Characterization of bacterial fluorescence: insight into rapid detection of bacteria in water

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ABSTRACT

Microbial contamination is one of the main risks affecting water safety. Traditional microbial detection methods tend to be time-consuming and labor-intensive. Thus, this study investigated a potential rapid and simple method for bacterial detection in water by excitation–emission matrix (EEM) fluorescence spectroscopy. Particularly, bacterial intrinsic fluorophores were divided into three regions, namely Region A (amino acids), Region N (NAD(P)H) and Region F (flavins). Afterwards, fluorescence characteristics of four pure bacterial species (Bacillus subtilis, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa) as well as indigenous bacteria in secondary effluent from two water reclamation plants were evaluated via fluorescence regional integration (FRI). Correlation analysis between fluorescence intensity (FI) integral and bacterial concentration was conducted, and principal component analysis (PCA) was applied to distinguish the fluorescence spectra of different bacteria. The results showed that most of the bacterial autofluorescence was emitted by amino acids and the FI integral of flavins had a good linear relationship ($R^2 > 0.9$) with bacterial concentration. PCA could distinguish varied bacterial species and bacteria from different secondary effluents. This study indicated that FRI was helpful for the characterization of bacterial fluorescence and the quantification of bacteria in water.

Key words: bacterial detection, fluorescence regional integration, fluorescence spectroscopy, principal component analysis

HIGHLIGHTS

• Fluorescence regional integration was used to characterize bacterial intrinsic fluorescence.
• Most of the bacterial intrinsic fluorescence was emitted by amino acids.
• Flavin fluorescence was well correlated with bacterial concentration.

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1. INTRODUCTION

Clean water is vital to public health as well as the sustainable development of society and economy (UN 2015; Shi et al. 2020). However, with the rapid population growth and the acceleration of urbanization and industrialization, water pollution is increasingly serious and has caused many detrimental health problems around the world (Clemens et al. 2017; Budnik & Casteleyn 2019; Chen et al. 2020). According to the fourth edition of the World Health Organization’s (WHO) Guidelines for drinking-water quality, most of the evident water-related health problems are caused by microbial contamination (WHO 2011). Microorganisms in water include bacteria, viruses and protozoa, which may not only lead to the occurrence of water-borne diseases, but also pose other risks such as pipeline corrosion and membrane fouling (Matin et al. 2011; Enning & Garrelfs 2014; Luo et al. 2021). To efficiently prevent these adverse effects, it is essential to detect microbial contamination in water.

Culture-based methods (e.g., plate counting technique) have been widely used for microbial detection since the last century. However, the detection process is quite time-consuming and labor-intensive. Moreover, the existence of viable but nonculturable microorganisms may lead to the underestimation of the actual number of microorganisms (Rollins & Colwell 1986). Besides the traditional culture-based methods, emerging methods such as molecular methods (e.g., polymerase chain reaction), flow cytometry (FCM) and adenosine triphosphate (ATP) bioluminescence are increasingly used for microbial detection (Rajapaksha et al. 2019). Polymerase chain reaction (PCR) is a DNA amplification technique which can provide reliable results in several hours owing to its high sensitivity, but viable and nonviable microorganisms could not be distinguished since both types of DNA can be amplified (Cangelosi & Meschke 2014). In recent years, propidium monoazide (PMA) has been used to prevent the amplification of DNA from nonviable microorganisms (Banishahemi et al. 2015). Although PMA enables the detection of viable microorganisms, the detection process is quite complicated and includes PMA staining and DNA extraction. Besides, FCM is an optical detection method which analyzes the scatter light and/or fluorescence emitted by suspended microbial particles (Safford & Bischel 2019). The absolute number of microorganisms can be determined in very short times by this method. However, sample pretreatment and fluorescent staining may be needed, and the cost of this method is relatively expensive. In addition, ATP bioluminescence is a biochemical method which detects microbial activity since ATP exists in all living cells (Bottari et al. 2015). Although the detection process can be finished in several minutes, biotic and abiotic ATP could not be distinguished and some matrix effects may interfere with the determination or inhibit luminescence (Ntziachristos et al. 2005). Therefore, a rapid, simple, reliable and cost-effective method for microbial detection is urgently demanded.
Fluorescence spectroscopy is a fast and noninvasive technique which has been widely used for the characterization of organic matters and has a broad prospect in the real-time online detection of microorganisms (Carstea et al. 2016). Live bacteria contain many intrinsic fluorophores such as aromatic amino acids and coenzymes, which exhibit fluorescence at specific excitation and emission wavelengths (Ammor 2007). The relationship among excitation wavelengths, emission wavelengths and fluorescence intensity (FI) can serve as an intrinsic bacterial fingerprint. Early studies on bacterial fluorescence remained at a qualitative level, which focused on the identification and classification of bacterial species (Giana et al. 2003; Sohn et al. 2009). In recent years, fluorescence spectroscopy is increasingly used for the quantitative detection of bacteria in water. However, most relevant studies were based on tryptophan-like fluorescence, which was determined at a single excitation and emission wavelength (Baker et al. 2015; Bridgeman et al. 2015; Sorensen et al. 2015). A study of bacterial detection in groundwater compared the detection capability among a single excitation and emission wavelength pair, two-dimensional emission spectra and three-dimensional excitation–emission matrix (EEM), concluding that better quantification could be achieved based on partial least squares analysis of EEM and the detection limit was as low as 10 CFU/ml (Nakar et al. 2020). Relative to a single excitation and emission wavelength pair, EEM provides more abundant information, which is beneficial to improve the sensitivity of detection (Li et al. 2020). Fluorescence regional integration (FRI) is a quantitative method for analyzing EEM, which was first used to characterize fluorescence spectra of dissolved organic matter (DOM) in water (Chen et al. 2003). The principle of this method is to divide an EEM contour map into several regions which correspond to different fluorophores and calculate the FI integral of each region. Some studies have reported that FRI was an effective method which could be used to assess the formation potentials of disinfection by-products and the humification degree (Yang et al. 2008; He et al. 2011). Nevertheless, few studies so far have applied FRI to characterize fluorescence spectra of bacteria. Consequently, the objective of this study was to analyze the fluorescence characteristics of four different bacterial species and indigenous bacteria in secondary effluent from two water reclamation plants (WRPs) and develop a quantification method on bacterial autofluorescence by FRI, thus providing insight into the rapid detection and quantification of bacteria in water.

2. MATERIALS AND METHODS

2.1. Bacterial samples preparation

Two Gram-positive bacteria, Bacillus subtilis (CICC 10275) and Staphylococcus aureus (CGMCC 1.12409), and two Gram-negative bacteria, Escherichia coli (CGMCC 1.5373) and Pseudomonas aeruginosa (CGMCC 1.12483), were used for analysis. For each experiment, bacterial cultures stored in 20% glycerol at ~80 °C were inoculated in 20 ml of Luria Bertani (LB) broth and incubated overnight with shaking (150 rpm) at 37 °C. Bacterial cells were harvested by centrifugation (14,400 g) for 5 min at 4 °C and resuspended in 20 ml of 0.9% sterile saline solution. After repeating the washing steps twice, the final bacterial suspensions were 10-fold diluted serially with 0.9% sterile saline solution and used for fluorescence detection. Bacterial concentration was determined by plating 0.1 ml of bacterial solutions in triplicate on LB agar plate and counting colony-forming units (CFU) after overnight incubation at 37 °C.

2.2. Secondary effluent sample preparation

Secondary effluent was sampled from two large-scale WRPs in Beijing, China. Plant A has a daily treatment capacity of 1 × 10⁶ m³/d and a service area of 97 km², which undertakes the wastewater treatment of the downtown area and the eastern area of Beijing. Plant B has a daily treatment capacity of 550,000 m³/d, which undertakes the wastewater treatment of the western area of Beijing. 200 ml of water samples were filtered through a 0.22 μm sterile filter membrane and bacteria enriched on the membrane were eluted with 20 ml of 0.9% sterile saline solution. 10-fold serial dilutions of bacterial eluents were taken with 0.9% sterile saline solution and used for fluorescence detection. Bacterial concentration was determined by plating 0.1 ml of bacterial solutions in triplicate on an R2A agar plate and counting CFU after incubation for 48 h at 25 °C.

2.3. Fluorescence EEM measurements

Fluorescence EEM measurements were carried out by a fluorescence spectrophotometer (F-7100, Hitachi, Japan). The excitation wavelength (EX) ranged from 200 to 500 nm with 5-nm intervals, and the emission wavelength (EM) ranged from 250 to 600 nm with 2-nm intervals. The excitation and emission slits were 5 nm, and the voltage of photomultiplier was 600 V. Each sample was measured in duplicate, and the scanning time of each measurement was about 2 min. All measurements were finished at room temperature (20 ± 2 °C).
2.4. Data processing and interpretation

Overall, 10,736 data points were obtained for each EEM, and some data points (EM < EX or EM > 2 EX) were removed considering first-order and second-order Rayleigh scattering. Besides, the Raman scattering intensity of 0.9% saline solution was deducted accordingly. The EEM contour map was divided into three regions, and the FI integral of each region was calculated by MATLAB software (R2020b, MathWorks, USA). The correlation analysis between the logarithm of the bacterial concentration and the average logarithm of the FI integral was carried out with Excel software (2019, Microsoft, USA). Principal component analysis (PCA) was applied to differentiate the emission spectra at the excitation wavelength of 280 nm using Origin software (2021, OriginLab, USA).

3. RESULTS AND DISCUSSION

3.1. Fluorescence characteristics of four bacterial species

According to the literature, bacterial intrinsic fluorophores mainly include aromatic amino acids (i.e., tryptophan, tyrosine and phenylalanine) and coenzymes (Ammor 2007; Faassen & Hitzmann 2015). Particularly, tryptophan fluorescence (maximal EX: 280 nm, maximal EM: 350 nm) is the most intense among the three aromatic amino acids, which has been widely used in the detection and quantification of bacteria in water (Baker et al. 2015; Sorensen et al. 2015). Tyrosine fluorescence (maximal EX: 275 nm, maximal EM: 300 nm) and phenylalanine fluorescence (maximal EX: 260 nm, maximal EM: 280 nm) usually account for less than 10% of the total fluorescence from amino acids (Dalterio et al. 1986). Coenzymes in bacteria contain reduced nicotinamide adenine dinucleotide (NADH), reduced NADH phosphate (NADPH) and flavin adenine dinucleotide (FAD). Notably, NADH (maximal EX: 351 nm, maximal EM: 460 nm), NADPH (maximal EX: 356 nm, maximal EM: 464 nm) and FAD (maximal EX: 450 nm, maximal EM: 535 nm) also play an important role in the autofluorescence of bacteria since these coenzymes fluorophores are closely related to bacterial metabolism, which can be used to distinguish bacteria from inanimate materials (Estes et al. 2003). Therefore, this study proposed a new FRI approach by dividing bacterial intrinsic fluorophores into three regions, namely Region A (EX: 200–300 nm, EM: 250–400 nm) which is related to amino acids, Region N (EX: 300–400 nm, EM: 400–500 nm) which is related to NAD(P)H and Region F (EX: 400–500 nm, EM: 500–600 nm) which is related to flavins (Figure 1). Compared to five EEM regions of DOM (Chen et al. 2003), this new FRI method based on bacterial intrinsic fluorophores enlarged the ranges of EX and EM and added Regions N and F to better reflect the autofluorescence of bacteria, which is beneficial for comprehensive detection of bacterial contamination in water.

Figure 1 | EEM regions of bacterial intrinsic fluorophores.
Based on the new FRI division depicted in Figure 1, the EEM contours of four typical bacterial species were further measured (Figure 2). The results showed that most of the bacterial autofluorescence was emitted by amino acids (Region A), while coenzymes fluorescence (Regions N and F) was much less intense. This is probably because the main constituents of bacterial cells are proteins, which are made up of amino acids. However, the content of coenzymes in bacteria is relatively low. There were two strong fluorescence peaks in Region A, although their maximal EX was different (around 230 and 280 nm), and the maximal EM was the same (around 330 nm). Dartnell et al. (2013) investigated the fluorescence characteristics of several clinically important bacteria and found a double-peak fluorescence associated with tryptophan, which was consistent with the results of this study. To prove this conclusion, the EEM contours of the standard tryptophan were measured, and a double-peak fluorescence was also observed (Supplementary Figure S1). Although the four bacterial species all exhibited tryptophan fluorescence in Region A, the FI of different bacterial species was different. For example, the FI values of B. subtilis and S. aureus in Region A were higher than those of E. coli and P. aeruginosa, indicating that B. subtilis and S. aureus were more abundant in proteins. This may be due to the difference in cell wall structure between Gram-positive and Gram-negative bacteria. Especially, although the concentration of B. subtilis was much lower than that of the other three bacterial species, the FI of B. subtilis was comparable among the four bacterial species, which could be attributed to the content of extracellular polymeric substance (EPS) excreted by bacteria since proteins in EPS also exhibited fluorescence (Marvasi et al. 2010). Moreover, P. aeruginosa also showed strong fluorescence in Region N due to the siderophore.
pyoverdine in EPS (Cornelis 2010). Overall, all the four bacterial species exhibited a double-peak fluorescence in Region A, but the FI varied largely among different bacterial species.

3.2. Correlation analysis between FI integral and bacterial concentration

The relationship between FI integral and bacterial concentration was further analyzed, as shown in Figure 3. With the increase of bacterial concentration, the FI integral of each region also increased. The FI integral of Region A was one or two orders of magnitude higher than that of Regions N and F except for P. aeruginosa, which was consistent with EEM contours. Table 1 lists the determination coefficient of linear regression analysis ($R^2$) and slope between the logarithm of FI integral and the logarithm of bacterial concentration. For each bacterial species, $R^2$ of Region F were all above 0.9, indicating that the FI integral of Region F had a good linear relationship with bacterial concentration. Although tryptophan fluorescence is widely used in the quantification of bacteria, the results from this study suggested that flavin fluorescence could offer a better indication of bacterial concentration. Furthermore, tryptophan fluorescence is not suitable for characterizing bacteria in the case of high organic contents in actual water and wastewater samples since organic matters such as proteins also exhibit fluorescence in Region A, which makes it difficult for distinguishing between organic matters and bacteria. However, the detection limit based on the FI integral of Region F varied among the four bacterial species in this study. For example, the detection limit of B. subtilis was as low as 10 CFU/ml, while E. coli could be detected only if the concentration was above $10^6$ CFU/ml. This is because the content of flavins in bacteria depends on the type and metabolic state of the bacteria.

![Figure 3](http://iwaponline.com/jwrd/article-pdf/doi/10.2166/wrd.2021.040/921464/jwrd2021040.pdf)
and coenzyme fluorescence is not always detectable (Estes et al. 2003). Therefore, although flavin fluorescence was well correlated with bacterial concentration, it is still needed to strengthen its fluorescence response and lower the detection limit.

3.3. Fluorescence detection of bacteria in secondary effluents

To explore the feasibility of applying fluorescence spectroscopy to bacterial detection in actual water samples, the EEM contours of indigenous bacteria in secondary effluent from two WRPs were measured (Figure 4). Similar to pure bacteria, amino acids accounted for most of the bacterial fluorescence and a double-peak fluorescence could also be observed in Region A. Although the concentration of bacteria from Plants A and B was on the same order of magnitude, the FI of bacteria from Plant A was much higher than that from Plant B. This is probably because of the differences in bacterial community structure between the two plants (Supplementary Figure S7). Although the biological treatment process of the two plants is the same (i.e., Anaerobic–Anoxic–Oxic, A2/O), the source of wastewater is different, which results in different dominant bacteria in water. Plant A receives wastewater mainly from the downtown area, while Plant B receives wastewater from the western area of Beijing. Figure 5 shows the relationship between FI integral and bacterial concentration. Table 2 lists the determination coefficient of linear regression analysis ($R^2$) and slope between the logarithm of FI integral and the logarithm of bacterial concentration. As for Plant A, there was a good linear correlation ($R^2 > 0.9$) between the FI integral of three regions and bacterial concentration. However, as for Plant B, the FI integral of Region A had a poor correlation ($R^2 = 0.6597$) with bacterial concentration, and the detection limit based on Region N was above $10^3$ CFU/ml. Flavin fluorescence seemed to correlate well with bacterial concentration in both plants. Therefore, the double-peak tryptophan fluorescence in Region A can be used to characterize the existence of bacteria, while the FI integral of Region F can be used to characterize bacterial concentration in secondary effluent. It is noted that the bacterial community structure is different in different types of water, and thus even with the same bacterial concentration, the FI may vary greatly. The quantification method based on

**Table 1** | Determination coefficient of linear regression analysis ($R^2$) and slope between the logarithm of FI integral and the logarithm of bacterial concentration

| Region | Statistics | B. subtilis | S. aureus | E. coli | P. aeruginosa |
|--------|------------|-------------|-----------|---------|---------------|
| A      | $R^2$      | 0.9579      | 0.9552    | 0.8527  | 0.8642        |
|        | Slope      | 0.43        | 0.38      | 0.35    | 0.36          |
| N      | $R^2$      | 0.8813      | 0.9323    | 0.7175  | 0.9504        |
|        | Slope      | 0.48        | 0.56      | 0.44    | 0.74          |
| F      | $R^2$      | 0.9330      | 0.9654    | 0.9999  | 0.9420        |
|        | Slope      | 0.59        | 0.63      | 1.06    | 0.65          |

**Figure 4** | EEM contours of bacteria in secondary effluent from (a) Plant A ($6.2 \times 10^5$ CFU/ml) and (b) Plant B ($1.6 \times 10^5$ CFU/ml).
FRI can be used for long-term monitoring of a certain type of water, which has an early warning effect to bacterial concentration exceeding standard limits and/or potential microbial risks.

3.4. PCA for differentiation of bacteria

PCA was applied to the emission spectra (EX: 280 nm) of these four bacterial species and indigenous bacteria from two WRPs (Figure 6). As mentioned above, tryptophan fluorescence is the characteristic autofluorescence of bacteria. However, the four bacterial species and bacteria from secondary effluents all exhibited a double-peak tryptophan fluorescence in Region A, which makes it difficult to differentiate them. To explore the possibility of whether fluorescence spectroscopy could distinguish different bacteria, PCA was used to extract feature information from fluorescence spectra. In the PCA diagram, the distance between any two points represents the similarity of their fluorescence spectra. Particularly, PC1 (64.69%) and PC2 (33.20%) explained the major difference among the fluorescence spectra of bacteria. It was observed that the distance between Gram-positive and Gram-negative bacteria was relatively far, indicating that their fluorescence spectra varied greatly.

The points of Plants A and B lay in the points of Gram-positive and Gram-negative bacteria. This is because the components in secondary effluent are complicated and diverse, bacteria from Plants A and B were a mixture of varied Gram-positive and Gram-negative bacteria. It is noted that the point of Plant A was closer to the point of Gram-positive bacteria, while the point of Plant B was closer to the point of Gram-negative bacteria, which was consistent with the above conclusion that the FI of the former was higher than that of the latter. Sohn et al. (2009) also used PCA to analyze the emission spectra (EX: 280 nm) of E. coli, Salmonella and Campylobacter and found that different bacterial species as well as their concentrations could be successfully classified. According to the PCA results from this study, different bacterial species could be distinguished, and the composition of bacterial community structure in secondary effluent from different WRPs could be roughly estimated.

![Figure 5](image_url) Relationship between FI integral and bacterial concentration: (a) Plant A and (b) Plant B.

**Table 2** | Determination coefficient of linear regression analysis ($R^2$) and slope between the logarithm of FI integral and the logarithm of bacterial concentration

| Region | Statistics | Plant A | Plant B |
|--------|------------|---------|---------|
| A      | $R^2$      | 0.9117  | 0.6597  |
|        | Slope      | 0.47    | 0.17    |
| N      | $R^2$      | 0.9366  | 0.9997  |
|        | Slope      | 0.60    | 1.11    |
| F      | $R^2$      | 0.9975  | 0.9355  |
|        | Slope      | 0.79    | 0.50    |
according to the distance between Gram-positive and Gram-negative bacteria. Thus, fluorescence spectroscopy coupled with PCA analysis will be helpful for the rapid determination of microbial water quality fluctuations.

3.5. Discussion

3.5.1. Possible applications

Fluorescence spectroscopy has a good prospect in the rapid detection of bacteria in water, and there are three possible applications. First, real-time online monitoring of bacterial concentration in bacteria-rich water samples (e.g., wastewater and reclaimed water) can be achieved by automatic measurement of fluorescence EEM of water samples after filtration. With the relationship between the FI integral of Region F and bacterial concentration, the quantitative detection of bacteria in water samples can be achieved by automatically calculating the FI integral of water samples. PCA can be further used to determine whether bacterial community structure has changed or not. Traditional culture-based methods are time-consuming and labor-intensive, and accidental hazardous events may have happened before the results are obtained. Therefore, the serious lag of culture-based methods makes them unsuitable for real-time monitoring. Comparatively, the measurement of fluorescence EEM only takes 2–3 min without a high level of expertise compared to PCR and FCM techniques, which also reduces the generation of human errors. Secondly, fluorescence spectroscopy can be used to detect bacterial contamination in relatively clean water (e.g., drinking water). According to the results in this study, once the highly purified water is contaminated with bacteria, substantial changes of fluorescence signals in Region A can be observed. Therefore, in addition to the quantitative monitoring of bacterial concentration, the qualitative detection of bacterial contamination is also feasible. Thirdly, a portable spectrometer based on the detection of fluorescence peaks can be exploited for more field investigation on water quality. In fact, fluorescence spectroscopy can be used for the characterization of both organic matters and microorganisms (Bridgeman et al. 2015). Although the results by portable spectrometer may not be as accurate as fluorescence EEM, portability and faster detection time make it superior when it comes to early warning of water contamination.

3.5.2. Current limitations and future research directions

Although fluorescence spectroscopy has more advantages in the detection of bacteria in water compared to other methods, there are some limitations. For one thing, the content of intrinsic fluorophores, especially for coenzymes in bacteria, is relatively low. Thus, in this study, the detection limit based on the FI integral of Regions N and F varied greatly among different bacterial species. In other words, the FRI method may not be sensitive enough when it comes to low bacterial concentration, which makes it difficult for the quantification of bacteria in drinking water. Although bacteria can be enriched through...
filtration, the additional step may also increase the detection error. Therefore, enhancing the coenzyme fluorescence in bacteria can be further explored for quantitative detection when it comes to low bacterial concentration (Chowdhury et al. 2011). For another, organic matters (e.g., proteins) in water also emit fluorescence, and sometimes the FI is much higher than the bacterial intrinsic autofluorescence, which may cause interference or even masking to bacterial detection (Sherchan et al. 2018). Although fluorescence spectroscopy can be used to quantify bacterial concentration in relatively polluted water, fluorescence signals of organic matters under high DOM concentration could not be ignored. Further work is required to distinguish bacteria from organic matters. For example, the fluorescence data processing can be used to correct the fluorescence internal filtration effect and remove the redundant data to find out the characteristic information of bacteria (Sgroi et al. 2020). In addition, parallel factor analysis (PARAFAC) is also a data dimension reduction method which has been increasingly used for the identification of various fluorophores (Murphy et al. 2013). Fluorescence spectroscopy coupled with FRI, PCA and PARAFAC can be a research area of concern in the field of the rapid detection and quantification of bacteria in water.

4. CONCLUSIONS
In this study, a quantitative method for bacteria by EEM FRI was developed, and bacterial intrinsic fluorophores were divided into three regions, including Region A (amino acids), Region N (NAD(P)H) and Region F (flavins). The autofluorescence of four bacterial species (B. subtilis, S. aureus, E. coli and P. aeruginosa) was identified and mainly concentrated in Region A, implying that the main bacterial intrinsic fluorophores were amino acids. It was found that the FI integral of Region F had a good linear relationship (R^2 > 0.9) with bacterial concentration. In addition, the study demonstrated that PCA could distinguish varied bacterial species and bacteria from different secondary effluents. In conclusion, fluorescence spectroscopy shows great potential in the rapid detection of bacteria in water, and further work is still needed to improve the sensitivity and applicability of the FRI method.

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DATA AVAILABILITY STATEMENT
All relevant data are included in the paper or its Supplementary Information.

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