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# Using Fourier Transform Infrared (FT-IR) Absorbance Spectroscopy and Multivariate Analysis To Study the Effect of Chlorine-Induced Bacterial Injury in Water

HAMZAH M. AL-QADIRI,*,† NIVIN I. AL-ALAMI,§ MURAD A. AL-HOLY,# AND BARBARA A. RASCO⊥

Department of Nutrition and Food Technology, Faculty of Agriculture, and Water and Environment Research and Study Center, The University of Jordan, Amman 11942 Jordan; Department of Clinical Nutrition and Dietetics, Faculty of Allied Health Sciences, Hashemite University, Zarqa, Jordan; and School of Food Science, P.O. Box 646376, Washington State University, Pullman, Washington 99164-6376

The effect of chlorine-induced bacterial injury on spectral features using Fourier transform infrared (FT-IR) absorbance spectroscopy was studied using a mixed bacterial culture of (1:1) ca. 500 CFU/mL each *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 15442 in 0.9% saline. Bacterial cells were treated with 0, 0.3, or 1.0 ppm of initial free chlorine (21 °C, 1 h of contact time). Chlorine-injured and dead bacterial cells retained the ATR spectral properties of uninjured or live cells in the region of C−O−C stretching vibrations of polysaccharides, indicative of the cell wall peptidoglycan layer and lipopolysaccharide outer leaflet. This confirms the observations of others that extensive bacterial membrane damage is not a key factor in the inactivation of bacteria by chlorine. The bactericidal effect of chlorine caused changes in the spectral features of bacterial ester functional groups of lipids, structural proteins, and nucleic acids, with apparent denaturation reflected between 1800 and 1300 $cm^{-1}$ for injured bacterial cells. Three-dimensional principal component analysis (PCA) showed distinct segregation and clustering of chlorine-treated and untreated cells. Cells exposed to chlorine at 0.3 or 1.0 ppm could be distinguished from the untreated control 73 and 80% of the time, respectively, using soft independent modeling of class analogy (SIMCA) analysis. This study suggests that FT-IR spectroscopy may be applicable for detecting the presence of injured and viable but not culturable (VBNC) waterborne pathogens that are underestimated or not discernible using conventional microbial techniques.

**KEYWORDS: FT-IR; spectroscopy; drinking water; chlorination; *E. coli*; *P. aeruginosa***

## INTRODUCTION

The U.S. Environmental Protection Agency (EPA) recommends that the heterotrophic plate count (HPC) in finished drinking water not exceed 500 bacterial colonies per milliliter. However, epidemiological studies indicate that a significant regrowth of heterotrophic bacteria could be the source of gastrointestinal illness (*1*). An estimated 6 billion to 60 billion cases of gastrointestinal illness occur annually due to contaminated drinking water. Free residual chlorine is the most commonly used disinfectant worldwide (*2, 3*), and when it is used as a disinfectant in water piped distribution systems, a level of 0.2−0.5 mg/L free residual chlorine throughout the system is recommended to reduce the risk of microbial regrowth and risk of recontamination (*2*).

Rapid and accurate screening methods for the detection and identification of waterborne pathogens in water remain a great challenge to public health and environmental microbiology. Fourier transform infrared (FT-IR) spectroscopy in the range of 4000−600 $cm^{-1}$ is a potential method for rapid detection and identification of bacterial cells and yeasts (*4*−*7*). Recently, FT-IR was used to detect and identify *Pseudomonas aeruginosa* and *Escherichia coli* as pure and mixed cultures in bottled drinking water (*6*) and to study spectral changes resulting from bacterial sublethal injuries (*8, 9*). Infrared attenuated total reflection (ATR) spectra of microbial cells reflect the biochemical structure and composition of the cellular constituents that include water, fatty acids, proteins, polysaccharides, and nucleic acids for different microbial strains (*6, 7, 10, 11*) and which are unique for specific types of cell injury (*8, 9*).

The main objective of this study was to examine how the spectral features of heterotrophic bacteria changed as a result

* Corresponding author [telephone (+962 6) 5355000, ext. 22422; fax (+962 6) 5355577; e-mail alqadiri@wsu.edu, h.qadiri@ju.edu.jo].
† Department of Nutrition and Food Technology, The University of Jordan.
§ Water and Environment Research and Study Center, The University of Jordan.
# Hashemite University.
⊥ Washington State University.

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**Table 1.** Average Viable Bacterial Count ($n = 6$) and Chlorine Measurement for the Four Water Treatments

| | sample treatment | | | |
|---|---|---|---|---|
| | control[a] | 0 ppm | 0.3 ppm | 1.0 ppm |
| bacterial count[b] (CFU/100 mL) | | | | |
| total count | 0 | $530 \times 10^2$ | 5 | 0 |
| *E. coli* ATCC 25922 | 0 | $310 \times 10^2$ | 3 | 0 |
| *P. aeruginosa* ATCC 15442 | 0 | $210 \times 10^2$ | 2 | 0 |
| chlorine measurement[c] (ppm) | | | | |
| total | | | 0.2 | 0.9 |
| free residual | | | 0.1 | 0.6 |

[a] Noninoculated sample treatment. [b] Samples were enumerated in duplicate using membrane filtration technique after incubation at 37 °C for 48 h. [c] Total and free chlorine were measured after 1 h of contact time.

**Table 2.** SIMCA Classification Results for Each Sample Treatment

| | correctly classified spectra | |
|---|---|---|
| sample treatment | no. | % |
| control | 50 | 83 |
| 0 ppm | 49 | 82 |
| 0.3 ppm | 44 | 73 |
| 1.0 ppm | 48 | 80 |

of chlorine-induced injury and to evaluate the potential of FT-IR spectroscopy to discriminate between chlorine-injured, dead, and intact bacterial cells.

## MATERIALS AND METHODS

**Bacterial Strains and Growth Conditions.** Bacterial strains were obtained from the culture collection in the Department of Food Science and Human Nutrition, Washington State University. *E. coli* ATCC 25922 (a primary indicator of water fecal pollution) and *P. aeruginosa* ATCC 15442 (an indicator of water hygienic quality) were studied. To activate bacteria, cells from both strains were transferred from refrigerated slants to tryptic soy agar (TSA) (Difco, Sparks, MD) followed by incubation of culture plates at 37 °C for 24 h. A well-isolated single colony of each strain was then inoculated into 50 mL of brain heart infusion (BHI) broth (Bacto, Sparks, MD), and bacteria were aerobically at 37 °C for 24 h to reach the stationary phase (count of approximate ca. $1 \times 10^9$ CFU/mL).

After the 24 h incubation in BHI broth, 5 mL broth of each strain was transferred to a 50 mL sterile centrifuge tube. The tubes were then centrifuged at room temperature for 15 min at 5000 rpm (Fisher Scientific, Fisher AccuSpin model 400 benchtop centrifuge, Pittsburgh, PA) to harvest bacterial cells. To eliminate any effect of chlorine demand by media components and bacterial metabolites, the resultant pellets were resuspended in 10 mL of sterile 0.9% saline solution and centrifuged as before (repeated twice). The supernatants were discarded, and the washed pellets were then ready for inoculation into water samples. Serial dilutions of a 1:1 v/v mixed culture were prepared in 0.9% saline.

**Inoculation of Water Samples.** Bottled distilled drinking water (treated by microfiltration) was purchased from a local grocery store the day that the experiment was conducted. Distilled microfiltered drinking water was used because it had not been treated with chlorine and/or did not contain traces of organic matter. The purchased water was filtered through sterile cellulose−nitrate membrane filters (0.45 $\mu$m pore size, 47 mm diameter, Sartorius type filters) to remove bacterial cells that may have been present. After filtration, NaCl was added to obtain a concentration of 0.9% (w/v), which would limit osmotic lysis of bacterial cells during the subsequent chlorine treatment. After this, the saline solutions were autoclaved at 121 °C for 20 min and then cooled at room temperature. Inoculated water samples (1 L) ($n = 3$) were prepared by adding a mixed culture (1:1 v/v), described above, at a concentration of ca. 500 CFU/mL. An uninoculated control was also prepared. Samples were incubated at 37 °C for 48 h.

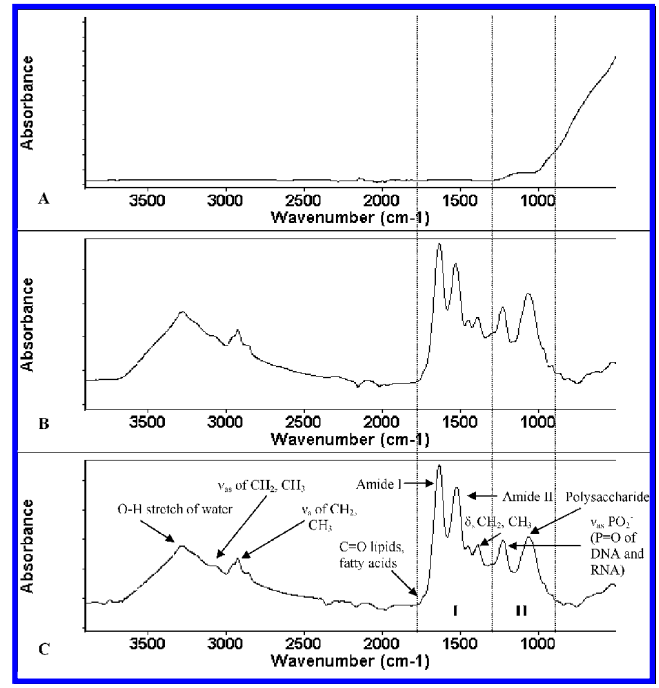


**Figure 1.** Representative ATR spectra (4000−600 $cm^{-1}$) of control (**A**), nonchlorinated (**B**), and chlorinated (1.0 ppm) (**C**) mixed bacterial cultures of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15442. $\nu_{as}$ = asymmetric stretch, $\delta_s$ = symmetric stretch, and $\delta_s$ = symmetric deformation.

**Stock Chlorine Solution and Chlorination of Inoculated Water Samples.** A stock chlorine solution was prepared from reagent grade chemicals (5% sodium hypochlorite, Acros Organics, Geel, Belgium). A freshly prepared free chlorine stock solution [100 mg/L (ppm)] was made using sterile deionized water that had no chlorine demand. After 48 h, the incubation described above, chlorine was added to provide a concentration of 0, 0.3, or 1.0 ppm at 21 °C. The total and free chlorine residuals were determined after 1 h of contact time in the treated water samples (Total and Free Chlorine Micro Checks, HF Scientific, Inc., Fort Myers, FL).

**Recovery of Bacterial Cells and Enumeration.** Before recovery of the bacterial cells following chlorine treatment, the inoculated water samples were treated with freshly prepared sterile 3% sodium thiosulfate solution (1.0 mL/L) to neutralize any residual chlorine. Sodium thiosulfate is considered to be a satisfactory dechlorinating agent for neutralizing residual chlorine and inhibiting bactericidal action during sample analysis (*12*). Following sodium thiosulfate treatment, samples were then placed in a refrigerator for 15 min.

A membrane filtration technique was used to recover bacterial cells (*12*). Under aseptic conditions, 100 mL of the control and chlorine-treated inoculated samples were filtered through a gridded sterile cellulose−nitrate membrane filter (0.45 $\mu$m pore size, 47 mm diameter, Sartorius type filters) under partial vacuum in duplicate. The membrane filters were immediately removed with sterile forceps and placed on the following culture media with rolling motion to avoid air entrapment: TSA for culturing and enumeration of total bacterial count, m-Endo agar LES (Difco) for selective detection and enumeration of *E. coli*, and M-PA-C agar (BBL, Sparks, MD) for selective recovery and enumeration of *P. aeruginosa*. Samples were then incubated at 37 °C for 48 h.

**FT-IR Spectral Measurements.** One liter of each sample treatment ($n = 3$) was filtered through an aluminum oxide membrane filter (0.2 $\mu$m pore size, 25 mm OD) (Anodisc, Whatman Inc., Clifton, NJ) using a Whatman vacuum glass membrane filter holder (Whatman catalog no. 1960-032) to harvest bacterial cells. The anodisc membrane filter does not contribute significantly to the bacterial spectra and has no absorbance in the range of 4000−600 $cm^{-1}$; it also provides a smooth and flat surface onto which a bacterial film can form (*6, 7*). Following filtration and with the filter still in place, the interior surface of the filtration funnel was rinsed with 20 mL of sterile 0.9% saline solution.

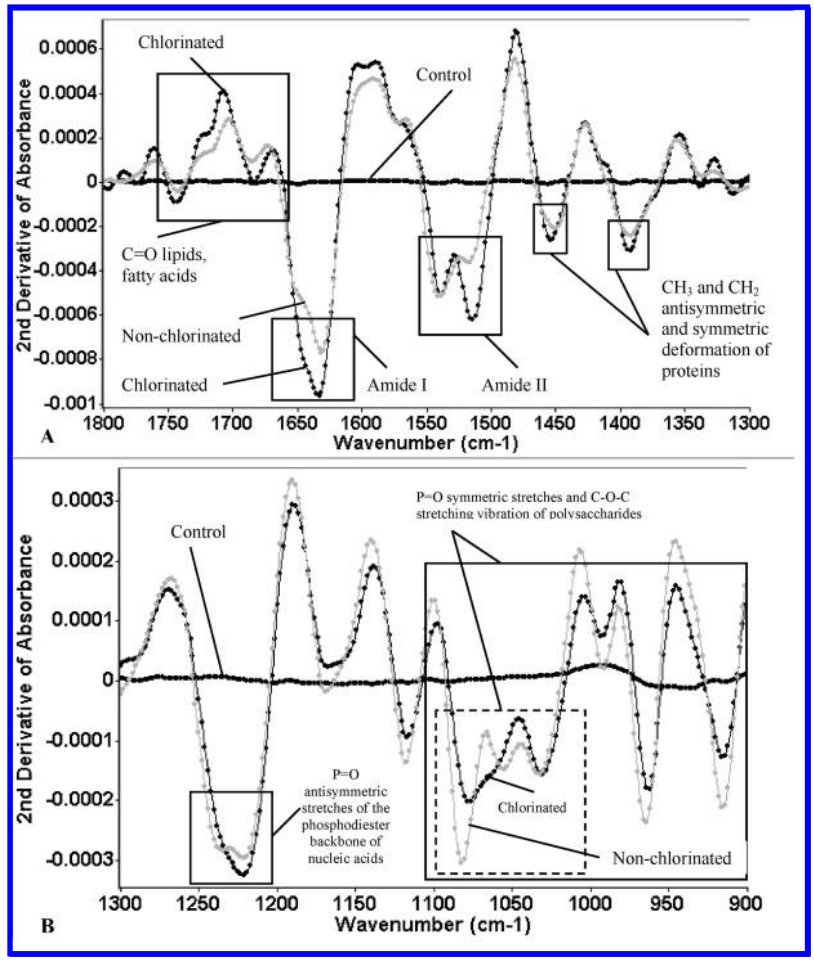


**Figure 2.** Representative second-derivative ATR spectra 1800−1300 $cm^{-1}$ (**A**) and 1300−900 $cm^{-1}$ (**B**) of control (heavy black line), nonchlorinated (gray diamonds), and chlorinated (black diamonds) (1.0 ppm) mixed bacterial cultures of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15442.

The anodisc filters were then removed from the filtration apparatus and air-dried under laminar flow at room temperature for 5 min to allow a homogeneous dried film of bacterial cells to form. This method gives a more homogeneous distribution of cells and more reliable spectral results than recovering cells by centrifugation and then applying them to the membrane, thereby increasing the reproducibility of the spectroscopic method (*6, 7*).

FT-IR bacterial spectra of the recovered bacterial cells were collected using a Thermo Nicolet 380 FT-IR spectrometer (Thermo Electron Corp.). The anodisc membrane filters coated with a layer of bacterial cells were positioned in direct contact with an attenuated total reflection (ATR) diamond crystal (30000−200 $cm^{-1}$) (*5−7, 13*). ATR spectra were recorded from 4000 to 600 $cm^{-1}$ at a resolution of 2 $cm^{-1}$. Twenty spectra were acquired at room temperature for each sample to yield 60 spectra for each treatment. Each spectrum was acquired by adding together 128 interferograms. ATR spectra were automatically baseline corrected and normalized so that the intensity of the amide 1 band was set equal to unity.

**Multivariate Analysis.** OMNIC (Thermo Electron Inc., San Jose, CA) and Delight version 3.2.1 (Textron Systems, Wilmington, MA) software packages were used to analyze the ATR spectra. Data preprocessing algorithms of binning by 4 $cm^{-1}$, boxcar averaging of every second datum, smoothing with a Gaussian function of 15 $cm^{-1}$ full width at half-height, followed by a second-derivative transformation (15 $cm^{-1}$ gap) were employed (*14−16*) along with mean centering.

Spectral data were statistically analyzed by principal component analysis (PCA) and soft independent modeling of class analogy (SIMCA) (*16−18*). PCA provided graphical representations of similarities and differences in spectral data between treatments (*11, 19, 20*) by removing random variation and generating natural clustering within a data set (*5, 20, 21*). The first principal component (PC1) expresses the largest amount of spectral variation followed by the second principal component (PC2), which explains the next largest part of remaining variation, and so on (*18*).

SIMCA classifies samples according to their analogy to a set of training samples (*22*). SIMCA is based upon disjoint modeling of principal components. Samples can be classified on the basis of their similarity to members within a training set (*23*).

## RESULTS AND DISCUSSION

**Table 1** shows the average viable bacterial count (CFU/mL) and chlorine measurements for the treated water samples. At 1.0 ppm of free chlorine, bacterial cells were inactivated and not recoverable. At 0.3 ppm, a small number of cells were able to recover and grow, indicating that 0.3 ppm of free chlorine was not enough to completely inactivate bacterial cells. Therefore, it is essential to maintain sufficient free residual chlorine (>0.5 ppm) in water distribution systems to prevent bacterial cell regrowth and to reduce the risk from postchlorination recontamination.

Representative ATR spectra (4000−600 $cm^{-1}$) of the control (noninoculated treatment), nonchlorinated, and chlorinated treatments of the mixed bacterial culture of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15442 are shown in panels **A**, **B**, and **C**, respectively, of **Figure 1**. Spectral variations were observed between nonchlorinated and chlorinated bacteria. Bacterial cell concentrations of $<10^4$ CFU/mL may not provide an adequate ATR absorbance signal (*6, 24*). Because the water samples here had cell concentrations of ca. 500 CFU/mL, filtration was required to concentrate the cells. However, the absorbance was

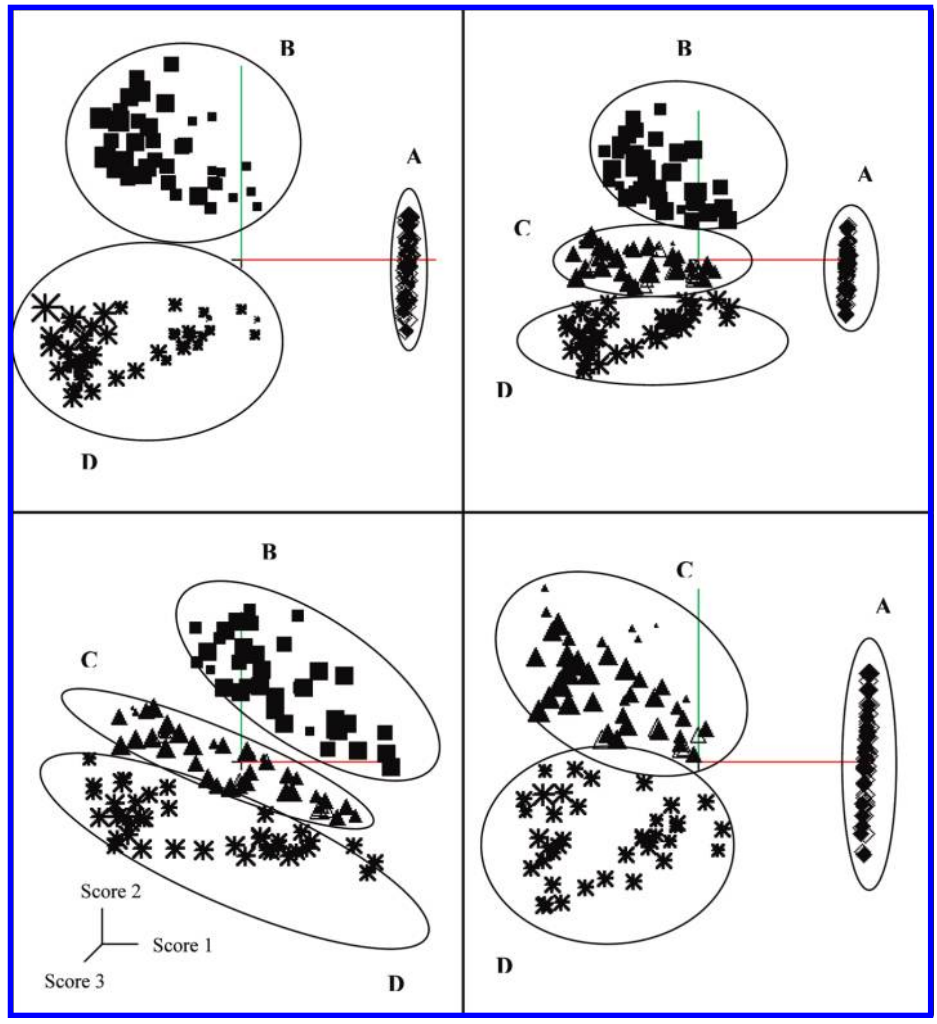


**Figure 3.** Principal component analysis (PCA) combinations of control (**A**), 0 ppm (**B**), 0.3 ppm (**C**), and 1.0 ppm (**D**) sodium hypochlorite treatments for mixed bacterial cultures of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15442.

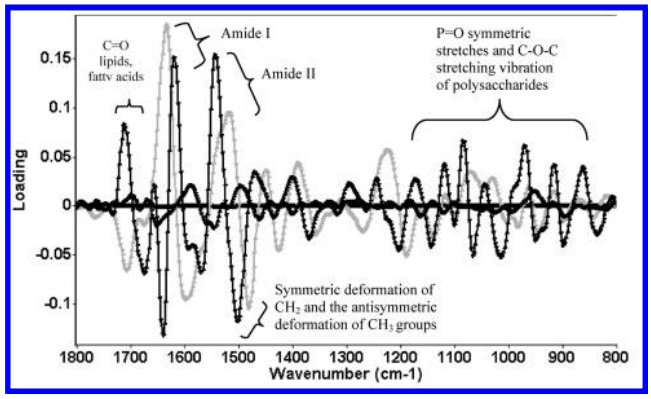


**Figure 4.** Loadings plot of the first (gray diamonds), second (black diamonds), and third (heavy black line) principal components (PCs) obtained from PCA of ATR spectra for chlorine-treated and control treatments.

relatively weak compared to the absorbance of more concentrated bacterial cultures (*6*).

Alteration in the permeability of the bacterial cytoplasmic membrane could be a possible key target in bacterial inactivation by chlorine (*25*). Membrane permeabilization has been observed when bacteria were exposed to chlorine concentrations as high as 50 ppm, which is several times higher than the concentration required for cell inactivation (*26*). This indicates that extensive cytoplasmic membrane damage may not be the most important factor leading to cell death from chlorine treatment (*26*). For chlorine to be an effective bactericide, it must be able to penetrate the cell envelopes and attain a concentration at the target site high enough to exert antimicrobial activity. More subtle effects such as uncoupling of the electron transport chain or enzyme inactivation either in the membrane or in the cell interior are involved in the bactericidal mechanism of chlorine (*25*−*27*). This explains why the ATR spectral features of bacterial cell wall and cell membrane were maintained in the region of C−O−C stretching vibrations of polysaccharides (1200−900 $cm^{-1}$) from the cell wall peptidoglycan layer and lipopolysaccharide outer leaflet (*6, 7, 11, 28, 29*). The bactericidal effect of chlorine on bacterial cells causes changes in spectral features associated with ester functional groups of lipids and fatty acids, proteins, and nucleic acids, in the region between 1800 and 1300 $cm^{-1}$. Differences in the spectra of samples treated at different chlorine concentrations are clearly apparent for the mixed bacterial culture studied here, indicating that both chlorine-injured and dead bacterial cells could be detected using this method.

The major bands between 1800 and 1300 $cm^{-1}$ (denoted region I) include the C═O of ester functional groups of lipids at 1740 $cm^{-1}$. The amide I band at ∼1650 $cm^{-1}$ is assigned to the C═O stretching vibrations of amide groups of proteins (*6, 7, 11, 28, 29*). The amide II band at ∼1540 $cm^{-1}$ represents the amide N−H deformation and C−N stretch, and the bending modes of $CH_3$ and $CH_2$ groups are present in all biomolecules. The band

at ~1455 $cm^{-1}$ is assigned to the symmetric deformation of $CH_2$ and the antisymmetric deformation of $CH_3$ groups. The symmetric deformation of $CH_3$ groups (*6, 7, 11*) may be at ~1398 $cm^{-1}$, but often this band is about 20 ± 5 $cm^{-1}$ lower, so this band assignment is not definitive.

The spectral region between 1300 and 900 $cm^{-1}$ (denotated region II) is characterized by vibrational features of cellular proteins, nucleic acids, cell membrane, and cell wall components (*10*). The main bands in this region include the antisymmetric (~1242 $cm^{-1}$) and symmetric (~1080 $cm^{-1}$) P═O stretching modes of the phosphodiester backbone of nucleic acids and the C−O−C stretching vibrations of polysaccharides (1200−900 $cm^{-1}$) in the cell wall peptidoglycan layer and lipopolysaccharide outer leaflet (*6, 7, 11, 28, 29*).

Due to the relatively minor compositional and structural differences in bacterial cellular constituents, ATR bacterial spectra were preprocessed to amplify the spectral variations (*6, 7, 11, 30*). Binning and smoothing can eliminate high-frequency instrumental noise when normalization balances the differences in path length strength (*31*). Spectra were converted to their second derivative to decrease the variability between replicate spectra from baseline offsets (*11*). Conversion to the second derivative makes it easier to discriminate spectral features (*32, 33*).

The second-derivative ATR spectra of the chlorine-treated mixed culture of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15442 are shown in **Figure 2**. The variations in the spectral patterns (1800−1300 $cm^{-1}$, **Figure 2A**) at different levels of chlorine treatment were distinctive and appear to be associated with ester functional groups of lipids and bacterial proteins in the amide I and amide II regions. Minor differences were observed in the $CH_3$ and $CH_2$ deformations.

Similarly, there are differences between treatments in the region between 1300 and 900 $cm^{-1}$ (**Figure 2B**), indicating nucleic acid denaturation associated with the P═O antisymmetric stretching mode of the phosphodiester backbone of nucleic acids (~1242 $cm^{-1}$) and effects on nucleic acid ribose or deoxyribose structure as observed in the difference between treatments in the P═O symmetric stretching mode (~1080 $cm^{-1}$) (*11, 29*). Chlorine may also affect the structure of bacterial envelope polysaccharides as observed by differences in C−O−C stretching vibrations (~1100−~950 $cm^{-1}$).

A mean-centered PCA was conducted on the second-derivative ATR spectra over the range of 1800−900 $cm^{-1}$ (**Figure 3**). Distinct segregation and clustering between the four sample treatments is apparent. This differentiation most likely reflects compositional and structural impacts of the bacteriocidal effect of chlorine on nucleic acids, proteins, phospholipids, and peptidoglycans.

To predict which spectral regions provide the most significant contributions to data variation, loadings plots were used to highlight the contribution of each variable (wavenumber) to each principal component (*6, 11*). The first, second, and third principal components from PCA over the range of 1800−900 $cm^{-1}$ are shown in **Figure 4**. Large positive or negative loadings are generally associated with spectral regions that are responsible for bacterial differentiation (*31*). Over the range of 1800−900 $cm^{-1}$, principal components 1 (PC1), 2 (PC2), and 3 (PC3) accounted for 85% of the total variability (66, 15, and 4%, respectively). The major contributions to spectral variation were the C═O of ester functional groups of lipids and fatty acids at 1740 $cm^{-1}$, the amide I band at ~1650 $cm^{-1}$, the amide II band at ~1540 $cm^{-1}$, symmetric deformation of $CH_2$ and antisymmetric deformation of $CH_3$ groups of protein components around 1455 $cm^{-1}$, P═O of the phosphodiester backbone of nucleic acids around 1242 and 1080 $cm^{-1}$, and polysaccharide compounds of the bacterial envelopes around 1100− 950 $cm^{-1}$.

**Table 2** shows SIMCA classification for bacterial spectra from each treatment developed using a model constructed from spectra of the other test treatments. Using these models, chlorine treatment levels of 0.3 and 1.0 ppm could be predicted correctly at least 73 and 80% of the time, respectively.

In conclusion, FT-IR spectroscopy may have applications for determining the presence of injured waterborne pathogens that could be underestimated and/or not recoverable by conventional microbial techniques. Furthermore, this method may be useful for validating the effectiveness of chlorine treatments and for predicting the degree of cell injury or death. In future investigations, the sensitivity of FT-IR for the detection of bacterial cells will be studied in addition to examining the effect of other disinfection treatments on waterborne pathogens such as solar and UV irradiation and ozone processing.

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