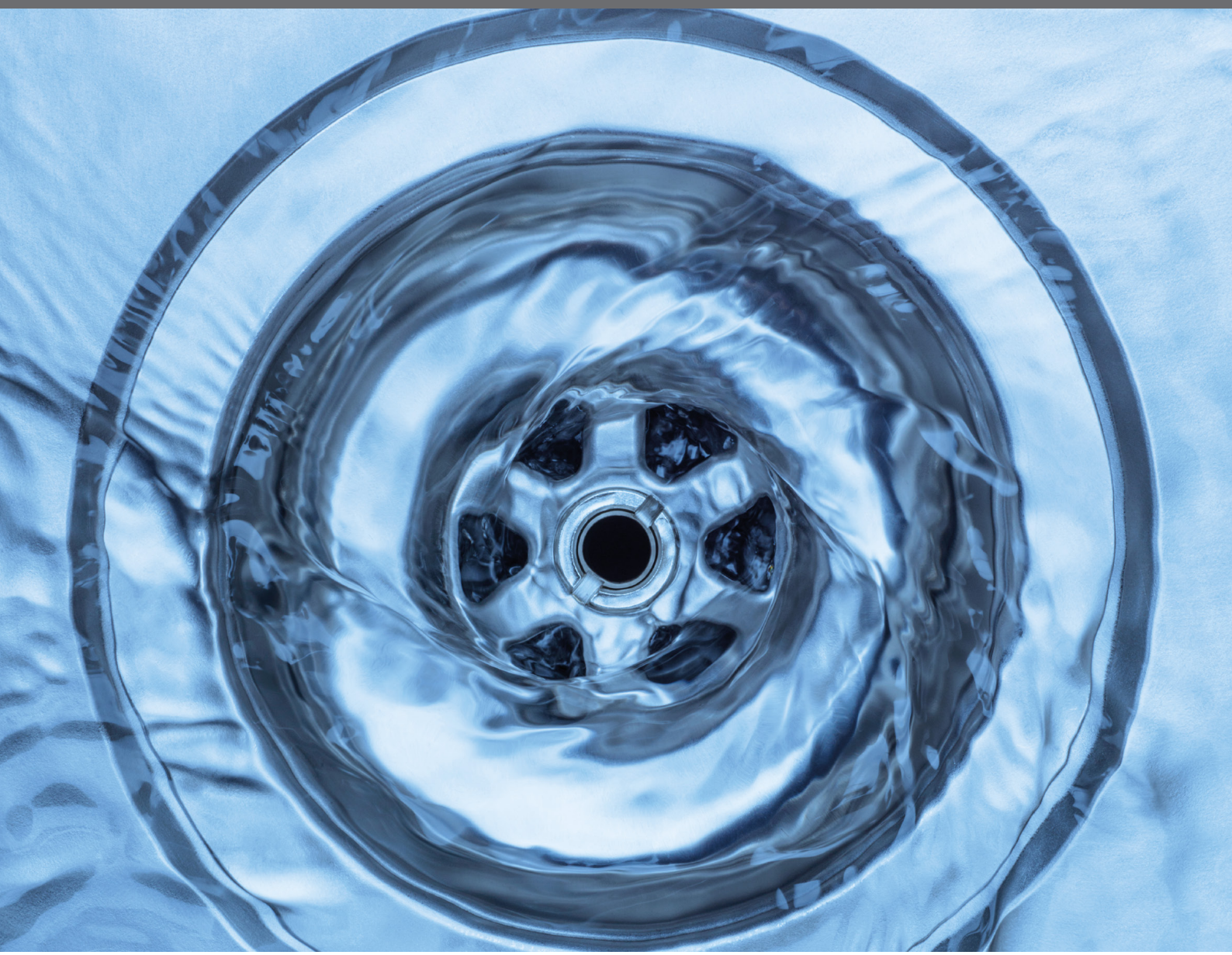
MICROBIAL SAFETY IN WATER RESOURCES

EDITED BY: Pei-Ying Hong, Timothy R. Julian and Muhammad Raihan Jumat
PUBLISHED IN: Frontiers in Microbiology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-756-4
DOI 10.3389/978-2-88945-756-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

MICROBIAL SAFETY IN WATER RESOURCES

Topic Editors:
Pei-Ying Hong, King Abdullah University of Science and Technology, Saudi Arabia
Timothy R. Julian, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Switzerland
Muhammad Raihan Jumat, King Abdullah University of Science and Technology, Saudi Arabia

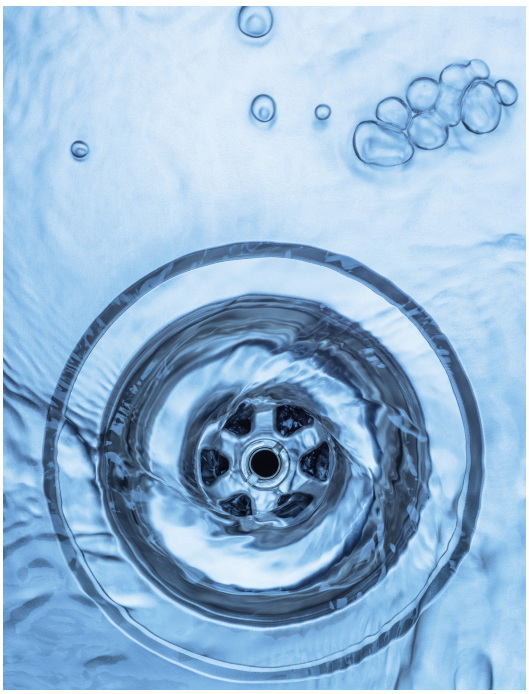


Image: rkafoto/Shutterstock.com

As more countries become water-scarce, alternative water sources like treated wastewaters will be used to meet the demands of the domestic, agriculture and industrial sectors. However, the use of treated wastewater is only justified when it is without any detrimental impacts on public health, food safety and water quality. To minimize impacts, well-operated treatment plants are important barriers that reduce the amount of contaminants disseminated from wastewaters into the environment during reuse events. Continuous, accurate and comprehensive monitoring on our water further safeguards the public against potential risks. This eBook looks into topics that close the knowledge gaps in these mentioned areas.

Citation: Hong, P.-Y., Julian, T. R., Jumat, M. R., eds. (2019). Microbial Safety in Water Resources. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-756-4

Table of Contents

frontiers in Microbiology

EDITORIAL
published: 10 December 2018
doi: 10.3389/fmicb.2018.03064

Editorial: Microbial Safety in Water Resources

Pei-Ying Hong[1]*, *Timothy R. Julian*[2,3,4] *and Muhammad Raihan Jumat*[1]

[1] Water Desalination and Reuse Center, Biological and Environmental Science & Engineering Division, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia, [2] Eawag, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland, [3] Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland, [4] University of Basel, Basel, Switzerland

Keywords: wastewater treatment, water reuse, water quality, molecular methods, microbial contaminants

Editorial on the Research Topic

Microbial Safety in Water Resources

The scientific community can help to advance wastewater reuse in two important ways: First, through the exploration of new treatments and technologies that allow use of safe water supply alternatives, and second, through development and use of new methods that improve insights on water quality. Examples of these two contributions are provided here in the Frontiers Research Topic *Microbial Safety in Water Resources*.

Wastewater treatment technologies serve as important engineering barriers to remove majority of the contaminants from wastewater, hence achieving safe water reuse or disposal to the natural environment. The conventional wastewater treatment process include clarifiers or sedimentation tanks, activated sludge processes, and disinfection. In recent years, membranes (e.g., microfiltration) are also retrofitted into activated sludge tanks to form aerobic membrane bioreactors, which in turn improve solid-liquid separation and hence achieve high effluent quality.

In most instances, the above described wastewater treatment processes are operated as centralized facilities. However, decentralized facilities are gaining in favor due to decreased capital costs, reduced reliance on sewage infrastructure, and potential for resource recovery at a local-scale. Depending on the treatment process, a well-operated decentralized process can perform as well as that of centralized treatment. To exemplify, Nguyen et al. evaluated on-site treatment of wastewater using granular activated carbon, chlorination and electrolysis, demonstrating 5-log inactivation of *Escherichia coli*. The study also demonstrates how modular technologies should be explored to tackle emerging contaminants present in untreated wastewaters. If a wastewater treatment process is designed with modularity in mind, the process can adapt dynamically to meet the current needs. This concept of modularity is also reviewed by Barancheshme and Munir in a discussion of treatment options to combat antimicrobial resistance threats arising from wastewaters.

Clearly, a well-operated treatment plant remains an important barrier to reduce contaminant dissemination from wastewaters into the environment. However, recent studies showed that some bacterial strains developed strategies to survive treatment and environmental stressors (Al-Jassim et al., 2017; Mantilla-Calderon and Hong, 2017; Jumat et al., 2018). Trigui et al. discussed this concept by examining differences in viability rates between two *Campylobacter jejuni* strains. The *C. jejuni* strain isolated from oligotrophic water was able to survive better in a freshwater medium than the other strain, potentially due to the observed higher resistance to oxidative stress and bile

OPEN ACCESS

Edited by:
Qiang Wang,
Chinese Academy of Sciences, China

Reviewed by:
Abid Ali Khan,
Jamia Millia Islamia, India

Correspondence:
Pei-Ying Hong
peiying.hong@kaust.edu.sa

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *22 August 2018*
Accepted: *28 November 2018*
Published: *10 December 2018*

Citation:
Hong P-Y, Julian TR and Jumat MR (2018) Editorial: Microbial Safety in Water Resources. Front. Microbiol. 9:3064. doi: 10.3389/fmicb.2018.03064

salts. Interestingly, genes involved in resisting against oxidative stress and bile salts were induced, hence conferring a protective effect.

The results from Trigui et al. and many other studies demonstrate that understanding the microbial ecosystems and the microbial behaviors will improve effective mitigation measures downstream of wastewater treatment. Such measures include storing treated wastewaters in an evaporation pond and exposing the waters to natural sunlight. Sunlight achieves antimicrobial effect via both direct DNA damage and radical oxidative species (ROS)-mediated damage. Depending on the depth of the pond, solar exposure can occur either under oxic or anoxic conditions, resulting in differences in decay rates and gene expression for the same bacterium. This concept is explored by McClary and Boehm, who used *Staphylococcus aureus* to demonstrate a different response toward oxygen-dependent and oxygen-independent photostress.

Treatment plants can also utilize the more conventional chlorine or other disinfectants (e.g., chlorine dioxide, UV radiation, and heat lysis) to further inactivate remnant microbial contaminants. Different disinfection strategies inactivate microbial contaminants via different mechanisms. Zhong et al. explained that chlorine and chlorine dioxide inactivate Echovirus 11 by inhibiting the binding interactions between viruses and host cells. Echovirus strains that are resistant to chlorine exhibit cross resistance to chlorine dioxide but were susceptible to UV, sunlight and heat treatment due to the differences in disinfection strategy. Their findings suggest a need to complement different disinfection strategies for improved viral removal.

The scientific community can also help advance wastewater reuse through the development and use of novel methods for water quality monitoring. Novel methods are needed to gain insight into the presence, quantity, and dynamics of new and emerging pollutants, better characterize microbial populations including pathogen ecology, and improve specificity and sensitivity of existing, primarily culture-based, tools. For example, water quality monitoring often requires monitoring for culturable bacteria, such as *E. coli*, fecal coliforms, total coliforms, and/or heterotrophs. Culture-based methods suffer numerous limitations, including limited or no correlation with the presence of pathogens, and susceptibility to false-positives. Recent work has highlighted that US-EPA approved media for detection of fecal coliforms and *E. coli* can be particularly prone to false-positives (Olstadt et al., 2007; Zhang et al., 2015). This can generate unnecessary alarm and operational costs for water utilities.

Four of the papers published in this Research Topic demonstrate and/or implement new water quality monitoring technologies to gain new understanding on water quality. Specifically, online flow cytometry, 16S rRNA gene-based amplicon sequencing, genome characterization, and metagenomics. Besmer et al. demonstrated the use of online flow cytometry with an optimized monitoring strategy to detect precipitation-induced microbial peak loads in karstic spring waters. This method could potentially be useful when applied to climate-change induced precipitation that may differ from predictable rainfall patterns on a local scale. Similarly, Uprety et al. utilized 16S rRNA gene-based amplicon sequencing to monitor microbial dynamics in groundwater before and after an earthquake. By looking into the relative abundances of microbial groups, the authors could determine a short-term perturbation on the indigenous groups that eventually restore with time after implementation of sanitation practices.

In recent years, decreasing costs in next generation sequencing have resulted in an increase in the use of metagenomics to elucidate water quality. Ng et al. utilized metagenomics to elucidate the diversities and average relative abundance of antibiotic resistance genes present in hospitals and untreated municipal wastewaters. The authors further assembled scaffolds from the raw sequencing reads, and identified that most of the ARGs are associated with mobile genetic elements that can aid in horizontal transfer of resistance genes among bacterial populations.

This technique of assembling scaffolds was further exemplified by Zhang et al. By first performing very deep metagenomics sequencing on drinking water samples, sufficient coverage was achieved to assemble raw reads into complete draft genomes of 9 bacterium. Further annotation of the draft genomes revealed pathogenic characteristics and for some, CRISPR-Cas genetic signatures, present in the drinking water samples. Such insights would not have been discovered by conventional culture techniques, hence reiterating the usefulness of exploring new molecular methods.

In summary, articles in this Research Topic exemplify how the scientific community can work toward addressing water scarcity by a two-pronged approach—first, to explore alternative water supplies and ensuring that these new waters are safe for use; second, utilizing new methods to provide comprehensive insights on water quality, which would in turn advance water reuse and management programs in a safe and sustainable manner.

AUTHOR CONTRIBUTIONS

P-YH conceived the outline, wrote and edited the manuscript. TJ and MJ contributed to the writing and editing of the manuscript. All authors contributed to the overall framing, writing, and revision of this manuscript.

FUNDING

This work is partly supported by KAUST Baseline funding BAS/1/1033-01-01 awarded to P-YH.

REFERENCES

Al-Jassim, N., Mantilla-Calderon, D., Wang, T., and Hong, P. Y. (2017). Inactivation and gene expression of a virulent wastewater *Escherichia coli* strain and the nonvirulent commensal *Escherichia coli* DSM1103 strain upon solar irradiation. *Environ. Sci. Technol.* 51, 3649–3659. doi: 10.1021/acs.est.6b05377

Jumat, M., Haroon, M., Al-Jassim, N., Cheng, H., and Hong, P.-Y. (2018). An increase of abundance and transcriptional activity for *Acinetobacter junii* post wastewater treatment. *Water* 10:436. doi: 10.3390/w10040436

Mantilla-Calderon, D., and Hong, P. Y. (2017). Fate and persistence of a pathogenic NDM-1-positive *Escherichia coli* strain in anaerobic and aerobic sludge microcosms. *Appl. Environ. Microbiol.* 83:e00640-17. doi: 10.1128/AEM.00640-17

Olstadt, J., Schauer, J. J., Standridge, J., and Kluender, S. (2007). A comparison of ten USEPA approved total coliform/*E. coli* tests. *J Water Health* 5, 267–282. doi: 10.2166/wh.2007.008b

Zhang, Y., Hong, P. Y., LeChevallier, M. W., and Liu, W. T. (2015). Phenotypic and phylogenetic identification of coliform bacteria obtained using 12 coliform methods approved by the U.S. environmental protection agency. *Appl. Environ. Microbiol.* 81, 6012–6023. doi: 10.1128/AEM.01510-15

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 19 December 2017
doi: 10.3389/fenvs.2017.00090

Controlling Bacterial Pathogens in Water for Reuse: Treatment Technologies for Water Recirculation in the Blue Diversion Autarky Toilet

*Mi T. Nguyen[1,2], Lukas Allemann[1], Christopher Ziemba[1,3], Odile Larivé[1,4], Eberhard Morgenroth[1,3] and Timothy R. Julian[1]**

[1] Eawag, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland, [2] Nguyen Tat Thanh Hi-Tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam, [3] ETH Zürich, Institute of Environmental Engineering, Zurich, Switzerland, [4] EPFL, School of Architecture, Civil and Environmental Engineering, Lausanne, Switzerland

Highlight

- Bacterial growth in fecally-contaminated water is highly variable and dependent on several factors.
- Regrowth occurs after chlorination (low doses, no residual).
- Indigenous microbial communities variably impact bacterial growth.
- A combination of treatments can both inactivate and inhibit growth.

The Blue Diversion AUTARKY Toilet is a urine-diverting toilet with on-site treatment. The toilet is being developed to provide a safe and affordable sanitation technology for people who lack access to sewer-based sanitation. Water used for personal hygiene, hand washing, and flushing to rinse urine- and feces-collection bowls is treated, stored, and recycled for reuse to reduce reliance on external water supplies. The system provides an opportunity to investigate hygiene of water for reuse following treatment. Treatment in the toilet includes a Biologically Activated Membrane Bioreactor (BAMBi) followed by a secondary treatment technology. To identify effective secondary treatment, three options, including granular activated carbon (GAC) only, GAC+chlorine (sodium hypochlorite), and GAC+electrolysis are considered based on the bacterial inactivation and growth inhibition efficiency. Four different hygiene-relevant bacteria are tested: *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa,* and *Salmonella typhimurium*. Our evaluation demonstrates that—despite treatment of water with the BAMBi—*E. coli, P. aeruginosa*, and *S. typhimurium* have the potential to grow during storage in the absence of microbial competition. Including the indigenous microbial community influences bacterial growth in different ways: *E. coli* growth decreases but *P. aeruginosa* growth increases relative to no competition. The addition of the secondary treatment options considerably improves water quality. A column of GAC after the BAMBi reduces *E. coli* growth potential by 2 $\log_{10}$, likely due to the reduction of carbon sources. Additional treatments including chlorination and electrolysis provide further safety margins, with

OPEN ACCESS

Edited by:
Pascal E. Saikaly, King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Pierre Le Clech, University of New South Wales, Australia
Moustapha Harb, University of Southern California, United States

***Correspondence:**
Timothy R. Julian
tim.julian@eawag.ch

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Environmental Science

Received: *29 September 2017*
Accepted: *04 December 2017*
Published: *19 December 2017*

Citation:
Nguyen MT, Allemann L, Ziemba C, Larivé O, Morgenroth E and Julian TR (2017) Controlling Bacterial Pathogens in Water for Reuse: Treatment Technologies for Water Recirculation in the Blue Diversion Autarky Toilet. Front. Environ. Sci. 5:90. doi: 10.3389/fenvs.2017.00090

more than 5 $\log_{10}$ inactivation of *E. coli*. However, reactivation and/or regrowth of *E. coli* and *P. aeruginosa* occurs under in the absence of residual disinfectant. Treatment including the BAMBi, GAC, and electrolysis appear to be promising technologies to control bacterial growth during storage in water intended for reuse.

Keywords: water for reuse, pathogen, inactivation, regrowth, biologically active membrane bioreactor, biostability

INTRODUCTION

Two-thirds of the world's population suffer from water scarcity (United Nations, 2012; Global Water Institute, 2013; Mekonnen and Hoekstra, 2016). Water recovery and reuse from diverse sources (i.e., graywater, wastewater, stormwater) can help to increase water efficiency and reduce impacts of scarcity. However, reuse may pose risks to environmental and human health, especially when source water is fecally-contaminated (Christova-Boal et al., 1996; Gross et al., 2005; Wiel-Shafran et al., 2006). Fecally-contaminated water (water containing feces, such as blackwater, brownwater, and wastewater) may contain high concentrations of microbial contamination, including fecal bacteria (e.g., *E. coli*, enterococci), enteric pathogens (e.g., *Salmonella typimurium, Cryptosporidium* spp., *Giardia* spp.), and/or opportunistic pathogens (e.g., *Pseudomonas aeruginosa*) (Christova-Boal et al., 1996; Albrechtsen, 2002; O'Toole et al., 2012; Katukiza et al., 2015). Exposures during reuse like inhaling aerosols generated from toilet flushing, indirect ingestion via hand-to-mouth contacts, unintentional ingestion (Christova-Boal et al., 1996), or consumption of plants irrigated using fecally-contaminated water (Shuval et al., 1997; Mara et al., 2007) may contribute to disease transmission (Morel and Diener, 2006).

Risks from fecally-contaminated water reuse can be mitigated through safe management, including chemical and physical disinfection. Disinfection reduces the concentration of pathogens in water and helps prevent pathogen growth during subsequent distribution and/or storage. Chlorine disinfection (1.4 mg L^{-1} with 30 min exposure, or 42 mg min L^{-1}) was shown to be effective in inactivating fecal coliforms in treated graywater, with no regrowth (or growth following disinfection) (Friedler et al., 2006). Membrane filtration (e.g., MBR, ultrafiltration) used to treat graywater achieved up to 4 $\log_{10}$ removal of fecal coliforms (Friedler et al., 2006). UV irradiation (25–40 mJ cm^{-2}, unreported water thickness) was also shown to be effective in lowering total coliforms (to 2–500 CFU/100 mL) and fecal coliforms (to 2–30 CFU/100 mL) to meet German quality guidelines for graywater reuse (Nolde, 2000). In another study, Gilboa and Friedler (2008) report observing no regrowth of fecal coliforms, *P. aeruginosa,* or *Staphylococcus aureus* after exposure to UV disinfection (0–439 mJ cm^{-2}, unreported water thickness) up to 6 h (Gilboa and Friedler, 2008). Although treatment processes can significantly reduce the concentration of bacteria, regrowth can occur in treated fecally-contaminated water during storage and distribution due to the availability of assimilable organic carbon (AOC) and the loss of disinfectant residual (Jjemba et al., 2010; Thayanukul et al., 2013; Lin et al., 2016). Other factors can affect the growth of pathogens in disinfected water, including type and concentration of available nutrients, type and concentration of residual disinfectant, presence of indigenous community, water age, pipe materials, and environmental conditions (Wang et al., 2014; Prest et al., 2016).

In this study, risks from reuse of fecally-contaminated water in the Blue Diversion Autarky Toilet (BDAT, http://www.autarky.ch) were investigated. The BDAT was developed by Eawag (Dübendorf, Switzerland) and designed by EOOS (Vienna, Austria) to provide a sanitation option that is safe, affordable, and off-the-grid (Larsen et al., 2015). As a source-separating toilet, the BDAT has urine and feces separately collected and treated for resource recovery. The BDAT also provides and recycles running water, with a design flow rate of 75 L/day for 10 users (Larsen et al., 2015). Despite the source separation strategy, between 1 and 2% of the urine and feces produced by the users enter into the water recycling system. After the BDAT is primed with water from local sources, the water is filtered through a biologically activated membrane bioreactor (BAMBi) composed of aerated flatsheet polyethersulfone membranes with a nominal cutoff of 150 kDa. The BAMBi achieves low, but stable, gravity-driven flux, with the stability attributed to biological activation of the membrane surface (Künzle et al., 2015). After filtration through the BAMBi, permeate water is stored in a 30 L clean water tank to be used for toilet flushing, hand washing, and personal hygiene before being collected and recirculated back to the BAMBi. This permeate water still contains 40–50 mg L^{-1} of dissolved organic carbon (DOC). The addition of a granular activated carbon (GAC) filter into the top of the clean water tank has demonstrated the ability to reduce this DOC concentration significantly without impacting system operation. The compatibility between permeate water either with or without the GAC treatment and any pathogens that may enter the system is not well-understood, and therefore represents a potential obstacle to the safe utilization of the BDAT.

The specific goal of this study was to evaluate bacterial growth, inactivation, and reactivation/regrowth in treated water during storage within the BDAT. Growth is defined, here, as an increase in cell concentration by either total cell count (TCC), or colony forming units (CFU). Reactivation or regrowth is defined, here, as growth following an observed decrease in cell concentration due to treatment processes. Two fecal indicators (*Escherichia coli* and *Enterococcus faecalis*) and two pathogens (*Pseudomonas aeruginosa* and *Salmonella typhimurium*) were chosen to study

Abbreviations: AOC, Assimilable Organic Carbon; ANOVA, Analysis of Variance; BAMBi, Biologically Activated Membrane Bioreactor; BDAT, Blue Diversion AUTARKY Toilet; CFU, Colony Forming Units; DOC, Dissolved Organic Carbon; FCM, Flow Cytometry; GAC, Granular Activated Carbon; GDM, Gravity Driven Membrane; ICC, Intact Cell Count; RPM, Revolutions Per Minute; TCC, Total Cell Count; VBNC, Viable But Non-culturable.

behaviors of waterborne pathogens. Bacterial concentrations were measured using culture method (spreading on selective-media plates) and flow cytometry [TCC and intact cell counts (ICC)]. Results from this study improve the understanding of inactivation and growth/regrowth of fecal indicators and pathogens in water for reuse in the context of the BDAT and other water recycling systems.

METHODS

Two sets of experiments were performed: (1) growth potential assays to assess bacterial growth within stored water, and (2) disinfection assays to assess bacterial inactivation and reactivation. Both sets of experiments were performed using aliquots of water collected from within the BDAT following treatment by either the BAMBi alone or the BAMBi and the GAC (BAMBi+GAC).

Blue Diversion AUTARKY Toilet

Water for the experimental assays was collected from full-scale BDATs using recreated influent to mimic expected flush water, hand wash water, and personal hygiene water. The BDAT design and operation was previously described by Larsen et al. (2015); and the BAMBi was previously described by Künzle et al. (2015). The recreated influent contained 12.5 g feces, 25 mL urine, and 2.5 g hand soap (Consumerline Products, Kenya) in 1 L of tap water. This influent mixture was prepared every 3–4 days, refrigerated at 4°C and stirred at ∼50 RPM. The feces and urine were collected from a urine-diverting dry toilet at Eawag. Feces were collected weekly, homogenized with a blender, and stored at −20°C for up to 60 days. Urine was stored at room temperature for up to 48 h. In the BAMBi tank, the recreated influent is mixed with recirculated, treated water from the storage tank at a ratio of 1:20.

The full-scale BDAT treatment included a 50-L BAMBi tank with nine panels of polyethersulfone ultrafiltration membrane UP150 (Microdyn Nadir, Wiesbaden, Germany) with a nominal cut-off of 150 kDa, seeded with 0.5 L of municipal wastewater sludge. Aeration was achieved by pumping air (0.2-m^3 h^{-1}) through a perforated pipe underneath the membrane to maintain aerobic conditions on the exterior of the biofilm within the BAMBi tank. Water filtered through the BAMBI is then pumped into a 30-L clean water tank, which housed one or more of the post-treatment options: (1) GAC—to treat the remaining of organic matter and to reduce color (pore size of 0.6–1 mm, Norit Americas Inc., USA), (2) chlorination—to disinfect water using sodium hypochlorite (Fluka Chemie, Switzerland), and (3) electrolysis—to provide on-site chlorine for disinfection (Condias, Germany).

Collecting and Preparing Water Samples

Water samples (20 L) were collected from the clean water tanks of two BDATs: one operating with only the BAMBi (after BAMBi), and one with the addition of a 6 L-GAC column after the BAMBi (after BAMBi+GAC). The samples were prepared followed a method modified from Vital et al. (2008) to remove indigenous microorganisms. Indigenous microorganisms were removed to preserve water quality at the time of collection so that a consistent source water could be used for all studies. Otherwise, storage of water with indigenous community intact would have led to water quality changes due to continued microbial activity and may have interfered with growth of target bacteria. Specifically, samples were filtered through a 1-μm pressurized filter (Geberit, Germany), pasteurized at 80°C for 60 min, and filtered again through a 0.2-μm membrane filter (Whatman, UK).

Notably, pretreatment did not substantially affect the concentration or characterization of DOC, as shown from chromatograms measured using an LC-OCD instrument for water samples before and after the procedure of filtration and pasteurization [Figure S1, Supporting Information (SI)]. All samples were processed within 6 h after collection and stored at 4°C until used. In this study, prepared water is referred to water after mentioned pasteurization and filtration steps. Characteristics of prepared water samples, including concentration of anions (NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, Cl^-) and cations (Ca^{2+}, Mg^{2+}, Na^+, K^+, $Fe^{3+/2+}$, Mn^{2+}, Cu^{2+}, Ba^{2+}), were analyzed by the Engineering Analytical Laboratory at Eawag.

Bacterial Strains

Four bacteria used in this study include two isolated indicators from wastewater [*Escherichia coli* (GenBank KU737538) and *Enterococcus faecalis* (GenBank KU737539)] and two pathogens [*Pseudomonas aeruginosa* PAO1 (ATCC 15692) and *Salmonella typhimurium* SB300 (provided by Dr. M. Suar from Institute of Microbiology, ETH Hönggerberg, Switzerland)]. The wastewater isolates were confirmed as *E. coli* and *Ent. faecalis* using 16sRNA sequencing (Microsynth, Switzerland). All experiments involved the four bacteria of interest were conducted under proper procedures in a Biosafety Level 2 laboratory at Eawag. Further details of sequencing and bacterial preparation are provided in the SI.

Growth Potential Experiments

The experiments were set up following the pathogen growth potential assay of Vital et al. (2010). Target bacteria were grown overnight and seeded at an initial concentration of $\sim 10^3$ cells mL^{-1} with 20 ml prepared water samples (20 mL) into 40 mL carbon-free glass vials. Vials were then incubated at 30°C in 72 h in the dark to reach stationary phase. Samples (1 mL) were taken at the beginning, end and intermittently throughout to measure bacterial concentration using both flow cytometry and culture. Sterilized distilled water was included as negative controls. AOC concentration was defined by the growth of the indigenous community: 1 μg AOC $L^{-1} = 10^7$ cells L^{-1} (Hammes and Egli, 2005).

Bacterial Competition Assays

To measure growth of bacteria in competition with the indigenous community, vials with prepared water samples and bacteria of interest were inoculated with raw water samples such that the indigenous community was seeded to a concentration of 10^3 cells mL^{-1}.

Nutrient Limitation Assays

Nutrient limitations to growth for the bacteria were determined by supplementing with additional sources of carbon (C) [300 mg L^{-1} sodium acetate (CH_3COONa, Fluka Chemie, Switzerland)], nitrogen (N) [5 mg L^{-1} ammonium sulfate ($(NH_4)_2SO_4$, Sigma-Aldrich, Germany)], phosphorus (P) [80 mg L^{-1} sodium phosphate dibasic (Na_2HPO_4, Sigma-Aldrich, USA)], and iron (Fe) [5 mg L^{-1} ferric chloride ($FeCl_3$, Sigma-Aldrich, USA)]. The concentrations were chosen to be at least 10 times higher than in concentrations of C, N, P, and Fe measured in the water after BAMBi.

Nutrient limitations for *Ent. faecalis* were further evaluated by supplementation of minimal media Davis broth (7 g L^{-1} of K_2HPO_4, 2 g L^{-1} of KH_2PO_4, 0.5 g L^{-1} of sodium citrate, 0.1 g L^{-1} of $MgSO_4$, 1 g L^{-1} of NH_4SO_4, supplemented with 100 mg L^{-1} of thiamine, 0.1% glucose), vitamins [biotin, calcium, pantothenic acid, and pyridoxine (each at 20 μg mL^{-1}), nicotinic acid and riboflavin (each at 2 μg mL^{-1}), folic acid (0.2 μg mL^{-1})], and 20 amino acids (each at 20 μg mL^{-1}) (Murray et al., 1993). All experiments were conducted in triplicates.

Inactivation and Regrowth Experiments

Inactivation Experiments

Water after BAMBi+GAC was used for the inactivation and regrowth experiments. *E. coli* and *P. aeruginosa* were tested in the inactivation experiments due to their ability to grow in water after BAMBi+GAC without the need for additional nutrients (See Results section Growth of Bacteria in Waters from the BDAT). Overnight incubated cells were added to prepared water samples to reach final concentration of $\sim 10^5$ cells mL^{-1}, and then exposed to a disinfectant (chlorine or electrolysis). When chlorine was used directly as disinfectant, sodium hypochlorite (10%, Fluka Chemie, Switzerland) was added to the mixture of 100 mL of water after BAMBi+GAC to establish initial concentrations of 0.07, 0.14, 0.2, 0.5, 1.7, and 3.4 mg Cl_2 mL^{-1}. The initial concentrations of chlorine were 1×, 2×, 3×, 7×, 24× and 49× higher than the total chlorine demand of the water after BAMBi+GAC. The polyvinylidene fluoride electrolysis unit (Condias, Germany) used to produce disinfectants has a dimension of 4.5 × 3.5 × 22.5 cm (l × w × h) and four niobium substrate electrodes, each with a boron-doped diamond coating. The electrolysis unit was run at 0.5, 5, and 20 W at a recirculating flow rate of 7 L h^{-1} in total of 30 min. Samples were collected at 0, 5, 15, and 30 min after disinfection exposure. Free and total chlorine concentrations were measured using the DBD method (Hach LCK 310, Germany) at the same time as bacterial concentrations (Table S1). After each time point, sodium thiosulfate solution ($Na_2S_2O_3$, stock concentration of 46 g L^{-1}) was added to samples to quench residual chlorine prior to bacterial quantification. All experiments were conducted in triplicates.

Reactivation/Regrowth Experiments

To determine whether bacteria can reactivate or regrow after inactivation, water samples after 30 min of each inactivation experiment were aliquoted in 40 mL carbon free glass vials and incubated at 30°C in the dark for 72 h. A subset of the samples exposed to direct chlorination and electrolysis were treated with sodium thiosulfate solution (46 g L^{-1}) to quench residual chlorine before regrowth experiments to isolate the impact of treatment from residual chlorine on reactivation or regrowth. An increase in bacterial concentration of more than 10 cell mL^{-1} after 72 h of incubation was considered as reactivation or regrowth. Additionally, to determine the effect of each post-treatment on bacterial growth potential, overnight incubated bacterial cells were also added to water samples after each inactivation experiment (initial concentration of 10^3 cells mL^{-1}). All experiments were conducted in triplicates.

Bacterial Concentration Measurements

Culture and flow cytometry (FCM) methods were used to measure concentrations of bacteria in water samples. Samples were analyzed immediately after collection. Further details are in SI.

Calculating Net Growth/Growth Potential and Growth Coefficient

Net Growth/Growth Potential

Net growth of bacteria or growth potential of a water sample is defined here as the difference between the final cell concentration in the stationary phase and the initial cell concentration at the beginning when the bacterial inoculum was introduced in the water sample (Vital et al., 2010). Bacteria were considered to grow in a water sample only when the minimum net growth was 10^3 cells mL^{-1} (Vital et al., 2010).

Growth Coefficient

A sigmoid function was used to fit an S-shaped growth curve to each growth potential data set shown in **Figure 1** (Zwietering et al., 1990). Parameters of the sigmoid function for each data set are shown in Table S1.

$$y = y_{\min} + \frac{\left(y_{\max} - y_{\min}\right)}{1 + 10^{\left(logEC50 - t\right) \times r_j^i}} \quad (1)$$

where $y_{\min}$ and $y_{\max}$ are minimum and maximum values of log concentration, respectively. t is time (h), and $logEC50$ is t-value when y-value is halfway between $y_{\min}$ and $y_{\max}$. r_j^i is the slope of the exponential phase, or growth coefficient of bacterium i (i = E for *E. coli*, P for *P. aeruginosa*, S for *S. typhimurium*, Ent for *Ent. faecalis*) in water sample j (j = BAMBi for water after BAMBi, and GAC for water after BAMBi+GAC).

Statistical Analysis

GraphPad Prism 6.0.1 (GraphPad Software, USA) was used to perform statistical tests. Non-linear regression was performed to fit the sigmoid function to each bacterial growth curve and calculate growth coefficients. Comparison of net growth, growth coefficients, and water characteristics was conducted using either paired t-tests or two-way ANOVA. All statistical tests assumed an alpha of 0.05.

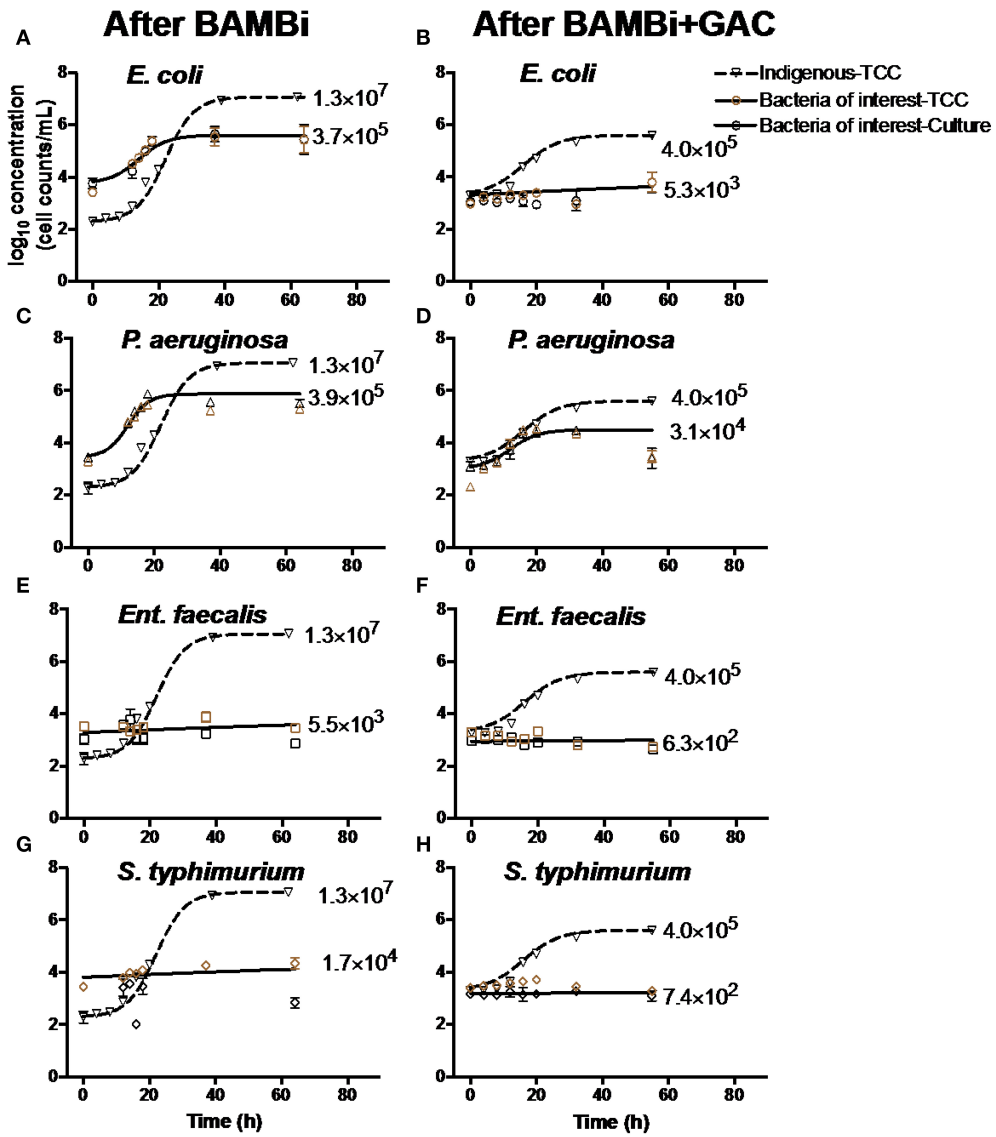


FIGURE 1 | Growth curves of indigenous communities, **(A,B)** *E. coli,* **(C,D)** *P. aeruginosa*, **(E,F)** *Ent. faecalis,* and (**G,H**) *S. typhimurium* in water after BAMBi and after BAMBi+GAC. Concentrations of bacteria were measured using culture method and FCM for TCC. Growth curves of the indigenous bacteria (▽) were added in each figure to compare with the growth of the bacteria of interest (○ for TCC, and ○ for culture method). Negative controls and samples with *Ent. faecalis* and *S. typhimurium* did not show significant change in bacterial concentration during the course of the experiments. Net growth of each bacteria (CFU/mL) is shown next to each growth curve. More details of the growth curves can be found in Table S1.

RESULTS

Water Characteristics

Water treated with the BAMBi had generally higher nutrient and metal ion concentrations than water treated with the BAMBi and GAC (**Table 1**). Specifically, DOC, PO_4^{3-}, SO_4^{2-}, and Cu^{2+} were statistically significantly lower in the BAMBi+GAC than the BAMBi (Student's *t*-test with Sidak correction, $p < 0.05$, **Table 1**). Although reductions were observed for pH, AOC, NO_3^-, Cl^-, Na^+, K^+, Ca^{2+}, and Mg^{2+}, the reduction was not statistically significant (Student's *t*-test with Sidak correction, $p > 0.05$).

Growth of Bacteria in Waters from the BDAT

The indigenous microbial community, *E. coli,* and *P. aeruginosa*, grew in both waters during storage, whereas *Ent. faecalis* and *S. typhimurium* only grew in water after treated with BAMBi (**Figure 1**). The indigenous microbial community grew to a higher concentration in water after treatment with the BAMBi alone ($Net\ growth_{BAMBi} = 1.3 \times 10^7$ cells mL^{-1}) as compared to the BAMBi+GAC (4.0×10^5 cells mL^{-1}, **Figure 1**). The indigenous community also grew faster in water after BAMBi

TABLE 1 | Characteristics of water after BAMBi and after BAMBi+GAC.

Parameters	After BAMBi	After BAMBi+GAC	*p*-value (Student's *t*-test)	Adj. *p*-value (Sidak correction)
pH	8.3 ± 0.0	7.9 ± 0.1	8×10^{-3}	0.09
DOC (mg L^{-1})	36.5 ± 1.3	7.9 ± 0.1	2.5×10^{-8}	3×10^{-7}
AOC (mg L^{-1})	0.3 ± 0.1	0.1 ± 0.1	0.53	1.00
NO_2^--N (mg L^{-1})	<2	<2	NA	NA
NO_3^--N (mg L^{-1})	25 ± 1.0	21.1 ± 1.1	0.07	0.60
PO_4^{3-}-P (mg L^{-1})	16.5 ± 1.8	11.5 ± 1.2	4.3×10^{-4}	5×10^{-3}
SO_4^{2-}-S (mg L^{-1})	74.5 ± 2.9	100.7 ± 6.7	1.4×10^{-5}	1.7×10^{-4}
Cl^- (mg L^{-1})	133.8 ± 2.5	126.3 ± 2.4	0.022	0.24
NH_4^+-N (mg L^{-1})	<0.2	<0.2	NA	NA
Ca^{2+} (mg L^{-1})	2.5 ± 0.1	2.3 ± 0.1	0.21	0.94
Mg^{2+} (mg L^{-1})	3.7 ± 0.1	3.6 ± 0.2	0.81	1.00
Na^+ (mg L^{-1})	78.4 ± 0.1	71.9 ± 4.9	0.22	0.95
K^+ (mg L^{-1})	100.5 ± 1.8	96.7 ± 6.3	0.57	1.00
$Fe^{3+/2+}$ (μg L^{-1})	<5	<5	NA	NA
Mn^{2+} (μg L^{-1})	<5	<5	NA	NA
Cu^{2+} (μg L^{-1})	11.8 ± 0.7	<5	8.5×10^{-6}	1×10^{-4}
Ba^{2+} (μg L^{-1})	<5	<5	NA	NA

AOC concentration was determined from the growth of indigenous bacteria. Data is shown as mean ± standard error. Sample size per measurement is 6. Student's t-test was used to determine significant differences between BAMBi and BAMBi+GAC, as shown with p-value and adj. p-value, where adj. p-value is Sidak correction for familywise error rates. NA refers to Not Applicable, and is used when samples were below the lower limit of detection.

($r_{BAMBi} = 0.1\ h^{-1}$) than BAMBI+GAC ($r_{GAC} = 0.08\ h^{-1}$, Table S1). Similarly, the growth of both *P. aeruginosa* and *E. coli* in water treatment with BAMBi was higher than in water treated with BAMBi+GAC (56% more for *P. aeruginosa*, and 38% more for *E. coli,* Table S1). While *P. aeruginosa* had similar growth coefficient in both waters (Table S1), *E. coli* grew faster in water after BAMBi compared to in water after BAMBi+GAC ($r^{E.\ coli}_{BAMBi} = 10r^{E.\ coli}_{GAC}$).

There was a good agreement between TCC data and culture data for all four bacteria of interest in both water samples (two-way ANOVA, $p > 0.05$).

Factors Influencing Bacterial Growth

Nutrient Addition

The addition of all four nutrients (C, N, P, and Fe) individually or in combination increased the net growth of *E. coli* and *S. typhimurium* compared to the control samples (two-way ANOVA, $p < 0.05$, **Figure 2**). The growth of *P. aeruginosa* significantly increased when C, N, and P were added (two-way ANOVA, $p < 0.05$, **Figure 2**). The addition of C appeared to dramatically increase the growth of *E. coli* and *P. aeruginosa* (approximately two times higher compared to the addition of other nutrients). The growth of *S. typhimurium* approximately doubled with the addition of Fe compared to other nutrients.

Although the addition of C, N, P, and Fe did not show any positive effect on the growth of *Ent. faecalis* in water after BAMBi (**Figure 2D**), at least some bacterial growth was observed in the presence of minimal media (Davis media), amino acids, or vitamins with the largest effect size observed in the presence of all three (Figure S3).

There was a good agreement between TCC data and culture data for *E. coli*, *Ent. faecalis*, and *S. typhimurium* (two-way ANOVA, $p > 0.05$).

Indigenous Communities

The presence of an indigenous microbial community typically, but not always, reduced the extent of growth of the target bacteria *E. coli*, *Ent. faecalis*, and *S. typhimurium*, but supported the growth of *P. aeruginosa* (**Figure 3**). Inhibition of *E. coli* appeared greatest: *E. coli* was not detected ($N_t <$ 10 cell mL^{-1}) after 72 h of incubation with the indigenous communities in both waters despite growth in the absence of the indigenous community. The indigenous community increased the growth of *P. aeruginosa* (two-way ANOVA, $p < 0.05$), with the BAMBi+GAC community having a bigger effect on the bacterial growth than the BAMBi community (**Figure 3**).

Disinfection Processes

Inactivation

Chlorination and electrolysis treatment options were tested for the efficiency of inactivation of *E. coli* and *P. aeruginosa*. Inactivation of *E. coli* was measured at various initial chlorine concentrations (0.07–3.4 mg Cl_2 L^{-1}, **Figures 4A,B**). As chlorine demand of water after BAMBi+GAC was 0.14 mg Cl_2 L^{-1}, there was no observed inactivation of *E. coli* when chlorine concentrations were lower or equal to 0.14 mg Cl_2 mL^{-1}. At higher initial chlorine concentrations (≥0.5 mg Cl_2 L^{-1}), more than 5 $\log_{10}$ inactivation of culturable *E. coli* was achieved after 5 min. Electrolysis at different intensities (0.5–20 W) was also shown to be effective in inactivating *E. coli* (>5 $\log_{10}$ inactivation after 5 min at all tested intensities of electrolysis, **Figure 4C**). Chlorination and electrolysis were shown to be effective in inactivating *P. aeruginosa* (>5 $\log_{10}$ inactivation after 5 min, **Figures 5A,B**). Concentration of free and total chlorine during each electrolysis and chlorination experiment is shown in Figure S2 and Table S1, respectively.

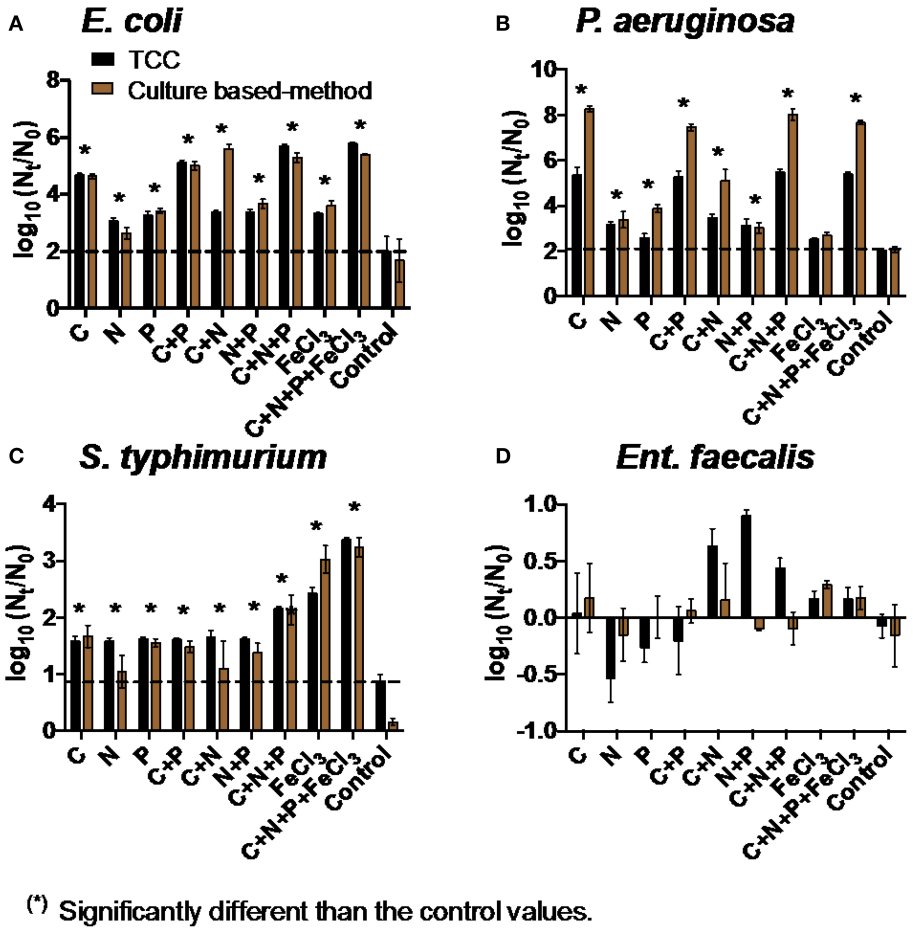


FIGURE 2 | Net growth [$\log_{10}(N_t/N_0)$] of **(A)** *E. coli*, **(B)** *P. aeruginosa*, **(C)** *S. typhimurium*, and **(D)** *Ent. faecalis* in the addition of C, N, P, and Fe in water after BAMBi after 72 h. Controls refer to water after BAMBi without any nutrient supplementation. Dashed lines were drawn for comparison with the highest value of the controls in each experiment. Star symbols indicated that the values were significantly higher than the corresponding control values according to two-way ANOVA test for both TCC and culture data ($p < 0.05$). No significant differences were observed for *Ent. faecalis*.

There was a significant discrepancy between results from culture and FCM methods for every disinfection treatment: Viable cells shown by culture data were significantly lower than ICC data (two-way ANOVA, $p < 0.05$).

Reactivation/Regrowth of bacteria after disinfection

Different disinfection methods variably influenced the likelihood of reactivation and/or regrowth of bacteria. For chlorination, *E. coli* inactivated when exposed to low concentrations of chlorine (from 0.14 to 0.5 mg Cl_2 L^{-1}) reactivated in the presence of $Na_2S_2O_3$–a residual chlorine quencher (**Figure 6A**). No reactivation/regrowth of *E. coli* was observed after exposure to higher concentration of chlorine (3.4 mg Cl_2 L^{-1}) or electrolysis at various intensities, regardless of adding $Na_2S_2O_3$ (**Figure 6A**). We observed increases in intact cell count—but not culturable cell count—in water exposed to 1.7 mg Cl_2 L^{-1}. This result—an outlier—is inconsistent with the rest of the data, and though we can speculate on the cause (i.e., flow cytometry error, incorrect gating by flow cytometer) we do not have evidence to support these speculations.

A similar trend was observed for *P. aeruginosa*: reactivation occurred after exposure to low concentration of chlorine in the presence of $Na_2S_2O_3$ (1.7 mg Cl_2 L^{-1}) (**Figure 6B**). Electrolysis seemed to be effective in inhibiting reactivation of *P. aeruginosa* even in the presence of $Na_2S_2O_3$.

Growth of bacteria in disinfected water

Bacterial growth was occasionally observed in disinfected water, influenced by bacterial species as well as level and type of treatment. For example, *P. aeruginosa* grew in waters after exposure to electrolysis and chlorine, but *Ent. faecalis* was not able to grow in any conditions (**Figures 7B,D**). *E. coli* and *S. typhimurium* were unable to grow after chlorination and electrolysis treatments (**Figures 7A,C**).

The TCC data and culture data in experiments with *E. coli* and *P. aeruginosa* were significantly different (two-way ANOVA, $p < 0.05$), but not in experiments with *S. typhimurium* and *Ent. faecalis* (two-way ANOVA, $p > 0.05$).

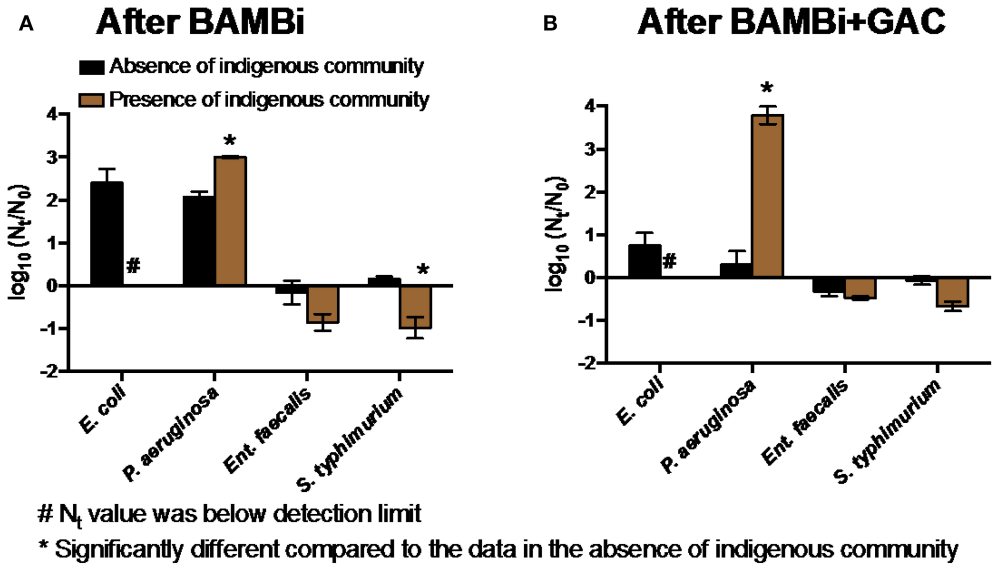


FIGURE 3 | Net growth [$\log_{10}(N_t/N_0)$] of four tested bacteria in the presence and absence of **(A)** the BAMBi indigenous community in water after BAMBi and **(B)** the GAC indigenous community in water after BAMBi+GAC. Concentration at $t = 72$ h of *E. coli* in the presence of the indigenous communities was below detection limit ($N_t < 10$ CFU mL^{-1}). Star symbols indicated that the values in the presence and absence of the indigenous communities were significantly different (two-way ANOVA test, $p < 0.05$).

DISCUSSION

Treatment of water for reuse is a promising strategy to increase water safety. However, hygienic risks increase during storage. Here, we demonstrate that bacterial species, water quality characteristics, and level and type of treatment all influence hygiene risks of water stored for reuse.

Effects of Water Characteristics on Bacterial Growth

Nutrients

Nutrient availability strongly influenced bacterial growth in water stored for reuse. C was an important nutrient in our study. Growth of the indigenous community (which was used to indicate AOC concentration) decreased following water treatment with GAC (**Table 1**). The growth of *E. coli* and *P. aeruginosa* increased dramatically with the addition of C, indicating that C was the main limiting factor for bacterial growth (Morita, 1993; Vital et al., 2008). The maximum concentrations of *E. coli* and *P. aeruginosa* were always lower than the indigenous communities (**Figure 1**). This result aligns with Vital et al. (2008) reporting that the source, type and composition of C has strong effects on bacterial growth. In relation to the water treatment system in the BDAT, this finding emphasizes the need for an efficient removal of DOC in the feed water. The combination of BAMBi and GAC reduces ∼95% of total DOC concentration, which helps limit bacterial growth during storage.

For *Ent. faecalis,* C (in the form of acetate), N, P, or Fe did not improve growth. Addition of Davis media, vitamins, or amino acids were required for growth, implying growth was limited by lack of specific nutrients, not by inhibitory substances. For *S. typhimurium*, the limiting nutrient was Fe, confirming findings of a previous study showing increased growth of *S. typhimurium* and other enteric pathogens in Fe-supplemented water (Kortman et al., 2012). Within the BDAT, blood (i.e., menstrual blood) may introduce iron into the system. The impacts of the introduction of blood as a source of iron should be monitored, as our results suggest *S. typhimurium* (and potentially other pathogens) is limited by Fe.

Our study also highlighted that growth in water stored for reuse depends on the characteristics of the bacteria. Only *E. coli* and *P. aeruginosa* were able to grow in water samples following treatment with BAMBi and GAC. *P. aeruginosa* had a faster growth coefficient and higher final concentration than *E. coli*, indicating that *P. aeruginosa* was more ubiquitous and had a larger nutrient pool than *E. coli*. This result agrees with findings from previous studies showing that *P. aeruginosa* were able to grow relatively fast to reach high cell concentration in waters with limited nutrients (e.g., distilled water from hospitals, tap water; Favero et al., 1971; van der Kooij et al., 1982). Because *P. aeruginosa* is ubiquitous in the environment, it is likely that it will colonize the BDAT system during operation. Our results highlight that carbon control via the BAMBi and GAC treatments will not be sufficient: additional controls (i.e., electrolysis) are needed.

Indigenous Microbial Community

We demonstrated that the indigenous microbial community is generally—but not always—antagonistic to the growth of pathogens in the water (**Figure 3**). Previous studies showed the same antagonism (Chandran and Mohamed Hatha, 2005; Vital et al., 2012; Van Nevel et al., 2013). Antagonism may arise

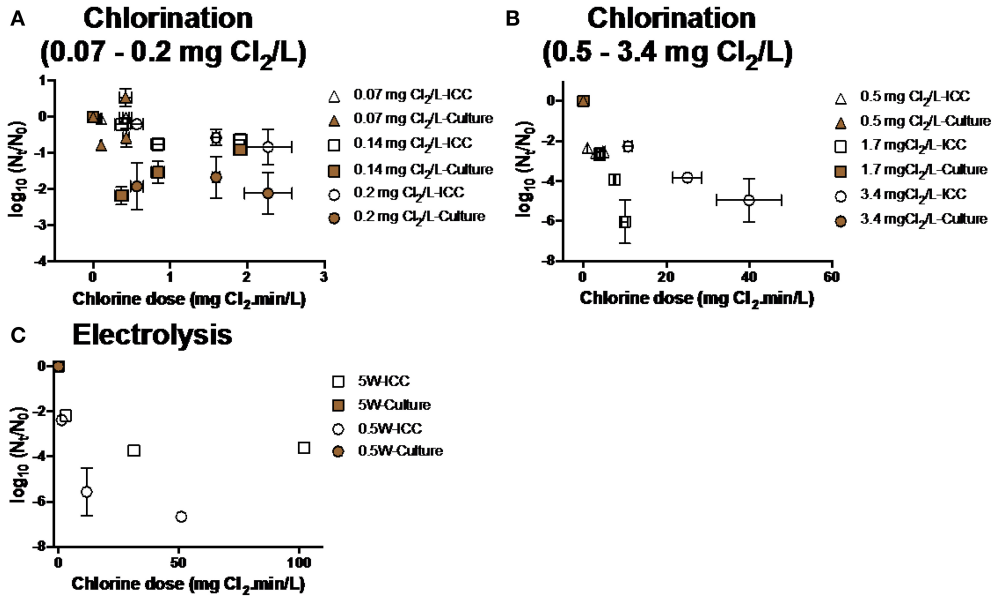


FIGURE 4 | Inactivation of *E. coli* at various conditions during **(A,B)** chlorination and **(C)** electrolysis. Note that samples were measured using both FCM and culture based method data at every single time point. Culture data of some of the time points was not shown because their N_t-values were below detection limit (<10 CFU mL^{-1}).

from nutrient (like C) competition, production of inhibitory compounds (e.g., antibiotics, bacteriocins), or predation and parasitism (e.g., bacteriophage, protozoa, invertebrates) (Hibbing et al., 2010; Vital et al., 2012; Wang et al., 2013). However, we also observed a protagonistic effect for *P. aeruginosa,* as also shown elsewhere for both *E. coli* and *Klebsiella pneumonia* (Moreira et al., 1994; Kerr et al., 1999). One potential explanation is that *P. aeruginosa* was able to use intermittent or end products that were synthesized by the indigenous community (Sherr and Sherr, 2002).

It should be noted that the water samples had been filtered and pasteurized to remove the original indigenous community to preserve the water quality during the course of the study (see section Collecting and Preparing Water Samples). In the BDAT system, the indigenous community is expected to be always present in the planktonic phase and/or in the biofilms. As a result, the interaction between the indigenous community and the pathogens (e.g., nutrient competition, production of inhibitory compounds, and predation) in the BDAT system likely differs from that observed. Additional research is needed to confirm dynamics observed in the laboratory align with dynamics observed in the BDAT under operating conditions (Ziemba et al., in preparation).

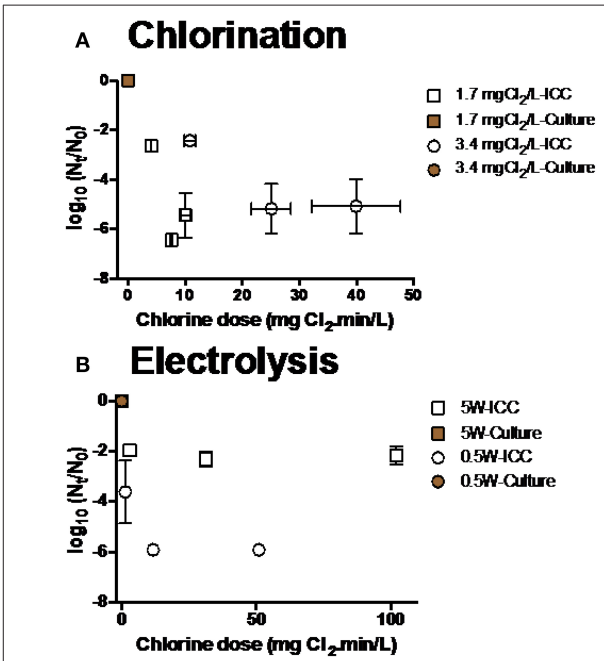


FIGURE 5 | Inactivation of *P. aeruginosa* at various conditions during **(A)** chlorination and **(B)** electrolysis. Culture data of time points ($n = 6$) was not shown because their N_t-values were below detection limit (<10 CFU mL^{-1}).

Effects of Inactivation Processes on Reactivation and Growth of Bacteria

The reactivation/regrowth of *E. coli* and *P. aeruginosa* depended on the water and disinfectant characteristics (**Figure 6**). Bacterial reactivation occurred after exposed to low concentrations of chlorine without residual, as observed in previous studies (Jjemba et al., 2010; Li et al., 2013). *E. coli* and *P. aeruginosa* might enter the viable but non-culturable (VBNC) state under certain conditions during chlorination (Oliver, 2010), and therefore be able to reactivate.

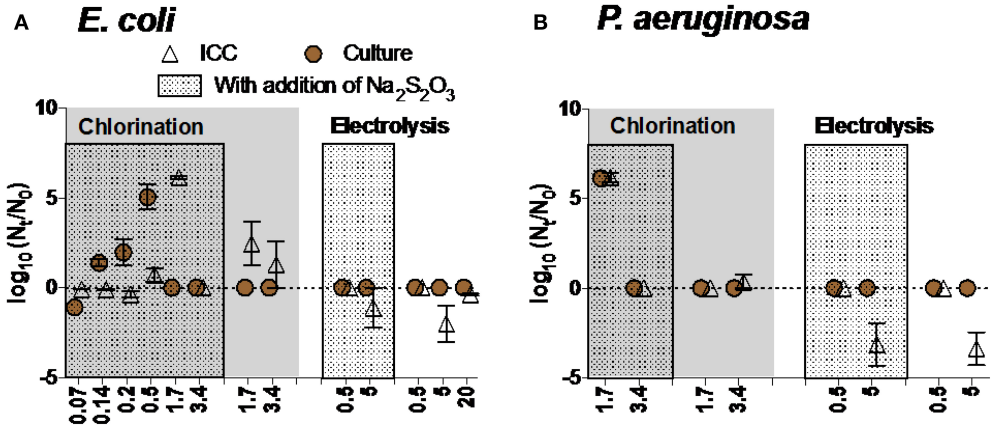


FIGURE 6 | Reactivation and/or regrowth of **(A)** *E. coli* and **(B)** *P. aeruginosa* after inactivation experiments (chlorination and electrolysis). Data within boxes were from experiments in which $Na_2S_2O_3$ was added to quench residual chlorine. Note that no bacteria were added after disinfection and before reactivation/regrowth experiments. Negative values reflect continued inactivation or loss of bacteria during incubation time.

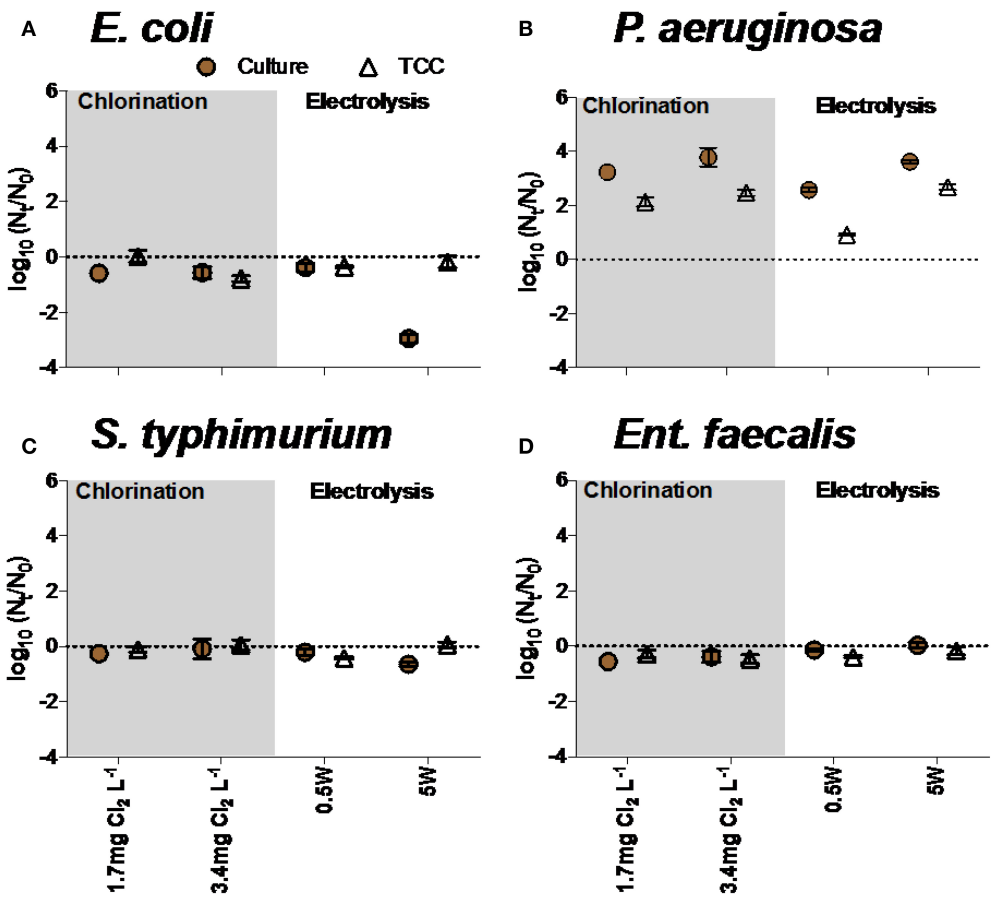


FIGURE 7 | Growth of **(A)** *E. coli*, **(B)** *P. aeruginosa*, **(C)** *S. typhimurium*, and **(D)** *Ent. faecalis* in water after chlorination and electrolysis. $Na_2S_2O_3$ was added in all experiments to quench residual chlorine. Note that negative values reflect continued inactivation or loss of bacteria during incubation time.

No reactivation was observed after electrolysis, even in the presence of chlorine quencher. This result may be due, in part, to production of disinfectants (e.g., hydroxyl, nitrate radical, or phosphate radical besides chlorine that prevent formation of a reactivating VBNC state (Kerwick et al., 2005; Guitaya et al., 2015). We also suspect that the presence of reactive oxidants may

change carbon structure and thereby remove available carbon required for regrowth.

As *P. aeruginosa* was shown to grow after being spiked into disinfected water without residual chlorine, pathogen contamination is potentially a high risk that can affect the system hygienic performance besides regrowth/reactivation. During storage, pathogen contamination can happen via multiple events, including malfunction of one or more treatment processes, access to storage tank by hosts that contain pathogens (e.g., aquatic organisms, insects, birds, rodents, contaminated human hands). Therefore, to ensure the safety of reusing treated fecal-contaminated water, it is important to manage the access of the storage tank carefully, maintain a cleaning procedure for the tank regularly, and have a proper residual of disinfectant constantly.

In this study, we investigated inactivation and reactivation/regrowth of *E. coli* and *P. aeruginosa* in the absence of an indigenous community. The indigenous microbiota were removed to prevent microbial degradation of source water during storage and to allow quantification of bacteria using culture-based methods and flow cytometry simultaneously. The indigenous community may influence inactivation and/or reactivation/regrowth rates of bacteria. For example, in studies of chlorination of both reclaimed and drinking water, inactivation using chlorine has been shown to result in tailing of intact cell counts of the indigenous microbial community (Ramseier et al., 2011; Li et al., 2013). This phenomenon has been attributed to a resistant sub-population and/or shielding through bacterial aggregation or particle adsorption (Ramseier et al., 2011). However, Li et al. (2013) demonstrated inactivation of *in situ* total coliforms, enterococci, and *Salmonella* spp. in reclaimed water at rates similar to those observed here. We therefore expect similar pathogen inactivation and reactivation/regrowth kinetics in studies including the indigenous communities, but further research is warranted to conclusively demonstrate this.

Comparison between Culture and FCM Methods

In general, conclusions from culture and FCM methods aligned for experiments measuring bacterial growth, but differed for inactivation experiments. The discrepancy between culture and FCM methods observed for inactivation was likely because ICC measures intact cell membranes for bacteria that may not be culturable. The ICC measurement relies on cell staining with Propidium Iodide which only stains cells with damaged membranes (Berney et al., 2007). Some dead or unculturable cells may have intact membranes, so ICC data is considered more conservative (i.e., overestimate concentrations of surviving cells) than culture (Joux and Lebaron, 2000; Bosshard et al., 2010).

Implication for Water Reuse

Concerns related to pathogen growth in drinking water and reclaimed water have been raised in previous studies (van der Kooij, 2003; Oesterholt et al., 2007; Jjemba et al., 2010; Weinrich et al., 2010). In this study, we demonstrated that the growth of pathogens in water stored for reuse in an onsite sanitation technology (i.e., the BDAT) is a potential concern and, therefore, increases health risks. A bacterial indicator (*E. coli*) and an opportunistic pathogen (*P. aeruginosa*) were able to grow in water stored for reuse after multiple, different effective treatment processes. Water characteristics, including nutrient concentrations and indigenous bacterial communities, were shown to have strong effects on the bacterial growth. Additionally, we demonstrated the complex and non-uniform influence of indigenous communities on the growth of pathogens.

We also demonstrated that the choice of treatment processes was a key factor influencing bacterial growth. The presence of residual disinfectants (e.g., chlorine and/or other reactive active species) contributed to water biostability. Among three disinfection options, electrolysis appeared to be the most effective method to inhibit pathogen growth, followed by chlorination with high chlorine concentrations (e.g., >0.5 mg Cl_2 L^{-1} for *E. coli* and >1.7 mg Cl_2 L^{-1} for *P. aeruginosa*).

Limitation of the Study

There were notable limitations to this study. First, we only tested growth, inactivation, and reactivation of four bacteria of interest. There is heterogeneity in both growth and inactivation coefficients of the four tested bacterial species, so future research is likely needed to investigate other pathogens (i.e., *Legionalla* spp.) to ensure safety of water reuse.

Second, the study focused on the growth and inactivation of bacteria in the planktonic phase. Biofilms in storage tanks, like the clean water tank where water is stored for reuse in the BDAT, likely also influence growth and inactivation of bacteria. As aggregates of microbial cells attach to surfaces, biofilm members have advantages of being protected from disinfectants and predators compared to planktonic cells (LeChevallier et al., 1988; Flemming and Wingender, 2010). In addition, the matrix of hydrated extracellular polymeric substances (EPS) helps biofilm members consume complex substrates (e.g., humic acids) as food, which are not bioavailable for planktonic bacteria (Fischer, 2003; Flemming and Wingender, 2010). More research is needed to understand the growth and inactivation of bacteria in biofilms in water stored for reuse, especially in the context of the BDAT.

Third, there is a discrepancy between conditions in the laboratory and in the real BDAT system. In our laboratory setup, all experiments were conducted in batch reactors, in which the concentration of nutrients, residual disinfectant, and bacteria drastically changes in the course of the experiments in 72 h. These conditions are different to those in the BDAT, where water after BAMBi is frequently added into the clean water tank and treated water is frequently removed upon every usage. The continuous flow conditions in the BDAT create a certain level of consistency in the bulk-phase concentration of nutrients and bacteria for prolonged periods of time. The concentration of residual disinfectant in the clean water tank of the BDAT is also kept constant with a regular dosing interval. Another difference between laboratory setup and real-life conditions is that the indigenous community is constantly present in the BDAT system whereas it was removed from the water samples for the subset of laboratory experiments on inactivation

and reactivation/regrowth. The indigenous community in the BDAT may impact the inactivation and reactivation/regrowth of pathogens, for example by shielding and/or providing. Results learnt from inactivation experiments, for example the disinfectant demand for a sufficient pathogen removal, likely need to be adapted in the presence of the indigenous community in the BDAT conditions.

CONCLUSIONS

Reuse of treated fecally-contaminated water is a promising strategy to safely increase water efficiency. However, water must be sufficiently treated to reduce hygiene risks associated with reuse. Storage and distribution of water for reuse, in particular, pose potential health risks. We demonstrated these risks through the following observations:

- Both *E. coli* and opportunistic pathogen *P. aeruginosa* grow in water following treatment unless there is sufficient disinfectant residual.
- Growth during storage is influenced by both water quality and bacterial species: although neither *Ent. faecalis* nor *S. typhimurium* grew in treated water, the addition of limiting nutrients was able to initiate growth.
- GAC combined with chlorination (sufficiently high concentrations, with residual) or electrolysis is effective additional treatment that both reduce microorganisms in the water and limits regrowth potential.

AUTHOR CONTRIBUTIONS

MN: contributed to the conception, design, data acquisition, analysis, and interpretation of the work, and drafted and critically revised the manuscript. LA, CZ, and OL: contributed to the data acquisition, analysis, and interpretation of the work, and critically revised the manuscript. EM: contributed to the conception, design and interpretation of the work, and critically revised the manuscript. TJ: contributed to the conception, design, data analysis, and interpretation of the work, and critically revised the manuscript.

ACKNOWLEDGMENTS

This work was carried out in the context of the Blue Diversion AUTARKY-Project, funded by the Bill and Melinda Gates Foundation (OPP1111293). We thank Konstanze Schiessl, Ana K. Pitol, Lea Caduff, Frederik Hammes, Stefan Koetzsch, Juerg Sigrist, Adriano Joss, Richard Fankhauser, Julian Fleiner, and Sara Beck for help with lab work, field work and useful discussions. We thank Kai Udert and Steffi Enssle for help with managing the project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2017.00090/full#supplementary-material

REFERENCES

Albrechtsen, H. J. (2002). Microbiological investigations of rainwater and graywater collected for toilet flushing. *Water Sci. Technol.* 46, 311–316. Available online at: http://wst.iwaponline.com/content/46/6-7/311

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.-U., and Egli, T. (2007). Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Appl. Environ. Microbiol.* 73, 3283–3290. doi: 10.1128/AEM.02750-06

Bosshard, F., Bucheli, M., Meur, Y., and Egli, T. (2010). The respiratory chain is the cell's Achilles' heel during UVA inactivation in *Escherichia coli*. *Microbiol. Read. Engl.* 156, 2006–2015. doi: 10.1099/mic.0.038471-0

Chandran, A., and Mohamed Hatha, A. A. (2005). Relative survival of *Escherichia coli* and *Salmonella typhimurium* in a tropical estuary. *Water Res.* 39, 1397–1403. doi: 10.1016/j.watres.2005.01.010

Christova-Boal, D., Eden, R. E., and McFarlane, S. (1996). An investigation into greywater reuse for urban residential properties. *Desalination* 106, 391–397. doi: 10.1016/S0011-9164(96)00134-8

Favero, M. S., Carson, L. A., Bond, W. W., and Petersen, N. J. (1971). *Pseudomonas aeruginosa*: growth in distilled water from hospitals. *Science* 173, 836–838. doi: 10.1126/science.173.3999.836

Fischer, H. (2003). "The role of biofilms in the uptake and transformation of dissolved organic matter," in *Aquatic Ecosystems* (Elsevier), 285–313. Available Online at: http://linkinghub.elsevier.com/retrieve/pii/B9780122563713500135 (Accessed May 22, 2016).

Flemming, H.-C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633. doi: 10.1038/nrmicro2415

Friedler, E., Kovalio, R., and Ben-Zvi, A. (2006). Comparative study of the microbial quality of greywater treated by three on-site treatment systems. *Environ. Technol.* 27, 653–663. doi: 10.1080/09593332708618674

Gilboa, Y., and Friedler, E. (2008). UV disinfection of RBC-treated light greywater effluent: kinetics, survival and regrowth of selected microorganisms. *Water Res.* 42, 1043–1050. doi: 10.1016/j.watres.2007.09.027

Global Water Institute (2013). *Future Water (In)Security: Facts, Figures, and Predictions*.

Gross, A., Azulai, N., Oron, G., Ronen, Z., Arnold, M., and Nejidat, A. (2005). Environmental impact and health risks associated with greywater irrigation: a case study. *Water Sci. Technol.* 52, 161–169. Available online at: http://wst.iwaponline.com/content/52/8/161

Guitaya, L., Drogui, P., and Blais, J. F. (2015). *In situ* reactive oxygen species production for tertiary wastewater treatment. *Environ. Sci. Pollut. Res. Int.* 22, 7025–7036. doi: 10.1007/s11356-014-3907-3

Hammes, F. A., and Egli, T. (2005). New method for assimilable organic carbon determination using flow-cytometric enumeration and a natural microbial consortium as inoculum. *Environ. Sci. Technol.* 39, 3289–3294. doi: 10.1021/es048277c

Hibbing, M. E., Fuqua, C., Parsek, M. R., and Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* 8, 15–25. doi: 10.1038/nrmicro2259

Jjemba, P. K., Weinrich, L. A., Cheng, W., Giraldo, E., and Lechevallier, M. W. (2010). Regrowth of potential opportunistic pathogens and algae in reclaimed-water distribution systems. *Appl. Environ. Microbiol.* 76, 4169–4178. doi: 10.1128/AEM.03147-09

Joux, F., and Lebaron, P. (2000). Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes Infect.* 2, 1523–1535. doi: 10.1016/S1286-4579(00)01307-1

Katukiza, A. Y., Ronteltap, M., Niwagaba, C. B., and Kansiime, F., and Lens P. N. L. (2015). Grey water characterisation and pollutant loads in an urban

slum. *Int. J. Environ. Sci. Technol.* 12, 423–436. doi: 10.1007/s13762-013-0451-5

Kerr, M., Fitzgerald, M., Sheridan, J. J., McDowell, D. A., and Blair, I. S. (1999). Survival of *Escherichia coli* O157:H7 in bottled natural mineral water. *J. Appl. Microbiol.* 87, 833–841. doi: 10.1046/j.1365-2672.1999.00928.x

Kerwick, M. I., Reddy, S. M., Chamberlain, A. H. L., and Holt, D. M. (2005). Electrochemical disinfection, an environmentally acceptable method of drinking water disinfection? *Electrochim. Acta* 50, 5270–5277. doi: 10.1016/j.electacta.2005.02.074

Kortman, G. A. M., Boleij, A., Swinkels, D. W., and Tjalsma, H. (2012). Iron availability increases the pathogenic potential of *Salmonella typhimurium* and other enteric pathogens at the intestinal epithelial interface. *PLoS ONE* 7:e29968. doi: 10.1371/journal.pone.0029968

Künzle, R., Pronk, W., Morgenroth, E., and Larsen, T. A. (2015). An energy-efficient membrane bioreactor for on-site treatment and recovery of wastewater. *J. Water Sanit. Hyg. Dev.* 5, 448–455. doi: 10.2166/washdev.2015.116

Larsen, T. A., Gebauer, H., Gründl, H., Künzle, R., Lüthi, C., Messmer, U., et al. (2015). Blue diversion: a new approach to sanitation in informal settlements. *J. Water Sanit. Hyg. Dev.* 5, 64–71. doi: 10.2166/washdev.2014.115

LeChevallier, M. W., Cawthon, C. D., and Lee, R. G. (1988). Factors promoting survival of bacteria in chlorinated water supplies. *Appl. Environ. Microbiol.* 54, 649–654.

Li, D., Zeng, S., Gu, A. Z., He, M., and Shi, H. (2013). Inactivation, reactivation and regrowth of indigenous bacteria in reclaimed water after chlorine disinfection of a municipal wastewater treatment plant. *J. Environ. Sci.* 25, 1319–1325. doi: 10.1016/S1001-0742(12)60176-4

Lin, Y., Li, D., Gu, A. Z., Zeng, S., and He, M. (2016). Bacterial regrowth in water reclamation and distribution systems revealed by viable bacterial detection assays. *Chemosphere* 144, 2165–2174. doi: 10.1016/j.chemosphere.2015.10.071

Mara, D. D., Sleigh, P. A., Blumenthal, U. J., and Carr, R. M. (2007). Health risks in wastewater irrigation: comparing estimates from quantitative microbial risk analyses and epidemiological studies. *J. Water Health* 5, 39–50. doi: 10.2166/wh.2006.055

Mekonnen, M. M., and Hoekstra, A. Y. (2016). Four billion people facing severe water scarcity. *Sci. Adv.* 2:e1500323. doi: 10.1126/sciadv.1500323

Moreira, L., Agostinho, P., Morais, P. V., and da Costa, M. S. (1994). Survival of allochthonous bacteria in still mineral water bottled in polyvinyl chloride (PVC) and glass. *J. Appl. Bacteriol.* 77, 334–339. doi: 10.1111/j.1365-2672.1994.tb03082.x

Morel, A., and Diener, S. (2006). *Greywater Management in Low and Middle-Income Countries. Review of Different Treatment Systems for Households or Neighbourhoods.* Duebendorf: Sandec, EAWAG. Available online at: http://www.susana.org/_resources/documents/default/2-947-en-greywater-management-2006.pdf.

Morita, R. Y. (1993). "Bioavailability of energy and the starvation state," in *Starvation in Bacteria*, ed S. Kjelleberg (Boston, MA: Springer), 1–23.

Murray, B. E., Singh, K. V., Ross, R. P., Heath, J. D., Dunny, G. M., and Weinstock, G. M. (1993). Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. *J. Bacteriol.* 175, 5216–5223. doi: 10.1128/jb.175.16.5216-5223.1993

Nolde, E. (2000). Greywater reuse systems for toilet flushing in multi-storey buildings – over ten years experience in Berlin. *Urban Water* 1, 275–284. doi: 10.1016/S1462-0758(00)00023-6

Oesterholt, F., Medema, G., van der Kooij, D., and Martijnse, G. (2007). Health risk assessment of non-potable domestic water supplies in the Netherlands. *J. Water Supply Res. Technol.* 56, 171–179. doi: 10.2166/aqua.2007.043

Oliver, J. D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* 34, 415–425. doi: 10.1111/j.1574-6976.2009.00200.x

O'Toole, J., Sinclair, M., Malawaraarachchi, M., Hamilton, A., Barker, S. F., and Leder, K. (2012). Microbial quality assessment of household greywater. *Water Res.* 46, 4301–4313. doi: 10.1016/j.watres.2012.05.001

Prest, E. I., Hammes, F., van Loosdrecht, M. C. M., and Vrouwenvelder, J. S. (2016). Biological stability of drinking water: controlling factors, methods, and challenges. *Food Microbiol.* 7:45. doi: 10.3389/fmicb.2016.00045

Ramseier, M. K., von Gunten, U., Freihofer, P., and Hammes, F. (2011). Kinetics of membrane damage to high (HNA) and low (LNA) nucleic acid bacterial clusters in drinking water by ozone, chlorine, chlorine dioxide, monochloramine, ferrate(VI), and permanganate. *Water Res.* 45, 1490–1500. doi: 10.1016/j.watres.2010.11.016

Sherr, E. B., and Sherr, B. F. (2002). Significance of predation by protists in aquatic microbial food webs. *Anton. Van Leeuwen.* 81, 293–308. doi: 10.1023/A:1020591307260

Shuval, H., Lampert, Y., and Fattal, B. (1997). Development of a risk assessment approach for evaluating wastewater reuse standards for agriculture. *Water Sci. Technol.* 35, 15–20.

Thayanukul, P., Kurisu, F., Kasuga, I., and Furumai, H. (2013). Evaluation of microbial regrowth potential by assimilable organic carbon in various reclaimed water and distribution systems. *Water Res.* 47, 225–232. doi: 10.1016/j.watres.2012.09.051

United Nations (2012). *Managing Water under Uncertainty and Risk.*

van der Kooij, D., Oranje, J. P., and Hijnen, W. A. (1982). Growth of *Pseudomonas aeruginosa* in tap water in relation to utilization of substrates at concentrations of a few micrograms per liter. *Appl. Environ. Microbiol.* 44, 1086–1095.

van der Kooij, D. (2003). "Managing regrowth in drinking- water distribution systems," in *WHO, Heterotrophic Plate Counts and Drinking-Water Safety the Significance of HPCs for Water Quality and the Human Health,* eds J. Bartram, J. Cotruvo, M. Exner, C. Fricker, and A. Glasmacher (London, UK: IWA Publishing), 199–232.

Van Nevel, S., De Roy, K., and Boon, N. (2013). Bacterial invasion potential in water is determined by nutrient availability and the indigenous community. *FEMS Microbiol. Ecol.* 85, 593–603. doi: 10.1111/1574-6941.12145

Vital, M., Hammes, F., and Egli, T. (2008). *Escherichia coli* O157 can grow in natural freshwater at low carbon concentrations. *Environ. Microbiol.* 10, 2387–2396. doi: 10.1111/j.1462-2920.2008.01664.x

Vital, M., Hammes, F., and Egli, T. (2012). Competition of *Escherichia coli* O157 with a drinking water bacterial community at low nutrient concentrations. *Water Res.* 46, 6279–6290. doi: 10.1016/j.watres.2012.08.043

Vital, M., Stucki, D., Egli, T., and Hammes, F. (2010). Evaluating the growth potential of pathogenic bacteria in water. *Appl. Environ. Microbiol.* 76, 6477–6484. doi: 10.1128/AEM.00794-10

Wang, H., Masters, S., Edwards, M. A., Falkinham, J. O., and Pruden, A. (2014). Effect of disinfectant, water age, and pipe materials on bacterial and eukaryotic community structure in drinking water biofilm. *Environ. Sci. Technol.* 48, 1426–1435. doi: 10.1021/es402636u

Wang, H., Pryor, M. A., Edwards, M. A., Falkinham, J. O., and Pruden, A. (2013). Effect of GAC pre-treatment and disinfectant on microbial community structure and opportunistic pathogen occurrence. *Water Res.* 47, 5760–5772. doi: 10.1016/j.watres.2013.06.052

Weinrich, L. A., Jjemba, P. K., Giraldo, E., and LeChevallier, M. W. (2010). Implications of organic carbon in the deterioration of water quality in reclaimed water distribution systems. *Water Res.* 44, 5367–5375. doi: 10.1016/j.watres.2010.06.035

Wiel-Shafran, A., Ronen, Z., Weisbrod, N., Adar, E., and Gross, A. (2006). Potential changes in soil properties following irrigation with surfactant-rich greywater. *Ecol. Eng.* 26, 348–354. doi: 10.1016/j.ecoleng.2005.12.008

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., and van 't Riet, K. (1990). Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* 56, 1875–1881.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 17 January 2018
doi: 10.3389/fmicb.2017.02603

Strategies to Combat Antibiotic Resistance in the Wastewater Treatment Plants

*Fateme Barancheshme and Mariya Munir**

Department of Civil and Environmental Engineering, University of North Carolina at Charlotte, Charlotte, NC, United States

The main goal of this manuscript is to review different treatment strategies and mechanisms for combating the antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) in the wastewater environment. The high amount of antibiotics is released into the wastewater that may promote selection of ARB and ARGs which find their way into natural environments. Emerging microbial pathogens and increasing antibiotic resistance among them is a global public health issue. The propagation and spread of ARB and ARGs in the environment may result in an increase of antibiotic resistant microbial pathogens which is a worldwide environmental and public health concern. A proper treatment of wastewater is essential before its discharge into rivers, lake, or sewage system to prevent the spread of ARB and ARGs into the environment. This review discusses various treatment options applied for combating the spread of ARB and ARGs in wastewater treatment plants (WWTPs). It was reported that low-energy anaerobic–aerobic treatment reactors, constructed wetlands, and disinfection processes have shown good removal efficiencies. Nanomaterials and biochar combined with other treatment methods and coagulation process are very recent strategies regarding ARB and ARGs removal and need more investigation and research. Based on current studies a wide-ranging removal efficiency of ARGs can be achieved depending on the type of genes present and treatment processes used, still, there are gaps that need to be further investigated. In order to find solutions to control dissemination of antibiotic resistance in the environment, it is important to (1) study innovative strategies in large scale and over a long time to reach an actual evaluation, (2) develop risk assessment studies to precisely understand occurrence and abundance of ARB/ARGs so that their potential risks to human health can be determined, and (3) consider operating and environmental factors that affect the efficiency of each treatment mechanism.

Keywords: antibiotic resistant genes, antibiotic resistant bacteria, treatment strategies, wastewater treatment, nanomaterial, coagulation, biochar, disinfection

OPEN ACCESS

Edited by:
Muhammad Raihan Jumat, King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Magdalena Popowska, University of Warsaw, Poland
Guadalupe Virginia Nevárez-Moorillón, Autonomous University of Chihuahua, Mexico

***Correspondence:**
Mariya Munir
mmunir@uncc.edu

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *31 July 2017*
Accepted: *14 December 2017*
Published: *17 January 2018*

Citation:
Barancheshme F and Munir M (2018) Strategies to Combat Antibiotic Resistance in the Wastewater Treatment Plants. Front. Microbiol. 8:2603. doi: 10.3389/fmicb.2017.02603

INTRODUCTION

Environmental Impact

Recently, World Health Organization (WHO) announced that antibiotic resistance is growing, and we are fast running out of treatment options (Lawe-Davies and Bennett, 2017). Antibiotics serve as selective pressure and the development of antibiotic resistant bacteria (ARB) is linked with the type of antibiotic and the bacterial species (Kolár et al., 2001) therefore measuring

TABLE 1 | Antibiotics concentrations in the WWTP and receiving river (Xu et al., 2015).

Site Location	Tetracyclines (ng L^{-1})	Sulfonamides (ng L^{-1})	Quinolones (ng L^{-1})
WWTP influent	1615.8	2263.0	3664.0
WWTP effluent	195.0	2001.0	3866.0
Upstream	265.2	648.1	728.8
Downstream	345.1	1111.0	2769.0
Removal efficiency	**87.9%**	**11.6%**	**Increased**[a]

[a] *The release of adsorbed Ofloxacin from sludge or suspended particles may contribute to the high level of quinolones at this site.*

the concentration of antibiotics in wastewater, the effluent of WWTPs, and natural water is important. The concentrations of various antibiotics in an effluent-receiving river in Beijing China were characterized where samples were collected from the upstream before the WWTP and the downstream after the WWTP. It was observed that the concentration of tetracycline in the downstream of river was equal to the effluent of WWTP and the concentration of total sulfonamides in the effluent was around 2-fold higher than in the receiving river water suggesting that the effluent containing antibiotics are contaminating the natural water bodies (**Table 1**; Xu et al., 2015). In another study in Spain occurrence of nine antibiotics was measured at different points to assess the effect of hospitals and WWTPs effluents on a river. In this study, antibiotics, namely ofloxacin, azithromycin, trimethoprim, and metronidazole, were detected at high concentrations in downstream river samples (up to 131.0 ng/L for ofloxacin) with no detection in upstream of the WWTP discharge, and ciprofloxacin and sulfamethoxazole showed around ten-fold higher concentrations in downstream rather than in upstream samples (Rodriguez-Mozaz et al., 2015). These studies suggest that discharge of antibiotic through effluent into the natural environment can lead to selective pressure for the occurrence of antibiotic resistance.

The abuse of antibiotics for a long time has resulted in multi-resistant bacteria which carry multiple resistance genes (Icgen and Yilmaz, 2014; Lv et al., 2015; Xu et al., 2017). Multiple drug resistant pathogens are emerging with alarming rate. On September 20, 2017, WHO announced the antibiotic-resistant infections as the greatest risk to health, expected to reach a time in future when people fear common infections and threat their lives from minor surgery. It is reported that drug-resistant tuberculosis kills around 250,000 people each year (WHO, 2017). Antimicrobial resistance claims 25,000 lives in Europe and 23,000 in the US every year (Sachdeva et al., 2017). ARB cause serious disease, for example, methicillin-resistant *Staphylococcus aureus* (MRSA) can cause skin infections (a pimple, impetigo, and scalded skin syndrome), pneumonia, endocarditis, and toxic shock syndrome. Other ARB that can cause life-threatening disease are vancomycin-resistant *Enterococcus*, multi-drug-resistant *Mycobacterium tuberculosis*, and carbapenem-resistant *Enterobacteriaceae gut* bacteria. WHO scientists in July 2017 warned that antibiotic-resistant gonorrhea is growing and gonorrhea can cause very serious complications and sometimes is impossible to treat. Drug-resistant *Salmonella* species are a serious problem for public health worldwide (Su et al., 2004). The emergence of multiple drug resistant pathogenic species are even more problematic. Antibiotic resistance challenge is getting worse while new antibiotics exploration is decreasing, hence it is possible that there would be no defenses against infection in the future (McKinney and Pruden, 2012).

Increasing levels of ARB carrying antibiotic resistance genes (ARGs) in the environment, especially in water and wastewater is a human health issue (Rizzo et al., 2013; Devarajan et al., 2015; Sharma et al., 2016; Li H. et al., 2017). Diverse ARGs have reduced susceptibility of pathogens to different antibiotics like sulfonamide (*sul*), tetracycline (*tet*), fluoroquinolone (*qnr*), macrolide (*erm*), chloramphenicol (*cml, flo*), methicillin (*mec*), and b-lactam (*bla*). High concentrations of ARB and ARGs in industrial, community, clinical, and farming wastewaters are threats to the ecosystems (Devarajan et al., 2015). Surprisingly, based on a study in China, the total ARGs and ARB concentrations in sludge from hospitals were 3 to 4 orders of magnitude higher in residential area samples (Li et al., 2015).

A study on a WWTP using activated sludge and chlorination in their treatment trail showed that the concentration of total tetracycline resistance genes was 6.4×10^5 copies/mL in biosolids and 6.4×10^3 copies/mL in the effluent (Al-Jassim et al., 2015). In another study, at WWTP which applied activated sludge, chlorination and UV irradiation, the concentrations of *tet*(Q) and *tet*(G) in biosolid and effluent was reported to be 2.2×10^9 and 3.4×10^4 copies/mL, respectively. In addition, the copies of resistance genes normalized to the number of bacterial 16S rRNA genes at different sites of a natural river (Cache La Poudre River) ranged from 10^{-7} to 10^{-3} for ARGs [*sul*(1), *sul*(2), *tet*(W), and *tet*(O)](Pei et al., 2006). Therefore, the concentration of ARGs in the effluent of WWTPs is often more than the concentration of ARGs in the natural rivers, and discharge of WWTPs' effluent in the natural rivers lead to dissemination of ARGs in the environment. The study of ARGs occurrence in sediments, lakes, rivers, and soils prove this correlation (Sharma et al., 2016). In another study conducted by Xu et al. (2015) on a river that received the effluent of a WWTP and it was shown that the measured ARGs in the river were identical as found in the WWTP including nine tetracycline resistance genes, four sulfonamide resistance genes, and six quinolone resistance genes (Xu et al., 2015). Additionally, in another study, microbial analysis of water samples collected from 12 stations along Kizilirmak river showed that all the isolates have the multi antibiotic resistant ability. Resistance to aztreonam (63%), pefloxacin (54%), trimethoprim-sulfamethoxazole (54%), gentamicin (50%), oxacillin (46%), penicillin (38%), piperacillin (38%), and ampicillin (38%) were very common (Icgen and Yilmaz, 2014).

On February 2017, WHO published its first ever list of antibiotic-resistant "priority pathogens." The list includes three classes sorted by the urgency (critical, high and medium priority) with which new antibiotics are needed. Overall, 12 families of

TABLE 2 | WHO priority pathogens that need new antibiotics (Lawe-Davies and Bennett, 2017).

Bacteria	Antibiotic resistance
PRIORITY 1: CRITICAL	
Acinetobacter baumannii	Carbapenem
Pseudomonas aeruginosa	Carbapenem
Enterobacteriaceae	Carbapenem, ESBL[a]-producing
PRIORITY 2: HIGH	
Enterococcus faecium	Vancomycin
Staphylococcus aureus	Methicillin, vancomycin-intermediate, and resistant
Helicobacter pylori	Clarithromycin
Campylobacter spp.	Fluoroquinolone
Salmonellae	Fluoroquinolone
Neisseria gonorrhoeae	Cephalosporin, fluoroquinolone
PRIORITY 3: MEDIUM	
Streptococcus pneumoniae	Penicillin-non-susceptible
Haemophilus influenzae	Ampicillin
Shigella spp.	Fluoroquinolone

[a]*Extended Spectrum Beta-Lactamases. The ESBL enzyme breaks down and destroys most antibiotics causing them to be inactive, which is why they are not effective against infections caused by these types of bacteria.*

bacteria are the greatest threat to human health, as shown in **Table 2**.

Sources of ARGs

A low-level antibiotic resistance can occur via natural selection, however, the high level of ARB and ARGs in the environment is due to human activities. ARGs enter the environments from various sources, mainly human and animal sources. WWTPs are among the main anthropogenic sources for occurrence and spread of ARGs while land applications of manure are animal sources (Kemper, 2008; Rizzo et al., 2013). WWTPs receive the discharges from various sources and are hotspots for ARGs that are associated with clinical pathogens (Gao et al., 2012; Pruden et al., 2012; Riquelme et al., 2013; Devarajan et al., 2015; Di Cesare et al., 2016). Many studies demonstrated the presence of ARGs in wastewater that are associated with clinical pathogens (Tseng et al., 2009; Huerta et al., 2013; Rizzo et al., 2013; Devarajan et al., 2015; von Wintersdorff et al., 2016). These ARGs can move along the water cycle by means of wastewater discharge into other aquatic environments (Rizzo et al., 2013). Aquatic ecosystems are ideal sites for occurrence and spread of ARGs since they are constantly polluted by antimicrobial compounds resulting from anthropogenic activities (Rodriguez-Mozaz et al., 2015).

The genetic reactor is the term given to the places where genetic evolution occurs frequently and possibly evolving antibiotic resistance. There are four major places where the genetic evolution occurs frequently and antibiotic resistance evolves; 1. Human and animal microbiota, 2. Hospitals and longstanding care facilities, 3. Wastewater and any form of biological residues and 4. Soil and the surface or groundwater environments (Baquero et al., 2008).

The urban wastewaters may not ideally undergo an appropriate treatment, so the receiving environments may be impacted by wastewater discharges and antimicrobial compounds can be present at detectable concentration (Rodriguez-Mozaz et al., 2015). There are many rivers that have high concentrations of antibiotic, ARB, and ARGs in their sediment samples. Most of these rivers are impacted by urban wastewater while their pristine origins have no antibiotic or antibiotic resistant contaminations (Pei et al., 2006).

Agriculturally influenced regions, such as broiler feedlots and fishponds are known as important sources of ARGs in the environment (He et al., 2014; Yu et al., 2016). Researchers at the Arizona State University's Biodesign Institute inspected antibiotic use in shrimp, salmon, catfish, trout, tilapia, and swai, originating from 11 countries. In this study, the 47 antibiotics were assessed and researchers discovered traces of five antibiotics (Done and Halden, 2015). Animal husbandry is a major subscriber to the environmental burden of ARGs. Pig manure, with its abundant and diverse ARGs and sheer volume, is a major source of ARGs (Zhu et al., 2013). A study led by He et al. (2014) suggested investigating molecular signatures of more common ARGs like *tet*(W) and *sul*(1) or the ratio of *tet*(W): *sul*(1) to track probable sources of ARGs in complex aquatic environments. Based on these studies consideration of an array of ARGs on behalf of various classes will be beneficial to tracing anthropogenic sources of ARGs (He et al., 2014).

Another hotspot for the ARGs is constructed a wetland, and ARGs accumulated in constructed wetlands sediments are an imperative source of aqueous ARGs because of the regular release of microbes from sediments to water. The variation in patterns and concentrations of ARGs is greatly dependent on the operational and environmental factors of the constructed wetlands rather than to the pollutant source and the major source of ARGs in the constructed wetland is observed to be domestic sewage. In this regard, an integrated surface flow constructed wetland has been studied by Fang et al. (2017), and *sul*(1), *sul*(3), *tet*(A), *tet*(C), *tet*(E), and *qnr*(S) was observed. In this study, the microbial species, the presence of ARB, and the amount of absorbed contaminate like antibiotics and metals have been investigated (Fang et al., 2017). In another study, six mesocosm-scale constructed wetland with three flow types (surface flow, horizontal subsurface flow, and vertical subsurface flow) were set up. Based on this study, *sul*(1), *sul*(2), *sul*(3), *tet*(G), *tet*(M), *tet*(O), *tet*(X), *erm*(B), *erm*(C), *cml*(A) and *flo*(R) were observed in the wetlands (Chen et al., 2016).

Healthcare centers and hospitals are most important facilities with regard to antibiotic consumption and they are sources of ARB and ARGs (Devarajan et al., 2015; Rowe et al., 2017). Many ARGs including *tet*(M), *tet*(O), *tet*(S), *tet*(Q), *tet*(W), and *mec*(A) have been identified in microbial communities of hospital wastewaters due to the wide consumption of human antibiotics in the environments of the hospital (Zhang et al., 2009). The study on three hospital wastewaters showed that fluoroquinolones (among antibiotic families), and bla_{TEM}, *qnr*(S), *erm*(B), *sul*(1) and *tet*(W) (among ARGs) were detected at the highest concentration (Rodriguez-Mozaz et al., 2015). Devarajan et al. (2015) studied fate of a WWTP effluent that was receiving a wastewater from quite a few health care centers like Centre Hospitalier Universitaire Vaudois which is one of

the biggest and the most significant facilities. The effluent of this WWTP was discharged in Lake Geneva and qPCR quantification of fecal indicator bacteria and ARGs in sediment of that lake showed that the average of total bacterial load is 2.75×10^{11} copy number per each gram of dry sediment, and the relative abundance of bla_{CTX-M} and bla_{SHV} in core samples, top layers samples, and surface of core samples were 1.97×10^{-3}, 1.30×10^{-3}, and 3.64×10^{-6}. The reason for these high concentrations of ARGs is related to continuing medical usage of antibiotics like penicillin and aminoglycosides (Devarajan et al., 2015). Wastewater from hospitals is possibly the main source of pathogenic and antibiotic-resistant organisms and as well as the ARGs that are released into the environment. A study was conducted in Oslo city hospitals showed that hydrophobic antibiotics, like tetracycline or ciprofloxacin, were detected in all sludge samples of the hospital and fluoroquinolones were consistently found in hospital effluents (Baquero et al., 2008). The preliminary disinfection of hospital wastewater before its discharge into the sewage system or rivers can prohibit the spread ARGs into the environment.

Many studies aimed at the detection and quantification of ARGs in the aquatic and terrestrial environment like soil, surface waters, constructed wetlands, and WWTPs (Kemper, 2008; Pruden et al., 2013; Fang et al., 2017; Zheng et al., 2017). Among the main sources of ARB and ARGs, more attention should be given to leachates of municipal solid waste landfills. The high amount of antibiotics can dominate in municipal solid waste landfill leachates and may be a source of ARB and ARGs to the environment. The presence of antibiotics, metals, and organic pollutants in municipal solid waste and landfill leachate possibly will intensify the persistence of ARGs (Wu et al., 2015).

Spread

WHO classified ARB and ARGs as two major threats to public health in the twenty-first century, and spread of ARGs in aquatic ecosystems as an increasing concern (Rodriguez-Mozaz et al., 2015). One of the reasons why ARGs bear great concern is that they are related to mobile genetic elements and can easily pass between microorganisms by horizontal gene transfer (HGT). The HGT is one of the most important mechanisms leading to the distribution of antibiotic resistance in the environment. The transfer can happen from donor bacteria, phages, free DNA, or even from the dead cells to living cells (McKinney and Pruden, 2012; Sharma et al., 2016). There are four different mechanisms of HGT:

A. conjugation that is a process at which DNA is transferred from the donor cell to the recipient cell via sexual pilus and requires cell-to-cell contact. Then the recipient cell that was susceptible bacteria previously, become resistant as coded by these freshly acquired resistance genes,
B. transformation includes uptake, integration, and functional expression of naked DNA by naturally transformable bacteria,
C. transduction that is transfer of DNA from one bacterium into another through bacteriophages,
D. and gene transfer agents (GTAs) are bacteriophage-like elements made by several bacteria. GTAs carry random segments of DNA present in the host bacterium, which can be transduced to a recipient cell. GTA particles can be free through cell lysis and spread to a recipient cell (von Wintersdorff et al., 2016).

Aquatic ecosystems are ideal sites for occurrence and spread of ARGs since they are constantly polluted by antimicrobial compounds resulting from anthropogenic activities (Rodriguez-Mozaz et al., 2015). These ARGs accumulate in the ecosystem and transfer to clinical pathogens through HGT, causing the failure of antibiotic treatment in the future (Zhou et al., 2016). A study led by Zhou et al. (2016) explored prevalence of ARGs in dairy farms and detected a variety of ARGs and mobile genetic elements (transposase) in feces and soil samples. The results showed the positive correlation ($p < 0.001$) between the total amount of transposase genes and ARGs and suggested the high mobility of ARGs (Zhou et al., 2016).

Considerable amounts of one type of ARGs, such as class 1 integron gene (*intI1*), may lead to subsequent HGT in the WWTP and the spread and occurrence of ARGs and multi-resistant microorganisms (Du et al., 2015). It has been illustrated through a case study on observing variation of ARGs in municipal WWTP that adopted anaerobic/anoxic/aerobic membrane biological reactor (MBR) (Du et al., 2015). It is notable that different genes encoding for specific antibiotic are often located in the same position of chromosomes or mobile genetic elements which lead to multiple resistances (Xu et al., 2017). Therefore, mobile genetic elements for instance plasmids, transposons, and integrons play a significant role in the emergence and spread of ARGs (Zhu et al., 2013). The transposases mostly belong to a family of insertion sequences and are typically found flanking an array of resistance genes. Integrons contain resistance cassettes encoding different ARGs including aminoglycoside and sulfonamide resistance genes, as well as *qacEΔ1* efflux pump genes (Zhu et al., 2013). These multi-gene cassettes can encode different ARGs under a mutual promoter and help co-selection of ARGs, hence, selection pressure applied by one antibiotic may select for ARGs associated with diverse antibiotics within the gene cassette of the integron (Miller et al., 2013; Di Cesare et al., 2016). MRSA is a relevant example of the gaining a gene cassette that results in the transfer of multiple ARG simultaneously (Sharma et al., 2016). Trimethoprim resistance mechanism is also the replacement of a trimethoprim-sensitive dihydrofolate reductase by a plasmid-, transposon-, or cassette-borne trimethoprim-resistant dihydrofolate reductase (Zhang et al., 2009).

Although the spread and distribution of ARGs in different environmental systems are well studied, the ecological properties that could result in the selection of ARGs are lesser known, for example, the presence of heavy metals in the environment can cause co-selection of antibiotic and heavy metal resistance. Typical heavy metals such as Cu and Zn are used widely in industry and play important roles in increasing the abundance of certain ARGs (Li H. et al., 2017). Heavy metals are natural compounds present in different ecosystems and are known as a selective pressure on antibiotic resistance and heavy metals exposure may be responsible for antibiotic resistance in either the absence or the presence of antibiotics themselves (Peltier et al., 2010). Understanding heavy metal resistance in natural

ecosystems may help to understand antibiotic resistance in the environment since the molecular mechanisms influencing the selection of genes are similar and there are relations between the occurrence of heavy metal resistance genes (HMRGs) and ARGs (Knapp et al., 2017). The elements involved in the resistance to heavy metals are encoded in the chromosomes of bacteria like *Ralstonia metallidurans* (Mergeay et al., 2003), which is well adapted for surviving in naturally heavy metals-rich habitats (e.g., volcanic soils).

The fate of various ARGs and HMRGs have been studied and the results show that these genes can be divided into two groups. The first group includes genes that co-presence does not hint to their co-occurrence. For example, co-presence of *tet*(A), *qnr*(S) (ARGs), and *ars*(B), *czc*(A) (HMRGs) in WWTPs is just because WWTPs are hotspots of different microbial communities of both gram-positive and gram-negative bacteria. The second group of ARGs and HMRGs is showing a strong correlation to each other. For example, Di Cesare and his co-workers obtained a strong correlation between *sul*(2) and *czc*(A), however, the potential mechanisms of such co-selection were never explored (Di Cesare et al., 2016).

Environmental factors are significant subscribers to any types of ecosystems to the transport and spread of antibiotic resistance in the community (Riquelme et al., 2013). It is important to understand the mechanisms that lead to antibiotic resistance and detect factors that provide selective pressures in wastewater habitats (Rizzo et al., 2013). For example, antibiotics, quaternary ammonium compounds or high concentrations of heavy metals resulted in the selection of class 1 RIs-harboring bacteria (Rizzo et al., 2013). Total organic carbon (TOC) concentrations that is one of the environmental factors can affect the selection for some certain genes [*tet*(A), *erm*(B), and *qnr*(S)]. Hence an effluent of a WWTP with high TOC concentrations can change the magnitude and distribution of ARGs in receiving environments (Di Cesare et al., 2016).

TREATMENT STRATEGIES

The high amount of ARGs and antibiotics can dominate in WWTPs, landfills, municipal solid waste leachates, the soil of dairy farms, and surface waters. In order to limit the occurrence and spread of antibiotic resistance, treatment methods should be able to destroy ARGs in addition to inactivating pathogens (McKinney and Pruden, 2012). Efforts that have been made to combat ARGs are summarized in **Table 3** and are further discussed in this section.

Anaerobic and/or Aerobic Treatment Reactors

Aerobic and anaerobic treatment processes are low energy and environmentally friendly strategies which are mostly used to treat chemical oxygen demand (COD), moreover, they can successfully remove ARB and ARGs (Christgen et al., 2015). The aerobic treatment processes occur in the presence of air and microorganisms which use oxygen to convert organic contaminants to carbon dioxide, water, and biomass (aerobes). The anaerobic treatment processes, on the other hand, take place in the lack of air and microorganisms which do not require air to convert organic contaminants to methane and carbon dioxide gas and biomass (anaerobes) (Grady et al., 1999).

Samples of a municipal WWTP has been studied to evaluate the variation of five ARGs [*tet*(G), *tet*(W), *tet*(X), *sul*(1), and *intI*(1)] in the influent and effluent of each treatment unit. The WWTP possessed the anaerobic/anoxic/aerobic MBR process. The concentration of ARGs in wastewater diminished in the anaerobic and anoxic effluent, while increment in the aerobic effluent was observed. Later the ARGs concentration declined in the MBR discharge. Based on this study, it was concluded that anaerobic and anoxic treatments are much more successful to remove ARGs rather than aerobic treatment since microorganism has lower bioactivity under anaerobic condition and the propagation of resistance genes are inhibited (Du et al., 2015). There was significant positive correlation observed between the reduction of *tet*(W), *intI*(1), and *sul*(1) and the reduction of 16S rDNA in the wastewater treatment process (Du et al., 2015).

Anaerobic–aerobic sequence (AAS) bioreactors also is a low energy treatment option including an anaerobic treatment to diminish carbon concentration as a pretreatment and then aerobic treatment. Metagenomics studies of this treatment method showed the effect of this approach on antibiotic resistance in general and ARG in particular. AAS removed more than 85% of ARGs in the influent which means it was more efficient compared with aerobic and anaerobic units (83 and 62%, respectively; Christgen et al., 2015).

In another study, occurrence and release of tetracycline-resistant and sulfonamide-resistant bacteria, as well as three genes [*sul*(1), *tet*(W), and *tet*(O)] in the effluent of five WWTPs was studied, and performance of different processes was compared. ARGs and ARB removal ranged 2.57-log to 7.06-log in MBR, and 2.37-log to 4.56- log in activated sludge, oxidative ditch and rotatory biological contactors (Munir et al., 2011).

Removal of antibiotics including sulfamethazine, sulfamethoxazole, trimethoprim, and lincomycin had been studied in five different WWTPs using aerobic/anaerobic treatment methods (Behera et al., 2011). It was found that the removal efficiency of antibiotics was low compared with other pharmaceutical compounds. The removal efficiency of sulfamethazine, sulfamethoxazole, trimethoprim, and lincomycin was 13.1, 51.9, 69.0, and −11.2%, respectively. High load of lincomycin led to its negative removal efficiency (Behera et al., 2011).

To sum it up, biological treatment methods can remove antibiotics, ARB, and ARGs successfully if anaerobic and aerobic reactors operate in sequence, and aerobic reactors alone are not effective. If membrane-based technologies, like MBR, can be used in combination with biological treatment the better ARG removal efficiency would be achieved.

Constructed Wetlands

Constructed Wetlands are small semi-aquatic ecosystems, in which a great population of different microbial community multiplies and various physical-chemical reactions happen. Over the past years, man-made wetlands have been designed and they are known as attractive municipal, industrial and agricultural

TABLE 3 | Removal of ARGs by different treatment processes.

Target	Log removal	References
ANAEROBIC AND/OR AEROBIC TREATMENT REACTORS		
tet(G), *tet*(W), *tet*(X), *sul*(1), and *intI*(1)	–	Du et al., 2015
sul(1), *tet*(W), and *tet*(O)	2.37 to 7.06[a]	Munir et al., 2011
BIOCHAR		
sul genes	1.21	Ye et al., 2016
CONSTRUCTED WETLANDS		
sul(1), *sul*(2), *sul*(3), *tet*(G), *tet*(M), *tet*(O), *tet*(X), *erm*(B), *erm*(C), *cml*(A) and *flo*(R)	0.44 to 0.80	Chen et al., 2016
sul(1), *tet*(A), *tet*(C), *tet*(E), *qnr*(S), *sul*(1), *sul*(3), *tet*(A), *tet*(C), *tet*(E), and *qnr*(S)	0.39 to 0.65 removal rates of total 14 targeted ARGs	Fang et al., 2017
DISINFECTION		
tet(C), *tet*(G), *tet*(W), *tet*(X), *sul*(2), *drfA1*, *drfA7*, *erm*(B), *erm*(F), *erm*(Q), and *erm*(X)	0.1 to 2.3	Li H. et al., 2017
ere(A), *ere*(B), *erm*(A), *erm*(B), *tet*(A), *tet*(B), *tet*(M), and *tet*(O)	0.42 and 0.10 removal of *erm* and *tet* genes, respectively	Yuan et al., 2015
sul(1), *tet*(X), *tet*(G), *intI*(1), and 16S rRNA	1.30 to 1.49	Sharma et al., 2016
COAGULATION		
sul, *tet*, and integrase genes	0.5 to 3.1	Li N. et al., 2017

[a]*Conventional treatment plants and MBR facility.*

wastewater treatment approaches because of their simplicity, cost efficiency, and effect on eliminating ARGs (Fang et al., 2017). Characteristics of constructed wetland can affect ARB and ARGs removal efficiency. These characteristics are namely, flow configuration, plant species and flow types including (surface flow, horizontal subsurface flow, and vertical subsurface flow). Biodegradation, substrate adsorption, and plant uptake all play a certain role in decreasing the loadings of nutrients, antibiotics, and ARGs in the constructed wetlands, however, biodegradation is the most vital process in the removal of these pollutants (Chen et al., 2016).

Constructed Wetlands can efficiently remove aqueous ARGs however they can also act as reservoirs for specific ARGs. A study led by Fang et al. (2017) suggested constructed wetland as a domestic sewage treatment method. They attained 77.8 and 59.5% removal rates of total 14 targeted ARGs in the integrated surface flow constructed wetlands in the winter and summer season, respectively. The results of this study also found strong positive correlations between concentrations of intI1 and ARGs, indicating that mobile genetic elements affect the dissemination of ARGs in a constructed wetlands (Fang et al., 2017).

The removal of ARB and ARGs in raw domestic wastewater by differently constructed wetland has been investigated and 8 antibiotics and 12 genes, and 16S rRNA (bacteria) were studied in different matrices. The aqueous removal efficiencies of total antibiotics ranged from 75.8 to 98.6%, while those of total ARGs fluctuated between 63.9 and 84.0% by the constructed wetland. The presence of plants was beneficial to the removal of pollutants, and the subsurface flow constructed wetland had higher pollutant removal than the surface flow constructed wetlands, particularly for antibiotics (Chen et al., 2016).

Disinfection

Disinfection of water and wastewater is a process that kills a significant percentage of pathogenic organisms that may cause bacterial, viral or parasitic diseases. The most popular disinfection process in wastewater treatment is chlorination since it is available and effective, however, ozone and UV radiation also are employed. WWTPs are hotspots for ARB and ARGs, and there are so many heterotrophic bacteria in the effluents exhibiting resistance to multiple antibiotics (Pang et al., 2016). Effective disinfection is a vital and regular process for disruption of ARB and ARGs and inactivation of harmful microorganisms.

Effect of Chlorination on the removal of different antibiotics such as cephalexin, ciprofloxacin, chloramphenicol, erythromycin, gentamicin, rifampicin, sulfadiazine, tetracycline, and vancomycin have been previously monitored along with its effect on inactivation of ARB and ARGs. Advanced oxidation processes utilizing ozone, UV irradiation, Fenton reagent, and photocatalytic systems have also been applied to disinfection process. Some studies were also extended to two or more processes in regard to making a comparison of their efficacy and mechanism (Keen and Linden, 2013; Sharma et al., 2016).

Yuan et al. (2015), studied fates of nine different ARB and two series of ARGs [*ere*(A), *ere*(B), *erm*(A), *erm*(B), *tet*(A), *tet*(B), *tet*(M), and *tet*(O)] in treated wastewater using chlorination. Their detailed quantitative real-time PCR examination and analysis showed 60 and 20% removal of these genes, respectively, applying various doses of chlorine ranging from 15 to 300 $\frac{mg\ Cl_2}{min.\ L}$. All the bacteria, other than sulfadiazine- and erythromycin-resistant bacteria, were inactivated fully by just 15 $\frac{mg\ Cl_2}{min.\ L}$. Sulfadiazine- and erythromycin-resistant bacteria were inactivated when the chlorine dose was more than 60 $\frac{mg\ Cl_2}{min.\ L}$ (Yuan et al., 2015).

A recent study explored three disinfection methods including chlorination, single UV irradiation, and sequential UV/chlorination to compare their efficiency to combat ARGs in municipal WWTP (Sharma et al., 2016). ARGs including *sul*(1),

tet(X), *tet*(G), *intI*(1), and 16S rRNA were considered and the results proved a positive relationship between the inactivation of ARG and the parameters involving dosage of chlorine, contact time, and the maximum inactivation. Based on the results, maximum inactivation was in the range from 1.30-log to 1.49-log at 30 $\frac{mg}{min.\ L}$ of Cl_2 (Sharma et al., 2016). Single UV irradiation was less effective while sequential UV/chlorination had the maximum ARGs removal efficiency.

A similar lab-scale research was established to investigate the inactivation of *sul*(1), *tet*(G), and *intI*(1) by three different disinfection methods involving Chlorination, UV irradiation, and Ozonation. Samples for lab test were obtained from a municipal WWTP effluent and gene copies of *sul*(1), *tet*(G), *intI*(1), and 16S rDNA was measured (Zhuang et al., 2015). Results from this study suggest that chlorination was most effective in inactivation of ARGs compared to other methods (Zhuang et al., 2015).

Nanomaterial

Many diverse combinations of nanomaterial have proved that antimicrobial nanotechnology can be effective defenses against antibiotic resistance, ARB, and ARGs. Two different mechanisms are probable when nanoparticles treat antibiotic resistance; in the first mechanism a functionalized nanomaterial is combined with antibiotics and nanomaterial enters inside ARB and then release considerable amounts of toxic ions. In the second mechanism, a combination of antibiotic and nanomaterials result in synergistic effects, that is they combat ARGs separately (Aruguete et al., 2013).

The potential for antimicrobial nanomaterials to restrict the propagation of multi-drug resistant pathogens while avoiding the generation of new nanomaterial-resistant organisms was studied by a group of researchers led by Aruguete et al. (2013). They prepared a combination of nanomaterials functionalized with molecular antibiotics. This combination consisted of liposomes, dendrimers, and an antibiotic that is inside of a polymer nanoparticles capsules, and inorganic nanoparticles with antibiotic molecules attached to the surfaces (Aruguete et al., 2013). In this study, silver nanoparticles coated with a water-soluble polymer called polyvinylpyrrolidone were used to combat nanomaterial-resistant organisms (Aruguete et al., 2013). This experiment proved that nanomaterial combinations are able to perform like an antibiotic and are toxic to *Pseudomonas aeruginosa* bacteria which was resistant to multiple drugs (Aruguete et al., 2013).

Nanomaterials have been considered as a defense against multiple drug resistance because of their antimicrobial activity (Shahverdi et al., 2007; Monteiro et al., 2009; Lam et al., 2016; Yu et al., 2016). Antibacterial activities of nanoparticles depend on two fundamental elements, physicochemical properties of nanoparticles and type of target bacteria. Despite the fact that there is a great correlation in a few aspects of the antibacterial activity of nanoparticles, individual studies are challenging to generalize because the majority of researchers perform experiments in light of accessible nanoparticles and bacteria, instead of targeting particular and preferred nanoparticles or bacteria (Hajipour et al., 2012). Nanoparticles which are used in lab-scale studies are not well-known and correlating them with basic physicochemical properties for full-scale production is difficult.

Recently, a mixture of nanomaterials and molecular antibiotics attracts much attention since they are effective in killing multi-drug resistant isolates of pathogenic bacterial species and combating a broad spectrum of ARB and ARGs (Aruguete et al., 2013; Sharma et al., 2016). A few studies in regard to nanoparticles and their role in combating ARB and ARG are summarized in **Table 4**.

Nanomaterials play controversial roles regarding antibiotic resistance; on one hand, as mentioned before, they have been considered as a defense against multiple drug resistance because of their antimicrobial activity. On the other hand, nanomaterials can encourage the development of antibiotic resistance in the environment (Aruguete et al., 2013; Miller et al., 2013) and also some of them have shown toxic effects on fauna, flora, and human beings, such as infection, cytotoxicity, tissue ulceration, and reduction of cell capability (Srivastava et al., 2015). In this regard, silver nanoparticles which are tremendously used for its application in drug delivery, like Citrate-coated silver nanoparticles, demonstrated genotoxicity and cytotoxicity *in vitro* and *in vivo*. Besides, iron and iron oxide nanoparticles are widely used because of their magnetic target drug delivery potential, however, their *in vitro* cytotoxicity has been established (Srivastava et al., 2015).

Overall, more information is needed concerning the mechanisms behind the antimicrobial activity of nanomaterials and their potential for influencing the development of resistance and their toxic effect on the proper microflora of water or soil and more importantly on plants, animals, and humans.

Coagulation

Coagulation is an active method to remove colloidal particles in water and treat turbidity, color, natural organic matter, and heavy metals (Zainal-Abideen et al., 2012). Colloidal particles mostly have negative electrical charges while coagulants carry a positive charge. A coagulant neutralizes particles and makes them stick together when contact is made. The coagulation process as the tertiary treatment process in WWTPs is broadly utilized for improving water quality and removing contaminants.

Different types of organic and inorganic coagulants have been prepared based on the target contaminants. A study has been conducted to treat drinking water and assess removal of the natural organic matter by zirconium coagulant (Jarvis et al., 2012). Zirconium coagulant has improved water quality and provided lower dissolved organic carbon residual rather than iron coagulant (Jarvis et al., 2012). Furthermore, the flocs formed by using zirconium coagulant were larger and stronger compared to iron and aluminum salts coagulant. For example, floc sizes were 930, 710, and 450 μm for zirconium, iron and aluminum coagulant, respectively (Jarvis et al., 2012). Phosphorus contamination also can be removed by coagulation. Residual phosphorus in the effluent of WWTPs can result in

TABLE 4 | Nanoparticles combating ARB and ARGs.

Types of Nanoparticles	Source	Target	References
Nanosilver and sulfamethoxazole	Sulfamethoxazole Anaerobic Digester Sludge	ARGs: *tet*(O), *tet*(W), *sul*(1), *sul*(2), and *intl*(1)	Miller et al., 2013
Silver nanoparticles	An aquatic environment with Fe^{3+} or Fe^{2+} ions and natural organic matter	ARB: *Enterococcus faecalis* *Staphylococcus aureus* *Staphylococcus epidermidis* *Pseudomonas aeruginosa* *Klebsiella pneumoniae*	Adegboyega et al., 2014
Silver	Aqueous solution	ARB: *Mycobacterium tuberculosis* Multi-drug resistant *S. pneumoniae*	Singh et al., 2014
Nitric oxide releasing nanoparticles	Aqueous solution	ARB: *Acinetobacter baumanii* *methicillin-resistant S. aureus* *Klebsiella pneumoniae* *Pseudomonas aeruginosa*	
Silver nanoparticles with NOM and Iron	Aqueous solution	ARB: methicillin-resistant *S. aureus*	Sharma et al., 2014
Nanoparticles including copper oxide (CuO), zinc oxide (ZnO), and TiO_2	Aqueous environment	ARB: *E. coli* and *B. subtilis*	Pavithra et al., 2015
Ceftriaxone- ZnO Nanorods	Aquatic solutions of E. coli with ceftriaxone, ZnO nanorods and ceftriaxone-ZnO nanorods with phosphate-buffered solution	ARB: *E. coli*	Luo et al., 2013
Superparamagnetic iron oxide nanoparticles (conjugation of iron, zinc, and silver)	Treatment of medical device infections	ARB: multi- drug resistant *S. aureus* and antibiotic-resistant biofilms ARGs: *Staphylococcus*	Taylor et al., 2012
Nano alumina	Water	ARB: *E. coli* and *Salmonella* spp.	Qiu et al., 2012
Gold nanoparticles with vancomycin	Aqueous solution of polyvinyl alcohol	ARB: vancomycin resistant *S. aureus*, *E. coli* strain	Mohammed Fayaz et al., 2011
Iron oxide nanoparticles	Aqueous solution	ARB: *S. aureus*	Tran et al., 2010

eutrophication and affect the environment. A research has been directed that used electrochemical coagulation and to remove phosphorus. The results showed 97% phosphorus removal regardless of initial concentration (Tran et al., 2012). A study on the coagulation behavior of polyferric chloride showed that it can influence density, the stability of flocs and improve removal of algal cell, turbidity, color, and humic acid in eutrophicated water (Lei et al., 2009). Persistent organic pollutants in surface water, like perfluorooctane sulfonate and perfluorooctanoate, can be removed from drinking water by using alum and ferric chloride as a coagulant (Xiao et al., 2013). Solution pH, coagulant dosage, coagulants, natural organic matter concentration, turbidity, and flocculation time can affect the removal efficiency (Xiao et al., 2013).

In recent years, the effectiveness of coagulation technology in the removal of ARGs from treated wastewater has been investigated Li N. et al. (2017). Coagulation is an active method for ARG removal from WWTP effluent. Li H. et al. (2017) used inorganic coagulant $FeCl_3$ and inorganic polymer coagulant poly ferric chloride (PFC) and examined the removal of sul genes, tet genes, and integrase genes. Significant removal correlations were detected between dissolved NH_3-N and DOC[1] and ARGs. ARGs removal efficiency ranged from 0.5-log to 3.1-log reductions (Li N. et al., 2017).

Biochar

Biochar is active charcoal derived from the pyrolysis of carbon-rich biomass. Biochar is a porous material that has rich mineral elements and large specific surface area (Ye et al., 2016), and thus provide the most sites to be filled by sorption of contaminants.

IUPAC[2] classification categorized the porosity of biochar into micropores (<2 nm), mesopores (2–50 nm), and macropores (>50 nm) (Gray et al., 2014). The main treatment mechanism of biochar is sorption and base on the properties of contaminant, micropore-filling or macropore-filling would dominant for sorption on biochar. Moreover, electrostatic repulsion is another sorption mechanism that is attributed to complex properties of biochar. Different parameters including various proportions of carbon and inorganic fractions, pyrolytic temperature, and source of a compound that are used to prepare biochar would affect properties of biochar (Zheng et al., 2013).

The effect of biochar on ARGs has been studied recently and based on the results significant change in the microbial communities was observed after addition of biochar in the soil. Various types of biochar prepared from different feedstocks cause different changes in microbial community structures. Change in bacterial phylogenetic compositions can result in a change of ARGs, and thus the addition of different biochar to the manure

[1]Dissolved organic carbon.

[2]International Union of Pure and Applied Chemistry.

composting may have the effects on the relative abundance of ARGs (Cui et al., 2016).

Biochar amendment has been recently studied to assess its effects on preventing antibiotic, ARB, and ARGs from accumulating in tissues of vegetable that was cultivated in contaminated soil. Ye et al. (2016) conducted experiments in pots and cultivated lettuce on a soil contaminated by sulfonamides. They concluded that lettuce cultivation with biochar amendment can dissipate sulfonamides from soil and offer the highest growth indices. Furthermore, the concentration of sulfonamides and bacterial endophytes resistant to sulfonamides in roots and leaves were reduced by one to two orders of magnitude compared to vegetables grown without biochar amendment (Ye et al., 2016).

In a research study, antibiotic removal has been studied using biochars produced at different temperatures. The K_d for the sorption of sulfamethoxazole was found to be ranging from 30 to 1,675 L kg^{-1} showing that biochar has the potential for remediation of soil or water containing sulfonamides (Zheng et al., 2013).

Overall, using biochar is a practical strategy that can treat antibiotics contaminated soils and also prevent antibiotics, ARB, and ARGs from accumulating in the vegetation (Ye et al., 2016).

CONCLUSION AND RECOMMENDATION

The accelerating antibiotic resistance development among bacteria is a challenging issue that requires improvement of next-generation treatment processes. It has been observed that Low-energy anaerobic–aerobic treatment reactors reduce high concentrations of various ARGs from domestic wastewater. Over the past years, constructed wetlands with different flow configurations or plant species have been designed and they are known as attractive wastewater treatment approaches on eliminating ARGs from raw domestic wastewater. Most of the studies on the inactivation of ARG by disinfection have been conducted using chlorination, however, some studies were also extended to ultraviolet irradiation, allowing an evaluation of the two processes regarding their efficacy and mechanism. Recently, nanoparticles are known as antimicrobial agents that are effective to remove ARG and are known as a novel defense against ARB when accompanied by antibiotics. The coagulation process as the tertiary treatment process in WWTPs has recently known as an active method for ARGs removal and the use of biochar makes a notable change in the microbial communities and inactivate ARGs after addition of biochar amendment in the soil.

The emergence of antibiotic resistance among pathogens increases the demand for novel treatment strategies. Even though significant efforts have been made to investigate the treatment methods combating ARB and ARGs, there are considerable gaps to fill in:

- Studies have mainly been lab-scale or pilot-scale and over short operation time. It is important to conduct large-scale testing on real samples derived from the environment.
- Develop risk assessment studies to estimate precise values of the abundance of ARB and ARGs in WWTP discharges that do not trigger human health issues.
- Considering the effect of the operating conditions (pH, free available chlorine, HRT, SRT, Biomass concentration), environmental factors (temperature, COD, BOD, water flow), and mechanisms (mutation, selection, mechanisms of genetic exchange including conjugation, transduction, and transformation) that may increase antibiotic resistance bacteria and genes while treating water or wastewater in plants.
- Assessing the feasibility of implementing modifications to improve existing water and wastewater treatment facilities to increase ARBs and ARGs in the effluent of plants.
- Conduct further studies to determine the fate of ARBs and ARGs that encode the more extensive spectrum of antibiotics namely fluoroquinolone, ertapenem, and levofloxacin resistance in municipal wastewater.
- Future studies on the effect of stress conditions on ARG horizontal transfer and fate of ARGs at anaerobic digesters and aerobic reactors.
- Consider seasonal changes in ARB and ARGs amounts and perform experiments over sufficient time while using constructed wetlands as a treatment method. Exact factors controlling ARG levels and patterns in sediments of the constructed wetland system must be studied since they affect variation and patterns of ARGs.
- Investigating advanced treatment systems and combined disinfection methods to discover a suitable and cost-effective method to remove ARGs from WWTP effluents. Since the current disinfection units at WWTPs can only partially remove ARGs.
- Gain more information to figure out the mechanisms behind the antimicrobial activity of nanomaterials and their probable effect on the increase of resistance in the environment. For example, molecular mechanisms causing antimicrobial activity in nanomaterials and microbial interactions with nanomaterials should be studied to prevent the development of microbial resistance nanomaterials.
- Current practices ensured that some coagulants partially eliminate ARGs, however, further removal of ARGs from WWTPs should be examined because coagulation is an essential part of treatment plants and their potential to treat ARB and ARGs would be practical for many existing plants.
- Case studies on using biochar for treating water and wastewater, for example, evaluating filtration applications of biochar.

In light of all above-mentioned gaps in treatment strategies to combat ARB and ARGs, conducting further research in this area is essential to provide the opportunity to increase the efficiency of existing strategies or innovate new approaches.

AUTHOR CONTRIBUTIONS

FB and MM conceived, designed and formulated the outline of this manuscript. FB wrote the manuscript and MM provided feedback and discussion on the manuscript.

FUNDING

We acknowledge funding support from the University of North Carolina-Charlotte start-up funds.

ACKNOWLEDGMENTS

We thank Dr. Minal Zagade for critical reading of the manuscript.

REFERENCES

Adegboyega, N. F., Sharma, V. K., Siskova, K. M., Vecerova, R., Kolar, M., Zboril, R., et al. (2014). Enhanced formation of silver nanoparticles in Ag^{+}-NOM-iron(II, III) systems and antibacterial activity studies. *Environ. Sci. Technol.* 48, 3228–3235. doi: 10.1021/es405641r

Al-Jassim, N. M. I., Ansari, H. M., and Hong, P. Y. (2015). Removal of bacterial contaminants and antibiotic resistance genes by conventional wastewater treatment processes in Saudi Arabia: is the treated wastewater safe to reuse for agricultural irrigation? *Water Res.* 73, 277–290. doi: 10.1016/j.watres.2015.01.036

Aruguete, D. M., Kim, B., Hochella, M. F. Jr., Ma, Y., Cheng, Y., Hoegh, A., et al. (2013). Antimicrobial nanotechnology: its potential for the effective management of microbial drug resistance and implications for research needs in microbial nanotoxicology. *Environ. Sci. Process. Impacts* 15, 93–102. doi: 10.1039/c2em30692a

Baquero, F., Martínez, J. L., and Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265. doi: 10.1016/j.copbio.2008.05.006

Behera, S. K., Kim, H. W., Oh, J. E., and Park, H. S. (2011). Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. *Sci. Total. Environ.* 409, 4351–4360. doi: 10.1016/j.scitotenv.2011.07.015

Chen, J., Ying, G. G., Wei, X. D., Liu, Y. S., Liu, S. S., Hu, L. X., et al. (2016). Removal of antibiotics and antibiotic resistance genes from domestic sewage by constructed wetlands: effect of flow configuration and plant species. *Sci. Total. Environ.* 571, 974–982. doi: 10.1016/j.scitotenv.2016.07.085

Christgen, B., Yang, Y., Ahammad, S. Z., Li, B., Rodriquez, D. C., Zhang, T., et al. (2015). Metagenomics shows that low-energy anaerobic-aerobic treatment reactors reduce antibiotic resistance gene levels from domestic wastewater. *Environ. Sci. Technol.* 49, 2577–2584. doi: 10.1021/es505521w

Cui, E., Wu, Y., Zuo, Y., and Chen, H. (2016). Effect of different biochars on antibiotic resistance genes and bacterial community during chicken manure composting. *Bioresour. Technol.* 203, 11–17. doi: 10.1016/j.biortech.2015.12.030

Devarajan, N., Laffite, A., Graham, N. D., Meijer, M., Prabakar, K., Mubedi, J. I., et al. (2015). Accumulation of clinically relevant antibiotic-resistance genes, bacterial load, and metals in freshwater lake sediments in Central Europe. *Environ. Sci. Technol.* 49, 6528–6537. doi: 10.1021/acs.est.5b01031

Di Cesare, A., Eckert, E. M., D'Urso, S., Bertoni, R., Gillan, D. C., Wattiez, R., et al. (2016). Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Res.* 94, 208–214. doi: 10.1016/j.watres.2016.02.049

Done, H. Y., and Halden, R. U. (2015). Reconnaissance of 47 antibiotics and associated microbial risks in seafood sold in the United States. *J. Hazard. Mater.* 282, 10–17. doi: 10.1016/j.jhazmat.2014.08.075

Du, J., Geng, J., Ren, H., Ding, L., Xu, K., and Zhang, Y. (2015). Variation of antibiotic resistance genes in municipal wastewater treatment plant with A2O-MBR system. *Environ. Sci. Pollut. Res.* 22, 3715–3726. doi: 10.1007/s11356-014-3552-x

Fang, H., Zhang, Q., Nie, X., Chen, B., Xiao, Y., Zhou, Q., et al. (2017). Occurrence and elimination of antibiotic resistance genes in a long-term operation integrated surface flow constructed wetland. *Chemosphere* 173, 99–106. doi: 10.1016/j.chemosphere.2017.01.027

Gao, P., Munir, M., and Xagoraraki, I. (2012). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci. Tot. Environ.* 421–422, 173–183. doi: 10.1016/j.scitotenv.2012.01.061

Grady, C. P. L., Daigger, G. T., and Lim, H. C. (1999). "Biological wastewater treatment, in *Hazard. Waste,* Avaliable online at: http://www.loc.gov/catdir/enhancements/fy0647/98037263-d.html

Gray, M., Johnson, M. G., Dragila, M. I., and Kleber, M. (2014). Water uptake in biochars: the roles of porosity and hydrophobicity. *Biomass Bioenergy* 61, 196–205. doi: 10.1016/j.biombioe.2013.12.010

Hajipour, M. J., Fromm, K. M., Ashkarran, A. A., Jimenez de Aberasturi, D., de Larramendi, I. R., Rojo, T., et al. (2012). Antibacterial properties of nanoparticles. *Trends Biotechnol.* 30, 499–511. doi: 10.1016/j.tibtech.2012.06.004

He, L. Y., Liu, Y. S., Su, H. C., Zhao, J. L., Liu, S. S., Chen, J., et al. (2014). Dissemination of Antibiotic resistance genes in representative broiler feedlots environments: identification of indicator ARGs and correlations with environmental variables. *Environ. Sci. Technol.* 48, 13120–13129. doi: 10.1021/es5041267

Huerta, B., Marti, E., Gros, M., López, P., Pompêo, M., Armengol, J., et al. (2013). Exploring the Links between antibiotic occurrence, antibiotic resistance, and bacterial communities in water supply reservoirs. *Sci. Total Environ.* 456–457, 161–170. doi: 10.1016/j.scitotenv.2013.03.071

Icgen, B., and Yilmaz, F. (2014). Co-Occurrence of antibiotic and heavy metal resistance in Kizilirmak river isolates. *Bull. Environ. Contam. Toxicol.* 93, 735–743. doi: 10.1007/s00128-014-1383-6

Jarvis, P., Sharp, E., Pidou, M., Molinder, R., Parsons, S. A., and Jefferson, B. (2012). Comparison of coagulation performance and floc properties using a novel zirconium coagulant against traditional ferric and alum coagulants. *Water Res.* 46, 4179–4187. doi: 10.1016/j.watres.2012.04.043

Keen, O. S., and Linden, K. G. (2013). Degradation of antibiotic activity during UV/H2O2 advanced oxidation and photolysis in wastewater effluent. *Environ. Sci. Technol.* 47, 13020–13030. doi: 10.1021/es402472x

Kemper, N. (2008). Vetrinary antibiotics in the aquatic and terrestrial environment. *Ecol. Indic.* 8, 1–13. doi: 10.1016/j.ecolind.2007.06.002

Knapp, C. W., Callan, A. C., Aitken, B., Shearn, R., Koenders, A., and Hinwood, A. (2017). Relationship between antibiotic resistance genes and metals in residential soil samples from Western Australia. *Environ. Sci. Pollut. Res.* 24. 2484–2494. doi: 10.1007/s11356-016-7997-y

Kolár, M., Urbánek, K., and Látal, T. (2001). Antibiotic selective pressure and development of bacterial resistance. *Int. J. Antimicrob. Agents* 17, 357–363. doi: 10.1016/S0924-8579(01)00317-X

Lam, S. J., O'Brien-Simpson, N. M., Pantarat, N., Sulistio, A., Wong, E. H., Chen, Y. Y., et al. (2016). Combating multidrug-resistant gram-negative bacteria with structurally nanoengineered antimicrobial peptide polymers. *Nat. Microbiol.* 1:16162. doi: 10.1038/nmicrobiol.2016.162

Lawe-Davies, O., and Bennett, S. (2017). *WHO - List of Bacteria for Which New Antibiotics Are Urgently Needed*. WHO Department of Communications.

Lei, G., Ma, J., Guan, X., Song, A., and Cui, Y. (2009). Effect of basicity on coagulation performance of polyferric chloride applied in eutrophicated raw water. *Desalination* 247 518–529. doi: 10.1016/j.desal.2008.06.026

Li, H., Duan, M., Gu, J., Zhang, Y., Qian, X., Ma, J., et al. (2017). Effects of bamboo charcoal on antibiotic resistance genes during chicken manure composting. *Ecotoxicol. Environ. Saf.* 140, 1–6. doi: 10.1016/j.ecoenv.2017.01.007

Li, J., Cheng, W., Xu, L., Strong, P. J., and Chen, H. (2015). Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, Hospitals, and a municipal wastewater treatment plant system. *Environ. Sci. Pollut. Res.* 22, 4587–4596. doi: 10.1007/s11356-014-3665-2

Li, N., Sheng, G. P., Lu, Y.-Z., Zeng, R. J., and Yu, H.-Q. (2017). Removal of antibiotic resistance genes from wastewater treatment plant effluent by coagulation. *Water Res.* 111, 204–212. doi: 10.1016/j.watres.2017.01.010

Luo, Z., Wu, Q., Xue, J., and Ding, Y. (2013). Selectively enhanced antibacterial effects and ultraviolet activation of antibiotics with Zno nanorods against *Escherichia coli*. *J. Biomed. Nanotechnol.* 9, 69–76. doi: 10.1166/jbn.2013.1472

Lv, L., Yu, X., Xu, Q., and Ye, C. (2015). Induction of bacterial antibiotic resistance by mutagenic halogenated nitrogenous disinfection byproducts. *Environ. Pollut.* 205, 291–298. doi: 10.1016/j.envpol.2015.06.026

McKinney, C. W., and Pruden, A. (2012). Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater. *Environ. Sci. Technol.* 46, 13393–13400. doi: 10.1021/es303652q

Mergeay, M. S., Monchy, T., Vallaeys, V., Auquier, A., Benotmane, P., Bertin, S., et al. (2003). *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS Microbiol. Rev.* 27, 385–410. doi: 10.1016/S0168-6445(03)00045-7

Miller, J. H., Novak, J. T., Knocke, W. R., Young, K., Hong, Y., Vikesland, P. J., et al. (2013). Effect of silver nanoparticles and antibiotics on antibiotic resistance genes in anaerobic digestion. *Water Environ. Res.* 85, 411–421. doi: 10.2175/106143012X13373575831394

Mohammed Fayaz, A., Girilal, M., Mashihur, R., Venkatesan, R., and Kalaichelvan, P. T. (2011). Biosynthesis of silver and gold nanoparticles using thermophilic bacterium *Geobacillus stearothermophilus*. *Process Biochem.* 46, 1958–1962. doi: 10.1016/j.procbio.2011.07.003

Monteiro, D. R., Gorup, L. F., Takamiya, A. S., Ruvollo-Filho, A. C., de Camargo, E. R., and Barbosa, D. B. (2009). The growing importance of materials that prevent microbial adhesion: antimicrobial effect of medical devices containing silver. *Int. J. Antimicrob. Agents* 34, 103–110. doi: 10.1016/j.ijantimicag.2009.01.017

Munir, M., Wong, K., and Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* 45, 681–693. doi: 10.1016/j.watres.2010.08.033

Pang, Y., Huang, J., Xi, J., Hu, H., and Zhu, Y. (2016). Effect of ultraviolet irradiation and chlorination on ampicillin-resistant *Escherichia coli* and its ampicillin resistance gene. *Front. Environ. Sci. Eng.* 10, 522–530. doi: 10.1007/s11783-015-0779-9

Pavithra, M., Menezes, R., Suarez, J., and Santra, S. (2015). Antimicrobial properties of copper and silver loaded silica nanomaterials. *Acta Mater.* 46, 17–26. doi: 10.1002/9781118217511.ch6

Pei, R., Kim, S. C., Carlson, K. H., and Pruden, A. (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding Antibiotic Resistance Genes (ARG). *Water Res.* 40, 2427–2435. doi: 10.1016/j.watres.2006.04.017

Peltier, E., Vincent, J., Finn, C., and Graham, D. W. (2010). Zinc-induced antibiotic resistance in activated sludge bioreactors. *Water Res.* 44, 3829–3836. doi: 10.1016/j.watres.2010.04.041

Pruden, A., Arabi, M., and Storteboom, H. N. (2012). Correlation between upstream human activities and riverine antibiotic resistance genes. *Environ. Sci. Technol.* 46, 11541–11549. doi: 10.1021/es302657r

Pruden, A., Larsson, D. G. J., Amézquita, A., Collignon, P., Brandt, K. K., Graham, D. W., et al. (2013). Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ. Health Perspect.* 121, 878–885. doi: 10.1289/ehp.1206446

Qiu, Z., Yu, Y., Chen, Z., Jin, M., Yang, D., Zhao, Z., et al. (2012). Nanoalumina promotes the horizontal transfer of multiresistance genes mediated by plasmids across genera. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4944–4949. doi: 10.1073/pnas.1107254109

Riquelme, B., Maria, V., Novak, J. T., Vikesland, P. J., and Pruden, A. (2013). Effect of wastewater colloids on membrane removal of antibiotic resistance genes. *Water Res.* 47, 130–140. doi: 10.1016/j.watres.2012.09.044

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., et al. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–360. doi: 10.1016/j.scitotenv.2013.01.032

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., et al. (2015). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* 69, 234–242. doi: 10.1016/j.watres.2014.11.021

Rowe, W. P. M., Baker-Austin, C., Verner-Jeffreys, D. W., Ryan, J. J., Micallef, C., Maskell, D. J., et al. (2017). Overexpression of antibiotic resistance genes in hospital effluents over time. *J. Antimicrob. Chemother.* 72, 1617–1623. doi: 10.1093/jac/dkx017

Shahverdi, A. R., Fakhimi, A., Shahverdi, H. R., and Minaian, S. (2007). Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*. *Nanomedicine* 3, 168–171. doi: 10.1016/j.nano.2007.02.001

Sharma, V. K., Johnson, N., Cizmas, L., McDonald, T. J., and Kim, H. (2016). A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes. *Chemosphere* 150, 702–714. doi: 10.1016/j.chemosphere.2015.12.084

Sharma, V. K., Siskova, K. M., Zboril, R., and Gardea-Torresdey, J. L. (2014). Organic-coated silver nanoparticles in biological and environmental conditions: fate, stability and toxicity. *Adv. Colloid Interface Sci.* 204, 15–34. doi: 10.1016/j.cis.2013.12.002

Sachdeva, S., Palur, R. V., Sudhakar, K. U., and Rathinavelan, T. (2017). *E. Coli* group 1 capsular polysaccharide exportation nanomachinary as a plausible antivirulence target in the perspective of emerging antimicrobial resistance. *Front. Microbiol.* 8:70. doi: 10.3389/fmicb.2017.00070

Singh, R., Smitha, M. S., and Singh, S. P. (2014). The role of nanotechnology in combating multi-drug resistant bacteria. *J. Nanosci. Nanotechnol.* 14, 4745–56. doi: 10.1166/jnn.2014.9527

Srivastava, V., Gusain, D., and Sharma, Y. C. (2015). Critical review on the toxicity of some widely used engineered nanoparticles. *Ind. Eng. Chem. Res.* 54, 6209–6233. doi: 10.1021/acs.iecr.5b01610

Su, L. H., Chiu, C. H., Chu, C., and Ou, J. T. (2004). Antimicrobial resistance in nontyphoid salmonella serotypes: a global challenge. *Clin. Infect. Dis.* 39, 546–551. doi: 10.1086/422726

Taylor, E. N., Kummer, K. M., Durmus, N. G., Leuba, K., Tarquinio, K. M., and Webster, T. J. (2012). Superparamagnetic iron oxide nanoparticles (SPION) for the treatment of antibiotic-resistant biofilms. *Small* 8, 3016–3027. doi: 10.1002/smll.201200575

Tran, N., Drogui, P., Blais, J. F., and Mercier, G. (2012). Phosphorus removal from spiked municipal wastewater using either electrochemical coagulation or chemical coagulation as tertiary treatment. *Sep. Purif. Technol.* 95, 16–25. doi: 10.1016/j.seppur.2012.04.014

Tran, N., Mir, A., Mallik, D., Sinha, A., Nayar, S., and Webster, T. J. (2010). Bactericidal effect of iron oxide nanoparticles on *Staphylococcus aureus*. *Int. J. Nanomed.* 5, 277–283.

Tseng, Y. S., Wu, D. C., Chang, C. Y., Kuo, C. H., Yang, Y. C., Jan, C. M., et al. (2009). Amoxicillin resistance with β-lactamase production in *Helicobacter pylori*. *Eur. J. Clin. Invest.* 39, 807–812. doi: 10.1111/j.1365-2362.2009.02166.x,

von Wintersdorff, C. J., Penders, J., Van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., et al. (2016). Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front. Microbiol.* 7:173. doi: 10.3389/fmicb.2016.00173

WHO (2017). *Antibacterial Agents in Clinical Development: An Analysis of the Antibacterial Clinical Development Pipeline, Including Tuberculosis*. World Health Organization. WHO/EMP/IAU/2017.11.

Wu, D., Huang, Z., Yang, K., Graham, D., and Xie, B. (2015). Relationships between antibiotics and antibiotic resistance gene levels in municipal solid waste leachates in Shanghai, China. *Environ. Sci. Technol.* 49, 4122–4128. doi: 10.1021/es506081z

Xiao, F., Simcik, M. F., and Gulliver, J. S. (2013). Mechanisms for Removal of Perfluorooctane Sulfonate (PFOS) and Perfluorooctanoate (PFOA) from drinking water by conventional and enhanced coagulation. *Water Res.* 47, 49–56. doi: 10.1016/j.watres.2012.09.024

Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., et al. (2015). Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. *Chemosphere* 119, 1379–1385. doi: 10.1016/j.chemosphere.2014.02.040

Xu, Y. B., Hou, M. Y., Li, Y. F., Huang, L., Ruan, J. J., Zheng, L., et al. (2017). Distribution of tetracycline resistance genes and AmpC β-lactamase genes in representative non-urban sewage plants and correlations with treatment processes and heavy metals. *Chemosphere* 170, 274–281. doi: 10.1016/j.chemosphere.2016.12.027

Ye, M., Sun, M., Feng, Y., Wan, J., Xie, S., Tian, D., et al. (2016). Effect of Biochar amendment on the control of soil sulfonamides, antibiotic-resistant

bacteria, and gene enrichment in lettuce tissues. *J. Hazard. Mater.* 309, 219–227. doi: 10.1016/j.jhazmat.2015.10.074

Yu, W., Zhan, S., Shen, Z., Zhou, Q., and Yang, D. (2016). Efficient removal mechanism for antibiotic resistance genes from aquatic environments by graphene oxide nanosheet. *Chem. Eng. J.* 313, 836–846. doi: 10.1016/j.cej.2016.10.107

Yuan, Q. B., Guo, M. T., and Yang, J. (2015). Fate of antibiotic resistant bacteria and genes during wastewater chlorination: implication for antibiotic resistance control. *PLoS ONE* 10:e0119403. doi: 10.1371/journal.pone.0119403

Zainal-Abideen, M., Aris, A., Yusof, F., Abdul-Majid, Z., Selamat, A., and Omar, S. I. (2012). Optimizing the coagulation process in a drinking water treatment plant - comparison between traditional and statistical experimental design Jar tests. *Water Sci. Technol.* 65, 496–503. doi: 10.2166/wst.2012.561

Zhang, X. X., Zhang, T., and Fang, H. H. (2009). Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol.* 82, 397–414. doi: 10.1007/s00253-008-1829-z

Zheng, H., Wang, Z., Zhao, J., Herbert, S., and Xing, B. (2013). Sorption of antibiotic sulfamethoxazole varies with biochars produced at different temperatures. *Environ. Pollut.* 181, 60–67. doi: 10.1016/j.envpol.2013.05.056

Zheng, J., Su, C., Zhou, J., Xu, L., Qian, Y., and Chen, H. (2017). Effects and mechanisms of ultraviolet, chlorination, and ozone disinfection on antibiotic resistance genes in secondary effluents of municipal wastewater treatment plants. *Chem. Eng. J.* 317, 309–316. doi: 10.1016/j.cej.2017.02.076

Zhou, B., Wang, C., Zhao, Q., Wang, Y., Huo, M., Wang, J., et al. (2016). Prevalence and dissemination of antibiotic resistance genes and coselection of heavy metals in chinese dairy farms. *J. Hazard. Mater.* 320, 10–17. doi: 10.1016/j.jhazmat.2016.08.007

Zhu, Y. G., Johnson, T. A., Su, J. Q., Qiao, M., Guo, G. X., Stedtfeld, R. D., et al. (2013). Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3435–3440. doi: 10.1073/pnas.1222743110

Zhuang, Y., Ren, H., Geng, J., Zhang, Y., Zhang, Y., Ding, L., et al. (2015). Inactivation of antibiotic resistance genes in municipal wastewater by chlorination, ultraviolet, and ozonation disinfection. *Environ. Sci. Pollut. Res.* 22, 7037–7044. doi: 10.1007/s11356-014-3919-z

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 September 2017
doi: 10.3389/fmicb.2017.01781

Phenotypic and Transcriptomic Responses of *Campylobacter jejuni* Suspended in an Artificial Freshwater Medium

Hana Trigui[1], Kristen Lee[1], Alexandre Thibodeau[2], Simon Lévesque[3], Nilmini Mendis[1], Philippe Fravalo[2], Ann Letellier[2] and Sébastien P. Faucher[1]**

[1] Department of Natural Resource Sciences, Faculty of Agricultural and Environmental Sciences, McGill University, Sainte-Anne-de-Bellevue, QC, Canada, [2] Research Chair in Meat Safety, Department of Pathology and Microbiology, University of Montreal, Saint-Hyacinthe, QC, Canada, [3] Laboratoire de Santé Publique du Québec (LSPQ)/Institut National de Santé Publique du Québec, Sainte-Anne-de-Bellevue, QC, Canada

OPEN ACCESS

Edited by:
Peiying Hong,
King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Christopher L. Hemme,
University of Rhode Island, United States
Michael Iakiviak,
University of Illinois at Urbana–Champaign, United States

***Correspondence:**
Hana Trigui
hanatrigui@gmail.com
Sébastien P. Faucher
sebastien.faucher2@mcgill.ca

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: 21 June 2017
Accepted: 01 September 2017
Published: 20 September 2017

Citation:
Trigui H, Lee K, Thibodeau A, Lévesque S, Mendis N, Fravalo P, Letellier A and Faucher SP (2017) Phenotypic and Transcriptomic Responses of Campylobacter jejuni Suspended in an Artificial Freshwater Medium. Front. Microbiol. 8:1781. doi: 10.3389/fmicb.2017.01781

Campylobacter jejuni is the leading cause of campylobacteriosis in the developed world. Although most cases are caused by consumption of contaminated meat, a significant proportion is linked to ingestion of contaminated water. The differences between *C. jejuni* strains originating from food products and those isolated from water are poorly understood. Working under the hypothesis that water-borne *C. jejuni* strains are better equipped at surviving the nutrient-poor aquatic environment than food-borne strains, the present study aims to characterize these differences using outbreak strains 81116 and 81-176. Strain 81116 caused a campylobacteriosis outbreak linked to consumption of water, while strain 81-176 was linked to consumption of raw milk. CFU counts and viability assays showed that 81116 survives better than 81-176 at 4°C in a defined freshwater medium (Fraquil). Moreover, 81116 was significantly more resistant to oxidative stress and bile salt than strain 81-176 in Fraquil. To better understand the genetic response of 81116 to water, a transcriptomic profiling study was undertaken using microarrays. Compared to rich broth, strain 81116 represses genes involved in amino acid uptake and metabolism, as well as genes involved in costly biosynthetic processes such as replication, translation, flagellum synthesis and virulence in response to Fraquil. In accordance with the observed increase in stress resistance in Fraquil, 81116 induces genes involved in resistance to oxidative stress and bile salt. Interestingly, genes responsible for cell wall synthesis were also induced upon Fraquil exposure. Finally, twelve unique genes were expressed in Fraquil; however, analysis of their distribution in animal and water isolates showed that they are not uniquely and ubiquitously present in water isolates, and thus, unlikely to play a major role in adaptation to water. Our results show that some *C. jejuni* strains are more resilient than others, thereby challenging current water management practices. The response of 81116 to Fraquil serves as a starting point to understand the adaptation of *C. jejuni* to water and its subsequent transmission.

Keywords: *C. jejuni*, microarrays, survival, oxidative stress, sodium choleate, starvation, cell wall

INTRODUCTION

Campylobacter jejuni is the leading cause of bacterial food-borne diarrheal disease in the developed world (Dasti et al., 2010). Acute *C. jejuni* infection causes watery to bloody diarrhea, with fever, nausea, and vomiting, and can be fatal to vulnerable individuals (Butzler and Skirrow, 1979; Walker et al., 1986). Although the infection is often self-limiting, it has been reported to lead to the development of secondary autoimmune disorders such as the Guillain-Barré or Miller-Fisher syndromes (Wassenaar and Blaser, 1999; Young et al., 2007). *C. jejuni* is a prevalent human pathogen but is usually viewed as commensal in livestock, particularly in poultry (Inglis and Kalischuk, 2004; Boes et al., 2005; Gormley et al., 2008). The majority of human infections occur directly through consumption of raw or undercooked contaminated animal products, such as meat and milk, or indirectly through cross-contamination events in the consumer kitchen. Nevertheless, animal products are not the sole route of transmission of *C. jejuni* to humans. Analysis of waterborne outbreaks and sporadic cases show that water is an important environmental reservoir for *C. jejuni* (Bolton et al., 1982; Thomas et al., 1999; Huang et al., 2015). Contamination of surface water and well water may occur due to direct deposition of animal feces, sewage discharge and farmland run-offs (Vogt et al., 1982; Lind et al., 1996; Clark et al., 2003; O'Reilly et al., 2007; Bronowski et al., 2014; Huang et al., 2015).

Campylobacter jejuni is a microaerophilic bacterium that grows best at temperatures ranging from 37 to 42°C, and requires a rich growth medium (Skirrow, 1991). In the host digestive tract, *C. jejuni* encounters various challenges, such as acidity, antimicrobial bile salts, resident microorganisms, fluctuations in osmolarity, and effectors of the immune system (Fordtran and Locklear, 1966; Stintzi et al., 2005). Once expelled from one host, *C. jejuni* is exposed to and must survive a different set of stress conditions before colonizing another host (Bronowski et al., 2014). The conditions encountered within the different transmission routes are variable; transmission to humans through contaminated meat subjects the bacterium to stresses that are different from those found when its transmission occurs through water (Bronowski et al., 2014). Potential stresses encountered in water include nutrient scarcity, extreme temperatures, disinfectant and osmotic stresses (Thomas et al., 1999; Murphy et al., 2006; Jackson et al., 2009). *C. jejuni* must therefore overcome these challenges to survive and use water as a transmission route. Presumably, strains that survive the best in water are the most likely to be succesfully transmitted between hosts through this medium. Supporting this hypothesis, some *Campylobacter* multilocus sequence type (ST) complexes (ST-2381, ST-45, and ST-1225) were found to be more commonly associated to water (Sopwith et al., 2008; Carter et al., 2009). The high incidence of ST-45 in river water isolates and in human infections could indicate that it is well adapted to environmental transmission routes (Murphy et al., 2005; Lévesque et al., 2013). Indeed, *C. jejuni* strains assigned to ST-45 survive heat, aerobic and oxidative stresses better than other sequence types (Habib et al., 2010).

The multiple transmission routes of *C. jejuni* suggest that some strains may possesses effective mechanisms that allow it to sense and cope with a variety of stresses present in a given niche (Bronowski et al., 2014). Contributing to the survival success of *C. jejuni* is its ability to persist in natural environments by adapting lifestyles other than the planktonic form. Consequently, *C. jejuni* can be found as a free-living member of complex multispecies biofilm (Nguyen et al., 2011), internalized within some waterborne protozoa (Axelsson-Olsson et al., 2005; Snelling et al., 2005; Baffone et al., 2006), excreted within multilamellar bodies (MLBs) by ciliates (Trigui et al., 2016) and viable but non-culturable (VBNC) cells (Rollins and Colwell, 1986; Murphy et al., 2006). Given that water is a vehicle for the spread of *C. jejuni*, many studies have focused on the survival of *Campylobacter* in different types of water, such as tap water (Buswell et al., 1998; Cools et al., 2003), bottled mineral water (Tatchou-Nyamsi-König et al., 2007), artificial seawater (ASW) (Baffone et al., 2006; Trigui et al., 2015b) and a defined freshwater medium (Fraquil) (Trigui et al., 2015b). Notably, several *Campylobacter* strains were found to enter the VBNC state after exposure to the aforementioned water sources (Cools et al., 2003; Baffone et al., 2006; Trigui et al., 2015b). VBNC *C. jejuni* cells were able to maintain their ability to adhere to intestinal cells after 3 weeks in freshwater at 4°C (Patrone et al., 2013). In addition, *Campylobacter*-naïve chicks that consumed water contaminated by VBNC *C. jejuni* were successfully colonized by the bacterium (Pearson et al., 1993). Therefore, VBNC *C. jejuni* are considered a threat to public health (Murphy et al., 2006).

Some of the *C. jejuni* stress response mechanisms and their regulators that have been studied to-date are distinct from those in other enteric Gram-negative pathogens, while others remain poorly understood. For example, full genome analyses of *C. jejuni* strains suggest that this pathogen has a relatively small genome (Parkhill et al., 2000) and lacks many classical stress tolerance regulators, such as the stationary phase sigma factor RpoS, the oxidative stress response regulators SoxRS and OxyR, and the osmotic shock regulator BetAB (Murphy et al., 2006). In contrast, genes related to iron metabolism and oxidative stress defense, which are controlled by the ferric uptake regulator (Fur) and the peroxide responsive regulator (PerR), respectively, are key factors for *C. jejuni*'s survival *in vivo* (Palyada et al., 2009; Flint et al., 2014; Butcher et al., 2015).

Recently, we investigated the survival of *C. jejuni* chicken cecal isolates in Fraquil, artificial sea water and Fraquil supplemented with salt (Trigui et al., 2015b). Fraquil is an artificial freshwater medium used to study the behavior of bacteria in water (Mendis et al., 2015). The strains tested varied significantly in their ability to survive in the three aforementioned water systems, presumably due to genetic differences between the isolates. In the present study, the survival of two additional model strains was evaluated in Fraquil. Strain 81116 remained culturable and viable longer than strain 81-176 in Fraquil. Moreover, strain 81116 was more resistant to oxidative stress and exposure to bile salts after incubation in water relative to 81-176. Given that 81116 was better adapted to surviving this aquatic environment, we performed a microarray analysis to uncover its transcriptomic response when exposed to water.

MATERIALS AND METHODS

Bacterial Strains and Media

The *C. jejuni* strains used in this study are listed in **Table 1**. *C. jejuni* was stored at −80°C in Brucella broth containing 10% glycerol. *C. jejuni* was routinely grown on TSA-blood plates (1.5% pancreatic digest of casein, papaic digest of soybean, 0.5% sodium chloride, 1.5% agar and 5% defibrinated sheep blood) for 2 days at 42°C under a microaerophilic atmosphere generated with the CampyGen system (Oxoid). For liquid culture, *C. jejuni* was grown in Brucella broth (BD Biosciences) containing 10 g/L pancreatic digest of casein, 10 g/L peptic digest of animal tissue, 1 g/L dextrose, 2 g/L yeast extract, 5 g/L sodium chloride, 0.1 g/L sodium bisulfite.

Survival in Fraquil

The survival of *C. jejuni* strains 81116 and 81-176 was evaluated in the artificial freshwater media Fraquil as described previously (Trigui et al., 2015b). The composition of Fraquil is 0.004% (wt/vol) $CaCl_2$, 0.004% $MgSO_4$, 0.001% $NaHCO_3$, 0.0002% K_2HPO_4, 0.004% $NaNO_3$, 10 nM $FeCl_3$, 1 nM $CuSO_4$, 0.22 nM $(NH_4)_6Mo_7O_{24}$, 2.5 nM $CoCl_2$, 23 nM $MnCl_2$ and 4 nM $ZnSO_4$ (Morel et al., 1975). Bacteria grown on agar plates were suspended in Fraquil in a 5 ml plastic tube (Sarstedt), washed three times with Fraquil, and the optical density was adjusted to 0.1 at 600 nm (OD_{600}). Centrifugation for the washing steps were performed at 5,000 *g* for 10 min at room temperature. The suspensions were then further diluted 1:5 in Fraquil, and then incubated at 4°C or 25°C. CFU counts were determined periodically on TSA-blood plates.

LIVE/DEAD Staining

The BacLight™ LIVE/DEAD® bacterial viability kit (Life Technologies) was used to stain *C. jejuni* in Fraquil according to the manufacturer's protocol. A Guava easyCyte flow cytometer (EMD Millipore) was used to analyze stained cells as described previously (Trigui et al., 2015a). Sterile Fraquil containing the LIVE/DEAD stain was used as a blank. Freshly cultured *C. jejuni* was used as the live control and *C. jejuni* incubated in boiling water for 10 min was used as the dead control for data analysis. Controls were performed for each strain. Both controls and samples were diluted to an OD_{600} of 0.01 before staining and analysis by flow cytometry.

Stress Resistance Tests

The procedure to test the sensitivity of *C. jejuni* to sodium hypochlorite, hydrogen peroxide and sodium choleate were adapted from a previous study (Levi et al., 2011). Briefly, one milliliter aliquots of 81116 and 81-176 were retrieved from 4h-old Fraquil or Brucella broth suspensions incubated at 4°C and transferred to a 24-well plate (Sarstedt). Each strain was treated in triplicate for 1 h with 500 μM of H_2O_2 (Sigma–Aldrich) or 100 mg/ml of Na-choleate (Sigma–Aldrich). For the sodium hypochlorite test, Clorox bleach solution containing 10.3% of sodium hypochlorite was added to the wells at different final concentrations (0.0001, 0.00013, 0.0002, and 0.0003%). No treatment controls were also included. The samples were incubated at 4°C for 1 h prior to plating on TSA-blood agar plates for CFU enumeration. The differences in CFU counts between the controls and the treatments were calculated for each strain and stress condition.

TABLE 1 | Strains used in this study.

Name	Origin	Condition of isolation	Reference
81116	Human	Clinical isolate (water-borne outbreak)	Korlath et al., 1985
81-176	Human	Clinical isolate (raw milk-borne outbreak)	Palmer et al., 1983
NCTC11168_H	Human	Clinical isolate	Ahmed et al., 2002
RM1221_C	Chicken	Store-bought chicken carcass	Miller et al., 2000
L2003a_C	Chicken	Caecal content at time of slaughter	Thibodeau et al., 2015
T2003a_C	Chicken	Caecal content at time of slaughter	Thibodeau et al., 2015
D2008aC	Chicken	Caecal content at time of slaughter	Thibodeau et al., 2015
F2008d_C	Chicken	Caecal content at time of slaughter	Thibodeau et al., 2015
F2008a_C	Chicken	Caecal content at time of slaughter	Thibodeau et al., 2015
G2008b_C	Chicken	Caecal content at time of slaughter	Thibodeau et al., 2015
A2008a_C	Chicken	Caecal content at time of slaughter	Thibodeau et al., 2015
006A0089_B	Bovine	Fresh feces sample picked at the farm	Lévesque et al., 2013
007A0289_W	Water	Environmental surface water	Lévesque et al., 2013
007A0333_W	Water	Environmental surface water	Lévesque et al., 2013
007A0418_W	Water	Environmental surface water	Lévesque et al., 2013
007A0613_W	Water	Environmental surface water	Lévesque et al., 2013
007A1045_W	Water	Environmental surface water	Lévesque et al., 2013
007A1078_W	Water	Environmental surface water	Lévesque et al., 2013
007A1431_W	Water	Environmental surface water	Lévesque et al., 2013
012A0093_SG	Snow Goose	Fresh feces sample picked from the soil	Lévesque et al., 2013
012A0094_G	Gull	Fresh feces sample picked from the soil	Lévesque et al., 2013

Transcriptomic Analysis by Microarray

Strain 81116 cultured on TSA-Blood agar at 42°C for 2 days was suspended in 100 ml of Fraquil or Brucella broth at an OD_{600} of 1 in triplicate, and washed three times with either Fraquil or Brucella broth, respectively. The suspensions were then incubated at 4°C for 4 h. Samples for RNA extraction, Live/Dead staining and CFU count were collected from each replicate. For RNA extraction, the cells were pelleted by centrifugation, suspended in 40 μl of Tris-EDTA, and lysed by the addition of 1 ml of TRIzol reagent. RNA extraction was performed with TRIzol reagent according to the manufacturer's protocol. The RNA was subsequently treated with Turbo DNase (Ambion) and purified by acid-phenol extraction. The purity and concentration of RNA were determined by UV spectrophotometry. The integrity of extracted RNA was confirmed on a formaldehyde-agarose gel. 15 μg of RNA was labeled with amino-allyl dUTP (Sigma) during reverse transcription (Superscript II; Invitrogen) using random hexamers (Invitrogen) as previously described (Hovel-Miner et al., 2009; Faucher and Shuman, 2013). Genomic DNA was used as a reference channel and labeled by random priming using Klenow fragments, amino-allyl dUTP, and random primers as described previously (Faucher and Shuman, 2013). DNA was subsequently coupled to the succinimidyl ester fluorescent dye (Invitrogen) Alexa Fluor 647 (for cDNA) or Alexa Fluor 546 (for gDNA) according to the manufacturer's protocols.

The microarray slides designed and produced by Mycroarray for *C. jejuni* strain 81116 was used (GEO accession numbers GPL23071). Pre-hybridization, hybridization and washing were carried out as described previously (Trigui et al., 2015a). Data acquisition was performed with an InnoScan 710 microarray scanner and data analysis was performed as previously described (Trigui et al., 2015a). Background signal was subtracted and the ratio between Fraquil and broth (F/B) was calculated for each probe. The ratio was considered differentially expressed when the log2 ratio was higher than 1 or lower than −1, and the student's *t*-test *P*-value was lower than 0.05. The complete dataset was deposited in GEO (GSE94930).

Reverse Transcription-Quantitative PCR (RT-qPCR)

RNA was extracted and purified from 81116 exposed to Fraquil or Brucella broth for 4 h at 4°C. Three biological replicates were tested. One μg of RNA was used for reverse transcription reactions along with a negative control without reverse transcriptase. qPCR was performed on an iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad) using iTaq universal SYBR green supermix (Bio-Rad) according to manufacturer's protocol. Gene-specific primer sets were designed with the IDT primer design software (Bachman and Swanson, 2001) (**Table 2**) and their amplification efficiency was determined experimentally to be >85%. The 16S rRNA gene was used as a reference to normalize the data. Fold change was calculated as described previously (Livak and Schmittgen, 2001) and presented as a log2 ratio.

Distribution of the Unique Genes of 81116 in *C. jejuni* Isolates

The presence of genes unique to 81116 and expressed in Fraquil was evaluated by PCR. Genomic DNA was isolated from *C. jejuni* using the Wizard Genomic DNA Purification Kit. Primer sets were designed with IDT-PrimerQuest (**Table 2**). PCR was performed on 10 ng of gDNA using OneTaq polymerase (NEB). The PCR products were analyzed on a 0.7% agarose gel. Strains 81116 and 81-176 served as positive and negative controls, respectively.

RESULTS

Comparative Survival of *C. jejuni* 81116 and 81-176 in Fraquil

Here, we compared the survivorship in Fraquil of two widely used reference strains, 81116 and 81-176 which were originally isolated from two human outbreaks. Strain 81116 was the etiological agent of a water-borne outbreak (Palmer et al., 1983) and strain 81-176 caused an outbreak due to consumption of raw milk (Korlath et al., 1985). Given its origin from a water-source, we hypothesized that strain 81116 would better retain viability and culturability in water compared to strain 81-176. To this end,

TABLE 2 | Primers used in this study.

Gene	Primer name	Primer sequence
C8J_0133	C8J0133-F	TATTGCTGGGCATAGGAAAGG
	C8J0133-R	TCTAGCAGCTTCTCTTGGAGTA
C8J_0398	C8J0398-F	GCAACATCTACCGTGATGCTAA
	C8J0398-R	ACATATCTACAATCCACCAAATCCA
C8J_0648	C8J0648-F	GTATCAGCAGACATAAGACAAGG
	C8J0648-R	TGCTTTCTTCTAGGTACTCTTTATC
C8J_1333	C8J1333-F	TGAGCTTGCACAAGATGATACC
	C8J1333-R	GCACCAGAATACAAACCCTTCT
C8J_1342	C8J1342-F	GTTGATTTAGTGGCAGTTGGTG
	C8J1342-R	CTCTTTCTACTGCTCCTTGAATACT
C8J_1423	C8J1423-F	AAATTTATGCGCGTGCTTT
	C8J1423-R	AACTATGCCACCAAGCAAA
C8J_1619	C8J1619-F	CCAAAGTGGATAGTATTGCAAGAATTAG
	C8J1619-R	GACGACTTAAAGAACTTGAAACTGG
frdA	qfrdA-F	GTGTGCCTTGGACTAGAGTTAC
	qfrdA-R	CTGCGATATAGCAAGTTCTCCA
ccpA-2	qccpA-2-F	GTGGTATCATTTCTTGTAATACCTGTC
	qccpA-2-R	TGATGAGGATTTGCTGTCCAT
racR	qracR-F	ACGGATACAGCGTTTCAAGAG
	qracR-R	ACTCTTAAGCGACCGATGATAAC
flhB	qflhB-F	GGAAGGAGATCCTCAGGTTAAAG
	qflhB-R	GCATAATGCGTTGGGTTTGT
kpsM	qkpsM-F	TGTGGAACCTTTAAGAACTTTGC
	qkpsM-R	AAGCAAAGGACGAGGAGTTAG
cmeB	qcmeB-F	GCCATAGGGATCGTTGTAGATG
	qcmeB-R	CTATCCAAGCGATGCAAGAAGT
16S rRNA	16S-qF	AGAGATGCATTAGTGCCTTCGGGA
	16S-qR	ACTAAGGATAAGGGTTGCGCTCGT

81116 and 81-176 were suspended in Fraquil and incubated at 25°C and 4°C, the refrigeration temperature known to favor the survival of *C. jejuni* in water (Buswell et al., 1998; Thomas et al., 1999; Tatchou-Nyamsi-König et al., 2007; Trigui et al., 2015b). As expected, both strains showed a steep decline at 25°C (**Figure 1A**). At 4°C, 81116 survived better in Fraquil than 81-176 (**Figure 1B**). After 10 days in water, 50% of the 81116 population were culturable compared to only 3% of the 81-176 population. By day 21, the percent culturability was 0.2 and 0.003% for 81116 and 81-176, respectively, falling to 0% thereafter. To determine whether loss of culturability on agar plates was due to cell death, the viability of each population was assessed using the LIVE/DEAD kit and flow cytometry, as previously described (Trigui et al., 2015b). Freshly grown *C. jejuni* was used as a live control, while heat-killed *C. jejuni* served as a dead control. In contrast to the sharp decline in culturable cells, the viability of the *C. jejuni* strains decreased slowly over time. Nonetheless, 81116 showed a small but significantly higher viability compared to 81-176 (**Figure 1**). It is not clear whether incubation in water for 80 days produced authentic viable-but-non-culturable cells, since resuscitation was not attempted. Nevertheless, 81116 survived in Fraquil better than 81-176.

81116 Is More Resistant Than 81-176 to a Variety of Stresses in Fraquil

Adaptation to the low nutrient content of Fraquil could mediate cross-adaptation to other stresses. Indeed, starved *Escherichia coli* cells are more resistant to osmotic stress and oxidative stress (Jenkins et al., 1988, 1990). We investigated whether a similar adaptation occurred in *C. jejuni* after a short-term exposure to Fraquil, compared to short-term exposure to rich broth. Since chlorine and other oxidative disinfectants are routinely used to control the presence of *C. jejuni* in potable water and in slaughterhouse water chillers (Kameyama et al., 2012), the resistance of 81116 and 81-176 toward hydrogen peroxide and sodium hypochlorite after exposure to Fraquil was investigated. During the infection process, *C. jejuni* is exposed to bile salts in the small intestine (Begley et al., 2005). The *C. jejuni* capsule increases resistance to bile salts, but also contributes to avoiding complement-mediated killing, increasing bacterial colonization and bacterial persistence within the chicken host (Wong et al., 2015). As such, an increased resistance to bile salts after water exposure may indicate a strain's host colonization potential. Therefore, the resistance of 81116 and 81-176 to sodium choleate, containing the main constituents of bile (Begley et al., 2005), was also tested. To determine the relative resistance of *C. jejuni* when faced with the aforementioned stresses, 81116 and 81-176 were suspended in Fraquil and rich broth, and incubated for 4 h at 4°C. Each strain were then added to the suspension and CFUs were determined by serial dilution and plating on TSA-blood plates before adding the stresses and after 1 h of exposure to the different stresses.

Sodium hypochlorite had little effect on the survival of the strains suspended in broth. However, 81-176 suspended in Fraquil showed a marked decreased in survival with increasing sodium hypochlorite concentration (**Figure 2A**, circles), whereas, the survival of 81116 was only slightly affected by sodium hypochlorite exposure in water (**Figure 2A**, squares). Upon exposure to hydrogen peroxide in rich broth, the CFU counts of both strains decreased by 5 logs after 1 h (**Figure 2B**). In contrast, the strains suspended in Fraquil showed a greater resistance; the CFU counts of 81-176 decreased by 4 logs, while 81116 showed a mere 1 log decrease in CFUs. Overall, these results suggest that 81116 is more resistant to oxidative stress than 81-176 when suspended in Fraquil, but both strains exhibit a similar sensitivity to hydrogen peroxide in Brucella broth.

Strain 81-176 was also significantly more sensitive to sodium choleate relative to strain 81116. While the latter strain showed no difference in its survival when challenged with sodium choleate in broth compared to the same stress in water, 81-176 was almost 100-fold more sensitive to bile salts in water relative to the rich medium, however this difference was not statistically significant (**Figure 3**). As such, we conclude that 81116 is more resistant than 81-176 to bile salt.

Transcriptomic Response of 81116 to Fraquil

Taken together, the phenotypic analysis indicates that strain 81116 is better equipped to induce a genetic response that promotes its survival in Fraquil. Therefore, the transcriptomic response of this strain in response to Fraquil was elucidated using microarray analysis. To this end, 81116 was exposed to either Fraquil or Brucella broth (rich medium) for 4 h at 4°C, in triplicate. RNA was extracted and the transcriptomic profiles were analyzed using DNA microarrays. To identify the genes differentially expressed in water, the transcriptome in Fraquil was compared to that in broth (F/B). In order to ensure reliable quantitative measurements of gene expression, most genes were represented by three different, non-overlapping probes. A few genes could only accommodate one or two probes, because of their small size or homology with other genes. Genes were considered differentially expressed when the following conditions were met; (1) all probes show a twofold change in the same direction and (2) at least 50% of the probes have a *P*-value less than 0.05. Differentially expressed genes were classified into Gene Ontology (GO) groups according to their cellular functions (**Figure 4**). **Table 3** contains the fold-change expression of selected genes and the complete dataset is presented in Supplementary Table S1. Exposure to Fraquil leads to the induction of 187 genes and the repression of 149 genes. *cmeB*, coding for an efflux pump, and *ccpA-2*, coding for a cytochrome peroxidase, are among the stress response genes that are induced in 81116 upon exposure to Fraquil. Genes involved in enterobactin uptake, such as *ceuDE* and *cfrB*, were also strongly induced in 81116. Notably, many genes involved in the metabolism of amino acids, including *aspA*, *sdaA*, *glnA*, and *ggt* are repressed in water (**Figure 4**).

Validation of the Expression of Selected Genes by RT-qPCR

To validate the transcriptomic data, the expression profile of five genes was confirmed by reverse transcription-quantitative

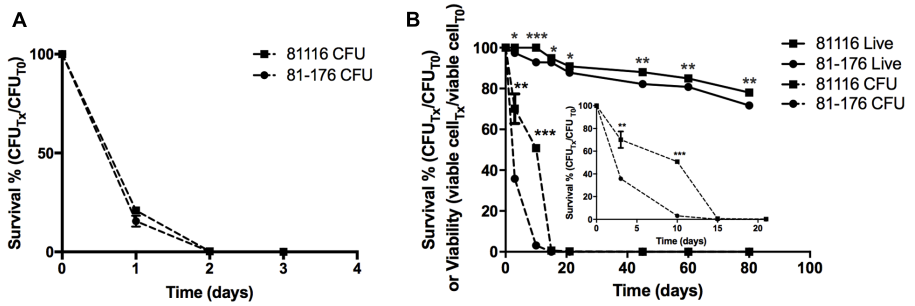


FIGURE 1 | Survival of 81116 and 81-176 in Fraquil. Strains 81116 (square) and 81-176 (circle) were grown on TSA-blood, suspended in Fraquil and incubated at 25°C **(A)** and 4°C **(B)**. The number of cells was evaluated by CFU counts (dashed line). The viability of each strain (solid line) was evaluated using the LIVE/DEAD staining kit (Invitrogen) and flow cytometry. We used an unpaired Student's *t*-test to assess statistical significance for 81116, vs. 81-176 (*$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.0005$).

PCR (RT-qPCR) using 16S rRNA as an internal control. The genes tested by RT-qPCR were selected from five different gene ontology groups: energy metabolism (*frdA*), oxidative stress (*ccpA-2*), regulation (*racR*), motility (*flhB*), and multidrug efflux pumps (*cmeB*). Consistent with the microarray data, the RT-qPCR analysis confirmed induction of *ccpA-2*, *cmeB*, and *flhB*, and repression of *frdA and racR* (**Figure 5**). Overall, the correlation between microarray values and RT-qPCR values is 0.88, which validates the transcriptomic data (Draghici et al., 2006).

Strain 81116 Expresses Unique Genes in Fraquil

Since 81116 survives better in water than strain 81-176, we hypothesized that genes unique to 81116 could contribute to this phenotype. The Pan-genome analysis tool (Miele et al., 2011; Vallenet et al., 2013) on the MicroScope website revealed that the two strains used in this study have 1473 genes in common. Strain 81116 possesses 165 unique genes, while 81-176 encodes 296 unique genes, including those encoded on the pVir and the pTet plasmids (Hofreuter et al., 2006). Twelve genes that are unique to 81116 were strongly expressed in Fraquil compared to rich broth (**Table 3**). Their presence was subsequently tested in 19 *C. jejuni* strains isolated from various water and animal sources. We hypothesized that strains originating from aquatic environments would harbor these unique genes, while those isolated from animals would lack them. Despite being classified as genes unique to 81116, PCR analysis revealed that 5 sets of primers tested amplified a product in 81-176. This is likely due to the amplification of a homologous gene, an annotation error, or low primer specificity. The distribution of the remaining seven genes was tested by PCR using chicken, bovine, human, snow goose, gull and water isolates, as well as the model strains NCTC 11168 and RM 1221 (**Figure 6**). Six genes encode hypothetical proteins or proteins with a putative function, while C8J_1423 codes for a CRISPR-associated protein called Cas2. Four isolates, including 81116, possess the full set of genes tested. None of the genes tested were present solely in water isolates. C8J_1423 was only absent in 81-176, suggesting that the CRISPR system is widely distributed in *C. jejuni* as previously reported (Pearson et al., 2015).

DISCUSSION

Transmission of campylobacteriosis to humans occurs via consumption of contaminated foods or water. The ability of *C. jejuni* to survive in water determines its ability to be transmitted by water to humans, or from one animal reservoir to another (Bronowski et al., 2014). The ability to survive in water varies greatly between *C. jejuni* strains (Buswell et al., 1998; Thomas et al., 1999; Tatchou-Nyamsi-König et al., 2007; Trigui et al., 2015b), resulting in some sequence types (ST) being isolated more frequently from water than others (Sopwith et al., 2008; Carter et al., 2009; Lévesque et al., 2013). This suggests that some strains are better adapted for utilizing water as a vehicle for transmission. This study aims at identifying phenotypes associated with the ability to survive in freshwater, and determining the transcriptomic response of a strain adapted to survive in water. Since the composition of tap water is variable, the experiments were carried out in the chemically defined freshwater medium Fraquil, which approximates the composition of freshwater of North America (Morel et al., 1975; Trigui et al., 2015b). First, the ability of each strain to survive in Fraquil was assessed using CFU counts and the LIVE/DEAD assay. 81116, a strain isolated during a water-borne outbreak of campylobacteriosis (Palmer et al., 1983), was found to survive in water better than 81-176, an epidemic strain isolated from contaminated milk (**Figure 1**) (Korlath et al., 1985). We hypothesized that the capacity of each strain to survive in different environmental conditions explains their mode of transmission and their respective outbreaks (Bronowski et al., 2014).

In addition, we report that exposure to Fraquil also affects the sensitivity of these strains to oxidative stress and bile salts (**Figures 2**, **3**). Oxidative disinfectants, such as chlorine, are routinely used in processing plant chiller water to prevent cross contamination and reduce *C. jejuni* loads on carcasses (Kameyama et al., 2012). Our results suggest that some strains,

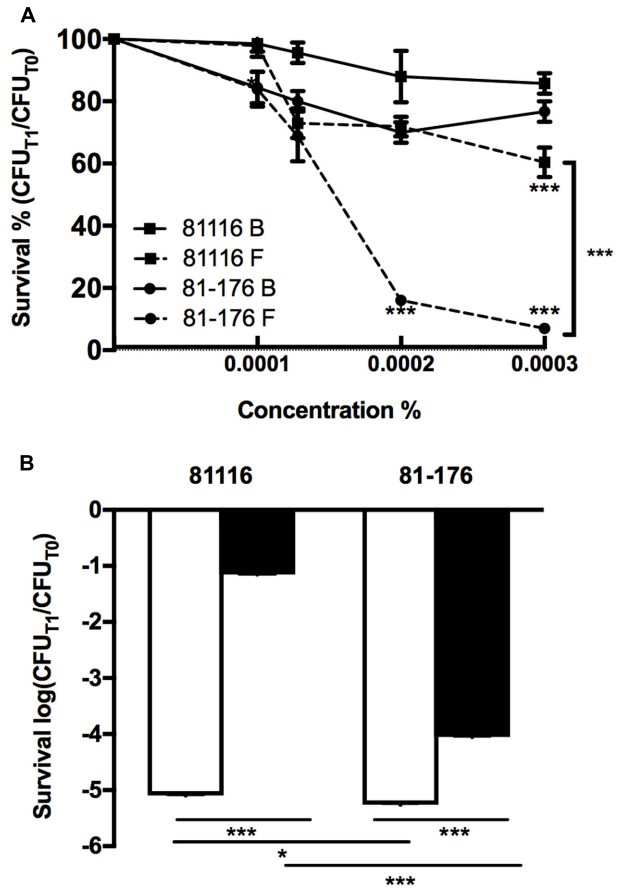


FIGURE 2 | Resistance of 81116 and 81-176 strains to oxidative stresses. The strains were suspended in Brucella broth (B, white bar) and Fraquil (F, black bar) for 4 h at 4°C and then, exposed to **(A)** a dilution series of sodium hypochlorite and **(B)** 500 μM hydrogen peroxide (H_2O_2). CFU counts were determined before exposure to stress (CFU_{T0}) and after 1 h of treatment (CFU_{T1}). Error bars represent the standard deviation from three independent biological replicates. An unpaired Student's *t*-test was used to assess statistical significance between broth and Fraquil in **(A)**, unless noted otherwise, and between each condition for **(B)** (*$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.0005$).

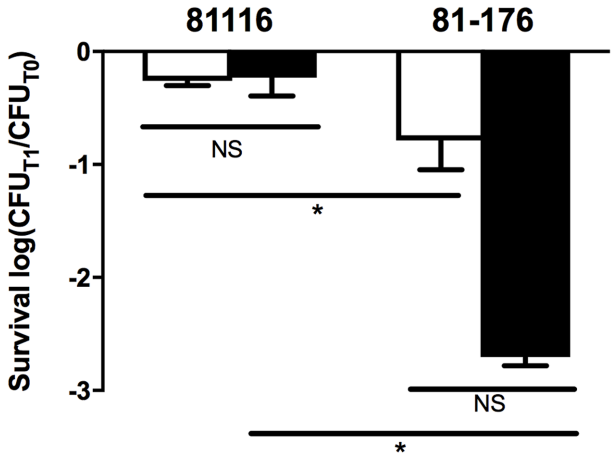


FIGURE 3 | Resistance of 81116 and 81-176 to sodium choleate. The strains were suspended in Brucella broth (white bars) and Fraquil (black bars) for 4 h at 4°C and then exposed to 100 mg ml^{-1} of sodium choleate. CFU was determined before exposure to stress (CFU_{T0}) and after 1 h of treatment (CFU_{T1}). Error bars represent the standard deviation from three independent biological replicates. An unpaired Student's *t*-test was used to assess statistical significance (NS, not significant, *$P \leq 0.05$).

such as 81116, are better than others at resisting these disinfection procedures during chilling and are more likely to contaminate the final product. A recent study reports that some *flaA* genotypes are selected by the slaughtering process and appear more frequently on the finished product (Kudirkienė et al., 2011).

The resistance of strain 81116 to bile salts was not affected by exposure to Fraquil or rich broth (**Figure 3**). In contrast, 81-176 was markedly more sensitive to this stress (**Figure 3**). The CmeABC efflux pump mediates resistance to bile salt and is essential for colonization of the intestinal tract (Lin et al., 2003, 2005, 2007; Gibreel et al., 2007; Young et al., 2007; Caldwell et al., 2008). While 81116 induced the expression of *cmeB* in response to Fraquil, the remaining components of the efflux system were not differentially expressed in water relative to rich broth, suggesting that this system is expressed at the same level in water and in broth (Supplementary Table S1). Possibly, the expression of this efflux pump is reduced in 81-176, which results in lower resistance to bile salts. *C. jejuni* is unlikely to be exposed to sodium choleate at concentrations used in this study in the natural environment. Nevertheless, the increased sensitivity of 81-176 to bile salts after exposure to water (**Figure 3**) suggests that some strains are better at colonizing the intestinal tract following transmission by water compared to other strains.

To better understand the genetic elements that contribute to the enhanced survival of 81116 in water, its transcriptome in Fraquil was compared to that in Brucella broth. To this end, 81116 was exposed to Fraquil at 4°C since it survives better at this temperature compared to 25°C (**Figure 1**). The exposure time was limited to 4 h because transcriptional changes are known to happen quickly in response to a new condition (Hinton et al., 2004). Moreover, the rate of transcription is reduced dramatically during starvation (Srivatsan and Wang, 2008), as evidenced in *Legionella pneumophila* exposed to water (Li et al., 2015). Since phenotypic differences were observed between Fraquil and rich Brucella broth (**Figures 2**, **3**), suspension in rich Brucella broth at 4°C for 4 h was used as the control condition. In addition, this control allows the study of the starvation response of *C. jejuni*, which is likely necessary to survive in the nutrient-poor water environment. Starvation of *C. jejuni* in Ringer solution induces heat-shock resistance and affects the expression of catalases and superoxide dismutases (Klancnik et al., 2009). Our analysis revealed that 336 genes are differentially regulated upon exposure to Fraquil. *C. jejuni* uses amino acids, and to a lesser extent, short chain fatty acids as carbon and energy sources (Stahl et al., 2012). Since Fraquil is devoid of these nutrients, expression of transporters and enzymes involved in their catabolism should be repressed. Indeed, the amino acid transporters *pebA* and *pebC*,

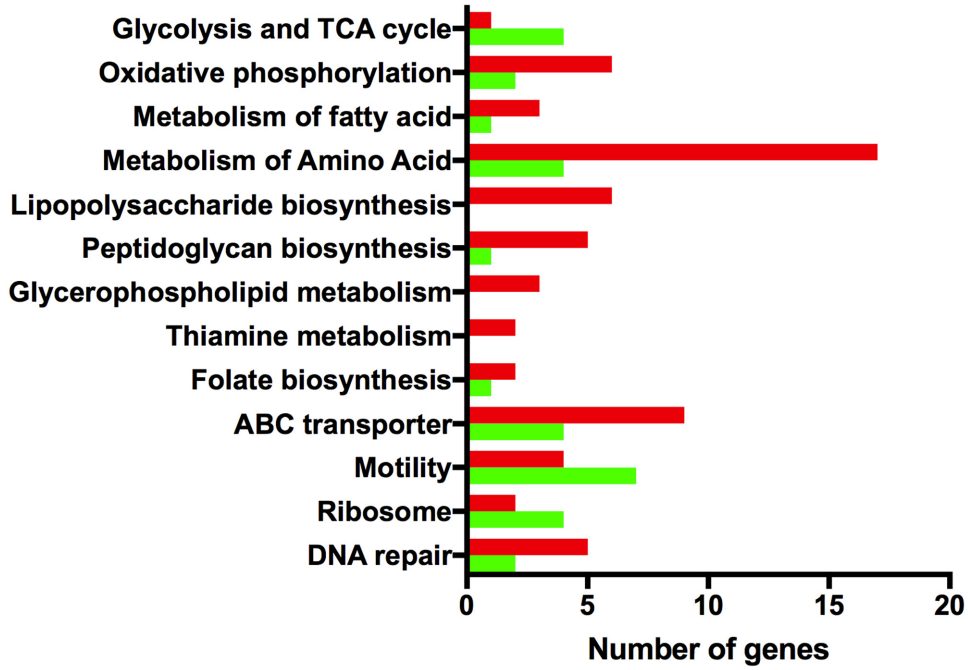


FIGURE 4 | Gene ontology analysis of differentially expressed genes. The Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/kegg2.html) BRITE Hierarchy was used to classify differentially expressed genes into categories. The *x*-axis shows the number of genes that are induced (red) and repressed (green) in each group.

as well as the aspartate ammonia-lyase *aspA* and the gamma glutamyltransferase *ggt* required for the utilization of aspartate, glutamate and glutamine were repressed in Fraquil (**Table 3**). The serine ammonia-lyase *sdaA* followed a similar expression pattern (**Table 3**). In contrast, genes involved in the biosynthesis of various amino acids were induced in Fraquil (**Figure 4**). Genes involved in the catabolism of short-chain fatty acid, such as *acs* and *lctP* were also repressed following exposure to Fraquil (**Table 3**); however, *fabH* and *fabF* involved in the biosynthesis of fatty acids were induced.

Costly biosynthetic processes such as glycosylation (*pglJ*) and production of the flagellum (*flaG, fliS, flgG, flgI*) were repressed in Fraquil (**Table 3**). Repression of flagella genes was expected since *C. jejuni* is known to repress them in the post-exponential phase of growth, when nutrients are limiting and waste accumulates (Wright et al., 2009). Adhesins used during infection, including *cadF* and *jlpA* were strongly repressed in Fraquil.

The need to repress costly metabolic systems in water is supported by recent analyses of the survival of *L. pneumophila* in water (Li et al., 2015). *L. pneumophila* is transmitted to humans by inhalation of contaminated aerosols (Mittal et al., 2013). Transcriptomic profiling revealed that *L. pneumophila* represses most systems in Fraquil, including transcription, translation, flagellum synthesis, and virulence factors (Li et al., 2015). In *L. pneumophila*, this is mediated by the stringent response and the sigma factor RpoS (Trigui et al., 2015a). *C. jejuni* does not possess RpoS and only codes three sigma factors (RpoD, FliA, and RpoN) within its genome (Parkhill et al., 2000). RpoD is the housekeeping sigma factor, while FliA and RpoN regulated flagella synthesis and defense against various stresses, respectively (Hwang et al., 2011). The stringent response is initiated by the production of guanosine tetraphosphate (ppGpp), a cellular alarmone signaling starvation (Mittenhuber, 2001). In general, Gram-negative bacteria employ two enzymes to regulate cellular levels of ppGpp; RelA which synthesizes the alarmone and the dual acting SpoT which has low synthase and high hydrolase activities (Mittenhuber, 2001; Gaynor et al., 2005). However, ppGpp levels in *C. jejuni* are regulated solely by SpoT. Deletion of *spoT* affects multiple phenotypes in *C. jejuni*, including interaction with host cells, resistance to rifampicin and oxygen, and survival in the stationary phase (Gaynor et al., 2005). *C. jejuni* survives poorly in stationary phase compared to model bacteria, presumably because it lacks an *rpoS* homolog, the stationary phase sigma factor (Kelly et al., 2001). It is likely that survival of *C. jejuni* in water is mediated mainly by the stringent response. Transcriptomic analysis of the *spoT* mutant at different growth phases in rich broth revealed that 30 genes are regulated by the stringent response in *C. jejuni* (Gaynor et al., 2005). The transcriptome of 81116 in Fraquil showed limited similarities to the *spoT* mutant in broth, which suggests that, in Fraquil, only a few genes are under the control of the stringent response. For example, *clpB* is repressed in Fraquil, but induced in the *spoT* mutant, while and the putative ferredoxin *napH* was induced in Fraquil, but repressed in the *spoT* mutant. Attempts at deleting *spoT* in 81116 in order to confirm the role of the stringent response in water was unsuccessful. Additional studies

TABLE 3 | Select genes differentially expressed in water.

Gene	Name	Product	F/B (log2)
Amino acid transporter			
C8J_0858	*pebA*	Amino acid ABC transporter, periplasmic amino	−5.08
C8J_0859	*pebC*	Putative polar amino acid transport system	−4.82
C8J_0951	*livF*	Branched-chain amino acid transport system	−2.01
C8J_0953	*livM*	Branched-chain amino acid transport system	5.44
C8J_0954	*livH*	Branched-chain amino acid transport system	3.80
Amino acid metabolism			
C8J_1526	*sdaA*	L-serine ammonia-lyase	−3.03
C8J_0079	*aspA*	Aspartate ammonia-lyase	−4.77
C8J_0666	*glnA*	Glutamine synthetase, type I	−4.99
C8J_0033	*ggt*	Gamma-glutamyltransferase	−9.53
Fatty acid metabolism			
C8J_0305	*fabH*	3-oxoacyl-(acyl-carrier-protein) synthase III	2.82
C8J_0417	*fabF*	3-oxoacyl-(acyl-carrier-protein) synthase II	7.42
C4-dicarboxylate transporter			
C8J_1136	*dctA*	Putative C4-dicarboxylate transport protein	3.00
Metabolism of short chain fatty acid			
C8J_1436	*acs*	Acetyl-coenzyme A synthetase	−4.54
C8J_0069	*lctP*	L-lactate permease	−3.77
Translation			
C8J_1498	*rplQ*	50S ribosomal protein L17	2.66
C8J_1605	*rpsC*	30S ribosomal protein S3	−2.20
C8J_0288	*rplY*	50S ribosomal protein L25	−2.09
C8J_0346	*rpsU*	30S ribosomal protein S21	−4.41
Iron uptake			
C8J_1312	*feoB*	Ferrous iron transport protein	3.47
C8J_1311	*feoA*	Ferrous iron transport protein	7.63
C8J_1270	*ceuD*	Enterochelin transport system ATP-binding	7.14
C8J_1271	*ceuE*	Enterochelin transport system substrate-binding	2.51
C8J_0419	*cfrB*	Enterobactin transporter	3.95
Other metal transporter			
C8J_1438		Tungsten ABC transporter, permease protein	5.64
C8J_0240	*zupT*	Zinc transporter	5.20
Oxidative stress			
C8J_0165	*sodB*	Superoxide dismutase (Fe)	−7.35
C8J_0311	*ahpC*	Alkyl hydroperoxide reductase	−3.79
C8J_0335	*ccpA-2*	Cytochrome C551 peroxidase	2.45
C8J_0730	*tpx*	Thiol peroxidase	−5.92
Efflux pump			
C8J_0342	*cmeB*	CME efflux system, inner membrane transporter	6.50
C8J_1294		Multidrug resistance efflux transporter	−3.25
C8J_1131	*arsB*	Putative arsenical pump membrane protein	3.83
Regulator			
C8J_1205	*racR*	Two-component regulator	−2.87
C8J_1206	*racS*	Sensor histidine kinase	3.39
C8J_1044	*csrA*	Carbon storage regulator-like protein	−3.77
Adhesins			
C8J_1383	*cadF*	Outer membrane fibronectin-binding protein	−4.67
C8J_0922	*jlpA*	42-kDa lipoprotein	−3.70
Flagella			
C8J_0296	*fliG*	Flagellar motor switch protein	2.79
C8J_0312	*flhB*	Flagellar biosynthetic protein	2.31

(Continued)

TABLE 3 | Continued

Gene	Name	Product	F/B (log2)
C8J_0508	*flaG*	Possible flagellar protein	−5.08
C8J_0510	*fliS*	Flagellar protein	−4.85
C8J_0664	*flgG2*	Putative flagellar basal-body rod protein	−4.21
C8J_0665	*flgG*	Flagellar basal-body rod protein	−2.11
C8J_0687	*flaC*	Flagellin	−3.91
C8J_0767	*fliP*	Flagellar biosynthesis protein	2.65
C8J_1368	*flgI*	Flagellar P-ring protein FlgI	−2.84
C8J_1576	*fliQ*	Flagellar biosynthetic protein	3.88
Glycosylation			
C8J_1067	*pglJ*	General glycosylation pathway protein	−3.71
Lipopolysaccharide (lipooligosaccharide) synthesis			
C8J_1251	*neuB*	*N*-acetylneuraminic acid synthetase	3.40
C8J_0762	*lpxK*	Tetraacyldisaccharide 4′-kinase	4.84
C8J_1095	*gmhA-1*	Phosphoheptose isomerase	4.00
C8J_1096	*waaE*	Putative ADP-heptose synthase	3.50
C8J_0262		UDP-2,3-diacylglucosamine hydrolase	2.74
C8J_0264	*lpxB*	Lipid-A-disaccharide synthase	2.75
C8J_1074	*waaM*	Lipid A biosynthesis lauroyl acyltransferase	2.56
Peptidoglycan synthesis			
C8J_0407	*murD*	UDP-*N*-acetylmuramoylalanine-D-glutamate ligase	3.68
C8J_0408	*mraY*	Phospho-*N*-acetylmuramoyl-pentapeptide-transferase	3.94
C8J_0749	*ddlA*	D-alanine-D-alanine ligase	3.47
C8J_0746	*murF*	UDP-*N*-acetylmuramoyl-tripeptide—D-alanyl-D-alanine ligase	−2.73
C8J_0843	*pgp2*	LD-carboxypeptidase	3.79
C8J_1261	*pgp1*	DL-carboxypeptidase	4.14
Unique genes of 81116			
C8J_0133		Putative DNA-methyltransferase	2.97
C8J_0398		Protein of unknown function	4.15
C8J_0648		Hypothetical protein	1.63
C8J_1333		Putative CMP-NeuAc synthase	3.32
C8J_1342		Hypothetical protein	4.51
C8J_1423	*cas2*	CRISPR-associated protein Cas2	3.51
C8J_1619		Hypothetical protein	4.75

are required to fully appreciate the role of the stringent response in the survival of *C. jejuni* in water.

Strain 81116 induces 187 genes in Fraquil, which may provide useful functions for its survival. Genes involved in the synthesis of the cell envelope were induced in Fraquil (**Figure 4**), including peptidoglycan synthesis (*ddlA*, *murD*, and *mraY*) and lipopolysaccharide (lipooligosaccharide) synthesis (lpxB, lpxK, waaE, and waaM). Genes involved in iron transport (*feoA*, *feoB*, *ceuDE*, and *cfrB*), as well as other transport systems and porins were also induced. Some of the genes induced in Fraquil code for hypothetical proteins or proteins with a putative function. The potential contribution of the aforementioned genes to the survival of *C. jejuni* in water is discussed in the following paragraph.

The peptidoglycan layer plays a role in maintaining the turgor pressure of the cell, and is required for cell growth and division (Egan et al., 2016). *C. jejuni* cell morphology changes from a rod or spiral shape in the exponential phase to a coccoid form in the stationary phase of growth (Ikeda and Karlyshev, 2012). The amount of peptidoglycan in the coccoid form is about one third of the amount present in the spiral form (Amano and Shibata, 1992). Therefore, *C. jejuni* modifies the amount of peptidoglycan according to growth conditions. The spiral shape is produced by the action of two peptidoglycan modifying enzymes, Pgp1 and Pgp2 (Frirdich et al., 2012, 2014). Both genes are induced in *C. jejuni* upon exposure to Fraquil (**Table 3**). Since *C. jejuni* does not replicate in Fraquil, the induction of peptidoglycan synthesis and modification genes likely serves an ulterior purpose, such as resistance to hypoosmotic stress or differentiation into the VBNC state. The freshwater medium Fraquil used in this study is hypoosmotic relative to Brucella broth. The latter contains 5 g/L of sodium chloride in addition to other osmolytes. *C. jejuni* may induce peptidoglycan synthesis in Fraquil to increase the strength of the peptidoglycan mesh, which in turn, resists the influx of water and maintains cell shape. To our knowledge, the hypoosmotic response of *C. jejuni* has never been studied. Exposure of *C. jejuni* to moderate hyperosmotic stress results in cell elongation and induces the expression of chaperones,

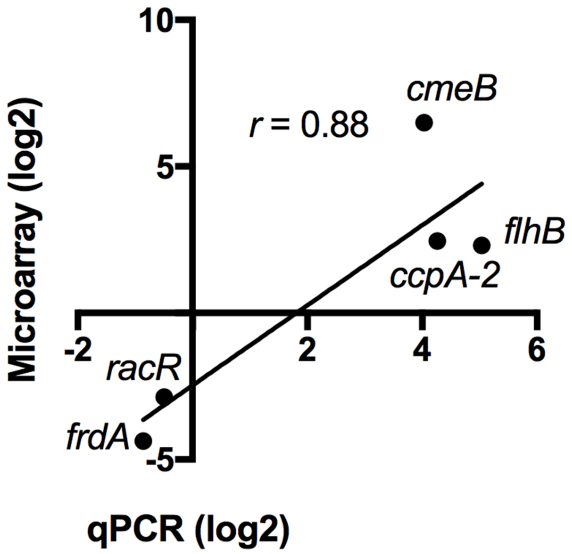


FIGURE 5 | Validation of the transcriptomic data by RT-qPCR. The expression of five differentially regulated genes was validated by RT-qPCR. The values obtained by microarray and by qPCR are shown. The correlation (*r*) between each data set is 0.88.

oxidative stress response genes and amino acid synthesis genes (Cameron et al., 2012). The present study and others have shown that exposure of *C. jejuni* to water triggers differentiation into the VBNC state (Cools et al., 2003; Baffone et al., 2006; Trigui et al., 2015b). In *E. coli*, the peptidoglycan layer undergoes extensive modification upon entry into VBNC state (Signoretto et al., 2002). Similarly, VBNC *Enterococcus faecalis* cells contain high level of O-acetylated peptidoglycan (Pfeffer et al., 2006). In addition, VBNC *E. faecalis* are more resistant to mechanical stress than actively growing cells, likely due to increased peptidoglycan cross-linking (Signoretto et al., 2000). It is tempting to postulate that induction of peptidoglycan-related genes in Fraquil are necessary to modify the cell wall of *C. jejuni* during differentiation into the VBNC state, protecting cells against stresses, including hypoosmotic stress, encountered in the water environment. The population of *C. jejuni* cells exposed to Fraquil enters the VBNC state progressively (**Figure 1**); however, the prerequisite cellular modifications likely occur relatively quickly after exposure to Fraquil, when cells have sufficient energy and supplies to do so.

C. jejuni lipooligosaccharide (LOS) consists of a lipid A moiety, an inner core composed of a conserved trisaccharide and a strain-variable outer core consisting of various sugars (Karlyshev et al., 2005). LOS is similar in structure and function to lipopolysaccharide, but lacks the O-antigen. Modification of LPS is important for many pathogens to evade the host immune defenses (Whitfield and Trent, 2014). Similarly, mutations affecting the length of the outer core of LOS in *C. jejuni* reduce resistance to complement-mediated killing and colonization of mice (Naito et al., 2010). In addition, abnormal LOS results in increased susceptibility to polymyxin B and sodium dodecyl sulfate, but increase biofilm formation (Naito et al., 2010). The effect of temperature on LOS length is strain-dependent, but growth at 42°C favors the production of a shorter LOS (Semchenko et al., 2010). Induction of LOS synthesis genes in Fraquil suggests that modification of the LOS sheath is important for survival in water and/or for differentiation into the VBNC state.

Genes involved in the acquisition of iron were induced in Fraquil, including the ferrous iron transporters *feoAB* and the siderophore transporters encoded by *ceuDE* and *cfrB*. Presumably, the iron-poor environment of Fraquil leads to induction of genes encoding functions related to iron homeostasis. Oxidative stress resistance genes are repressed by iron in *C. jejuni* during growth in minimum essential medium (Palyada et al., 2004; Butcher and Stintzi, 2013); however, they

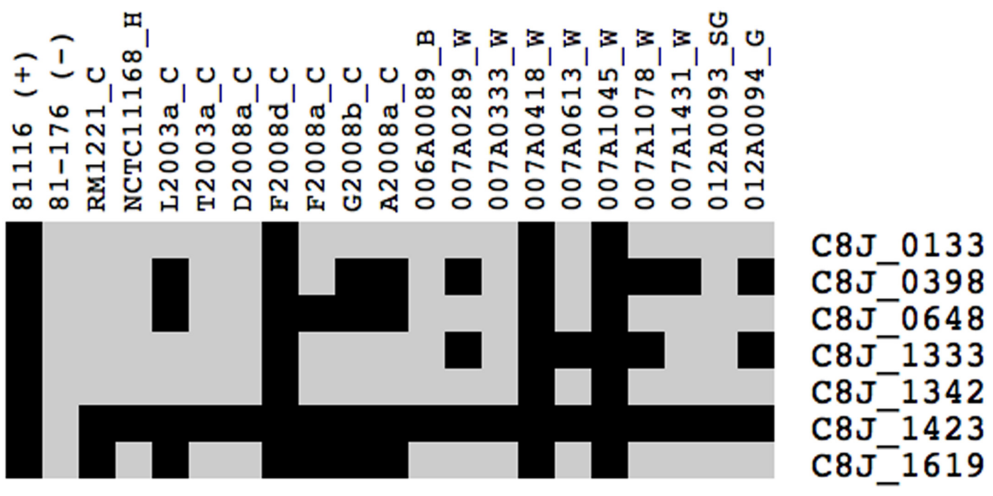


FIGURE 6 | Presence of genes unique to 81116 induced in water in different isolates of *C. jejuni*. The presence of the select genes was tested by PCR using strains 81116 and 81-176 as positive and negative controls, respectively. Black indicates that the gene is present in the strain, whereas light gray indicates that the gene is absent. The letter following the name of each isolate indicates its source of isolation: H, human; C, chicken; B, bovine; SG, snow goose; G, gull; W, water.

are induced during the metabolic switch from acetate production to acetate uptake, and also between the exponential phase and the stationary phase (Wright et al., 2009). Therefore, it was expected that genes involved in the oxidative stress response would also be induced in Fraquil, since it mimicks the low nutrient condition that is found in stationary phase. Unexpectedly, we found that strain 81116 represses three oxidative stress defense genes, *sodB*, *ahpC,* and *tpx* in Fraquil. The absence of nutrient and toxic metabolic waste in Fraquil compared to the stationary phase in broth probably leads to the repression of oxidative stress response genes, which could explain the discrepancies with previous studies. Since iron mediates the formation of reactive oxygen species (ROS) inside cells (Imlay, 2003), it is expected that oxidative stress would be reduced in an iron-limiting condition. This speculation is supported by the findings from Folsom et al. (2014) where several enzymes associated with oxidative stress and ROS in *E. coli* were down-regulated under iron limitation, including superoxide dismutases (Sod). Nevertheless, despite the down-regulation of these oxidative stress defense genes in Fraquil, strain 81116 is more tolerant to H_2O_2 stress than in rich broth (**Figure 2B**). Interestingly, strain 81116 induces the expression of *ccpA-2*, which encodes for a cytochrome peroxidase enzyme (Kim et al., 2015). It has been shown that loss of *ccpA-2* in *C. jejuni* NCTC 11168 resulted in increased sensitivity to H_2O_2 compared to the wild-type (Flint et al., 2014), suggesting that the contribution of *ccpA-2* to oxidative stress response is strain-dependent. Strain 81-176 is more sensitive to oxidative stress in Fraquil than 81116, suggesting differential expression of genes involved in resistance to oxidative stress.

A fraction (32 genes, 9.5%) of the differentially expressed genes codes for hypothetical proteins without known or putative functions. Of these, 10 were induced in Fraquil and could encode functions necessary for 81116 to survive in water. In addition, seven genes induced in Fraquil are absent in 81-176 genome, which suggests that they may contribute to 81116's ability to better survive in water. Therefore, their presence in water-isolated strains, as well as strains isolated from other sources was tested. The presence of the genes was not correlated with the source of the strain (**Figure 6**) suggesting that the unique genes tested did not contribute significantly to its survival. Alternatively, enhanced survival of 81116 in Fraquil is likely due to the induction of multiple regulatory systems that promote adaptation to water. This is likely due to subtle difference in the regulation of gene expression. Indeed, Dugar et al. (2013) reported that strain-specific transcriptome structure could modulate phenotypic variation among *C. jejuni* strains. This could be due to acquisition of specific regulators, effectors or organization of the genome.

CONCLUSION

The transcriptomic profiling of 81116 in water suggest that its ability to survive in water for extended periods of time is due to multiple adaptations that shut down nutrient uptake systems, and costly metabolic pathways, including synthesis of the flagellum. Moreover, the induction of stress response pathways, genes involved in detoxification and cell wall synthesis in response to water enhances the resistance of 81116 to multiple stresses in the aquatic environment. Unique genes do not contribute to its enhanced fitness. Strains with a similar genetic background as 81116 are likely better at transmission via water and more resistant to current disinfection processes.

AUTHOR CONTRIBUTIONS

HT, AT, PF, AL, and SF designed the experiments. HT, KL, and AT performed the experiments. AT, SL, PF, and AL provided strains of *C. jejuni*. HT, AT, NM, and SF analyzed the data. HT, NM, and SF wrote the manuscript. All authors edited the manuscript and approved the final version.

ACKNOWLEDGMENTS

This project was funded by a New Initiative Grant from the Centre de Recherche en Infectiologie Porcine et Aviaire (CRIPA), a Fond de Recherche du Québec – Nature et Technologie (FRQNT) strategic cluster and Programme Innov'Action Agroalimentaire IA113123: "Ces travaux ont été réalisés grâce à une aide financière du Programme Innov'Action agroalimentaire, un programme issu de l'accord Cultivons l'avenir 2 conclu entre le ministre de l'Agriculture, des Pêcheries et de l'Alimentation, et Agriculture et Agroalimentaire Canada."

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01781/full#supplementary-material

REFERENCES

Ahmed, I. H., Manning, G., Wassenaar, T. M., Cawthraw, S., and Newell, D. G. (2002). Identification of genetic differences between two *Campylobacter jejuni* strains with different colonization potentials. *Microbiology* 148, 1203–1212. doi: 10.1099/00221287-148-4-1203

Amano, K., and Shibata, Y. (1992). Structural studies of peptidoglycans in *Campylobacter* species. *Microbiol. Immunol.* 36, 961–967. doi: 10.1111/j.1348-0421.1992.tb02099

Axelsson-Olsson, D., Waldenström, J., Broman, T., Olsen, B., and Holmberg, M. (2005). Protozoan *Acanthamoeba polyphaga* as a potential reservoir for *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 71, 987–992. doi: 10.1128/AEM.71.2.987-992.2005

Bachman, M. A., and Swanson, M. S. (2001). RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. *Mol. Microbiol.* 40, 1201–1214. doi: 10.1046/j.1365-2958.2001.02465

Baffone, W., Casaroli, A., Citterio, B., Pierfelici, L., Campana, R., Vittoria, E., et al. (2006). *Campylobacter jejuni* loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. *Int. J. Food Microbiol.* 107, 83–91. doi: 10.1016/j.ijfoodmicro.2005.08.015

Begley, M., Gahan, G. M., and Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiol. Rev.* 29, 625–651. doi: 10.1016/j.femsre.2004.09.003

Boes, J., Nersting, L., Nielsen, E., Kranker, S., Enøe, C., Wachmann, H., et al. (2005). Prevalence and diversity of *Campylobacter jejuni* in pig herds on farms with and without cattle or poultry. *J. Food Prot.* 68, 722–727. doi: 10.4315/0362-028X-68.4.722

Bolton, F., Coates, D., Hinchliffe, P., and Robertson, L. (1982). A most probable number method for estimating small numbers of campylobacters in water. *J. Hyg.* 89, 185–190. doi: 10.1017/S0022172400070716

Bronowski, C., James, C. E., and Winstanley, C. (2014). Role of environmental survival in transmission of *Campylobacter jejuni*. *FEMS Microbiol. Lett.* 356, 8–19. doi: 10.1111/1574-6968.12488

Buswell, C. M., Herlihy, Y. M., Lawrence, L. M., McGuiggan, J. T., Marsh, P. D., Keevil, C. W., et al. (1998). Extended Survival and Persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and-rRNA staining. *Appl. Environ. Microbiol.* 64, 733–741.

Butcher, J., Handley, R. A., van Vliet, A. H., and Stintzi, A. (2015). Refined analysis of the *Campylobacter jejuni* iron-dependent/independent Fur-and PerR-transcriptomes. *BMC Genomics* 16:498. doi: 10.1186/s12864-015-1661-7

Butcher, J., and Stintzi, A. (2013). The transcriptional landscape of *Campylobacter jejuni* under iron replete and iron limited growth conditions. *PLoS ONE* 8:e79475. doi: 10.1371/journal.pone.0079475

Butzler, J., and Skirrow, M. (1979). *Campylobacter enteritis*. *Acta Paediatr. Belg.* 32, 89.

Caldwell, D. B., Wang, Y., and Lin, J. (2008). Development, stability, and molecular mechanisms of macrolide resistance in *Campylobacter jejuni*. *Antimicrob. Agents Chemother.* 52, 3947–3954. doi: 10.1128/AAC.00450-08

Cameron, A., Frirdich, E., Huynh, S., Parker, C. T., and Gaynor, E. C. (2012). Hyperosmotic stress response of *Campylobacter jejuni*. *J. Bacteriol.* 194, 6116–6130. doi: 10.1128/JB.01409-12

Carter, P., McTavish, S., Brooks, H., Campbell, D., Collins-Emerson, J., Midwinter, A., et al. (2009). Novel clonal complexes with an unknown animal reservoir dominate *Campylobacter jejuni* isolates from river water in New Zealand. *Appl. Environ. Microbiol.* 75, 6038–6046. doi: 10.1128/AEM.01039-09

Clark, C. G., Price, L., Ahmed, R., Woodward, D. L., Melito, P. L., Rodgers, F. G., et al. (2003). Characterization of waterborne outbreak–associated *Campylobacter jejuni*, Walkerton, Ontario. *Emerg. Infect. Dis.* 9, 1232–1241. doi: 10.3201/eid0910.020584

Cools, I., Uyttendaele, M., Caro, C., D'Haese, E., Nelis, H., and Debevere, J. (2003). Survival of *Campylobacter jejuni* strains of different origin in drinking water. *J. Appl. Microbiol.* 94, 886–892. doi: 10.1186/s40064-015-1595-1

Dasti, J. I., Tareen, A. M., Lugert, R., Zautner, A. E., and Groß, U. (2010). *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. *Int. J. Med. Microbiol.* 300, 205–211. doi: 10.1016/j.ijmm.2009.07.002

Draghici, S., Khatri, P., Eklund, A. C., and Szallasi, Z. (2006). Reliability and reproducibility issues in DNA microarray measurements. *Trends Genet.* 22, 101–109. doi: 10.1016/j.tig.2005.12.005

Dugar, G., Herbig, A., Förstner, K. U., Heidrich, N., Reinhardt, R., Nieselt, K., et al. (2013). High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. *PLoS Genet.* 9:e1003495. doi: 10.1371/journal.pgen.1003495

Egan, A. J. F., Cleverley, R. M., Peters, K., Lewis, R. J., and Vollmer, W. (2016). Regulation of bacterial cell wall growth. *FEBS J.* 284, 851–867. doi: 10.1111/febs.13959

Faucher, S. P., and Shuman, H. A. (2013). Methods to study *Legionella* transcriptome in vitro and in vivo. *Legionella* 954, 567–582. doi: 10.1007/978-1-62703-161-5_35

Flint, A., Sun, Y.-Q., Butcher, J., Stahl, M., Huang, H., and Stintzi, A. (2014). Phenotypic screening of a targeted mutant library reveals *Campylobacter jejuni* defenses against oxidative stress. *Infect. Immun.* 82, 2266–2275. doi: 10.1128/IAI.01528-13

Folsom, J. P., Parker, A. E., and Carlson, R. P. (2014). Physiological and proteomic analysis of *Escherichia coli* iron-limited chemostat growth. *J. Bacteriol.* 196, 2748–2761. doi: 10.1128/JB.01606-14

Fordtran, J. S., and Locklear, T. W. (1966). Ionic constituents and osmolality of gastric and small-intestinal fluids after eating. *Am. J. Dig. Dis.* 11, 503–521. doi: 10.1007/BF02233563

Frirdich, E., Biboy, J., Adams, C., Lee, J., Ellermeier, J., Davis Gielda, L., et al. (2012). Peptidoglycan-modifying enzyme Pgp1 is required for helical cell shape and pathogenicity traits in *Campylobacter jejuni*. *PLoS Pathog.* 8:e1002602. doi: 10.1371/journal.ppat.1002602

Frirdich, E., Vermeulen, J., Biboy, J., Soares, F., Taveirne, M. E., Johnson, J. G., et al. (2014). Peptidoglycan LD-carboxypeptidase Pgp2 influences *Campylobacter jejuni* helical cell shape and pathogenic properties, and provides the substrate for the DL-carboxypeptidase Pgp1. *J. Biol. Chem.* 289, 8007–8018. doi: 10.1074/jbc.M113.491829

Gaynor, E. C., Wells, D. H., MacKichan, J. K., and Falkow, S. (2005). The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. *Mol. Microbiol.* 56, 8–27. doi: 10.1111/j.1365-2958.2005.04525.x

Gibreel, A., Wetsch, N. M., and Taylor, D. E. (2007). Contribution of the CmeABC efflux pump to macrolide and tetracycline resistance in *Campylobacter jejuni*. *Antimicrob. Agents Chemother.* 51, 3212–3216. doi: 10.1128/AAC.01592-06

Gormley, F. J., MacRae, M., Forbes, K. J., Ogden, I. D., Dallas, J. F., and Strachan, N. J. (2008). Has retail chicken played a role in the decline of human campylobacteriosis? *Appl. Environ. Microbiol.* 74, 383–390. doi: 10.1128/AEM.01455-07

Habib, I., Uyttendaele, M., and De Zutter, L. (2010). Survival of poultry-derived *Campylobacter jejuni* of multilocus sequence type clonal complexes 21 and 45 under freeze, chill, oxidative, acid and heat stresses. *Food Microbiol.* 27, 829–834. doi: 10.1016/j.fm.2010.04.009

Hinton, J. C., Hautefort, I., Eriksson, S., Thompson, A., and Rhen, M. (2004). Benefits and pitfalls of using microarrays to monitor bacterial gene expression during infection. *Curr. Opin. Microbiol.* 7, 277–282. doi: 10.1016/j.mib.2004.04.009

Hofreuter, D., Tsai, J., Watson, R. O., Novik, V., Altman, B., Benitez, M., et al. (2006). Unique features of a highly pathogenic *Campylobacter jejuni* strain. *Infect. Immun.* 74, 4694–4707. doi: 10.1128/IAI.00210-06

Hovel-Miner, G., Pampou, S., Faucher, S. P., Clarke, M., Morozova, I., Morozov, P., et al. (2009). σ^S controls multiple pathways associated with intracellular multiplication of *Legionella pneumophila*. *J. Bacteriol.* 191, 2461–2473. doi: 10.1128/JB.01578-08

Huang, H., Brooks, B. W., Lowman, R., and Carrillo, C. D. (2015). *Campylobacter* species in animal, food, and environmental sources, and relevant testing programs in Canada. *Can. J. Microbiol.* 61, 701–721. doi: 10.1139/cjm-2014-0770

Hwang, S., Jeon, B., Yun, J., and Ryu, S. (2011). Roles of RpoN in the resistance of *Campylobacter jejuni* under various stress conditions. *BMC Microbiol.* 11:207. doi: 10.1186/1471-2180-11-207

Ikeda, N., and Karlyshev, A. V. (2012). Putative mechanisms and biological role of coccoid form formation in *Campylobacter jejuni*. *Eur. J. Microbiol. Immunol.* 2, 41–49. doi: 10.1556/EuJMI.2.2012.1.7

Imlay, J. A. (2003). Pathways of oxidative damage. *Annu. Rev. Microbiol.* 57, 395–418. doi: 10.1146/annurev.micro.57.030502.090938

Inglis, G. D., and Kalischuk, L. D. (2004). Direct quantification of *Campylobacter jejuni* and *Campylobacter lanienae* in feces of cattle by real-time quantitative PCR. *Appl. Environ. Microbiol.* 70, 2296–2306. doi: 10.1128/AEM.70.4.2296-2306.2004

Jackson, D. N., Davis, B., Tirado, S. M., Duggal, M., van Frankenhuyzen, J. K., Deaville, D., et al. (2009). Survival mechanisms and culturability of *Campylobacter jejuni* under stress conditions. *Antonie Van Leeuwenhoek* 96, 377–394. doi: 10.1007/s10482-009-9378-8

Jenkins, D. E., Chaisson, S. A., and Matin, A. (1990). Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. *J. Bacteriol.* 172, 2779–2781. doi: 10.1128/jb.170.9.3910-3914.1988

Jenkins, D. E., Schultz, J. E., and Matin, A. (1988). Starvation-induced cross protection against heat or H2O2 challenge in *Escherichia coli*. *J. Bacteriol.* 170, 3910–3914. doi: 10.1128/jb.170.9.3910-3914.1988

Kameyama, M., Chuma, T., Nishimoto, T., Oniki, H., Yanagitani, Y., Kanetou, R., et al. (2012). Effect of cooled and chlorinated chiller water on *Campylobacter* and coliform counts on broiler carcasses during chilling at a middle-size poultry processing plant. *J. Vet. Med. Sci.* 74, 129–133. doi: 10.1292/jvms.11-0167

Karlyshev, A. V., Ketley, J. M., and Wren, B. W. (2005). The *Campylobacter* glycome. *FEMS Microbiol. Rev.* 29, 377–390. doi: 10.1016/j.fmrre.2005.01.003

Kelly, A. F., Park, S. F., Bovill, R., and Mackey, B. M. (2001). Survival of *Campylobacter jejuni* during stationary phase: evidence for the absence of a

phenotypic stationary-phase response. *Appl. Environ. Microbiol.* 67, 2248–2254. doi: 10.1128/AEM.67.5.2248-2254.2001

Kim, J. C., Oh, E., Kim, J., and Jeon, B. (2015). Regulation of oxidative stress resistance in *Campylobacter jejuni*, a microaerophilic foodborne pathogen. *Front. Microbiol.* 6:751. doi: 10.3389/fmicb.2015.00751

Klancnik, A., Guzej, B., Jamnik, P., Vuckovic, D., Abram, M., and Mozina, S. S. (2009). Stress response and pathogenic potential of *Campylobacter jejuni* cells exposed to starvation. *Res. Microbiol.* 160, 345–352. doi: 10.1016/j.resmic.2009.05.00

Korlath, J. A., Osterholm, M. T., Judy, L. A., Forfang, J. C., and Robinson, R. A. (1985). A point-source outbreak of campylobacteriosis associated with consumption of raw milk. *J. Infect. Dis.* 152, 592–596. doi: 10.1093/infdis/152.3.592

Kudirkienė, E., Bunevičienė, J., Brøndsted, L., Ingmer, H., Olsen, J. E., and Malakauskas, M. (2011). Evidence of broiler meat contamination with post-disinfection strains of *Campylobacter jejuni* from slaughterhouse. *Int. J. Food Microbiol.* 145, S116–S120. doi: 10.1016/j.ijfoodmicro.2010.06.024

Lévesque, S., Fournier, E., Carrier, N., Frost, E., Arbeit, R. D., and Michaud, S. (2013). Campylobacteriosis in urban versus rural areas: a case-case study integrated with molecular typing to validate risk factors and to attribute sources of infection. *PLoS ONE* 8:e83731. doi: 10.1371/journal.pone.0083731

Levi, A., Folcher, M., Jenal, U., and Shuman, H. A. (2011). Cyclic diguanylate signaling proteins control intracellular growth of *Legionella pneumophila*. *mBio* 2:e00316-10. doi: 10.1128/mBio.00316-10

Li, L., Mendis, N., Trigui, H., and Faucher, S. P. (2015). Transcriptomic changes of *Legionella pneumophila* in water. *BMC Genomics* 16:637. doi: 10.1186/s12864-015-1869-6

Lin, J., Cagliero, C., Guo, B., Barton, Y. W., Maurel, M. C., Payot, S., et al. (2005). Bile salts modulate expression of the CmeABC multidrug efflux pump in *Campylobacter jejuni*. *J. Bacteriol.* 187, 7417–7424. doi: 10.1128/JB.187.21.7417-7424.2005

Lin, J., Sahin, O., Michel, L. O., and Zhang, Q. (2003). Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of *Campylobacter jejuni*. *Infect. Immun.* 71, 4250–4259. doi: 10.1128/IAI.71.8.4250-4259.2003

Lin, J., Yan, M., Sahin, O., Pereira, S., Chang, Y. J., and Zhang, Q. (2007). Effect of macrolide usage on emergence of erythromycin-resistant *Campylobacter* isolates in chickens. *Antimicrob. Agents Chemother.* 51, 1678–1686. doi: 10.1128/AAC.01411-06

Lind, L., Sjögren, E., Melby, K., and Kaijser, B. (1996). DNA fingerprinting and serotyping of *Campylobacter jejuni* isolates from epidemic outbreaks. *J. Clin. Microbiol.* 34, 892–896.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Mendis, N., McBride, P., and Faucher, S. P. (2015). Short-term and long-term survival and virulence of *Legionella pneumophila* in the defined freshwater medium Fraquil. *PLoS ONE* 10:e0139277. doi: 10.1371/journal.pone.0139277

Miele, V., Penel, S., and Duret, L. (2011). Ultra-fast sequence clustering from similarity networks with SiLiX. *BMC Bioinformatics* 12:116. doi: 10.1186/1471-2105-12-116

Miller, W. G., Bates, A. H., Horn, S. T., Brandl, M. T., Wachtel, M. R., and Mandrell, R. E. (2000). Detection on surfaces and in Caco-2 cells of *Campylobacter jejuni* cells transformed with new gfp, yfp, and cfp marker plasmids. *Appl. Environ. Microbiol.* 66, 5426–5436. doi: 10.1128/AEM.66.12.5426-5436.2000

Mittal, R., Agrawal, A., Roach, R., Buckley, T., and Tiwari, A. K. (2013). Acquired Legionnaire's disease through aerosolization of water from natural sources. *Trans. Clin. Biol.* 1, 6–9. doi: 10.14259/tcb.v1i1.61

Mittenhuber, G. (2001). Comparative genomics and evolution of genes encoding bacterial (p) ppGpp synthetases/hydrolases (the Rel. RelA and SpoT proteins). *J. Mol. Microbiol. Biotechnol.* 3, 585–600.

Morel, F. M., Westall, J. C., Reuter, J., and Chaplick, J. P. (1975). *Description of the Algal growth Media 'Aquil' and 'Fraquil'*. Technical Report No. 16. Cambridge, MA: Massachusetts Institute of Technology.

Murphy, C., Carroll, C., and Jordan, K. (2006). Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol.* 100, 623–632. doi: 10.1111/j.1365-2672.2006.02903.x

Murphy, C., Carroll, C., and Jordan, K. N. (2005). The effect of different media on the survival and induction of stress responses by *Campylobacter jejuni*. *J. Microbiol. Methods* 62, 161–166. doi: 10.1016/j.mimet.2005.02.005

Naito, M., Frirdich, E., Fields, J. A., Pryjma, M., Li, J., Cameron, A., et al. (2010). Effects of sequential *Campylobacter jejuni* 81–176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. *J. Bacteriol.* 192, 2182–2192. doi: 10.1128/JB.01222-09

Nguyen, V. T., Turner, M. S., and Dykes, G. A. (2011). Influence of cell surface hydrophobicity on attachment of *Campylobacter* to abiotic surfaces. *Food Microbiol.* 28, 942–950. doi: 10.1016/j.fm.2011.01.004

O'Reilly, C. E., Bowen, A. B., Perez, N. E., Sarisky, J. P., Shepherd, C. A., Miller, M. D., et al. (2007). A waterborne outbreak of gastroenteritis with multiple etiologies among resort island visitors and residents: Ohio, 2004. *Clin. Infect. Dis.* 44, 506–512. doi: 10.1086/511043

Palmer, S. R., Gully, P. R., White, J. M., Pearson, A. D., Suckling, W. G., Jones, D. M., et al. (1983). Water-borne outbreak of *Campylobacter* gastroenteritis. *Lancet* 1, 287–290. doi: 10.1016/S0140-6736(83)91698-7

Palyada, K., Sun, Y. Q., Flint, A., Butcher, J., Naikare, H., and Stintzi, A. (2009). Characterization of the oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. *BMC Genomics* 10:481. doi: 10.1186/1471-2164-10-481

Palyada, K., Threadgill, D., and Stintzi, A. (2004). Iron acquisition and regulation in *Campylobacter jejuni*. *J. Bacteriol.* 186, 4714–4729. doi: 10.1128/JB.186.14.4714-4729.2004

Parkhill, J., Wren, B., Mungall, K., Ketley, J., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665–668. doi: 10.1038/35001088

Patrone, V., Campana, R., Vallorani, L., Dominici, S., Federici, S., Casadei, L., et al. (2013). CadF expression in *Campylobacter jejuni* strains incubated under low-temperature water microcosm conditions which induce the viable but non-culturable (VBNC) state. *Antonie Van Leeuwenhoek* 103, 979–988. doi: 10.1007/s10482-013-9877-5

Pearson, A., Greenwood, M., Healing, T., Rollins, D., Shahamat, M., Donaldson, J., et al. (1993). Colonization of broiler chickens by waterborne *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 59, 987–996.

Pearson, B. M., Louwen, R., Van Baarlen, P., and Van Vliet, A. H. (2015). Differential distribution of Type II CRISPR-Cas systems in agricultural and nonagricultural *Campylobacter coli* and *Campylobacter jejuni* isolates correlates with lack of shared environments. *Genome Biol. Evol.* 7, 2663–2679. doi: 10.1093/gbe/evv174

Pfeffer, J. M., Strating, H., Weadge, J. T., and Clarke, A. J. (2006). Peptidoglycan O acetylation and autolysin profile of *Enterococcus faecalis* in the viable but nonculturable state. *J. Bacteriol.* 188, 902–908. doi: 10.1128/JB.188.3.902-908.2006

Rollins, D., and Colwell, R. (1986). Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl. Environ. Microbiol.* 52, 531–538.

Semchenko, E. A., Day, C. J., Wilson, J. C., Grice, I. D., Moran, A. P., and Korolik, V. (2010). Temperature-dependent phenotypic variation of *Campylobacter jejuni* lipooligosaccharides. *BMC Microbiol.* 10:305. doi: 10.1186/1471-2180-10-305

Signoretto, C., Lleò, M. M., and Canepari, P. (2002). Modification of the peptidoglycan of *Escherichia coli* in viable but nonculturable state. *Curr. Microbiol.* 44, 125–131. doi: 10.1007/s00284-001-0062-0

Signoretto, C., Lleò, M. M., Tafi, M. C., and Canepari, P. (2000). Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. *Appl. Environ. Microbiol.* 66, 1953–1959. doi: 10.1128/AEM.66.5.1953-1959.2000

Skirrow, M. (1991). Epidemiology of *Campylobacter enteritis*. *Int. J. Food Microbiol.* 12, 9–16. doi: 10.1016/0168-1605(91)90044-P

Snelling, W., McKenna, J., Lecky, D., and Dooley, J. (2005). Survival of *Campylobacter jejuni* in waterborne protozoa. *Appl. Environ. Microbiol.* 71, 5560–5571. doi: 10.1128/aem.71.9.5560-5571.2005

Sopwith, W., Birtles, A., Matthews, M., Fox, A., Gee, S., Painter, M., et al. (2008). Identification of potential environmentally adapted *Campylobacter jejuni* Strain, United Kingdom. *Emerg. Infect. Dis.* 14, 1769–1773.

Srivatsan, A., and Wang, J. D. (2008). Control of bacterial transcription, translation and replication by (p)ppGpp. *Curr. Opin. Microbiol.* 11, 100–105. doi: 10.1016/j.mib.2008.02.001

Stahl, M., Butcher, J., and Stintzi, A. (2012). Nutrient acquisition and metabolism by *Campylobacter jejuni*. *Front. Cell. Infect. Microbiol.* 2:5. doi: 10.3389/fcimb.2012.00005

Stintzi, A., Marlow, D., Palyada, K., Naikare, H., Panciera, R., Whitworth, L., et al. (2005). Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. *Infect. Immun.* 73, 1797–1810. doi: 10.1128/IAI.73.3.1797-1810.2005

Tatchou-Nyamsi-König, J. A., Moreau, A., Federighi, M., and Block, J. C. (2007). Behaviour of *Campylobacter jejuni* in experimentally contaminated bottled natural mineral water. *J. Appl. Microbiol.* 103, 280–288. doi: 10.1111/j.1365-2672.2006.03239.x

Thibodeau, A., Fravalo, P., Yergeau, É., Arsenault, J., Lahaye, L., and Letellier, A. (2015). Chicken caecal microbiome modifications induced by *Campylobacter jejuni* colonization and by a non-antibiotic feed additive. *PLoS ONE* 10:e0131978. doi: 10.1371/journal.pone.0131978

Thomas, C., Hill, D., and Mabey, M. (1999). Evaluation of the effect of temperature and nutrients on the survival of *Campylobacter* spp. in water microcosms. *J. Appl. Microbiol.* 86, 1024–1032. doi: 10.1046/j.1365-2672.1999.00789.x

Trigui, H., Dudyk, P., Oh, J., Hong, J. I., and Faucher, S. P. (2015a). A regulatory feedback loop between RpoS and SpoT supports the survival of *Legionella pneumophila* in water. *Appl. Environ. Microbiol.* 81, 918–928. doi: 10.1128/AEM.03132-14

Trigui, H., Thibodeau, A., Fravalo, P., Letellier, A., and Faucher, S. P. (2015b). Survival in water of *Campylobacter jejuni* strains isolated from the slaughterhouse. *Springerplus* 4, 799. doi: 10.1186/s40064-015-1595-1

Trigui, H., Paquet, V. E., Charette, S. J., and Faucher, S. P. (2016). Packaging of *Campylobacter jejuni* into multilamellar bodies by the ciliate *Tetrahymena pyriformis*. *Appl. Environ. Microbiol.* 82, 2783–2790. doi: 10.1128/AEM.03921-15

Vallenet, D., Belda, E., Calteau, A., Cruveiller, S., Engelen, S., Lajus, A., et al. (2013). MicroScope—an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res.* 41, D636–D647. doi: 10.1093/nar/gks1194

Vogt, R. L., Sours, H. E., Barrett, T., Feldman, R. A., Dickinson, R. J., and Witherell, L. (1982). *Campylobacter enteritis* associated with contaminated water. *Ann. Intern. Med.* 96, 792–796. doi: 10.7326/0003-4819-96-3-292

Walker, R., Caldwell, M., Lee, E., Guerry, P., Trust, T. J., and Ruiz-Palacios, G. M. (1986). Pathophysiology of *Campylobacter enteritis*. *Microbiol. Rev.* 50, 81–94.

Wassenaar, T. M., and Blaser, M. J. (1999). Pathophysiology of *Campylobacter jejuni* infections of humans. *Microbes Infect.* 1, 1023–1033. doi: 10.1016/S1286-4579(99)80520-6

Whitfield, C., and Trent, M. S. (2014). Biosynthesis and export of bacterial lipopolysaccharides. *Annu. Rev. Biochem.* 83, 99–128. doi: 10.1146/annurev-biochem-060713-035600

Wong, A., Lange, D., Houle, S., Arbatsky, N. P., Valvano, M. A., Knirel, Y. A., et al. (2015). Role of capsular modified heptose in the virulence of *Campylobacter jejuni*. *Mol. Microbiol.* 96, 1136–1158. doi: 10.1111/mmi.12995

Wright, J. A., Grant, A. J., Hurd, D., Harrison, M., Guccione, E. J., Kelly, D. J., et al. (2009). Metabolite and transcriptome analysis of *Campylobacter jejuni* in vitro growth reveals a stationary-phase physiological switch. *Microbiology* 155, 80–94. doi: 10.1099/mic.0.021790-0

Young, K. T., Davis, L. M., and DiRita, V. J. (2007). *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat. Rev. Microbiol.* 5, 665–679. doi: 10.1038/nrmicro1718

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 February 2018
doi: 10.3389/fmicb.2018.00249

Transcriptional Response of *Staphylococcus aureus* to Sunlight in Oxic and Anoxic Conditions

*Jill S. McClary and Alexandria B. Boehm**

Civil and Environmental Engineering, Stanford University, Stanford, CA, United States

The transcriptional response of *Staphylococcus aureus* strain Newman to sunlight exposure was investigated under both oxic and anoxic conditions using RNA sequencing to gain insight into potential mechanisms of inactivation. *S. aureus* is a pathogenic bacterium detected at recreational beaches which can cause gastrointestinal illness and skin infections, and is of increasing public health concern. To investigate the *S. aureus* photostress response in oligotrophic seawater, *S. aureus* cultures were suspended in seawater and exposed to full spectrum simulated sunlight. Experiments were performed under oxic or anoxic conditions to gain insight into the effects of oxygen-mediated and non-oxygen-mediated inactivation mechanisms. Transcript abundance was measured after 6 h of sunlight exposure using RNA sequencing and was compared to transcript abundance in paired dark control experiments. Culturable *S. aureus* decayed following biphasic inactivation kinetics with initial decay rate constants of 0.1 and 0.03 m^2 kJ^{-1} in oxic and anoxic conditions, respectively. RNA sequencing revealed that 71 genes had different transcript abundance in the oxic sunlit experiments compared to dark controls, and 18 genes had different transcript abundance in the anoxic sunlit experiments compared to dark controls. The majority of genes showed reduced transcript abundance in the sunlit experiments under both conditions. Three genes (*ebpS*, *NWMN_0867*, and *NWMN_1608*) were found to have the same transcriptional response to sunlight between both oxic and anoxic conditions. In the oxic condition, transcripts associated with porphyrin metabolism, nitrate metabolism, and membrane transport functions were increased in abundance during sunlight exposure. Results suggest that *S. aureus* responds differently to oxygen-dependent and oxygen-independent photostress, and that endogenous photosensitizers play an important role during oxygen-dependent indirect photoinactivation.

Keywords: *Staphylococcus*, sunlight, photoinactivation, transcription, RNA, sequencing

OPEN ACCESS

Edited by:
Peiying Hong,
King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Sebastien P. Faucher,
McGill University, Canada
Bin Cao,
Nanyang Technological University, Singapore

****Correspondence:***
Alexandria B. Boehm
aboehm@stanford.edu

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *28 November 2017*
Accepted: *31 January 2018*
Published: *23 February 2018*

Citation:
McClary JS and Boehm AB (2018) Transcriptional Response of Staphylococcus aureus to Sunlight in Oxic and Anoxic Conditions. Front. Microbiol. 9:249. doi: 10.3389/fmicb.2018.00249

INTRODUCTION

In the United States, pollution of recreational waters led to 23,481 beach closures in 2011 (National Resources Defense Council, 2012), and contact with polluted recreational waters can cause gastrointestinal illness, respiratory infections, and skin ailments (Cabelli et al., 1982; Haile et al., 1999; Colford et al., 2007). To prevent excess exposure to microbial pollution, recreational

waters are traditionally monitored by the detection of culturable fecal indicator bacteria (FIB), such as *Escherichia coli* and enterococci, which requires processing times of ∼18–24 h (US EPA, 2012). However, FIB concentrations are known to fluctuate on short timescales due to factors such as sunlight exposure and tides (Boehm et al., 2009; Russell et al., 2013; Corsi et al., 2016), calling into question the utility of FIB measurements that require long processing times. To address this issue, rapid detection methods and water quality modeling techniques have begun to be applied in recreational water quality monitoring (Wade et al., 2008; Thoe et al., 2015; He et al., 2016; Tryland et al., 2016). However, an incomplete understanding of the mechanisms leading to bacterial decay in coastal environments limits our ability to include these factors in water quality models and points to a need for improved understanding of these mechanisms.

Photoinactivation, or inactivation due to sunlight exposure, is an important process that modulates bacterial concentrations in environmental waters (Sassoubre et al., 2015) and can occur by both direct and indirect mechanisms. Direct photoinactivation involves the absorption of photons by vital cell components, like nucleic acids, which leads to cellular damage (Sinha and Häder, 2002). In contrast, during indirect photoinactivation, photons are absorbed by sensitizers (either endogenous or exogenous to the cell) which become excited and subsequently damage vital cell components either directly or through generation of reactive oxygen species (ROS) (Curtis et al., 1992). Several studies have identified ROS as one of the most important factors influencing photoinactivation of both bacteria and viruses in natural waters (Kohn and Nelson, 2007; Sassoubre et al., 2012; Maraccini et al., 2016b). However, the relative importance of direct and indirect photoinactivation mechanisms in environmental systems remains poorly understood. In engineered systems, advanced oxidation unit processes, which combine UV treatment with ROS or ROS precursors, are being increasingly considered for use in wastewater reuse treatment trains (Sun et al., 2016). The combination of ROS precursors and light exposure is also the basis of photodynamic therapy, which can be used for localized treatment of bacterial infections (Sabbahi et al., 2008). Due to the importance of photoinactivation in a range of contexts, a better understanding of direct and indirect photoinactivation mechanisms is needed.

Investigation into the transcriptional response of bacteria to sunlight stress can provide insights into photoinactivation mechanisms. Microarrays and RNA sequencing (RNA-seq) have been used to investigate the effects of sunlight exposure on gene expression in FIB, including *Enterococcus faecalis* (Sassoubre et al., 2014) and *E. coli* (Berney et al., 2006; Al-Jassim et al., 2017). A range of cellular processes are triggered by sunlight exposure, including DNA repair, oxidative stress response, virulence, and SOS response (Berney et al., 2006; Sassoubre et al., 2014; Al-Jassim et al., 2017). Evidence to date suggests that different species transcribe different genes in response to sunlight exposure. For example, following sunlight exposure, genes coding for superoxide dismutase, a highly conserved enzyme involved in oxidative stress response, were identified as upregulated in *E. faecalis* (Sassoubre et al., 2012, 2014) but downregulated in *E. coli* (Berney et al., 2006; Al-Jassim et al., 2017). This information allows us to gain insight into cells' ability to repair or respond to sunlight exposure and advances our understanding of bacterial fate in sunlight-exposed waters.

One bacterial pathogen of concern in recreational waters is *Staphylococcus aureus*, which is commonly detected in recreational beach water and sand (Charoenca and Fujioka, 1993; Goodwin et al., 2012; Levin-Edens et al., 2012; Hower et al., 2013) and can cause gastrointestinal, respiratory, and skin infections. Epidemiological studies have identified associations between recreational water contact and various skin ailments (Wade et al., 2008; Yau et al., 2009; Sinigalliano et al., 2010). Some studies have further identified relationships between staphylococci concentrations in beach water and skin ailments (Prüss, 1998), and between *S. aureus* skin infections and recreational water contact (Charoenca and Fujioka, 1995), indicating that recreational beaches may be a reservoir for pathogenic *S. aureus* in the environment. Recently, concern regarding particular strains of antibiotic resistant *S. aureus* that are able to spread within the community has grown. Compared to healthcare-associated strains, community-associated *S. aureus* have also been shown to be more virulent in mouse models, partially due to their ability to resist ROS-mediated killing by neutrophils (Voyich et al., 2005).

The present study investigates the transcriptional response of *S. aureus* suspended in clear seawater to sunlight exposure in order to gain insight into photoinactivation mechanisms and bacterial stress response. Experiments were performed under both oxic and anoxic conditions in order to differentiate between photostress responses associated with oxygen-mediated and non-oxygen-medated photoinactivation mechanisms. To our knowledge, this is the first study to evaluate genome-wide transcriptional response of a pathogenic bacterium under both oxygen-dependent and oxygen-independent photostress conditions.

MATERIALS AND METHODS

Photoinactivation Experiments

Staphylococcus aureus photoinactivation under oxic and anoxic conditions was evaluated using an experimental design identical to a previously published study (McClary et al., 2017). In brief, *S. aureus* subsp. *aureus* str. Newman (ATCC 25904) was grown in chemostat cultures filled with 20 mL 25% Brain Heart Infusion (BHI) broth (Fluka Analytical, Steinheim, Germany). *S. aureus* was grown in chemostats in order to improve reproducibility between experimental replicates (Maraccini et al., 2015). After reaching a stable growth rate, bacteria were washed twice and resuspended in ∼1 L sterile simulated seawater for a concentration of $\sim 10^7$ CFU/mL. The composition of simulated seawater was derived from Parker et al. (2013) and consisted of 424 mM sodium chloride, 0.87 mM sodium bromide, 29.2 mM sodium sulfate, 0.27 mM sodium carbonate, 1.83 mM sodium bicarbonate, 10.5 mM potassium chloride, 54.8 mM magnesium chloride, and 10.7 mM calcium chloride. The initial concentration of $\sim 10^7$ CFU/mL of *S. aureus* was chosen to allow for sufficient masses of mRNA to be extracted for sequencing. For

experiments performed under anoxic conditions, the bacteria-seawater suspension was divided into two black PVC pipe reactors (described previously McClary et al., 2017), one experimental and one control. Reactors were sealed by fixing quartz glass plates to the top of the reactors with silicone sealant and were then sparged with nitrogen through rubber septa to remove oxygen from the water column and headspace. After sparging for ∼30 min, reactors were held in the dark at 15°C with constant stirring for 12 h to acclimate to a cool, oligotrophic environment. For experiments performed under oxic conditions, reactors were set up identically but with quartz glass plates secured loosely with tape and without nitrogen sparging.

After 12 h of incubation at 15°C, the experimental reactor (oxic or anoxic) was placed in a 15°C recirculating water bath in a solar simulator (Atlas Suntest XLS+; Chicago, IL) equipped with a 1.1 kW xenon arc lamp and a glass filter to generate full spectrum sunlight (see Maraccini et al., 2015 for solar simulator light spectra). Reactors were exposed to 6 h of full spectrum sunlight. Six hours of sunlight exposure was chosen based on previous data showing significant changes in gene expression at this exposure duration (McClary et al., 2017). The control reactor was kept in the dark at 15°C during the photoinactivation experiments. Both reactors were constantly stirred, and samples were taken from the reactors as described below. For experiments performed under anoxic conditions, an equal volume of nitrogen was injected into the reactors during sampling events to keep the reactors anoxic and at constant pressure. Triplicate experiments were performed in both oxic and anoxic conditions to generate three biological replicates for each condition.

Culturability

To track *S. aureus* photoinactivation during experiments, 0.5-mL samples were taken from the experimental reactor every hour and from the control reactor every 3 h to determine culturability. Samples were diluted as necessary and appropriate dilutions were spread plated in duplicate on Brain Heart Infusion agar (BD Difco, Sparks, MD). After incubation at 37°C for 18–24 h, colonies were counted and sample concentrations were calculated in CFU/mL. Only dilutions resulting in countable colonies on duplicate plates were used to calculate sample concentrations. Inactivation rate constants were determined by non-linear regression using a biphasic first-order inactivation model:

$$\ln\left(\frac{C}{C_0}\right) = \ln\left[(1-f)\,e^{-k_1 F_{UVA+UVB}} + f e^{-k_2 F_{UVA+UVB}}\right]$$

where $ln(C/C_0)$ is the natural log-transformed relative concentration, f is the subpopulation fraction, k_1 and k_2 are the inactivation rate constants for the first and second phases, respectively, and $F_{UVA+UVB}$ is fluence in kJ/m^2. Fluence was calculated as has been done previously based on wavelengths in the UVA & UVB spectra (280–400 nm) (Maraccini et al., 2016a; McClary et al., 2017). Rate constants were also determined using log-linear and shoulder log-linear decay models (Geeraerd et al., 2005), but the biphasic model resulted in the best fit as determined by minimizing residual standard error and so was used for all subsequent analysis.

RNA Stabilization, Extraction, and rRNA Removal

At the end of each experiment (i.e., after 6 h of sunlight exposure), 200-mL samples were taken from both the experimental and control reactors for RNA extraction. Samples were immediately centrifuged for 10 min at 10,000 × *g*, and bacterial pellets were treated with RNAProtect Bacterial Reagent (Qiagen, Hilden, Germany). After 5 min of incubation at room temperature, samples were centrifuged again and the supernatant discarded. Stabilized bacterial pellets were stored at −80°C until RNA extraction.

RNA extractions were performed as described previously (McClary et al., 2017). In brief, stored bacterial pellets were resuspended in 0.2 mg/mL lysostaphin (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C to lyse cells. Further lysis was performed by addition of a 100:1 vol:vol solution of Buffer RLT (Qiagen) and β-mercaptoethanol (Sigma-Aldrich), followed by bead beating in Lysis Matrix B tubes with a FastPrep-24 cell homogenizer (MP Biomedicals, Solon, OH). After brief centrifugation, lysate was transferred to new tubes, 470 μL ethanol was added to each sample, and RNA was extracted using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. After elution in 60 μL of RNase-free water warmed to 60°C, extracts were DNase-digested using the RNase-free DNase Set (Qiagen), following the manufacturer's instructions. Samples were then cleaned up using the RNeasy Mini Kit, with final elution in 40 μL of RNase-free water warmed to 60°C. DNase digestion was confirmed by a qPCR assay targeting the *rexA* gene of *S. aureus* as described previously (McClary et al., 2017). Primer and probe sequences for the qPCR assay are provided in **Table 1**. For each set of extractions, an extraction blank was processed in parallel to verify lack of contamination from protocol reagents.

Total RNA samples were precipitated by adding 0.1 volume 3 M sodium acetate, 2.5 μL of 2 mg/mL glycogen, and 2.5 volumes 100% ethanol. The mixture was left overnight at −20°C before recovering precipitated RNA by centrifuging at 12,000 × *g* for 30 min at 4°C. RNA pellets were then washed twice in 1 mL ice cold 70% ethanol and recollected by centrifuging at 12,000 × *g* for 10 min at 4°C. After two ethanol washes, the RNA pellet was dissolved in 25 μL TE buffer. RNA precipitates were then depleted of rRNA using the MICROBExpress mRNA Enrichment Kit (Life Technologies, Carlsbad, CA), following the manufacturer's instructions. Five microliters from each extraction blank was also pooled and carried through the precipitation and rRNA-removal procedures as a negative control. Total RNA extracts, RNA precipitates, and rRNA-depleted samples were quantified on a Qubit v2.0 fluorometer or Nanodrop 1000, and RNA quality was confirmed on an Agilent 2100 Bioanalyzer at the Stanford Protein and Nucleic Acid Facility.

Library Preparation and Sequencing

Indexed sequencing libraries were prepared from rRNA-depleted samples, including the negative control, using the ScriptSeq v2 RNA-Seq Library Preparation Kit and ScriptSeq Index PCR

TABLE 1 | Summary of primer and probe sequences used for RTqPCR reactions.

Gene target	Gene name	Primer/probe	Sequence	Product size (bp)
NWMN_0838	*rexA*	Forward primer	GATTTGGACTGACGCGCAA	142
		Reverse primer	ATCGACATCAATGCCATCACG	
		Probe	TGTTGCAGCCGCGGCAGGTTCAGGT	
NWMN_1240	*metL*	Forward primer	GCAGGCAGTTTAGCAACAGGTA	134
		Reverse primer	GAATCCATCATTTCCCGTGTTT	
		Probe	TGAATCAGATTTACACACATTGCCACCACA	
NWMN_1723	*hemY*	Forward primer	GAAGTCTGATAAAAGGTATGAAGGATGAG	122
		Reverse primer	TTCAATAAATGAGCTTAAACCATGCT	
		Probe	CCTGGCGCACCGAAAGGACAA	
NWMN_2341		Forward primer	CACCTGTTAAAGGTTCTGAATTTGC	122
		Reverse primer	CGCTTTAAACTTCTCATTGCTTACG	
		Probe	TCAACCTGCGCAACCATTTGAACG	
NWMN_2439	*cidB*	Forward primer	ACTGGCGTCATGCTGAATTTC	116
		Reverse primer	TCGATACCTACTGCGGCTGTT	
		Probe	ACGTCATTGTAACGTTATTGCCCCGATCT	

Primers (Epicentre, Madison, WI) following the manufacturer's instructions. PCR amplification of the indexed libraries was performed for 14 cycles. An additional positive control sample was also included, consisting of a 101 nt RNA sequence coding for a portion of the *grpE* gene of *Methanobacterium* sp. MB1, obtained from Integrated DNA Technologies (San Diego, CA). This sequence was chosen as a control as it would not be expected to occur in any of the experimental samples. Library preparation of the positive control followed the manufacturer's instructions for Severely Fragmented RNA. Amplified indexed libraries were quantified on an Agilent 2100 Bioanalyzer at the Stanford Functional Genomics Facility.

A total of 14 indexed libraries were generated, with each index corresponding to an individual sample (**Table 2**). The 12 oxic & anoxic sample libraries were combined in equimolar ratios to generate a pooled library. The positive control was added to the pooled library at a 10-fold lower molar ratio. The average volume of the oxic & anoxic sample libraries that were pooled was calculated, and this volume of negative control was also added to the pooled library. The pooled library was then sequenced on an Illumina MiSeq machine at the Stanford Functional Genomics Facility, generating 75 bp paired-end reads.

TABLE 2 | Description of samples included for RNA sequencing.

Sample number	Experiment number	Condition	Treatment
1	1		Light
2			Dark
3	2	Oxic	Light
4			Dark
5	3		Light
6			Dark
7	4		Light
8			Dark
9	5	Anoxic	Light
10			Dark
11	6		Light
12			Dark
13	Positive control		
14	Negative control		

Each sample corresponds to an individual treatment and condition, and each sample was indexed separately before pooling into a single sequencing library.

Sequencing Data Analysis

Raw sequencing data was demultiplexed and quality scored by Illumina MiSeq software to generate fastq files for forward and reverse reads of each indexed sample library. Initial read quality was assessed in FastQC version 0.11.4. Adapter trimming and quality filtering was performed for paired-end reads using Trimmomatic version 0.36 with provided adapter Fasta files for TruSeq3, removing low quality bases from the beginning and end of reads, and dropping reads shorter than 75% of the amplicon length or with quality scores <30 (Bolger et al., 2014).

Following quality filtering, RNA-seq reads were aligned to the *S. aureus* genome using STAR version 2.5.3a with default settings (Dobin et al., 2013), and count matrices were generated from the alignment output using the Bioconductor GenomicAlignments package (Gentleman et al., 2004; Lawrence et al., 2013). The *S. aureus* genome and gene annotation information used for alignment and read counting, respectively, were obtained from Ensembl (taxid: 426430). Separate count matrices were generated for oxic and anoxic experiments, and each count matrix was filtered to remove genes with low or no counts (i.e., counts ≤ 1 across all samples) and to remove counts mapped to rRNA genes. Data from the count matrices were then analyzed using

DESeq2 (Love et al., 2014). First, the regularized-logarithm (rlog) transformation was applied to the count matrices and used to calculate Euclidean distances between samples. Visualization of the sample-to-sample distances using a distance matrix revealed that samples from one experiment (Experiment #4, Samples 7 & 8, **Table 2**) were outliers (Supplementary Figure 1), and so this experiment was dropped from further analysis. Next, non-transformed count matrices were used to determine differential expression between light and dark conditions using DESeq2. DESeq2 is capable of evaluating differential expression on as few as two biological replicates (Love et al., 2014; Sekulovic and Fortier, 2015), making this method most appropriate for use in this study. Genes with a false discovery rate (FDR) < 25% were considered significantly differentially expressed. After identifying differentially expressed genes, gene functions were explored using the KEGG pathways database. All sequencing data analysis was performed in Linux and R version 3.4.1. RNA-seq data are deposited in the NCBI sequence read archive (SRA) under accession number SRP125691.

Reverse transcription qPCR (RTqPCR) confirmation of RNA-seq results was performed for four selected genes: *metL*, *hemY*, *cidB*, and NWMN_2341 (**Table 1**). These genes were selected based on (1) their observed expression changes from RNA-seq data analysis, and (2) the ability to develop efficient qPCR assays for these genes. Differential expression between light and dark samples by RTqPCR was based on calculating a relative expression ratio (*R*) using the Pfaffl method (Pfaffl, 2001) with *rexA* as the reference gene. *rexA* was used as a reference because we previously developed an RTqPCR assay for this gene (McClary et al., 2017) and the RNA sequencing data analysis demonstrated that *rexA* was not significantly differentially expressed. Significant differential expression was determined if *R* was ≥ 2 or ≤ 0.5 and if $R \pm$ standard error (SE) did not include 1. Further details on RTqPCR assays are provided in the Supplementary Material.

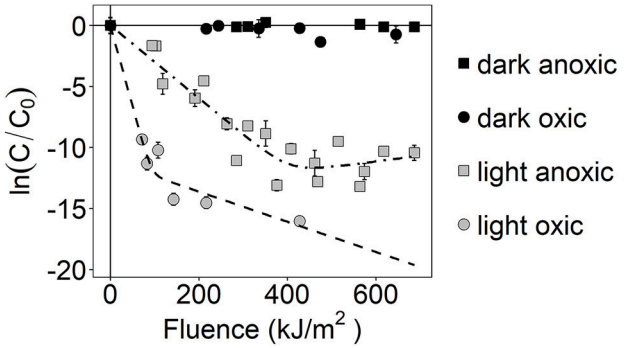


FIGURE 1 | Photoinactivation kinetics of *S. aureus*, as measured by loss of culturability. $\ln(C/C_0)$ is the natural-log transformed relative concentration. Error bars = ± standard deviation of technical replicates. Dashed lines are modeled biphasic inactivation curves.

TABLE 3 | Modeled inactivation rate constants of *S. aureus* under sunlight exposure.

Condition	Inactivation rate constants (m^2 kJ^{-1})	
	k_1	k_2
Oxic	0.1 ± 0.01	0.01 ± 0.005
Anoxic	0.03 ± 0.002	−0.005 ± 0.007

Inactivation was fit to a biphasic model, and reported rate constants represent the first (k_1) and second (k_2) phases of inactivation.

RESULTS

Staphylococcus aureus Photoinactivation Kinetics in Oxic and Anoxic Conditions

Inactivation of *S. aureus* was observed during sunlight exposure under both oxic and anoxic conditions, as shown in **Figure 1**, and was discussed in our previous publication (McClary et al., 2017). Inactivation kinetics are biphasic under both conditions, displaying relatively fast inactivation followed by a period of slow or no inactivation. Non-linear regression was used to fit the observed data to biphasic first-order inactivation curves, and inactivation rate constants are presented in **Table 3**. The first-order rate constant during the initial phase of inactivation was larger in the oxic compared to anoxic condition ($k_1 \pm$ SE = 0.1 ± 0.01 m^2 kJ^{-1} in oxic conditions vs. 0.03 ± 0.002 m^2 kJ^{-1} in anoxic; Z-test, $P < 0.05$). These rate constants are in agreement with those presented in our previous work (McClary et al., 2017). The first-order rate constants during the second phase of inactivation (k_2) were 0.01 ± 0.005 m^2 kJ^{-1} and −0.005 ±0.007 m^2 kJ^{-1} in oxic and anoxic conditions, respectively. *S. aureus* continued to slowly decay following the initial rapid decay in oxic conditions. In anoxic conditions, k_2 is not different from 0. No inactivation was observed in dark controls, suggesting that all observed inactivation was due to sunlight exposure. After 6 h of sunlight exposure [i.e., fluence ($F_{UVA+UVB}$) between 427 and 687 kJ/m^2], the concentration of cultivatable cells was at or below the limit of detection (i.e., ≤ 20 CFU/mL) in oxic experiments and was ∼700 CFU/mL for anoxic sunlight experiments. After 6 h of dark incubation, the concentration of cultivatable cells in the control oxic and anoxic experiments remained steady at $\sim 10^7$ CFU/mL. These samples were used to investigate gene expression changes in sunlight-exposed experiments vs. dark controls.

Differential Gene Expression Due to Sunlight Exposure in Oxic and Anoxic Conditions

RNA sequencing was used to investigate changes in *S. aureus* gene expression as a result of sunlight exposure under oxic and anoxic conditions. A summary of sample-specific data generated by RNA sequencing is presented in **Table 4**. Sequencing resulted in ∼21 million total reads, with an average of ∼900,000 read pairs per sample. Quality filtering removed between 7 and 39% of read pairs per sample, and the resulting filtered reads aligned to the *S. aureus* genome at rates of at least 92%. As described in the Materials and Methods, based on Euclidean sample-to-sample distances generated from rlog-transformed count matrices, samples from one experiment (Experiment #4) clustered far

TABLE 4 | Summary of sample-specific data generated by RNA sequencing.

Condition	Oxic						Anoxic					
Experiment number	**1**		**2**		**3**		**4**		**5**		**6**	
Sample	**Light**	**Dark**	**Light**	**Dark**	**Light**	**Dark**	**Light**	**Dark**	**Light**	**Dark**	**Light**	**Dark**
Data generated (MB)	480	405	468	340	316	392	171	423	467	306	157	368
Total read pairs	1157836	971815	1132694	823982	843365	950993	412592	1022242	1127263	748010	430231	891451
Total read pairs after Trimmomatic	1077294	906926	1035527	752140	567485	867647	385046	943652	1044373	665765	260350	816798
Reads mapped (%)	98.7	99.1	99.2	99	97.3	98.3	99.1	97.4	99.4	98.8	92.2	94.5

from all other samples; samples from this experiment were subsequently removed from further gene expression analyses.

To determine the effects of sunlight exposure on gene expression, differential expression analysis was carried out comparing sunlight-exposed samples from a single experimental condition (oxic or anoxic) to corresponding controls prepared identically and kept in the dark. Using this framework, a total of 71 differentially expressed genes were identified from oxic experiments (**Table 5**) and 18 from anoxic experiments (**Table 6**). Of these, three genes were differentially expressed under sunlight exposure in both the oxic and anoxic conditions: NWMN_1608 was increased in expression, while *ebpS* and NWMN_0867 were decreased in expression. Under both conditions, most differentially expressed genes showed reduced expression under sunlight-exposed conditions compared to the dark control; nine genes and two genes were significantly increased in abundance in sunlit oxic and anoxic conditions, respectively. Of the total number of differentially expressed genes, the proportions of genes showing increased expression under oxic and anoxic conditions are similar.

Functional Classification of Differentially Expressed Genes

The genome of *S. aureus* subsp. *aureus* str. Newman contains genes encoding 2,624 proteins, of which 1,051 are classified as hypothetical meaning that their function is unknown or unconfirmed. In the oxic condition, 30 differentially expressed genes (42% of 71) were assigned to functional pathways whereas for the anoxic condition, three differentially expressed genes (17% of 18) were assigned (**Figure 2**). Functional pathways with decreased expression due to sunlight exposure in both the oxic and anoxic conditions involved metabolism, environmental information processing, genetic information processing, cellular processes, and human disease. Expression of other genes involved in metabolism and environmental information processing were also induced by sunlight exposure in the oxic condition. Neither of the genes induced by sunlight exposure in the anoxic condition was assigned to functional pathways in KEGG.

Differential Gene Expression Not Categorized to Functional Pathways

Differentially expressed genes not assigned to pathways include genes with no annotated function or with predicted functions not yet linked to specific *S. aureus* cell reactions or networks. In the oxic condition, 39 genes were differentially expressed but not assigned to KEGG functional pathways. Five hypothetical proteins showed increased expression; the remaining 34 differentially expressed genes not assigned to functional pathways in the oxic condition were decreased in expression following sunlight exposure. These included a glycolytic operon regulator (*gapR*), a subunit of Clp protease (NWMN_0845), an ATPase family protein (NWMN_1529), a component of RNase P (*rnbP*), an ABC transporter (NWMN_0250), a sporulation protein (*spoVG*), staphylococcal accessory regulator A (*sarA*), elastin binding protein (*ebpS*), an alkaline shock protein (NWMN_2086), holin-like protein CidB (*cidB*), a CsbD-like superfamily protein (NWMN_0783), sigma 54 modulation protein (NWMN_0721), and 22 hypothetical proteins.

In the anoxic condition, 15 genes were differentially expressed and not assigned to functional pathways. These included an epimerase/dehydratase family protein (NWMN_2341), a Na+/H+ antiporter (*mnhA*), a polyribonucleotide nuleotidyltransferase (NWMN_0470), and 12 hypothetical proteins. Of these, NWMN_2341 and a conserved hypothetical protein (NWMN_1608) were increased in expression; the expression of the remaining 13 genes was decreased following sunlight exposure.

Confirmation of Gene Expression with RTqPCR

Expression changes in the same samples analyzed by RNA sequencing were also measured using RTqPCR assays targeting four different genes: *cidB*, *hemY*, *metL*, and NWMN_2341. Fold changes of these genes detected by RTqPCR and RNA sequencing are shown for the oxic and anoxic cases in **Figures 3**, **4**, respectively. As RTqPCR and RNA-seq use different methods to normalize the "baseline" expression level in samples, we opted not to compare the specific fold change values but rather to compare whether statistical analysis of each method concluded an increase, decrease, or no change in expression of the gene of interest. With this treatment of the data, RTqPCR and RNA-seq results were in agreement in most cases: 2/4 genes are in agreement in the oxic condition and 3/4 genes are in agreement in the anoxic condition. Exceptions were for *metL* in the anoxic samples, and *cidB* and *hemY* in the oxic samples. RNA sequencing detected significant decreases in expression for *metL*

TABLE 5 | List of significantly differentially expressed genes from oxic experiments.

Gene name	Gene description	Fold change	FDR (%)
hemY	Protoporphyrinogen oxidase	5.97	3.1
NWMN_1978	Conserved hypothetical protein	5.64	13.0
NWMN_0650	Conserved hypothetical protein	4.50	5.5
NWMN_1466	Conserved hypothetical protein	4.16	7.9
vraB	Acetyl-CoA C-acetyltransferase VraB	3.57	9.3
NWMN_1608	Conserved hypothetical protein	3.54	5.0
NWMN_2520	Conserved hypothetical protein	3.35	5.3
narG	Nitrate reductase, alpha subunit	2.78	18.8
glk	Glucokinase	2.70	13.0
thrS	Threonyl-tRNA synthetase	0.46	21.6
gudB	NAD-specific glutamate dehydrogenase	0.43	20.2
NWMN_1689	Conserved hypothetical protein	0.42	14.5
agrC	Staphylococcal accessory gene regulator protein C	0.41	12.1
NWMN_1806	Conserved hypothetical protein	0.39	24.8
NWMN_2026	Aldehyde dehydrogenase family protein	0.38	12.6
glnA	Glutamine synthetase	0.38	8.6
gapR	Glycolytic operon regulator	0.36	13.0
citC	Isocitrate dehydrogenase, NADP-dependent	0.36	19.2
NWMN_1263	Aconitate hydratase	0.35	21.7
NWMN_0845	ATP-dependent Clp protease, ATP-binding subunit ClpB	0.34	5.7
NWMN_1529	ATPase AAA family protein	0.34	6.4
NWMN_2210	Formate dehydrogenase homolog	0.31	4.4
sdhA	Succinate dehydrogenase flavoprotein subunit	0.31	6.5
NWMN_0377	Conserved hypothetical protein	0.31	5.3
glmS	Glucosamine-fructose-6-phosphate aminotransferase, isomerizing	0.30	23.8
rnbP	RNase P RNA component class B	0.30	9.0
NWMN_0475	Cysteine synthase homolog	0.30	18.4
NWMN_0250	ABC transporter, permease protein	0.28	2.8
spoVG	Stage V sporulation protein G homolog	0.28	8.8
NWMN_0460	Conserved hypothetical protein	0.28	14.2
NWMN_2262	Conserved hypothetical protein	0.27	7.9
gapA	Glyceraldehyde 3-phosphate dehydrogenase 1	0.27	2.4
pdhD	Dihydrolipoamide dehydrogenase: subunit E3	0.26	2.4
pgm	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	0.26	5.3
spa	Immunoglobulin G binding protein A precursor (protein A)	0.26	8.0
NWMN_1195	Conserved hypothetical protein	0.26	6.1
dnaK	Chaperone protein DnaK	0.26	3.2
sarA	Staphylococcal accessory regulator A	0.25	13.0
NWMN_0585	Conserved hypothetical protein	0.25	8.8
pdhC	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	0.25	2.5
NWMN_0163	Conserved hypothetical protein	0.24	9.9
NWMN_1371	Conserved hypothetical protein	0.24	7.2
NWMN_0366	Conserved hypothetical protein	0.24	6.4
NWMN_2392	Conserved hypothetical protein	0.24	12.6
NWMN_2282	Conserved hypothetical protein	0.23	5.0
NWMN_1477	Conserved hypothetical protein	0.23	10.1
clfA	Clumping factor A	0.22	1.8
hutG	Formiminoglutamase	0.22	1.8
NWMN_0735	Conserved hypothetical protein	0.22	5.0
NWMN_2088	Conserved hypothetical protein	0.22	9.9

(Continued)

TABLE 5 | Continued

Gene name	Gene description	Fold change	FDR (%)
ebpS	Elastin binding protein	0.22	0.5
NWMN_2597	Conserved hypothetical protein	0.22	8.8
citZ	Citrate synthase II	0.21	9.9
NWMN_2548	Conserved hypothetical protein	0.21	3.2
qoxC	Quinol oxidase polypeptide III	0.21	2.4
NWMN_2087	Conserved hypothetical protein	0.21	11.1
katA	Catalase	0.20	2.0
poxB	Pyruvate oxidase	0.20	2.4
tpi	Triosephosphate isomerase	0.19	0.5
NWMN_2086	Alkaline shock protein 23	0.19	2.5
NWMN_1746	Conserved hypothetical protein	0.19	2.5
cidB	Holin-like protein CidB	0.18	0.5
NWMN_1631	Conserved hypothetical protein	0.18	2.5
NWMN_0783	CsbD-like superfamily protein	0.17	1.1
NWMN_1526	Hypothetical protein	0.17	1.5
NWMN_0867	Conserved hypothetical protein	0.16	0.7
NWMN_1989	Conserved hypothetical protein	0.12	3.5
NWMN_0721	Sigma 54 modulation protein	0.11	2.5
NWMN_1527	Conserved hypothetical protein	0.11	0.5
NWMN_2406	Conserved hypothetical protein	0.11	0.9
NWMN_0868	Conserved hypothetical protein	0.07	0.5

in the anoxic condition and *cidB* in the oxic condition, whereas RTqPCR did not detect any significant expression changes. Similarly, RNA sequencing detected a significant increase in expression of *hemY* in the oxic condition, while the fold change generated by RTqPCR was not significant. Others have also found that RTqPCR results do not always agree with RNA-seq or microarray results, usually in cases where significance is detected by one method but not by the other (Song et al., 2016; Al-Jassim et al., 2017).

DISCUSSION

To better understand the ways in which *S. aureus* responds to oxygen-mediated and non-oxygen-mediated photoinactivation, we used RNA sequencing to identify gene expression changes between oxic and anoxic sunlit reactors and their corresponding dark controls. After 6 h of sunlight exposure, concentrations of cultivatable *S. aureus* were reduced by more than four orders of magnitude in both oxic and anoxic conditions, and were reduced to levels at or below the limit of detection in the sunlit oxic treatment. Despite significant reduction in cultivatable cell concentration after 6 h of sunlight exposure, our previous work showed only slight reduction in the intact cell concentration during the same exposure period, as measured by fluorescence microscopy (McClary et al., 2017). The combination of intact cell membranes and detectable mRNA concentrations in these samples suggests the possibility that *S. aureus* entered a viable but non-culturable (VBNC) state under the sunlight stress condition, and these metrics have been used in previous studies to conclude the presence of VBNC cells (Liu et al., 2009; Chaisowwong et al., 2012; Pasquaroli et al., 2013). Additionally, samples collected after 6 h, which were analyzed by RNA sequencing, were collected during the second phase of the observed biphasic inactivation. This second phase of inactivation is often assumed to represent a resistant subpopulation of the bacterial community, a shift to a resistant phenotype, and/or a shift to a VBNC state, which could be triggered by environmental stresses (Brouwer et al., 2016). While the existence of a VBNC state is generally accepted within the scientific community, there remains uncertainty regarding what specific metrics must be used to define this state and differentiate from other non-growing states (Hammes et al., 2011; Ramamurthy et al., 2014; Pinto et al., 2015). Future work to characterize the transition of *S. aureus* into a VBNC state during sunlight exposure should include attempts at resuscitation of non-culturable cells.

To identify gene expression changes associated with oxic and anoxic photostress conditions, we used RNA sequencing and differential expression analysis with DESeq2 to compare mRNA transcript abundances between sunlight-exposed samples and control samples under either oxic or anoxic conditions, separately. To identify significant differential expression, we chose to consider genes identified by the DESeq2 program with FDR $< 25\%$. Significant expression thresholds based on FDR are highly variable among previous microarray and RNA-seq studies, often ranging between 5 and 30%, while other studies base results on nominal p-values without correction for multiple hypothesis testing (Graham et al., 2005; Bore et al., 2007; Stasiewicz et al., 2011; Dhanjal et al., 2014; Sassoubre et al., 2014). We opted to consider significance based on FDR due to

TABLE 6 | List of significantly differentially expressed genes from anoxic experiments.

Gene name	Gene description	Fold change	FDR (%)
NWMN_2341	NAD dependent epimerase/dehydratase family protein	8.30	7.4
NWMN_1608	Conserved hypothetical protein	2.17	19.6
NWMN_1804	Conserved hypothetical protein	0.38	19.6
mnhA	Na+/H+ antiporter, MnhA component	0.30	19.6
atpA	ATP synthase F1, alpha subunit	0.30	7.4
NWMN_0470	Polyribonucleotide nucleotidyltransferase	0.27	19.6
NWMN_1123	Conserved hypothetical protein	0.27	7.4
NWMN_1800	Conserved hypothetical protein	0.26	11.3
NWMN_1008	Conserved hypothetical protein	0.23	7.5
NWMN_0759	Conserved hypothetical protein	0.21	0.3
NWMN_0867	Conserved hypothetical protein	0.21	12.6
ebpS	Elastin binding protein	0.19	9.1
metL	Homoserine dehydrogenase	0.19	3.4
NWMN_0860	Conserved hypothetical protein	0.16	0.4
NWMN_0748	Conserved hypothetical protein	0.14	12.6
NWMN_1913	Conserved hypothetical protein	0.13	9.1
NWMN_1004	Conserved hypothetical protein	0.13	0.1
NWMN_1101	Conserved hypothetical protein	0.09	0.1

the importance of multiple hypothesis testing in detecting gene expression changes across the full genome, and we chose to set a somewhat liberal threshold at FDR $< 25\%$ based on our goals in this study to identify and explore overall transcriptional response to photostress conditions.

Overall, we identified 71 and 18 genes which were significantly differentially expressed after 6 h of sunlight exposure in oxic and anoxic conditions, respectively. This is comparable to the number of differentially expressed genes identified in *E. faecalis* during sunlight exposure using microarrays (Sassoubre et al., 2014), but is a smaller amount of genes than those identified in *E. coli* during sunlight exposure using RNA sequencing (Al-Jassim et al., 2017). Of the genes identified as differentially expressed, most showed significantly decreased expression in sunlight exposed reactors compared to their dark controls: 87 and 89% in oxic and anoxic conditions, respectively. Due to the fact that experiments were performed in oligotrophic conditions, it is possible that *S. aureus* in the sunlit experiments were forced to shut down transcription of cell functions not immediately necessary for combating the damaging effects of sunlight. In contrast, while control dark reactors were similarly oligotrophic, *S. aureus* in these reactors were exposed only to starvation stress and therefore were able to maintain a higher level of transcription in contrast to the sunlight-exposed cells. Additionally, sunlight exposure may lead to the direct mutation and degradation of mRNA transcripts in the sunlight-exposed samples. While the effects of UVA+UVB exposure on DNA have been more comprehensively investigated (Sinha and Häder, 2002; Rastogi et al., 2010), UVA+UVB can lead to degradation of RNA molecules through similar mechanisms (Swenson and Setlow, 1964; Qiao and Wigginton, 2016). It is therefore possible that mRNA transcripts were able to persist longer in the dark control reactors than in the sunlight-exposed reactors, and this differential persistence could also have an effect on the overall decreased gene expression detected in sunlight-exposed reactors. Another factor that may have influenced the overall changes in gene expression is a transition to a viable but non-culturable state. As mentioned previously, samples collected following sunlight exposure exhibited substantially reduced culturable cell numbers compared to those in dark controls. However, our previous work demonstrated that *S. aureus* cells remain intact in these samples (McClary et al., 2017), suggesting that cells remain viable but may be transitioning to a non-culturable state in the sunlight-exposed system. The difference between non-culturable cells in the sunlight-exposed samples and largely culturable cells in the dark control samples could control some of the transcriptome changes observed.

Due to the significant losses in *S. aureus* culturability observed after 6 h of sunlight exposure, genes identified with increased expression in the sunlight-exposed reactors relative to dark controls are hypothesized to be of great importance to the *S. aureus* photostress response. For the oxic case, genes with increased expression included *hemY*, *vraB*, *narG*, *glk*, and five conserved hypothetical proteins. The gene *hemY*, which was expressed in the oxic sunlight-exposed experiments ∼6-fold more than in the dark controls, codes for a protoporphyrinogen oxidase and is involved in porphyrin metabolism. Porphyrins are well-known photosensitizers, and the use of synthetic or naturally occurring porphyrins for the enhancement of photoinactivation in applications like photodynamic therapy has been studied for many years (Jori and Brown, 2004; Ferro et al., 2007; Khlebtsov et al., 2013; Nakonieczna et al., 2016). Specifically, hemY catalyzes the oxidation of protoporphyrinogen (or coproporphyrinogen), yielding protoporphyrin (or coproporphyrin) and hydrogen peroxide. Despite the fact that this reaction yields potentially damaging hydrogen peroxide as well as the photosensitizer protoporphyrin, the enhancement of protoporphyrinogen oxidase activity would be required to metabolize and subsequently reduce the overall levels of endogenous porphyrins. A previous study in mice found that the use of a protoporphyrinogen oxidase inhibitor led to the buildup of endogenous porphyrin molecules and subsequently enhanced the effects of photodynamic therapy (Fingar et al., 1997). Additionally, in *Bacillus subtilis*, a Gram-positive bacterium with very similar hemY structure to that of *S. aureus* (Lobo et al., 2015), hemY mutants were found to accumulate endogenous coproporphyrin (Hansson and Hederstedt, 1994). In contrast, a recent study found that activation of hemY led to increased photosensitization in *S. aureus* (Surdel et al., 2017). Interestingly, of the four *S. aureus* strains tested in that study, activation of hemY in *S. aureus* Newman led to the least significant reduction in cell viability following light exposure (Surdel et al., 2017). We therefore hypothesize that oxygen-mediated indirect photoinactivation mechanisms in *S. aureus* are strongly dependent on levels

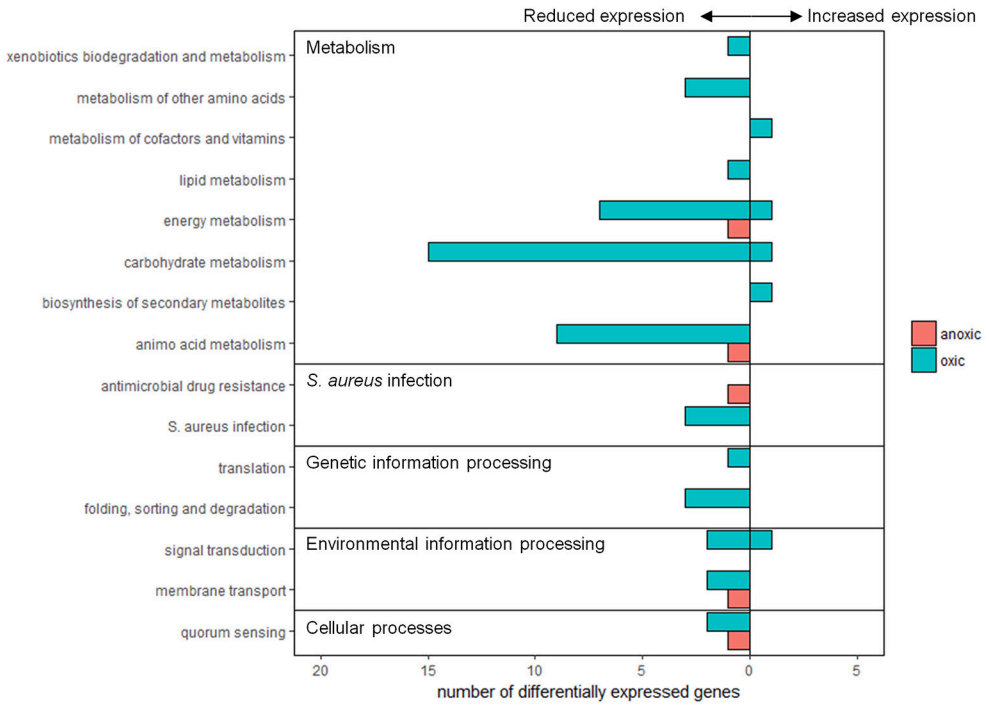


FIGURE 2 | Summary of differentially expressed genes assigned to functional groups according to KEGG pathways. Pink bars represent expression from anoxic experiments, and blue bars represent expression from oxic experiments. Values to the left and right of the y-axis indicate genes with reduced or increased expression, respectively.

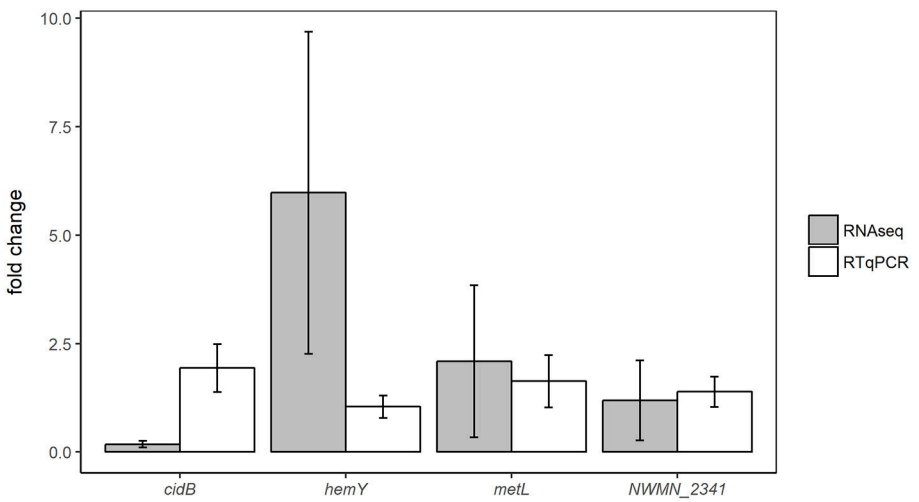


FIGURE 3 | Comparison of differential expression results from oxic experiments using RNA-seq or RTqPCR. Error bars = ± SE.

of endogenous photosensitizers within the cells, and that the metabolism of photosensitizing porphyrins is potentially a more efficient stress response method under starvation conditions than the expression of antioxidant enzymes. This hypothesis should be explored in future work using mutants for specific genes in the porphyrin metabolism pathway, such as *hemY*, or by quantifying and identifying intracellular porphyrins (Nitzan and Kauffman, 1999; Fyrestam et al., 2015).

In addition to the increased expression of *hemY*, *S. aureus* also increased expression of *vraB*, *narG*, and *glk* following exposure to sunlight in oxic conditions. *vraB* codes for an acetyl-CoA acetyltransferase and is involved in the TCA cycle. Expression of *vraB* in *S. aureus* was previously found to

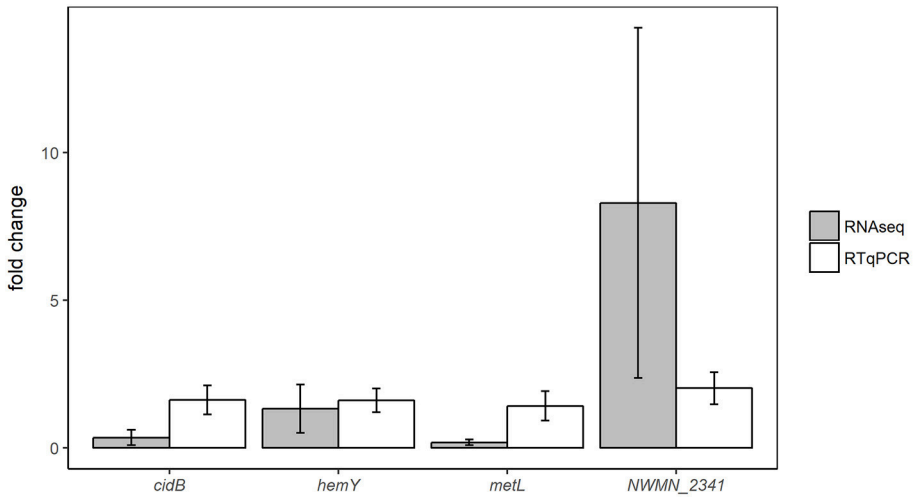


FIGURE 4 | Comparison of differential expression results from anoxic experiments using RNA-seq or RTqPCR. Error bars = ± SE.

be induced by other stresses, including treatment with the antibacterial compound berberine chloride (Wang et al., 2008) and exposure to Cr(VI) (Zhang et al., 2014), suggesting expression of *vraB* could be important for general *S. aureus* stress response. *narG* codes for the alpha subunit of nitrate reductase, a membrane-bound oxidoreductase enzyme. While *narG* is typically only regulated during anaerobic metabolism (Richardson et al., 2001), nitrate can also serve as an important precursor to reactive oxygen species like hydroxyl radical (Brezonik and Fulkerson-Brekken, 1998). *S. aureus* may therefore increase expression of *narG* in order to manage the potentially damaging effects of nitrate to the cell. *S. aureus* also increased expression of *glk*, coding for glucokinase, following sunlight exposure in oxic conditions. Glucokinase is involved in a range of metabolic functions, including metabolism of galactose and sucrose, as well as the biosynthesis of streptomycin. While overall more metabolism genes were observed to be decreased in expression following sunlight exposure, the increased expression of *glk* suggests that *S. aureus* remains metabolically active. Future work to identify *S. aureus* metabolism of specific substrates following sunlight exposure is warranted.

In the sunlit anoxic treatments, fewer genes were identified as differentially expressed. This could be because bacteria in anoxic experiments had been exposed to less overall stress due to the fact that oxygen-mediated photostress was not present in these systems. *S. aureus* in the anoxic experiments also decayed more slowly and better tracked the cell numbers in the dark controls, further pointing to the anoxic treatment being less stressful than the oxic. However, despite the fact that fewer differentially expressed genes were identified, we would like to stress the fact that, by using true biological replicates and carefully considered metrics of significant expression, the genes identified as differentially expressed are likely those that show the greatest expression changes and are most consistently differentially expressed in the anoxic photostress condition.

In the anoxic condition, two genes were identified as significantly increased in expression: NWMN_2341, coding for a NAD dependent epimerase/dehydratase family protein, and NWMN_1608, coding for a conserved hypothetical protein identified as a probable membrane transporter according to the UniProt database. NWMN_1608 is also the only gene identified as significantly increased in expression during sunlight exposure in both the oxic and anoxic conditions, suggesting its importance for the *S. aureus* photostress response. The increased expression of a probable membrane transporter could indicate that *S. aureus* are responding to membrane damage, or that the cells are attempting to increase the removal of toxic species from inside the cell. Cell membrane damage due to sunlight exposure could occur in an anoxic environment due to non-ROS radicals generated from endogenous cell components or direct UV damage of intermembrane proteins (Oppezzo et al., 2001; Kalisvaart, 2004). Our previous work suggests that sunlight exposure in anoxic conditions does lead to increased membrane damage in *S. aureus* (McClary et al., 2017). Additionally, previous work on the photostress response of *E. coli* confirmed the importance of efflux pumps in protecting *E. coli* from critical damage (Al-Jassim et al., 2017).

In conclusion, we have investigated gene expression changes associated with oxic and anoxic photostress in *S. aureus* in clear oligotrophic seawater. Results suggest that the photostress responses associated with oxygen-mediated and non-oxygen-mediated photoinactivation mechanisms are different from each other. Additionally, the increased expression of *hemY* in the oxic photostress condition suggests the importance of porphyrin metabolism for combating oxygen-mediated photoinactivation. While further work is needed to confirm that the gene expression changes described here correspond to protein level changes as well, this study helps to identify genes of importance for responding to different types of photostress. In particular, future work should focus on improving our

understanding of types and concentrations of endogenous photosensitizers present in bacterial pathogens and fecal indicators, as these appear to play an important role in photoinactivation.

AUTHOR CONTRIBUTIONS

AB and JM conceived and designed the study; JM wrote the manuscript, conducted experiments, and analyzed the data; AB and JM edited the manuscript; AB supervised the project; AB and JM read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation (NSF) grants CBET-1334359. JM was supported by a NSF Graduate Research Fellowship (DGE-114747).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00249/full#supplementary-material

REFERENCES

Al-Jassim, N., Mantilla-Calderon, D., Wang, T., and Hong, P.-Y. (2017). Inactivation of a virulent wastewater *Escherichia coli* and non-virulent commensal *Escherichia coli* DSM1103 strains and their gene expression upon solar irradiation. *Environ. Sci. Technol.* 51, 3649–3659. doi: 10.1021/acs.est.6b05377

Berney, M., Weilenmann, H.-U., and Egli, T. (2006). Gene expression of *Escherichia coli* in continuous culture during adaptation to artificial sunlight. *Environ. Microbiol.* 8, 1635–1647. doi: 10.1111/j.1462-2920.2006.01057.x

Boehm, A. B., Yamahara, K. M., Love, D. C., Peterson, B. M., McNeill, K., and Nelson, K. L. (2009). Covariation and photoinactivation of traditional and novel indicator organisms and human viruses at a sewage-impacted marine beach. *Environ. Sci. Technol.* 43, 8046–8052. doi: 10.1021/es9015124

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bore, E., Langsrud, S., Langsrud, Ø., Rode, T. M., and Holck, A. (2007). Acid-shock responses in *Staphylococcus aureus* investigated by global gene expression analysis. *Microbiology* 153, 2289–2303. doi: 10.1099/mic.0.2007/005942-0

Brezonik, P. L., and Fulkerson-Brekken, J. (1998). Nitrate-induced photolysis in natural waters: controls on concentrations of hydroxyl radical photo-intermediates by natural scavenging agents. *Environ. Sci. Technol.* 32, 3004–3010. doi: 10.1021/es9802908

Brouwer, A. F., Eisenberg, M. C., Remais, J. V., Collender, P. A., Meza, R., and Eisenberg, J. N. (2016). Modeling biphasic environmental decay of pathogens and implications for risk analysis. *Environ. Sci. Technol.* 51, 2186–2196. doi: 10.1021/acs.est.6b04030

Cabelli, V. J., Dufour, A. P., McCabe, L. J., and Levin, M. A. (1982). Swimming-associated gastroenteritis and water quality. *Am. J. Epidemiol.* 115, 606–616. doi: 10.1093/oxfordjournals.aje.a113342

Chaisowwong, W., Kusumoto, A., Hashimoto, M., Harada, T., Maklon, K., and Kawamoto, K. (2012). Physiological characterization of *Campylobacter jejuni* under cold stresses conditions: its potential for public threat. *J. Vet. Med. Sci.* 74, 43–50. doi: 10.1292/jvms.11-0305

Charoenca, N., and Fujioka, R. S. (1993). Assessment of *Staphylococcus bacteria* in Hawaii's marine recreational waters. *Water Sci. Technol.* 27, 283–289.

Charoenca, N., and Fujioka, R. S. (1995). Association of staphylococcal skin infections and swimming. *Water Sci. Technol.* 31, 11–17.

Colford, J. M., Wade, T. J., Schiff, K. C., Wright, C. C., Griffith, J. F., Sandhu, S. K., et al. (2007). Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. *Epidemiology* 18, 27–35. doi: 10.1097/01.ede.0000249425.32990.b9

Corsi, S. R., Borchardt, M. A., Carvin, R. B., Burch, T. R., Spencer, S. K., and Lutz, M. A., et al. (2016). Human and Bovine Viruses and bacteria at three great lakes beaches: environmental variable associations and health risk. *Environ. Sci. Technol.* 50, 987–995. doi: 10.1021/acs.est.5b04372

Curtis, T. P., Mara, D. D., and Silva, S. A. (1992). Influence of pH, oxygen, and humic substances on ability of sunlight to damage fecal coliforms in waste stabilization pond water. *Appl. Environ. Microbiol.* 58, 1335–1343.

Dhanjal, S., Singh, A. K., and Cameotra, S. S. (2014). Global gene expression analysis of bacterial stress response to elevated concentrations of toxic metalloids—selenium and arsenic. *Geomicrobiol. J.* 31, 480–492. doi: 10.1080/01490451.2013.822613

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635

Ferro, S., Ricchelli, F., Monti, D., Mancini, G., and Jori, G. (2007). Efficient photoinactivation of methicillin-resistant *Staphylococcus aureus* by a novel porphyrin incorporated into a poly-cationic liposome. *Int. J. Biochem. Cell Biol.* 39, 1026–1034. doi: 10.1016/j.biocel.2007.02.001

Fingar, V. H., Wieman, T. J., McMahon, K. S., Haydon, P. S., Halling, B. P., Yuhas, D. A., et al. (1997). Photodynamic therapy using a protoporphyrinogen oxidase inhibitor. *Cancer Res.* 57, 4551–4556.

Fyrestam, J., Bjurshammar, N., Paulsson, E., Johannsen, A., and Östman, C. (2015). Determination of porphyrins in oral bacteria by liquid chromatography electrospray ionization tandem mass spectrometry. *Anal. Bioanal. Chem.* 407, 7013–7023. doi: 10.1007/s00216-015-8864-2

Geeraerd, A. H., Valdramidis, V. P., and Van Impe, J. F. (2005). GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. *Int. J. Food Microbiol.* 102, 95–105. doi: 10.1016/j.ijfoodmicro.2004.11.038

Gentleman, R., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5:R80. doi: 10.1186/gb-2004-5-10-r80

Goodwin, K. D., McNay, M., Cao, Y., Ebentier, D., Madison, M., and Griffith, J. F. (2012). A multi-beach study of *Staphylococcus aureus*, MRSA, and enterococci in seawater and beach sand. *Water Res.* 46, 4195–4207. doi: 10.1016/j.watres.2012.04.001

Graham, M. R., Virtaneva, K., Porcella, S. F., Barry, W. T., Gowen, B. B., Johnson, C. R., et al. (2005). Group A *Streptococcus* transcriptome dynamics during growth in human blood reveals bacterial adaptive and survival strategies. *Am. J. Pathol.* 166, 455–465. doi: 10.1016/S0002-9440(10)62268-7

Haile, R. W., Witte, J. S., Gold, M., Cressey, R., McGee, C., Millikan, R. C., et al. (1999). The health effects of swimming in ocean water contaminated by storm drain runoff. *Epidemiology* 10, 355–363. doi: 10.1097/00001648-199907000-00004

Hammes, F., Berney, M., and Egli, T. (2011). Cultivation-independent assessment of bacterial viability. *Adv. Biochem. Eng. Biotechnol.* 124, 123–150. doi: 10.1007/10_2010_95

Hansson, M., and Hederstedt, L. (1994). *Bacillus subtilis* HemY is a peripheral membrane protein essential for protoheme IX synthesis which can oxidize coproporphyrinogen III and protoporphyrinogen IX. *J. Bacteriol.* 176, 5962–5970. doi: 10.1128/jb.176.19.5962-5970.1994

He, C., Post, Y., Dony, J., Edge, T., Patel, M., and Rochfort, Q. (2016). A physical descriptive model for predicting bacteria level variation at a dynamic beach. *J. Water Health* 14, 617–629. doi: 10.2166/wh.2016.206

Hower, S., Phillips, M. C., Brodsky, M., Dameron, A., Tamargo, M. A., Salazar, N. C., et al. (2013). Clonally related methicillin-resistant *Staphylococcus aureus* isolated from short-finned pilot whales (*Globicephala macrorhynchus*), human volunteers, and a bayfront cetacean rehabilitation

facility. *Microb. Ecol.* 65, 1024–1038. doi: 10.1007/s00248-013-0178-3

Jori, G., and Brown, S. B. (2004). Photosensitized inactivation of microorganisms. *Photochem. Photobiol. Sci.* 3, 403–405. doi: 10.1039/b311904c

Kalisvaart, B. F. (2004). Re-use of wastewater: preventing the recovery of pathogens by using medium-pressure UV lamp technology. *Water Sci. Technol.* 50, 337–344.

Khlebtsov, B. N., Tuchina, E. S., Khanadeev, V. A., Panfilova, E. V., Petrov, P. O., Tuchin, V. V., et al. (2013). Enhanced photoinactivation of *Staphylococcus aureus* with nanocomposites containing plasmonic particles and hematoporphyrin. *J. Biophotonics* 6, 338–351. doi: 10.1002/jbio.201200079

Kohn, T., and Nelson, K. L. (2007). Sunlight-mediated inactivation of MS2 coliphage via exogenous singlet oxygen produced by sensitizers in natural waters. *Environ. Sci. Technol.* 41, 192–197. doi: 10.1021/es061716i

Lawrence, M., Huber, W., Pagès, H., Aboyoun, P., Carlson, M., Gentleman, R., et al. (2013). Software for computing and annotating genomic ranges. *PLoS Comput. Biol.* 9:e1003118. doi: 10.1371/journal.pcbi.1003118

Levin-Edens, E., Soge, O. O., No, D., Stiffarm, A., Meschke, J. S., and Roberts, M. C. (2012). Methicillin-resistant *Staphylococcus aureus* from Northwest marine and freshwater recreational beaches. *FEMS Microbiol. Ecol.* 79, 412–420. doi: 10.1111/j.1574-6941.2011.01229.x

Liu, Y., Wang, C., Tyrrell, G., Hrudey, S. E., and Li, X. F. (2009). Induction of *Escherichia coli* O157:H7 into the viable but non-culturable state by chloraminated water and river water, and subsequent resuscitation. *Environ. Microbiol. Rep.* 1, 155–161. doi: 10.1111/j.1758-2229.2009.00024.x

Lobo, S. A., Scott, A., Videira, M. A., Winpenny, D., Gardner, M., Palmer, M. J., et al. (2015). *Staphylococcus aureus* haem biosynthesis: characterisation of the enzymes involved in final steps of the pathway. *Mol. Microbiol.* 97, 472–487. doi: 10.1111/mmi.13041

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 1–34. doi: 10.1186/s13059-014-0550-8

Maraccini, P. A., Mattioli, M. C. M., Sassoubre, L. M., Cao, Y., Griffith, J. F., Ervin, J. S., et al. (2016a). Solar inactivation of enterococci and *Escherichia coli* in natural waters: effect of water absorbance and depth. *Environ. Sci. Technol.* 50, 5068–5076. doi: 10.1021/acs.est.6b00505

Maraccini, P. A., Wang, D., McClary, J. S., and Boehm, A. B. (2015). Growth-dependent photoinactivation kinetics of *Enterococcus faecalis*. *J. Appl. Microbiol.* 118, 1226–1237. doi: 10.1111/jam.12773

Maraccini, P. A., Wenk, J., and Boehm, A. B. (2016b). Photoinactivation of eight health-relevant bacterial species: determining the importance of the exogenous indirect mechanism. *Environ. Sci. Technol.* 50, 5050–5059. doi: 10.1021/acs.est.6b00074

McClary, J. S., Sassoubre, L. M., and Boehm, A. B. (2017). *Staphylococcus aureus* strain newman photoinactivation and cellular response to sunlight exposure. *Appl. Environ. Microbiol.* 83, 1–14. doi: 10.1128/AEM.01052-17

Nakonieczna, J., Kossakowska-Zwierucho, M., Filipiak, M., Hewelt-Belka, W., Grinholc, M., and Bielawski, K. P. (2016). Photoinactivation of *Staphylococcus aureus* using protoporphyrin IX: the role of haem-regulated transporter HrtA. *Appl. Microbiol. Biotechnol.* 100, 1393–1405. doi: 10.1007/s00253-015-7145-5

National Resources Defense Council (2012). *Testing the Waters: A Guide to Water Quality at Vacation Beaches*. New York, NY.

Nitzan, Y., and Kauffman, M. (1999). Endogenous porphyrin production in bacteria by δ-aminolaevulinic acid and subsequent bacterial photoeradication. *Lasers Med. Sci.* 14, 269–277. doi: 10.1007/s101030050094

Oppezzo, O. J., and Pizarro, R. A. (2001). Sublethal effects of ultraviolet A radiation on *Enterobacter cloacae*. *J. Photochem. Photobiol. B Biol.* 62, 158–165. doi: 10.1016/S1011-1344(01)00180-4

Parker, K. M., Pignatello, J. J., and Mitch, W. A. (2013). Influence of ionic strength on triplet-state natural organic matter loss by energy transfer and electron transfer pathways. *Environ. Sci. Technol.* 47, 10987–10994. doi: 10.1021/es401900j

Pasquaroli, S., Zandri, G., Vignaroli, C., Vuotto, C., Donelli, G., and Biavasco, F. (2013). Antibiotic pressure can induce the viable but non-culturable state in *Staphylococcus aureus* growing in biofilms. *J. Antimicrob. Chemother.* 68, 1812–1817. doi: 10.1093/jac/dkt086

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, 2002–2007. doi: 10.1093/nar/29.9.e45

Pinto, D., Santos, M. A., and Chambel, L. (2015). Thirty years of viable but nonculturable state research: unsolved molecular mechanisms. *Crit. Rev. Microbiol.* 41, 61–76. doi: 10.3109/1040841X.2013.794127

Prüss, A. (1998). Review of epidemiological studies on health effects from exposure to recreational water. *Int. J. Epidemiol.* 27, 1–9. doi: 10.1093/ije/27.1.1

Qiao, Z., and Wigginton, K. R. (2016). Direct and indirect photochemical reactions in viral RNA measured with RT-qPCR and mass spectrometry. *Environ. Sci. Technol.* 50, 13371–13379. doi: 10.1021/acs.est.6b04281

Ramamurthy, T., Ghosh, A., Pazhani, G. P., and Shinoda, S. (2014). Current perspectives on Viable but Non-Culturable (VBNC) pathogenic bacteria. *Front. Public Heal.* 2:103. doi: 10.3389/fpubh.2014.00103

Rastogi, R. P., Richa, Kumar, A., Tyagi, M. B., and Sinha, R. P. (2010). Molecular mechanisms of ultraviolet radiation-induced dna damage and repair. *J. Nucleic Acids* 2010, 1–32. doi: 10.4061/2010/592980

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S., and Taylor, C. J. (2001). Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cell. Mol. Life Sci.* 58, 165–178. doi: 10.1007/PL00000845

Russell, T. L., Sassoubre, L. M., Wang, D., and Boehm, A. B. (2013). A coupled modeling and molecular biology approach to microbial source tracking at cowell beach, santa cruz, CA, United States. *Environ. Sci. Technol.* 47, 10231–10239. doi: 10.1021/es402303w

Sabbahi, S., Alouini, Z., Jemli, M., and Boudabbous, A. (2008). The role of reactive oxygen species in *Staphylococcus aureus* photoinactivation by methylene blue. *Water Sci. Technol.* 58, 1047–1054. doi: 10.2166/wst.2008.471

Sassoubre, L. M., Nelson, K. L., and Boehm, A. B. (2012). Mechanisms for photoinactivation of *Enterococcus faecalis* in seawater. *Appl. Environ. Microbiol.* 78, 7776–7785. doi: 10.1128/AEM.02375-12

Sassoubre, L. M., Ramsey, M. M., Gilmore, M. S., and Boehm, A. B. (2014). Transcriptional response of *Enterococcus faecalis* to sunlight. *J. Photochem. Photobiol. B Biol.* 130, 349–356. doi: 10.1016/j.jphotobiol.2013.12.013

Sassoubre, L. M., Yamahara, K. M., and Boehm, A. B. (2015). Temporal stability of the microbial community in sewage-polluted seawater exposed to natural sunlight cycles and marine microbiota. *Appl. Environ. Microbiol.* 81, 2107–2116. doi: 10.1128/AEM.03950-14

Sekulovic, O., and Fortier, L. C. (2015). Global transcriptional response of *Clostridium difficile* carrying the ϕCD38-2 prophage. *Appl. Environ. Microbiol.* 81, 1364–1374. doi: 10.1128/AEM.03656-14

Sinha, R. P., and Häder, D.-P. (2002). UV-induced DNA damage and repair: a review. *Photochem. Photobiol. Sci.* 1, 225–236. doi: 10.1039/b201230h

Sinigalliano, C. D., Fleisher, J. M., Gidley, M. L., Solo-Gabriele, H. M., Shibata, T., Plano, L. R., et al. (2010). Traditional and molecular analyses for fecal indicator bacteria in non-point source subtropical recreational marine waters. *Water Res.* 44, 3763–3772. doi: 10.1016/j.watres.2010.04.026

Song, Y., Rundberget, J. T., Evenseth, L. M., Xie, L., Gomes, T., Høgåsen, T., et al. (2016). Whole-organism transcriptomic analysis provides mechanistic insight into the acute toxicity of emamectin benzoate in *Daphnia magna*. *Environ. Sci. Technol.* 50, 11994–12003. doi: 10.1021/acs.est.6b03456

Stasiewicz, M. J., Wiedmann, M., and Bergholz, T. M. (2011). The transcriptional response of *Listeria monocytogenes* during adaptation to growth on lactate and diacetate includes synergistic changes that increase fermentative Acetoin production. *Appl. Environ. Microbiol.* 77, 5294–5306. doi: 10.1128/AEM.02976-10

Sun, P., Tyree, C., and Huang, C.-H. (2016). Inactivation of *E. coli*, Bacteriophage MS2 and *Bacillus* Spores under UV/H2O2 and UV/Peroxydisulfate Advanced Disinfection Conditions. *Environ. Sci. Technol.* 50, 4448–4458. doi: 10.1021/acs.est.5b06097

Surdel, M. C., Horvath, D. J., Lojek, L. J., Fullen, A. R., Simpson, J., Dutter, B. F., et al. (2017). Antibacterial photosensitization through activation of coproporphyrinogen oxidase. *Proc. Natl. Acad. Sci. U.S.A.* 114, E6652–E6659. doi: 10.1073/pnas.1700469114

Swenson, P. A., and Setlow, R. B. (1964). β-Galactosidase: inactivation of its messenger RNA by ultraviolet irradiation. *Science* 146, 791–795. doi: 10.1126/science.146.3645.791

Thoe, W., Gold, M., Griesbach, A., Grimmer, M., Taggart, M. L., and Boehm, A. B. (2015). Sunny with a chance of gastroenteritis: predicting swimmer risk at California beaches. *Environ. Sci. Technol.* 49, 423–431. doi: 10.1021/es504701j

Tryland, I., Braathen, H., Wennberg, A. C.,Eregno, F., and Beschorner, A.-L. (2016). Monitoring of β -D-Galactosidase activity as a surrogate parameter for rapid detection of sewage contamination in urban recreational water. *Water* 8:65. doi: 10.3390/w8020065

US EPA (2012). *Recreational Water Quality Criteria*.

Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Whitney, A. R., Saïd-Salim, B., Porcella, S. F., et al. (2005). Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J. Immunol.* 175, 3907–3919. doi: 10.4049/jimmunol.175.6.3907

Wade, T. J., Calderon, R. L., Brenner, K. P., Sams, E., Beach, M., Haugland, R., et al. (2008). High sensitivity of children to swimming-associated gastrointestinal illness. *Epidemiology* 19, 375–383. doi: 10.1097/EDE.0b013e318169cc87

Wang, D., Yu, L., Xiang, H., Fan, J., He, L., Guo, N., et al. (2008). Global transcriptional profiles of *Staphylococcus aureus* treated with berberine chloride. *FEMS Microbiol. Lett.* 279, 217–225. doi: 10.1111/j.1574-6968.2007.01031.x

Yau, V., Wade, T. J., de Wilde, C. K., and Colford, J.r., J. M. (2009). Skin-related symptoms following exposure to recreational water: a systematic review and meta-analysis. *Water Qual. Exp. Heal.* 1, 79–103. doi: 10.1007/s12403-009-0012-9

Zhang, X., Wu, W., Virgo, N., Zou, L., Liu, P., and Li, X. (2014). Global transcriptome analysis of hexavalent chromium stress responses in *Staphylococcus aureus* LZ-01. *Ecotoxicology* 23, 1534–1545. doi: 10.1007/s10646-014-1294-7

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 October 2017
doi: 10.3389/fmicb.2017.01928

Cross-Resistance of UV- or Chlorine Dioxide-Resistant Echovirus 11 to Other Disinfectants

*Qingxia Zhong, Anna Carratalà, Rachele Ossola†, Virginie Bachmann and Tamar Kohn**

Laboratory of Environmental Chemistry, School of Architecture, Civil and Environmental Engineering, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

OPEN ACCESS

Edited by:
Muhammad Raihan Jumat, King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Thanh Nguyen, University of Illinois at Urbana–Champaign, United States
Xavier Abad, Centre de Recerca en Sanitat Animal, Spain

***Correspondence:**
Tamar Kohn
tamar.kohn@epfl.ch

†Present Address:
Rachele Ossola, Environmental Chemistry Group, Department of Environmental Systems Science, Swiss Federal Institute of Technology in Zurich (ETH), Zurich, Switzerland

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *28 July 2017*
Accepted: *21 September 2017*
Published: *04 October 2017*

Citation:
Zhong Q, Carratalà A, Ossola R, Bachmann V and Kohn T (2017) Cross-Resistance of UV- or Chlorine Dioxide-Resistant Echovirus 11 to Other Disinfectants. Front. Microbiol. 8:1928. doi: 10.3389/fmicb.2017.01928

The emergence of waterborne viruses with resistance to disinfection has been demonstrated in the laboratory and in the environment. Yet, the implications of such resistance for virus control remain obscure. In this study we investigate if viruses with resistance to a given disinfection method exhibit cross-resistance to other disinfectants. Chlorine dioxide (ClO_2)- or UV-resistant populations of echovirus 11 were exposed to five inactivating treatments (free chlorine, ClO_2, UV radiation, sunlight, and heat), and the extent of cross-resistance was determined. The ClO_2-resistant population exhibited cross-resistance to free chlorine, but to none of the other inactivating treatments tested. We furthermore demonstrated that ClO_2 and free chlorine act by a similar mechanism, in that they mainly inhibit the binding of echovirus 11 to its host cell. As such, viruses with host binding mechanisms that can withstand ClO_2 treatment were also better able to withstand oxidation by free chlorine. Conversely, the UV-resistant population was not significantly cross-resistant to any other disinfection treatment. Overall, our results indicate that viruses with resistance to multiple disinfectants exist, but that they can be controlled by inactivating methods that operate by a distinctly different mechanism. We therefore suggest to utilize two disinfection barriers that act by different mechanisms in order to control disinfection-resistant viruses.

Keywords: environmental virology, virus disinfection, echovirus 11, cross-resistance, water treatment

INTRODUCTION

Waterborne and foodborne viruses are typically efficiently controlled by chemical (e.g., free chlorine and ozone) or physical [e.g., ultraviolet (UV) radiation] disinfectants. However, it is well-documented that viruses may evolve to exhibit tolerance to disinfection. For example, poliovirus isolated from chlorinated drinking water was found to be chlorine-resistant (Shaffer et al., 1980). Similarly, isolates of coxsackievirus B5 from sewage or tap water were more resistant to chlorination compared to their corresponding lab strain (Payment et al., 1985). Finally, resistant viruses can also readily be generated in the laboratory by experimental evolution (Bates et al., 1977; Maillard et al., 1998; Zhong et al., 2016).

While the occurrence of disinfection resistance among virus populations has thus been established, information is lacking regarding the prevalence of such resistant viruses in water distribution system and the environment, or about their overall fitness and contribution to waterborne infections. Given the challenges associated with isolating and identifying such viruses, it is currently unlikely that such information will be routinely obtained in the near future. As such,

it appears advisable to design treatment strategies that can control disinfection-resistant viruses, to avoid their proliferation in the first place.

To inactivate a virus, a disinfectant must inhibit one or more of its vital functions, which include host binding, host entry and genome replication. Different disinfectants can target different viral functions. For example, the inactivation of MS2 bacteriophage by UV at 254 nm (UV_{254}) is mainly driven by genome damage, which results in the inability of the virus to successfully replicate (Wigginton et al., 2012). In contrast, MS2 inactivation by chlorine dioxide (ClO_2) is dominated by damage to the protein capsid, leading to the inability of the virus to bind to its host (Wigginton et al., 2012). A possible treatment strategy for viruses with resistance to a given disinfectant may therefore be the application of a disinfectant with a different mode of action (Ballester and Malley, 2004). This approach, however, can only work if a virus does not exhibit cross-resistance to other inactivation mechanisms.

In this work we determined if UV_{254}- or ClO_2-resistant strains of the echovirus 11 (E11) can be controlled by inactivating agents with a different mode of action. Echoviruses are enteric pathogens with clinical manifestations ranging from mild symptoms to more severe diseases such as meningitis, encephalitis, myocarditis, and hemorrhagic conjunctivitis (Knipe and Howley, 2007). They are members of the *Enterovirus* genus, which is frequently detected in the aqueous environment (Fong and Lipp, 2005 and references therein). Due to *Enterovirus*' potential risk to public health via contaminated water (Ford, 1999), this genus was included in the USEPA's Drinking Water Contaminant Candidate List (CCL) (USEPA, 2016). Here, we investigated the susceptibility of resistant E11 populations to treatments commonly applied to control pathogens in water, wastewater and food [free chlorine (FC), ClO_2, UV_{254}, and heat], as well as to an important environmental stressor, namely sunlight.

The action of several disinfectants on members of the *Enterovirus* genus have been previously investigated, yet the dominant inactivation mechanisms remain debated. Different experimental conditions used in different studies with respect to disinfectant concentration, contact time, temperature, pH, or ionic strength, further complicate a comparison of the different findings. For example, Nuanualsuwan and Cliver (2003b) suggested that the poliovirus genome is the primary target of FC inactivation, though in another study conducted at a lower FC working concentration, they observed binding loss for poliovirus and hepatitis A virus (Nuanualsuwan and Cliver, 2003a). Similarly, these researchers reported the genome to be the major target during poliovirus inactivation by UV_{254} (Nuanualsuwan and Cliver, 2003b), though in a different study they also observed binding loss (Nuanualsuwan and Cliver, 2003a). For ClO_2, the discrepancy in experimental conditions used in different studies also led to a lack of consensus regarding its mode of action. Olivieri et al. (1985) demonstrated that the genomes of inactivated poliovirus were still infectious, thus suggesting that the genome was not the main target of ClO_2. In contrast, Simonet and Gantzer (2006), who worked with high ClO_2 exposures (5 mg/L during 120 min), reported that viral RNA did degrade, but did not fully account for inactivation. Genome damage, specifically damage to the 5′ non-coding region, was found to be the main target for the treatment of enterovirus 71 and Hepatitis A virus at ClO_2 exposures of 13.5 mg/L*min or higher (Li et al., 2004; Jin et al., 2013). These authors also reported a similar finding for the inactivation of poliovirus by lower ClO_2 exposures (0.1–1.2 mg/L during 1–12 min) (Jin et al., 2012). Similarly, an additional study of poliovirus by ClO_2 at a low ClO_2 exposure (1 mg/L for 2 min) concluded that a loss in genome replicability was the main mode of ClO_2 action, whereas loss in host binding was ruled out (Alvarez and O'Brien, 1982). In contrast, our previous work on inactivation of E11 at similarly low ClO_2 exposures (up to 1.5 mg/L* min) revealed that inactivation coincided with a decrease in host binding, though the loss in this function did not fully account for inactivation (Zhong et al., 2017). Combined, these studies highlight that a comprehensive understanding of the mechanisms of action of these disinfectants, and their dependence on the experimental conditions, is thus still lacking.

Here, we determined the kinetics of inactivation of ClO_2- or UV_{254}-resistant E11 populations by ClO_2, FC, heat, UV_{254}, and sunlight, and compared them to the inactivation kinetics of the corresponding wild-type E11. This allowed us to determine the occurrence and extent of cross-resistance among the disinfection methods tested. We then investigated the main inactivation mechanisms acting on E11 during treatment by these disinfection methods, to evaluate if cross-resistance only affected disinfectants acting by a similar mechanism, or if resistance was a general trait. Finally, we interpreted the results in the context of possible implications for the control of resistant viruses.

EXPERIMENTAL SECTION

Cells and Viruses

BGMK cells, *Escherichia coli*, E11 and bacteriophage MS2 were cultured and maintained as described previously (Zhong et al., 2016, 2017). Infective E11 concentrations were enumerated as most probable number of cytopathic units per mL (MPNCU/mL), and infective MS2 concentrations were determined as plaque forming units per mL (PFU/mL) (Suess, 1982).

Resistant Echovirus and Their Corresponding Wild-Types

ClO_2-resistant and UV_{254}-resistant E11 populations were obtained by experimental evolution (**Figure 1**). The production of ClO_2-resistant populations was described in detail in Zhong et al. (2017). Briefly, the E11 laboratory strain, here denoted as "wild-type" (WT), was subjected to 20 passages of directed evolution. During each passage, the virus population was exposed to ClO_2 up to an exposure of 6 mg/L*min, resulting in an inactivation of at least 3 log_{10}, before the inactivation was halted and the remaining virus population was regrown on BGMK cells. The resulting ClO_2-resistant population is henceforth referred to as E_ClO_2 ("exposed to ClO_2"). A similar approach was used to obtain UV_{254}-resistant E11, except that a different ancestral population of E11 was used. Specifically, prior to any

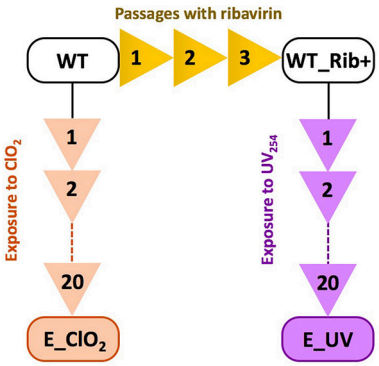


FIGURE 1 | Overview over the experimental evolution lineages to produce resistant populations E_ClO_2 and E_UV from their wild-types WT or WT Rib+.

exposure to UV_{254}, the E11 lab strain (WT) was first subjected to three cell culture adaptation passages in the presence of the mutagen ribavirin (Fluorochem), to enhance the genetic diversity of the starting population (Crotty et al., 2000). This population (WT_Rib+) was then subjected to 20 passages of titer reduction by UV_{254} followed by regrowth. The details of the UV_{254} setup are given in the following section. The resulting UV_{254}-resistant population is named E_UV hereafter. Both evolved populations at their last (20th) passage as well as the corresponding wild-types were sequenced as described below and the mutations of the evolved populations are listed in **Table 1**.

TABLE 1 | Heat map of the frequency of alleles that changed from minor to major or from major to fixed in the evolved populations E_ClO_2 and E_UV.

10 — 100

	NT	AA	E_ClO_2	E_UV
VP4	A849T[a]	Y33F	**0**	**100**
VP2	G1373C[c]	G139R	**100**	**100**
VP1	A2835G[b]	K126R	61	0
	C2844A[a,d]	P129Q	63	**100**
	T2849A	S131N	60	0
	C2850A	S131N	60	0
	C3162T	T235I	63	0
	A3170G[b]	M238V	63	**100**
	A3233G[b]	K239E	63	**99**
2C	T4200C	V40A	0	**100**
3D	T6006C[d]	M19T	**98**	**100**
	A6989G	T347A	1	**99**

Only non-synonymous mutations are included. The location of the mutation, and the resulting change in nucleotide (NT) and amino acid (AA) are listed. Mutations that reached fixation (≥99%) are indicated by bold fonts. Shared major alleles among E_ClO_2 and E_UV are shaded in gray.

[a] These mutations caused an amino acid substitution from ClO_2-reactive to stable ones;

[b] These mutations caused an amino acid substitution from FC-reactive to less reactive ones;

[c] At this position, the ancestral WT of E_ClO_2 already has a cytosine as a major allele while its frequency increased by more than 30% in E_ClO_2. Virus WT_Rib+ has a guanosine.

[d] At these positions, the mutations were already fixed in the ancestral WT_Rib+ of E_UV.

Cross-Resistance Experiments

All inactivation experiments were performed in phosphate-buffered saline [PBS; 5 mM Na_2HPO_4 (99%, Acros), 10 mM NaCl (99.5%, Acros), pH 7.4], at a starting E11 concentration of 10^6–10^7 MPNCU/mL. The specific inactivation assays associated with the different methods tested are summarized below. Kinetic analyses were performed by monitoring the loss of infective E11 as a function of disinfectant exposure (oxidants), dose (UV_{254} and solar radiation), or time (heat).

Chlorine Dioxide (ClO_2)

Concentrated ClO_2 was produced as described previously (Zhong et al., 2016) and stored in the refrigerator at 4°C. Working solutions were prepared in PBS immediately prior to the experiment.

Inactivation by ClO_2 was conducted in continuously stirred 10 mL beakers on ice containing 2 mL of PBS and an initial ClO_2 concentration of 1 mg/L. Samples were withdrawn periodically at time intervals of 10 s to 1 min over the course of up to 5 min, were mixed with sodium thiosulfate (98%, Sigma-Aldrich) to quench the residual ClO_2, and the virus titer (N) was enumerated. Control experiments showed that the addition of sodium thiosulfate did not affect virus infectivity, viral functions, or genome extraction in the subsequent experimental procedures. The ClO_2 concentration was monitored at the beginning ($C_{ClO2,0}$) and periodically throughout the experiment (C_{ClO2}) using the chlorophenol red (Sigma-Aldrich) method described by Fletcher and Hemmings (1985). The ClO_2 decay throughout the experiment was first order, and the associated decay rate constant k_d (min^{-1}) was determined as:

$$\ln\left(\frac{C_{ClO2}}{C_{ClO2,0}}\right) = -k_d t \quad (1)$$

The ClO_2 exposure at any time point during the inactivation experiment was estimated from the cumulative area under the curve of C_{ClO2} vs. t:

$$ClO_2\ exposure = \int_0^t C_{ClO2}\,dt \quad (2)$$

Kinetic inactivation parameters were obtained by fitting the data to the modified Hom model (Haas and Joffe, 1994):

$$\ln\frac{N}{N_0} = -k_{ClO2} C_{ClO2,0}^{n} t^{m} \left(\frac{1-\exp\left(-\frac{k_d t}{m}\right)}{\frac{k_d t}{m}}\right)^{m} \quad (3)$$

Here N_0 and N are the virus titers at times 0 and t, respectively, and k_{ClO2} is the Hom inactivation rate constant [$mg^{-n}L^{n}min^{-m}$]. Model parameters m and n were treated as constant across all experiments (Zhong et al., 2016) and corresponded to 0.30 and 0.46 respectively.

Free Chlorine (FC)

Inactivation experiments by FC were conducted analogously to ClO_2 experiments. The initial FC concentrations ranged from 1 to 2 mg/L, and were prepared by diluting NaClO (13–14%, Reactorlab SA) in PBS (pH 7.4). Samples were taken periodically at time intervals of 10 or 15 s over the course of up to 90 s. The FC concentration (C_{FC}) was measured in every sample using the N,N-diethyl-p-phenylenediamine (Sigma-Aldrich) colorimetric method (Rice et al., 2012). The chlorine exposure was determined from the cumulative area under the curve of C_{FC} vs. t:

$$FC\ exposure\ = \int_0^t C_{FC} dt \tag{4}$$

The inactivation rate constant k_{FC} was then determined using a first-order Chick-Watson model:

$$\ln\left(\frac{N}{N_0}\right) = -k_{FC} \int_0^t C_{FC} dt \tag{5}$$

Ultraviolet Radiation (UV_{254})

Continuously stirred 10 mL beakers containing 2 mL PBS (solution depth: 0.6 cm) were spiked with E11 and were placed under a low-pressure 18 W UV-C lamp (TUV T8 Philips) emitting light at 253.7 nm. All solutions were optically dilute, such that the transmission of UV_{254} throughout the reactor was >95%. The fluence rate (I_{UV}) was determined by actinometry using a solution of iodide (Alfa Aesar) and iodate (Acros) in borate buffer (Acros) (Rahn, 1997), and corresponded to 1.7 W/m^2. The UV_{254} dose was determined from the product of the fluence rate and time ($I_{UV}{}^*t$). Samples (100 μL) were taken at 1 min intervals over the course of 7 min, and BGMK cells were immediately infected in order to enumerate the concentration of infective E11. The UV_{254} inactivation rate constants (k_{UV}) were determined from model fits of the data to a first-order Chick-Watson model:

$$\ln\left(\frac{N}{N_0}\right) = -k_{UV}\, I_{UV} t \tag{6}$$

Sunlight

Inactivation experiments with sunlight were performed as described elsewhere (Bosshard et al., 2013). In brief, reactors containing 2 mL virus solutions at $5 \times 10^4 - -1 \times 10^6$ MPNCU/mL were placed under a solar simulator (Sun 2000, ABET Technologies) equipped with a 1,000 W Xenon lamp, an Air mass 1.5 filter, and an atmospheric edge filter. All solutions were optically dilute. The inactivation experiments were conducted in a thermostatic bath at 20°C with magnetic bars constantly stirring the reactors. Samples were taken periodically at time intervals of 1–3 h over the course of 24 h. The solar fluence rate (I_{sun}) was determined by a radiometer (ILT-900-R, International Light) over the range of 290–315 nm and the inactivation rate constant k_{sun} was obtained by fitting the data to a first-order Chick-Watson model:

$$\ln\left(\frac{N}{N_0}\right) = -k_{sun}\, I_{sun} t \tag{7}$$

Heat

Tolerance to heat was assessed by comparing the decay temperatures of the different E11 populations. Experiments were performed by thermal shift using a PCR thermal cycler (PCR System 9700, GeneAmp). PCR tubes (250 μL) each containing 90 μL of PBS were prepared. Ten microliters of virus solution were injected to the first tube, and this tube was immediately cultured to quantify the starting titer of E11. Starting from 38°C, each thermal shift was set to a 2°increase in temperature. At each shift, one tube containing PBS was preheated in the thermal cycler for 2 min before 10 μL virus solution were injected into the tube. The solution was then kept at this temperature for 1 min and thereafter immediately put on ice until enumeration. Segmental linear regression was applied to determine the decay temperature Td, at which the infective virus concentration started to decline at a rate that corresponded to the slope of the second segment (S):

$$\begin{cases} \ln\left(\frac{N}{N_0}\right) = 0,\ if\ T < Td \\ \ln\left(\frac{N}{N_0}\right) = -S\left(T - Td\right),\ if\ T \geq Td \end{cases} \tag{8}$$

Identification of Virus Functions Inhibited by Disinfectants

In order to identify the main viral functions affected during inactivation, we quantified the effect of the different disinfectants on genome replication and on host binding. To this end, E11 WT was inactivated by several orders of magnitude by ClO_2, FC, heat, UV_{254}, or sunlight. Isothermal conditions were applied for heat inactivation where viruses were incubated at 56°C in a water bath for 5 min. Samples were collected and divided into two aliquots. The first aliquot was diluted and infectious units of the sample were determined by infectivity assay. The other aliquot was subjected to the genome replication or host binding assays described below.

Genome Replication

The ability of a genome to replicate after inactivation was examined by quantitative reverse transcription-PCR (qRT-PCR). Viral RNA was extracted from initial and inactivated samples as described previously (Pecson et al., 2009). Prior to extraction, ∼10^7 PFU/mL MS2 was added to each sample as an internal reference to correct for differences in the genome extraction efficiency between the initial and inactivated samples. In each viral extract, the copy numbers of four E11 genome segments of approximate 550-base length each (549–1,080, 2,685–3,254, 4,227–4,793, 5,854–6,364, using primer sets 3F/4R, 11F/12R, 17F/18R, and 23F/24R as specified previously by Zhong et al., 2017) were quantified. Combined, these segments covered ∼30% of the E11 genome. In addition, the number of MS2 genome copies in each sample was determined using primer set 5′-CCGCTACCTTGCCCTAAAC-3′ and 5′-GACGACAACCATGCCAAAC-3′ as described previously (Pecson et al., 2009). The extraction efficiency in each sample was calculated as:

$$efficiency = \frac{g\_MS2}{g\_MS2_0} \tag{9}$$

where *g_MS2* and g_MS2_0 are the MS2 genome copy number in the inactivated and the initial samples, respectively. The extraction efficiency in each sample was used to correct the corresponding copy numbers of the different E11 segments. Finally, the intact, PCR-replicable proportion of each E11 genome segment *i* after disinfection, $(\frac{g_i}{g_{i0}})$, was determined, and was used to estimate the loss in PCR replicability of the whole E11 genome, $\left(\frac{G}{G_0}\right)$ using the following extrapolation (Wigginton et al., 2012):

$$\log\left(\frac{G}{G_0}\right) = \log\left[\left(\prod \frac{g_i}{g_{i0}}\right)^{\frac{whole\ genome\ length}{total\ length\ of\ all\ PCR\ segments}}\right] \quad (10)$$

Further information pertaining to genome extraction and qRT-PCR quality control can be found in the Supplementary Material.

Host Binding

Initial and inactivated (by ∼5 $\log_{10}$) samples were subjected to two binding assays as described in detail elsewhere (Zhong et al., 2017). First, flow cytometry was used to quantify the proportion of BGMK cells with bound viruses before and after inactivation according to a method modified from literature (Triantafilou et al., 2001). This method provided a rapid identification of the disinfecting methods that caused a loss in host binding. Briefly, BGMK cells were harvested and fixed in fixing buffer (4% paraformaldehyde, Alfa Aesar). Cells were then incubated in blocking buffer (PBS with 1% bovine serum albumin, Sigma-Aldrich) for 30 min before virus samples were added and incubated for 1 h at room temperature. After incubation, the solution with unbound viruses was discarded and cells were then sequentially incubated with anti-E11 primary antibody (LSBio) and secondary antibody conjugated with FITC (Sigma-Aldrich) on a rotator. Staining was measured using a CyFlow® SL flow cytometer (Partec) and the proportion of cells with viruses bound was analyzed by counting cells with green fluorescence emitted by FITC using FlowMax.

This flow cytometry assay offers a straight-forward method to track host cells with bound viral capsids. However, the method is less suited for direct quantification of bound viruses, and it cannot distinguish between intact virions and empty viral capsids. Therefore, the flow cytometry results were confirmed and refined by directly quantifying the number of viruses bound to cells before and after disinfection by qRT-PCR. In contrast to the flow cytometry assay, this approach targets the viral genome. Briefly, virus samples were inoculated onto BGMK cell monolayers on ice. After 40 min, any unbound viruses were removed by washing with PBS. The cell monolayer was then subjected to three freeze-thaw cycles and subjected to chloroform treatment. Bound viruses (N_b) were harvested and quantified by qRT-PCR as described previously (Zhong et al., 2017) and as further detailed in the Supplementary Material. The difference in qRT-PCR signal between untreated and inactivated virus samples results from a reduction in bound virus, plus the decrease in genome integrity of the targeted segment due to exposure to disinfectants (Wigginton et al., 2012). Hence, the observed binding loss was corrected for the genome decay due to disinfectant exposure. Heat-treated viruses served as a control to assess the extent of non-specific binding of inactivated viruses.

Genome Sequencing

The genomes of virus populations of interest were extracted, and sequencing libraries were prepared as described previously (Zhong et al., 2017). The whole genomes were then sequenced by Next Generation Sequencing (NGS) at the Lausanne Genomics Technologies Core Facility. Briefly, 300 bp PCR amplicons were purified and pooled to obtain 100 ng of nucleic acids per sample. Libraries of 100 bp double-stranded cDNA were constructed using the TruSeq Stranded mRNA Library Prep kit (Illumina). The library nucleic acid concentrations were measured by Nanodrop 2000 (Thermal Fisher Scientific Inc.) and the cDNA quality was checked by Fragment AnalyzerTM (Advanced Analytical). An Illumina HiSeq 2500 platform was used to sequence up to 6 independent, barcoded and pooled libraries. Reads of 100 nucleotides were trimmed and cleaned for further bioinformatics analysis. Only the genomic positions for which the number of reads exceeded 100 were included in downstream analysis. Single-end reads were aligned to E11 Gregory strain using HTS station (David et al., 2014) to call the nucleotide/base at each position The sequence of WT was used as reference to identify the fixed mutations in all evolved populations.

Statistical Analyses

To determine if the evolved (E_ClO_2 and E_UV) populations were more resistant than their corresponding wild-types, their inactivation rate constants were compared first by paired *t*-test analysis. The comparison of ClO_2 inactivation kinetics was done by Likelihood Ratio (LR) test (Haas et al., 2014), where the test statistics were determined by Chi-squared distribution table. For all tests the threshold *p*-value for statistical significance was 0.05. The goodness-of-fit was evaluated based on the coefficient of determination (R^2), which was determined by GraphPad Prism (Version 6.01, 2012). Unpaired *t*-test or regular one-way ANOVA was applied to compare all other parameters between populations or treatments.

RESULTS

Inactivation of E11 WT by Different Treatments

Example inactivation curves of E11 WT by ClO_2, FC, UV_{254}, sunlight, and heat are shown in **Figure 2**. The inactivation of E11 WT by to FC, $UV_{254,}$ and sunlight were first-order with respect to the disinfectant exposure over the inactivation range tested. The inactivation curve of ClO_2, in contrast, tailed off at higher ClO_2 exposures. Despite this tail, a 3 $\log_{10}$ (99.9%; 6.9 ln) reduction in the infective viral load could be readily achieved (within minutes) by ClO_2 at the disinfectant exposures tested. Inactivation by FC and UV_{254} at the conditions employed herein were similarly effective. In contrast, inactivation by simulated sunlight proceeded more slowly, such that a 3 $\log_{10}$ inactivation required several hours of exposure. Finally, inactivation by thermal shift revealed that the decay temperature of E11 was around 41°C. Beyond this temperature, 3 $\log_{10}$ virus decay

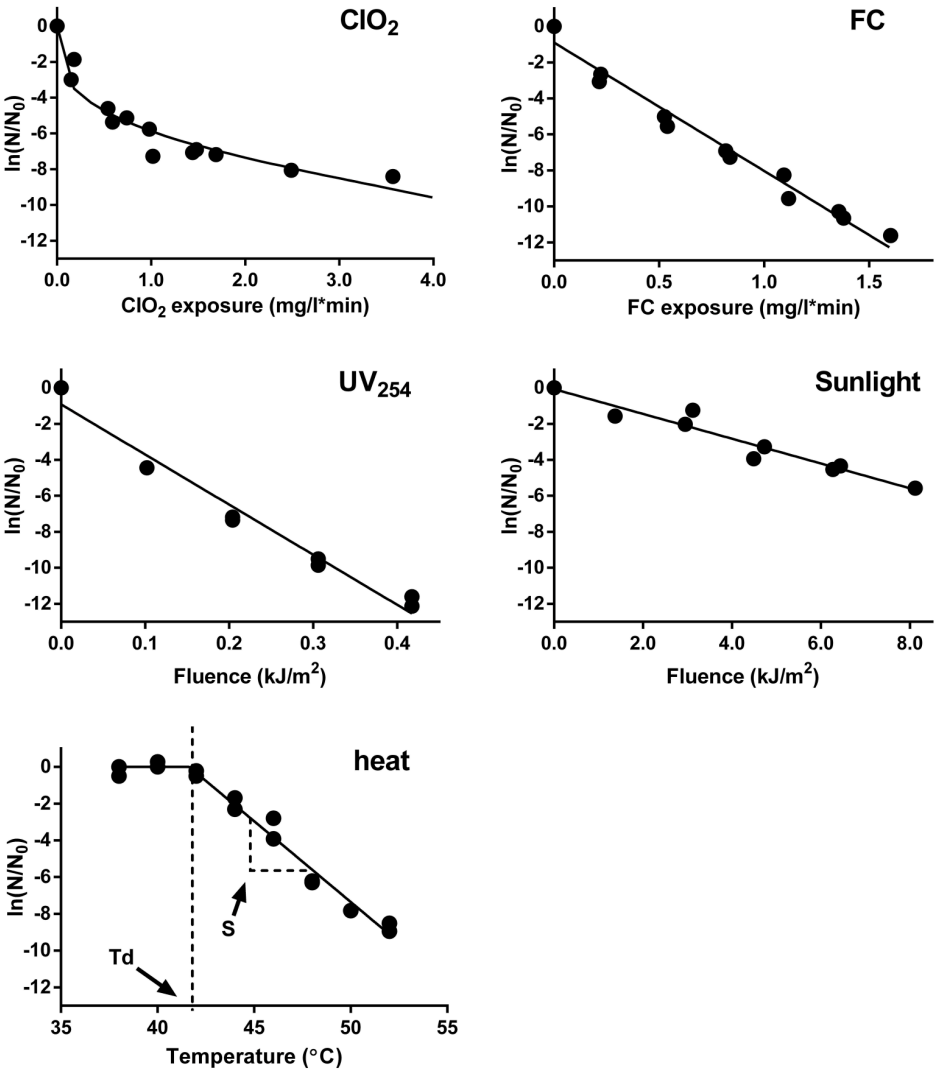


FIGURE 2 | Inactivation of E11 (WT) by ClO_2, FC, UV_{254}, sunlight, and heat. Infectivity loss (ln (N/N_0)) is plotted against disinfectant exposure for ClO_2 and FC exposure, fluence dose for UV_{254} and sunlight, and temperature for heat inactivation. Experimental data was fitted using the modified Hom model (ClO_2), Chick-Watson model (FC, UV_{254} and sunlight), and segmental linear regression (heat). Model fits are shown as solid lines. Results from duplicate experiments are presented.

was achieved within four temperature shifts during which the temperature increased to 50°C.

Cross-Resistance of ClO_2- and UV_{254}-Tolerant Populations to Other Disinfectants

The susceptibilities of the ClO_2- and UV_{254}-tolerant E11 populations, as well as their corresponding wild-type were tested for the five inactivating treatments considered. **Figure 3** shows the extent of resistance to the original stressor (ClO_2 for E_ClO_2 and UV_{254} for E_UV), along with the extent of cross-resistance to each additional treatment considered. The data are presented as the inactivation rate constants of the E_ClO_2 and E_UV populations relative to their respective wild-types, such that a value below unity indicates greater resistance than the wild-type. The absolute inactivation rate constants are reported in Supplementary Tables 1–4. For heat inactivation, the difference in decay temperature to the corresponding wild-type is shown, with the original decay temperatures reported in Supplementary Table 5.

E_ClO_2 exhibited a 43% reduction in k_{ClO2}compared to its wild-type ($p < 0.0001$; **Figure 3A**). E_ClO_2 was furthermore cross-resistant to FC, with a 19% reduction in k_{FC} compared to its wild-type ($p < 0.0001$). Compared to ClO_2, resistance to FC was thus less pronounced. Finally, no significant resistance was observed toward UV_{254}, sunlight or heat.

E_UV exhibited resistance to UV_{254} inactivation, though its extent was low. Specifically, the reduction in k_{UV} compared to its wild-type was 15% ($p = 0.0008$; **Figure 3B**). In addition, E_UV

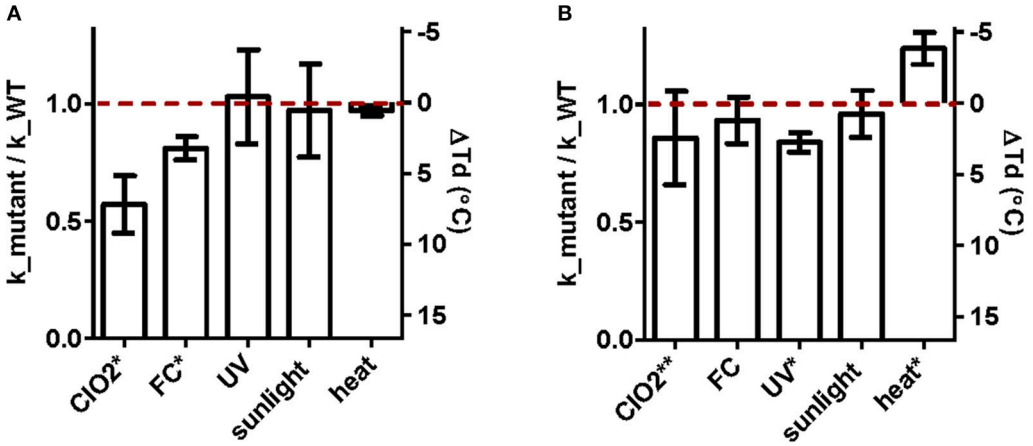


FIGURE 3 | Extent of (cross-) resistance of ClO_2-resistant populations E_ClO_2 **(A)** and UV_{254}-resistant E_UV **(B)** upon inactivation by ClO_2, FC, UV_{254}, sunlight, and heat. Except heat, results are presented as the ratio of the inactivation rate constants of the resistant populations to those of the corresponding wild-types (left y-axis). Cross-resistance to heat was determined from the differences in the decay temperature (ΔTd) between the resistant populations and their wild-type (right y-axis). The red dashed line indicates unity. Bars extending above the red line correspond to reduced resistance, and bars below indicate enhanced resistance compared to the wild-type. Asterisks indicates significant difference in resistance compared to the wild-type at the 95% (*) or 90% (**) confidence level. Error bars represent the propagated standard errors from the model fits to pooled duplicate or triplicate experiments.

exhibited a 14% reduction in susceptibility to ClO_2 compared to the wild type, though the difference was not significant at the 95% confidence level ($p = 0.0977$). Finally, this population was more susceptible than its wild-type to heat, yielding a 4°decrease in Td ($p = 0.0070$), whereas no measurable resistance to FC or sunlight was observed.

Viral Functions Inhibited by Different Disinfectants

Effect of Inactivating Treatments on Genome Integrity

Genome replication by the host cell could not be quantified as an isolated process, as it is preceded by host binding and internalization. We instead determined how the different inactivating treatments affect the ability of the E11 genome to be amplified by PCR. Specifically, the reduction in the qRT-PCR signal upon exposure to ClO_2, FC, heat, UV_{254}, and sunlight was determined for different genome segments (Supplementary Figure 2), and was used to estimate the loss in PCR-replicability of the entire genome (G/G_0, equation 10) of E11 (WT) (**Figure 4A**).

As is evident from **Figure 4A**, heat did not cause a measurable loss in PCR-replicable genomes. In contrast, exposure to UV_{254} caused a rapid decrease in G/G_0 and the rate of this decrease corresponded to that of the corresponding decrease in infectivity (**Figure 4B**). For sunlight-, FC-, ClO_2- inactivated viruses, the loss in PCR-replicable genome exceeded infectivity loss. Sunlight exposure caused G/G_0 to decrease by 7.4 ± 0.8 $\log_{10}$ for a 2.3 ± 0.54 $\log_{10}$ of infectivity loss. For FC, the decrease in G/G_0 was 35 ± 11 $\log_{10}$, compared to an infectivity loss of roughly 5 $\log_{10}$. Finally, for ClO_2, the loss in genome replicability was further investigated at different levels of inactivation (**Figure 5**). Interestingly, log G/G_0 roughly corresponded to extent of inactivation (log N/N_0) at low ClO_2 exposures, but increasingly exceeded inactivation with increasing ClO_2 exposure. Ultimately, a 5 $\log_{10}$ inactivation of E11 infectivity by ClO_2 resulted in a reduction of log G/G_0 of 14.1 ± 1.0 $\log_{10}$.

These data indicate that PCR is more sensitive to genome damage induced by chemical oxidants or sunlight than the host cell. This may be rationalized by considering that the enzymes used in PCR are selected to have a low error tolerance. In BGMK cells, in contrast, only a fraction of the genome damage incurred led to inhibition of genome replication and hence inactivation. Alternatively, genome damage could also be rescued by genome recombination in the cells (Mattle and Kohn, 2012). The extent by which PCR and BGMK cells differ in their ability to replicate damaged genomes disinfectants is currently not well-understood. Despite this limitation of the assay, the PCR results are consistent with a contribution of genome damage, and a resulting loss in the replication function, to inactivation by UV_{254}, FC, ClO_2, and sunlight.

Effect of Inactivating Treatments on Host Binding

The effect of inactivation on the ability of E11 to bind to BGMK cells was first cursorily screened by flow cytometry. Hereby we determined how inactivation affected the load of viruses bound to BGMK cells. Results revealed that the different disinfection methods affect host binding to varying extents (**Figure 4C** and Supplementary Figure 1). After roughly 5 $\log_{10}$ of infectivity loss by ClO_2 or heat, no cells carrying viruses could be detected, indicating a strong inhibition of host binding by these two treatments. A small fraction of cells with bound viruses was observed after treatment by FC, though this fraction was below the instrumental limit of quantification. Finally, host binding was only minimally affected by UV_{254} disinfection, resulting in negligible reduction in the observation of cells with bound viruses after treatment.

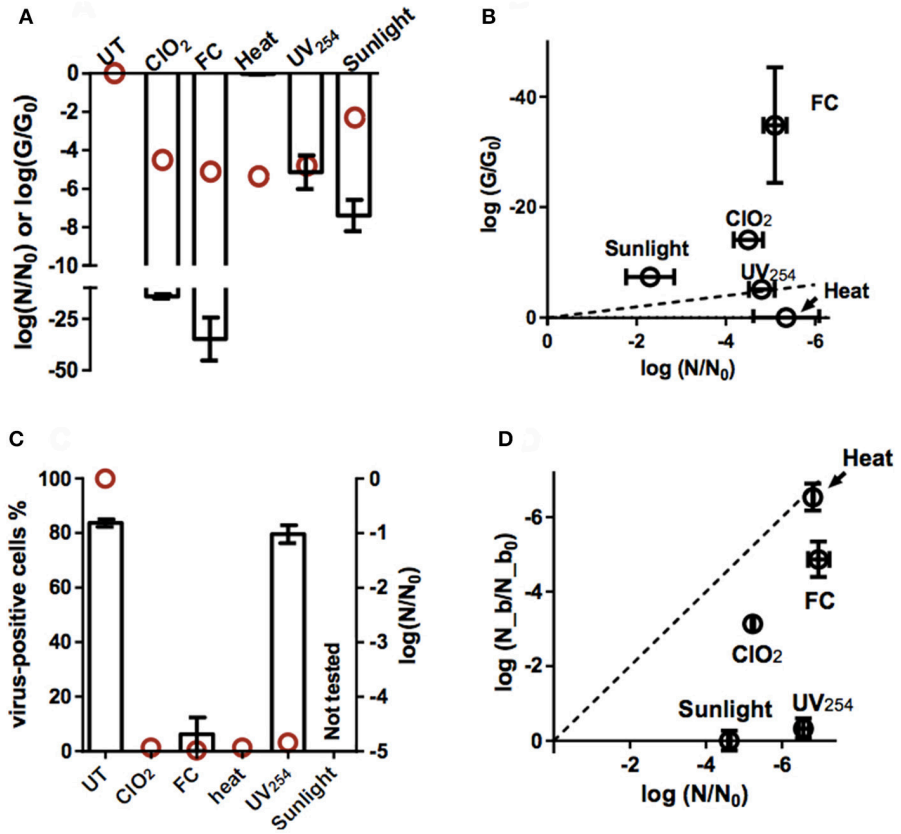


FIGURE 4 | Effect of inactivation by ClO_2, FC, heat, UV_{254}, and sunlight on viral functions. **(A)** Loss of PCR-replicable genome upon inactivation ($\log(G/G_0)$; bars). Red circles indicate the corresponding loss in inactivation ($\log(N/N_0)$). Errors bars represent the standard deviations associated with G/G_0 (Ku, 1966). **(B)** Loss of PCR-replicable genome, plotted against infectivity loss. The dashed line represents the 1:1 correlation between genome loss and infectivity loss. Errors bars represent the the MPN enumeration error (horizontal) or standard deviations associated with G/G_0 (vertical). **(C)** Percentage of cells with bound viruses, determined by flow cytometry (bars, left y-axis, UT: untreated E11 sample). Red circles indicate the corresponding virus infectivity loss (right y-axis). **(D)** Residual fraction of bound viruses ($\log(N\_b/N\_b_0)$) measured by PCR and plotted against infectivity loss. The dashed line represents the 1:1 correlation between binding loss and infectivity loss. Error bars represent the MPN enumeration error (horizontal) or range of duplicate experiments (vertical).

These findings were further refined by directly quantifying the concentration of viruses bound to cells (**Figure 4D**) by qRT-PCR. The loss of binding capacity due to heat (6.54 ± 0.36 $\log_{10}$) corresponded to the observed infectivity loss (6.81 ± 0.02 $\log_{10}$). In contrast, for FC and ClO_2, the loss in binding was smaller than infectivity loss (4.87 ± 0.48 vs. 7.0 ± 0.28 $\log_{10}$ for FC; 3.14 ± 0.10 vs. 5.23 ± 0.03 $\log_{10}$ for ClO_2). Finally, UV_{254} and sunlight manifested less than half a log of binding loss for an infectivity loss was 6.56 ± 0.14 $\log_{10}$ and 4.61 ± 0.03 $\log_{10}$ respectively.

Assuming a first-order rate of loss in host binding (Wigginton et al., 2012; Zhong et al., 2017), and given the approximate first-order inactivation over the range of disinfection exposures considered, the contribution of binding loss to overall inactivation can be estimated from a log-log plot of binding loss vs. infectivity loss. In **Figure 4D**, the dotted 1:1 line indicates the region where binding loss can account for all the infectivity loss. The region below the line signifies that binding loss is smaller than infectivity loss, such that other viral functions must also be affected by a given disinfectant. **Figure 4D** reveals that for heat, binding loss fully accounts for inactivation, whereas for FC and ClO_2, the contribution to inactivation is ∼70 and 60% respectively. Finally, for UV_{254} and sunlight, host binding remains unaffected. Note, however, that the extent of residual binding may be overestimated for most treatments, as it was not possible to control for non-specific binding of inactivated viruses. The only exception is heat, where binding loss was proportional to infectivity loss, such that non-specific binding was unlikely.

Comparison of the Mutation Spectrum of the Resistant Populations

Disinfection resistance arises from mutations to the viral genome which confer an advantage with respect to withstanding disinfection. A comparison of the mutations fixed in each of the resistant populations, along with their likely effects on the viral phenotype, may further aid in understanding the occurrence or absence of cross-resistance.

A total of 12 non-synonymous mutations were identified (**Table 1**). Of these, five were shared by both resistant populations, and may be a result of adaptation to cell culturing.

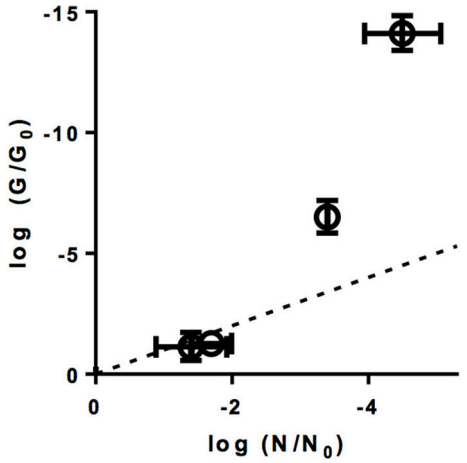


FIGURE 5 | Genome loss log(G/G_0) at different levels of inactivation log(N/N_0) by ClO_2. The dotted line represents the 1:1 correlation between inactivation and genome loss. Errors bars represent the MPN enumeration error (horizontal) or the standard deviation associated with G/G_0 (vertical) (Ku, 1966).

E_ClO_2 furthermore exhibited unique non-synonymous mutations in the structural protein VP1, whereas unique non-synonymous mutations in E_UV were located in the replication-related proteins 2C and 3D, as well as in structural protein VP4. Given that there was no cross-resistance of E_ClO_2 to UV_{254} (**Figure 3A**) and only minor cross-resistance of E_UV to ClO_2, these unique mutations serve as candidate mutations responsible for the resistance of E_UV to UV_{254} and of E_ClO_2 to ClO_2 respectively.

DISCUSSION

The occurrence of viral multi-resistance to disinfectants with different modes of action appears limited in the virus populations tested herein. Among the 10 combinations of resistances and disinfectants tested, only one significant cross-resistance was observed between two chemical oxidants, resulting in a reduced sensitivity of ClO_2-resistant virus to free chlorine. To rationalize the cross-resistance patterns observed, we investigated the mechanisms of action of each disinfectant. Hereby we hypothesized that cross-resistance was a result of a shared mechanism of action of two disinfectants, whereas the absence of cross-resistance is found among treatments acting by different mechanisms. As discussed above, most studies to date report genome damage and inhibition of host binding as the main action of most disinfectants. We therefore focused our mechanistic investigation on these two traits.

Mechanisms of E11 Inactivation

Based on our findings of reduction in genome integrity (**Figures 4A,B**) and inhibition of host binding (**Figures 4C,D**), an overview of the main mechanisms of inactivation is presented in **Figure 6**. Roughly, the modes of action of the different disinfectants can be categorized into three groups, depending on the major viral function impaired. First, for heat, inactivation can be attributed entirely to a loss in host binding. Accordingly, no other viral functions are implicated in inactivation and all genomes remain as replicable as in the untreated samples. Second, for UV_{254} and sunlight, no or minimal binding loss was detected. Inactivation must thus be due to losses in other viral functions, such as genome internalization, replication or virion assembly. While these functions were not tested individually, both UV_{254} and sunlight resulted in a considerable decrease in the fraction of PCR-replicable genome copies. The extensive genome degradation observed by PCR supports the conclusion that the main mechanism of inactivation by both UV_{254} and sunlight involves genome damage, and hence inhibition of replication. Third, inactivation by the oxidants ClO_2 and FC cannot be attributed to loss in a single virus function. Their mode of action mainly involves a reduction in host binding, yet losses in other functions also contribute significantly. As observed for UV_{254} and sunlight, treatment by FC and ClO_2 also leads to extensive genome degradation, which likely causes a loss of genome replication.

The proposed mechanisms agree with previous studies on enterovirus that demonstrated that heat and FC inhibited host binding of poliovirus (Nuanualsuwan and Cliver, 2003a) and that UV_{254}'s primary target is the genome (Helentjaris and Ehrenfeld, 1977; Nuanualsuwan and Cliver, 2003b). The proposed mechanisms of inactivation of E11 are also are largely consistent with those previously described for MS2 (Wigginton et al., 2012). Major discrepancies were only found for ClO_2: this disinfectant was previously reported to have no effect on the genome integrity of MS2 (Wigginton et al., 2012), and no effect on host binding for poliovirus (Alvarez and O'Brien, 1982), whereas both these functions were inhibited in E11. The disagreement may be linked to differences in the viral species investigated and their binding motifs, as well as to the disinfectant exposures and solution conditions considered. Furthermore, the inactivation curve of E11 by ClO_2 exhibits a pronounced tail (**Figure 2A**). This feature has previously been reported for virus inactivation by ClO_2, and has been attributed to multiple causes, including the presence of resistant subpopulations or the gradual accumulation of protein oxidation products that form a protective layer on the viral capsid (Berman and Hoff, 1984; Chen and Vaughn, 1990; Thurston-Enriquez et al., 2005; Lim et al., 2010; Jin et al., 2013; Sigstam et al., 2013). The tailing inactivation curve may cause the extent of genome damage by ClO_2 to not scale linearly with inactivation, but instead to increasingly exceed the extent of inactivation (**Figure 5**). As such, it is likely that the relative contribution of genome damage to inactivation by ClO_2 depends on the ClO_2 exposure and the extent of inactivation considered.

Cross-Resistance of E11 to Different Disinfectants Is Specific to the Mechanism of Inactivation

In the ClO_2-resistant population E_ClO_2, we previously identified that resistance was rooted in the ability to utilize an additional host receptor, which was in turn linked to mutations

in VP1 (Zhong et al., 2017). This trait allowed the resistant population to better maintain host binding in the presence of ClO_2, and hence to tolerate higher ClO_2 exposure. Cross-resistance of E_ClO_2 may thus be expected to any disinfectant that inhibits host binding (**Figure 6**). Consistent with this hypothesis, E_ClO_2 also exhibited resistance to FC. In contrast, no cross-resistance to heat was observed, even though this treatment also affects host binding. This result can be rationalized by considering that ClO_2 and FC both oxidize viral proteins, whereas heat acts by denaturation (Rombaut et al., 1994; Dodd, 2012). While the mutations in E_ClO_2 protected the virus from oxidation by allowing alternative receptor use, and by replacing oxidation-reactive by stable amino acids (**Table 1**) (Sharma and Sohn, 2012), they may not yield the same benefits for protection from denaturation. Finally, the absence of cross-resistance to UV_{254} and sunlight, which do not act on host binding, supports that resistance to ClO_2 is a mechanism-specific trait.

In population E_UV, resistance to UV_{254} implies a greater ability of the resistant population to deal with mutations accumulated through the action of UV_{254}. This ability should also extend to sunlight, since solar UVB (280–315 nm) is also known to have mutagenic action (Pfeifer et al., 2005). Yet, population E_UV did not demonstrate measurable cross-resistance to sunlight. This observation can be explained by considering that the UV_{254}-resistance of this population was relatively mild, and that UV at different wavelengths have distinct mutational specificity (Pfeifer et al., 2005).

No cross-resistance of E_UV was observed for heat, which is consistent with its mode of action being entirely protein dependent. Instead, E_UV exhibited enhanced susceptibility to heat. We tentatively attribute this feature to mutation Y33F, which was only found in E_UV, and which is located on the N-terminus of structural protein VP4. The intertwining N-terminus extension of VP1, VP3, and VP4 form a network of protein-protein interactions on the interior of the capsid that is crucial to viral stability (Knipe and Howley, 2007). Therefore, we argue that Y33F on VP4 rendered the E_UV less structurally stable and hence more heat-sensitive. This proposition is supported by the prediction of protein stability changes upon single point mutations using I-Mutant2.0 (Capriotti et al., 2005, 2008). Mutation Y33F was estimated to yield a Gibbs free energy change of −0.76 at 45°C compared to the wild-type. Therefore, Y33F is destabilizing the protein. I-Mutant, however, considers only single proteins, hence free energy calculations that take into consideration inter-protein interactions are needed to validate the result.

Finally, E_UV was slightly cross-resistant to ClO_2, even though these two disinfectants act by drastically different mechanisms. This finding indicates that populations with a more general resistance spectrum can exist. However, the inverse cross-resistance was not found: E_ClO_2 remained susceptible to UV_{254}. This supports the notion that the multi-resistance of E_UV is not linked to the resistance to UV_{254} *per se*, but may be induced by the experimental evolution assay used to produce the evolved populations. Specifically, resistant populations were produced by repeated and drastic reduction of their population numbers by either ClO_2 or UV_{254} exposure, followed by regrowth. This action likely selected for those variants that most efficiently proliferated under the experimental conditions used. Efficient proliferation may be aided by enhanced host binding, which in turn is also beneficial to resistance to ClO_2. Interestingly, both evolved populations shared mutations that are confirmed or probable sites associated with a host receptor switch (VP2-G139R, VP1-M238V, and VP1-K259E; **Table 1**) (Zhong et al., 2017). This supports the notion that analogous to E_ClO_2, the ClO_2 resistance manifested in E_UV is also associated with a better use of an alternative cell receptor.

Given the significant cross-resistance of ClO_2 and FC observed in E_ClO_2, the presence of cross-resistance of E_UV to ClO_2 but absence of cross-resistance to FC is surprising. However, resistance of E_ClO_2 to FC is less pronounced compared to ClO_2 (**Figure 3A**), and cross-resistance to ClO_2 in E_UV was only slight (**Figure 3B**). Combined, these two factors likely rendered any cross-resistance of E_UV to FC too small to be experimentally measured.

Overall, this study supports the hypothesis that cross-resistance is mainly found among disinfectants that act by a similar mechanism. To confirm this result, future studies should include viruses with resistance to less specific stressors, such as ozone or free chlorine, which significantly target both viral proteins and genomes. It is conceivable that viruses evolved under pressure of such non-selective disinfectants evolve more general resistances that extend to both genome- and protein-active disinfectants.

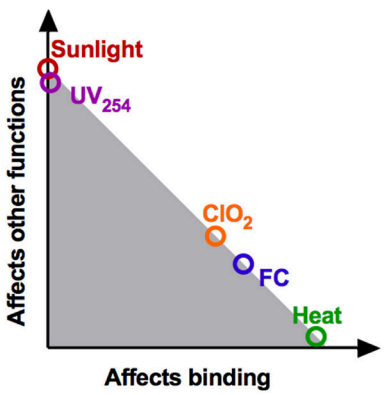


FIGURE 6 | Schematic summary of the contributions of binding loss and other viral functions to overall inactivation. This location of the data points correspond to the ratio of log(N_b/N_b_0): log(N/N_0) (see **Figure 4D**). The shaded region represents the additive effects of loss of binding, genome replication and potentially other functionalities.

Implications for Virus Control

As discussed in the introduction, the presence of disinfection-resistant viruses in the environment and is already well-established, though the origin of their resistance is not always known. A potential new source of resistant viruses may be the increasing practice of direct potable reuse of wastewater. In these systems, waterborne viruses may remain in the

"treatment-consumption-excretion-treatment" cycle, where they can become subjected to iterate disinfectant exposures and cause new infections. In such a setting, we should be conscious of the potential emergence of disinfection-resistant viruses, and evaluate the best approaches to control their occurrence.

Our results to date suggest that viruses with resistance to a given disinfectant can be controlled by a disinfectant with a different mode of action. This may be achieved by implementing a double disinfection barrier that uses different disinfectants in sequence. From our study on echovirus, a smart choice of disinfectant to include in a double barrier setup is UV_{254}. First, UV_{254} is a rather non-selective disinfectant that acts on all genetic material to a roughly similar extent (Lytle and Sagripanti, 2005). This is a stark contrast to a disinfectant like ClO_2, which only efficiently targets specific amino acids, namely cysteine, tyrosine, tryptophan, histidine, and proline (Tan et al., 1987; Sharma and Sohn, 2012). Compared to ClO_2, it is thus unlikely that any virus will ever fully escape the pressure of UV_{254}. Second, the resistance to UV_{254} was slight compared to that to ClO_2 (though we cannot exclude that different experimental approaches to produce the resistant virus result in greater resistance). Even if the resistance is minor, however, it remains necessary to include an additional disinfection step using a different disinfectant, such as free chlorine, to control UV_{254}-resistant organisms.

The efficiency of different double disinfection barriers to control resistant viruses remains to be tested in future work. In particular, this approach should be validated for additional viruses, as their inactivation mechanisms by the disinfectants tested may differ from that of E11. Furthermore, research should identify ideal combinations of disinfectants and optimal treatment regimes. Ultimately, such a setup should be able to successfully inactivate resistant viruses while avoiding the emergence of multi-resistant viruses.

AUTHOR CONTRIBUTIONS

QZ and TK designed the experimental plan. QZ, AC, VB, and RO conducted the experiments. QZ, TK, and RO analyzed the data. QZ, AC, and TK wrote the manuscript.

FUNDING

This work was funded by the Swiss National Foundation (project numbers 31003A_138319 and 31003A_163270).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01928/full#supplementary-material
Additional details on the qRT-PCR protocol, figures on analysis of flow cytometer output, reduction of PCR replicable genome segments and absolute inactivation rate constants.

REFERENCES

Alvarez, M. E., and O'Brien, R. T. (1982). Mechanisms of inactivation of poliovirus by chlorine dioxide and iodine. *Appl. Environ. Microbiol.* 44, 1064–1071.

Ballester, N. A., and Malley, J. P. (2004). Sequential disinfection of adenovirus type 2 with UV-chlorine-chloramine. *J. Am. Water Work Assoc.* 2, 97–103.

Bates, R. C., Shaffer, P. T., and Sutherland, S. M. (1977). Development of poliovirus having increased resistance to chlorine inactivation. *Appl. Environ. Microbiol.* 34, 849–853.

Berman, D., and Hoff, J. C. (1984). Inactivation of simian rotavirus sa11 by chlorine, chlorine dioxide, and monochloramine. *Appl. Environ. Microbiol.* 48, 317–323.

Bosshard, F., Armand, F., Hamelin, R., and Kohn, T. (2013). Mechanisms of human adenovirus inactivation by sunlight and UVC light as examined by quantitative PCR and quantitative proteomics. *Appl. Environ. Microbiol.* 79, 1325–1332. doi: 10.1128/AEM.03457-12

Capriotti, E., Fariselli, P., and Casadio, R. (2005). I-Mutant2.0: predicting stability changes upon mutation from the protein sequence or structure. *Nucleic Acids Res.* 33, W306–W310. doi: 10.1093/nar/gki375

Capriotti, E., Fariselli, P., Rossi, I., and Casadio, R. (2008). A three-state prediction of single point mutations on protein stability changes. *BMC Bioinformatics* 9(Suppl. 2):S6. doi: 10.1186/1471-2105-9-S2-S6

Chen, Y. S., and Vaughn, J. M. (1990). Inactivation of human and simian rotaviruses by chlorine dioxide. *Appl. Environ. Microbiol.* 56, 1363–1366.

Crotty, S., Maag, D., Arnold, J. J., Zhong, W., Lau, J. Y., Hong, Z., et al. (2000). The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. *Nat. Med.* 6, 1375–1379. doi: 10.1038/82191

David, F. P. A., Delafontaine, J., Carat, S., Ross, F. J., Lefebvre, G., Jarosz, Y., et al. (2014). HTSstation: a web application and open-access libraries for high-throughput sequencing data analysis. *PLoS ONE* 9:e85879. doi: 10.1371/journal.pone.0085879

Dodd, M. C. (2012). Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. *J. Environ. Monit.* 14, 1754–1771. doi: 10.1039/c2em00006g

Fletcher, I. J., and Hemmings, P. (1985). Determination of chlorine dioxide in potable waters using chlorophenol red. *Analyst* 110, 695–699. doi: 10.1039/an9851000695

Fong, T.-T., and Lipp, E. K. (2005). Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiol. Mol. Biol. Rev.* 69, 357–371. doi: 10.1128/MMBR.69.2.357

Ford, T. E. (1999). Microbiological safety of drinking water: United States and global perspectives. *Environ. Health Perspect.* 107, 191–206. doi: 10.2307/3434483

Haas, C. N., and Joffe, J. (1994). Disinfection under dynamic conditions: modification of Hom's model for decay. *Environ. Sci. Technol.* 28, 1367–1369. doi: 10.1021/es00056a028

Haas, C. N., Rose, J. B., and Gerba, C. P. (2014). *Quantitative Microbial Risk Assessment, 2nd Edn.* Hoboken, NJ: John Wiley and Sons, Inc.

Helentjaris, T., and Ehrenfeld, E. (1977). Inhibition of host cell protein synthesis by UV-inactivated poliovirus. *J. Virol.* 21, 259–267.

Jin, M., Shan, J., Chen, Z., Guo, X., Shen, Z., Qiu, Z., et al. (2013). Chlorine dioxide inactivation of enterovirus 71 in water and its impact on genomic targets. *Environ. Sci. Technol.* 47, 4590–4597. doi: 10.1021/es305282g

Jin, M., Zhao, Z.-G., Wang, X.-W., Shen, Z.-Q., Xu, L., Yu, Y.-M., et al. (2012). The 40–80 nt region in the 5-NCR of genome is a critical target for inactivating poliovirus by chlorine dioxide. *J. Med. Virol.* 84, 526–535. doi: 10.1002/jmv.23221

Knipe, D. M., and Howley, P. M. (Eds) (2007). *Fields Virology, 5th Edn.* Philadelphia, PA: Lippincott Williams & Wilkins.

Ku, H. H. (1966). Notes on the use of propagation of error formulas. *J. Res. Natl. Bur. Stand. Sect. C Eng. Instrum.* 70C, 263–273. doi: 10.6028/jres.070C.025

Li, J. W., Xin, Z. T., Wang, X. W., Zheng, J. L., and Chao, F. H. (2004). Mechanisms of inactivation of hepatitis A virus in water by chlorine dioxide. *Water Res.* 38, 1514–1519. doi: 10.1016/j.watres.2003.12.021

Lim, M. Y., Kim, J.-M., and Ko, G. (2010). Disinfection kinetics of murine norovirus using chlorine and chlorine dioxide. *Water Res.* 44, 3243–3251. doi: 10.1016/j.watres.2010.03.003

Lytle, C. D., and Sagripanti, J.-L. (2005). Predicted inactivation of viruses of relevance to biodefense by solar radiation. *J. Virol.* 79, 14244–14252. doi: 10.1128/JVI.79.22.14244-14252.2005

Maillard, J.-Y., Hann, A. C., and Perrin, R. (1998). Resistance of *Pseudomonas aeruginosa* PAO1 phage F116 to sodium hypochlorite. *J. Appl. Microbiol.* 85, 799–806. doi: 10.1046/j.1365-2672.1998.00578.x

Mattle, M. J., and Kohn, T. (2012). Inactivation and tailing during UV254 disinfection of viruses: contributions of viral aggregation, light shielding within viral aggregates, and recombination. *Environ. Sci. Technol.* 46, 10022–10030. doi: 10.1021/es302058v

Nuanualsuwan, S., and Cliver, D. O. (2003a). Capsid functions of inactivated human picornaviruses and feline calicivirus. *Appl. Environ. Microbiol.* 69, 350–357. doi: 10.1128/AEM.69.1.350

Nuanualsuwan, S., and Cliver, D. O. (2003b). Infectivity of RNA from inactivated poliovirus. *Appl. Environ. Microbiol.* 69, 1629–1632. doi: 10.1128/AEM.69.3.1629

Olivieri, V. P., Hauchman, F. S., Noss, C. I., and Vasl, R. (1985). "Mode of action of chlorine dioxide on selected viruses," in *Water Chlorination Chemistry: Environmental Impact and Health Effects*, *Vol 5*, ed R. L. Jolley (Chelsea, MI: Lewis Publishers), 619–634.

Payment, P., Tremblay, M., and Trudel, M. (1985). Relative resistance to chlorine of poliovirus and coxsackievirus isolates from environmental sources and drinking water. *Appl. Environ. Microbiol.* 49, 981–983.

Pecson, B. M., Martin, L. V., and Kohn, T. (2009). Quantitative PCR for determining the infectivity of bacteriophage MS2 upon inactivation by heat, UV-B radiation, and singlet oxygen: advantages and limitations of an enzymatic treatment to reduce false-positive results. *Appl. Environ. Microbiol.* 75, 5544–5554. doi: 10.1128/AEM.00425-09

Pfeifer, G. P., You, Y., and Besaratinia, A. (2005). Mutations induced by ultraviolet light. *Mutat. Res.* 571, 19–31. doi: 10.1016/j.mrfmmm.2004.06.057

Rahn, R. (1997). Potassium iodide as a chemical actinometer for 254 nm radiation : use of iodate as an electron scavenger. *Photochem. Photobiol.* 66, 450–455. doi: 10.1111/j.1751-1097.1997.tb03243.x

Rice, E. W., Baird, R. B., Eaton, A. D., and Clesceri, L. S. (2012). *Standard Methods for the Examination of Water and Wastewater, 22nd Edn*. Washington, DC: American Public Health Association; American Water Works Association; Water Environment Federation.

Rombaut, B., Verheyden, B., Andries, K., and Boeyé, A. (1994). Thermal inactivation of oral polio vaccine: contribution of RNA and protein inactivation. *J. Virol.* 68, 6454–6457.

Shaffer, P. T., Metcalf, T. G., and Sproul, O. J. (1980). Chlorine resistance of poliovirus isolants recovered from drinking water. *Appl. Environ. Microbiol.* 40, 1115–1121.

Sharma, V. K., and Sohn, M. (2012). Reactivity of chlorine dioxide with amino acids, peptides, and proteins. *Environ. Chem. Lett.* 10, 255–264. doi: 10.1007/s10311-012-0355-5

Sigstam, T., Rohatschek, A., Zhong, Q., Brennecke, M., and Kohn, T. (2013). On the cause of the tailing phenomenon during virus disinfection by chlorine dioxide. *Water Res.* 48, 82–89. doi: 10.1016/j.watres.2013.09.023

Simonet, J., and Gantzer, C. (2006). Degradation of the poliovirus 1 genome by chlorine dioxide. *J. Appl. Microbiol.* 100, 862–870. doi: 10.1111/j.1365-2672.2005.02850.x

Suess, M. J. (Ed) (1982). *Biological, Bacteriological and Virological Examination: A Reference Handbook*, *1st Edn*. Oxford: Pergamon Press Ltd.

Tan, H. K., Wheeler, W. B., and Wei, C. I. (1987). Reaction of chlorine dioxide with amino acids and peptides: kinetics and mutagenicity studies. *Mutat. Res.* 188, 259–266. doi: 10.1016/0165-1218(87)90002-4

Thurston-Enriquez, J. A., Haas, C. N., Jacangelo, J., and Gerba, C. P. (2005). Inactivation of enteric adenovirus and feline calicivirus by chlorine dioxide. *Appl. Environ. Microbiol.* 71, 3100–3105. doi: 10.1128/AEM.71.6.3100

Triantafilou, M., Wilson, K. M., and Triantafilou, K. (2001). Identification of echovirus 1 and coxsackievirus A9 receptor molecules via a novel flow cytometric quantification method. *Cytometry* 43, 279–289. doi: 10.1002/1097-0320(20010401)43:4<279::AID-CYTO1060>3.0.CO;2-B

USEPA (2016). Drinking water contaminant candidate list 4-final. *Fed. Regist.* 81, 81099–81114.

Wigginton, K. R., Pecson, B. M., Sigstam, T., Bosshard, F., and Kohn, T. (2012). Virus inactivation mechanisms: impact of disinfectants on virus function and structural integrity. *Environ. Sci. Technol.* 46, 12069–12078. doi: 10.1021/es3029473

Zhong, Q., Carratalà, A., Nazarov, S., Guerrero-Ferreira, R. C., Piccinini, L., Bachmann, V., et al. (2016). Genetic, structural, and phenotypic properties of MS2 coliphage with resistance to ClO2 disinfection. *Environ. Sci. Technol.* 50, 13520–13528. doi: 10.1021/acs.est.6b04170

Zhong, Q., Carratalà, A., Shim, H., Bachmann, V., Jensen, J. D., and Kohn, T. (2017). Resistance of echovirus 11 to ClO2 is associated with enhanced host receptor use, altered entry routes and high fitness. *Environ. Sci. Technol.* 51, 10746–10755. doi: 10.1021/acs.est.7b03288

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 November 2017
doi: 10.3389/fmicb.2017.02229

Evaluating Monitoring Strategies to Detect Precipitation-Induced Microbial Contamination Events in Karstic Springs Used for Drinking Water

Michael D. Besmer[1,2], *Frederik Hammes*[1]*, *Jürg A. Sigrist*[1] and *Christoph Ort*[3]

[1] Department of Environmental Microbiology, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland, [2] Department of Environmental Systems Science, Institute of Biogeochemistry and Pollutant Dynamics, ETH Zürich, Zurich, Switzerland, [3] Department of Urban Water Management, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland

OPEN ACCESS

Edited by:
Muhammad Raihan Jumat, King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Pascal E. Saikaly, King Abdullah University of Science and Technology, Saudi Arabia
Anna Leena Maria Neubeck, Stockholm University, Sweden

***Correspondence:**
Frederik Hammes
frederik.hammes@eawag.ch

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *31 July 2017*
Accepted: *30 October 2017*
Published: *22 November 2017*

Citation:
Besmer MD, Hammes F, Sigrist JA and Ort C (2017) Evaluating Monitoring Strategies to Detect Precipitation-Induced Microbial Contamination Events in Karstic Springs Used for Drinking Water. Front. Microbiol. 8:2229. doi: 10.3389/fmicb.2017.02229

Monitoring of microbial drinking water quality is a key component for ensuring safety and understanding risk, but conventional monitoring strategies are typically based on low sampling frequencies (e.g., quarterly or monthly). This is of concern because many drinking water sources, such as karstic springs are often subject to changes in bacterial concentrations on much shorter time scales (e.g., hours to days), for example after precipitation events. Microbial contamination events are crucial from a risk assessment perspective and should therefore be targeted by monitoring strategies to establish both the frequency of their occurrence and the magnitude of bacterial peak concentrations. In this study we used monitoring data from two specific karstic springs. We assessed the performance of conventional monitoring based on historical records and tested a number of alternative strategies based on a high-resolution data set of bacterial concentrations in spring water collected with online flow cytometry (FCM). We quantified the effect of increasing sampling frequency and found that for the specific case studied, at least bi-weekly sampling would be needed to detect precipitation events with a probability of >90%. We then proposed an optimized monitoring strategy with three targeted samples per event, triggered by precipitation measurements. This approach is more effective and efficient than simply increasing overall sampling frequency. It would enable the water utility to (1) analyze any relevant event and (2) limit median underestimation of peak concentrations to approximately 10%. We conclude with a generalized perspective on sampling optimization and argue that the assessment of short-term dynamics causing microbial peak loads initially requires increased sampling/analysis efforts, but can be optimized subsequently to account for limited resources. This offers water utilities and public health authorities systematic ways to evaluate and optimize their current monitoring strategies.

Keywords: water quality monitoring, sampling, microbial dynamics, drinking water, spring water, early warning systems, risk assessment

INTRODUCTION

Adequate monitoring of drinking water quality is one of the key components ensuring that safe and clean drinking water is produced and provided to customers. Short-term microbial dynamics at the scale of minutes to weeks are to be expected in drinking water systems. This can result from natural fluctuations in raw water sources (e.g., precipitation events, snowmelt) as well as operational changes (e.g., filter backwashing, intermittent flow) during treatment (Stevenson, 1997; Pronk et al., 2006; Madrid and Zayas, 2007; Stadler et al., 2008; Bakker et al., 2013). Short-term dynamics and especially peak concentrations strongly influence water quality—and the infection risk in the case of pathogens—especially in raw water but also in treated water (Gauthier et al., 2001; Kistemann et al., 2002; Vreeburg et al., 2004; Farnleitner et al., 2005; Signor and Ashbolt, 2006; Astrom et al., 2007; Pronk et al., 2007; Stadler et al., 2008). Furthermore, many small water utilities using spring water or groundwater have either no or very limited water treatment in place and are thus directly exposed to changes and associated risks in raw water quality. In spite of this, current monitoring practice is often not designed to detect short-term dynamics (Stadler et al., 2008). In fact, it is not uncommon for small utilities to sample on a quarterly or monthly frequency only. This is due to limited financial and logistic resources but also due to the limited existing knowledge on microbial short-term dynamics *per se*.

The general problem of a low sampling frequency is that it represents a system's dynamics insufficiently and especially does not reflect transient changes in water quality. This was considered previously for seasonal changes and water quality violations in river water (Loftis and Ward, 1980; Casey et al., 1983). More recent studies on chemical water quality monitoring in surface waters included optimization strategies for quarterly and monthly sampling (Do et al., 2013; Naddeo et al., 2013; Liu et al., 2014) and illustrations of the large uncertainties remaining even with weekly sampling (Skeffington et al., 2015). Similarly, many dynamics in drinking water production systems occur at short time scales and can thus be easily missed by conventional sampling regimes (i.e., infrequent, manual grab sampling) (Signor and Ashbolt, 2006; Madrid and Zayas, 2007). For example, systems treating surface water tend to be driven by diurnal cycles and thus dynamics have a time scale of hours to days (Besmer et al., 2014). Technical systems that are influenced by human activity can have dynamics of virtually any time scale and different dynamics are often superimposed on each other (Besmer et al., 2016). Many of the dynamics are diurnal or otherwise periodic (i.e., regular) because technical systems include defined, regular operational procedures (e.g., backwashing of filters) and the typical time scale is minutes to hours (Besmer and Hammes, 2016). Arguably, both periodic and even more so aperiodic deviations/peaks in microbial quality can be viewed as time periods of increased contamination risk and hence should be investigated in more detail to verify or exclude contamination. From a practical point of view, it is particularly relevant to know if/when a contamination event occurs and what its magnitude is.

One obvious solution is online monitoring. For drinking water, Janke et al. (2006) showed the advantage of physicochemical online monitoring over conventional monitoring with sampling frequencies below 24 h in the context of deliberate sabotage. Emerging online monitoring tools were further summarized by Storey et al. (2011) and emerging technologies for measuring microbial variables online and at high frequency have been demonstrated in various settings and include flow cytometry (FCM), enzymatic activity, and optical detection (Besmer et al., 2014; Ryzinska-Paier et al., 2014; Hojris et al., 2016). While promising, it is highly unlikely that widespread routine application of microbial online monitoring will be implemented in the near future, due to financial constraints and legal limitations. Therefore, we argue that smarter and more efficient monitoring strategies, based on available and/or affordable equipment, are needed. To optimize monitoring strategies, the drivers and relevant time scales of the dynamic need to be understood (ISO, 2006). To our knowledge, this has not been done adequately for microbial monitoring in spring water, partially due to the lack of high-resolution data sets to date.

The present study focuses on karstic springs, which are used as drinking water sources throughout Europe (Scheidleder, 1999). The porous nature of the karstic geology enables microbial contamination of the spring water with infiltrating surface water after localized precipitation events (Field and Nash, 1997; Farnleitner et al., 2005). We assessed historical records of conventional monitoring data of karstic spring water and compared them with newly collected high-frequency data sets. The purpose was to systematically assess the temporal variability of spring water microbial quality, and to evaluate suitable monitoring strategies to accurately capture those dynamics. The specific goals of this study were: (1) to assess limitations of the current monitoring practice of regular but infrequent grab sampling for microbial water quality control; (2) to illustrate the effect of sampling frequency on the probabilities of detecting precipitation-induced microbial events in karstic spring water; and (3) to suggest a targeted sampling strategy for microbial water quality changes in karstic spring water after precipitation events. The novelty of this study is the investigation of the effect of different monitoring strategies on the information gained from sampling, based on systematic analysis of temporally highly resolved measurements of bacterial concentrations.

MATERIALS AND METHODS

Study Sites, Samples, and Data Sets

Data was collected from two springs (A and B) in a karstic region in the Northeast of Switzerland. The focus was on raw spring water prior to any treatment. An overview of the experimental work and data sets is given in **Table 1**. Auto-sampler campaigns and subsequent detection with manual FCM and plating for both heterotrophic plate count (HPC) and indicator organisms were carried out for this study specifically in spring A, during two subsequent weeks. Within this period, two dry-weather periods were sampled every hour for 24 h each. In addition, a 48-h sampling campaign was carried out with samples taken every hour on two consecutive days after a precipitation event. An online FCM data set was generated for spring B, of which a subset was published in Besmer

TABLE 1 | Overview table of sampling campaigns and data sets used for the different analyses in this study.

Data sets	Spring A		Spring B	
Auto-sampler campaign Total cell concentration Heterotrophic plate count Indicator organisms	December 2014 3 × 24 h every 1 h	**Figure 1**	June 2015 1 × 48 h every 1 h	Figure S1
Local precipitation	2014/2015 2 years every 30 min	**Figure 1**	2014/2015 2 years every 30 min	**Figure 2**, Figures S1–S3
Spring discharge	Aug 2014–Jul 2015 1 year every 30 min	**Figure 1**	March–July 2015 99 days every 30 min	Figure S1
Online flow cytometry Total cell concentration	–	–	March–July 2015 99 days every 15 min	**Figure 2**, Figures S1–S3
Conventional monitoring Heterotrophic plate count Indicator organisms	2002–2015 14 years quarterly/monthly	**Figure 1**, **Table 2**	2002–2015 14 years quarterly/monthly	Figure S1
Regional precipitation	2002–2015 14 years every 10 min	in text	2002–2015 14 years every 10 min	in text

and Hammes (2016). Here, the full 99-day data set is used and the focus is on systematic analysis. In addition, two long-term data sets (2002–2015) of conventional monitoring based on infrequent (i.e. quarterly/monthly) grab sampling and cultivation-based detection methods were provided by the Food Safety and Veterinary Office Basel-Landschaft (FSVO BL) for springs A and B. Precipitation data in parallel to the intensive microbial measurements (2014–2015) was available from a temporary meteorological station located close to the two investigated springs. Additional, long-term precipitation data (2002–2015) was obtained from the Swiss Federal Office of Meteorology and Climatology (MeteoSwiss) for the permanent meteorological station closest to the study region. Spring discharge measurements were provided by the water utilities.

Sampling

Grab samples were taken according to the standard procedures of the FSVO BL, which is an accredited state agency for inspection in accordance with standard ISO 17020:2012 (ISO, 2012) as well as an accredited testing laboratory in accordance with standard ISO 17025:2015 (ISO, 2005). In short, water samples were collected from disinfected (flame treatment of taps prior to sampling), flowing taps or directly from the spring outflow. A portable and programmable auto-sampler (ISCO 6712, Teledyne ISCO Inc., Lincoln, USA) was used for automated sampling. Samples (800 ml) were drawn every hour into sterilized plastic bottles [rinsed thoroughly with hypochlorite solution (1% active chlorine) and 3 times with nanopure water (deionized, 0.22 μm filtered) water before pasteurization at 60°C for 1 h]. The sampling tube was automatically rinsed and purged three times before each sample to avoid stagnation and cross contamination. All samples were transported and stored at 4°C and processed within 24 h.

Manual Detection Methods

Heterotrophic plate count (HPC) plating was done in accordance with the standard ISO 4833:2003 (ISO, 2003) spread plating method by an accredited laboratory. In short, 1 ml of a water sample was evenly distributed on an agar plate and then incubated for 72 h at 30°C. The number of formed colonies was subsequently counted. For indicator organism plating, the standard 9308-1:2000 (ISO, 2000) and 1406.1 (SLMB, 2007) membrane filtration and plating methods for the enumeration of *Escherichia coli* (*E. coli*) and Enterococcus respectively were used. In short, 100 ml of a water sample were filtered through a 0.45 μm filter, which was then placed on an agar plate and incubated for 24 h at 37°C. The number of formed colonies was subsequently counted.

Manual FCM measurements of total cell concentration (TCC) were done based on the reference method 333.1 (SLMB, 2012). In short, 500 μl of the water samples were pre-warmed for 3 min at 37°C and then stained with the fluorescent stain SYBR Green I (Life Technologies, Eugene OR, USA; final concentration 1:10,000). After 10 min of incubation at 37°C in the dark, 100 μl of a sample were measured on an Accuri C6 flow cytometer (BD Accuri, San Jose CA, USA) at a flow rate of 66 μl min^{-1} with a lower threshold on the green fluorescence (FL1-H) channel of 1,000. Fixed gates were applied in the Accuri C6 CFlow software to separate bacteria from background signals (Prest et al., 2013).

Automated Detection Method: Online Flow Cytometry

For online FCM, water was sampled directly from a bypass with continuous flow by an automated sampling, staining, and incubation module connected to an Accuri C6 flow cytometer (BD Accuri, San Jose CA, USA) as described previously (Besmer et al., 2014). In short, water samples were drawn discretely every

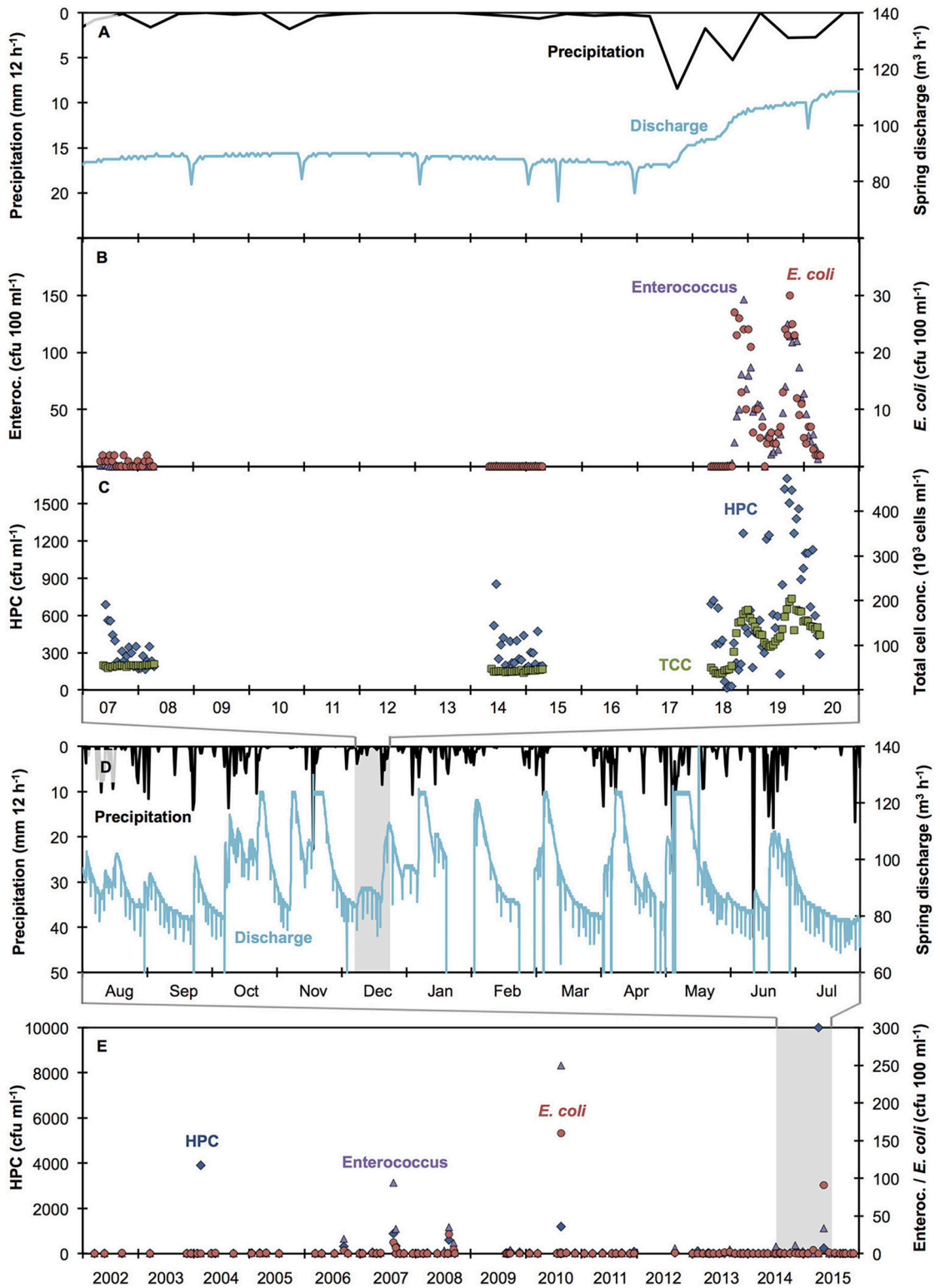


FIGURE 1 | Evaluation of raw spring water quality (Spring A) over different time scales: **(A,D)** precipitation and spring discharge measurements [hourly measurements; 2 weeks **(A)** and one year **(D)** respectdively], **(B,C)** auto-sampler measurements analyzed with conventional plating methods for the indicator organisms Enterococcus (purple triangles), *E. coli* (red circles), and HPC (blue diamonds) as well as flow cytometric total cell concentration (green squares), **(E)** conventional grab sampling (quarterly to monthly; 14 years; $n = 100$) analyzed with conventional plating methods for the indicator organisms Enterococcus (purple triangles), *E. coli* (red circles), and HPC (blue diamonds). Short-term drops in spring discharge are due to water being discarded for operational reasons. Maximum spring discharge was 125 m^3 h^{-1} for operational reasons (excessive water was discarded).

15 min and mixed with a fluorescent stain [SYBR Green I (Life Technologies, Eugene OR, USA); final concentration 1:10,000]. This mixture was incubated for 10 min at 37°C before transfer to the flow cytometer for measurement at a flow rate of 66 µl min^{-1} for 90 s with a lower threshold on the green fluorescence (FL1-H) channel of 1,000. After each sampling and measurement cycle, the staining module was rinsed with nanopure water (deionized, 0.22 µm filtered). In addition, an extended cleaning cycle with hypochlorite and detergent was performed after every 100 samples. For data analysis, files were exported for batch processing with custom software. Fixed gates were applied to separate bacteria from background signals (Prest et al., 2013).

Systematic Analysis of Monitoring Strategies

Event Definition

A preliminary analysis of high resolution TCC and precipitation data in spring B indicated substantial TCC increases after precipitation events with total volumes exceeding 10 mm within 24 h. Due to the time scale of the system response (i.e., TCC increase/decrease after precipitation), we added a second criterion that no new precipitation event should start within 48 h.

Evaluation of Different Monitoring Strategies

We tested three different monitoring strategies to assess their efficacy in *TCC event detection* and *TCC peak concentration estimation*: (1) sampling at pre-defined, constant time intervals, (2) random grab samples taken during working hours only (Skeffington et al., 2015), and (3) targeted sampling (triggered by precipitation events). The analysis was performed by subsampling the high-resolution TCC data set, which was assumed to represent the "true" temporal evolution of bacterial concentration. Based on the definition of relevant precipitation events above, the TCC data set was divided into separate TCC events to be evaluated. Then, strategies 1 and 2 were tested for five different sampling frequencies over the entire 99-day monitoring period to detect these defined TCC events (with different total numbers of samples): quarterly (1 sample), monthly (3 samples), weekly (14 samples), bi-weekly (28 samples) and daily [99 (all days, strategy 1) and 70 (all working days, strategy 2)]. For strategy 1, the maximum number of possible realizations (resulting from sampling interval and TCC event duration) was evaluated. For strategy 2, 10,000 random realizations were evaluated. For strategy 3, three (sub-)samples were taken, 24, 48, and 72 h after the criterion for the precipitation volume was met.

Statistical Analysis

Two criteria were assessed for the evaluation of the different monitoring strategies for each of the 11 events defined above: (1) The efficacy in *detecting a TCC event*. This was quantified for each event by the probability of taking a sample during an event. (2) The *accuracy in estimating the TCC peak concentration*. This was quantified for each event by the ratio (R) of the sampled maximum divided by the true maximum. Subsequently, for the comparison of monitoring strategies the 25%, 50% (median), and 75% quartiles were used. The three quartiles of all realizations were calculated for each individual event for each sampling frequency and both sampling strategies 1 and 2. In the case of the targeted monitoring strategy (3), the second step was performed with the highest measurement (i.e., closest to the true maximum) of the three samples per individual event. The second step was additionally performed excluding events 7 and 8, which showed no substantial/relevant TCC increase despite fulfilling the sampling trigger criterion (10 mm within 24 h).

Software

All data analysis was carried out in R (R Development Core Team, 2008) using standard packages (the full code is available in the Supplementary Information).

RESULTS AND DISCUSSION

The overall goal of this study was to systematically assess the temporal variability of karstic spring water microbial quality and suitable monitoring strategies to accurately capture the prevalent dynamics. To this end, we investigated two karstic springs from the same geographical area based on the availability of a large historical data set (Spring A) and the opportunity to install new online monitoring equipment (Spring B). The investigations in Spring A (section *Precipitation-Induced Dynamics and Current Grab Sampling Practice*) cover the effect of precipitation events and the implications resulting from infrequent grab sampling practices by (1) illustrating the link between precipitation events, increased spring discharge, and microbial contamination, (2) establishing the suitability of flow cytometric TCC as a useful parameter to follow bacterial dynamics in these springs, and (3) estimating how many precipitation-induced contamination events are missed by conventional monitoring. A detailed analysis of online FCM data from Spring B (section *Increased Sampling Frequency Improves Contamination Event Detection*) illustrates how increasing the sampling frequency increases the probability of detecting microbial contamination events. From this data, we argue for an optimized, targeted monitoring strategy with event-based triggering and appropriate sampling intervals (section *Optimizing Contamination Event Detection Through Targeted Sampling*).

Precipitation-Induced Dynamics and Current Grab Sampling Practice (Spring A)

Time-resolved data from Spring A shows that localized precipitation in excess of 10 mm in 24 h causes increased discharge and microbial contamination of karstic spring water (**Figures 1A–C**), in agreement with previous studies (Stadler et al., 2008; Goldscheider et al., 2010; Butscher et al., 2011; Page et al., 2017) and analogous measurement campaigns in other springs in this region and at other times of the year (Figure S1; other data not shown). As such, multiple precipitation events will result in multiple contamination events, characterized by both the frequency and magnitude of increases in relevant microbial variables. During dry-weather periods (**Figure 1A**), low concentrations of indicator organisms (0–2 cfu 100 ml^{-1}) were detected (**Figure 1B**), suggesting a minor input from precipitation-independent sources. In contrast, the 48-h sampling after a localized precipitation event revealed two

distinct peaks in both Enterococcus and *E. coli* concentrations (up to 150 and 30 cfu 100 ml^{-1} respectively, **Figure 1B**). Time series of both indicator organisms followed a clear trend, with rapid increases and slightly slower decreases after peaking (**Figure 1B**). HPC exceeded 1,700 cfu ml^{-1} after precipitation events and were lower (314.5 ± 149.7 cfu ml^{-1}) during the dry weather periods (**Figure 1C**). Compared to results for indicator organisms (above), HPC results were more variable between consecutive time points, making the contamination event difficult to track. TCC was low (48,600 ± 6,400 cells ml^{-1}) during dry weather periods and reached more than 200,000 cells ml^{-1} after precipitation events (**Figure 1C**). Of the four microbial measurements, TCC evolved most consistently (i.e., lowest variation between consecutive time points). From this we draw a first conclusion that TCC data is particularly suitable to describe both dry weather conditions as well as precipitation-induced dynamics in bacterial concentrations in karstic springs. Importantly, the temporal evolution of indicator organisms and TCC was comparable, although no direct proportionality was found (data not shown).

When expanding the observation period to a detailed set of precipitation and discharge data during 12 consecutive months (2014–2015), it is evident that a total of 31 major precipitation events occurred, which exceeded 10 mm in 24 h (**Figure 1D**). All of these precipitation events caused noticeable increases in spring discharge (**Figure 1D**). Hence, for this spring and this time period, precipitation events were frequent and thus precipitation-induced contamination events can be expected to be equally frequent. From these combined observations, we infer that historical precipitation data can reasonably be used to estimate the number of contamination events in the spring water.

Based on this argument, we subsequently evaluated regional precipitation data during 14 years (2002–2015) and found that 380 major precipitation events (>10 mm within 24 h) occurred (data not shown). In the same historical period, a total of 100 water samples was analyzed by the responsible authority in the course of routine monitoring campaigns of this spring (quarterly samples from 2002 to 2012 and monthly samples from 2013 to 2015) (**Table 2**, **Figure 1E**). Of these conventional grab samples, <30% tested positive for indicator organisms, **Table 2**). Based on the historical data, Spring A appears to have experienced rather few contamination events and most of these were of moderate magnitude (**Figure 1E**). Furthermore, because the number of grab samples with elevated bacterial concentrations was low, it is conceivable that they may be (falsely) considered to be outliers due to contamination during sampling or analysis. In stark contrast, the results from the auto-sampler campaign (**Figures 1A–C**) strongly suggest that (1) the spring actually experienced substantial bacterial peak loads after precipitation events and (2) the high concentrations of Enterococcus and *E. coli* occasionally detected with grab sampling (**Figure 1E**) were probably real detections of precipitation-induced contamination events.

On the above-discussed premise that the 380 major precipitation events between 2002 and 2015 most likely caused substantial increases in spring discharge and bacterial concentrations, the quarterly sampling strategy (2002–2012) only detected at most 6% (18 measured samples >0 cfu 100 ml^{-1} vs. 292 major precipitation events). When taking into account the observation of occasionally low detection of indicator organisms during dry-weather periods (**Figure 1B**) and the median values for the samples above 0 cfu 100 ml^{-1} being similarly low (**Table 2**, **Figure 1E**), the actual detection of precipitation-induced contamination events was probably even lower. Analogously, the monthly sampling strategy (2013–2015) detected at most 10% of contamination events (9 measured samples >0 cfu 100 ml^{-1} while 88 major precipitation events were recorded).

In summary, the data shows that the conventional monitoring strategy based on infrequent grab sampling was ineffective in detecting the frequency of precipitation-induced contamination events in karstic springs and failed to quantify the magnitude of these events. Importantly, these findings were not limited to this specific spring (Spring A) and were confirmed in a similar assessment of Spring B, with a known record of generally high microbial loads (Figure S1).

Increased Sampling Frequency Improves Contamination Event Detection (Spring B)

An obvious strategy to improve the probability of detecting and correctly quantifying contamination events in any system is to sample more frequently. In this respect, continuous online microbial monitoring presents an interesting future solution (Besmer et al., 2014; Besmer and Hammes, 2016; Page et al., 2017). Online FCM data from Spring B (7,878 measurements at 15 min interval in 3 months) shows the frequency and magnitude of TCC increases during precipitation-induced contamination events (**Figure 2A**). Based on the precipitation event definition above (>10 mm in 24 h), a total of 11 precipitation events, each followed by an increase in TCC, were identified (**Figure 2A**). We subsequently performed a theoretical sub-sampling of this online TCC data set to evaluate different monitoring strategies. The probability to detect elevated TCC as a result of precipitation events was assessed for (1) constant sampling intervals and (2) random sampling during working hours, at frequencies of quarterly (1 sample), monthly (3 samples), bi-weekly (28 samples), weekly (14 samples), and daily (99 samples) sampling.

The monitoring strategy with constant sampling intervals performed slightly but consistently better compared to the same number of samples taken randomly during working hours, but the differences were small (**Table 2**, **Figures 2B,C**). For the widespread conventional monitoring strategy of quarterly or monthly sampling, the average probability to detect an individual event of elevated TCC was 9.6 and 28.9% respectively at constant sampling intervals. This probability increased to 85.5% for bi-weekly and to 98.6% for weekly sampling and reached 100% for daily sampling (**Table 3**, **Figure 2**, Figure S2). If samples were taken randomly but during working hours only, the probabilities to detect the TCC events were consistently lower for the same number of samples but reached 100% for daily sampling as well (**Table 3**, Figure S3). While daily sampling is effective in detecting the events, it is not a logistically, practically or financially realistic strategy for

TABLE 2 | Monitoring data for indicator organisms during 14 years as part of conventional monitoring of drinking water microbial quality by responsible authorities based on infrequent quarterly (Q) (2002–2012) and monthly (M) (2013–2015) grab sampling in Spring A, displayed in **Figure 1**.

Detected organisms	Number of samples analyzed		Samples >0 cfu 100 ml^{-1}									
			Number of positives		Concentration							
					Median		Average		Std. dev.		Maximum	
	Q	M	Q	M	Q	M	Q	M	Q	M	Q	M
Enterococcus	63	37	18 (29%)[a]	9 (24%)[a]	3.0	3.0	26.3	7.8	60.3	10.5	250.0	34.0
E. coli	63	37	14 (22%)[a]	8 (22%)[a]	1.5	2.0	16.3	13.3	42.0	31.4	160.0	91.0
Both	63	37	9 (14%)[a]	4 (11%)[a]	–	–	–	–	–	–	–	–

[a]*Percentage of samples >0 cfu 100 ml^{-1} in all samples analyzed.*

routine applications. At least bi-weekly sampling was needed to reach detection probabilities >90% for this specific spring (**Table 2**, **Figures 2B,C**), which is still a very resource-intensive approach. Nevertheless, we used this sampling frequency as the example for further comparison with optimization strategies.

For risk evaluation, it is important to not only detect periods of elevated bacterial concentrations, but also to quantify the peak concentration of a given event to judge the magnitude of pollution (Kistemann et al., 2002; Signor and Ashbolt, 2006). Therefore, we used the accuracy of estimating TCC peaks after precipitation as a second performance criterion for evaluating different monitoring strategies. In the following assessment, we evaluated the ratio R, i.e., the sampled maximum divided by the true maximum (**Figure 2**, **Table 3**, Figures S2, S3). As can be seen from **Figure 3** and **Table 3**, the median peak estimation improved with increasing numbers of samples. For a bi-weekly sampling strategy, we found the median underestimation of the true peak concentration (i.e., 1–R) to be 36% for constant sampling intervals and 39% for random sampling during working hours (**Table 3**, **Figure 3**) with some variation between individual TCC events (**Figures 2B,C**, Figures S2, S3). Increasing the sampling frequency also increased the values for the 25 and 75% quartiles but strong underestimation was still observed in some realizations (**Figure 3**). This is due to the fact that the peaks in TCC were often sharp (in the range of hours) and thus even with a daily sampling strategy, the chances of not sampling close to the peak remained substantial. In tendency, sampling at constant intervals had a narrower range between the 25 and 75% quartiles compared to random sampling during working hours (**Figure 3**).

From the above analysis of different monitoring strategies applied to this particular spring, the following observations can be summarized:

(1) Increasing the sampling frequency strongly increased the probability of detecting precipitation-induced TCC events, decreased the median underestimation of peak concentrations, and narrowed the range of this underestimation (**Table 3**, **Figure 3**, Figures S2, S3, Table S1).
(2) A bi-weekly sampling strategy resulted in average detection probabilities >90% for TCC events and median underestimation of peak concentrations below 39%.
(3) With a few specific exceptions, there was no substantial difference in performance between the strategies of constant sampling intervals (irrespective of working hours) and random grab samples during working hours for the same number of samples. This concurs with similar findings on chemical measurements in surface waters (Skeffington et al., 2015).

Although frequent sampling can achieve high detection probabilities and reliable peak estimations, large labor and cost requirements for these monitoring strategies renders them unrealistic for most practical applications. Hence, given limited resources and thus a limited number of samples that can be processed, sampling strategies must be optimized to focus on "meaningful" time periods. Furthermore, the goal of utilities and practitioners is not necessarily to detect every single contamination event, but to have the ability to detect any given event at any given time. In the following section, such a targeted monitoring strategy for precipitation-induced contamination events in karstic springs is considered.

Optimizing Contamination Event Detection Through Targeted Sampling

The basic idea of targeted sampling is to trigger sample collection with data from an affordable and continuously available measurement of a relevant variable. For the specific example of karstic springs, precipitation or spring discharge measurements can be used as an early-warning signal (**Figures 1A,D**, **2A**), indicating that a critical observation period is about to begin and thus (increased) sampling and analysis would be valuable (Madrid and Zayas, 2007; Stadler et al., 2008; Goldscheider et al., 2010). In the following analysis, we used precipitation events >10 mm in 24 h as the early warning criterion for triggering sampling. Subsequently a virtual sub-sampling of the online FCM data set (**Figure 2A**) was performed, with three samples collected at 24 h, 48 h, and 72 h after the event criterion was met.

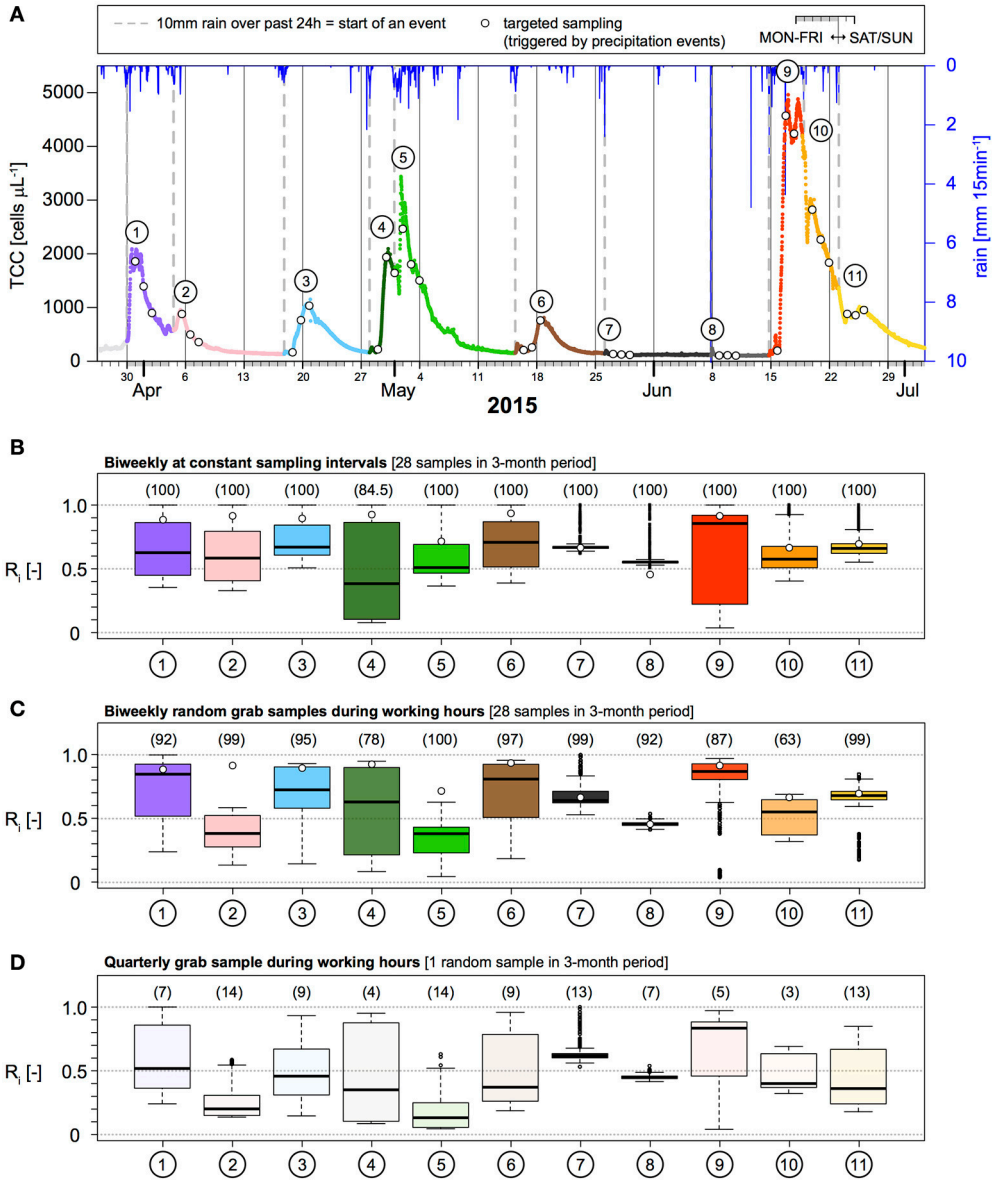


FIGURE 2 | Overview of the 3-month observation period for total cell concentration (TCC) and precipitation **(A)**. A minimum of 10 mm of rainfall recorded over 24 h marks the start of a TCC event (dashed gray lines). Each TCC event is numbered and color-coded throughout the figure. Circles indicate the three samples from the targeted sampling (i.e., 24, 48, and 72 h after the start of a TCC event). **(B–D)** Show the distribution of the accuracy of peak concentrations of TCC for different monitoring strategies based on multiple realizations. Numbers in brackets and opacity of boxes indicate the probability of TCC event detection. White circles indicate the best result of the targeted sampling for direct comparison. Boxes represent 25%, 50% (i.e., median, black lines), and 75% quartiles. Whiskers represent 1.5-fold interquartile ranges or minima/maxima when outside this range.

With this approach, the probability to detect an individual contamination event was particularly high. In reality, short (<24 h) TCC events, would be missed with this approach because they would be over before the first sample was collected (for example events 7 and 8 in **Figure 2**). It can be seen from **Table 3** (with and without inclusion of events 7 and 8) that the targeted sampling strategy (n = 33) exceeded the probability of detecting the TCC events achieved with the two bi-weekly sampling strategies (n = 28) samples in the same observation period.

The median underestimation of the true peak concentration of a TCC event (i.e., 1–R) was 11% based on the highest TCC sample (**Table 3**; range for individual TCC events: 6–54%, Table S1). Thus, the targeted sampling performed 25%-points better than the bi-weekly constant interval sampling (**Figure 3**, **Table 3**). For individual TCC events, the peak estimations of the targeted sampling were 3–34%-points closer to the

TABLE 3 | Overview of the different monitoring strategies and the resulting (1) probability to detect precipitation-induced TCC events and (2) accuracy of peak concentration estimations of bacteria in karstic spring water during a 3-month observation period (**Figure 2**, Spring B).

Monitoring strategy		Probability of TCC event detection			Estimation of TCC peak concentration (R = sampled maximum divided by true maximum)			
		n	Average	Range (%)	Median (%)[a]	25% Quartile (%)[a]	75% Quartile (%)[a]	Median range (%)[b]
Constant interval	Quarterly	1	10	3–16	43 (31)	19 (17)	61 (59)	14–84
	Monthly	3	29	10–48	44 (31)	19 (17)	62 (59)	14–84
	Weekly	14	86	42–100	54 (47)	34 (29)	67 (68)	33–84
	Bi-weekly	28	99	85–100	64 (64)	52 (49)	80 (84)	38–86
	Daily	99	100	–	87 (89)	72 (82)	93 (93)	56–94
Randomly (working hours)	Quarterly	1	9	2–15	41 (32)	20 (17)	62 (60)	16–83
	Monthly	3	24	9–37	43 (36)	21 (18)	63 (64)	16–83
	Weekly	14	71	35–91	51 (50)	32 (26)	68 (74)	23–85
	Bi-weekly	28	91	63–100	61 (62)	41 (38)	84 (86)	38–87
	Daily	70	100	–	81 (87)	55 (58)	92 (92)	46–93
Targeted		33	100	–	89 (90)	69 (72)	92 (92)	46–94

*For the constant sampling interval and random sampling, multiple possible realizations were statistically summarized whereas for the targeted sampling only one realization exists in this study. See **Figure 3**, Figures S2, S3 for graphical representations of the results and Table S1 for results of individual TCC events.*

[a] *For the combination of all realizations for all 11 TCC events in **Figure 3** (in brackets without events 7 and 8).*

[b] *For the 11 individual TCC events; see Table S1 for all values.*

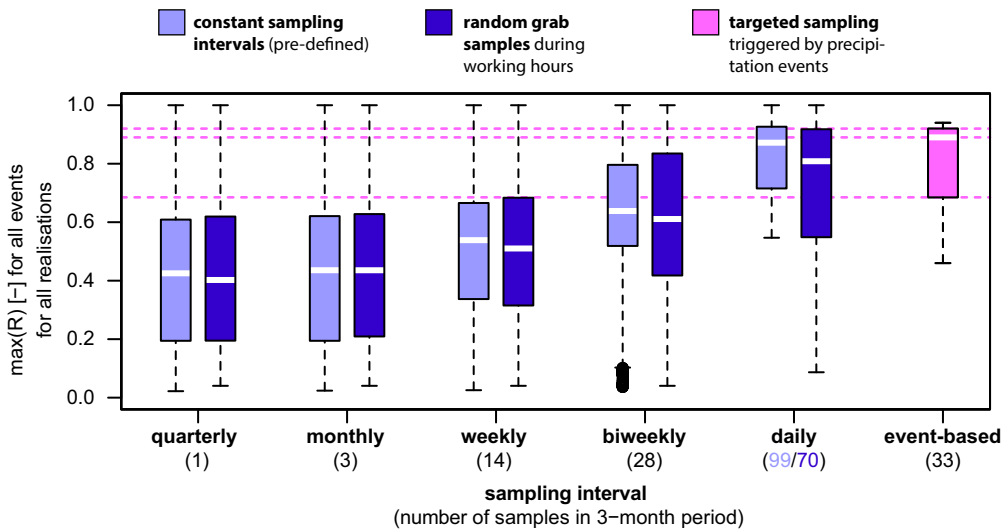


FIGURE 3 | Comparison of estimation of peak concentration (R = sampled maximum divided by true maximum) for different monitoring strategies and number of samples calculated for all realizations for all 11 TCC events (see **Table 3** and Table S1 for detailed values). For the targeted sampling, the values were calculated for one realization only for all 11 TCC events and the best R values (i.e., closest to the true value) out of three samples taken per TCC event were used. White lines represent the median, boxes represent 25/75% quartiles, whiskers represent 1.5 times interquartile ranges (or minima/maxima). Horizontal dotted lines are 25%, 50% (median), and 75% quartiles of the targeted sampling for comparison with other strategies and numbers of samples.

true values except for the minor events 7 and 8 (where the targeted sampling performed equally well and 9%-points worse respectively) (Table S1). In addition, the targeted sampling had much higher values and a narrower range for the 25 and 75% quartiles, which the other monitoring strategies would only reach with daily sampling (**Figure 3**, **Table 3**). In summary, the targeted sampling achieved a moderately higher detection probability of TCC events and a considerably better estimation of peak concentrations with a similar number of samples.

In order to capture every single TCC event in our data set, the targeted sampling strategy required 33 samples to be taken and analyzed (compared to 28 samples for a bi-weekly strategy). However, the strength of the targeted sampling lies in that it provides the utility with the choice to sample any given contamination event with high accuracy, rather than necessarily

trying to detect every TCC event. Also, it is evident that if a system experiences fewer contamination events with longer periods in between events than seen in the above example, the targeted sampling will become considerably more efficient than the other two monitoring strategies.

Considerations on Generalization and System Specific Characteristics

The presented approach is considered to be generally valid for springs in geological settings and climatic regions that are frequently influenced by precipitation-induced contamination events (Stadler et al., 2008; Butscher et al., 2011; Delbart et al., 2014; Meus et al., 2014; Sinreich et al., 2014). However, the concepts discussed above are not limited to karstic springs, and can be developed for different systems (e.g., riverbank filtration, surface waters, treatment plants). In this regard, targeted sampling strategies always need to be adapted to the specific characteristics of the investigated system, and the following aspects should be considered:

(1) The best *variable* to serve as the trigger for targeted sampling should be identified based on an assessment of existing data sets (e.g., precipitation data, operational data, online measurements of abiotic variables) and ideally also initial high-frequency microbial measurements (e.g., online flow cytometry or auto-sampler campaigns), if available.
(2) The *threshold* of the trigger variable that leads to the start of sampling is crucial for the detection probability of events. Too low thresholds lead to unnecessary high numbers of samplings of baseline conditions whereas too high thresholds bare the risk of missing events. Initial high-frequency microbial measurements will support the identification of such thresholds.
(3) The *lag time* between exceeding the trigger variable threshold and first targeted sampling should be selected such that the latter ideally always occurs well before the peak of the contamination event. Again, the system-inherent lag times should ideally be extracted from initial high-frequency microbial measurements (see also Delbart et al., 2014).
(4) The *sampling interval* and the *number of samples* per event should be chosen such that the typical time scale of events in the investigated system are adequately covered. This means that the contamination peak always falls into the sampled period and thus depends on lag time, sampling interval and number of samples.

Implications and Practical Recommendations

The suitability of TCC as a microbial process variable for improved understanding of water resources shown previously (Vital et al., 2012; Gillespie et al., 2014; Helmi et al., 2014) was extended to the investigation of short-term dynamics in the present study (**Figure 1C**). This highlights the value of measuring TCC (or similar cultivation-independent variables) automatically at high temporal resolution for microbial monitoring (Brognaux et al., 2013; Besmer et al., 2014; Besmer and Hammes, 2016). While TCC is not a direct hygienic indicator, it is one of the most direct microbial variables that can be measured online and is seen as a useful process variable to detect microbial dynamics. Using online microbial measurements to drive a targeted sampling approach allows the use of more advanced methods, e.g., for specific fecal indicator organisms or direct pathogen and/or community detection, at meaningful points in time and comparison to long-term records (**Figures 1B,E**; Stadler et al., 2008; Goldscheider et al., 2010; Butscher et al., 2011).

While permanent online monitoring offers considerable advantages (Janke et al., 2006) it will probably not be practically and financially feasible for microbial water quality monitoring in the near future – especially for smaller utilities. However, the two examples in our study clearly show that after initial high-frequency measurements during a limited period, future targeted monitoring can be based on a moderate number of samples, which can be handled with an auto-sampler or even manual grab sampling and conventional detection methods (e.g., indicator organisms). Our findings clearly support the growing awareness that conventional water quality monitoring approaches need to be improved to better support risk assessment and system optimization (Petterson and Ashbolt, 2016) and further confirm the high value of automated, targeted sampling to this end (Stadler et al., 2008).

Consequently, we propose the following practical recommendations for improved monitoring of microbial short-term dynamics in raw and treated drinking water systems:

(1) Compile all available data and knowledge on possible dynamics in water quality (e.g., precipitation data; online measurements of discharge, conductivity; conventional monitoring records).
(2) Prioritize systems or locations within a system (e.g., raw water sources, treatment plants) with assumed or known high variability in water quality based on the above data.
(3) Perform monitoring at the highest possible temporal resolution for at least 10 events with available online tools for direct (e.g., TCC) or surrogate (e.g., turbidity, particle counter) detection of bacterial concentrations. In natural systems, such as karstic springs, the possible influence of seasonal differences should be taken into account when performing high-frequency monitoring campaigns.
(4) From the high-frequency data set, establish the causes and the typical time scales of microbial dynamics.
(5) Specifically, identify the most suitable early-warning variable (e.g., precipitation event, increase in spring discharge, increase in turbidity) as a trigger for targeted sampling.
(6) Based on this compiled knowledge on the dynamics, test different alternative monitoring strategies on the high-frequency data set as was demonstrated in this study.
(7) Implement the best alternative strategy that delivers sufficient information for the questions to be answered and is feasible with the resources available (see also: Ward et al., 1986; Harmel et al., 2006).

CONCLUSIONS

- Bacterial concentrations in karstic spring water are usually low during dry weather periods but increase substantially after localized precipitation events.
- Conventional monitoring strategies, which are based on infrequent grab sampling, substantially underestimate both the number of contamination events and peak concentrations of bacteria during such contamination events.
- TCC is a useful measurement to track precipitation-induced contamination events in spring water.
- Emerging automated TCC measurement devices allow for the collection of high-frequency data sets over extended periods that can be used for a systematic evaluation of short-term dynamics and monitoring strategies.
- Optimization of monitoring strategies should be site specific and based on (1) systematic analysis of existing data sets and (2) pilot studies with the highest possible temporal and spatial resolution and information depth to enable an informed optimization of a targeted monitoring strategy.
- While higher sampling frequencies generally improve both the probability of event detection and the estimation of microbial peak concentrations, targeted sampling is most efficient and effective and can be applied flexibly for individual contamination events.

AUTHOR CONTRIBUTIONS

Experimental design: MB, JS, and FH. Research: MB, JS, and FH. Data analysis: MB, FH, and CO. Writing/editing: MB, JS, FH, and CO.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from the Canton Basel-Landschaft, Switzerland in the framework of the project "Regionale Wasserversorgung Basel-Landschaft 21" as well as internal Eawag Discretionary Funding. We thank the Food Safety and Veterinary Office Basel-Landschaft and Timon Langenegger for laboratory and field support, and the local treatment plant operator and utility for their forthcoming collaboration. The meteorological station was operated in cooperation with the group for Meteorology, Climatology, and Remote Sensing (MCR) at the University of Basel.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02229/full#supplementary-material

REFERENCES

Astrom, J., Petterson, S., Bergstedt, O., Pettersson, T. J. R., and Stenstrom, T. A. (2007). Evaluation of the microbial risk reduction due to selective closure of the raw water intake before drinking water treatment. *J. Water Health* 5, 81–97. doi: 10.2166/wh.2007.139

Bakker, M., Vreeburg, J. H. G., Palmen, L. J., Sperber, V., Bakker, G., and Rietveld, L. C. (2013). Better water quality and higher energy efficiency by using model predictive flow control at water supply systems. *J. Water Supply Res. Technol. Aqua* 62, 1–13. doi: 10.2166/aqua.2013.063

Besmer, M. D., and Hammes, F. (2016). Short-term microbial dynamics in a drinking water plant treating groundwater with occasional high microbial loads. *Water Res.* 107, 11–18. doi: 10.1016/j.watres.2016.10.041

Besmer, M. D., Epting, J., Page, R. M., Sigrist, J. A., Huggenberger, P., and Hammes, F. (2016). Online flow cytometry reveals microbial dynamics influenced by concurrent natural and operational events in groundwater used for drinking water treatment. *Sci. Rep.* 6:38462. doi: 10.1038/srep38462

Besmer, M. D., Weissbrodt, D., G., Kratochvil, B., E., Sigrist, J., A., Weyland, M., S., and Hammes, F. (2014). The feasibility of automated online flow cytometry for *in-situ* monitoring of microbial dynamics in aquatic ecosystems. *Front. Microbiol.* 5:265. doi: 10.3389/fmicb.2014.00265

Brognaux, A., Han, S. S., Sorensen, S. J., Lebeau, F., Thonart, P., and Delvigne, F. (2013). A low-cost, multiplexable, automated flow cytometry procedure for the characterization of microbial stress dynamics in bioreactors. *Microb. Cell Fact.* 12:100. doi: 10.1186/1475-2859-12-100

Butscher, C., Auckenthaler, A., Scheidler, S., and Huggenberger, P. (2011). Validation of a numerical indicator of microbial contamination for karst springs. *Ground Water* 49, 66–76. doi: 10.1111/j.1745-6584.2010.00687.x

Casey, D., Nemetz, P. N., and Uyeno, D. H. (1983). Sampling frequency for water-quality monitoring-measures of effectiveness. *Water Resour. Res.* 19, 1107–1110. doi: 10.1029/WR019i005p01107

Delbart, C., Valdes, D., Barbecot, F., Tognelli, A., Richon, P., and Couchoux, L. (2014). Temporal variability of karst aquifer response time established by the sliding-windows cross-correlation method. *J. Hydrol.* 511, 580–588. doi: 10.1016/j.jhydrol.2014.02.008

Do, H. T., Lo, S. L., and Lan, A. P. T. (2013). Calculating of river water quality sampling frequency by the analytic hierarchy process (AHP). *Environ. Monit. Assess.* 185, 909–916. doi: 10.1007/s10661-012-2600-6

Farnleitner, A. H., Wilhartitz, I., Ryzinska, G., Kirschner, A. K. T., Stadler, H., Mach, R. L. et al. (2005). Bacterial dynamics in spring water of alpine karst aquifers indicates the presence of stable autochthonous microbial endokarst communities. *Environ. Microbiol.* 7, 1248–1259. doi: 10.1111/j.1462-2920.2005.00810.x

Field, M. S., and Nash, S. G. (1997). Risk assessment methodology for karst aquifers: (1) Estimating karst conduit-flow parameters. *Environ. Monit. Assess.* 47, 1–21.

Gauthier, V., Barbeau, B., Millette, R., Block, J.-C., and Prévost, M. (2001). Suspended particles in the drinking water of two distribution systems. *Water Sci. Technol.* 1, 237–245. Available online at: http://ws.iwaponline.com/content/1/4/237

Gillespie, S., Lipphaus, P., Green, J., Parsons, S., Weir, P., Juskowiak, K., et al. (2014). Assessing microbiological water quality in drinking water distribution systems with disinfectant residual using flow cytometry. *Water Res.* 65, 224–234. doi: 10.1016/j.watres.2014.07.029

Goldscheider, N., Pronk, M., and Zopfi, J. (2010). New insights into the transport of sediments and microorganisms in karst groundwater by continuous monitoring of particle-size distribution. *Geologia Croatica* 63, 137–142. doi: 10.4154/gc.2010.10

Harmel, R. D., King, K. W., Haggard, B. E., Wren, D. G., and Sheridan, J., M. (2006). Practical guidance for discharge and water quality data collection on small watersheds. *Trans. Asabe* 49, 937–948. doi: 10.13031/2013.21745

Helmi, K., Watt, A., Jacob, P. I., Ben-Hadj-Salah, Henry, A., Meheut, G., Charni-Ben-Tabassi, N., et al. (2014). Monitoring of three drinking water treatment plants using flow cytometry. *Water Sci. Technol.* 14, 850–856. doi: 10.2166/ws.2014.044

Hojris, B., Christensen, S. C. B., Albrechtsen, H. J., Smith, C., and Dahlqvist, M. (2016). A novel, optical, on-line bacteria sensor for monitoring drinking water quality. *Sci. Rep.* 6: 23935. doi: 10.1038/srep23935

ISO (2000). *Detection and Enumeration of Escherichia Coli and Coliform Bacteria-Part 1: Membrane Filtration Method*. Geneva: ISO. 9308-1:2000.

ISO (2003). *Horizontal Method for the Enumeration of Microorganisms-Colony-Count Technique at 30 Degrees*. Geneva: ISO. 4833:2003.

ISO (2005). *General Requirements for the Competence of Testing and Calibration Laboratories*. Geneva: ISO. 17025:2005.

ISO (2006). *Sampling-part 1: Guidance on the Design of Sampling Programmes and Sampling Techniques*. Geneva: ISO. 5667-1:2006.

ISO (2012). *Conformity Assessment-Requirements for the Operation of Various Types of Bodies Performing Inspection*. Geneva: ISO. 17020:2012.

Janke, R., Murray, R., Uber, J., and Taxon, T. (2006). Comparison of physical sampling and real-time monitoring strategies for designing a contamination warning system in a drinking water distribution system. *J. Water Resour. Plan. Manage.* 132, 310–313. doi: 10.1061/(ASCE)0733-9496(2006)132:4(310)

Kistemann, T., Classen, T., Koch, C., Dangendorf, F., Fischeder, R., Gebel, J., et al. (2002). Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. *Appl. Environ. Microbiol.* 68, 2188–2197. doi: 10.1128/AEM.68.5.2188-2197.2002

Liu, Y., Zheng, B. H., Wang, M., Xu, Y. X., and Qin, Y. W. (2014). Optimization of sampling frequency for routine river water quality monitoring. *Sci China* 57, 772–778. doi: 10.1007/s11426-013-4968-8

Loftis, J. C., and Ward, R. C. (1980). Water-quality monitoring-some practical sampling frequency considerations. *Environ. Manage.* 4, 521–526. doi: 10.1007/BF01876889

Madrid, Y., and Zayas, Z. P. (2007). Water sampling: traditional methods and new approaches in water sampling strategy. *Trac Trends Analyt. Chem.* 26, 293–299. doi: 10.1016/j.trac.2007.01.002

Meus, P., Moureaux, P., Gailliez, S., Flament, J., Delloye, F., and Nix, P. (2014). *In situ* monitoring of karst springs in Wallonia (southern Belgium). *Environ. Earth Sci.* 71, 533–541. doi: 10.1007/s12665-013-2760-x

Naddeo, V., Scannapieco, D., Zarra, T., and Belgiorno, V. (2013). River water quality assessment: implementation of non-parametric tests for sampling frequency optimization. *Land Use Policy* 30, 197–205. doi: 10.1016/j.landusepol.2012.03.013

Page, R. M., Besmer, M. D., Epting, J., Sigrist, J. A., Hammes, F., and Huggenberger, P. (2017). Online analysis: deeper insights into water quality dynamics in spring water. *Sci. Total Environ.* 599, 227–236. doi: 10.1016/j.scitotenv.2017.04.204

Petterson, S. R., and Ashbolt, N. J. (2016). QMRA and water safety management: review of application in drinking water systems. *J. Water Health* 14, 571–589. doi: 10.2166/wh.2016.262

Prest, E. I., Hammes, F., Kotzsch, S., van Loosdrecht, M. C. M., and Vrouwenvelder, J. S. (2013). Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method. *Water Res.* 47, 7131–7142. doi: 10.1016/j.watres.2013.07.051

Pronk, M., Goldscheider, N., and Zopfi, J. (2006). Dynamics and interaction of organic carbon, turbidity and bacteria in a karst aquifer system. *Hydrogeol. J.* 14, 473–484. doi: 10.1007/s10040-005-0454-5

Pronk, M., Goldscheider, N., and Zopfi, J. (2007). Particle-size distribution as indicator for fecal bacteria contamination of drinking water from karst springs. *Environ. Sci. Technol.* 41, 8400–8405. doi: 10.1021/es071976f

R Development Core Team (2008). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computin. Available online at: http://cran.r-project.org/

Ryzinska-Paier, G., Lendenfeld, T., Correa, K., Stadler, P., Blaschke, A. P., Farnleitner, A. H., et al. (2014). A sensitive and robust method for automated on-line monitoring of enzymatic activities in water and water resources. *Water Sci. Technol.* 69, 1349–1358. doi: 10.2166/wst.2014.032

Scheidleder, A. (1999). *Groundwater Quality and Quantity in Europe*. Copenhagen: European Environment Agency.

Signor, R. S., and Ashbolt, N. J. (2006). Pathogen monitoring offers questionable protection against drinking-water risks: a QMRA (Quantitative Microbial Risk Analysis) approach to assess management strategies. *Water Sci. Technol.* 54, 261–268. doi: 10.2166/wst.2006.478

Sinreich, M., Pronk, M., and Kozel, R. (2014). Microbiological monitoring and classification of karst springs. *Environ. Earth Sci.* 71, 563–572. doi: 10.1007/s12665-013-2508-7

Skeffington, R. A., Halliday, S. J., Wade, A. J., Bowes, M. J., and Loewenthal, M. (2015). Using high-frequency water quality data to assess sampling strategies for the EU Water Framework Directive. *Hydrol. Earth Syst. Sci.* 19, 2491–2504. doi: 10.5194/hess-19-2491-2015

SLMB (2007). *Method 1406.1: Detection of Enterococcus spp*. (Schweizerisches Lebensmittelhandbuch). Berne: Federal Office for Public Health.

SLMB (2012). *Method 333.1: Determining the Total Cell Count And Ratios Of High And Low Nucleic Acid Content Cells in Freshwater Using Flow Cytometry*. (Schweizerisches Lebensmittelhandbuch). Berne: Federal Office for Public Health.

Stadler, H., Skritek, P., Sommer, R., Mach, R. L., Zerobin, W., and Farnleitner, A. H. (2008). Microbiological monitoring and automated event sampling at karst springs using LEO-satellites. *Water Sci. Technol.* 58, 899–909. doi: 10.2166/wst.2008.442

Stevenson, D. G. (1997). *Water Treatment Unit Processes*. London: Imperial College Press.

Storey, M. V. van der Gaag, B., and Burns, B. P. (2011). Advances in on-line drinking water quality monitoring and early warning systems. *Water Res.* 45, 741–747. doi: 10.1016/j.watres.2010.08.049

Vital, M., Dignum, M., Magic-Knezeu, A., Ross, P., Rietueld, L., and Hammes, F. (2012). Flow cytometry and adenosine tri-phosphate analysis: alternative possibilities to evaluate major bacteriological changes in drinking water treatment and distribution systems. *Water Res.* 46, 4665–4676. doi: 10.1016/j.watres.2012.06.010

Vreeburg, J. H. G., Schaap, P. G., and van Dijk, J. C. (2004). "Particles in the drinking water system: from source to discolouration," in *4th World Water Congress: Innovation in Drinking Water Treatment*, Vol. 4 (Marrakesh; London), 431–438.

Ward, R. C., Loftis, J. C., and Mcbride, G. B. (1986). The data-rich but information-poor syndrome in water-quality monitoring. *Environ. Manage.* 10, 291–297.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer PS and handling Editor declared their shared affiliation.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 December 2017
doi: 10.3389/fmicb.2017.02380

The Effect of the 2015 Earthquake on the Bacterial Community Compositions in Water in Nepal

*Sital Uprety[1], Pei-Ying Hong[2], Nora Sadik[1], Bipin Dangol[3], Rameswor Adhikari[3], Antarpreet Jutla[4], Joanna L. Shisler[5], Patrick Degnan[5] and Thanh H. Nguyen[1]**

[1] Department of Civil and Environmental Engineering, University of Illinois at Urbana Champaign, Urbana, IL, United States, [2] Water Desalination and Reuse Center, Biological and Environmental Science and Engineering Division, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia, [3] Environment and Public Health Organization, Kathmandu, Nepal, [4] Department of Civil and Environmental Engineering, West Virginia University, Morgantown, WV, United States, [5] Department of Microbiology, University of Illinois at Urbana Champaign, Urbana, IL, United States

We conducted a study to examine the effect of seasonal variations and the disruptive effects of the 2015 Nepal earthquake on microbial communities associated with drinking water sources. We first characterized the microbial communities of water samples in two Nepali regions (Kathmandu and Jhapa) to understand the stability of microbial communities in water samples collected in 2014. We analyzed additional water samples from the same sources collected from May to August 2015, allowing the comparison of samples from dry-to-dry season and from dry-to-monsoon seasons. Emphasis was placed on microbes responsible for maintaining the geobiochemical characteristics of water (e.g., ammonia-oxidizing and nitrite-oxidizing bacteria and archaea and sulfate-reducing bacteria) and opportunistic pathogens often found in water (*Acinetobacter*). When examining samples from Jhapa, we identified that most geobiochemical microbe populations remained similar. When examining samples from Kathmandu, the abundance of microbial genera responsible for maintaining the geobiochemical characteristics of water increased immediately after the earthquake and decreased 8 months later (December 2015). In addition, microbial source tracking was used to monitor human fecal contamination and revealed deteriorated water quality in some specific sampling sites in Kathmandu post-earthquake. This study highlights a disruption of the environmental microbiome after an earthquake and the restoration of these microbial communities as a function of time and sanitation practices.

Keywords: microbial stability, perturbation, earthquake, opportunistic pathogens, Nepal

OPEN ACCESS

Edited by:
Qiang Wang,
Institute of Hydrobiology (CAS), China

Reviewed by:
Sarah-Jane Haig,
University of Michigan, United States
Amy Michele Grunden,
North Carolina State University, United States

***Correspondence:**
Thanh H. Nguyen
thn@illinois.edu

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *31 July 2017*
Accepted: *17 November 2017*
Published: *06 December 2017*

Citation:
Uprety S, Hong P-Y, Sadik N, Dangol B, Adhikari R, Jutla A, Shisler JL, Degnan P and Nguyen TH (2017) The Effect of the 2015 Earthquake on the Bacterial Community Compositions in Water in Nepal. Front. Microbiol. 8:2380. doi: 10.3389/fmicb.2017.02380

INTRODUCTION

Safe drinking water requires that the microbial community remains stable to minimize the risk of pathogen propagation and release (Rittmann, 1984; Hu et al., 1999; Prest et al., 2016). The biological stability of drinking water during common water treatment processes and water distribution has been examined (Lautenschlager et al., 2013; Prest et al., 2014). However, the variation in microbial community as a result of sudden changes, such as a natural disaster, remain understudied. Earthquakes are one form of natural disaster that can negatively impact human health and have high economic and environmental costs. The April 2015 earthquakes in Nepal caused more than

5 billion USD in damage (Government of Nepal, 2015; Upadhya and Seikh, 2015). These earthquakes caused 8,959 fatalities, a significant increase in waterborne infection incidence (Simkhada et al., 2015), limited water supply, sanitation, and hygiene resources (Uprety et al., in press). There was a 80% increase in communicable waterborne infections in the first 6 months of 2015, including the 2 months after the April earthquake, as compared to years 2013–2014 combined [Department of Health Services (DOHS) of Nepal, 2016]. There are only a few studies examining the microbial community in water in Nepal, and these studies show the presence of multiple pathogens and multi-drug resistance species of bacteria (Pokhrel and Viraraghavan, 2004; Tanaka et al., 2012). Waterborne infectious disease outbreaks are a result of many factors, including person-to-person transmission, food contamination, poor sanitation, and water contamination through fecal-oral route (Yan and Sadowsky, 2007; Grandesso et al., 2014; Ashbolt, 2015). More recently, it has been appreciated that environmental conditions that favor an increased load of pathogens in water also are crucial factors contributing to outbreaks of waterborne diseases, as was the case for Haiti in 2010 (Lobitz et al., 2000; Jutla et al., 2013). However, it is not known how the dynamics of water microbial communities change after a catastrophic earthquake that destroys sanitation and water infrastructure.

To fill a knowledge gap regarding changes in environmental microbial communities' due to the 2015 earthquake, we collected source drinking water samples in Kathmandu and Jhapa in Nepal, two regions that were affected and unaffected by earthquakes, respectively. We performed 16S rRNA gene sequencing on three sets of water samples. The first set of samples were collected 11 months prior to the earthquake, and the remaining sample sets were collected 1–3 months and then 8 months after the earthquake. Microbial source tracking was also performed using human and cow specific markers to better understand the change in sanitation practices along with the change in microbial community. To our knowledge, this is the first study that probes water microbiome dynamics with respect to earthquakes.

MATERIALS AND METHODS

Study Site

Water samples were collected at seven schools in Kathmandu (S1–S7) and four households in Jhapa (J2–J5) at four different time points occurring from May 2014 (**Figure 1**, **Table 1**). This is referred to as Batch 1. All schools in Kathmandu were selected because these schools' water sources historically contained high concentrations of fecal and total coliform counts. All schools (S1–S7) are in central Kathmandu in an urbanized area with high population density, and groundwater is the water source for all schools. Apart from S2, which has unprotected bore holes, all sites have unprotected dug wells. Students used the school's water source and water brought from home as drinking water. The seven schools are government-owned and accommodated children mostly from lower-middle class families.

Households in Jhapa (J2–J5) were also selected because their water source historically contained abundant fecal and total coliforms. In Jhapa, the drinking water source is river water, which is collected in a reservoir and piped to individual houses. Water samples (J2–J5) were taken from the household tanks piped from the river. Most of the families in the selected households relied on subsistence farming and had little or no formal education. However, due to various Water, Sanitation and Hygiene (WASH) campaigns conducted in the area, community members have been informed about basic sanitation and safe water practices.

After several earthquakes in April and May of 2015 (epicenters marked in **Figure 1**), additional water samples were collected at four time points from May to August 2015 (Batch 2) from the same locations in Kathmandu and Jhapa with some exceptions (**Figure 1**). The earthquake heavily affected Kathmandu, and as a result, two schools (S3 and S4) were not accessible for the second round of sampling. All sites in Jhapa were sampled during this same time frame because there were very limited effects of the earthquake on Jhapa compared to Kathmandu.

In December 2015, an additional water sample was collected again from the same sampling sites in Kathmandu (**Figure 1**). However, no samples were collected in Jhapa in December 2015 due to an ongoing fuel crisis in Nepal at the time that prohibited travel.

Sampling Protocol

Two-liter water samples were collected directly from faucet at each sampling site in sterile Whirl-pak® sampling bags (Nasco, WI) and were processed within 24 h of collection. Careful precautionary steps were taken during sampling to avoid cross contamination including changing of gloves between each sampling and sterilizing the cooler before and after each sampling. Samples of Kathmandu and Jhapa were collected and processed successively, so that there were no chances of cross contamination between the samples from two sites.

Samples in Kathmandu were collected directly from the well using the bucket provided and the samples in Jhapa were collected after a quick flush of 30 s. Samples were treated with 2.5 M $MgCl_2 \bullet 6H_2O$ (Sigma-Aldrich, St. Louis. MO) for 30 min to coagulate the microorganisms (Mattioli et al., 2013; Sadik et al., 2017). Next, coagulated water samples were vacuum-filtered through a 0.45 μm sterile cellulose acetate filter (GVS Maine, Sanford, ME) placed in 47 mm filtration funnel (Pall Corporation, New York, NY) for samples taken in 2014 (referred to as Batch 1 samples). However, this process clogged the 0.45 μm cellulose acetate filters very rapidly and was not feasible for practice on-site after the earthquake. Hence, water samples collected 1 year and year and half post-earthquake (Batch 2 and Batch 3, respectively) were vacuum-filtered through a 1.6 μm glass fiber membrane (Fisher Scientific, Hamptoon, NH) followed by a 0.45 μm cellulose acetate membrane after coagulation in a solution containing 25 mM magnesium chloride. During sample processing, the filtration unit was sterilized between each sample using disposable chlorine and ethanol wipes to avoid contamination. All working surface was thoroughly wiped with chlorine and ethanol wipe frequently during the sample processing. Sample membrane were then treated with RNAlater (Qiagen, Helden, Germany) and were stored in sterile

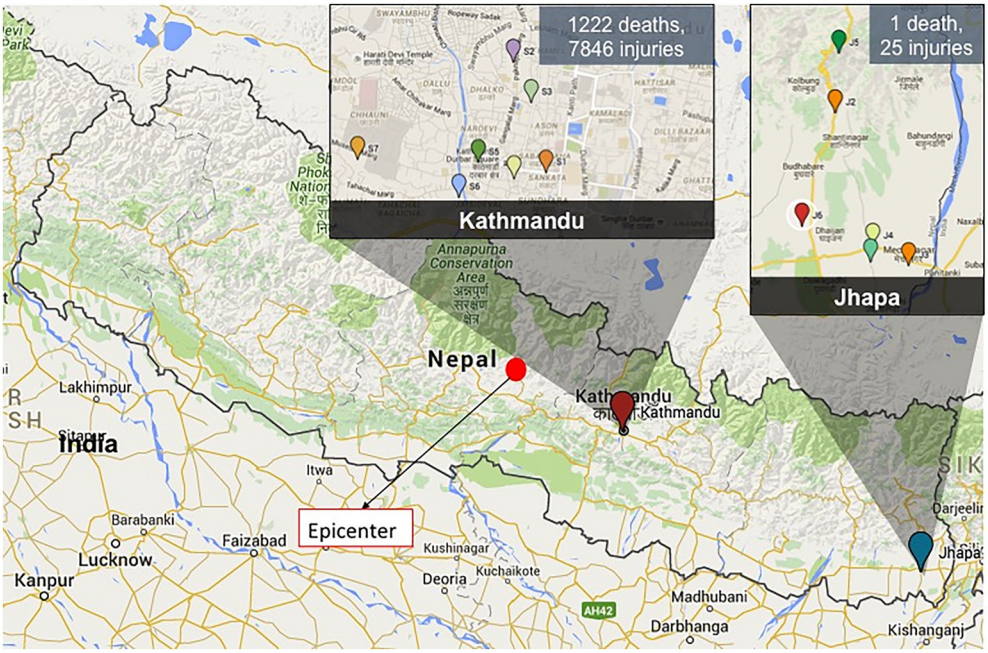


FIGURE 1 | A map showing the epicenter of the 2015 Nepal earthquake (filled red circle) and the sampling locations in Kathmandu and Jhapa. The magnitude of damage in two sampling locations is shown as well.

Whirlpak® bags at −20°C until transport to University of Illinois at Urbana Champaign (UIUC). At UIUC, samples were stored at −80°C until extraction.

DNA Extraction

Total DNA for the biomass retained on 0.45 μm membrane was extracted using the MoBio PowerWater RNA Isolation Kit (Yu and Morrison, 2004), removing the DNase step to ensure the collection of both DNA and RNA. RNA was then removed by treating the extracted nucleic acids with RNase, followed by standard sodium acetate–ethanol precipitation to concentrate the DNA. Total DNA for the biomass retained on 1.6 μm membrane was extracted using the MPI FastDNA Kit for Soil Extraction (Smith et al., 2012) with minor modifications. The minor modification includes the repeat of ethanol precipitation four times instead of once as recommended in the manufacture's protocol. Extra ethanol precipitation was needed to remove the high concentration of salts present in the RNAlater used to stabilize RNA during sample storage and transportation. For Batch 1 samples, DNA from the 0.45 μm filter membrane was used for analysis of microbial community. For Batch 2 and Batch 3 samples, combined DNA in equal volumes from both 1.6 and 0.45 μm filters was used for microbial community analysis to best approximate the total biomass that would have been captured by the coagulation–filtration protocol used for Batch 1 samples. All nucleic acid extractions of the samples were carried out in a sterile hood at the UIUC and all recommended precautionary steps were taken during extraction to avoid contamination. The only bacteria being grown in the lab at the time was *Legionella*, and since *Legionella* was not detected in any of the samples, we are confident that the steps taken to avoid contamination were successful.

PCR-Based Fecal Source Tracking

Microbial source tracking was performed using three primer pairs that target human-associated *Bacteroides uniformis, Bacteroides fragilis,* and *Bacteroides vulgatus* and a primer pair that targets cow-specific uncultivated *Bacteroidales.* Gene inserts were obtained from *B. vulgatus* BCRC12903, *B. uniformis* JCM5828, *B. fragilis* BCRC10619, and from a cow-specific uncultivated *Bacteroidales* clone obtained from an earlier study (Hong et al., 2009). qPCR standards were prepared by first cloning the gene inserts into pCR4 TOPO vector (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was extracted using PureYield™ Plasmid Miniprep System (Promega, Madison, WI, USA). The extracted plasmids were sequenced to verify the oligonucleotide sequences of gene inserts and quantified. PCR amplifications were performed with each plasmid to obtain standard curves. These experiments were performed in triplicate, while PCR amplification of experimental samples or negative controls was run in duplicates. Each PCR reaction volume of 20 μL contained 10 μL of FAST SYBR Green master mix, 0.4 μL of each primer (10 μM), 1 μL of DNA template (10–400 ng), and 8.2 μL molecular biology grade water. The Applied Biosystems 7900 HT Fast protocol was used for thermal cycling. The protocol includes 40 cycles of 1 s denaturation at 95°C and 60 s of annealing and extension. Dissociation curve analysis was included to detect non-specific amplification. The qPCR assays used in this study are the same as that previously reported (Zhang et al., 2014). The sensitivity and specificity assessment of these

TABLE 1 | Sampling location with GPS coordinates, water source type, level of earthquake damage and sampling Batches for each site.

Site	Location	GPS coordinates	Water source type	Location type	Earthquake damage	Sampling batches[a]
S1	Kathmandu	N27°42′44″ E 85°18′37″	Dug Shallow Well	School	High	Batch 1, 2, and 3
S2	Kathmandu	N27°42′53″ E 85*18′27″	Borehole Deep Well	School	High	Batch 1, 2, and 3
S3	Kathmandu	N 27°42′38″ E 85°18′37″	Dug Shallow Well	School	Damaged	Batch 1
S4	Kathmandu	N 27°42′10″ E 85°18′30″	Dug Shallow Well	School	Damaged	Batch 1
S5	Kathmandu	N 27°42′16″ E 85°18′15″	Dug Shallow Well	School	High	Batch 1, 2, and 3
S6	Kathmandu	N 27°42′03″ E 85°18′07″	Dug Shallow Well	School	High	Batch 1, 2, and 3
S7	Kathmandu	N 27°42′17″ E 85°17′25″	Dug Shallow Well	School	High	Batch 1, 2, and 3
J2	Jhapa	N 26°46′19″ E 88°04′13″	Surface water	Household	Low	Batch 1 and 2
J3	Jhapa	N 26°46′19″ E 88°04′19″	Surface water	Household	Low	Batch 1 and 2
J4	Jhapa	N 26°39′44″ E 88°06′20″	Surface water	Household	Low	Batch 1 and 2
J5	Jhapa	N 26°42″44″ E 88°05′21″	Surface water	Household	Low	Batch 1 and 2

[a]*Batch 1 = May-August 2014; Batch 2 = May-August 2015; Batch 3 = December 2015 water samples.*
High earthquake damage indicates severe damage in infrastructure; low earthquake damage indicates minimum to no damage in infrastructure and damaged indicates the sampling site was inaccessible after the earthquake.

assays were evaluated and the LOD for the human-associated Bacteriodales primer assays are 1.3×10^3, 1.9×10^3 and 1.7×10^3 copies/ng genomic DNA for Bvg, Bfrg and Bufm primer pairs respectively. Also, the LOD for the cow-specific primer assay was determined to be 4.7×10^2 copies/ng genomic DNA. The LOQ of human-associated Bacteriodales primer assays were 1.3×10^9, 1.9×10^8 and 1.7×10^8 copies/ng DNA for Bvg, Bfrg and Bufm primer pairs respectively and that for cow-specific Bacteriodales was 4.7×10^7 copies/ng DNA.

16S rRNA Gene-Based Amplicon Sequencing and Data Analysis and Statistics

Illumina MiSeq amplicon sequencing was performed for all the samples to provide information on the microbial community. To prepare the 16S rRNA gene amplicon libraries, 515F (5′-Illumina overhang-GTGYCAGCMGCCGCGGTAA-3′) and 907R (5′-Illumina overhang-CCCCGYCAATTCMTTTRAGT-3′) primers were modified to encode the overhang adaptor sequences, and used to amplify the 16S rRNA genes. The thermal cycling program included an initial denaturation stage at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, followed by a final extension period at 72°C for 5 min. PCR amplicons were purified by AMPure XP beads (Beckman Coulter, CA, USA) prior to the index PCR assay. Nextera XT Index (Illumina, CA, USA) was incorporated into each of the individual samples during PCR. The thermal cycling program included denaturation stage at 95°C for 3 min, followed by eight cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, followed by a final extension period at 72°C for 5 min. The final indexed PCR amplicons were again purified by AMPure XP beads, and nucleic acid concentrations were quantified using Invitrogen Qubit® 2.0 fluorometer. The controls for all PCR reactions were negative for amplification. Purified amplicons were submitted to KAUST Genomics Core lab for unidirectional sequencing read on an Illumina MiSeq platform. The sequences are deposited in the European Nucleotide Archive (ENA) under accession number PRJEB14325.

Raw sequences were first trimmed to remove the primers, barcodes, and adaptor sequences. Trimmed sequences that were <300 nt in length and with Phred score <20 were removed. Chimeras were identified using UCHIME (Edgar et al., 2011) by referencing to a core set that was downloaded from Greengenes (i.e., gold strains gg16—aligned.fasta, last modified on 19 March 2011). Chimeras were then removed from future analyses. The relative abundances of the bacterial and archaeal genera were then calculated, collated, and square-root transformed. The transformed data sets were computed for their Bray–Curtis similarities and represented graphically for spatial distribution and vector analysis in a non-metric multidimensional scaling (MDS) plot using Primer-E version 7.

Finally, two-way ANOVA test to analyze the statistical significance was tested for samples collected in several time periods for both Kathmandu and Jhapa samples. Samples were tested for May 2015–May 2015 samples, May 2014–July 2015

samples, and May–August and December 2015 samples. Significant change between two sampling periods were considered for $p < 0.05$. This statistical comparison suggested if the change in microbial communities were because of natural or seasonal variation or because of the earthquake.

RESULTS AND DISCUSSION

Although the incidence of waterborne diseases usually increases dramatically after major natural disasters (Ivers and Ryan, 2006; Watson et al., 2007), there is very limited research on the direct impact on the changes in microbial communities of water and the potential impact of these changes on public health arising from earthquakes. Instead of analyzing the changes in the microbial community of water longitudinally to determine the direct impact of an earthquake (as we did here), most studies tend to examine the indirect impact at a given time due to earthquakes or earthquake-triggered tsunamis. Metagenomic analysis of soil microbial communities after the 2011 earthquake and tsunami in Japan revealed the loss of siderophore-synthesis genes from *Arthrobacter* strains, an over-representation of denitrification related genera of microbes, and the presence of pathogenic bacteria (Hiraoka et al., 2016). Similarly, a soil microbial ecology study conducted 7 years after the tsunami in the Phang Nga province in Thailand revealed the presence of more *Bacteriodes* and other pathogenic microbes as compared sites that were not affected by the tsunami (Somboonna et al., 2014). In instances where studies examined the anthropogenic impact on water sources due to earthquake damage, these studies typically examined samples collected during one sampling event after an earthquake. For example, an increase in the amount of pathogenic bacteria were present in water samples collected from earthquake-affected area in Pakistan as compared to the areas that were not affected by earthquake (Rasheed et al., 2009). Even though these studies provide some insight about disturbance in the microbial communities after an extreme natural event, the emphasis is largely on the detection of fecal indicators and pathogenic microorganisms at one time point. There still exists a knowledge gap for understanding the dynamics of microbial communities in response to natural disasters and this study begins to fill this gap. Our strategy to fill this gap was to analyze and compare changes in the microbial community of water longitudinally, both before and after events like monsoons and earthquakes.

Characterization of Microbial Communities of Water Prior to the 2015 Earthquake

We first determined microbial communities in water samples that were taken from Kathmandu and Jhapa in May 2014, a time prior to the earthquake, by using 16S rRNA sequencing. The relative abundance of known bacterial genera and unclassified bacterial groups in each water sample was compared to other samples using their Bray–Curtis similarities (**Figure 2**). These data revealed that the microbial communities of all four Jhapa water sources (J2–J5) shared 55% similarity and formed one cluster. We analyzed the bacterial communities using a non-metric multidimensional scaling plot coupled with vector-based analysis to confirm data from the Bray–Curtis similarities (**Figure 2**). We observed four bacterial populations that were prevalent in all four samples from Jhapa (**Figure 2**). For example, members of the order *Burkholderiales* accounted for 13, 25, 30, and 47% of total microbial community for samples J2, J3, J4, and J5, respectively (**Figure 2**). Members of family *Comamonadaceae* accounted from between 5 and 27% of the bacterial population in the water samples from Jhapa (**Figure 2**). The remaining two dominant bacterial population present in all Jhapa samples were members of Moraxellaceae family (3–18%) and *Flavobacterium* genus (1–7%) (data not shown in the plot).

In contrast to the water samples from Jhapa, the seven water samples collected in Kathmandu (S1–S7) clustered into three different groups when using Bray–Curtis similarities (**Figure 2**). Samples S1, S5, and S6 were clustered in one group and samples S3, S4, and S7 were clustered in another group (**Figure 2**). Sample S2 was in its own cluster. Bray–Curtis analyses also revealed that samples S3, S4, and S7 shared 51% similarity. Each of these three samples possessed members from the genus *Flavobacterium* and *Polynucleobacter,* which contributed on average to 18–8% of microbial community, respectively. However, each sample also possessed unique bacterial populations, which is why the Bray–Curtis score was not higher. For example, in sample S3, members of the *Comamondaceae* family (8.5% of the total microbial population) predominated, followed by members of the order *Burkholderiales* (6% of the total microbial community). In contrast, the predominant bacterial genera present in S4 were *Flavobacterium* and *Polynucleobacter* (26 and 11% of the population, respectively). In sample S7, family *Planctomycetaceae* and members of family *Comamonadaceae* were predominant, accounting for 18 and 13% of the total microbial community. Thus, although samples S3, S4, and S7 shared some bacterial members, the predominance of different bacterial orders, families, and genera in each community have less similarity as compared to Jhapa samples. When examining similarities in between water samples taken in Kathmandu, Bray–Curtis analyses revealed that samples S1, S5, and S6 clustered together with 43% similarity. In these samples, members of Gammaproteobacteria and Betaproteobacteria are the most abundant (23 and 20%, respectively). In contrast, populations of Gammaproteobacteria and Betaproteobacteria were 8 and 7% abundant, respectively, in S6. This is one reason why there was a decreased percentage similarity in this cluster.

Sample S2 shared only 38% similarity to all other Kathmandu samples (**Figure 2**), indicating a major difference in microbial communities between S2 and other Kathmandu sites. This low similarity was because members of the order *Burkholderiales* and the family *Comamonadaceae,* which were not predominant in other Kathmandu samples, accounted for 60% of the microbial community in total for S2 samples. S2 also possessed bacterial genera like *Azospira* (4%) and *Zoogloea* (3%), genera that were absent in other Kathmandu water samples.

It was also observed that there were differences in the abundance of bacterial or archaeal genera routinely known to be important for geobiochemical characteristics of water in water

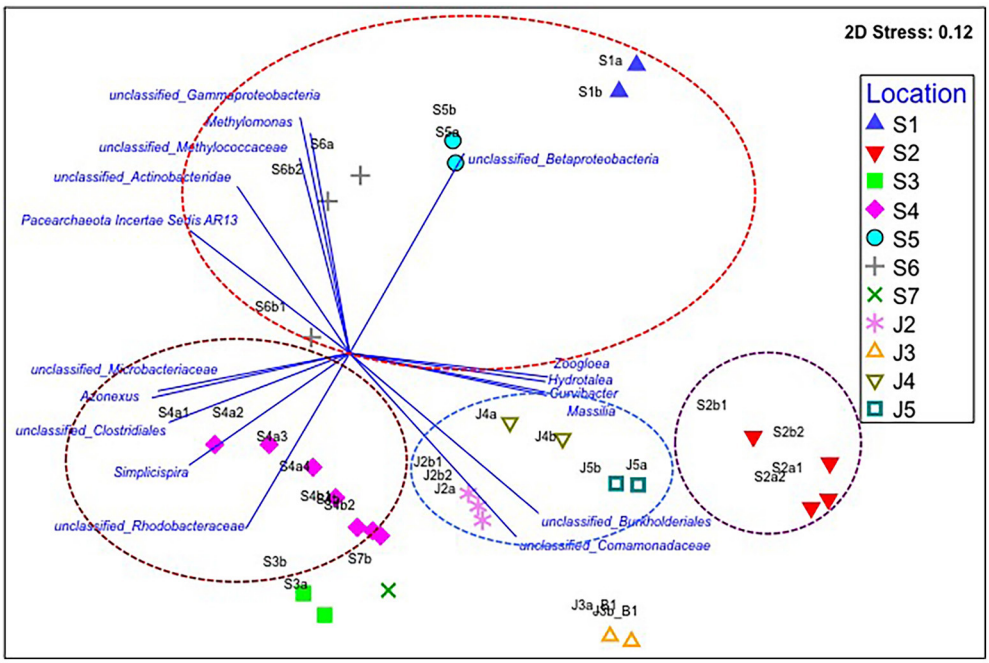


FIGURE 2 | Non-metric multidimensional scaling (NMDS) plot for the averaged microbial communities in each Kathmandu (S1–S7) and Jhapa (J2–J5) water sample that was taken in (Batch 1). Vector-based analysis (blue lines and text) overlay the bacterial population that showed significant correlation with the clustering patterns. The letters a, b, c, d (e.g., S2a, S2b, S2c, S2d) represent different filter membranes used for each sample collected. More filter membranes were used at some sites (S2) compared to other sites (J3) because water turbidity was higher. Similar bacterial populations are indicated by circles of different colors.

TABLE 2 | The average relative abundance of genera associated with geochemical characteristics of water in Kathmandu (S1–S7) and Jhapa (J2–J5).

Bacterial/Archaeal genera	Type of Bacteria/Archaea[a]	Average Kathmandu	Average Jhapa[b]
Nitrospira	NOB	0.182%	0.086%
Nitrososphaera	AOA	0.007%	0.003%
Nitrosopumilus	AOA	0.045%	ND
Methylobacter	MOB	0.005%	ND
Methylomonas	MOB	0.025%	ND
Desulfovibrio	SRB	0.004%	ND

Samples were collected from May to August 2014.
[a]*NOB, Nitrite Oxidizing Bacteria; AOA, Ammonia Oxidizing Archaea; MOB, Methane Oxidizing Bacteria; SRB, Sulfate Reducing Bacteria.*
[b]*ND, not detected.*

collected from Jhapa vs. Kathmandu (Azam and Smith, 1991). Water samples from both locations possessed *Nitrospira* and *Nitrososphaera* (**Table 2**). However, genera like *Nitrosopumilus*, *Methylobacter*, *Methylomonas,* and *Desulfovibrio* were only detectable in water samples from Kathmandu (**Table 2**). Together these data indicate that drinking water microbiomes in Jhapa are (i) more similar to each other than those in Kathmandu, and (ii) distinct from those in Kathmandu.

The differences in microbial communities in samples from Jhapa vs. Kathmandu pre-earthquake are likely reflective of the different water sources used by each community, different climate conditions, and different human activities. Source water in Kathmandu is from a single aquifer (Khatiwada et al., 2002), which is then accessed by a deep or shallow well. In contrast, households in Jhapa rely on river water that is stored and distributed through a shared reservoir. Surface water and groundwater environments have distinct indigenous microbial communities (Griebler and Lueders, 2009).

We also observed distinct microbial communities in water samples taken in Kathmandu (**Figure 2**). All sampling sites in Kathmandu were shallow/dug wells, except S2 which is a deep borehole (24 m) well. Thus, the microbial communities from the S2 samples were distinct from other urban samples (**Figure 2**). Furthermore, among the samples from the shallow wells, the formation of different clusters by S1, S5, and S6 vs. S3, S4, and S7, could be due to differences in sanitation practices at these locations. Notably, S3 and S7 samples were from wells that are only ∼10 and ∼4 m, respectively, from pit latrines, where there could be seepage of bacteria from human waste into drinking water sources.

Comparison of Microbial Communities from Samples Collected in May 2014 vs. May 2015

Only Jhapa samples J3 and J4, and Kathmandu samples S2 and S5 were reliably accessible throughout all three sampling periods (as some schools were destroyed after May 2015 earthquake in Kathmandu). For these reasons, we focused on these samples for the analyses shown in **Table 3**. Namely, we

TABLE 3 | Fold- difference in relative abundance of bacterial genera in Kathmandu and Jhapa water samples collected in May 2014 vs. May 2015 (dry season), or May 2015 vs. July 2015 (dry to wet season transition).

Bacterial genera	Fold change in S2 (dry season)	Fold change in S5 (dry season)	Fold change in J3 (dry season)	Fold change in J4 (dry season)	Fold change in S2 (dry to wet season)	Fold change in S5 (dry to wet season)	Fold change in J3 (dry to wet season)	Fold change in J4 (dry to wet season)
Methylobacter	N/A (ND to ND)	0.45 (0.028 to 0.014%)	N/A (ND to ND)	N/A (ND to ND)	N/A (ND to 0.50%)	94.8 (0.03 to 2.7%)	N/A (ND to ND)	N/A (ND to ND)
Desulfovibrio	593.47 (0.008 to 4.71%)	N/A (ND to ND)	N/A (ND to ND)	N/A (ND to ND)	42.4 (0.008 to 0.337%)	N/A (ND to 0.016%)	N/A (ND to ND)	N/A (ND to ND)
Nitrospira	N/A (ND to ND)	4.64 (0.014 to 0.065%)	0.48 (0.078 to 0.037%)	N/A (ND to ND)	N/A (ND to 0.027%)	2.4 (0.0139 to 0.033%)	4.50 (0.037 to 0.165%)	N/A (ND to 0.26%)
Methylomonas	N/A (ND to ND)	2.13 (0.042 to 0.091%)	N/A (ND to ND)	N/A (ND to ND)	N/A (ND to 0.08%)	3.14 (0.042 to 0.133%)	N/A (ND to ND)	N/A (ND to ND)
Acinetobacter	1.71 (0.063 to 0.180%)	N/A (ND to 1.772%)	4.25 (0.131 to 0.560%)	0.66 (0.174 to 0.116%)	2.6 (0.06 to 0.162%)	N/A (ND to 0.43%)	0.07 (4.717 to 0.337%)	121.04 (0.12 to 13.97%)
Aeromonas	N/A (0.007% to ND)	N/A (ND to 0.71%)	N/A (0.020% to ND)	N/A (ND to ND)	N/A (0.007% to ND)	N/A (ND to 0.433%)	N/A (ND to 0.147%)	N/A (ND to 0.03%)
Legionella	N/A (ND to ND)	N/A (ND to ND)	1.03 (0.012 to 0.013%)	N/A (ND to ND)	N/A (ND to 0.013%)	N/A (ND to ND)	2.35 (0.012 to 0.03%)	N/A (ND to 0.01%)

N/A, not applicable, fold change cannot be calculated due to the absence of a genus in one of the samples. ND, not detected.

examined the microbial communities from the same water sources 12 months later (in May 2015; **Figure 1**) to ask if bacterial communities changed longitudinally. We performed two-way ANOVA analyses to determine statistical significance for all samples collected in May 2014 and May 2015 in both Kathmandu and Jhapa (**Table 4**, columns 1 and 2). Results showed that that difference was not significant between Kathmandu samples collected in May 2014 and May 2015 ($p > 0.05$) except for *Methylomonas* (**Table 4**). For Jhapa samples, there were no statistically significant changes ($p > 0.05$, **Table 4**, column 2) for all selected genera except *Nitrospira, Legionella,* and *Aeromonas.*

In addition to examining bacteria associated with biostability of water, shown in **Table 2**, we also investigated if *Legionella*, *Aeromonas,* and *Acinetobacter* were present because they are opportunistic pathogens commonly found in water (Madigan et al., 2008). For Jhapa water samples, there was an increase in population for only one of the three bacterial genera that cause opportunistic infections (4.25-fold increase in *Acinetobacter* populations; **Table 3**, column 3). Also, there was no dramatic change in the abundance of the bacterial genera associated with geobiochemical characteristics of water (**Table 3**, columns 3 and 4). In the Kathmandu water samples, the relative abundance of several bacterial populations increased between May 2014 and May 2015 (**Table 3**, columns 1 and 2). The largest increase was that of *Desulfovibrio* spp., for which there was a 593-fold increase in S2 comparing May 2014 to May 2015 samples. For S5, a 4.64-fold increase in *Nitrospira* spp. and a 2.13-fold increase in *Methylomonas* spp. were observed between 2014 and 2015 (**Table 3**). The absence of geobiochemically-relevant bacteria, *Methylobacter*, *Desulfovibrio*, and *Methylomonas*, in Jhapa water samples collected almost a year apart (**Table 3**) suggested negligible methyl-oxidation and sulfate-reduction in water from Jhapa.

In contrast, we observed an increase in the relative abundance of bacterial genera that are responsible for maintaining geobiochemical characteristics (e.g., *Nitrospira, Desulfovibrio, Methylomonas*) of water post-earthquake for S2 and S5 in Kathmandu (**Figure 3**, **Table 3**). We suggest that the changes in these populations were a result of the April 2015 earthquake. Indeed, others have reported similar bacterial populations in water quality after a natural disaster (Ivers and Ryan, 2006; Rasheed et al., 2009; Hiraoka et al., 2016). Although not all locations were accessible for sampling, the analysis for all collected samples may not reflect the changes in microbiome before and after the earthquake, based on the available data we conclude that, in general, bacterial populations changed longitudinally to a greater degree in Kathmandu samples vs. Jhapa samples.

Seasonal Changes in Microbial Community Collected in May 2015 (Dry Season) and July 2015 (Monsoon Season)

We next compared the microbial communities of water samples collected in dry vs. monsoon seasons in 2015 to identify (i) the natural variation in water microbiomes collected from Jhapa, and (ii) the impact of the 2015 earthquake in water

TABLE 4 | Statistical significance for samples collected in different time periods.

Bacterial genera	*p*-value May 2014–May 2015 Kathmandu	*p*-value May 2014–May 2015 Jhapa	*p*-value May 2014–July 2015 Kathmandu	*p*-value May 2014–July 2015 Jhapa	*p*-value May-August and December 2015 Kathmandu
Nitrospira	0.07	0.01	0.90	0.09	0.04
Methylobacter	0.08	N/A	0.29	N/A	0.08
Desulfovibrio	0.27	0.17	0.41	0.18	0.28
Methylomonas	0.03	N/A	0.42	N/A	0.04
Legionella	0.79	0.01	0.35	0.54	0.02
Aeromonas	0.14	0.05	0.76	0.07	0.02
Acinetobacter	0.11	0.10	0.48	0.27	0.95

N/A, not applicable, p-value cannot be calculated due to the absence of a genus in one of the samples.

microbiomes from Kathmandu. We first conducted a two-way ANOVA analysis of data from May 2015 and July 2015 samples taken from both locations. There were no statistically significant changes in bacterial genera examined in May 2014–July 2015 for both Kathmandu and Jhapa samples (**Table 4**). Note again that only two sites from Jhapa (J3 and J4) and two sites from Kathmandu (S2 and S5) were selected for further analysis for bacteria associated with opportunistic infections. For the Jhapa samples J3 and J4, where the effect of the 2015 earthquake was minimal, only *Nitrospira* and *Acinetobacter* populations increased (**Table 3**). During this same transition period in Kathmandu, *Methylobacter* populations showed the largest change in relative abundance, for which there was a 94.8-fold increase in sample S5. *Desulfovibiro* populations increased by 42.4-fold in sample S2. Smaller increases in *Nitrospira* and *Aceintobacter* populations were observed in samples S5 and S2, respectively. Thus, the changes in populations of bacterial genera associated with geobiochemical characteristics of water were more pronounced in Kathmandu vs. Jhapa samples. In addition, changes in microbial communities were more pronounced when comparing pre- vs. post-earthquake to dry vs. wet season communities.

Microbial Community Dynamics in Kathmandu 6 Months Post-earthquake

In December 2015, samples were collected from S2 and S5 locations in Kathmandu to determine if the microbial communities approximated toward the relative abundances of microbes detected in 2014 samples (**Figure 3**). Jhapa water samples were not collected in December 2015 because results from **Table 3** suggested that there were minimal changes in microbial communities over time in Jhapa.

In the S2 sample, *Methylobacter* was not detected in 2014 samples. By May–August 2015, *Methylobacter* contributed to 0.3% of total microbial community. By December 2015, it contributed to 0.07%. Similarly, *Desulfovibrio* spp. contributed to 0.006% of the microbial community in 2014 samples. This contribution increased to 1.32% in May–August 2015 samples, and then decreased to 0.025% by December 2015. Thus, it appeared that the population of *Desulfovibrio* spp. was returning to levels observed pre-earthquake. The trend was also observed in bacteria that are opportunistic pathogens. In the S5 sample, *Acinetobacter* was not detected in 2014 samples but contributed to 0.73% of total microbial community in the samples collected 3 months after the earthquake. However, *Acinetobacter* was not detected in December 2015. Similarly, *Aeromonas* was not detected in 2014 was detected in May–August 2015 (0.21%) but decreased to 0.009% in December 2015, returning closer to 2014 samples.

In addition, there was an increase in relative abundance of other bacterial genera throughout the sampling periods. For example, the relative abundance of *Methylomonas* in the S5 samples increased from 0.04% to 0.65% to 1.063% in Batch 1, Batch 2, and Batch 3, respectively. In addition, *Methylobacter* increased from 0.028 to 1.23% over time. In summary, the microbial communities in Kathmandu water shifted after the earthquake. In some cases, populations of waterborne bacteria returned to the levels observed in 2014.

The earthquake also changed in human activities and behaviors, changes will also alter microbial communities in Kathmandu. For example, the creation of and changes in population sizes for temporary settlements (which were in response to the earthquake) may affect water microbiomes. Indeed, human settlement related to mining in Brazil drives the abundance of nitrifying bacteria and archaea (Reis et al., 2015). Similarly, human activities cause disturbances of methane-oxidizing bacteria like *Methylomonas* (Holmes et al., 1999) and also ammonia oxidizers (Ying et al., 2010). Thus, we speculated that the increase in geobiochemically relevant bacterial genera in Kathmandu samples may be related to human activities.

Two-way ANVOA analyses showed no statistically significant differences between *Acinetobacter*, *Methylobacter,* and *Desulfovirbiro* ($p > 0.05$) in samples collected between May–July 2015 and December 2015. However, there was a statistically significant change ($p < 0.05$) in *Nitrospira, Methylomonas*, *Legionella,* and *Aeromonas* populations at that same time (**Table 4**). When examining samples S2 and S5, we observed a dramatic increase in geobiochemically relevant bacterial genera (e.g., *Desulfovibrio* and *Nitrospira*) in S2 and S5 samples soon after the 2015 earthquake. These bacterial populations decreased to pre-earthquake levels by December 2015 (**Figure 3**). Similar trends were observed for *Acinetobacter* and *Aeromonas* in these same water samples (**Figure 3**). This observation indicates that, despite the shift in the microbial community that occurred immediately after the earthquake, the microbial community was returning to a profile similar to those observed prior to the

earthquake. We speculate that this return was due mostly to the closing of temporary settlements, which would decrease unsafe sanitation practices and the nitrate and ammonia load in water, providing an environment that is conducive to proliferation of indigenous microbiota.

PCR-Based Fecal Source Tracking

Host-associated Bacteroidales is used as bacterial indicator to identify an originating source of fecal contamination (Jenkins et al., 2009). Using this system, a sample is considered positive for human fecal contamination when two or more human-associated *Bacteroides* spp. are present in a sample (Hong et al., 2009). We used this approach as an additional method to indicate water quality and sanitation conditions for samples S2 and S5 longitudinally. Results are shown in **Table 5**. Samples from S2 were negative for all three human-associated *Bacteroides* spp. markers examined both pre- and post-earthquake. However, these same samples were positive for a cow-specific *Bacteroidales* (Hong et al., 2009) marker only at one time point (immediately after the earthquake), implying the presence of cow feces near this sampling site. For site S5, human fecal contamination was detected at all-time points (**Table 5**). However, cow-specific *Bacteroidales* markers were not detected in any of the S5 samples. It is to be noted that S2 water comes from a deep well, whereas S5 water comes from a shallow well. Moreover, these data suggest that there was more fecal contamination after the earthquake in Kathmandu.

The increase in human-specific *Bacteroides* and/or cow-specific *Bacteroidales* detected in the water samples collected post-earthquake indicated compromised sanitation practices. Sites S2 and S5 were being used as temporary camps for the victims of the earthquake. Open defecation due to the lack of toilets near the camps is expected to introduce fecal contamination to the water sources. One expectation is that the human and animal-associated Bacteroides will decrease over time, as the people of Kathmandu rebuild infrastructure.

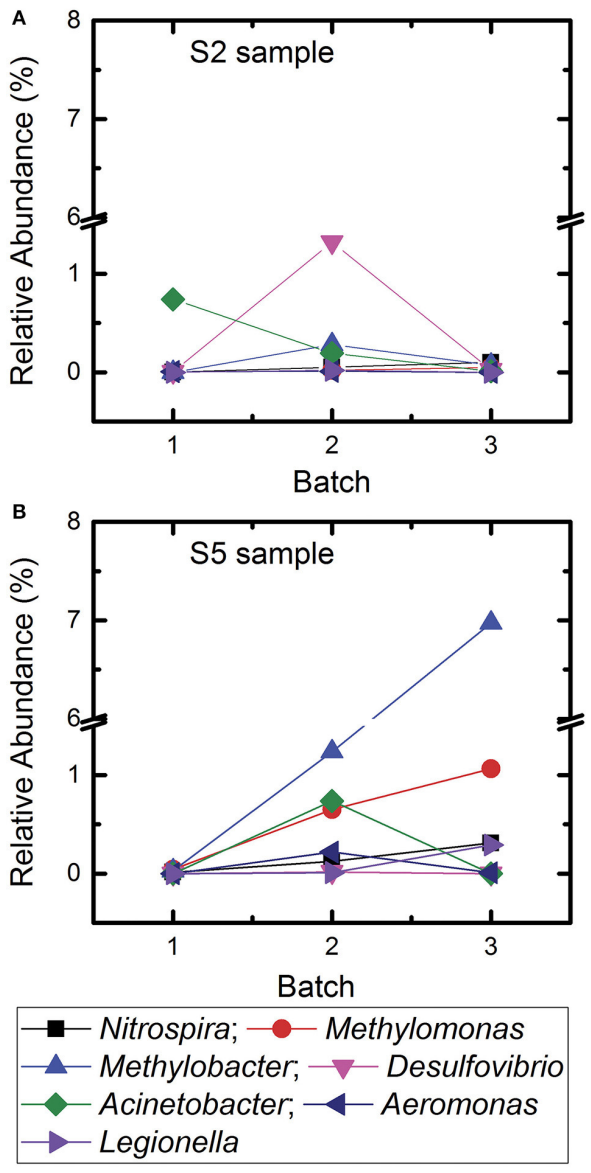


FIGURE 3 | Change of relative abundances of different bacterial genera associated with biogeochemical characteristics of water and genera associated with opportunistic pathogens in samples S2 and S5 for Batch 1, Batch 2, and Batch 3. **(A)** Sample site S2 and **(B)** sample site S5.

Study Limitation

This study presents new knowledge on the dynamics of water microbiota after the Nepal 2015 earthquake and demonstrates the restoration of the water microbiome over time. There were limitations to this study. First, although 16S rRNA gene-based sequencing can mostly characterize bacterial genera, information related to viruses and eukaryotes including fungal and parasitic genera are not included. Second, 16S rRNA gene-based amplicon sequencing also does not provide information related to the functional genes, which play important roles in the overall nutrient and biogeochemical cycling and those related to virulence-associated genes. Third, although this study aims to assess the degree of perturbation as a function of time, sampling immediately after the earthquake and 8 months after the earthquake may not be enough to comprehensively characterize all important genera as restoration properties may differ among genera. Fourth limitation of this study is on the sequencing control. While we conducted the sample

TABLE 5 | Presence or absence of human-associated *Bacteroides* spp. and cow-specific *Bacteroidales*.

	Sample	Positive for 2 or more human markers	Positive for cow marker
Batch 1 (May 2014)	S2	–	–
	S5	+	–
Batch 2 (July 2015)	S2	–	+
	S5	+	–
Batch 3 (Dec. 2015)	S2	–	–
	S5	+	–

extraction to the best of our ability, complete avoidance of contamination was not confirmed. The sequencing control was done in accordance to the specifications suggested by Illumina for low diversity libraries such as amplicon libraries. Specifically, PhiX was added at 20% to provide a spike-in internal control to monitor sequencing quality based on cluster density, base alignment error rates. All samples were monitored based on these parameters and those sequencing libraries that do not meet the quality control are discarded. Results in this study are those that pass the sequencing control quality check. However, since PhiX was used as Illumina's internal sequence, sequencing negative controls with samples collected throughout the study is a more reliable way to check on contamination due to reagents and laboratory condition, as suggested in Salter et al. (2014). In our study, we did not observe a microbial population that occurred consistently throughout all samples to indicate background contamination. For future study, we recommend including sequencing of negative control throughout the sample extraction and preparation for sequencing. To overcome these limitations, future studies will use shotgun metagenomics sequencing of samples collected longitudinally to understand the overall microbial diversity, including viruses, rather than be limited to 16S, 18S, and 23S rRNA genes (Riesenfeld et al., 2004; Edwards and Rohwer, 2005; Tringe et al., 2005). In addition to 16S rRNA sequencing, viability assay, metagenomics, and metatranscriptomics will allow a more comprehensive understanding of the microbial communities and their functions. Future studies will also aim to increase the frequency of sampling post-earthquake to better understand the kinetics of restoration of a microbial community in the source water. Despite the limitations, the results of this study provide an improved understanding on the change in microbial communities of water under the influence of seasonal variation and a large-scale earthquake.

AUTHOR CONTRIBUTIONS

SU: Field and lab work, manuscript writing and reviewing, data analysis. P-YH: Lab work, data analysis, manuscript writing and reviewing. NS: Field and lab work, manuscript writing and reviewing. BD: Manuscript writing and reviewing. RA: Field work, manuscript writing and reviewing. AJ, JS, and PD: Manuscript writing and reviewing, technical support. TN: Corresponding author, manuscript writing and reviewing, technical support, data analysis.

ACKNOWLEDGMENTS

Civil and Environmental Engineering (CEE) Rapid Response Grant, NSF IRES 1559530, University of Illinois travel grant and National Science Foundation Graduate Research Fellowship (GRFP). Costs and manpower incurred for 16S rRNA gene sequencing and qPCR are supported by KAUST baseline funding BAS/1/1033-01-01 awarded to P-YH.

REFERENCES

Ashbolt, N. J. (2015). Microbial contamination of drinking water and human health from community water systems. *Curr. Environ. Health Rep.* 2, 95–106. doi: 10.1007/s40572-014-0037-5

Azam, F., and Smith, D. C. (1991). Bacterial influence on the variability in the ocean's biogeochemical state: a mechanistic view. *Par. Anal. Oceanogr.* 27, 213–236.

Department of Health Services (DOHS) of Nepal (2016). *Report on Communicable Waterborne Infections*

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Edwards, R. A., and Rohwer, F. (2005). Viral metagenomics. *Nature* 3, 504–510. doi: 10.1002/rmv.532

Government of Nepal (2015). *Nepal Disaster Risk Reduction Portal* [Online], Government of Nepal. Available online at: http://drrportal.gov.np/home; http://drrportal.gov.np (accessed September 27, 2017).

Grandesso, F., Allan, M., Jean-Simon, P. S., Boncy, J., Blake, A., Pierre, R., et al. (2014). Risk factors for cholera transmission in Haiti during inter-peak periods: insights to improve current control strategies from two case-control studies. *Epidemiol. Infect.* 142, 1625–1635. doi: 10.1017/S0950268813002562

Griebler, C., and Lueders, T. (2009). Microbial biodiversity in groundwater ecosystems. *Freshw. Biol.* 54, 649–677. doi: 10.1111/j.1365-2427.2008.02013.x

Hiraoka, S., Machiyama, A., Ijichi, M., Inoue, K., Oshima, K., Hattori, M., et al. (2016). Genomic and metagenomic analysis of microbes in a soil environment affected by the 2011 Great East Japan Earthquake tsunami. *BMC Genomics* 17:53. doi: 10.1186/s12864-016-2380-4

Holmes, A. J., Roslev, P., McDonald, R. I., Iversen, N., Henriksen, K., and Murrell, J. C. (1999). Characterization of methanotrophic bacterial populations in soils showing atmospheric methane uptake. *Appl. Environ. Microbiol.* 65, 3312–3318.

Hong, P. Y., Wu, J. H., and Liu, W. T. (2009). A high-throughput and quantitative hierarchical oligonucleotide primer extension (HOPE)-based approach to identify sources of faecal contamination in water bodies. *Environ. Microbiol.* 11, 1672–1681. doi: 10.1111/j.1462-2920.2009.01892.x

Hu, Y. J., Wang, Z. S., Ng, W. J., and Ong, L. (1999). The effect of water treatment process on the biological stability of potable water. *Water Res.* 33, 2587–2592. doi: 10.1016/S0043-1354(98)00482-5

Ivers, L. C., and Ryan, E. T. (2006). Infectious diseases of severe weather-related and flood-related natural disasters. *Curr. Opin. Infect. Dis.* 19, 488–414. doi: 10.1097/01.qco.0000244044.85393.9e

Jenkins, M. W., Tiwari, S., Lorente, M., Gichaba, C. M., and Wuertz, S. (2009). Identifying human and livestock sources of fecal contamination in Kenya with host-specific Bacteroidales assays. *Water Res.* 43, 4956–4966. doi: 10.1016/j.watres.2009.07.028

Jutla, A., Whitcombe, E., Hasan, N., Haley, B., Akanda, A., Huq, A., et al. (2013). Environmental factors influencing epidemic cholera. *Am. J. Trop. Med. Hyg.* 89, 597–607. doi: 10.4269/ajtmh.12-0721

Khatiwada, N. R., Takizawa, S., Tran, T. V., and Inoue, M. (2002). Groundwater contamination assessment for sustainable water supply in Kathmandu Valley, Nepal. *Water Sci. Technol.* 46, 147–154.

Lautenschlager, K., Hwang, C., Liu, W. T., Boon, N., Koster, O., Vrouwenvelder, H., et al. (2013). A microbiology-based multi-parametric approach towards assessing biological stability in drinking water distribution networks. *Water Res.* 47, 3015–3025. doi: 10.1016/j.watres.2013.03.002

Lobitz, B., Beck, L., Huq, A., Wood, B., Fuchs, G., Faruque, A. S., et al. (2000). Climate and infectious disease: use of remote sensing for detection of *Vibrio cholerae* by indirect measurement. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1438–1443. doi: 10.1073/pnas.97.4.1438

Madigan, M. T., Martinko, J. M., Stahl, D. A., and Clark, D. P. (2008). *Brock Biology of Microorganisms, 12th Edn*. Boston, MA: Benjamin Cummings.

Mattioli, M. C., Pickering, A. J., Gilsdorf, R. J., Davis, J., and Boehm, A. B. (2013). Hands and water as vectors of diarrheal pathogens in

Bagamoyo, Tanzania. *Environ Sci. Technol.* 47, 355–363. doi: 10.1021/es303878d

Pokhrel, D., and Viraraghavan, T. (2004). Diarrhoeal diseases in Nepal vis-à-vis water supply and sanitation status. *J. Water Health* 2:71.

Prest, E. I., El-Chakhtoura, J., Hammes, F., Saikaly, P. E., van Loosdrecht, M. C., and Vrouwenvelder, J. S. (2014). Combining flow cytometry and 16S rRNA gene pyrosequencing: a promising approach for drinking water monitoring and characterization. *Water Res.* 63, 179–189. doi: 10.1016/j.watres.2014.06.020

Prest, E. I., Hammes, F., van Loosdrecht, M. C., and Vrouwenvelder, J. S. (2016). Biological stability of drinking water: controlling factors, methods and challenges. *Front. Microbiol.* 7:45. doi: 10.3389/fmicb.2016.00045

Rasheed, F., Khan, A., and Kazmi, S. U. (2009). Bacteriological analysis, antimicrobial susceptibility and detection of 16S rRNA gene of Helicobacter pylori by PCR in drinking water samples of earthquake affected areas and other parts of Pakistan. *Malasiyan J. Microbiol.* 5, 123–127. doi: 10.21161/mjm.18609

Reis, M. P., Avila, M. P., Keijzer, R. M., Barbosa, F. A., Chartone-Souza, E., Nascimento, A. M., et al. (2015). The effect of human settlement on the abundance and community structure of ammonia oxidizers in tropical stream sediments. *Front. Microbiol.* 6:898. doi: 10.3389/fmicb.2015.00898

Riesenfeld, C. S., Schloss, P. D., and Handelsman, J. (2004). Metagenomics: genomic analysis of microbial communities. *Annu. Rev. Genet.* 38, 525–552. doi: 10.1146/annurev.genet.38.072902.091216

Rittmann, B. E. (1984). Achieving biologically stable drinking water. *J. Am. Water Works Assoc.* 76, 106–114.

Sadik, N. J., Uprety, S., Nalweyiso, A., Kiggundu, N., Banadda, N. E., Shisler, J. L., et al. (2017). Quantification of multiple waterborne pathogens in drinking water, drainage channels, and surface water in Kampala, Uganda, during seasonal variation. *GeoHealth* 1, 258–269. doi: 10.1002/2017GH000081

Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., et al. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.* 12:87. doi: 10.1186/s12915-014-0087-z

Simkhada, P., van Teijlingen, E., Pant, P. R., Sathian, B., and Tuladhar, G. (2015). Public health, prevention and health promotion in post-earthquake Nepal. *Nepal J. Epidemiol.* 5, 462–464. doi: 10.3126/nje.v5i2.12826

Smith, D. J., Timonen, H. J., and Jaffe, D. A., Griffin, D. W., Birmele, M. N., Perry, K. D. (2012). Intercontinental dispersal of bacteria and archaea by transpacific winds. *Appl. Environ. Microbiol.* 79, 1134–1139. doi: 10.1128/AEM.03029-12

Somboonna, N., Wilantho, A., Jankaew, K., Assawamakin, A., Sangsrakru, D., Tangphatsornruang, S., et al. (2014). Microbial ecology of Thailand tsunami and non-tsunami affected terrestrials. *PLoS ONE* 9:e94236. doi: 10.1371/journal.pone.0094236

Tanaka, Y., Nishida, K., Nakamura, T., Chapagain, S. K., Inoue, D., Sei, K., et al. (2012). Characterization of microbial communities distributed in the groundwater pumped from deep tube wells in the Kathmandu Valley of Nepal. *J. Water Health* 10:170. doi: 10.2166/wh.2011.086

Tringe, S. G., Mering, C. V., Kobayashi, A., Salamov, A. A., Chen, K., Chang, H. W., et al. (2005). Comparative metagenomics of microbial communities. *Science* 308, 554–557. doi: 10.1126/science.1107851

Upadhya, Y. D. R., and Seikh, S. M. (2015). *Nepal Quake Assessment Shows Need For Major Recovery Efforts* [Online]. Worldbank. Available online at: http://www.worldbank.org/en/news/press-release/2015/06/16/nepal-quake-assessment-shows-need-effective-recovery-efforts (Accessed February 4, 2016).

Uprety, S., Iwelunmor, J., Sadik, N., Dangol, B., and Nguyen, T. H. (in press). A qualitative case study of water, sanitation, and hygiene resources after Gorkha earthquake in Nepal. *Earthquake Spectra.* doi: 10.1193/112916EQS212M

Watson, J. T., Gayer, M., and Connolly, M. A. (2007). Epidemics after natural disasters. *Emerging Infect. Dis.* 13, 1–5. doi: 10.3201/eid1301.060779

Yan, T., and Sadowsky, M. J. (2007). Determining sources of fecal bacteria in waterways. *Environ. Monit. Assess.* 129, 97–106. doi: 10.1007/s10661-006-9426-z

Ying, J. Y., Zhang, L. M., and He, J. Z. (2010). Putative ammonia-oxidizing bacteria and archaea in an acidic red soil with different land utilization patterns. *Environ. Microbiol. Rep.* 2, 304–312. doi: 10.1111/j.1758-2229.2009.00130.x

Yu, Z., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* 36, 808–812. doi: 10.2144/3605A0808

Zhang, Y., Kelly, W. R., Panno, S. V., and Liu, W. T. (2014). Tracing fecal pollution sources in karst groundwater by Bacteroidales genetic biomarkers, bacterial indicators, and environmental variables. *Sci. Tot. Environ.* 490, 1082–1090. doi: 10.1016/j.scitotenv.2014.05.086

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 16 November 2017
doi: 10.3389/fmicb.2017.02200

Characterization of Metagenomes in Urban Aquatic Compartments Reveals High Prevalence of Clinically Relevant Antibiotic Resistance Genes in Wastewaters

*Charmaine Ng[1], Martin Tay[2], Boonfei Tan[2], Thai-Hoang Le[3], Laurence Haller[1], Hongjie Chen[1], Tse H. Koh[4], Timothy M. S. Barkham[5], Janelle R. Thompson[2] and Karina Y.-H. Gin[1,6]**

[1] Department of Civil and Environmental Engineering, National University of Singapore, Singapore, Singapore, [2] Centre for Environmental Sensing and Modeling, Singapore-MIT Alliance for Research and Technology Centre, Singapore, Singapore, [3] Department of Environmental Engineering, Ho Chi Minh City International University, Ho Chi Minh City, Vietnam, [4] Department of Pathology, Singapore General Hospital, Singapore, Singapore, [5] Department of Laboratory Medicine, Tan Tock Seng Hospital, Singapore, Singapore, [6] NUS Environmental Research Institute, National University of Singapore, Singapore, Singapore

OPEN ACCESS

Edited by:
Peiying Hong, King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Nur A. Hasan, University of Maryland Center for Bioinformatics and Computational Biology, United States
Ying Yang, University of Hong Kong, Hong Kong

***Correspondence:**
Karina Y.-H. Gin
ceeginyh@nus.edu.sg

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *26 July 2017*
Accepted: *26 October 2017*
Published: *16 November 2017*

Citation:
Ng C, Tay M, Tan B, Le T-H, Haller L, Chen H, Koh TH, Barkham TMS, Thompson JR and Gin KY-H (2017) Characterization of Metagenomes in Urban Aquatic Compartments Reveals High Prevalence of Clinically Relevant Antibiotic Resistance Genes in Wastewaters. Front. Microbiol. 8:2200. doi: 10.3389/fmicb.2017.02200

The dissemination of antimicrobial resistance (AMR) is an escalating problem and a threat to public health. Comparative metagenomics was used to investigate the occurrence of antibiotic resistant genes (ARGs) in wastewater and urban surface water environments in Singapore. Hospital and municipal wastewater ($n = 6$) were found to have higher diversity and average abundance of ARGs (303 ARG subtypes, 197,816 x/Gb) compared to treated wastewater effluent ($n = 2$, 58 ARG subtypes, 2,692 x/Gb) and surface water ($n = 5$, 35 subtypes, 7,985 x/Gb). A cluster analysis showed that the taxonomic composition of wastewaters was highly similar and had a bacterial community composition enriched in gut bacteria (*Bacteroides, Faecalibacterium, Bifidobacterium, Blautia, Roseburia, Ruminococcus*), the *Enterobacteriaceae* group (*Klebsiella, Aeromonas, Enterobacter*) and opportunistic pathogens (*Prevotella, Comamonas, Neisseria*). Wastewater, treated effluents and surface waters had a shared resistome of 21 ARGs encoding multidrug resistant efflux pumps or resistance to aminoglycoside, macrolide-lincosamide-streptogramins (MLS), quinolones, sulfonamide, and tetracycline resistance which suggests that these genes are wide spread across different environments. Wastewater had a distinctively higher average abundance of clinically relevant, class A beta-lactamase resistant genes (i.e., bla_{KPC}, bla_{CTX-M}, bla_{SHV}, bla_{TEM}). The wastewaters from clinical isolation wards, in particular, had a exceedingly high levels of bla_{KPC-2} genes (142,200 x/Gb), encoding for carbapenem resistance. Assembled scaffolds (16 and 30 kbp) from isolation ward wastewater samples indicated this gene was located on a Tn3-based transposon (Tn*4401*), a mobilization element found in *Klebsiella pneumonia* plasmids. In the longer scaffold, transposable elements were flanked by a toxin–antitoxin (TA) system and other metal resistant genes that likely increase the persistence, fitness and propagation of the plasmid in the bacterial host under conditions of stress. A few bacterial species

(*Enterobacter cloacae*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*) that were cultured from the isolation ward wastewaters on CHROMagar media harbored the bla_{KPC-2} gene. This suggests that hospital wastewaters derived from clinical specialty wards are hotspots for the spread of AMR. Assembled scaffolds of other mobile genetic elements such as IncQ and IncF plasmids bearing quinolone resistance genes (*qnrS1, qnrS2*) and the class A beta-lactamase gene (bla_{TEM-1}) were recovered in wastewater samples which may aid the transfer of AMR.

Keywords: comparative metagenomics, antibiotic resistant genes, wastewaters, hospital, municipal, water body, tributary, beta-lactamase resistant genes

INTRODUCTION

Antimicrobial resistance (AMR) is a growing global health threat due to concerns over the reduced clinically efficacy of current antibiotics in the treatment of bacterial infections, especially for hospital-acquired infections. The excessive use of last-resort antibiotics, such as extended-spectrum-beta-lactams (ESBLs) and carbapenems has resulted in an increased prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) which have spread globally due to poor infection control and a highly mobile and connected world (Queenan and Bush, 2007; Nordmann et al., 2011; Papp-Wallace et al., 2011). Gram-negative bacteria, specifically *Enterobacteriaceae* are common causes of community- and hospital acquired infections and frequently harbor multiple antibiotic resistance mechanisms (Vasoo et al., 2015). The drug resistance problem extends beyond the hospital setting. Extensive studies have demonstrated that wastewater, specifically hospital discharges, and wastewater treatment plants (WWTPs) are important reservoirs of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Volkmann et al., 2004; Zhang et al., 2009; Berglund et al., 2014; Al-Jassim et al., 2015; Li J. et al., 2015; Xu J. et al., 2015; Mao et al., 2015; Chagas et al., 2016). Wastewater contains an amalgam of human and animal excrement, commensal and pathogenic bacteria, high loads of nutrients, detergents, antimicrobial agents, and heavy metals that may harbor and preferentially select for ARB (Novo et al., 2013). Studies on removal rates of opportunistic pathogens in conventional wastewater treatment processes have shown the persistence of *Pseudomonas* spp. and *Aeromonas hydrophila* in chlorinated effluent and an increased abundance of *Mycobacterium* spp. (Al-Jassim et al., 2015). Documented evidence points to wastewater treatment processes inducing the propagation of selected ARG subtypes (Alexander et al., 2015; Li J. et al., 2015; Mao et al., 2015; Xu G. et al., 2015) and this has prompted investigations into assessing the microbial risk associated with reusing treated wastewater effluent in agricultural irrigation (Al-Jassim et al., 2015).

The globalization of medical healthcare, influx of travelers and a transient workforce has spawned the emergence of broader population consequences and the importation of pathogens harboring antibiotic resistance genes through carriage in the human microbiome. Singapore is a travel hub, receiving an average almost 15 million foreign tourists per year over the last 2 years (Singapore Tourism Board, 2014), with 70% of overseas visitors seeking medical treatment in Singapore hospitals coming from the South East Asian region (Horowitz and Rosensweig, 2007; Smith et al., 2009). Visitors engaging in medical tourism abroad may exacerbate the carriage and spread of pathogens and antibiotic resistant determinants upon returning home (Kennedy and Collignon, 2010; Kumarasamy et al., 2010; Lopez et al., 2010; Struelens et al., 2010; Rogers et al., 2011; Van der Bij and Pitout, 2012; Wilson and Chen, 2012; Chen and Wilson, 2013). Understanding the dynamics and occurrence of AR from hotspots (wastewater), through the wastewater treatment process and to the urban environment (surface waters) provides bearing on the extent of the spread of AMR in densely populated cities that are reliant on an urban water cycle.

The over-reliance of culture-based methods of single strains which is consider the standard in investigating clinical resistance has vastly underestimated and narrowed insights into the composition of resistomes in different environments (Dantas, 2017). Recently, there has been increased interest in utilizing metagenomics as a tool to evaluate antibiotic resistance in the human microbiota and different environments to assess the risk to human health (Bengtsson-Palme et al., 2017). The objective of this study was to characterize ARG profiles, and potential vectors of transfer such as mobile genetic elements (MGE) in wastewater; treated effluent and urban environmental surface waters using assembled metagenomes. Assembled metagenomes were interrogated against the comprehensive antibiotic resistant database (CARD[1]) and Resfam database[2] for the broad identification of ARGs in each ecological niche. The phylogenic composition of bacteria was compared between samples and scaffolds containing specific plasmid replicons were identified using PlasmidFinder[3]. Hospital wastewater is a high source of opportunistic pathogens, ARGs, antimicrobials and chemical agents. This work focuses on exploring the diversity of β-lactamase resistant genes and inspected the gene neighborhoods of scaffolds containing emergent carbapenem resistant genes (i.e., bla_{KPC}) to identify genetic elements that promote proliferation and persistence of these ARGs in the hospital setting.

[1]https://card.mcmaster.ca/

[2]http://www.dantaslab.org/resfams/

[3]https://cge.cbs.dtu.dk/services/PlasmidFinder/

MATERIALS AND METHODS

Samples and Sequencing

Five clinical wastewater samples were collected from two hospitals in Singapore. Two hospital blocks (i.e., block A of hospital 1 and block B of hospital 1) (1,597 beds) were sampled once a week over a period of 2 weeks from a manhole receiving direct sewage from each block. These two blocks were differentiated based on their ward types, with block A (H1, H2) consisting of clinical isolation wards and block B (H3, H4) consisting of general wards. For hospital 2 (H5; 1,500 beds), one sample was collected from the main manhole discharging mixed wastewater from the entire hospital. Three samples were collected at different treatment stages of a municipal wastewater treatment plant, an influent (WW) and effluent samples (TW1, TW2) from the Modified Ludzack-Ettinger (MLE) process whereby wastewaters undergo anoxic and aerobic treatment. Surface waters were collected from three urban tributaries (BH, BI, BB) and an urban water body (RA) located southeast of the island within the commercial district. As a comparison, surface waters were sampled from a forested water body in central part of Singapore (MA). More details of sampling sites are found in **Table 1**. A volume of 1 L of wastewater and 10 – 20 L of surface water was collected using sterile plastic bottles and transported to the laboratory for immediate processing. For DNA extraction, water samples were filtered on 0.45 μm cellulose nitrate membranes (Sartorius stedim, Goettingen, Germany) until the membrane was saturated for maximum biomass yield. DNA was extracted using a PowerWater DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, United States) according to the manufacturer's instructions. The quantity and quality of DNA was measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States).

Sequencing was performed at the Singapore Centre of Environmental Life Sciences and Engineering (SCELSE). Library preparation was performed according to Illumina's TruSeq Nano DNA Sample Preparation protocol. DNA samples were sheared on a Covaris S220 (Covaris, United States) to ∼450 bp following manufacturer's recommendation. Each library was uniquely tagged with one of Illumina's TruSeq DNA HT dual barcode combination to enable library pooling for sequencing. The finished libraries were quantitated using Invitrogen's Picogreen assay and the average library size was determined on a Bioanalyzer 2100, using a DNA 7500 chip (Agilent Technologies, United States). Library concentrations were normalized to 4 nM and validated by qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems, United States), using qPCR primers recommended in Illumina's qPCR protocol, and Illumina's PhiX control library as standard. These libraries were pooled at equimolar concentrations and sequenced in a lane on Illumina HiSeq2500 sequencer in rapid mode at a final concentration of 10 pM and a read length of 250 bp paired-end.

Metagenomic Assembly and ORF Prediction

The quality of the sequenced library was assessed using BBDuk (BBTools package) where reads were trimmed to remove adaptor sequences and base calls with Phred scores above Q20 were accepted. Only paired reads that were greater than 75 bp in length were retained. Subsequently BBDuk was also used for removal of reads homologous to PhiX phage, which is commonly used as a control on the Illumina sequencing platform. A total of 13 datasets were generated and the number of paired reads which passed the quality filtering ranged between 1,928,883 and 3,202,560 (Supplementary Table S1). Quality filtered paired reads were assembled using CLC workbench (Version 6.0.2, CLC Bio, Aarhaus, Denmark) using the default settings, and sequence assemblies were submitted to the Integrated Microbial Genomes and Microbiome Samples (IMG[4]) for ORF prediction and automated annotation. Metagenomic datasets were deposited under the IMG Genome IDs and raw sequence reads can be downloaded via the NCBI short read archive (SRA) under accession numbers SRR5997540 – SRR5997552 (**Table 1**).

[4]https://img.jgi.doe.gov/cgi-bin/mer/main.cgi

TABLE 1 | Water sample sources for antibiotic resistome profile analysis.

Source of sample	Sample description	Sample ID	IMG ID	SRA ID
Hospital wastewater discharge	Clinical isolation ward (Hospital 1, week 1)	H1	3300008488	SRR5997548
	Clinical isolation ward (Hospital 1, week 2)	H2	3300008070	SRR5997541
	General ward (Hospital 1, week 1)	H3	3300008069	SRR5997540
	General ward (Hospital 1, week 2)	H4	3300008487	SRR5997552
	Entire hospital (Hospital 2)	H5	3300008067	SRR5997551
Wastewater treatment plant	Municipal wastewater influent	WW	3300008071	SRR5997546
	Post anaerobic/aerobic treated effluent (Train 1)	TW1	3300008507	SRR5997542
	Post anaerobic/aerobic treated effluent (Train 2)	TW2	3300008065	SRR5997545
Surface waters	Urban tributary (Site 1)	BH	3300008066	SRR5997544
	Urban tributary (Site 2)	BI	3300008508	SRR5997549
	Urban tributary (Site 3)	BB	3300008509	SRR5997550
	Forested water body (Site 4)	MA	3300008072	SRR5997543
	Urban water body (Site 5)	RA	3300008510	SRR5997547

Plasmid Identification

To detect and characterize plasmids, which may function as vectors for the transfer of ARGs, assembled contigs were analyzed using PlasmidFinder 1.3[5] at a threshold of a 95% nucleotide sequence identity match. This database consists of 116 replicon sequences derived from 559 fully sequenced plasmids of multidrug resistant *Enterobacteriaceae*.

Identification of ARG-Like ORFs

Predicted ORFs were interrogated against an "Antimicrobial Resistance Protein Database" (AMRPD) using BLASTP (E-value $\leq 10^{-5}$). We created the AMRPD by combining protein sequences in the CARD database[6] and the Resfam AR Proteins database, v1.2[7], which yielded a total of 5,331 ARG protein sequences. A queried ORF was regarded as an ARG-like sequence under the criteria of >70% similarity with a coverage of >70%. All identified ARG-like ORFs were assigned to ARG subtypes (e.g., *sul1*, *sul2, sul3*) and subsequently organized into ARG types (antibiotic class, e.g., sulfonamides) using the CARD database as a reference. The number of ARG-like ORFs identified in each sample is presented in Supplementary Table S1. All ARG annotations and IMG gene IDs are found in Supplementary Table S8.

ARG Abundance Analysis

To estimate the abundance of ARG-like ORFs in the different water samples, coverage was calculated using the following formula as described in Ma et al. (2016).

Coverage of ARG-like ORF expressed as x/Gb:

$$\frac{(\text{Number of mapped read}) \times 250/\text{Length of ARG like ORF}}{\text{Size of metagenomic dataset (Gb)}}$$

Each ARG-like ORF was then assigned to an ARG subtype based on the BLASTP assignment according to the threshold mentioned previously, and the abundance for each of the ARG types was calculated by summing the coverage values of each ARG subtype classified to common antibiotic classes.

Microbial Community Structure

To characterize the microbial community structure between samples, predicted ORFs were aligned against the NCBI non-redundant (NR) protein database using DIAMOND with the default parameters. The similarity search results were analyzed through MEGAN 5[8] by assigning BLAST results to NCBI taxonomies with the lowest common ancestor (LCA) algorithm. Identified taxa are found in Supplementary Table S7.

Statistical Analysis

Primer version 7 (Clarke and Gorley, 2015) was used to analyze clustering patterns of the microbial community structure (at the genus level) in the various types of water samples. A log(X+1) transformation was applied to datasets and a resemblance matrix was calculated by Bray–Curtis analysis. Clustering patterns were statistically validated by an Analysis of Similarity (ANOSIM) procedure using 999 iterations to test the significance of the clustered groups. A SIMPER analysis was used to determine the similarities in microbial community composition between samples.

Phylogenetic Identification of *bla*$_{KPC}$ Bearing Bacteria in Wastewater Samples

CHROMagar^{TM} Orientation, CHROMagar^{TM} KPC and ESBL (CHROMagar, Paris, France) were used to isolate bacteria that were resistant to carbapenems and/or ESBLs in hospital wastewater. ESBL and KPC supplements were added to the base medium at a final concentration of 570 and 400 μg/mL according to manufacturer's instructions. Samples were serially diluted in 1x phosphate buffered saline (PBS, Vivantis Technologies, Malaysia) and 10 mL of sample was filtered on to 0.45 μm nitrocellulose membranes (Sartorius stedim, Goettingen, Germany). Plates were then incubated at 37°C for 24 h. Bacterial isolates that grew on plates were re-streaked onto fresh media to ensure pure cultures were obtained. Colony PCR was performed and 16S rRNA genes were amplified using 27F (5′-AGA GTT TGA TYM TGG CTC AG-3′) and 1492R (5′-GGY TAC CTT GTT ACG ACT T-3′), and primer set F (5′–ATG TCA CTG TAT CGC CGT CT-3′) and R (5′-TTT TCA GAG CCT TAC TGC CC-3′) to screen for the presence of the *bla*$_{KPC}$ gene (Bratu et al., 2005). PCR products were run on a 1% agarose gel and products purified using the Expin cleanup kit (GeneAll Biotechnology, Seoul, South Korea). Purified products were sent for capillary sequencing at AIT biotech (Singapore), and sequences were manually assessed using the Bioedit software and queried against the National Centre for Biotechnology Information (NCBI) 16S rRNA gene database for archaea and bacteria for taxonomic identification and the non-redundant database for *bla*$_{KPC}$ identification using BLASTN.

RESULTS

Characterization of the Antibiotic Resistome

A principal component analysis of microbial community structure at the genus level showed wastewater samples formed one cluster (H1, H2, H3, H4, H5, WW), which was significantly different ($p = 0.008$, ANOSIM) from treated effluent and surface water samples (TW1, TW2, BH, BI, BB, MA, RA) formed another (**Figure 1**). A SIMPER analysis showed an average dissimilarity of 62% in bacterial community structure between the two groups. Wastewaters were enriched with genera associated with gut microbiomes (*Bacteroides, Faecalibacterium, Bifidobacterium, Blautia, Roseburia,* and *Ruminococcus*), members of the *Enterobacteriaceae* group (*Klebsiella, Aeromonas, Enterobacter*) and other opportunistic pathogens (*Prevotella, Comamonas, Neisseria*) while the treated effluent and surface water group had less of these genera present, and more enriched in *Limnohabitans*, a planktonic bacteria. Metagenomes from

[5]https://cge.cbs.dtu.dk/services/PlasmidFinder/

[6]https://card.mcmaster.ca/

[7]http://www.dantaslab.org/resfams/

[8]https://ab.inf.uni-tuebingen.de/software/megan5

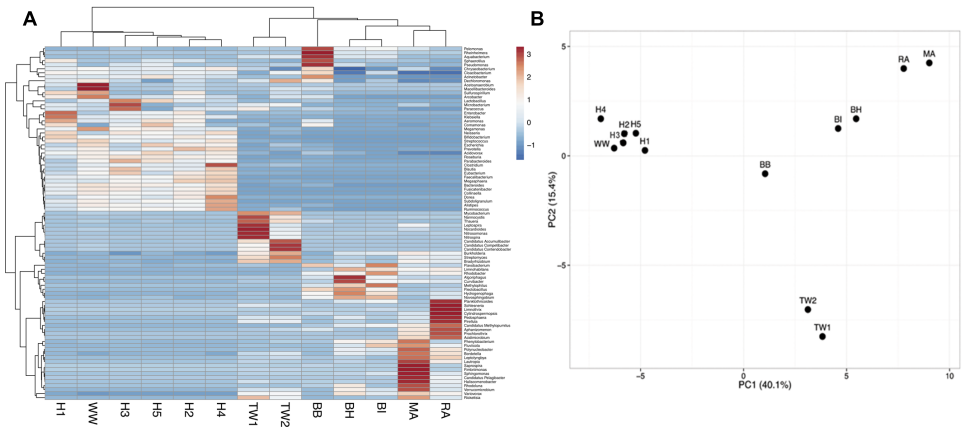


FIGURE 1 | (A) A heat map visualizing the distribution pattern of bacterial genera based on relative abundance (*z*-score). **(B)** Principal component analysis (PCA) of the bacterial community composition (genus-level) across all sample types. Only taxa present at >1% were included in the analysis. The bacterial community composition of wastewaters (H1-5, WW) was significantly different from treated effluent and surface waters (TW1-2, BH, BI, BB, MA, RA).

hospitals and municipal wastewater had a higher percentage of ARG-like ORFs identified (0.09–0.16%) compared to treated effluent and surface water (0.01–0.05%, Supplementary Table S1). These ORFs were assigned to 344 subtypes, which were classified into 22 main ARG types. Multidrug resistant efflux pumps, aminoglycoside and quinolone accounted for >66% of ARG subtypes identified in each sample (Supplementary Table S2).

The Shared Antibiotic Resistome

Wastewater had the highest diversity (303 subtypes) of ARGs followed by surface water (58 subtypes) and treated effluent (35 subtypes, Supplementary Figure S1). A core resistome of 21 ARG subtypes were shared between these three clusters, which included those encoding multidrug resistance efflux pumps (*adeJ*, *macB*, *mdtB*, *mexKT*, *msrE*, *pmrE*, RND antibiotic efflux pump), and resistance to aminoglycoside [*ant(3)*, *aph(3″)*, *aph(6)*, *ant(3″)-Ia*], macrolide (*ereA*), macrolide-lincosamide-streptogramin [MLS (*ermF*)], quinolone (fluoroquinolone resistant DNA topoisomerase, *qnrS2*), sulfonamides (*sul1*, *sul2*) or tetracycline (tet efflux pump, tet ribosomal protection protein, *tetX*) (Supplementary Table S3A). Genes encoding for *aph(3″)*, RND antibiotic efflux pumps and fluoroquinolone resistance DNA topoisomerase were detected in each sample with a coverage ranging between 73-87,965 x/Gb, 26-1,097 x/Gb, and 232-11,966 x/Gb, respectively (Supplementary Table S3A).

The wastewater and surface water shared 28 ARG subtypes, which spanned from genes encoding for resistance against aminoglycosides (*aph(3″)*, *aac(6′)-Ib9*), bacitracin (*bacA*), class A (*cfxA6*) and class D beta-lactamases (bla_{OXA}), chloramphenicol (*cat*), MLS (*cfr*), polymyxin (*arnA*), trimethoprim (*dfrAE*) and multidrug resistance efflux pumps (*crp, phoP, emrB, mdfA, mdtH, adeC-adeK-oprM, baeRS, msbA, adeGB, mexEFB, abeM, oprN, evgS*) (Supplementary Figure S1 and Table S3B). The treated effluents showed the least similarity with 9 and 1 ARG subtype ($bla_{\mathrm{OXA-198}}$) shared between the wastewater and surface waters (Supplementary Figure S1 and Table S3C). The wastewater and treated effluents had common genes encoding for resistance to aminoglycosides [p*aph(6)-Id*], multidrug resistance efflux pumps (ABC antibiotic efflux pump), MLS (*ermB*), beta-lactamases ($bla_{\mathrm{VEB-1a}}$, $bla_{\mathrm{OXA-347}}$), vancomycin (*vanX*), tetracycline (*tetO*), streptogramin (*vatB*) and sulfonamide (*sul3*) (Supplementary Table S3D).

Dominant ARG Subtypes across All Samples

The abundance of each of the 22 ARG types detected was calculated based on summing the coverage of ARG subtypes belonging to the same ARG type (Supplementary Table S4). A cluster analysis showed that wastewater samples had a higher similarity in antibiotic resistance profiles forming one cluster, while the treated effluent and surface water formed another (**Figure 2**). The wastewater cluster had a higher average abundance of ARGs (197,816 x/Gb) compared to the treated effluent (2,692 x/Gb) and surface water (7,985 x/Gb) clusters. Multidrug resistant efflux pumps, class A beta-lactamase and aminoglycoside resistance genes were the most abundant ARG types in the wastewater cluster, accounting for an average of 77,056, 53,034 and 24,524 x/Gb, respectively, while the treated effluent and surface water cluster was more abundant in aminoglycoside (2,626 x/Gb), multidrug resistant efflux pumps (1,857 x/Gb) and quinolone resistant genes (1,523 x/Gb).

The most abundant gene, $bla_{\mathrm{KPC-2}}$ (277,258 x/Gb) was detected in hospital sample H1 and at lower levels in hospital sample H2 (7,141 x/Gb) (Supplementary Table S5). Of the top 3 most abundant ARGs detected in each sample, two of the most commonly detected ARG was a quinolone resistant gene (fluoroquinolone resistant DNA topoisomerase) which was identified across 7 samples [H2 (11,966 x/Gb), TW1 (383 x/Gb), BH (464 x/Gb), BI (836 x/Gb), BB (2,887 x/Gb), MA (5,446 x/Gb), RA (232 x/Gb)] and aminoglycoside resistant gene *aph(3″)* gene was identified across six samples [H1 (87,964 x/Gb), TW2 (637 x/Gb), BI (1,025 x/Gb), BB (1,302 x/Gb), MA (14,200 x/Gb), RA (371 x/Gb), Supplementary Figure S2

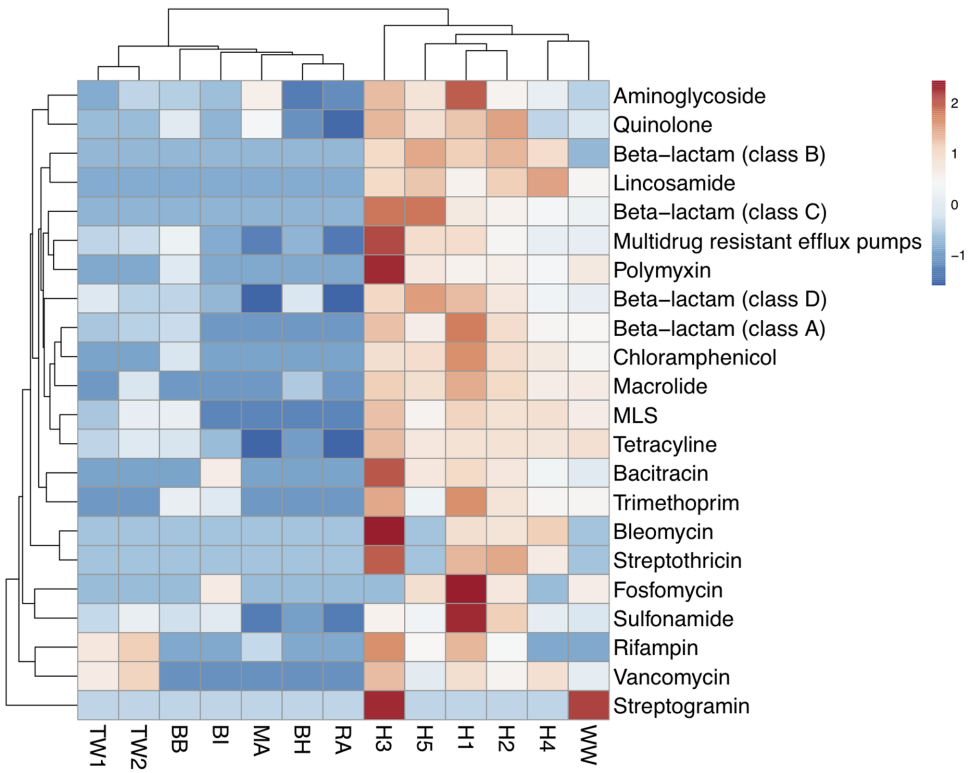


FIGURE 2 | Heat map of the abundance of ARG-like ORFs assigned to ARG types across wastewaters (WW, H1-5), treated effluents (TW1, 2) and surface waters (BI, BH, BB, RA, MA). Abundance values were transformed using log (x+1). Clustering based on euclidean distances showed two distinct groupings of the antibiotic resistomes of the wastewater samples, the treated effluents, and environmental waters.

and Table S3A]. These two genes were detected in all samples but at varying abundances (Supplementary Table S3A). Among these ARGs, other subtypes belonging to multidrug efflux pumps, aminoglycosidase, quinolone and beta-lactam resistant genes were also abundant in the hospital wastewater samples (Supplementary Figure S2).

The municipal wastewater was dominated by tetracycline resistance (*tet* ribosomal protein), class A beta-lactamase (*cfxA6*) and macrolide resistance genes (*ereA*), and treated wastewater consistently with vancomycin resistant genes (*vanX*) (Supplementary Figure S2).

Occurrence of β-Lactam Resistance Genes

Beta-lactamase genes in wastewater represented between 8 and 54% of total abundance of ARGs in wastewater samples, which was much higher compared to treated effluents (1–3%) and surface waters (0–8%). The clinically important β-lactamase gene variants identified in all samples is found in Supplementary Table S5. The bla_{OXA} genes were the most ubiquitous and detected in all samples except for surface waters MA and RA (Supplementary Figure S3 and Table S5). The highest abundance of bla_{OXA} genes was mainly in hospital wastewaters from H5 (7,094 x/Gb) and H1 (3,541 x/Gb, Supplementary Figure S3). For class C, the *AmpC* beta lactamase gene was only detected in wastewater samples at particularly high abundance in H5 (25,995 x/Gb) and H3 (23,568 x/Gb, Supplementary Figure S3). Class B beta-lactamases which consisted of bla_{IMP}, bla_{NDM} and bla_{VIM} were present in lower abundance and only in hospital wastewater samples (Supplementary Figure S3 and Table S5).

We detected $bla_{\mathrm{KPC-2}}$ and $bla_{\mathrm{NDM-1}}$, $bla_{\mathrm{NDM-2}}$, and $bla_{\mathrm{NDM-3}}$ in hospital discharge samples only (Supplementary Table S5). The $bla_{\mathrm{KPC-2}}$ gene was the most abundant ARG in hospital discharge samples, H1 (277,258 x/Gb), and the second most abundant ARG in H2 (7,141 x/Gb, Supplementary Figure S3 and Table S5). The bla_{NDM} (metallo-beta-lactamase, class B) genes were less abundant (8-19 x/Gb) but detected in 4 out of the 5 hospital wastewater samples (Supplementary Figure S3 and Table S5). Other metallo-beta-lactamase class B genes, bla_{VIM} (6-56 x/Gb) and bla_{IMP} (12-142 x/Gb) were detected in all hospital wastewater samples at higher abundance than bla_{NDM} genes. These included $bla_{\mathrm{VIM-2,3}}$, $bla_{\mathrm{VIM-11,12}}$, $bla_{\mathrm{VIM-18}}$, $bla_{\mathrm{VIM-26}}$, $bla_{\mathrm{VIM-30}}$, and $bla_{\mathrm{IMP-1}}$, $bla_{\mathrm{IMP-4}}$ $bla_{\mathrm{IMP-6}}$, $bla_{\mathrm{IMP-42}}$.

Hospital Wastewater Bacteria Bearing Dominant bla_{KPC} Genes

The scaffold carrying the $bla_{\mathrm{KPC-2}}$ gene in H1 (Ga0110937_100002322, 30,400 bp) and H2 (Ga0110938_100066613, 16,846 bp) samples were compared to *Klebsiella pneumoniae*

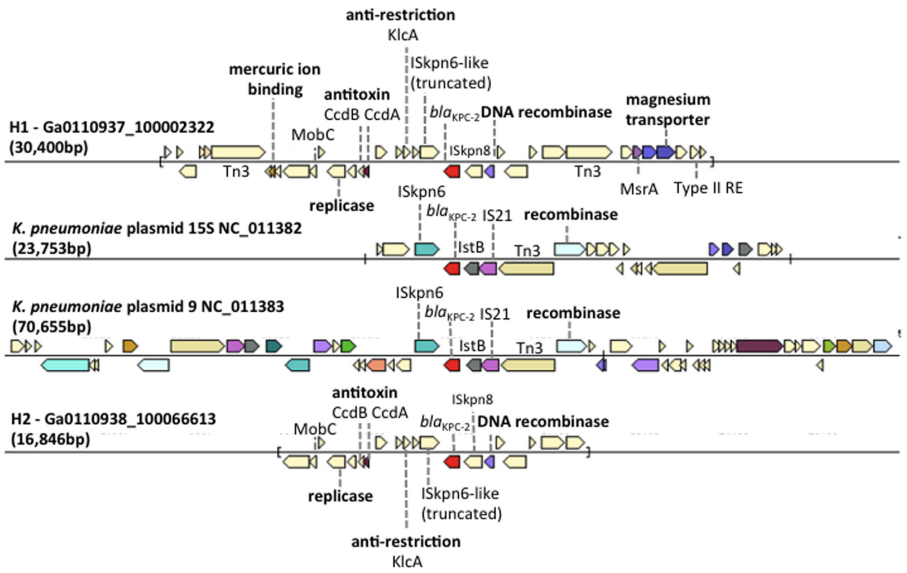


FIGURE 3 | Comparison of gene neighborhood in other genomes containing the bla_{KPC-2} gene in H1, *Klebsiella pneumoniae* plasmid 15S (NC_011382), *K. pneumoniae* plasmid 9 (NC_011383), and H2. Genes of similar colors belong to the same orthologous groups. The plasmid borne Tn4410 elements were labeled on *K. pneumoniae* plasmid 15S (NC_011382) and *K. pneumoniae* plasmid 9 (NC_011383).

plasmid sequences in the IMG database (**Figure 3**). The bla_{KPC-2} gene sequence and gene context had the best match to the *K. pneumoniae* plasmid 15S (NC_011382) and *K. pneumoniae* plasmid 9 (NC_011383) in the IMG database, both of which were isolated from *Klebsiella* strains from a hospital in New York. The genetic contexts of bla_{KPC-2} gene on the H1 and H2 scaffolds were identical suggesting that this scaffold is a partial plasmid sequence that may be responsible for the high genotypic resistance toward carbapenem antibiotics observed in the hospital wastewater samples. On the H1 and H2 scaffolds, genes encoding plasmid mobility (MobB), replication, antitoxin proteins (CcdA/B), anti-restriction encoded protein (KlcA) and a site-specific DNA recombinase site flanking the bla_{KPC-2} gene, were present. The slightly longer H1 scaffold possessed magnesium, mercuric ion binding genes. These genes likely play a role in mechanisms that allow the genetic modification of plasmids for increased fitness, metal/antibiotic resistance, and MGE acquisition for host propagation.

To verify species of bacteria carrying the bla_{KPC} gene, bacteria resistant to carbapenems (KPC) and extended-spectrum β-lactams (ESBL) were isolated from hospital wastewater samples. Bacterial strains belonging to *K. pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa,* and *Citrobacter freundii* that carried the bla_{KPC-2} gene had 100% sequence identity to the bla_{KPC-2} gene identified in the H1 and H2 samples (**Table 2**). These taxa of bacteria are candidates that may harbor the bla_{KPC-2} bearing plasmid.

TABLE 2 | Cultured bacterial isolates bearing the bla_{KPC-2} gene.

Sample	Isolate number	Media	Bacterial species
H1	H2	ESBL	*Klebsiella pneumoniae*
H1	H6	KPC	*Enterobacter cloacae*
H1	C20	OR	*Pseudomonas aeruginosa*
H2	H42	KPC	*Citrobacter freundii*

Abbreviations are as follows: CHROMagar orientation (OR), and CHROMagar supplemented with (ESBL) and (KPC).

Plasmids Associated with Antimicrobial Resistant *Enterobacteriaceae*

All wastewater and surface water sample BB had at least one contig matching replicon sequences of IncQ2 plasmid in Plasmidfinder (Supplementary Table S6). Plasmidfinder identified a total of 72 plasmids, in the assembled metagenomes; however, only 3 scaffolds were found to carry ARGs. This included the class A beta lactamase gene, bla_{TEM-1} (Ga0110939_10001761) in H3, and *qnrS1* genes in H1 (Ga0110937_100163213), and *qnrS2* in WW (Ga0110933_12248201) within the same scaffold as plasmid replicons (**Figure 4**).

DISCUSSION

The main findings of this study show that wastewater, particularly hospital discharge, are primary hotspots for AMR, and carry a high diversity and abundance of ARGs compared to surface waters and treated effluents. This is consistent with a recent *in silico* survey of ARGs from different ecological niches by Fitzpatrick and Walsh (2016) whereby the highest ARG diversity was described in the human gut microbiome, followed by hospital WWTP and non-hospital WWTP and environmental waters. Pal et al. (2016) investigated the structure and diversity of human, animal,

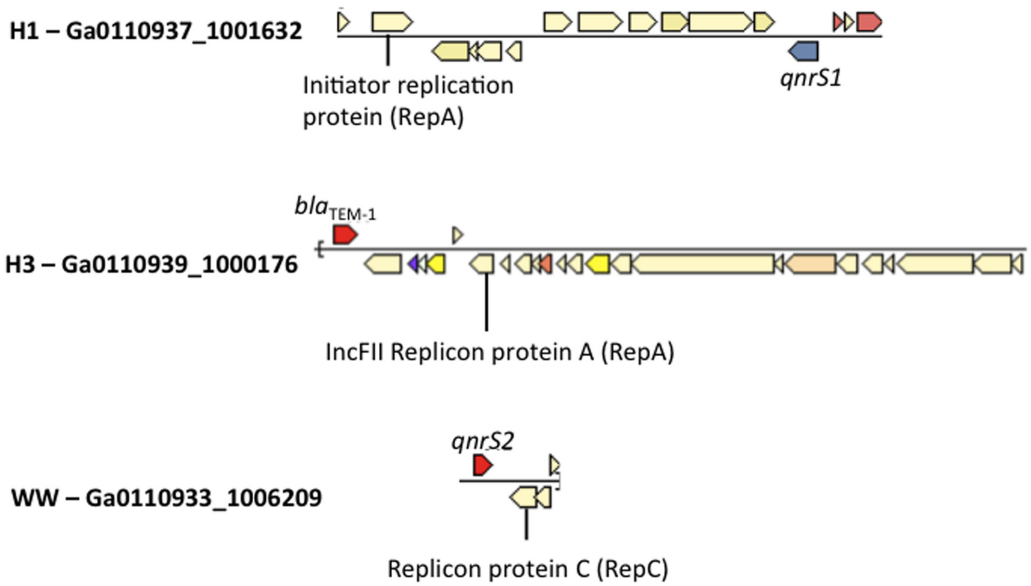


FIGURE 4 | Co-occurrence of *Enterobacteriaceae* plasmid replicons and ARGs in metagenomic scaffolds in H1, H3 and WW samples.

and environmental resistomes and found a similar trend. The wastewater cluster was enriched with commensal human gut microbiota (e.g., *Bacteroides, Faecalibacterium, Bifidobacterium, Blautia, Roseburia, Ruminococcus*) that is likely derived from the fecal shedding carrying traces of bacteria from the gastrointestinal tract (Lozupone et al., 2012; Pal et al., 2016; Pop et al., 2016). A study of multidrug resistant- and ESBL producing bacteria in Brazilian hospital effluent described resistant species belonging to *Klebsiella, Aeromonas, Enterobacter*, *Escherichia coli* (Chagas et al., 2016). The enrichment of the same *Enterobacteriaceae* (*Klebsiella, Aeromonas, Enterobacter*) taxa in the wastewaters cluster suggests that members within the community are potential carriers and a source of clinically important ARGs.

The ARG types detected in the core resistome have been reported in studies across environmental waters (sediments, soil, ocean), drinking water, and the influent and effluents of WWTP (Nesme et al., 2014; Li B. et al., 2015). The aminoglycoside resistant gene *aph(3″)* which was detected in all samples in this study, and was the top 3 most abundant ARG in 6 of the 13 samples appears to be widespread in different environments with variants of this same gene [*aph(3″)-IIa, aph(3′)-Ib*] described in other environmental datasets of similar nature to the ones in this study (Pal et al., 2016). The wastewater resistome had more ARGs in common with surface waters than treated effluent. The shared resistome between wastewater and surface waters were composed mainly of multidrug resistant efflux pumps, and genes resistant to aminoglycosides, bacitracin, beta-lactams (beta-lactamase class A and D), chloramphenicol, MLS, polymyxin and trimethoprim. In a recent study of ARGs of surface waters in Singapore, genes associated with a few these ARG types (multidrug resistant efflux pumps, beta-lactamase A–D) were detected in using the GeoChip (Low et al., 2016). Among the beta-lactamases detected in the wastewater, two genes which confer resistance to cephalosporins; class A beta-lactamase gene *cfxA6*, a gene commonly found in the human gut microbiome (Hu et al., 2013) and class D beta-lactamase gene bla_{OXA} which are plasmid mediated and associated with species of *Acinetobacter* (Evans and Amyes, 2014) were found in surface waters. Although the microbial community composition of surface waters have a relatively low representation of taxa (i.e., gut bacteria or opportunistic pathogens) which potentially carry these genes, traces of non-point source contamination coming from surface runoffs in the surrounding urban areas may facilitate contribute to beta-lactamases observed in the surface water resistomes.

Wastewaters had the highest diversity and abundance of clinically important β-lactam resistance genes. Some of these beta-lactamases confer resistance to ESBLs and carbapenems, which is a last resort antibiotic used in treating Gram-negative infections. The first KPC producing *K. pneumoniae* was identified in 2001 in North Carolina, which has since spread globally (Nordmann et al., 2011; Yigit et al., 2011). KPC producing *K. pneumonia* arrived in Asia in 2004 spreading from China to South Korea and Taiwan. In 2012, cases emerged in Singapore where two of four patients were found to harbor the China related strains. The detection in two other persons of non-Chinese origin with had no travel history suggesting possible community dissemination (Balm et al., 2012; Ling et al., 2015). National surveillance of AMR and antibiotic prescription has shown a dramatic increase in the occurrence of cephalosporin and carbapenem resistant *Enterobactericeae* (CRE) in local hospitals over the years (Hsu et al., 2007, 2010; Liew et al., 2011; Ng et al., 2014; Venkatachalam et al., 2014; Young et al., 2014). The CRE trend obtained from rectal screening of inpatients at local government hospitals

showed a positive CRE increase from an average of 16.0 per 100,000 patient-days from 2013 to 2015, in which bla_{KPC} was the predominant carbapenemase gene detected (Marimuthu et al., 2017). Hence, it is not surprising that a strikingly high abundance of carbapenemase gene bla_{KPC-2} was detected in hospital discharge from clinical isolation wards. Furthermore, isolates of *Enterobacteriaceae* (i.e., *K. pneumonia*, *E. cloacae,* and *C. freundii*) and *P. aeruginosa* harboring the bla_{KPC-2} gene were cultured from the same hospital wastewater samples indicating that this gene was present in a variety of different species.

Two scaffolds, assembled from the clinical isolation wastewater samples, carried the abundant bla_{KPC-2} gene on a plasmid borne *Tn4410* like element. Other metal resistant genes, (mercuric, magnesium) and a toxin–antitoxin (TA) system encoded genes (*ccdB*, *ccdA*) were also detected on the scaffold. Plasmid-based TA systems are involved in post-segregation killing or growth inhibition of daughter cells that do not inherit a plasmid copy during cell division (Hayes, 2003). The TA system is a form of bacterial persistence, a phenotype of dormant cells present at a low frequency in a growing population and characterized by tolerance to the presence of a variety of antibiotics (Balaban et al., 2004; Lewis, 2010). The occurrence of the TA system on a plasmid bearing the bla_{KPC-2} gene suggests that these functional genes are important mechanisms for the selection and persistence of KPC resistant phenotype in the hospital wastewaters.

The first case of NDM-1 was described in 2009 when a Swedish patient of Indian origin who traveled to New Delhi contracted a urinary tract infection resulting from NDM-1 producing *K. pneumoniae* (Yong et al., 2009). The first local cases of NDM-1 was identified in 2010 in an Indian and Bangladeshi national, and since then there has been a progressive increase of locally transmitted cases (Chien et al., 2012) mediated by bla_{NDM} bearing plasmids in *K. pneumoniae* and *E. coli* (Khong et al., 2016). In our study, a range of other carbapenemases including class B metallo-beta lactamases (bla_{NDM}, bla_{IMP}, bla_{VIM}), were detected at lower abundance than bla_{KPC-2}. The microbial community structure of wastewater was enriched in the *Enterobacteriaceae* group (*Klebsiella, Enterobacter*) and other opportunistic pathogens (*Comamonas*), which are the main taxa found carrying *bla-* genes as described in our previous study of hospital wastewaters (Le et al., 2016). The overrepresentation of these taxa in wastewaters offers an explanation for the high prevalence of beta-lactam resistance genes in the wastewater cluster. Fecal waste coming from patients colonized with ESBL and carbapenem resistant bacteria likely contribute to the high abundance of genes encoding ESBL (bla_{CTX}, bla_{TEM}, bla_{OXA}, bla_{SHV}) and carbapenem (bla_{KPC}, bla_{NDM}) resistance genes observed in hospital wastewaters. Our dataset also shows that community sewage may not have as large of an impact on the spread on ESBL and carbapenem genes given that these genes were present at lower abundance in municipal wastewaters, relative to hospital wastewater.

Partial plasmids sequences were assembled from wastewater datasets, one of which carried the bla_{TEM-1} gene (H3), and the two others quinolone resistance genes *qnrS1* (H1) and *qnrS2* (WW). IncQ plasmids bearing *qnrS2* genes are highly mobile and have been isolated in bacterial communities of WWTP elsewhere (Bonemann et al., 2005). The presence of these MGEs carrying quinolone resistance genes in local municipal wastewaters suggests the potential genetic exchange of quinolone resistant genes between bacterial species in community sewage. Although we were unable to detected ARGs on the IncQ2 plasmid in surface waters, which was perhaps due to low sequence coverage, hence poor scaffold assemblies, the detection of an IncQ2 plasmid in one of the surface water samples (BB) supports the notion that these plasmids may play a disseminating role of quinolone resistance in surface waters.

Wastewater treatment plants represent another significant hotspot for ARG transfer facilitated by high cell densities, and the mixing of sub-inhibitory concentrations of antibiotics (Rizzo et al., 2013; Fitzpatrick and Walsh, 2016). A review of urban WWTPs concluded that a large diversity of ARGs conferring resistance to almost all mechanisms of antibiotic resistance are capable of surviving the wastewater treatment process (Rizzo et al., 2013). The treatment process has been shown to induce the abundance of tetracycline (*tet* genes), sulfonamide (*sul* genes), quinolone (*gyr*, *qnr*, *par* genes), beta-lactam (bla_{VIM}, *ampC* genes), vancomycin (*vanA*), ARGs (Alexander et al., 2015; Li J. et al., 2015; Mao et al., 2015; Xu J. et al., 2015). We were unable establish the selective enrichment of specific ARGs in treated effluent due to insufficient coverage of low abundance ARGs in the untreated waters. However, within the context of our study the detection of a few ARGs in shared resistome of wastewater and treated effluents suggested incomplete removal of certain ARGs after the secondary treatment process. This included genes resistant to aminoglycosides [*aph(6)-ld*], multidrug efflux pumps (ABC efflux pumps), MLS (*ermB*), sulfonamide (*sul3*), tetracycline (*tetO*), beta-lactam (bla_{VEB-1a}, $bla_{OXA-347}$), vancomycin (*vanX*) and streptogramin (*vatB*).

CONCLUSION

The metagenomic approach used in this study has unveiled a vast array of ARGs within different ecological niches enabling a comparative analysis of resistomes to track of AMR dissemination patterns in Singapore. Although ARGs are found almost everywhere, all environments do not pose the same risk (Martinez et al., 2015). It is proposed that environments with higher abundance and diversity of ARGs are likely involved with higher probability of transfer due to increase chances of for potential donor strains to physically interact with suitable recipients (Bengtsson-Palme and Larsson, 2015). We conclude that wastewater habitats, particularly hospital wastewaters contain high loads of opportunistic pathogens, and higher diversity and abundance clinically important ESBL and carbapenem resistant genes. The detection of plasmid-encoded bla_{KPC} in other bacterial species in local hospital wastewaters is evidence that it is highly transmissible to other bacteria.

Countries including France, Portugal, Brazil, China are facing increased frequency of the recovery of KPC producing *Enterobacteriaceae* in environmental rivers (Woodford et al., 2014; Xu G. et al., 2015). This raises questions as to whether hospitals should develop waste management strategies and invests in pre-treatment membrane technology (ultra- or nanofiltration) prior to discharging hospital wastewaters into public sewers. This together with antibiotic stewardship programs could help reduce the propagation and dissemination of antibiotic resistance beyond the hospital setting (Hocquet et al., 2016). Efforts to monitor WWTP and surface waters should continue to gain better perspectives into dissemination within the general community.

AUTHOR CONTRIBUTIONS

CN wrote the manuscript and TK and TB provided clinical wastewater samples. CN, T-HL, LH, and HC conducted sampling and performed the experiments. CN, MT, and BT analyzed datasets. JT provided computational resources and supervision for data analysis. CN, MT, BT, and KG conceived and designed the experiments.

FUNDING

This research was funded by the Ministry of Education (MOE), Academic Research Fund (AcRF) Tier 2 grant (reference: MOE2015-T2-2-130), and The Public Utilities Board (PUB) Research & Development Project (reference: RND-WAQU-1510-0013). We thank the National University of Singapore (NUS) for supporting this research.

ACKNOWLEDGMENTS

We would like to thank Dr. Sally Partridge from The University of Sydney and Dr. Adrian Low from NUS Environmental Research Institute (NERI), National University of Singapore for helpful discussions on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02200/full#supplementary-material

REFERENCES

Alexander, J., Bollmann, A., Seitz, W., and Schwartz, T. (2015). Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria. *Sci. Total Environ.* 51, 316–325. doi: 10.1016/j.scitotenv.2015.01.046

Al-Jassim, N., Ansari, M. I., Harb, M., and Hong, P. Y. (2015). Removal of bacterial contaminants and antibiotic resistance genes by conventional wastewater treatment processes in Saudi Arabia: is the treated wastewater safe to reuse for agricultural irrigation? *Water Res.* 73, 277–290. doi: 10.1016/j.watres.2015.01.036

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L., and Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science* 305, 1622–1625. doi: 10.1126/science.1099390

Balm, M. N., Ngan, G., Jureen, R., Lin, R. T., and Teo, J. (2012). Molecular characterization of newly emerged blaKPC-2-producing *Klebsiella pneumoniae* in Singapore. *J. Clin. Microbiol.* 50, 475–476. doi: 10.1128/JCM.05914-11

Bengtsson-Palme, J., and Larsson, D. G. J. (2015). Antibiotic resistance genes in the environment and prioritizing risks. *Nat. Rev. Microbiol.* 13, 396–397. doi: 10.1038/nrmicro3399-c1

Bengtsson-Palme, J., Larsson, D. G. J., and Kristiansson, E. (2017). Using metagenomics to investigate human and environmental resistomes. *J. Antimicrob. Chemother.* doi: 10.1093/jac/dkx199 [Epub ahead of print].

Berglund, B., Fick, J., and Lindgren, P. E. (2014). Urban wastewater effluent increases antibiotic resistance gene concentrations in a receiving northern European river. *Environ. Toxicol. Chem.* 34, 192–196. doi: 10.1002/etc.2784

Bonemann, G., Stiens, M., Puhler, A., and Schluter, A. (2005). Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, qnrS2, isolated from the bacterial community of a wastewater treatment plant. *Antimicrob. Agents Chemother.* 50, 3075–3080. doi: 10.1128/AAC.00378-06

Bratu, S., Tolaney, P., Karumudi, U., Quale, J., Mooty, M., Nichani, S., et al. (2005). Carbapenemase-producing *Klebsiella pneumoniae* in Brooklyn, NY: molecular epidemiology and in vitro activity of polymyxin and other agents. *J. Antimicrob. Chemother.* 56, 128–132. doi: 10.1093/jac/dki175

Chagas, T. P. G., Seki, L. M., Cury, J. C., Oliveira, J. A. L., Davila, A. M. R., and Silva, D. M. (2016). Mutiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil. *J. Appl. Microbiol.* 111, 572–581. doi: 10.1111/j.1365-2672.2011.05072.x

Chen, L. H., and Wilson, M. E. (2013). The globalization of healthcare: implications of medical tourism for the infectious disease clinician. *Clin. Infect. Dis.* 57, 1752–1759. doi: 10.1093/cid/cit540

Chien, J. M., Koh, T. H., Chan, K. S., Chuah, T. H., and Tan, T. T. (2012). Successful treatment of NDM-1 *Klebsiella pneumoniae* bacteraemia in a neutropenic patient. *Scand. J. Infect. Dis.* 44, 312–314. doi: 10.3109/00365548.2011.633549

Clarke, K. R., and Gorley, R. N. (2015). *PRIMER v7: User Manual/Tutorial*. Plymouth: PRIMER-E, 296.

Dantas, G. (2017). Networks of exchanging antibiotic resistance between environmental, commensal, and pathogenic microbes. *FASEB J.* 1, 404–401.

Evans, B. A., and Amyes, S. G. (2014). OXA ß-Lactamases. *Clin. Microbiol. Rev.* 27, 241–263. doi: 10.1128/CMR.00117-13

Fitzpatrick, D., and Walsh, F. (2016). Antibiotic resistance genes across a variety of metagenomes. *FEMS Microb. Ecol.* 92, 1–6. doi: 10.1093/femsec/fiv168

Hayes, F. (2003). Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science* 301, 1496–1499. doi: 10.1126/science.1088157

Hocquet, D., Muller, A., and Bertrand, X. (2016). What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. *J. Hosp. Infect.* 93, 95–402. doi: 10.1016/j.jhin.2016.01.010

Horowitz, M. D., and Rosensweig, J. A. (2007). Medical tourism: globalization of the healthcare marketplace. *Med. Gen. Med.* 9:33.

Hsu, L. Y., Tan, T. Y., Jureen, R., Koh, T. H., Krishnana, P., Lin, R. T., et al. (2007). Antimicrobial drug resistance in Singapore hospitals. *Emerg. Infect. Dis.* 13, 1944–1947. doi: 10.3201/eid1312.070299

Hsu, L. Y., Tan, T. Y., Tam, V. H., Kwa, A., Fisher, D. A., and Koh, T. H. (2010). Network for antimicrobial resistance surveillance (Singapore). Surveillance and correlation of antibiotic prescription and resistance of gram-negative bacteria in Singaporean hospitals. *Antimicrob. Agents Chemother.* 54, 1173–1178. doi: 10.1128/AAC.01076-09

Hu, Y., Qin, J., Cheng, G., Wu, N., Pan, Y., Li, J., et al. (2013). Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat. Commun.* 4, 2151. doi: 10.1038/ncomms3151

Kennedy, K., and Collignon, P. (2010). Colonisation with *Escherichia coli* resistant to "critically important" antibiotics: a high risk for international travellers. *Eur. J. Clin. Microbiol. Infect. Dis.* 29, 1501–1506. doi: 10.1007/s10096-010-1031-y

Khong, W. X., Marimuthu, K., Teo, J., Ding, Y., Xia, E., Lee, J. J., et al. (2016). Tracking inter-institutional spread of NDM and identification of a novel NDM-positive plasmid, pSg1-NDM using next-generation sequencing approaches. *Antimicrob. Agents Chemother.* 11, 3081–3089. doi: 10.1093/jac/dkw277

Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., and Balakrishnan, R. (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect. Dis.* 10, 597–602. doi: 10.1016/S1473-3099(10)70143-2

Le, T. H., Ng, C., Chen, H., Yi, X. Z., Koh, T. S., Barkham, T. M. S. B., et al. (2016). Occurrences and characterization of antibiotic-resistant bacteria and genetic determinants of hospital wastewater in a tropical country. *Antimicrob. Agents Chemother.* 62, 7449–7456. doi: 10.1128/AAC.01556-16

Lewis, K. (2010). Persister cells. *Annu. Rev. Microbiol.* 64, 357–372. doi: 10.1146/annurev.micro.112408.134306

Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J. M., et al. (2015). Metagenomic and network analysis reveal wide distribution and co-occurance of environmental antibiotic resistance genes. *ISME J.* 9, 2490–2502. doi: 10.1038/ismej.2015.59

Li, J., Cheng, W., Xu, L., Strong, P. J., and Chen, H. (2015). Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. *Environ. Sci. Pollut. Res. Int.* 22, 4587–4596. doi: 10.1007/s11356-014-3665-2

Liew, Y. X., Krishnan, P., Yeo, C. L., Tan, T. Y., Lee, S. Y., Lim, W. P., et al. (2011). Network for antimicrobial resistance surveillance (Singapore). Surveillance of broad-spectrum antibiotic prescription in Singaporean hospitals: a 5-year longitudinal study. *PLOS ONE* 6:e28751. doi: 10.1371/journal.pone.0028751

Ling, M. L., Tee, Y. M., Tan, S. G., Amin, I. M., How, K. B., Tan, K. Y., et al. (2015). Risk factors for acquisition of carbapenem resistant *Enterobacteriaceae* in an acute tertiary care hospital in Singapore. *Antimicrob. Resist. Infect. Control* 4:26. doi: 10.1186/s13756-015-0066-3

Lopez, J. A., Correa, A., Navon-Venezia, S., Correa, A. L., Torres, J. A., Briceno, D. F., et al. (2010). Intercontinental spread from Israel to Colombia of a KPC-3-producing *Klebsiella pneumoniae* strain. *Clin. Microbiol. Infect.* 17, 52–56. doi: 10.1111/j.1469-0691.2010.03209.x

Low, A., Ng, C., and He, J. (2016). Identification of antibiotic resistant bacteria community and a GeoChip based study of resistome in urban watershed. *Water Res.* 106, 330–338. doi: 10.1016/j.watres.2016.09.032

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, K. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 13, 220–230. doi: 10.1038/nature11550

Ma, L., Xia, Y., Yang, Y., Li, L., Tiedje, J. M., and Zhang, T. (2016). Metagenomic assembly reveals hosts of antibiotic resistance genes and the shared resistome in pig, chicken and human feces. *Environ. Sci. Technol.* 50, 420–427. doi: 10.1021/acs.est.5b03522

Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., et al. (2015). Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Res.* 85, 458–466. doi: 10.1016/j.watres.2015.09.010

Marimuthu, K., Venkatachalam, I., Khong, W. X., Koh, T. H., Cherng, B. P. Z., La, M. V., et al. (2017). Clinical and molecular epidemiology of carbapenem-resistant *Enterobacteriaceae* among adult inpatients in Singapore. *Clin. Infect. Dis.* 64, 68–75. doi: 10.1093/cid/cix113

Martinez, J. L., Coque, T. M., and Baquero, F. (2015). What is a resistance gene? Ranking risk in resistomes. *Nat. Rev. Microbiol.* 13, 116–123. doi: 10.1038/nrmicro3399

Nesme, J., Cecillon, S., Delmont, T. O., Monier, J. M., Vogel, T. M., and Simonet, P. (2014). Large-scale metagenomic-based study of antibiotic resistance in the environment. *Curr. Biol.* 2, 1096–1100. doi: 10.1016/j.cub.2014.03.036

Ng, T. M., Liew, Y. X., Teng, C., Ling, L. M., Ang, B., Tai, H. Y., et al. (2014). An interactive, point-of- care computerised antibiotic prescription decision support improved quality of antibiotic prescription. *J. Global Antimicrob. Res.* 2, 127–128. doi: 10.1016/j.jgar.2014.03.001

Nordmann, P., Nass, T., and Poirel, L. (2011). Global spread of carbapenemase-producing *Enterobacteriaceae*. *Emerg. Infect. Dis.* 17, 1791–1798. doi: 10.3201/eid1710.110655

Novo, A., André, S., Viana, P., Nunes, O. C., and Manaia, C. M. (2013). Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. *Water Res.* 47, 1875–1887. doi: 10.1016/j.watres.2013.01.010

Pal, C., Bengtsson-Palme, J., and Kristiansson, E. (2016). The structure and diversity of human, animal and environmental resistomes. *Microbiome* 4:54. doi: 10.1186/s40168-016-0199-5

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A., and Bonoma, R. A. (2011). *Carbapenems:* past, present, and future. *Antimicrob. Agents Chemother.* 55, 4943–4960. doi: 10.1128/AAC.00296-11

Pop, M., Paulson, J. N., Chakraborty, S., Astrovskaya, I., Lindsay, B. R., Li, S., et al. (2016). Individual-specific changes in the human hut microbiota after challenge with enterotoxigentic *Escherichia coli* and subsequent ciprofloxacin treatment. *BMC Genomics* 17:440. doi: 10.1186/s12864-016-2777-0

Queenan, A. M., and Bush, K. (2007). Carbapenemases: the versatile β-lactamases. *Clin. Microbiol. Rev.* 20, 440–458. doi: 10.1128/CMR.00001-07

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., et al. (2013). Urban wastewater treatment plants as hotspsots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–360. doi: 10.1016/j.scitotenv.2013.01.032

Rogers, B. A., Aminzadeh, Z., Hayashi, Y., and Paterson, D. L. (2011). Country-to-country transfer of patients and the risk of multi-resistant bacterial inflection. *Clin. Infect. Dis.* 53, 49–56. doi: 10.1093/cid/cir273

Singapore Tourism Board (2014). *Annual Report on Tourism Statistics 2013*. Available at: https://www.stb.gov.sg/statistics-and-market-insights/Pages/statistics-Annual-Tourism-Statistics.aspx

Smith, R. D., Chandra, R., and Tangcharoensathien, V. (2009). Trade in health-related services. *Lancet* 373, 593–601. doi: 10.1016/S0140-6736(08)61778-X

Struelens, M. J., Monnet, D. L., Magiorakos, A. P., Santos, O., Connor, F., and Giesecke, J. (2010). New Delhi metallo-beta-lactamase 1-producing Enterobacteriaceae: emergence and response in Europe. *Euro Surveill.* 15, 19716.

Van der Bij, A. K., and Pitout, J. D. (2012). The role of international travel in the worldwide spread of multiresistant *Enterobacteriaceae*. *J. Antimicrob. Chemother.* 67, 2090–2100. doi: 10.1093/jac/dks214

Vasoo, S., Barreto, J. N., and Tosh, P. K. (2015). Emerging issues in gram-negative bacterial resistance: an update for the practicing clinician. *Mayo Clin. Proc.* 90, 395–403. doi: 10.1016/j.mayocp.2014.12.002

Venkatachalam, I., Yang, H. L., Fisher, D., Lye, D. C., Moi Lin, L., Tambyah, P., et al. (2014). Multidrug- resistant gram-negative bloodstream infections among residents of long-term care facilities. *Infect. Control Hosp. Epidemiol.* 35, 519–526. doi: 10.1086/675823

Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S., and Obst, U. (2004). Dection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J. Microbiol. Methods* 56, 277–286. doi: 10.1016/j.mimet.2003.10.014

Wilson, M. E., and Chen, L. H. (2012). NDM-1 and the role of travel in its dissemination. *Curr. Infect. Dis. Rep.* 14, 213–226. doi: 10.1007/s11908-012-0252-x

Woodford, N., Wareham, D. W., Guerra, B., and Teale, C. (2014). Carbapenemase-producing *Enterobacteriaceae* and non-*Enterobacteriaceae* from animals and the environment: an emerging public health risk of our own making? *J. Antimicrob Chemother.* 69, 287–291. doi: 10.1093/jac/dkt392

Xu, G., Jiang, Y., An, W., Wang, H., and Zhang, X. (2015). Emergence of KPC-2-producing *Escherichia coli* isolates in an urban river in Harbin, China. *World J. Microbiol. Biotechnol.* 31, 1443–1450. doi: 10.1007/s11274-015-1897-z

Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., et al. (2015). Occurence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. *Chemosphere* 119, 1379–1385. doi: 10.1016/j.chemosphere.2014.02.040

Yigit, H., Queenan, A. M., Anderson, G. J., Domenech-Sanchez, A., Biddle, J. W., Steward, C. D., et al. (2011). Novel carbapenem-hydrolyzing β-lactamase KPC-1 from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* 45, 1151–1161. doi: 10.1128/AAC.45.4.1151-1161.2001

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K., et al. (2009). Characterization of a new metallo-beta-lactamase gene bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure

in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob. Agents Chemother.* 53, 5046–5054. doi: 10.1128/AAC.00774-09

Young, B. E., Lye, D. C., Krishnan, P., Chan, S. P., and Leo, Y. S. (2014). A prospective observational study of the prevalence and risk factors for colonization by antibiotic resistant bacteria in patients at admission to hospital in Singapore. *BMC Infect. Dis.* 14:298. doi: 10.1186/1471-2334-14-298

Zhang, Y., Marrs, C. F., Simon, C., and Xi, C. (2009). Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. *Sci. Total Environ.* 407, 3702–3706. doi: 10.1016/j.scitotenv.2009.02.013

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 October 2017
doi: 10.3389/fmicb.2017.02036

Benefits of Genomic Insights and CRISPR-Cas Signatures to Monitor Potential Pathogens across Drinking Water Production and Distribution Systems

Ya Zhang[1], *Masaaki Kitajima*[2], *Andrew J. Whittle*[3] *and Wen-Tso Liu*[1]*

[1] Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, United States, [2] Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, Sapporo, Japan, [3] Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge, MA, United States

OPEN ACCESS

Edited by:
Timothy R. Julian,
Swiss Federal Institute of Aquatic Science and Technology, Switzerland

Reviewed by:
Claire Bertelli,
Simon Fraser University, Canada
David Gregory Weissbrodt,
Delft University of Technology, Netherlands

***Correspondence:**
Wen-Tso Liu
wtliu@illinois.edu

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *27 June 2017*
Accepted: *05 October 2017*
Published: *19 October 2017*

Citation:
Zhang Y, Kitajima M, Whittle AJ and Liu W-T (2017) Benefits of Genomic Insights and CRISPR-Cas Signatures to Monitor Potential Pathogens across Drinking Water Production and Distribution Systems. Front. Microbiol. 8:2036. doi: 10.3389/fmicb.2017.02036

The occurrence of pathogenic bacteria in drinking water distribution systems (DWDSs) is a major health concern, and our current understanding is mostly related to pathogenic species such as *Legionella pneumophila* and *Mycobacterium avium* but not to bacterial species closely related to them. In this study, genomic-based approaches were used to characterize pathogen-related species in relation to their abundance, diversity, potential pathogenicity, genetic exchange, and distribution across an urban drinking water system. Nine draft genomes recovered from 10 metagenomes were identified as *Legionella* (4 draft genomes), *Mycobacterium* (3 draft genomes), *Parachlamydia* (1 draft genome), and *Leptospira* (1 draft genome). The pathogenicity potential of these genomes was examined by the presence/absence of virulence machinery, including genes belonging to Type III, IV, and VII secretion systems and their effectors. Several virulence factors known to pathogenic species were detected with these retrieved draft genomes except the *Leptospira*-related genome. Identical clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins (CRISPR-Cas) genetic signatures were observed in two draft genomes recovered at different stages of the studied system, suggesting that the spacers in CRISPR-Cas could potentially be used as a biomarker in the monitoring of *Legionella* related strains at an evolutionary scale of several years across different drinking water production and distribution systems. Overall, metagenomics approach was an effective and complementary tool of culturing techniques to gain insights into the pathogenic characteristics and the CRISPR-Cas signatures of pathogen-related species in DWDSs.

Keywords: virulence, genomic analysis, drinking water distribution systems, *Legionella*, *Mycobacterium*, *Parachlamydia*, *Leptospira*, CRISPR

INTRODUCTION

Over 500 waterborne or water-based pathogens of potential concern in drinking water (e.g., *Legionella pneumophila*, *Escherichia coli* O157:H7, *Mycobacterium avium*, and *Cryptosporidium parvum*) have been included in the Candidate Contaminant List by the US Environmental Protection Agency (EPA; Ashbolt, 2015). The traditional approach to identify these pathogens is through cultivation and then biochemical/serological tests or 16S rRNA gene-based phylogeny analysis (Lye and Dufour, 1993; Edberg et al., 1996; Stelma et al., 2004). However, identifying pathogens at species level does not always translate into health risks as some strains of the same species are more pathogenic than others (Schmidt and Schaechter, 2012).

Alternatively, comparative genomic analysis has become an effective way to evaluate the pathogenicity potential. It is reported that pathogens infect host through a multi-step process from entering the host, adhering to host tissues, penetrating or evading host defenses, damaging host tissues, to exiting the host. As a result, various virulence factors (VFs) are required for pathogenic species during the infection process, which can be divided into several general groups based on the conservation of similar mechanisms, such as adhesins, invasins, toxins, protein secretion systems, and antibiotic resistance mechanisms (Finlay and Falkow, 1997; Wilson et al., 2002). Thus, the presence of a set of virulence machinery in a bacterial genome has been used to define pathogenic subpopulations (Chapman et al., 2006; Cazalet et al., 2008; Bouzid et al., 2013; Foley et al., 2013; Picardeau, 2017). The knowledge on virulence machinery and the functions of key VFs in the literature have facilitated the usage of virulence machinery to evaluate health risks associated with pathogens in drinking water distribution systems (DWDSs; Wu et al., 2008; Huang et al., 2014). Secretion systems are essential for the transportation of proteins (i.e., effectors) from the cytoplasm into host cells or host environments to enhance attachment to eukaryotic cells, scavenge resources in an environmental niche, and disrupt target cell functions (Green and Mecsas, 2016). Some secretion systems are dedicated for bacteria-host interaction, such as the type III secretion system (T3SS) in *Chlamydia* (Betts-Hampikian and Fields, 2010), the type IVB secretion system (T4BSS, Dot/Icm) in *Lg. pneumophila* (Voth et al., 2012), and the type VII secretion system (T7SS) in *Mycobacterium* (Costa et al., 2015). The deletion of these secretion systems could result in a substantial decrease in virulence (Costa et al., 2015). In addition, several other VFs have also been reported for pathogens including those facilitating attachment and invasion (e.g., cell wall, type IV pili) and endotoxins (i.e., lipopolysaccharides, LPS; Schroeder et al., 2010; Favrot et al., 2013; Tortora et al., 2013.

While the identification of pathogens of potential concern in DWDSs is an important task, recent studies have often detected pathogens simultaneously together with their closely related species, which are often present at higher abundance. These include, for example, *Lg. pneumophila*-related species such as *Lg. dumoffii* (Hsu et al., 1984), *Lg. sainthelensis* (Rodriguez-Martinez et al., 2015), and *Lg. jordanis* (Hsu et al., 1984; Kao et al., 2014), and *M. avium*-related species such as *Mycobacterium gordonae* (Falkinham et al., 2001; Lalande et al., 2001; Vaerewijck et al., 2005), *Mycobacterium immunogenum* (Gomez-Alvarez and Revetta, 2016a), and *Mycobacterium chelonae* (Gomez-Alvarez and Revetta, 2016b). Some of these species have been associated with illness and infections in clinical environments, including *Lg. dumoffii* (Yu et al., 2002), *M. gordonae* (Lalande et al., 2001), *M. immunogenum* (Wilson et al., 2001), and *M. chelonae* (Lowry et al., 1990). As pathogens and their closely related species often share ecological niches (predominantly in biofilms), genetic exchange through conjugation and transformation occurs between the two groups, sometimes involving VFs (Gimenez et al., 2011; Gomez-Valero et al., 2011). However, it is not clear whether they possess similar VFs as observed in pathogens.

Furthermore, in DWDSs, pathogens and their closely related species mostly reside within biofilms where protozoa predation and viral lysis occur more frequently than bulk water, and have developed mechanisms to resist predation by inhibiting phagosome acidification and lysosome fusion of protozoa (Hilbi et al., 2001; Tilney et al., 2001). Phage DNA can be integrated into bacterial genomes by horizontal gene transfer as prophages, which are major contributors to differences among individuals within a bacterial species (Bobay et al., 2014). To protect bacteria from phage lysis, encountered foreign DNA fragments can be integrated into a clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins (CRISPR-Cas) locus as spacers (Makarova et al., 2015). Through addition of spacers at one end of the CRISPR array and conservation of spacers at the other end (the leader distal end), the CRISPR-Cas system participates in a constant evolutionary battle between phages and bacteria (Deveau et al., 2010; Sun et al., 2016). This mechanism has been used as a vital tool for strain typing in epidemiology for the recognition of outbreaks and identification of infection sources (Horvath et al., 2008; Shariat and Dudley, 2014). Nevertheless, it is not clear how intracellular growth and phage integration might impact the genomic composition and virulence of pathogen-related species.

In this study, metagenomics analysis instead of cultivation based methods was carried out to investigate virulence machinery and genomic signatures as the result of phage integration in pathogens-related species in a drinking water production and distribution system. A groundwater-derived drinking water system studied previously (Ling et al., 2016; Zhang et al., 2017) was used as a model system. It consists of abstraction, softening, recarbonation, disinfection, filtration, and final distribution with a disinfectant residual (free chlorine). Samples of microbial biomass from 10 locations of the water production process and the distribution system were collected and community metagenomes sequenced (Zhang et al., 2017). Coupling digital droplet PCR (ddPCR) with metagenomics, draft genomes affiliated with known pathogen genera were recovered to reveal their abundance, diversity, potential pathogenicity, genetic exchange, and distribution across an urban drinking water system.

MATERIALS AND METHODS

Sampling and DNA Extraction

Microbial biomass samples from different stages of the treatment processes and different locations in the distribution system were collected from a groundwater-sourced drinking water system. Detailed description of the studied drinking water system can be found in a previous study (Zhang et al., 2017) and in Figure S1. Briefly, these samples were from raw water (RW), immediately before filtration and chlorination (BC), finished water (FW) prior to distribution, three taps (DS1-DS3), two retired water mains (PB1-PB2), 14 household water meters (WM, combined into one sample), and five premise plumbing pipe reactors (PR, combined into one sample). The three tap water sampling sites (DS1-3) were located approximately one mile apart from each other to represent different locations within the DWDS. For water-phase samples (including RW, BC, FW, and DS1-3), a 10-min flushing (the cold-water side) was carried out before each sampling event to minimize the influence of premise plumbing before installing point-of-use water purifiers (Toray Industries Inc. Japan). Approximately 2,000 L of water was filtered during each sampling event at each site over a time span of 48 hrs. Water purifiers were collected at the end of each sampling event and transported to the laboratory in cools (the Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign). They were disassembled after arriving at the laboratory and cells were washed off from the multilayer hollow fiber membrane with phosphate-buffered saline (PBS) through sonication (Symphony™ Ultrasonic Cleaners, VWR). The obtained mixture was filtered through 0.22 μm membranes and the membranes with cells were stored at −80°C. To obtain a better representation of the average composition, water-phase sampling was repeated four times, in June, July, August, and September 2014, except the BC sample due to membrane blockage (Zhang et al., 2017).

For biofilm samples, PB1 was a 2.25-inch cast iron water main installed in 1968 and PB2 was a 1.5-inch cast iron water main installed prior to 1927. Each pipe was cut into two 12-inch long pieces on site with an effort to minimize contamination. Additionally, 14 water meters were obtained through the local drinking water plant. For the PR sample, five galvanized pipes of the plumbing system of a dormitory were obtained within the service area of the studied system, which were installed before World War II (size = 2 inch, OD = 2.375 inch, ID = 2.067 inch, length = 14 feet). Detailed description and handling of these samples could be found in our previous study (Zhang et al., 2017). The biofilm samples were swabbed off the surfaces, re-suspended in PBS, and collected by filtering through 0.22 μm membranes. All the membranes with cells were stored at −80°C. Genomic DNA (gDNA) was extracted using FastDNA® SPIN Kit for Soil (MP Biomedicals, Carlsbad, CA, USA) from these membranes with cells following manufacturer's protocol with an elution volume of 50 μl. The effect of different DNA extraction methods on the quantity and quality of DNA yields from drinking water biofilms had been evaluated and published in a previous study (Hwang et al., 2012).

ddPCR and Real-Time PCR

ddPCR was used to quantify total *Bacteria* and *Archaea* 16S rRNA genes and pathogens of potential concern, including *Mycobacterium* spp., *M. tuberculosis* complex, *Legionella* spp., *Lg. pneumophila*, *Pseudomonas aeruginosa*, and *Aeromonas hydrophila*, in the combined samples submitted for metagenomic sequencing, except DS1 and DS3 due to not enough gDNA. TaqMan-based ddPCR assays using primer/probe sets specific to each target (Table S1) were performed with a QX200™ Droplet Digital™ PCR System using ddPCR™ Supermix for Probes (Bio-Rad, Pleasanton, CA, USA). In addition, three eukaryotic groups (amoebae), *Naegleria fowleri*, *Acanthamoeba* spp., and *Balamuthia madrillaris*, were tested with TaqMan-based real-time PCR assays using primer/probe sets specific to internal transcribed spacer (ITS)/18S rRNA gene of each target (Table S1). Real-time PCR was performed with a CFX96™ Real-Time PCR Detection System using SsoAdvanced™ Universal Probes Supermix (Bio-Rad, Pleasanton, CA, USA). Because of the large variations in the number of ITS/18S rRNA genes in different eukaryotic species, only cycle threshold (C_T) values were reported. Positive control (standard plasmid DNA) and negative control (H_2O) were included in every ddPCR and real-time PCR reaction to ensure the successful amplification and the absence of contamination, respectively.

Amplicon Sequencing and Metagenome Sequencing Analyses

16S rRNA gene amplicon analysis was carried out using a universal primer set targeting the V4-V5 hypervariable regions of both the Bacteria and Archaea domains (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 909R: 5′-CCCGTCAATTCMTTTRAGT-3′) using the Illumina Miseq platform with dual indexing strategy as described in a previous study (Zhang et al., 2017). DNA libraries for metagenomic sequencing were prepared by combining all the extracted gDNA from each sampling site due to the requirement of a relatively large amount of gDNA (>0.1 μg). The prepared library was paired-end sequenced on Illumina HiSeq2500 platforms (Illumina, Inc., San Diego, CA, USA) as described previously (Zhang et al., 2017).

16S rRNA Gene Sequencing Analysis

The obtained paired-end 16S rRNA gene sequences were aligned with Mothur (Kozich et al., 2013). The resulting sequences were screened for chimeras by the UCHIME algorithm implemented in USEARCH 6.1 and processed using the *de novo* OTU picking workflow in QIIME as described previously (Zhang et al., 2017). EMIRGE was used to reconstruct nearly full-length SSU genes in metagenomes (Miller, 2013).

Draft Genome Reconstruction

Draft genomes are presented as a set of sequence fragments or contigs, which are the most common form of genome assemblies obtained using metagenomics sequencing binning pipelines and account for two thirds of the bacterial genomes available in the GenBank database (Nagarajan et al., 2010; Edwards and Holt, 2013). **Figure 1** illustrates the workflow of

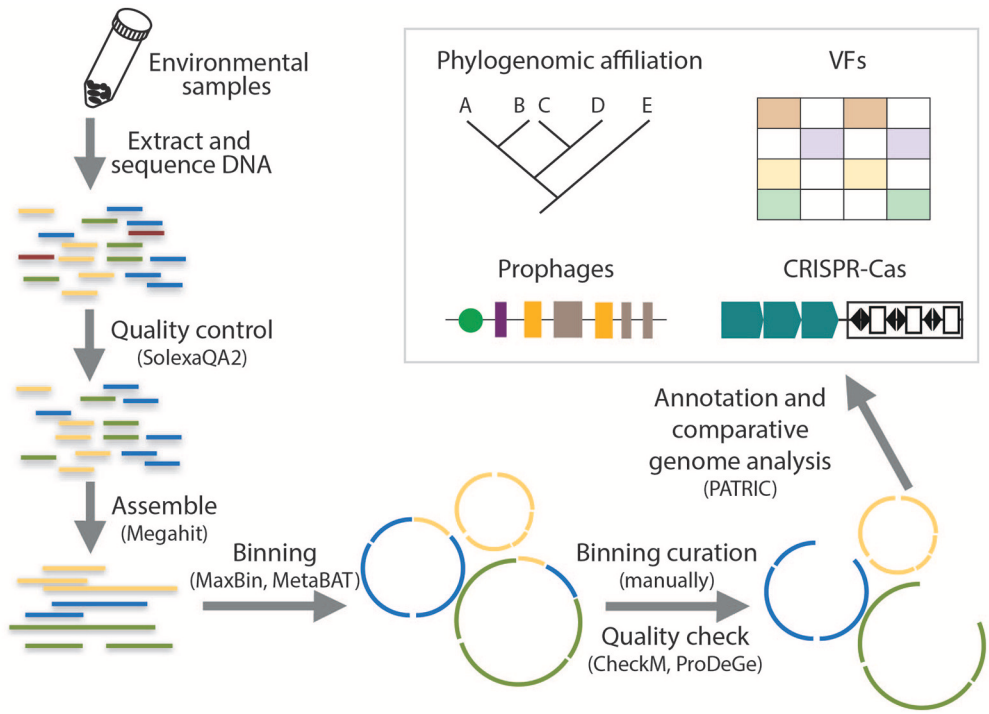


FIGURE 1 | Flowing chart on the analysis of metagenomic data for the reconstruction of draft genomes and the identification of genetic signatures.

draft genome recovery used in this study. All the metagenomic datasets were trimmed using SolexaQA2 based on a cutoff of 20 by phred scores (Cox et al., 2010) and assembled using Megahit (Li et al., 2015). High-quality contigs ($\sim$2.0 $\times$ 10^8 bp for each metagenome) were obtained at this step, to which >85.0% of the raw reads could be mapped except the RW sample. The longest contig in each metagenome was >4.0 $\times$ 10^5 bp. More details of the assemblies could be found in our previous study (Zhang et al., 2017). The obtained contigs were binned based on metagenomics read coverage, tetranucleotide frequency, and the occurrence of unique marker genes by using both MaxBin 2.0 (Wu et al., 2016) and MetaBAT (Kang et al., 2015) to minimize the contamination of each bin. These two binning methods employed different clustering methods for the determination of different bins: MaxBin compares the distributions of distances between and within the same bins whereas MetaBAT clusters contigs iteratively by modified K-methods algorithm. Bins of pathogen-related species from the two binning tools were compared and assessed with CheckM (Parks et al., 2015) and ProDeGe (Tennessen et al., 2016), followed by manual curation. The curated bins with $\geq$90% completeness and $\geq$15-fold coverage were finalized as draft genomes. Details of each step in the pipeline had been reviewed and summarized by Sangwan et al. (2016) and a step-by-step tutorial of the workflow supplied with a sample dataset had been available by Edwards and Holt (2013). Percentages of reads mapped over the refined genome bins were estimated by Burrow-Wheeler Aligner-mem (Li and Durbin, 2009). The entire workflow was computed on a high-performance workstation (DELL precision T7600) equipped with 136 GB memory.

Identification of VFs

Draft genomes of pathogen-related species retrieved were uploaded into PATRIC for annotation and feature identification (Wattam et al., 2014). VFs of different pathogens were collected from the literature and the VF database (VFDB, http://www.mgc.ac.cn/VFs/; Chen et al., 2012). Reported virulence genes within *Lg. pneumophila* included: the type II secretion system (T2SS, Lsp) for growth at low temperatures (Soderberg et al., 2008); the T4ASS (Lvh, F-type, and P-type) associated with conjugal DNA transfer and potentially in virulence (Gomez-Valero et al., 2011); the T4BSS (Dot/Icm) translocating several hundred effector proteins to support intracellular growth (Burstein et al., 2016); T4BSS-type effectors such as *ralF*, *lidA*, *sdhA*, and *lepAB* genes (Newton et al., 2010); type IV pili (*pilB,C,D*) involving in the entry to host cells, biofilm development, formation, type II protein secretion, and horizontal gene transfer (Schroeder et al., 2010); LPS transport (Lpt) proteins; and *mip* (macrophage infectivity potentiator) gene associated with the ability of *Lg. pneumophila* to replicate in eukaryotic cells (Newton et al., 2010).

For *M. tuberculosis*, the reported VFs included: the T7SS, also known as the ESX pathway (ESX-1 to ESX-5) to secrete proteins across their complex cell envelope (Houben et al., 2014);

early secretory antigenic target (ESAT6), *esxA*, *H*, and *N*; culture filtrate protein-10 kDa (CFP-10), *esxB*, *G*, and *M* (Li et al., 2005); *pe*/*ppe* genes unique to mycobacteria and abundant in pathogenic mycobacteria (Sampson, 2011); antigen 85 (*ag85*) complex and mycolic acid cyclopropane synthase (*pcaA*) required for the biosynthesis of major components of the cell envelope (Favrot et al., 2013); adhesin (*hbhA*); phospholipase C (*plcC*); and oxidative stress reducer (*ahpC*; Forrellad et al., 2013).

For leptospires, some potential VFs identified in the literature included: *lipL32*, *mce*, *invA*, *atsE*, *mviN*, *rfb* for attachment and invasion and *asd*, *trpE*, and *sphH* for amino acid biosynthesis (Ren et al., 2003; Ko et al., 2009; Fouts et al., 2016).

For *Parachlamydia,* known VFs included: negative regulator of the T3SS, SctW; protein kinase, Pkn5; translocated actin-recruiting phosphoprotein, *tarp*; inclusion membrane proteins IncA to IncG; translocator protein, CopB; modulation of host cell apoptosis, CADD; and Mip (Greub, 2009; Betts-Hampikian and Fields, 2010; Collingro et al., 2011; Croxatto et al., 2013). Furthermore, genes coding for nucleotide transporters that import host cell ATP in exchange for ADP (*ntt*) were part of the complex involving in bacteria-host interaction, but were generally not considered as VFs (Schmitz-Esser et al., 2004; Haferkamp et al., 2006).

Construction of Phylogenomic Tree

PhyloPhlAn (Segata et al., 2013) was used to construct phylogenomic trees based on draft genomes and reference genomes. The constructed trees were visualized using iTOL (Letunic and Bork, 2016).

Identification of Antibiotic Resistance Genes (ARGs) and CRISPR-Cas Loci

ARGs and CRISPR-Cas regions were screened with PATRIC. The identified CRISPR loci and ARGs were confirmed with CRISPRfinder (Grissa et al., 2007) and ResFinder (Zankari et al., 2012), respectively. Identified CRIPSR-Cas loci were classified into the current system consisting of two classes, five types, and 16 subtypes (type I-A to I-F and I-U, type II-A to II-C, type III-A to III-D, type IV, and type V) based on *cas* genes and additional signature genes (Makarova et al., 2015). Additionally, we investigated the possible targets (protospacers) of spacers in CRISPR-Cas arrays within the obtained draft genomes using CRISPRTarget to search against all the available databases (i.e., GenBank-Phage, GenBank-Environmental, RefSeq-Plasmid, RefSeq-Viral, and RefSeq-Bacteria), which was combined with the known features of each subtype that had been reported to be essential for target recognition, such as protospacer adjacent motifs (PAMs) and seed regions (Biswas et al., 2013). Extra weighting was given to known PAMs: 5′-GG-3′ for I-F (Mojica et al., 2009) at the 3′ region of protospacer and 5′-CCN-3′ for II-B (Fonfara et al., 2014) at the 5′ region of protospacer. Moreover, we also manually examined seed sequences (8-nt for Type I-F and 13-nt for Type II-B) within the match. PHAST was used to identify prophage sequences in these draft genomes (Zhou et al., 2011).

Genomic Data Depositing

The nine draft genomes reconstructed in this study are deposited in GenBank under the BioProject PRJNA323575 with BioSamples SAMN07572181- SAMN07572189.

RESULTS

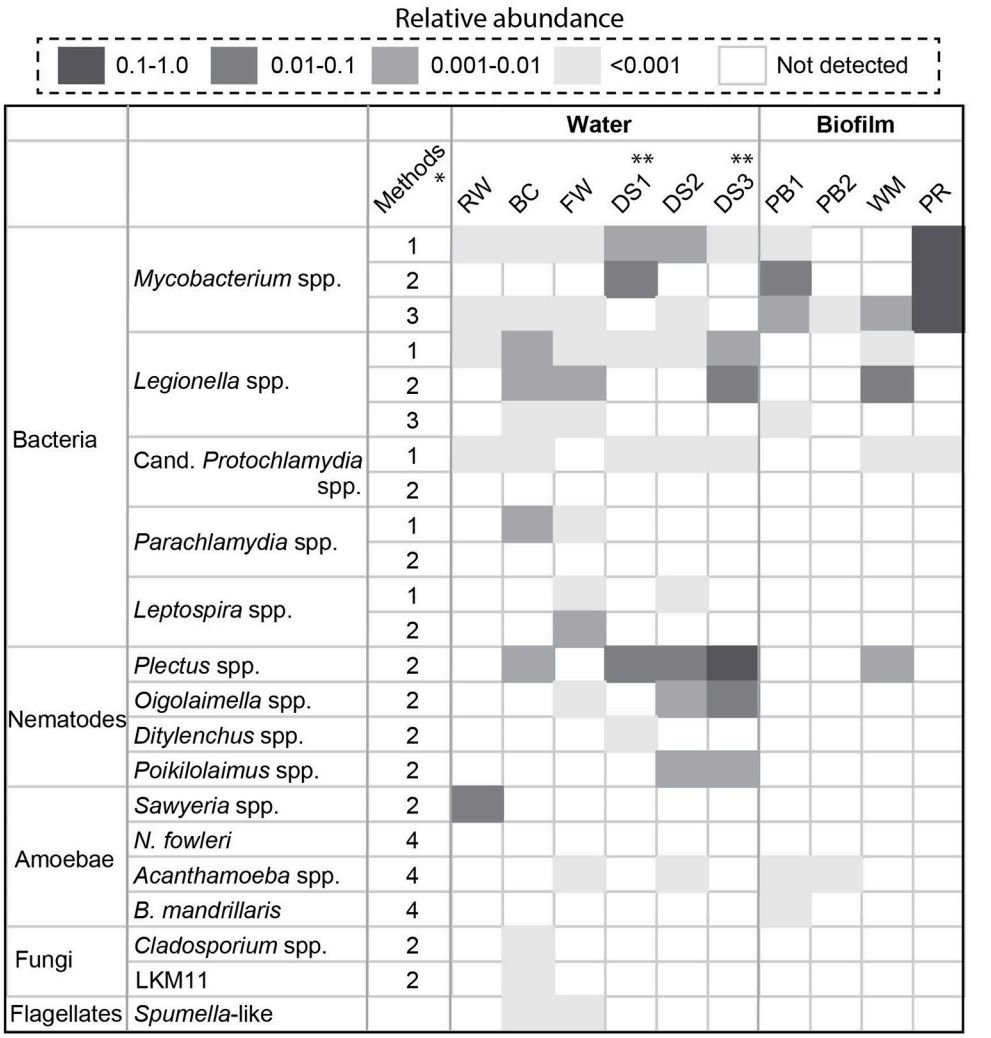
Detection of Pathogens of Potential Concern in the System

A combination of different molecular biological techniques, namely, 16S rRNA gene amplicon sequencing, metagenomics, and ddPCR/real-time PCR was employed to investigate the diversity and quantity of potential pathogens in the drinking water production and distribution system. Regarding prokaryotes, **Figure 2** shows that in general, the distribution system samples contained the highest relative abundance of *Mycobacterium* spp. and *Legionella* spp. in comparison with samples from the treatment process. The highest level of *Mycobacterium* spp. was detected with the PR sample with a relative abundance of 1.3×10^{-1} and an absolute concentration of 3.3×10^{4} copies/ng-gDNA by ddPCR (Table S2). The BC sample contained the highest level of *Legionella* spp.: a relative abundance of 4.7×10^{-3} based on 16S rRNA amplicon analysis and a concentration of 40.9 copies/ng-gDNA by ddPCR. Despite the occurrence of potential pathogens at the genus level, known pathogenic species, including *M. tuberculosis* complex, *Lg. pneumophila*, and *A. hydrophila* were not detected (Table S2). Additionally, sequences related to *Candidatus* Protochlamydia spp., *Parachlamydia* spp., and *Leptospira* spp. were also detected (**Figure 2**). *Candidatus Protochlamydia* spp. and *Parachlamydia* spp. were endosymbionts of amoeba and emerging agents of pneumonia (Greub, 2009). Notably, *Candidatus Protochlamydia* spp. were detected in all the distribution water phase samples.

Meanwhile, we could identify various eukaryotes, such as nematodes, amoebae, and flagellates with metagenomics and real-time PCR that co-existed with these potential pathogens. *Plectus* spp. were the most abundant nematodes detected in the system and present in half of the samples. For amoebae, *Acanthamoeba* spp. were observed in FW, DS2, PB1, and PB2 while *Sawyeria* spp. were only found in RW.

Characterization of Pathogen-Related Species through the Construction of Draft Genomes

Nine draft genomes closely related to known pathogens were successfully recovered from the metagenomes of BC, FW, DS1-3, and PR with ≥90% completeness and ≥15-fold coverage (**Table 1**). The phylogenomic tree in **Figure 3** showed that four draft genomes were affiliated with *Legionella* (BC.3.64, FW.3.37, DS3.009, BC.3.72; **Figure 3A**), three with *Mycobacterium* (DS1.3.26, DS2.013, PR.002; **Figure 3B**), one with *Leptospira* (FW.030; **Figure 3C**), and one with *Parachlamydia* (BC.030; **Figure 3D**). In **Figure 3A**, different species of *Legionella* were observed to co-exist in the same niche, i.e., BC.3.64 and BC.3.72 in the BC sample. FW.3.37 was observed to be 99.7% similar to BC.3.64 in the average nucleotide identity (ANI) based on 400



*Method 1 refers to 16S rRNA gene amplicon analyis , and the unit is abundance; Method 2 represents abundance information calculated from SSU genes extracted from metagenomics. Methods 3 and 4 refer to ddPCR and real-time PCR results, respectively. In Method 3, abundance is shown as determined by the concentration of certain target divided by the concentration of total *Bacteria* 16S rRNA genes. In Method 4, only C_T value was obtained and is shown with the lightest color if it is positive.

**DS1 and DS3 were not tested by ddPCR due to not enough gDNA

FIGURE 2 | Detected potential pathogens and eukaryotes (nematodes, amoebae, fungi, and flagellates) by 16S rRNA gene amplicon analysis (1), SSU genes extracted from metagenomes (2), ddPCR (3), and real-time PCR (4). Here, relative abundance was reported. The concentration of specific pathogens determined with ddPCR was summarized in Table S2. We divided the samples into water and biofilm phases.

marker genes. These three draft genomes probably represented new species of *Legionella* as they did not cluster together with any known species. A fourth draft genome, DS3.009, was affiliated with *Lg. drozanskii*. For *Mycobacterium* draft genomes, all three (DS1.3.26, DS2.013, PR.002) were closely related to *M. gordonae*. The *Leptospira* draft genome FW.030 was outside of the cluster containing mostly saprophytic species. Last, draft genome BC.030 fell between *Pa. acanthamoebae* and *Candidatus* Protochlamydia amoebophila. Collectively, five of the draft genomes retrieved were not closely related to any known isolated species, possibly due to the limitation of cultivation methods to recover microorganisms from drinking water systems so far.

VFs Detected in the Draft Genomes Recovered

Figure 4 (also see Table S3) indicated the presence and absence of VFs affiliated with secretion systems, effectors, attachment and invasion, endotoxins (e.g., lipopolysaccharides), and amino acid biosynthesis found in the recovered draft genomes and their related reference genomes. For *Legionella* in the secretion system category, the T2SS and T4BSS were the major pathogenesis

TABLE 1 | General features of the recovered genomes of pathogen-related species.

Bin ID	Source	Affiliation	Completeness	Coverage	No. of contigs	Genome size (bp)	G+C content (%)	No. of protein-coding genes	Possibly missing genes	Median sequence size	Longest contig size
BC.3.64	BC	*Legionella* sp.	94.44	30.13	62	2.27E+06	40.1	2112	5	31,419	150,921
BC.3.72	BC	*Legionella* sp.	94.51	23.78	22	1.95E+06	40.6	1829	11	74,242	336,208
FW.3.37	FW	*Legionella* sp.	94.15	27.68	63	2.10E+06	40.3	1926	14	18,840	221,613
DS3.009	DS3	*Legionella* sp.	98.83	45.78	140	3.36E+06	39.4	3159	39	16,314	165,891
DS1.3.26	DS1	*Mycobacterium* sp.	99.86	79.34	217	7.43E+06	66.8	6689	64	16,573	250,869
DS2.013	DS2	*Mycobacterium* sp.	99.86	23.74	219	7.96E+06	66.5	7334	77	15,428	244,689
PR.002	PR	*Mycobacterium* sp.	89.12	451.94	919	6.78E+06	67.0	6179	120	4,016	89,735
BC.030	BC	*Parachlamydia* sp.	100.00	24.81	39	3.04E+06	41.5	2763	15	54,962	289,998
FW.030	FW	*Leptospira* sp.	95.88	15.42	114	3.73E+06	35.1	3613	19	15,672	307,203

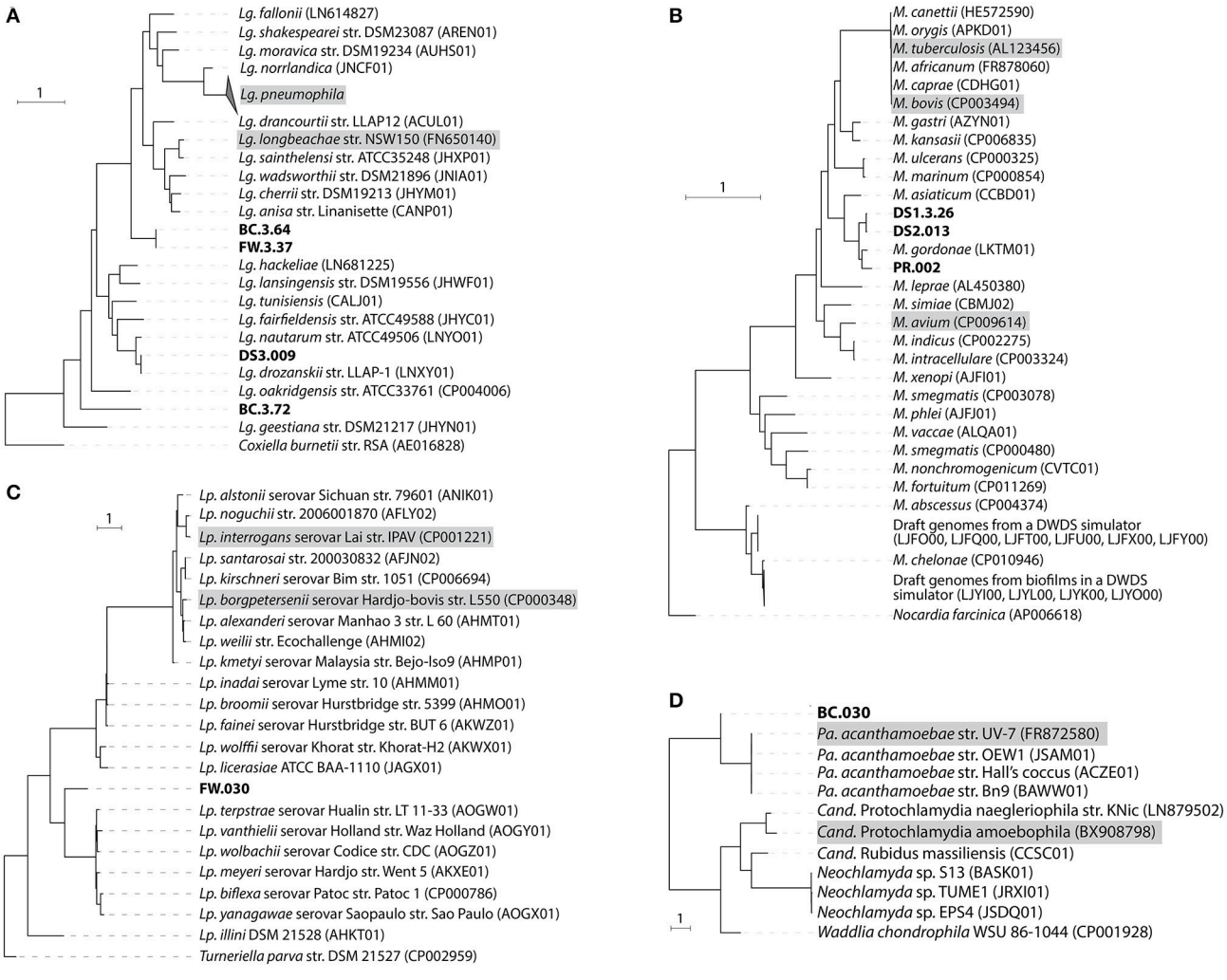


FIGURE 3 | Phylogenomic tree of recovered draft genomes constructed based on up to 400 conserved protein sequences. **(A)** *Legionella*; **(B)** *Mycobacterium*; **(C)** *Leptospira*; **(D)** *Parachlamydia*. The nine draft genomes recovered from this study were bold. Known pathogenic species were shaded with gray. Scale bar, 1 expected substitutions per site.

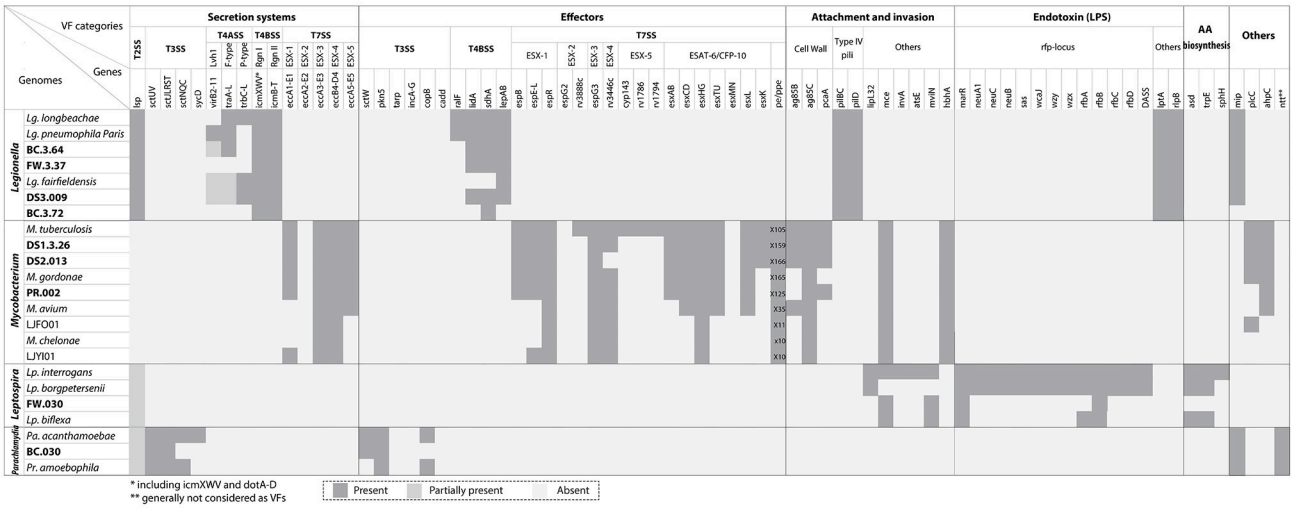


FIGURE 4 | VFs identified with the draft genomes recovered in this study and related genomes from public databases. VFs were grouped based on general categories (secretion systems and associated effectors, attachment and invasion, endotoxin, amino acid biosynthesis, and others). The genomes were organized by their taxonomic affiliations. There were some shared VFs among different genera, including T2SS among *Legionella, Leptospira*, and *Parachlamydia*, the *mip* gene between *Legionella* and *Parachlamydia*, and the *mce* gene between *Mycobacterium* and *Leptospira*. The accession number of known genomes were listed in Table S3.

systems observed in all draft genomes recovered. By contrast, the T4ASS, associated with conjugal DNA transfer, was detected in BC.3.64 and DS3.009 but absent in BC.3.72 and FW.3.37 possibly due to non-existence in these bacteria or the inability or poor efficiency to retrieve and assemble sequences pertaining to these hypervariable regions (Pop, 2009; Gomez-Valero et al., 2011). In the effectors category, T4BSS-assicated VFs including *lidA*, *sdhA*, and *lepAB* genes but not *ralF* were detected in three of the four draft genomes. In addition, all draft genomes contained LPS transport related genes, *lptA* and *lptE*. Last, the *mip* gene was observed in BC.3.64, FW.3.37, and DS3.009, but not BC.3.72.

For *Mycobacterium*, ESX-1, ESX-3, and ESX-5 T7SSs were observed in all *Mycobacterium* draft genomes recovered. Effectors belonging to ESX-1 and ESX-3 could also be detected, including *esxAB* and *TU*, but not effectors belonging to ESX-5 (*cyp143, rv1786, rv1794*, and *esxMN*). For the *pe*/*ppe* multigene family, all the recovered draft genomes contained more than 100 such genes, which was comparable to those observed in pathogenic species. Other VFs detected included cell envelop biosynthesis, *ag85* (except in PR.002) and *pca*A; adhesin, *hbhA*; phospholipase C, *plcC*; and oxidative stress reducer, *ahpC*. For *Leptospira*, the known VFs were mainly associated with the attachment and invasion, endotoxin and amino acid biosynthesis categories, and among them four (i.e., *mce1B, mviN*, *marR*, and *rfbD*) were detected in FW.030. The T2SS was partially present in *Leptospira* spp., including FW.030, but the association of the T2SS with virulence had not been experimentally tested (Picardeau, 2017). For *Parachlamydia*, VFs were mainly observed in the T3SS and associated effector categories. Two VFs, the T2SS (partially) and *mip* in the "others" category were also observed. As *Parachlamdia* spp. and *Candidatus Protochlamydia* spp. were intracellular bacteria of amoebae like *Legionella* spp., they also possessed T2SSs and Mip systems. Five *ntt* genes were observed with BC.030, putatively belonging to three NTT isoforms (NTT1-3) as shown in Figure S2 (Haferkamp et al., 2006). Last, several ARGs related to the resistance of aminoglycoside (moderate level), beta-lactam, and chloramphenicol (antimicrobial peptides) could be detected in the *Legionella* draft genome DS3.009. All the *Mycobacterium* recovered draft genomes possessed the *aac(2′)-Ic* gene, which was universally distributed among all *Mycobacterium* spp. (Ainsa et al., 1997; Table S4).

Usage of CRISPR-Cas Signatures to Monitor *Legionella* spp. across the Studied System

CRISPR-Cas genetic signatures, which are defense systems used by prokaryotes against viruses and not associated with pathogenicity, could be an effective tool to discriminate and monitor sub-lineages of pathogen-related species across the studied drinking water production and distribution system. **Figure 5** indicates the type of CRISPR-Cas systems identified in the draft genomes recovered and in several published *Lg. pneumophila* genomes. Among the three known subtypes of *Lg. pneumophila* (I-F, II-B, and I-C), this study detected type I-F with BC.3.64 and FW.3.37 based on *cas* gene clusters. The type I-F CRISPR-Cas observed in these two draft genomes was almost identical, i.e., 99% sequence similarity for *cas1* gene and 100% sequence similarity for the remaining *cas* genes (Table S5). Together with the findings of phylogenomic classification and genome similarity (99.7%; **Figure 3**), BC.3.64 and FW.3.37 were very likely to belong to a closely-related population originated from the same ancestor traveling from upstream (BC) to downstream (FW) of the studied drinking water production and distribution system. There was not enough information to

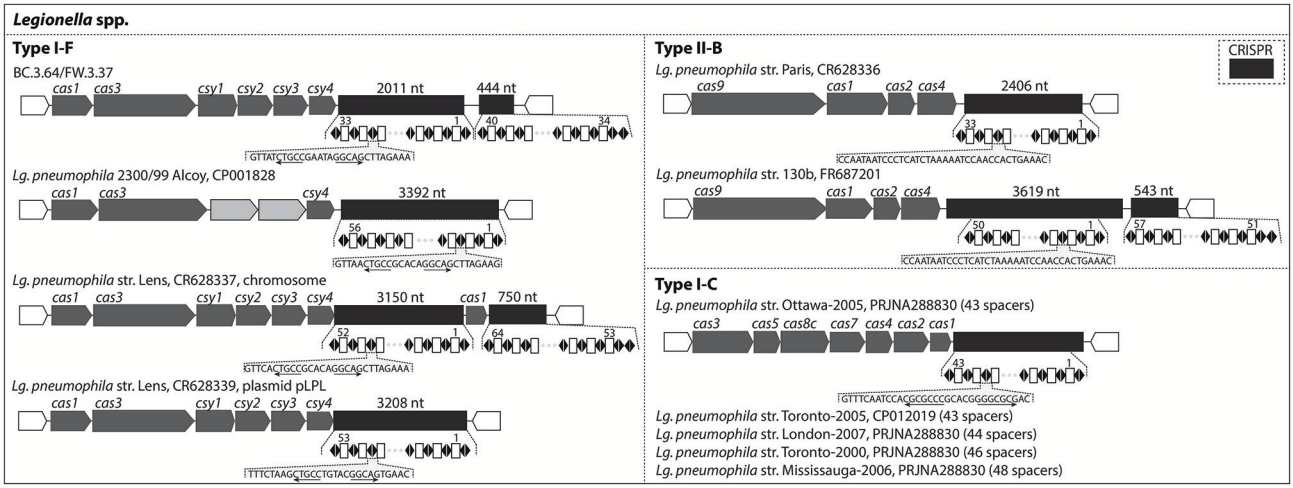


FIGURE 5 | CRISPR-Cas loci identified in the draft genomes recovered in this study and known genomes of *Legionella*. They were organized according to the subtypes (Type I-F, II-B, and I-C) of CRISPR-Cas loci.

determine whether the strain was alive at the BC site or whether filtration and chlorination had inactivated the strain in FW. Their *cas* gene clusters shared relatively low protein sequence similarities (from <40–76%) with other type I-F CRISPR-Cas loci of *Lg. pneumophila* (Table S5). Last, a Type II-B CRISPR-Cas locus was detected with *Leptospira* draft genome FW.030 (Figure S3).

Diversity of Prophage

Table 2 shows the types of prophages found in the recovered draft genomes. Initially, 36 potential prophage sequences were identified using PHAST (Figure S4) and they were reduced to 16 by considering the presence of genes encoding integrases and/or cI-type repressors (Fan et al., 2014; Figure S5). The lengths of prophage regions varied from 9.5 to 40.1 kbp. Six were associated with *Legionella* draft genomes, seven with *Mycobacterium* draft genome, and one each with *Parachlamydia* and *Leptospira*. An intact prophage (37.1 kbp) was recovered from PR.002. Shared prophage structures were observed between BC.3.64 and FW.3.37 and between DS1.3.26 and DS2.013. In addition, DS2.013 contained as many as five prophage sequences, which was rare for *Mycobacterium* genomes. Last, a prophage region identified in FW.030 showed sequence similarities to *Pandoravirus salinus* which was the largest virus reported so far with genomes up to 2.5 Mb and restricted to *Acanthamoeba* as hosts (Philippe et al., 2013).

DISCUSSION

Potential Virulence of Pathogen-Related Species

Virulence machinery characterized by genomic analysis has been used to define pathogenicity for many known pathogens, such as *E. coli* (Chapman et al., 2006), *Salmonella* (Foley et al., 2013), *Cryptosporidium* (Bouzid et al., 2013), *Lg. pneumophila* (Cazalet et al., 2008), and *Leptospira* (Picardeau, 2017). This approach is used here to evaluate the potential pathogenicity of those draft genomes of pathogen-related species recovered from an urban drinking water system. *Legionella*-related draft genomes found at two different locations of the water production process (i.e., BC.3.64 and FW.3.37) shared almost identical genomic sequences and possessed almost all known VFs to *Lg. pneumophila* and *Lg. longbeachae*. Another strain found during the water production process (i.e., BC.3.72) was clustered outside of known pathogenic *Legionella* clusters, and possessed fewer virulence genes than the other three recovered strains (i.e., BC.3.64, FW.3.37, and DS3.009). While the finding that most of the draft genomes encoded a high number of VFs may raises concerns on their pathogenicity, previous studies on closely related species/strains of pathogenic *Aeromonas* found no correlations between the presence/absence of VFs and extraintestinal infections (Havelaar et al., 1992; Lye et al., 2007). Thus, further studies combining microbiological (e.g., cultivation and animal models), genomic, and metabolic (e.g., transcriptomics and proteomics) methods should be carried out to understand the role of these VFs at the level of gene expression, protein function and regulation, and interaction with host immune system to confirm the virulence of these strains for immunocompromised individuals. This framework, once established, can be transferred into a novel pathogen surveillance program that enables virulence assessment of a broad range of heterotrophic bacteria found in potable water to possibly identify currently unknown pathogens.

All three *Mycobacterium*-related draft genomes recovered were closely related to *M. gordonae*, which is less virulent than *M. tuberculosis*, but contained a high number of genes (over 100) related to *pe/ppe* and T7SS. In comparison, genomes of *M. immunogenum* (LJFO01) and *M. chelonae* (LJYI01) isolated from a chloraminated DWDS simulator in previous studies (Gomez-Alvarez and Revetta, 2016a,b) lacked ESX-1 or ESX-5 and contained fewer *pe/ppe* genes. Due to the prevalence of *M. gordonae* in tap water and biofilms, particularly in

TABLE 2 | Prophages identified in the retrieved draft genomes.

Genera	Genomes	Regions	Length (kbp)	Possible phage
Legionella	BC.3.64	R1	9.5	*Salisaeta* icosahedral phage 1
		R2	31.1	*Stenotrophomonas* phage S1
	FW.3.37	R1	9.5	*Salisaeta* icosahedral phage 1
		R2	26.1	*Caulobacter* virus Karma
	DS3.009	R1	37.0	*Stenotrophomonas* phage S1
		R2	23.5	*Haemophilus* phage HP2
Mycobacterium	DS1.3.26	R1	19.0	*Mycobacterium* phage Adler
	DS2.103	R1	28.3	*Mycobacterium* phage RhynO
		R2	12.2	Molluscum contagiosum virus subtype 1
		R3	27.7	*Mycobacterium* phage Adler
		R4	31.6	*Mycobacterium* phage Adler
		R5	40.1	*Mycobacterium* phage Adler
	PR.002	R1	17.2	*Mycobacterium* phage Adler
		R2	37.1	*Mycobacterium* phage Milly
Leptospira	FW.030	R1	29.9	*Pandoravirus salinus*
Parachlamydia	BC.030	R1	19.3	*Cronobacter* phage vB_CsaM_GAP32

groundwater-derived drinking water systems (Vaerewijck et al., 2005), special attention to this group would be necessary. Pathogenic *Leptospira* are the causative agent of leptospirosis, which is the most widespread zoonotic disease infecting both human and animals (Evangelista and Coburn, 2010). In this study, the *Leptospira*-related genome FW.030 obtained did not contain most of the VFs known for *Lp. interrogans* and thus was likely not pathogenic. Among Parachlamydiaceae, only few strains such as *Pa. acanthamoebae* and *Candidatus Pr. naegleriophila* have been considered as emerging pathogens, causing mainly respiratory infections, while many others including *Neochlamydia hartmannellae* and *Pr. amoebophila* might be environmental strains or endosymbionts (Corsaro and Greub, 2006; Lamoth et al., 2011). Therefore, the pathogenic potential of *Parachlamydia*-related genome BC.030 remains to be determined.

Use of Spacers in CRISPR-Cas as Biomarkers for *Legionella* Subtyping

Due to the high genome plasticity of *Legionella* species, molecular typing by a single marker gene has been difficult. For instance, the *mip* gene is associated with the ability of *Lg. pneumophila* to replicate in eukaryotic cells, and has been extensively used as a biomarker to detect the presence/absence of *Lg. pneumophila* in a sample (Gomez-Valero et al., 2009). It was detected in three *Legionella*-related draft genomes constructed in this study: BC.3.64 and FW.3.37 were closely related to *Lg. fallonii*, and DS3.009 to *Lg. drozanskii* (Figure S6). However, the *mip* gene was limited in differentiating the *Lg. pneumophila* subspecies *fraseri* from other subspecies (Figure S4). Thus, the European Working Group for Legionella Infections (EWGLI) has suggested that a combination of several biomarkers, including *flaA, pilE, asd, mip, mompS*, *proA*, and *neuA*, should be used to effectively identify *Lg. pneumophila* (Fry et al., 2000; Gaia et al., 2005; Ratzow et al., 2007). However, phylogenetic incongruence (i.e., different lineages of the same strain indicated by different biomarkers) and limitations (i.e., the inability of some biomarkers to discriminate certain strains) in the discriminatory power of these multiple biomarkers could still occur because of differences in selection pressures associated with individual biomarkers.

Alternatively, spacers in CRISPR-Cas can be used as a biomarker in the monitoring of certain *Legionella* strains at an evolutionary scale of several years across drinking water production and distribution systems. The pattern of adding new spacers at one end of the CRISPR array and conserving spacers among common ancestors at the other end has been demonstrated with *Legionella* strains collected in Canada and Europe (CRISPR Type I-C and Type II-B; Ginevra et al., 2012; Lück et al., 2015; Rao et al., 2016). The longest time for these spacers to remain conserved among these strains and a *Leptospirillum* strain previously studied was reported to be 5 years or longer (Sun et al., 2016). As shown in **Figure 5**, Type I-F Cas loci were detected in the genomes of *Lg. pneumophila* str. 2300/99 Alcoy and str. Lens (both in the chromosome and plasmid). The two draft genomes recovered in our study, BC.3.64 and FW.3.37, also contained type I-F CRISPR-Cas loci, but the spacers were different from str. 2300/99 Alcoy and str. Lens. With 100% sequence similarity in CRISPR and high overall genomic similarity, these two genomes were likely derived from the same ancestor. Thus a specific CRISPR-Cas biomarker could be developed and used to monitor the distribution of this strain within the drinking water system studied. Furthermore, Types II-B and I-C were detected in a variety of *Lg. pneumophila* strains (**Figure 5**) and Type II-B was detected in 75.0% of the 400 *Lg. pneumophila* strains collected in a previous study (Ginevra et al., 2012). With more than 600 *Legionella* genomes available with NCBI's website and the diversity of CRISPR-Cas Types (I-C, I-F, and II-B) known among these strains, CRISPR-Cas spacers will be a promising biomarker for monitoring the distribution of *Legionella* at the strain level in samples taken from various drinking water systems, across different water bodies, and between patients over several years. However, cautions are needed when applying this method over a relatively large evolutionary scale as previous reports on *Yersinia pestis*, *Streptococcus thermophiles*, and *Leptospirillum* suggested that CRISPR loci could also evolve via internal deletion of spacers in the CRISPR array (Pourcel et al., 2005; Horvath et al., 2008; Sun et al., 2016).

Origin of Spacers in CRISPR-Cas of Pathogen-Related Genomes

The interaction between bacteria and viruses in drinking water systems or, more broadly, in oligotrophic environments is not well understood (Lehtola et al., 2004; Liu et al., 2015; Guidi et al., 2016). **Table 3** shows only 26 out of the 119 identified CRISPR-Cas spacers matched to entries in databases

TABLE 3 | Potential targets of CRISPR-Cas spacers in *Legionella*-related genomes.

Genomes	Spacer ID	Hits for spacers	Score	Number of mismatches within the spacer	PAMs**	Seed sequence mismatch position
BC.3.64	Sp6	Marine metagenome genome assembly TARA_030_DCM_0.22 (CENH01030675)	27	5	GG	8
Lgp* Lens	Chrm_Sp23	*Lg. pneumophila* serogroup 1, 30 kb instable genetic element (AJ277755)	35	1	GG	6
	Chrm_Sp35	*Paenibacillus* sp. FSL H7-0357, complete genome (CP009241)	27	5	GG	3
	Plsm_Sp22	Activated sludge metagenome contig16020 (AERA01015926)	37	0	GG	–
	Plsm_Sp46	*Lg. pneumophila* serogroup 1, 30 kb instable genetic element (AJ277755)	35	1	GG	7
	Plsm_Sp12	*Lg. pneumophila* 2300/99 Alcoy, complete genome (NC_014125)	31	3	GG	7
	Plsm_Sp12	*Lg. pneumophila* str. Corby, complete genome (NC_009494)	31	3	GG	7
	Plsm_Sp10	*Lg. pneumophila* str. Paris complete genome (NC_006368)	30	1	Not match	N/A
	Plsm_Sp8	Uncultured marine Microviridae clone SOG3-01 major capsid protein gene, partial cds (KC131005)	29	4	GG	1
	Plsm_Sp47	Activated sludge metagenome contig16020 (AERA01015926)	29	4	GG	–
	Plsm_Sp50	Marine metagenome 1096626097875, whole genome shotgun sequence (AACY023989113)	29	4	GG	5
	Plsm_Sp7	Activated sludge metagenome contig06523 (AERA01006474)	29	5	GG	3, 5
	Plsm_Sp13	*Lg. pneumophila* 2300/99 Alcoy, complete genome (NC_014125)	26	3	Not match	N/A
	Plsm_Sp32	*Lg. pneumophila* str. Lens plasmid pLPL, complete sequence (NC_006366)	24	4	Not match	N/A
	Plsm_Sp7	*Lg. pneumophila* str. Lens plasmid pLPL, complete sequence (NC_006366)	24	4	Not match	N/A
Lgp Alcoy	Sp32	Uncultured Gokushovirinae clone WSBWG10n1 major capsid protein gene (KF689311)	31	3	GG	8
	Sp28	Marine metagenome genome assembly TARA_122_SRF_0.1-0.22 (CETN01079705)	29	4	GG	–
	Sp3	*Lg. pneumophila* str. Lens plasmid pLPL (NC_006366)	26	3	Not match	N/A
Lgp Paris	Sp33	Activated sludge metagenome contig28417 (AERA01027227)	37	3	CCA	6, 9
	Sp4	*Schistocephalus solidus* genome assembly S_solidus_NST_G2 (LL901847)	29	5	CCA	–
	Sp15	*Lg. pneumophila* str. Lens plasmid pLPL (NC_006366)	28	3	Not match	N/A
	Sp14	*Lg. pneumophila* 130b draft genome (FR687201)	28	4	Not match	N/A
Lgp 130b	Sp40	*Lg. pneumophila* str. Paris complete genome (NC_006368)	37	0	CCA	–
	Sp41	Hypersaline lake metagenome ctg7180000052828 (APHM01003927)	30	5	CCA	10
	Sp27	*Lg. pneumophila* str. Corby, complete genome (NC_009494)	30	2	Not match	N/A
	Sp27	*Lg. pneumophila* 2300/99 Alcoy chromosome (NC_014125)	30	2	Not match	N/A

**Lgp, Lg. pneumophila; **PAMs, protospacer adjacent motifs.*

including GenBank-Phage, GenBank-Environmental, RefSeq-Plasmid, RefSeq-Viral, and RefSeq-Bacteria. Among them, 13 spacers matched sequences in other *Lg. pneumophila* strains. Two commonly observed targets were a 30-kb unstable genetic element previously identified in *Lg. pneumophila* str. RC1 and a 60-kb plasmid in *Lg. pneumophila* str. Lens. Likely, these elements were originated from bacteriophages in environments and incorporated into *Lg. pneumophila* genomes as mobile genetic elements such as prophages and plasmids. When the DNA of *Lg. pneumophila* was damaged or under other stress conditions, prophages could be excised, replicated, and ultimately used to lyse the host and spread into the environment. Ecologically, it would be rational for other *Lg. pneumophila* strains to incorporate their fragments into CRISPR systems so

that they had the ability to destroy them when being attacked (Rao et al., 2016).

We also observed near-perfect matches of four spacers in CRISPR-Cas to one activated sludge metagenome (AERA01; More et al., 2014). It has been reported that wastewater treatment plants (WWTPs) contained 10–1,000 times higher viral concentration than in natural aquatic environments, making WWTP an important reservoir and source of viruses (Edwards and Rohwer, 2005; Tamaki et al., 2012). In the studied drinking water production and distribution system, we estimated that the viral concentration was $\sim 10^4$ viruses/ml based on the bacterial cell counts published previously (Zhang et al., 2017) with the general rule that viral count is 10 times of the bacterial count (Maranger and Bird, 1995). Additionally, spacers detected in the BC.3.64 and FW.3.37 genomes recovered here and *Lg. pneumophila* 2300/99 Alcoy matched to contigs in marine metagenomes (AACY02; Venter et al., 2004). Although the matches are not perfect (except one) to organisms in WWTPs or marine environment, the evolving nature of spacers by mutations at CRISPR loci allows us to speculate that WWTPs and marine environments were possible sources of these spacers. Those *Legionella* strains could have come from water bodies under the influence of wastewater or seawater, such as flooded sewers or coastal groundwater.

Amoebae as a "Hub" Connecting Viruses and Intracellular Bacteria

This study observed that the prophage exhibiting high sequence similarity to *Pandoravirus* could co-exist with *Acanthamoeba* spp., *Parachlamydia* spp., *Legionella* spp., and *Mycobacterium* spp. in the FW sample. So far, free-living amoebae in drinking water systems are reported to be an ideal shelter to provide nutritional requirements for the growth of *Legionella* (Breiman et al., 1990; Dupuy et al., 2016), and are the only reported host of *Pandoravirus* (Philippe et al., 2013). Various giant viruses, including *Mimivirus*, *Mamavirus*, and *Pandoravirus*, have been detected in amoebae and were reported to be involved in lateral gene transfer between viruses and bacteria (La Scola et al., 2003, 2008; Philippe et al., 2013). While the detection of *Parachlamydia* in drinking water systems is rare (Thomas et al., 2008), previous studies have suggested that Chlamydiae were likely prevalent in aquatic environments (Barret et al., 2013; Lagkouvardos et al., 2014). These observations all support amoebae as the "hub" connecting viruses and intracellular bacteria, and facilitating the genetic exchange between pathogens and their closely related species (Gimenez et al., 2011; Gomez-Valero et al., 2011). Thus, developing control strategies to eukaryotic populations, e.g., filtration with 1 μm membranes, whose size is larger than bacteria but smaller than amoebae, could be an effective means to suppress the growth and spreading of pathogens in DWDSs (Wadowsky et al., 1988).

In summary, our study demonstrates that metagenomics analysis can be used to determine the presence of VFs in potential pathogens in drinking water production and distribution systems. Future studies combining microbiological, genomic, and metabolic methods at the level of gene expression, protein function and regulation, and bacteria-host interaction can help determine the relationship between the presence of these VFs and pathogenicity in immunocompromised individuals, especially for environmental strains recovered from drinking water systems. Furthermore, the development of genomics analysis can serve as a new platform for the detection, strain typing, and monitoring of pathogens, which can provide novel insights into the surveillance and control of waterborne or water-based pathogens. Characteristic regions in bacterial genomes, such as CRISPR-Cas studied here, can be used in combination with the traditional biomarkers to facilitate and simplify the subtyping of pathogens of potential concern and monitor the distribution of the same strains across different environmental niches.

AUTHOR CONTRIBUTIONS

YZ designed and carried out the experiments, analyzed the obtained data, and wrote the manuscript. MK and AW carried out the experiments to quantify the pathogens and participated in the manuscript writing process. WL designed and carried out the experiments, analyzed the obtained data, and revised the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02036/full#supplementary-material

REFERENCES

Ainsa, J. A., Perez, E., Pelicic, V., Berthet, F. X., Gicquel, B., and Martin, C. (1997). Aminoglycoside 2′-N-acetyltransferase genes are universally present in mycobacteria: characterization of the *aac*(2′)-*lc* gene from *Mycobacterium tuberculosis* and the aac(2′)-ld gene from *Mycobacterium smegmatis*. *Mol. Microbiol.* 24, 431–441. doi: 10.1046/j.1365-2958.1997.3471717.x

Ashbolt, N. J. (2015). Microbial contamination of drinking water and human health from community water systems. *Curr. Environ. Health Rep.* 2, 95–106. doi: 10.1007/s40572-014-0037-5

Barret, M., Egan, F., and O'Gara, F. (2013). Distribution and diversity of bacterial secretion systems across metagenomic datasets. *Environ. Microbiol. Rep.* 5, 117–126. doi: 10.1111/j.1758-2229.2012.00394.x

Betts-Hampikian, H. J., and Fields, K. A. (2010). The chlamydial type III secretion mechanism: revealing cracks in a tough nut. *Front. Microbiol.* 1:114. doi: 10.3389/fmicb.2010.00114

Biswas, A., Gagnon, J. N., Brouns, S. J. J., Fineran, P. C., and Brown, C. M. (2013). CRISPRTarget: bioinformatic prediction and analysis of crRNA targets. *RNA Biol.* 10, 817–827. doi: 10.4161/rna.24046

Bobay, L. M., Touchon, M., and Rocha, E. P. C. (2014). Pervasive domestication of defective prophages by bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12127–12132. doi: 10.1073/pnas.1405336111

Bouzid, M., Hunter, P. R., Chalmers, R. M., and Tyler, K. M. (2013). Cryptosporidium pathogenicity and virulence. *Clin. Microbiol. Rev.* 26, 115–134. doi: 10.1128/CMR.00076-12

Breiman, R. F., Fields, B. S., Sanden, G. N., Volmer, L., Meier, A., and Spika, J. S. (1990). Association of shower use with legionnaires-disease - possible role of amebas. *JAMA* 263, 2924–2926. doi: 10.1001/jama.1990.03440210074036

Burstein, D., Amaro, F., Zusman, T., Lifshitz, Z., Cohen, O., Gilbert, J. A., et al. (2016). Genomic analysis of 38 Legionella species identifies large and diverse effector repertoires. *Nat. Genet.* 48, 167–175. doi: 10.1038/ng.3481

Cazalet, C., Jarraud, S., Ghavi-Helm, Y., Kunst, F., Glaser, P., Etienne, J., et al. (2008). Multigenome analysis identifies a worldwide distributed epidemic *Legionella pneumophila* clone that emerged within a highly diverse species. *Genome Res.* 18, 431–441. doi: 10.1101/gr.7229808

Chapman, T. A., Wu, X. Y., Barchia, I., Bettelheim, K. A., Driesen, S., Trott, D., et al. (2006). Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *Appl. Environ. Microbiol.* 72, 4782–4795. doi: 10.1128/AEM.02885-05

Chen, L. H., Xiong, Z. H., Sun, L. L., Yang, J., and Jin, Q. (2012). VFDB 2012 update: toward the genetic diversity and molecular evolution of bacterial virulence factors. *Nucleic Acids Res.* 40, D641–D645. doi: 10.1093/nar/gkr989

Collingro, A., Tischler, P., Weinmaier, T., Penz, T., Heinz, E., Brunham, R. C., et al. (2011). Unity in variety-the pan-genome of the chlamydiae. *Mol. Biol. Evol.* 28, 3253–3270. doi: 10.1093/molbev/msr161

Corsaro, D., and Greub, G. (2006). Pathogenic potential of novel Chlamydiae and diagnostic approaches to infections due to these obligate intracellular bacteria. *Clin. Microbiol. Rev.* 19, 283–297. doi: 10.1128/CMR.19.2.283-297.2006

Costa, T. R. D., Felisberto-Rodrigues, C., Meir, A., Prevost, M. S., Redzej, A., Trokter, M., et al. (2015). Secretion systems in gram-negative bacteria: structural and mechanistic insights. *Nat. Rev. Microbiol.* 13, 343–359. doi: 10.1038/nrmicro3456

Cox, M. P., Peterson, D. A., and Biggs, P. J. (2010). SolexaQA: at-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics* 11:485. doi: 10.1186/1471-2105-11-485

Croxatto, A., Murset, V., Chassot, B., and Greub, G. (2013). Early expression of the type III secretion system of Parachlamydia acanthamoebae during a replicative cycle within its natural host cell Acanthamoeba castellanii. *Pathog. Dis.* 69, 159–175. doi: 10.1111/2049-632X.12065

Deveau, H., Garneau, J. E., and Moineau, S. (2010). CRISPR/Cas system and its role in phage-bacteria interactions. *Annu. Rev. Microbiol.* 64, 475–493. doi: 10.1146/annurev.micro.112408.134123

Dupuy, M., Binet, M., Bouteleux, C., Herbelin, P., Soreau, S., and Hechard, Y. (2016). Permissiveness of freshly isolated environmental strains of amoebae for growth of *Legionella pneumophila*. *FEMS Microbiol. Lett.* 363:fnw022. doi: 10.1093/femsle/fnw022

Edberg, S. C., Gallo, P., and Kontnick, C. (1996). Analysis of the virulence characteristics of bacteria isolated from bottled, water cooler, and tap water. *Microb Ecol Health D* 9, 67–77. doi: 10.3109/08910609609166445

Edwards, D. J., and Holt, K. E. (2013). Beginner's guide to comparative bacterial genome analysis using next-generation sequence data. *Microb. Inform. Exp.* 3:2. doi: 10.1186/2042-5783-3-2

Edwards, R. A., and Rohwer, F. (2005). Viral metagenomics. *Nat. Rev. Microbiol.* 3, 504–510. doi: 10.1038/nrmicro1163

Evangelista, K. V., and Coburn, J. (2010). Leptospira as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. *Future Microbiol.* 5, 1413–1425. doi: 10.2217/fmb.10.102

Falkinham, J. O., Norton, C. D., and LeChevallier, M. W. (2001). Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. *Appl. Environ. Microbiol.* 67, 1225–1231. doi: 10.1128/AEM.67.3.1225-1231.2001

Fan, X. Y., Xie, L. X., Li, W., and Xie, J. P. (2014). Prophage-like elements present in Mycobacterium genomes. *BMC Genomics* 15:243. doi: 10.1186/1471-2164-15-243

Favrot, L., Grzegorzewicz, A. E., Lajiness, D. H., Marvin, R. K., Boucau, J., Isailovic, D., et al. (2013). Mechanism of inhibition of *Mycobacterium tuberculosis* antigen 85 by ebselen. *Nat.Commun.* 4:2748. doi: 10.1038/ncomms3748

Finlay, B. B., and Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.* 61, 136–169.

Foley, S. L., Johnson, T. J., Ricke, S. C., Nayak, R., and Danzeisen, J. (2013). Salmonella pathogenicity and host adaptation in chicken-associated serovars. *Microbiol. Mol. Biol. Rev.* 77, 582–607. doi: 10.1128/MMBR.00015-13

Fonfara, I., Le Rhun, A., Chylinski, K., Makarova, K. S., Lecrivain, A. L., Bzdrenga, J., et al. (2014). Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems. *Nucleic Acids Res.* 42, 2577–2590. doi: 10.1093/nar/gkt1074

Forrellad, M. A., Klepp, L. I., Gioffre, A., Garcia, J. S. Y., Morbidoni, H. R., Santangelo, M. D., et al. (2013). Virulence factors of the *Mycobacterium tuberculosis* complex. *Virulence* 4, 3–66. doi: 10.4161/viru.22329

Fouts, D. E., Matthias, M. A., Adhikarla, H., Adler, B., Amorim-Santos, L., Berg, D. E., et al. (2016). What makes a bacterial species pathogenic? comparative genomic analysis of the genus Leptospira. *PLoS Negl. Trop. Dis.* 10:e0004403. doi: 10.1371/journal.pntd.0004403

Fry, N. K., Bangsborg, J. M., Bernander, S., Etienne, J., Forsblom, B., Gaia, V., et al. (2000). Assessment of intercentre reproducibility and epidemiological concordance of *Legionella pneumophila* serogroup 1 genotyping by amplified fragment length polymorphism analysis. *Eur. J. Clin. Microbiol. Infect. Dis.* 19, 773–780. doi: 10.1007/s100960000359

Gaia, V., Fry, N. K., Afshar, B., Luck, P. C., Meugnier, H., Etienne, J., et al. (2005). Consensus sequence-based scheme for epidemiological typing of clinical and environmental isolates of *Legionella pneumophila*. *J. Clin. Microbiol.* 43, 2047–2052. doi: 10.1128/JCM.43.5.2047-2052.2005

Gimenez, G., Bertelli, C., Moliner, C., Robert, C., Raoult, D., Fournier, P. E., et al. (2011). Insight into cross-talk between intra-amoebal pathogens. *BMC Genomics* 12:542. doi: 10.1186/1471-2164-12-542

Ginevra, C., Jacotin, N., Diancourt, L., Guigon, G., Arquilliere, R., Meugnier, H., et al. (2012). *Legionella pneumophila* sequence Type 1/Paris pulsotype subtyping by spoligotyping. *J. Clin. Microbiol.* 50, 696–701. doi: 10.1128/JCM.06180-11

Gomez-Alvarez, V., and Revetta, R. P. (2016a). Draft genome sequences of six *Mycobacterium immunogenum* strains obtained from a chloraminated drinking water distribution system simulator. *Genome Announc.* 4:e01538-15. doi: 10.1128/genomeA.01538-15

Gomez-Alvarez, V., and Revetta, R. P. (2016b). Whole-genome sequences of four strains closely related to members of the *Mycobacterium chelonae* group, isolated from biofilms in a drinking water distribution system simulator. *Genome Announc.* 4:e01539-15. doi: 10.1128/genomeA.01539-15

Gomez-Valero, L., Rusniok, C., and Buchrieser, C. (2009). *Legionella pneumophila*: population genetics, phylogeny and genomics. *Infect. Genet. Evol.* 9, 727–739. doi: 10.1016/j.meegid.2009.05.004

Gomez-Valero, L., Rusniok, C., Jarraud, S., Vacherie, B., Rouy, Z., Barbe, V., et al. (2011). Extensive recombination events and horizontal gene transfer shaped the *Legionella pneumophila* genomes. *BMC Genomics* 12:536. doi: 10.1186/1471-2164-12-536

Green, E. R., and Mecsas, J. (2016). Bacterial secretion systems: an overview. *Microbiol. Spectr.* 4. doi: 10.1128/microbiolspec.VMBF-0012-2015

Greub, G. (2009). Parachlamydia acanthamoebae, an emerging agent of pneumonia. *Clin. Microbiol. Infect.* 15, 18–28. doi: 10.1111/j.1469-0691.2008.02633.x

Grissa, I., Vergnaud, G., and Pourcel, C. (2007). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Res.* 35, W52–W57. doi: 10.1093/nar/gkm360

Guidi, L., Chaffron, S., Bittner, L., Eveillard, D., Larhlimi, A., Roux, S., et al. (2016). Plankton networks driving carbon export in the oligotrophic ocean. *Nature* 532, 465–470. doi: 10.1038/nature16942

Haferkamp, I., Schmitz-Esser, S., Wagner, M., Neigel, N., Horn, M., and Neuhaus, H. E. (2006). Tapping the nucleotide pool of the host: novel nucleotide carrier proteins of Protochlamydia amoebophila. *Mol. Microbiol.* 60, 1534–1545. doi: 10.1111/j.1365-2958.2006.05193.x

Havelaar, A. H., Schets, F. M., van Silfhout, A., Jansen, W. H., Wieten, G., and van der Kooij, D. (1992). Typing of Aeromonas strains from patients with diarrhoea and from drinking water. *J. Appl. Bacteriol.* 72, 435–444. doi: 10.1111/j.1365-2672.1992.tb01857.x

Hilbi, H., Segal, G., and Shuman, H. A. (2001). Icm/dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. *Mol. Microbiol.* 42, 603–617. doi: 10.1046/j.1365-2958.2001.02645.x

Horvath, P., Romero, D. A., Coute-Monvoisin, A. C., Richards, M., Deveau, H., Moineau, S., et al. (2008). Diversity, activity, and evolution of

CRISPR loci in *Streptococcus thermophilus*. *J. Bacteriol.* 190, 1401–1412. doi: 10.1128/JB.01415-07

Houben, E. N. G., Korotkov, K. V., and Bitter, W. (2014). Take five - Type VII secretion systems of Mycobacteria. *Biochim. Biophys. Acta* 1843, 1707–1716. doi: 10.1016/j.bbamcr.2013.11.003

Hsu, S. C., Martin, R., and Wentworth, B. B. (1984). Isolation of Legionella species from drinking water. *Appl. Environ. Microbiol.* 48, 830–832.

Huang, K. L., Zhang, X. X., Shi, P., Wu, B., and Ren, H. Q. (2014). A comprehensive insight into bacterial virulence in drinking water using 454 pyrosequencing and Illumina high-throughput sequencing. *Ecotoxicol. Environ. Saf.* 109, 15–21. doi: 10.1016/j.ecoenv.2014.07.029

Hwang, C. C., Ling, F. Q., Andersen, G. L., LeChevallier, M. W., and Liu, W. T. (2012). Evaluation of methods for the extraction of DNA from drinking water distribution system biofilms. *Microbes Environ.* 27, 9–18. doi: 10.1264/jsme2.ME11132

Kang, D. W. D., Froula, J., Egan, R., and Wang, Z. (2015). MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ* 3:e1165. doi: 10.7717/peerj.1165

Kao, P. M., Hsu, B. M., Hsu, T. K., Ji, W. T., Huang, P. H., Hsueh, C. J., et al. (2014). Application of TaqMan fluorescent probe-based quantitative real-time PCR assay for the environmental survey of *Legionella* spp. and *Legionella pneumophila* in drinking water reservoirs in Taiwan. *Sci. Tot. Environ.* 490, 416–421. doi: 10.1016/j.scitotenv.2014.04.103

Ko, A. I., Goarant, C., and Picardeau, M. (2009). Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nat. Rev. Microbiol.* 7, 736–747. doi: 10.1038/nrmicro2208

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi: 10.1128/AEM.01043-13

La Scola, B., Audic, S., Robert, C., Jungang, L., de Lamballerie, X., Drancourt, M., et al. (2003). A giant virus in amoebae. *Science* 299, 2033–2033. doi: 10.1126/science.1081867

La Scola, B., Desnues, C., Pagnier, I., Robert, C., Barrassi, L., Fournous, G., et al. (2008). The virophage as a unique parasite of the giant mimivirus. *Nature* 455, 100–104. doi: 10.1038/nature07218

Lagkouvardos, I., Weinmaier, T., Lauro, F. M., Cavicchioli, R., Rattei, T., and Horn, M. (2014). Integrating metagenomic and amplicon databases to resolve the phylogenetic and ecological diversity of the Chlamydiae. *ISME J.* 8, 115–125. doi: 10.1038/ismej.2013.142

Lalande, V., Barbut, F., Varnerot, A., Febvre, M., Nesa, D., Wadel, S., et al. (2001). Pseudo-outbreak of *Mycobacterium gordonae* associated with water from refrigerated fountains. *J. Hosp. Infect.* 48, 76–79. doi: 10.1053/jhin.2000.0929

Lamoth, F., Jaton, K., Vaudaux, B., and Greub, G. (2011). Parachlamydia and Rhabdochlamydia: emerging agents of community-acquired respiratory infections in children. *Clin. Infect. Dis.* 53, 500–501. doi: 10.1093/cid/cir420

Lehtola, M. J., Miettinen, K. T., Keinanen, M. M., Kekki, T. K., Laine, O., Hirvonen, A., et al. (2004). Microbiology, chemistry and biofilm development in a pilot drinking water distribution system with copper and plastic pipes. *Water Res.* 38, 3769–3779. doi: 10.1016/j.watres.2004.06.024

Letunic, I., and Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res.* 44, W242–W245. doi: 10.1093/nar/gkw290

Li, D. H., Liu, C. M., Luo, R. B., Sadakane, K., and Lam, T. W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 31, 1674–1676. doi: 10.1093/bioinformatics/btv033

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

Li, L. L., Bannantine, J. P., Zhang, Q., Amonsin, A., May, B. J., Alt, D., et al. (2005). The complete genome sequence of *Mycobacterium avium* subspecies paratuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12344–12349. doi: 10.1073/pnas.0505662102

Ling, F. Q., Hwang, C. A., LeChevallier, M. W., Andersen, G. L., and Liu, W. T. (2016). Core-satellite populations and seasonality of water meter biofilms in a metropolitan drinking water distribution system. *ISME J.* 10, 582–595. doi: 10.1038/ismej.2015.136

Liu, H., Yuan, X. C., Xu, J., Harrison, P. J., He, L., and Yin, K. D. (2015). Effects of viruses on bacterial functions under contrasting nutritional conditions for four species of bacteria isolated from Hong Kong waters. *Sci. Rep.* 5:14217. doi: 10.1038/srep14217

Lowry, P. W., Becksague, C. M., Bland, L. A., Aguero, S. M., Arduino, M. J., Minuth, A. N., et al. (1990). *Mycobacterium chelonae* infection among patients receiving high-flux dialysis in a hemodialysis clinic in California. *J. Infect. Dis.* 161, 85–90. doi: 10.1093/infdis/161.1.85

Lück, C., Brzuszkiewicz, E., Rydzewski, K., Koshkolda, T., Sarnow, K., Essig, A., et al. (2015). Subtyping of the *Legionella pneumophila* "Ulm" outbreak strain using the CRISPR–Cas system. *Int. J. Med. Microbiol.* 305, 828–837. doi: 10.1016/j.ijmm.2015.08.001

Lye, D. J., and Dufour, A. P. (1993). Virulence characteristics of heterotrophic bacteria commonly isolated from potable water. *Environ Toxic Water* 8, 13–23. doi: 10.1002/tox.2530080103

Lye, D. J., Rodgers, M. R., Stelma, G., Vesper, S. J., and Hayes, S. L. (2007). Characterization of Aeromonas virulence using an immunocompromised mouse model. *Curr. Microbiol.* 54, 195–198. doi: 10.1007/s00284-006-0381-2

Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., et al. (2015). An updated evolutionary classification of CRISPR-Cas systems. *Nat. Rev. Microbiol.* 13, 722–736. doi: 10.1038/nrmicro3569

Maranger, R., and Bird, D. F. (1995). Viral abundance in aquatic systems - a comparison between marine and fresh-waters. *Mar. Ecol. Prog. Ser.* 121, 217–226. doi: 10.3354/meps121217

Miller, C. S. (2013). Assembling full-length rRNA genes from short-read metagenomic sequence datasets using EMIRGE. *Method Enzymol.* 531, 333–352. doi: 10.1016/B978-0-12-407863-5.00017-4

Mojica, F. J. M., Diez-Villasenor, C., Garcia-Martinez, J., and Almendros, C. (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 155, 733–740. doi: 10.1099/mic.0.023960-0

More, R. P., Mitra, S., Raju, S. C., Kapley, A., and Purohit, H. J. (2014). Mining and assessment of catabolic pathways in the metagenome of a common effluent treatment plant to induce the degradative capacity of biomass. *Bioresour. Technol.* 153, 137–146. doi: 10.1016/j.biortech.2013.11.065

Nagarajan, N., Cook, C., Di Bonaventura, M., Ge, H., Richards, A., Bishop-Lilly, K. A., et al. (2010). Finishing genomes with limited resources: lessons from an ensemble of microbial genomes. *BMC Genomics* 11:242. doi: 10.1186/1471-2164-11-242

Newton, H. J., Ang, D. K. Y., van Driel, I. R., and Hartland, E. L. (2010). Molecular pathogenesis of infections caused by *Legionella pneumophila*. *Clin. Microbiol. Rev.* 23, 274–298. doi: 10.1128/CMR.00052-09

Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., and Tyson, G. W. (2015). CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* 25, 1043–1055. doi: 10.1101/gr.186072.114

Philippe, N., Legendre, M., Doutre, G., Coute, Y., Poirot, O., Lescot, M., et al. (2013). Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. *Science* 341, 281–286. doi: 10.1126/science.1239181

Picardeau, M. (2017). Virulence of the zoonotic agent of leptospirosis: still terra incognita? *Nat. Rev. Microbiol.* 15, 297–307. doi: 10.1038/nrmicro.2017.5

Pop, M. (2009). Genome assembly reborn: recent computational challenges. *Brief. Bioinformatics* 10, 354–366. doi: 10.1093/bib/bbp026

Pourcel, C., Salvignol, G., and Vergnaud, G. (2005). CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* 151, 653–663. doi: 10.1099/mic.0.27437-0

Rao, C. T., Guyard, C., Pelaz, C., Wasserscheid, J., Bondy-Denomy, J., Dewar, K., et al. (2016). Active and adaptive Legionella CRISPR-Cas reveals a recurrent challenge to the pathogen. *Cell. Microbiol.* 18, 1319–1338. doi: 10.1111/cmi.12586

Ratzow, S., Gaia, V., Helbig, J. H., Fry, N. K., and Luck, P. C. (2007). Addition of neuA, the gene encoding N-acylneuraminate cytidylyl transferase, increases the discriminatory ability of the consensus sequence-based scheme for typing *Legionella pneumophila* serogroup 1 strains. *J. Clin. Microbiol.* 45, 1965–1968. doi: 10.1128/JCM.00261-07

Ren, S. X., Gang, F., Jiang, X. G., Zeng, R., Miao, Y. G., Xu, H., et al. (2003). Unique physiological and pathogenic features of Leptospira interrogans revealed by whole-genome sequencing. *Nature* 422, 888–893. doi: 10.1038/nature01597

Rodriguez-Martinez, S., Sharaby, Y., Pecellin, M., Brettar, I., Hofle, M., and Halpern, M. (2015). Spatial distribution of *Legionella pneumophila* MLVA-genotypes in a drinking water system. *Water Res.* 77, 119–132. doi: 10.1016/j.watres.2015.03.010

Sampson, S. L. (2011). Mycobacterial PE/PPE proteins at the host-pathogen interface. *Clin. Dev. Immunol.* 2011: 497203. doi: 10.1155/2011/497203

Sangwan, N., Xia, F. F., and Gilbert, J. A. (2016). Recovering complete and draft population genomes from metagenome datasets. *Microbiome* 4:8. doi: 10.1186/s40168-016-0154-5

Schmidt, T. M., and Schaechter, M. (2012). *Topics in Ecological and Environmental Microbiology*. New York, NY: Academic Press.

Schmitz-Esser, S., Linka, N., Collingro, A., Beier, C. L., Neuhaus, H. E., Wagner, M., et al. (2004). ATP/ADP translocases: a common feature of obligate intracellular amoebal symbionts related to chlamydiae and rickettsiae. *J. Bacteriol.* 186, 683–691. doi: 10.1128/JB.186.3.683-691.2004

Schroeder, G. N., Petty, N. K., Mousnier, A., Harding, C. R., Vogrin, A. J., Wee, B., et al. (2010). *Legionella pneumophila* strain 130b possesses a unique combination of Type IV secretion systems and novel Dot/Icm secretion system effector proteins. *J. Bacteriol.* 192, 6001–6016. doi: 10.1128/JB.00778-10

Segata, N., Bornigen, D., Morgan, X. C., and Huttenhower, C. (2013). PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. *Nat. Commun.* 4, 2304. doi: 10.1038/ncomms3304

Shariat, N., and Dudley, E. G. (2014). CRISPRs: molecular signatures used for pathogen subtyping. *Appl. Environ. Microbiol.* 80, 430–439. doi: 10.1128/AEM.02790-13

Soderberg, M. A., Dao, J., Starkenburg, S. R., and Cianciotto, N. P. (2008). Importance of type II secretion for survival of *Legionella pneumophila* in tap water and in amoebae at low temperatures. *Appl. Environ. Microbiol.* 74, 5583–5588. doi: 10.1128/AEM.00067-08

Stelma, G. N., Lye, D. J., Smith, B. G., Messer, J. W., and Payment, P. (2004). Rare occurrence of heterotrophic bacteria with pathogenic potential in potable water. *Int. J. Food Microbiol.* 92, 249–254. doi: 10.1016/j.ijfoodmicro.2003.08.011

Sun, C. L., Thomas, B. C., Barrangou, R., and Banfield, J. F. (2016). Metagenomic reconstructions of bacterial CRISPR loci constrain population histories. *ISME J.* 10, 858–870. doi: 10.1038/ismej.2015.162

Tamaki, H., Zhang, R., Angly, F. E., Nakamura, S., Hong, P. Y., Yasunaga, T., et al. (2012). Metagenomic analysis of DNA viruses in a wastewater treatment plant in tropical climate. *Environ. Microbiol.* 14, 441–452. doi: 10.1111/j.1462-2920.2011.02630.x

Tennessen, K., Andersen, E., Clingenpeel, S., Rinke, C., Lundberg, D. S., Han, J., et al. (2016). ProDeGe: a computational protocol for fully automated decontamination of genomes. *ISME J.* 10, 269–272. doi: 10.1038/ismej.2015.100

Thomas, V., Loret, J. F., Jousset, M., and Greub, G. (2008). Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant. *Environ. Microbiol.* 10, 2728–2745. doi: 10.1111/j.1462-2920.2008.01693.x

Tilney, L. G., Harb, O. S., Connelly, P. S., Robinson, C. G., and Roy, C. R. (2001). How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. *J. Cell Sci.* 114, 4637–4650.

Tortora, G., Funke, B., and Case, C. (2013). *Microbiology: An Introduction, 11th Edn*. Yorkshire: Pearson.

Vaerewijck, M. J. M., Huys, G., Palomino, J. C., Swings, J., and Portaels, F. (2005). Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiol. Rev.* 29, 911–934. doi: 10.1016/j.femsre.2005.02.001

Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., et al. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304, 66–74. doi: 10.1126/science.1093857

Voth, D. E., Broederdorf, L. J., and Graham, J. G. (2012). Bacterial Type IV secretion systems: versatile virulence machines. *Future Microbiol.* 7, 241–257. doi: 10.2217/fmb.11.150

Wadowsky, R. M., Butler, L. J., Cook, M. K., Verma, S. M., Paul, M. A., Fields, B. S., et al. (1988). Growth-supporting activity for *Legionella-pneumophila* in tap water cultures and implication of hartmannellid amebas as growth-factors. *Appl. Environ. Microbiol.* 54, 2677–2682.

Wattam, A. R., Abraham, D., Dalay, O., Disz, T. L., Driscoll, T., Gabbard, J. L., et al. (2014). PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic Acids Res.* 42, D581–D591. doi: 10.1093/nar/gkt1099

Wilson, J. W., Schurr, M. J., LeBlanc, C. L., Ramamurthy, R., Buchanan, K. L., and Nickerson, C. A. (2002). Mechanisms of bacterial pathogenicity. *Postgrad. Med. J.* 78, 216–224. doi: 10.1136/pmj.78.918.216

Wilson, R. W., Steingrube, V. A., Bottger, E. C., Springer, B., Brown-Elliott, B. A., Vincent, V., et al. (2001). *Mycobacterium immunogenum* sp nov., a novel species related to Mycobacterium abscessus and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy. *Int. J. Syst. Evol. Microbiol.* 51, 1751–1764. doi: 10.1099/00207713-51-5-1751

Wu, H. J., Wang, A. H. J., and Jennings, M. P. (2008). Discovery of virulence factors of pathogenic bacteria. *Curr. Opin. Chem. Biol.* 12, 93–101. doi: 10.1016/j.cbpa.2008.01.023

Wu, Y. W., Simmons, B. A., and Singer, S. W. (2016). MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. *Bioinformatics* 32, 605–607. doi: 10.1093/bioinformatics/btv638

Yu, V. L., Plouffe, J. F., Pastoris, M. C., Stout, J. E., Schousboe, M., Widmer, A., et al. (2002). Distribution of Legionella species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. *J. Infect. Dis.* 186, 127–128. doi: 10.1086/341087

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhang, Y., Oh, S., and Liu, W.-T. (2017). Impact of drinking water treatment and distribution on the microbiome continuum: an ecological disturbance's perspective. *Environ. Microbiol.* 19, 3163–3174. doi: 10.1111/1462-2920.13800

Zhou, Y., Liang, Y. J., Lynch, K. H., Dennis, J. J., and Wishart, D. S. (2011). PHAST: a fast phage search tool. *Nucleic Acids Res.* 39, W347–W352. doi: 10.1093/nar/gkr485

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership