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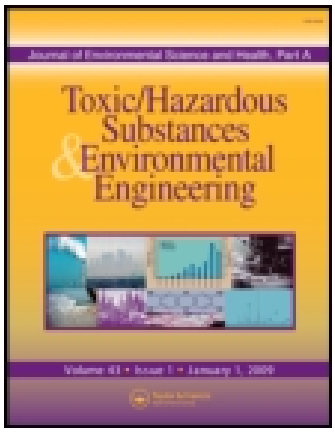

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Biochemical signature assay for use in a biosensor platform to detect bacteria in drinking water biofilms

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Biochemical signature assay for use in a biosensor platform to detect bacteria in drinking water biofilms

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The objective of the study was to identify the enzymatic-biochemical (enz-bio) signatures of *Escherichia coli* and *Salmonella* for rapid detection of these bacteria in drinking water biofilms. The relative potency of lipophilic, glucosidic, and proteolytic activities in biofilms containing single bacterial species and mixture of different bacterial was used to identify the enz-bio signatures of *Escherichia coli* and *Salmonella*. The enz-bio signatures identified were: Lipophilic < Glucosidic < Proteolytic (for *Escherichia coli*); and Glucosidic = Lipophilic < Proteolytic (for *Salmonella*). The enz-bio assays were performed sequentially for detecting *Escherichia coli* and *Salmonella* in pure and mixed biofilm cultures formed on the coupons incubated in a batch reactor. The results obtained were substantiated by culture-based assays indicating comparable data. The enz-bio sensing method described here is a proof of principle and the results of this study provide a platform for the fabrication of a biosensor for bacterial detection in biofilms. The detection time required for the biosensor platform versus culture methods ranged from 10 to 120 min and 24 to 48 h, respectively.

Keywords: Biofilm, Biosensor, Biochemical signature, *Escherichia coli*, *Salmonella.*

Introduction

Biofilms are ubiquitous in drinking water distribution systems with undesirable public health implications. Timely detection and control of pathogenic bacteria in biofilms safeguard the quality of drinking water. Drinking water delivered to end users is normally treated and disinfected before it is distributed via distribution networks. The microbial quality of drinking water at the point of use may be different from the quality of water at the point of entry in a distribution system. The decline or change in microbial quality of water can be attributed to multiple factors, including recovery and regrowth of sublethally injured bacteria, distribution system deficiencies such as cross connections; intrusion of contaminants/pathogens during pressure transients, pipe breaks, and repairs;[1,2] or biofilm formation and shedding of mature biofilms.[3,4]

The oligotrophic environment in water distribution networks provides appropriate conditions for the growth of bacteria, and biofilms are formed as a result of the accumulation of microorganisms and relevant extracellular products on the surfaces of the piping material.[5] In biofilms, the assembled microbial cells are enclosed in a matrix that is primarily composed of polysaccharide materials.[6] In addition to microorganisms, particulate matters such as mineral crystals, corrosion and clay or silt particles may also be found in the biofilm matrix. Mature biofilms are occasionally shed from the surfaces, possibly releasing entrapped microorganisms into flowing waters.

In drinking water distribution systems, the majority of bacteria occurs in biofilms rather than in the water phase.[5] Indicator bacteria as well as microbial pathogens are readily adsorbed to biofilms soon after they enter water distribution systems.[5,7,8] Approximately, 50% of *Escherichia coli* cells injected in a water distribution are adsorbed to biofilms within a few hours.[7] They have been reported to grow at temperatures common in distribution systems and within 2 weeks, their numbers can be higher than predictable numbers based on theoretical calculations.[7] Frequent detection of high numbers of coliforms in the distribution system without a contamination event has been associated with the growth of coliforms in biofilms formed on the surfaces of pipes and fittings.[9,10]

Despite significant improvements in water treatment and disinfection processes, there are still concerns about the microbiological safety of drinking water. A study conducted during the mid-1990s showed that every year in the United States, contaminated drinking water results in 35,000 cases of shigellosis, 59,000 cases of salmonellosis, 150,000 cases of infection with pathogenic *Escherichia coli*, and 320,000

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cases of campylobacteriosis.[11] In Missouri, an outbreak of *Salmonella enterica subsp. enterica* attributed to contaminated water sickened more than 650 persons and resulted in 7 deaths.[12]

To protect and maintain water quality from the source to the tap, it is critically important to consider a rapid method to identify indicator and pathogenic bacteria in biofilms. Conventionally, biofilm bacteria are detected using disruptive methods that include removal of biofilm by scraping pipe surfaces followed by analyses of samples using culture-based assays, lipid analyses, microscopic analyses (complemented with various staining procedures), and molecular techniques such as polymerase chain reaction and microarray. Non-disruptive methods such as *in situ* hybridization have been reported for the detection of microorganisms in biofilms; however, this technique is time consuming and labor intensive.

In a recent article, we have reported an image analysis based on non-destructive method for biofilm detection and quantification.[13] However, there remains a need for a rapid and easy method for the detection and identification of bacterial cells in biofilms. Such a method can facilitate in the detection of and the solution to water quality problems in a distribution network. We present the results of our study for the rapid detection of *Escherichia coli* and *Salmonella* cells in biofilm samples on the coupons.

Materials and methods

Bacterial preparation

Pure cultures of *Escherichia coli* (ATCC 25922) and *Salmonella enterica subsp. enterica* (ATCC 19585) were obtained from American Type Culture Collection (ATCC) and were grown in trypticase soy broth (TSB). Log phase bacterial cultures were prepared by incubating the bacterial suspension at 37°C in a C24 shaker-incubator (New Brunswick Scientific, Edison, NJ) at 150 rpm. The log phase bacterial cultures were centrifuged for 10 minutes at 1000 × *g*. After centrifugation, supernatant was discarded and pellet was washed (twice) with phosphate buffer saline (0.5 × PBS) and suspended in 2.0 mL of PBS. Optical densities were measured for aliquots of 10^{-1} to 10^{-3} serial dilutions using a spectrophotometer (Hach DR/4000U, Loveland, CO) at 600 nm. One mL of washed bacterial stocks (OD_{600} 0.2) was inoculated into each benchtop reactor for biofilm growth.

Glass coupons

In the present study, glass coupons were used as the standard surface for biofilm formation because; (1) it provides an inert surface that does not leach nutrients; (2) nonporous surface facilitates sample collection, thereby minimizing the data bias caused by sample variability due to surface roughness; and (3) it allows easy inference from other studies, as glass surfaces have been the common denominator in the majority of biofilm studies reported in the literature.[14,15] Glass coupons (1.25 cm × 3.75 cm ± 0.1 cm) were cut to fit the inner dimensions of the cuvette. These coupons were cleaned by soaking in 70% isopropanol for 1 h and then air dried. The coupons were rinsed in nanopure grade water, and autoclaved before use.

Formation of pure culture bacterial biofilms in bench-top reactors

Bench top reactors consisting of a plastic container fitted with inlet and outlet ports were used to grow pure culture biofilms. The reactors were thoroughly disinfected by soaking in 70% isopropanol for 12 h and then air dried. Prior to inoculation with pure cultures of selected bacterial isolates, reactors were rinsed three times with autoclaved nanopure grade water. Each reactor containing 325 mL of autoclaved tap water was spiked with 1.0 mL (OD_{600} 0.2) of washed pure bacterial culture. The inlet and outlet ports of the reactors were connected using Tygon tubing, and water was continuously circulated using peristaltic pumps (Cole-Palmer EW-78225, Vernon Hills, IL, USA) set at 6 rpm. Glass coupons mounted on sterilized nylon combs were placed in the reactors and incubated at room temperature for 3 weeks. Coupon samples were removed from the reactors and analyzed weekly for bacterial species in biofilms using enz-bio and culture-based assays.

Control biofilms were grown in reactors operated in parallel to the pure culture reactors. All the parameters for control reactors were the same except that instead of target bacteria they were inoculated with heterotrophic bacteria.

Formation of mixed culture biofilms on glass coupons

Glass coupons were incubated in two model water distribution systems (MDSs) and the biofilms on these coupons represented indigenous bacterial flora of these model distribution systems. Glass coupons were mounted on a plastic comb and incubated in the reservoirs of each MDS for 2 months. The laboratory scale MDSs consisted of two independent pipe loops: the cast-iron model water distribution system (CI-MDS) and the PVC model water distribution system (PVC-MDS) (Fig. 1). Each MDS had a 5.5 m (18-foot) long (5 cm/2-inch in diameter) distribution main (connected to a reservoir) and a 1.2 M (4-foot) long dead end. Distribution mains and dead end lines consisted of six and two removable pipe sections (0.6 M/2 feet in length), respectively.

The total volume of water contained in each MDS was 50 L. The water was continuously circulated in the main distribution line and reservoir using a self-priming, thermally protected magnetic-drive electric pump (Little Giant Pump Company, Oklahoma City, OK, USA). A rotameter and an inline ball valve were installed for controlling the flow at

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Fig. 1. Cast-iron and PVC model distribution systems.

0.3 M (1 foot) per second. The city of Tempe (Arizona) tap water was collected in an open tank to allow chlorine evaporation before the water was allowed to trickle under gravity to each of the reservoirs. The free chlorine level approaching 0 mg/L was achieved by allowing chlorine evaporation in the open tank. A turnover period of 24–72 h was maintained for each MDS. At the time of this study, each MDS was in operation for more than 5 years. The temperature in each MDS was maintained at 22°C–27°C.

Preparation of reagents

Stock solutions (40 mM) of β-Naphthylamine standard (βN) (Sigma-N8381, St. Louis, MO, USA) L-Leucine β-Naphthylamide Aminopeptidase substrate (LLβN; Sigma-L1635) and N-Phenyl-2-Naphthylamine (NPN; Sigma-178055) were prepared in analytical grade ethanol and pH was adjusted to 7.5 using HEPES buffer. Stock solutions (40 mM) of 4-Methylumbelliferone (MUF; Sigma-M1381) and 4-Methylumbelliferyl β-D-glucopyranoside substrate (8.87 mM; MUF-β; Sigma-69591) were prepared by dissolving in 2-Methoxyethanol (Sigma-284467). Stock solutions (4.8 mM) of fluoresceine diacetate (FDA; Sigma-31545) were prepared in analytical grade acetone. Stocks were further divided into 1 mL aliquots and stored at −20°C.

Culture-based assays

The heterotrophic plate counts (HPC) were determined by plating serial decimal dilutions of samples onto R2A agar (Oxoid, Lenexa, KS) and incubating at 28°C for 5 days. Colonies were counted daily for 5 days. *Escherichia coli* and *Salmonella* were analyzed by filtering one mL of the appropriate dilution through a 0.45-μM pore size membrane and plating on mEndo agar and Heckton agar (Hardy Diagnostics, Santa Maria, CA, USA), respectively.

Biochemical assays

Four enzymatic substrates were selected for identifying specific enz-bio signatures for *Escherichia coli* and *Salmonella*. These substrates are metabolized by specific and generic bacterial enzymes. Some of these enzyme and substrates have been reported in established culture/enzymatic methods for the identification of these bacteria in presence of large number of non target bacterial species.

Proteolytic assays

The proteolytic assays were carried out as originally described by Billén[16] with modifications suggested by Laurent and Servais.[17] The reaction was based on L-Leucine aminopeptidase, which hydrolyzes the substrate L-Leucine-β-Naphthylamide (LLβN) and releases the β-Naphthylamine (βN) fluorescent molecule through the following reaction:

$$\text{LL}\beta\text{N} + \text{Bacterial L-Leucine Aminopeptidase} \rightarrow \text{L-Leucine} + \beta\text{N}$$

Glucosidic assays

The glucosidic assays were performed using the method described by Holzapfel-Pschorn et al.[18] The reaction was based on β-D-Glucosidase that hydrolyzes the glucoside (glucopyranoside) substrate 4-Methylumbelliferyl β-D-glucopyranoside (MUF-β) and releases the 4-Methylumbelliferone (MUF) fluorescent molecule through the following reaction:

$$\text{MUF-}\beta + \text{Bacterial } \beta\text{-D-Glucosidase} \rightarrow \beta\text{-D-Glucoside} + \text{MUF}$$

Lipophilic assays

The lipophilic assays were performed as originally described by Martinez De Tejada and Moriyon[19] with the modifications suggested by Freer et al.[20] The reaction was based on lipophilic fluorescent probe N-Phenyl-2-Naphthylamine (NPN), the quantum yield of which increases upon transfer from a hydrophilic aqueous phase

(medium/cytoplasm) to a hydrophobic phase (cell membrane).

Metabolic esterase assays

The esterase assays were carried out as described by Holpzapfel-Pschorn et al.[18] Each reaction constituted 2.96 mL of HEPES buffer (pH 7.5) and 0.04 mL of 4.8 mM Fluorescein Diacetate (FDA). The reaction mixture was thoroughly mixed using a sterile plastic pipette, and the fluorescence intensity was recorded as a blank reading. The glass coupon with bacterial biofilm was aseptically transferred to the reaction cell (cuvette).

The solution in the reaction cell was mixed immediately using the sterile plastic pipette and the initial (time = 0 min) fluorescence intensity was recorded. The coupon was further incubated for a total of 10 min in the substrate solution. Fluorescence intensities were recorded at different time intervals during the incubation period to check for linearity of the fluorescence signals over time. At the end of the incubation period and after a desired linearity was achieved ($R^2 = 0.90$ or higher), the fluorescence signals were subtracted from the blank signal and reported as the final results. The fluorescence signal at the 10 minute assay time point was reported as a representative measurement of bacterial esterase activity.

Glass coupons with biofilms of pure bacterial cultures of *Escherichia coli* and *Salmonella* were analyzed for proteolytic, glucosidic, lipophilic and metabolic esterase activities over a period of 3 weeks. For each set of assays, fluorescence signals were collected at 2, 10, 30, and 420 min reaction for lipophilic, metabolic, proteolytic, and glucosidic assays, respectively. The enzymatic activity was quantified as relative fluorescence unit (RFU), as it provides an unbiased and useful tool to obtain a meaningful and quantifiable signal that can be used in the fabrication of a biosensor. The ratio of RFU was used to identify group-specific bacterial enz-bio signatures.

Instrumentation

Biosensor instrumentation was assembled in house. The optical and spectrometer components for the biosensor were obtained from Ocean Optics (Model # HR 2000, Ocean Optics, Dunedin, FL, USA). The xenon light source provides filtered excitation light at specific wavelengths to allow single excitation-, single emission detection of a specific fluorophore in each enz-bio assay. The excitation light spectrum appeared to have a ± 10 nm range around the peak maxima. This allowed all fluorescence assays to be carried out at a single excitation wavelength (350 ± 10 nm). The fluorescence signals (RFU) can be collected at single excitation, dual emission wavelength (SEDEW) settings.

Data analyses

For each data point three coupons were removed from reactor or model distribution system and analyzed in parallel for each enz-bio assay. The data were analyzed using Excel (Microsoft Corp, Redmond, WA, USA) for correlation between different parameters.

Results

Identification of enz-bio signatures for Escherichia coli *and* Salmonella *in pure culture biofilms*

The results of the experiment performed to identify patterns of enz-bio activities of *Escherichia coli* and *Salmonella* in pure culture biofilms are presented in Figures 2a and 2b. An incremental increase in enz-bio activities (measured as fluorescence signals) was observed with an increase in the number of bacterial cells and biofilm age (Figs. 2a and 2b). In the *Escherichia coli* biofilms, a 3- to 5-fold increase in enz-bio activities was noted in 3-week-old biofilms. During the same time period, *Escherichia coli* numbers in biofilms were also monitored. The bacterial counts doubled in the second week and at least quadrupled between the second and third week (Fig. 2a). The increase in enz-bio activities could be positively correlated with the increase in the bacterial numbers.

In the *Salmonella* biofilms, proteolytic and metabolic activities doubled by the end of the third week, whereas a 1.2-fold increase in glucosidic activity was observed during the same time period. Lipophilic activity slightly increased during the first week and no further increase in activity was observed between the second and the third week (Fig. 2b). In addition to enz-bio activities, biofilm samples were also assayed for bacterial numbers, revealing a 9-fold increase in the *Salmonella* concentration in biofilms during the same time frame.

Identification of enz-bio signatures for Escherichia *and* Salmonella *in mixed culture biofilms*

Results of a 3-week study of mixed culture biofilms (*Escherichia coli* and *Salmonella*) are presented in Figure 2c. Proteolytic, glucosidic, metabolic, and lipophilic activities in the biofilms increased over time. By the third week, most of the activity in the mixed culture *Escherichia coli*/*Salmonella* biofilms appeared to be contributed by *Escherichia coli*. Therefore, it is likely that this was due to a gradual inhibition of *Salmonella* activity caused by *Escherichia coli* dominating the biofilm development. At the microscale level this phenomenon could occur in other natural oligotrophic water environments where one predominant bacterial species competes with minor bacterial populations for survival in biofilms.

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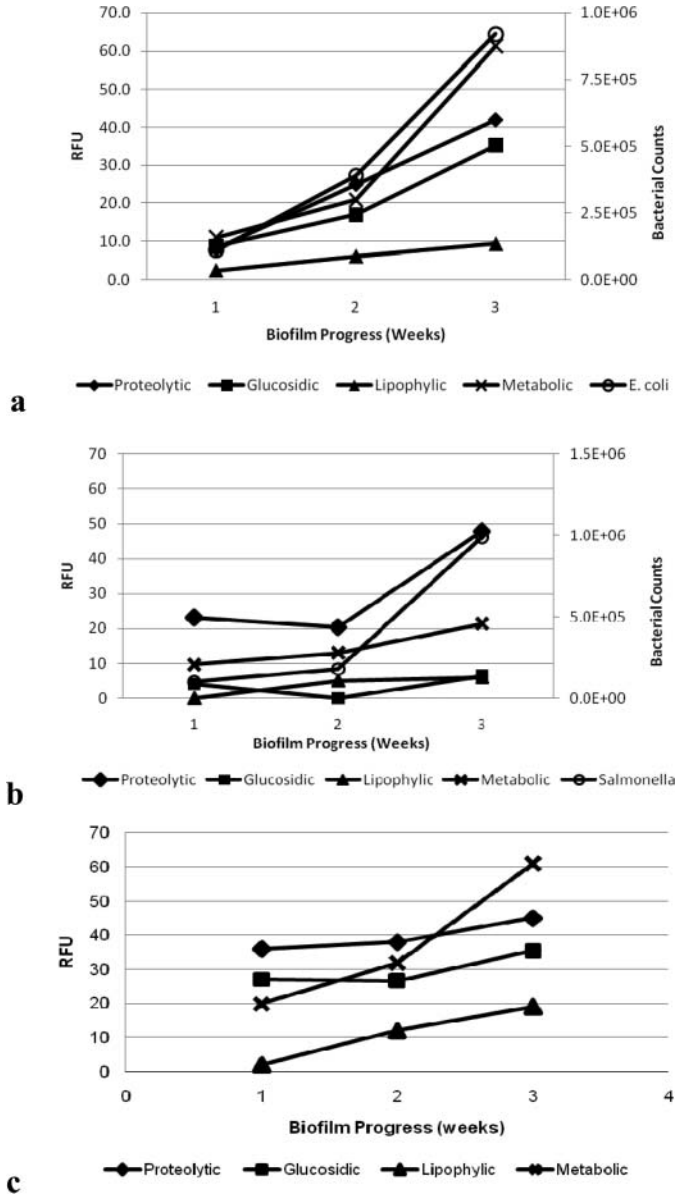

Fig. 2. The enz-bio signature of bacterial species in pure culture biofilms of: a) *Escherichia coli;* b) *Salmonella;* c) *Escherichia coli*/*Salmonella*. For control samples no clear trend in the relative progression of enz-bio signatures was noted and data is not plotted here. (All the data points are the average of three replicates. For lipophilic assays, metabolic assays, proteolytic assays, and glucosidic assays, all data represents readings at 55 sec, 10 min, 30 min and 7 h, respectively.)

Application of the enz-bio signatures for the detection and identification of mixed culture biofilms in water distribution systems

The applicability of bacterial enz-bio signatures for the identification of bacteria in water distribution systems was validated in this study. The comparative results of enz-bio signatures assays and cultural assays are presented in Figures 2a and b.

After 2-month incubation, no *Escherichia coli* was detected on the coupons placed in both MDS using culture-based and enz-bio signature assays. The concentrations of HPC bacteria were 6.4×10^4 and 5.8×10^2 cells/cm^2 in the PVC-MDS and the CI-MDS, respectively (Table 1).

The proteolytic assays showed that the rate of hydrolysis of Naphthylamine in mixed culture biofilms in the PVC-MDS and the CI-MDS was 19.9 nM/min and 0.41 nM/min, respectively (Fig. 3a). Based on the glucosidic assays the rate of Methylumbelliferone (MUF) hydrolysis was calculated to be 0.086 nM/min and 0.019 nM/min in biofilms in PVC-MDS and CI-MDS, respectively (Fig. 3b). The rate of proteolytic hydrolysis in biofilms was 49-fold higher in PVC-MDS than CI-MDS. A similar trend was observed for glucosidic (5-fold higher in PVC-MDS than CI-MDS), lipophilic (7-fold higher in PVC-MDS than CI-MDS), and overall metabolic activity (2-fold higher in PVC-MDS than CI-MDS) (Figs. 3b, 3c, and 3d). The enz-bio signature for lipophilic (55 sec), metabolic (10 min), proteolytic (30 min), and glucosidic (7 h) assays (at specific time points) are summarized in Table 2. The enz-bio signatures (Lipophilic < Glucosidic < Proteolytic) detected in both MDSs were similar (Table 2).

Discussion

Pure culture microbial studies are critical to identify basic phenomenon. The study of the temporal variations in the enz-bio signatures of *Escherichia coli* and *Salmonella* in pure culture biofilms over a 3-week time period revealed that in biofilms, different bacterial species manifest specific patterns of enz-bio activity. The pattern of enz-bio activity of a bacterial species is represented by the relative potency of its lipophilic, glucosidic, and proteolytic activities. This set of enz-bio activity tests can be performed sequentially with a sensitivity of 20 cfu (data not shown). On the microscale, nascent biofilms are mostly discrete patches of predominantly single bacterial types (data not shown). For instance, if a small biofilm sample (discrete patch) representing a predominant single bacterial species is analyzed, the enz-bio signature from such an assay will reflect the prevalent bacterial species in that patch of biofilm.

Under mixed culture conditions, the gradual exclusion phenomenon has been reported in a biofilm containing more than two bacterial species.[21] The rapid exclusion phenomenon has been reported in biofilms on the second day of development where only a single bacterial species was detected.[21] Under repeated chlorination (a condition representing a municipal water distribution system) after a period of 25 days, biofilm samples contained 90% of one bacterial species; and only 10% of another species survived in the mixed culture biofilms.[21]

In naturally occurring biofilms, it is expected that enzymatic activities would greatly depend on the ability of a mixed bacterial community to adapt and survive in such

Table 1. Concentration of bacteria in biofilm on coupons placed in PVC and cast iron water distribution systems.

	HPC		*Coliforms*	
Biofilm Age (8 week)	*PVC (CFU/cm²)*	*CI (CFU/cm²)*	*PVC (CFU/cm²)*	*CI (CFU/cm²)*
Bacterial counts	6.4E + 04	5.8E + 02	0	0
pH	7.2	8.0	7.2	8.0

environments. Verrhiest et al.[22] observed three successive steps and variations in glucosidic and proteolytic enzymatic activities of mixed species bacteria in natural sediments during a 5-week study. In the first step, initial adaptation of the bacterial population along with lower enzymatic activities was noted. In the second step, stabilization or increase in enzymatic activities of the adapted bacterial communities was recorded. Finally, limitation of nutrients and substrates was noted in the third step, resulting in reduced enzymatic activities.[22]

We have identified that 3-week-old bacterial biofilms in drinking water distribution systems have stable bacterial population patches (predominantly belonging to a single bacterial type). Other researchers have reported similar findings after 4 weeks.[21] In the present study the enz-bio signature recorded in 3-week-old biofilms was able to accurately identify the bacterial species in biofilm sample (Table 3) and the results were confirmed by culture-based analyses.

Even though the same coupon material was used in both systems, the number of bacteria in the PVC-MDS was almost 100-fold higher than the CI-MDS, suggesting the impact of pipe materials in biofilm formation in water distribution systems. Bacteria have been reported to accumulate in tubercles resulting from corrosion of CI pipes or in imperfections in the pipe material.[23] Pipe materials also influence the physicochemical quality of the water. The pH in the CI-MDS was more alkaline than that in the PVC-MDS (Table 3). Pipe linings are known for leaching of nutrients and potentially harmful compounds, as well as causing variations in the pH of the water, all of which may affect bacterial growth in biofilms.[23]

The results of culture analyses of the present study are concurrent with the previous studies.[24] For example, in a study of a non-chlorinated water distribution system, the entire surface of PVC pipe was covered by biofilms with bacterial cell densities of 5×10^6 cells/cm^2.[24] Another study reported growth of bacteria on steel and PVC coupons placed in a drinking water biofilm reactor.[25] Despite 0.1 mg/L of free chlorine being maintained in the water, a bacterial population of 4.9×10^6 cells/cm^2 was present after 167 days.

In the CI-MDS, the intensity of enz-bio signal was lower than in the PVC-MDS, which is consistent with the concentration of bacteria in both MDS. The physicochemical properties of water in both systems may also impact the results of these assays. In the CI-MDS, these interferences may originate from the corrosion products that can inhibit enzyme activities by masking catalytically active groups, denaturing protein conformations, or competing with metal ions involved in the enzyme-substrate complexes.[26]

Furthermore, in the CI-MDS, the pH of water was alkaline, which may induce metal precipitation and immobilization to surfaces. A slight alkalinization can affect enzyme-catalyzed reactions by altering ionization and the solubility of enzymes, substrates, and cofactors; by affecting the stability of active protein structures; or by reducing the solubility of metals acting as enzyme inhibitors.[26] If bacterial activities were inhibited due to heavy metals in the CI-MDS, this effect was probably enhanced by the proliferation of metal deposits on surfaces where bacterial cells were adsorbed and masked from participation in all enz-bio reactions. However, bacterial-specific enz-bio signatures were not impacted by the variations in the physicochemical properties of water in both MDSs, which indicates that the assays reported here have broader application in water distribution systems with diverse characteristics.

This proof of principle study shows that using enz-bio signature assay can be applied to detect bacterial species in drinking water biofilm within 10–120 min, while 24–48 h are needed to achieve comparable results using culture methods. The methodology described provides bases to rapidly and reliably detect selected bacterial species by analyzing their specific signals using a custom designed spectrophotometer.

Table 2. Enz-bio signatures of bacterial biofilms in water distribution systems.

	Intensity of different signals (RFU)				
Pipe loop system	*Metabolic*	*Glucosidic*	*Lipophilic*	*Proteolytic*	*Signature*
PVC	80	45	35	30	Prot < Lipo < Gluco
Cast Iron	30	9	7	2	Prot < Lipo < Gluco

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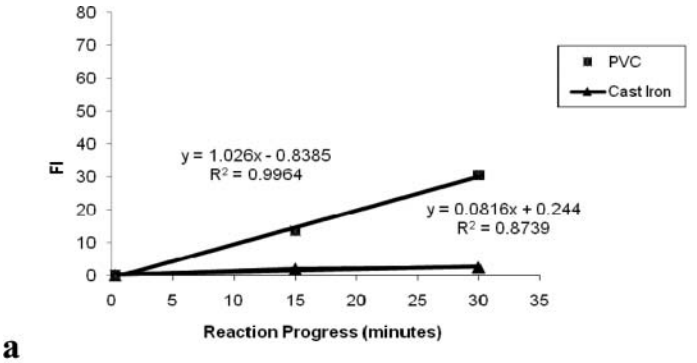

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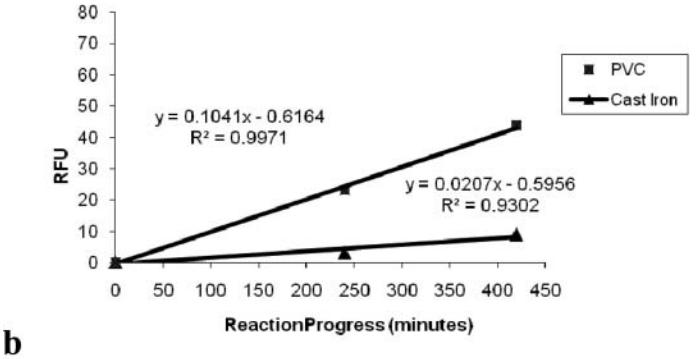

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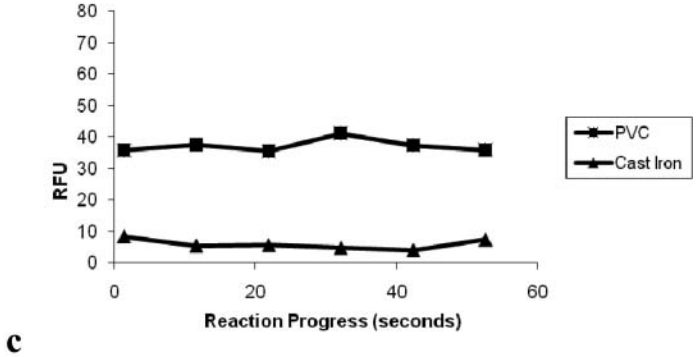

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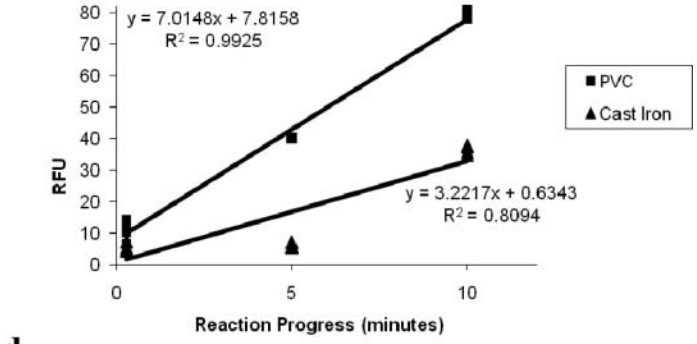

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Fig. 3. Enz-bio signature in 2 month old mixed culture biofilms grown in PVC and Cast Iron water distribution systems: a) proteolytic, b) glucosidic, c) lipophilic, d) esterase (total metabolic activity). (All the data points are average of three replicates. For lipophilic, esterase, proteolytic and glucosidic activities the data collected at 55 sec, 10 min, 30, min and 7 h, respectively, are used for generating biochemical signature specific to bacterial species).

Table 3. The enz-bio signatures of bacterial biofilms identified in bench top reactors.

Biofilm type	*Biochemical signature*	*Comments*
Escherichia coli	Lipophilic < Glucosidic < Proteolytic	
Salmonella	Glucosidic = Lipophilic < Proteolytic	
Mixed Culture	Lipophilic < Glucosidic < Proteolytic	*Identified as *Escherichia coli*

*Results confirmed by culture-based method.

Acknowledgments

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References

[1] Karim, M.; Abbaszadegan, M.; LeChevallier, M. Potential for pathogen intrusion during pressure transients. J. Am. Water Works Assoc. **2003**, *95*, 134–146.

[2] Craun, G.F.; Calderon, R.L. Waterborne disease outbreaks caused by distribution system deficiencies. J. Am. Water Works Assoc. **2001**, *93*, 64–75.

[3] Camper, A.K.; Hayes, J.T.; Jones, W.L.; Zelver, N. Persistence of coliforms in mixed population biofilms. In *Proceedings of the Water Quality Technology Conference*, Miami, FL, November 7–11, 1993; American Water Works Association: Denver, CO.

[4] Lee, D.G.; Kim, S.J. Bacterial species in biofilm cultivated from the end of the Seoul water distribution system. J. Appl. Microbiol. **2003**, *95*, 317–324.

[5] Szewzyk, U.; Szewzyk, R.; Manz, W.; Schleifer, K.H. Microbiological safety of drinking water. Ann. Rev. Microbiol. **2000**, *54*, 81–127.

[6] Donlan, R.M. Biofilms: Microbial Life on Surfaces. Emerging Infectious Diseases. 2002, (CDC) *8*. Available at http://www.cdc.gov/ncidod/EID/vol8no9/02-0063.htm (accessed Nov 2012).

[7] Fass, S.; Dincher, M.L.; Reasoner, D.J.; Gatel, D.; Block, J.C. Fate of *Escherichia coli* experimentally injected in a drinking water distribution pilot system. Water Res. **1996**, *30*, 2215–2222.

[8] Block, J.C.; Dutang, M.; Mailliard, J.; Reasoner, D.J. Growth of attached bacteria in water distribution systems. **1993**, 19th International Water Supply Congress SS1-8-1-12.

[9] LeChevallier, M.W.; Babcock, T.W.; Lee, R.G. Examination and characterization of distribution system biofilms. Appl. Environ. Microbiol. **1987**, *53*, 2714–2724.

[10] van der Wende, E.; Characklis, W.G.; Smith, D.B. Biofilms and bacterial drinking water quality. Water Res. **1989**, *23*, 1313–1322.

[11] Morris, R.D.; Levin, R. Estimating the incidence of waterborne infectious disease related to drinking water in the United States. In *Assessing and Managing Health Risks from Drinking Water Contamination: Approaches and Applications*; Reichard, E.G.; Zapponi, G.A., Eds.; International Association of Hydrological Sciences (IAHS), Publication#233: Wallingford, UK, 1995; 75–88.

[12] Angulo, F.J.; Tippen, S.; Sharp, D.J.; Payne, B.J.; Collier, C.; Hill, J.E.; Barrett, T.J.; Clark, R.M.; Geldreich, E.E.; Donnell, H.D.; Swerdlow, D.L. A community waterborne outbreak of salmonellosis and the effectiveness of a boil water order. Am. J. Publ. Heal **1997**, *87*, 580–584.

[13] Alum, A.; Mobasher, B.; Rashid, A.; Abbaszadegan, M. Image analyses-based non-disruptive method to quantify algal growth on concrete surfaces. Environ. Eng. **2009**, *135*, 185–190.
[14] van der Kooij, D.; Veenendaal, H.R.; Baars-Lorist, C.; Van der Klift, D.W.; Drost, Y.C. Biofilm formation on surfaces of glass and Teflon exposed to treated water. Water Res. **1995**, *29*, 1655–1662.
[15] Delanoue, A.; Holt, D.M.; Anderson, H.A.; McMath, S.M.; Smith, S.E.; Woodward, C.A.; Fraser, A.R.; Roe, M. Analysis of material (biofilm) present on the internal surfaces of a drinking water distribution systems. 1997, April 13–16, Proceedings of the Royal Society Chemical/CIWEM Conference on Aquatic Biofilms. University of Warwick, Coventry, UK.
[16] Billén, G. Protein degradation in aquatic environments. In *Microbial Enzymes in Aquatic Environments*; Chróst, R.J., Ed.; Springer-Verlag: New York, 1991; 123–143.
[17] Laurent, P.; Servais, P. Fixed bacterial biomass estimated by potential exoproteolytic activity. Can. J. Microbiol. **1995**, *41*, 749–752.
[18] Holpzapfel-Pschorn, A.; Obst, U.; Harberer, L. Sensitive methods for the determination of microbial activities in water samples using fluorogenic substrates. Fresen. J. Anal. Chem. **1987**, *327*, 521–523.
[19] Martinez De Tejada, G.; Moriyon, I. The outer membranes of *Brucella* spp. are not barriers to hydrophobic permeants. J. Bacteriol. **1993**, *175*, 5273–5275.
[20] Freer, E.; Moreno, E.; Moriyon, I.; Pizarro-Cerda, J.; Weitraub, A.; Gorvel, J.P. *Brucella-Salmonella* lippolysaccharide chimeras are less permeable to hydrophobic probes and more sensitive to cationic peptides and EDTA than are their native *Brucella* sp. counterparts. J. Bacteriol. **1996**, *178*, 5867–5876.
[21] Leriche, V.; Briandet, R.; Carpentier, B. Ecology of mixed biofilms subjected daily to a chlorinated alkaline solution: spatial distribution of bacterial species suggests a protective effect of one species to another. Environ Microbiol. **2003**, *5*, 64–71.
[22] Verrhiest, G.J.; Cortes, S.; Clement, B.; Montuelle, B. Chemical and bacterial changes during laboratory conditioning of formulated natural sediments. Chemosphere **2002**, *46*, 961–974.
[23] Conroy, P.; Fielding, M.; Wilson, I. Investigating the effect of pipelining materials on water quality. Water Supp. **1993**, *11*, 343–354.
[24] Characklis, W.G.; Goodman, D.; Hunt, W.A.; McFeters, G.A. *Bacterial regrowth in distribution systems*. American Water Works Association Research Foundation: Denver, CO, 1988.
[25] Pederson, K. Biofilm development on stainless steel and PVC surfaces in drinking water. Water Res. **1990**, *24*, 239–243.
[26] Gianfreda, L.; Bollac, J.M. Influence of natural and anthropogenic factors on enzyme activity in soil. In *Soil Biochemistry*; Stotzky, S.J., Bollac, J.M., Eds.; Marcel Dekker: New York, 1996; 123–193.

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