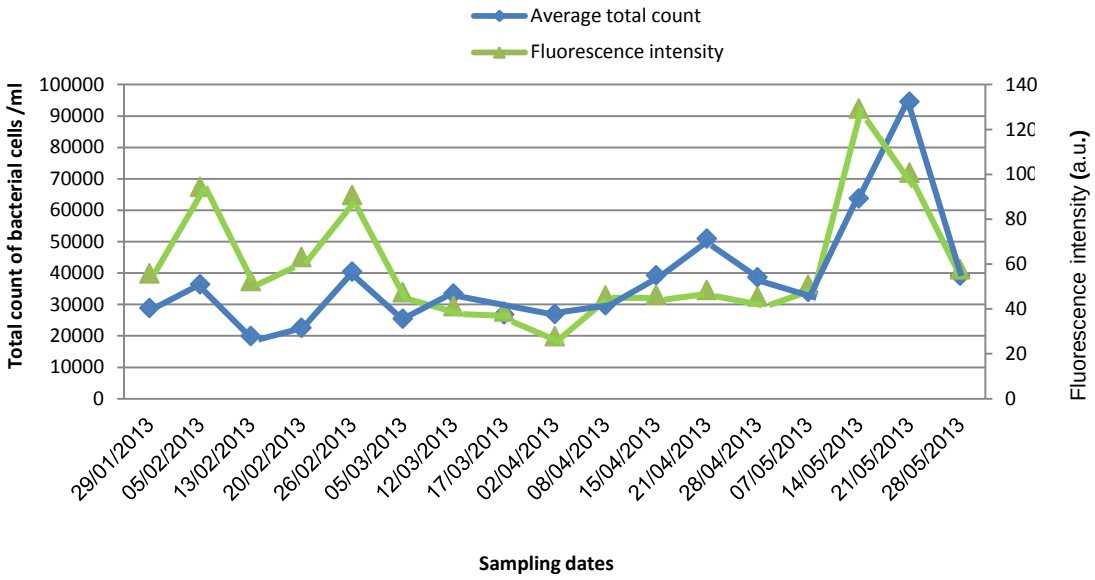

Fig. 26. Weekly monitoring of tap water fluorescence intensity (in arbitrary units, a.u.) at which excitation/emission wavelength 210/(368-370) nm and total bacteria. Total bacterial count data monitoring started with a delay; on the fifth week of the fluorescence monitoring.

Again, a regression analysis was performed (Fig. 27) and showed a linear correlation between the fluorescence intensity and the total counts.

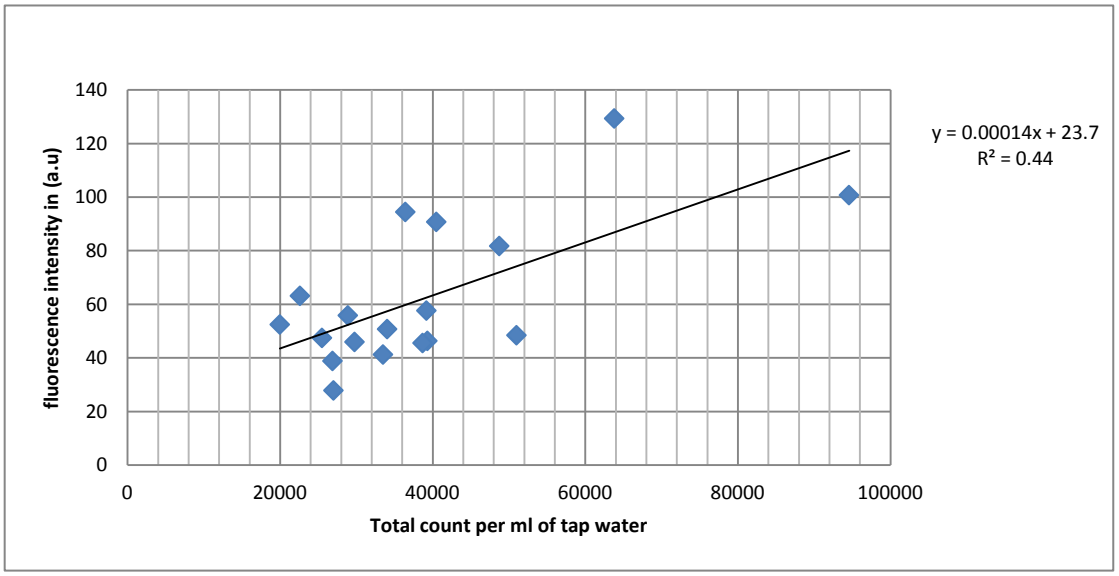

Fig. 27. Fluorescence intensity (in arbitrary units, a.u.) at excitation/emission wavelengths of 210/368-70 nm plotted against the total bacteria count per ml of tap water. The data were accumulated during the 18 week monitoring of in the Institute of

Soil, Water and Environmental Sciences building (The Volcani Center). The direct line represents the linear regression.

4.5 Summary of the parameters of linear regressions analyses

Table 7 summarizes the parameters of linear regressions relating to fluorescence emission intensity and the concentration of *E. coli*, *B. subtilis*, *P. aeruginosa*, natural water borne bacteria and L-tryptophan. For each correlation, Table 7 lists the slope and the intercept (with the standard errors and p-values), the correlation coefficient square and the number of points included into the regression.

The correlations found between fluorescence and the concentrations of laboratory-grown bacteria, water-borne bacteria and L-tryptophan, at the three excitation/emission peaks of interest, were strong with $R^2 > 0.8$ (Table 7).

Table 7. Summary of the parameters of linear regression analyses of fluorescence emission intensity upon the concentration of laboratory grown bacteria, natural water-borne bacteria and L- tryptophan in filtered tap water, at three excitation/emission[a] wavelength coordinates.[b]

	Coordinates of peak →	**Ex/Em 210/(368-370) nm**		**Ex/Em 225/(334-336) nm**		**Ex/Em 280/(332-336) nm**	
	statistical measures	Estimate	standard error[c]	Estimate	standard error	Estimate	standard error
***E.* coli**	slope	0.000088	0.000010	0.00018	0.000016	0.00015	0.000014
	Intercept	27	9	-12	14(0.4)	-21	12(0.09)
	R^2 (no. of points)	0.80 (20)		0.89 (20)		0.87 (20)	
	Coordinates of peak →	**Ex/Em 210/(368-370) nm**		**Ex/Em 225/(332-336) nm**		**Ex/Em 280/(332-336) nm**	
	statistical measures	Estimate	standard error	Estimate	standard error	Estimate	standard error
B. subtilis	Slope	0.00089	0.00010	0.0016	0.00012	0.0014	0.00011
	Intercept	12	19 (0.5)	-24	22(0.3)	-27	21(0.2)
	R^2 (no. of points)	0.83 (19)		0.92 (19)		0.91 (19)	
	Coordinates of peak →	**Ex/Em 210/(368-370) nm**		**Ex/Em 225/(334-336) nm**		**Ex/Em 280/(334-338) nm**	
	statistical measures	Estimate	standard error	Estimate	standard error	Estimate	standard error
***P.* aeruginosa**	Slope	0.00012	0.00000	0.00018	0.000016	0.00015	0.000016
	Intercept	20	4	-4	21(0.8)	-16	20(0.4)
	R^2 (no. of points)	0.99 (16)		0.89(16)		0.86 (16)	
	Coordinates of peak →	**Ex/Em 210/(368-370) nm**		**Ex/Em 225/(368-372) nm**		**Ex/Em 280/(438-444)**	
	statistical measures	Estimate	standard error	Estimate	standard error	Estimate	standard error
Natural water-borne bacteria	slope	0.0012	0.000031	0.00066	0.000027	0.000078	0.000000
	Intercept	32	4	41	4	39	1
	R^2 (no. of point)	0.98 (33)		0.95 (33)		0.88(33)	
	Coordinates of peak →	**Ex/Em 210/(368-370) nm**		**Ex/Em 220/(354-356) nm**		**Ex/Em 275/(354-356) nm**	
	statistical measures	Estimate	standard error	Estimate	standard error	Estimate	standard error
L-tryptophan	slope	47	5	101	9	97	12
	Intercept	-4	11(0.7)	-47	23(0.06)	-38	30(0.2)
	R^2 (no. of points)	0.90 (10)		0.91 (10)		0.83(10)	

[a]Concentrations of bacteria are in the number of cells per ml; concentration of L-tryptophan is in ppb.

[b]Data were analyzed with Statistica software (StatSoft, version 7) and Microsoft excel 2007.

[c]$P<0.01$ if not indicated differently in the parentheses near the standard error value.

5. Discussion

The major objective of the study was to test if fluorescence spectroscopy can be utilized to detect microbial quality of drinking water. To test this objective we needed to know whether fluorescence spectroscopy can detect, quantify and perhaps identify bacteria in drinking water.

5.1 Identification of the fluorescence peaks location

Two distinct fluorescence peaks were identified in tap water containing laboratory-grown bacteria (*E. coli, P. aeruginosa, B. subtilis*). The peaks were located at excitation/emission wavelengths of 225/(334-338) and 280/(332-338) nm (Figs 8-18 and Tables 2-4 and 7). For well-washed and aerosolized bacteria (*B. subtilis*, *E. herbicola*, and *E. coli*), Hill et al., (1999) have also observed a fluorescence at excitation/emission 226/330 nm. Their analysis revealed that the spectra of different bacteria were similar with a strong emission at around 330 nm originating from the contribution of tryptophan, and with the shoulders in the 400- to 500-nm regime.

It is well known that bacteria show fluorescence emission in the 300-400 nm range following excitation at 225-290 nm, due mainly to the fluorescence of the amino acid tryptophan present in bacterial proteins (Leblanc, 2002, Determann et al., 1998; Cantor et al., 1980). Tryptophan has two major fluorescence peaks due to excitation at 225 and 280 nm, which is in agreement with the location of the emission peaks in tap water containing lab-grown bacteria in our experiments. Thus, it is likely that the fluorescence of laboratory-grown bacteria in tap water was due to the presence of tryptophan.

In addition, in the experiments with laboratory-grown bacteria, there was a strong fluorescence, due to the excitation at 210 nm, which showed the emission maximum at around 368-370 nm. Since there was no possibility to apply the excitation at wavelengths shorter than 210 nm, the exact position of the excitation peak is unknown, and it can occur at wavelengths smaller than 210 nm.

When testing fluorescence of natural water-borne bacteria at excitation wavelengths of 225 and 280 nm, the fluorescence readings are at different emission locations (368-372) and (438-444), respectively, compared to the fluorescence peaks of lab-grown bacteria (Table 7).

A fluorescence characterized by the excitation/emission wavelengths of 210 and 330 nm, respectively, was also reported by Mopper and Schultz (1993) when examining the depth profiles of water fluorescence in the Sargasso Sea. They denoted this signal as protein-like fluorescence. It is likely that the strong fluorescence observed at excitation/emission wavelengths of 210/368-370 nm in natural water-borne bacteria may also be related to protein-like fluorophores in drinking water. Since the EEM of tryptophan does not display this peak, it is not clear what the specific source of this fluorescence is. Other fluorescing substances found often in water, such as humic-like matter, display fluorescence at the excitation/emission wavelengths in the ranges of 320-370/400-500 nm (Mounier et al, 1999; Artinger et al., 2000), which are different from those reported in this study.

5.2 Sensitivity of fluorescence measurements to bacterial concentration

During the experiments we used filtered tap water, which has negligible fluorescence emission intensity in order to determine the effect of exogenously added bacteria on fluorescence intensity. When increasing the concentrations of the lab-grown bacteria, such as *E. coli*, *B. subtilis*, *P. aeruginosa*, the fluorescence emission intensity demonstrated strong and positive linear relations with bacterial concentrations with R^2 ranging from 0.80 to 0.99 (see Fig 8-16 and Table 7).

Since the three bacterial species (laboratory-grown) may not represent the population of natural water-borne bacteria, which include large number of species, the majority are un-culturable, similar regression analyses have been made also with natural water-borne bacteria added to the filtered tap water. The presence of strong linear correlations (R^2 = 0.88 to 0.98) between fluorescence response and the concentrations of bacteria suggests that also in 'real-life' measuring the fluorescence emission intensity may be used to quantify the load of bacteria in drinking water.

The lowest detectable limit of fluorescence in all experiments was 10^4 bacteria per ml. Table 7 displays the slopes of the linear regressions for fluorescence intensity upon the bacterial concentrations which signifies the sensitivity of fluorescence. Based on variability of the slopes of linear regressions for fluorescence intensity versus bacterial concentration (Table 7), it is clear that bacteria demonstrate a different sensitivity of fluorescence to their concentration at various excitation/emission regions. The higher is the slope of the regression line, the greater is the sensitivity of fluorescence to the concentration of bacteria. The sensitivity of

fluorescence emission intensity to the bacterial concentration varies also among the tested bacteria. At excitation/emission wavelength pair of 225/(332-336) nm, *B. subtilis* has the highest sensitivity followed by *P. aeruginosa* and then *E. coli*. The sensitivity of fluorescence emission intensity at excitation/emission wavelength pair of 280/(332-338) nm, is also higher in *B. subtilis* followed with *P. aeruginosa* and *E. coli* sharing similar sensitivity. Finally, at excitation/emission wavelength pair of 210/(368-370) nm, the sensitivity of fluorescence emission intensity is higher in *B. subtilis* followed by *P. aeruginosa* and then *E. coli*. When comparing the fluorescence sensitivity of all studied bacteria (both laboratory-grown and natural water-borne) at excitation/emission wavelengths of 210/(368-370) nm, the sensitivity is higher in natural water-borne bacteria followed with *B. subtilis, P. aeruginosa*, and *E. coli* (the least sensitive).

5.3 Contribution of tryptophan to bacterial fluorescence

In order to evaluate the potential contribution of tryptophan to bacterial fluorescence at the indicated excitation/emission coordinates, we have calculated the amount of tryptophan (in ng) in the tested bacteria, assuming that the specific ability of tryptophan to fluoresce is the same regardless if it is free in water or incorporated in bacterial cells. The following formula was used:

$$T_c = (S_r/S_t)$$

where, T_c is the concentration of tryptophan (in ng) in one cell; S_r is the slope for the regression of fluorescence upon the cell number (per ml); S_t is the slope of the regression of fluorescence by tryptophan concentration (in µg per L). It is assumed that the intercepts in the regressions (Table 7) have a minor significance (if any) in controlling the measured fluorescence. Taking the slope of natural water-borne bacteria at excitation/emission 210/(368-370) nm (Table 7) as (S_r=0.0012), and the slope for tryptophan at the same location as (S_t=47), then: T_c = 0.0012 (mL/cell) /47 (L/µg) = 0.000025 ng/cell

Now, roughly assuming that the protein content and the amino acid usage in an average bacterial cell in tap water are similar to that in *E. coli*, and given that the weight of an average *E. coli* cell is about 10^{-12} g (Neidhardt, 1996 it seems that the calculated amount of tryptophan in a cell is around 2.6% by weight of this cell. Thus, it shows a meaningful content of tryptophan in a cell and supports the consideration that the bacterial fluorescence is related to the tryptophan fluorescence. At the same

time, this estimate of the tryptophan content is not indeed quantitative because the specific tryptophan fluorescence may be different depending on whether the amino-acid is inside of a cell or free in water. The fluorescence efficacy of tryptophan was reported to be affected by its environment, and in fact becomes enhanced in a less polar environment (as compared with water) (Vivian et al., 2001). Similarly, Determann et al. (1998) demonstrated that the average protein-specific fluorescence in bacterial cells is about five times higher than the efficiency fluorescence of protein dissolved in water.

Tryptophan is considered to be the major fluorophore in proteins since its quantum yield is hundred times higher than the quantum yield of the other two aromatic amino acids, tyrosine and phenylalanine (Cantor et al., 1980). Our findings regarding lab grown-bacteria suggest that tryptophan indeed contributes to their fluorescence. It is not clear why the two peaks associated to the regular tryptophan fluorescence do not appear in the EEMs of water-borne bacteria and what fluorophore(s) contributes to the observed fluorescence of waterborne bacteria.

5.4 Correlation between concentration of natural water-borne bacteria and fluorescence during weekly monitoring.

During the weekly monitoring of tap water, it has been noted that the tap water fluorescence at excitation/emission wavelengths of 210/(368-370) nm and the measured CFUs demonstrated a similar temporal dynamics (Fig. 24). Indeed, regression analysis showed a significant correlation between the fluorescence intensity and CFU ($R^2 = 0.84$; $p < 0.01$) (Fig. 25). These findings suggest that simple and rapid fluorescence measurements have the potential to be used as operational tool for monitoring variations in CFUs.

Culturable bacteria in drinking water only account for about 0.01% of the total number of bacteria, (Bartram et al., 2003). Moreover, considering that fluorescence might be contributed by both live and dead microorganisms, as well as by their degradation products, it was interesting to compare the relation between fluorescence intensity and the number of bacteria in tap water. Comparing tap water fluorescence and total number of bacteria determined by AO staining and microscopical observation showed also a similar temporal dynamics (Fig. 26), when measured at the excitation/emission wavelengths of 210/(368-70) nm. However, the correlation was weak ($R^2 = 0.44$; $p < 0.01$) (Fig. 27). The relatively low correlation of total number of

bacteria with fluorescence may be explained by the enumeration method used for total bacterial count, which requires bacterial staining. The staining is based on the assumption that mainly the bacterial cells will be stained by acridine orange. However, it is possible that other degradation products present in water might be stained, resulting in an overestimation of the total count. Sometime aggregation of cells may also occur, limiting the accuracy of the cell counts and resulting in underestimation of the real total number of bacteria contributing to the fluorescence. Also, it is possible that microorganisms other than bacteria, such as yeasts, algae, and protozoa also may contribute to fluorescence (although none was detected using the AO-staining), and affect the correlation between total count and fluorescence. Further studies on a larger number of samples are required to test this notion.

In summary, our study has shown that spectrofluorometry technique can be used for detection of bacteria in drinking water and has the potential to serve as an early warning system for a contamination events. Such system may enable prompt response, including a more detailed microbiological and chemical analyses to identify the specific contaminant. Future research may focus on determining why the two tryptophan peaks (225/334-336 and 280/334-336) are not observed with natural water-borne bacteria and what fluorophores contributes to the fluorescence of waterborne bacteria at (210/368-370) nm.

6. Conclusions

- The bacterial contamination of drinking water can be detected by fluorescence at the following excitation/emission wavelength pairs 210/(368-370) nm, 225/(334-338) and 280/(332-338).
- Fluorescence measurements can detect the presence of at least about 10^4 bacteria per ml in tap water with a low fluorescence background.
- *E. coli*, *B. subtilis* and *P. aeruginosa* display very similar fluorescence spectra, suggesting that it is problematic to distinguish between them in drinking water with the help of EEM fluorescence spectroscopy.

  Natural water-borne bacteria have a distinct dominant fluorescence emission peak at 368-370 nm when excited at 210 nm.

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תקציר

איכות מיקרוביאלית של מי שתייה נקבעת באופן שגרתי ע"י בדיקות בקטריאליות הכוללות ספירה של חיידקים ההטרוטרופיים וקוליפורמים. שיטות הבדיקה הקיימות דורשות כוח אדם מיומן וזמן הדגרה של לפחות 24 שעות עד קבלת התוצאות. רק לאחר קבלת תוצאות הספירות המיקרוביאליות, זמן רב לאחר שהמים נצרכו, ניתן לדעת שהמים היו תקינים מבחינה מיקרוביאלית. לכן, השיטות הקיימות לבדיקת האיכות המיקרוביאלית של מי שתייה אינן מסוגלות להתריע על סכנה לבריאות הצרכן בזמן אמת. זיהום של מי שתייה, אקראי או מכוון, מצריך זיהוי מידי של הזיהום שיאפשר טיפול וימנע סכנה לבריאות הציבור. המטרה המרכזית של המחקר הנוכחי הייתה לבחון האם ניתן בשיטת ספקטרוסקופיה פלואורסנטית לזהות ולכמת זיהום חיידקי במי שתייה. במידה שהשיטה תוכיח את יעילותה, היא יכולה לשמש כבסיס לפיתוח שיטה מהירה לבדיקת איכות מי שתייה בזמן אמת.

לצורך הבדיקה, נבנו מפות עירור/פליטה בטווח של 220-600 ננומטר עבור שלושה זני חיידקים (*Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa*) וזוהו שיאי העירור/פליטה. נמצאו שני שיאים כאלה באורכי גל (334-338)/225 ו- (332-338)/280 ננו מטר. בחינה של ריכוז החיידקים במים כפונקציה של עצמת הפלואורסנציה ע"י רגרסיה לינארית הראתה קורלציה מובהקת ($p<0.01$; R^2=0.8-0.99) בשני השיאים שנבדקו. היות וכל שלושת החיידקים הראו ספקטרום פלואורסנטי זהה, השיטה אינה מאפשרת אבחנה בין סוגי החיידקים השונים. בשיטה זו ניתן לזהות את נוכחות החיידקים כאשר מספרם גבוה מ- 10^4 CFU/ml .

אנליזת דומה נערכה גם עם פלורה טבעית של מי ברז. גם כאן, נצפתה קורלציה גבוהה בין מספר החיידקים (הטרוטרופיים) לבין עצמת השיאים הפלואורסנטיים. ניטור שבועי של חיידקים הטרוטרופיים ועצמת פלואורסנציה במי ברז הראה דינמיקת שינויים דומה, המצביעה על אפשרות להשתמש במדידת פלואורסנציה כמדד לאיכות המיקרוביאלית של מי שתייה.

לסיכום, המחקר הנוכחי מראה שמדידת הפלואורסנציה בשיאי עירור/פליטה המוזכרים לעיל, יכולה לשמש כשיטה מהירה לזיהוי של זיהום מיקרוביאלי של במי שתייה. לשיטה זו פוטנציאל יישומי לשמש כבסיס לפיתוח מכשיר לניטור איכות מיקרוביאלית של מי שתייה בזמן אמת.

שימוש בספקטרוסקופיה פלואורוסנטית לזיהוי של זיהום בקטריאלי במי שתייה

עבודת גמר

מוגשת לפקולטה לחקלאות , מזון ואיכות הסביבה על שם רוברט סמיט
של אוניברסיטה העברית בירושלים
לשם קבלת תואר "מוסמך במדעי המזון"

מאת

קוונלה סייבונגה סימנלה

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