USING NEXT-GENERATION METAGENOMIC TOOLS FOR MICROBIAL CHARACTERISATION OF DRINKING WATER.

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ABSTRACT (500 WORDS MAXIMUM)

Current Drinking Water Quality Assurance Rules 2022 refer to *Escherichia coli* and total coliforms as the primary methods for microbiological monitoring of water quality within drinking water distribution systems, water leaving treatment plants and source waters. Secondary investigations into source water contamination currently use faecal source tracking tools to identify sources of contamination to aid mitigation efforts.

This paper describes innovative research by ESR that attempts to improve monitoring and surveillance for water resource management by implementing next-generation sequencing and metagenomic tools to characterise microbial contaminants. These tools allow us to investigate the entire bacterial community associated with a water sample rather than relying on single bacterial species (*E. coli*) as sentinels of contamination. Therefore, we can identify which microbes are present naturally (i.e., environmental), which are influenced by contamination events (e.g., flooding and rainfall), and how they are altered by treatment and processing. This approach creates a clearer picture of areas of concern in the drinking water supply, and when testing should be increased. Our innovative approach allows us to perform timely identification as well as longer-term studies of chronic pollution to mitigate contamination events and protect water resources.

This drinking water research is aspirational and builds on ESR's well-established research background characterising faecal sources and microbial contaminants in surface waters such as rivers. However, in comparison to surface waters drinking water and groundwater provide more challenging environments due to typically lower levels of contaminants. These lower concentrations of bacterial and/or faecal contaminants still represent a health risk but require innovative sequencing techniques to identify microbes of interest. We have applied amplicon metagenomic sequencing to the drinking water samples to increase confidence in identification of environmental bacteria and potential pathogens and improve the sensitivity of detection.

We first presented the methodological concept of this work at WaterNZ, 2022. Since then, we have established partnerships with drinking water suppliers, and here we present case studies profiling real-world samples from sampling locations throughout the network supply, literally taking a source to tap approach. The case studies illustrate our research towards implementing cost-effective, rapid metagenomic approaches that identify bacterial populations and characterise contamination events.

KEYWORDS

Metagenomics; water quality; health and safety; bacterial communities; pathogen detection; monitoring and surveillance.

PRESENTER PROFILE

Meg Devane is a microbiologist working at ESR as a senior scientist advising councils and iwi about faecal contamination in water. Implementation of safer drinking water requires new tools. Meg will present innovative DNA methods for investigating entire bacterial communities in water by using next-generation metagenomic tools.

INTRODUCTION

Current Drinking Water Quality Assurance Rules 2022 refer to *Escherichia coli* and total coliforms as the primary methods for monitoring microbial water quality within drinking water distribution systems, water leaving treatment plants and source waters.

This paper describes innovative research by ESR, that attempts to improve monitoring and surveillance for water resource management by implementing next-generation sequencing and metagenomic tools to characterise microbial contaminants. These tools allow us to investigate the entire bacterial community within a water sample and identify which microbes are present naturally (i.e., environmental), which are influenced by contamination events (e.g., flooding and rainfall), and how they are altered by treatment and processing. Our innovative approach allows us to perform timely identification and longer-term studies of chronic pollution to mitigate contamination events and protect water resources.

This drinking water research is aspirational and builds on ESR's well-established research characterising faecal sources and microbial contaminants in surface waters such as rivers. In comparison to surface waters, drinking water and groundwater provide more challenging environments due to typically lower levels of contaminants. These lower concentrations of bacterial and/or faecal contaminants still represent a health risk but require innovative sequencing techniques to identify microbes of interest. We will discuss methods including amplicon metagenomic sequencing which have been applied to the drinking water samples to increase confidence in identification of environmental bacteria and potential pathogens.

Here, we present case studies from network suppliers who asked us to investigate source water quality issues and total coliform detections. These case studies illustrate that groundwater is not sterile and network supplies contain a complex bacterial community highlighting the utility of the metagenomic approach for

characterising bacteria and contamination events. These case studies include: the effect of a manganese filter system on bacterial community changes, the impacts of chlorination on waters containing iron and manganese and the changes in bacterial communities pre- and post-chlorination events triggered by total coliform detections.

The case studies will illustrate our research towards implementing cost-effective, rapid metagenomic approaches that support network suppliers to mitigate contamination events, protect water resources, and improve surveillance and remediation practices.

DISCUSSION

2.1 CASE STUDY 1: THE BACTERIAL COMMUNITY ASSOCIATED WITH MANGANESE FILTERS

2.1.1 BACKGROUND

The drinking water source for Case Study 1 is from six groundwater wells, each 140–250 metres deep. These groundwater wells naturally contain manganese. Elevated levels of manganese can have a number of aesthetic and health impacts. Aesthetic impacts include creating dark brown or black stains on showers, sinks, bathtubs, laundry, and plumbing fixtures. Manganese can result in an unpleasant metallic taste in the water, and when this water is used for cooking can impart a metallic flavour to foods. Health issues from ongoing consumption of high levels of manganese can include cognitive problems such as motor, memory and attention issues, and manganese toxicity can cause multiple side effects such as muscle tremors, insomnia, hearing problems, loss of appetite, depression, and headaches. As a consequence, the Water Services (Drinking Water Standards for New Zealand) Regulations 2022 specify a maximum acceptable value (MAV) of <0.4 mg/L of manganese. For aesthetic purposes the limit guidance value (GV) is 0.04 mg/L (Aesthetic Values for Drinking Water Notice, 2022).

During February 2022 results of 0.04 to 0.1 mg/L were reported in these wells and levels greater than the MAV have been previously observed. To reduce the manganese levels, the water from these six wells was mixed and then processed in parallel through two biological filters. At the time the water sources were split into two streams, with one stream undergoing additional chlorine treatment prior to distribution while the other stream remained untreated. Typical manganese values after treatment have been <0.0005 mg/L. The biological manganese filters pass aerated water through a sand filter, allowing microorganisms to proliferate in biofilms within the void spaces. A natural enrichment of bacteria results in removal of the manganese from the water. The filter is back washed twice a week.

2.1.2 ANALYSIS

Samples were collected from 14 locations within the water supply on two separate occasions in 2022 (Appendix A). At the time of sampling there was no

chlorination in part of the network and no total coliforms or *E. coli* were detected in any samples. Nucleic acids were extracted and amplicon metagenomic analysis performed targeting the 16S rRNA gene using Oxford Nanopore Technology (ONT). Figure 1 presents the workflow pipeline for processing sequences and assigning bacterial taxonomies to each read; including using an expectation-maximization algorithm for potentially pathogenic species (Curry et al., 2022).

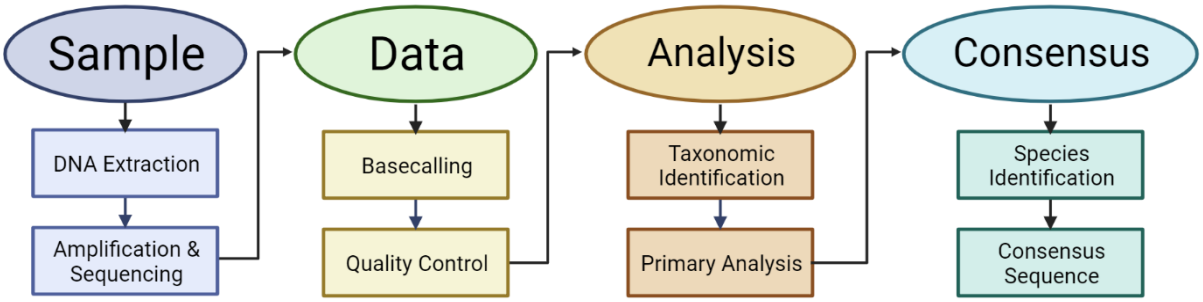

Figure 1: DNA analysis pipeline

2.1.3 RESULTS

There was a significant variation in the read counts of microorganisms detected in the groundwater wells. The highest read counts were in Well 2, while the lowest were in EQ2 and EQ3 wells (Figure 2). Read counts do not correlate directly to numbers of bacteria in a sample but provide an indication of the concentration of each type of bacteria. Compositionally, the bacterial communities in the wells were stable between time points (Figure 2). The wells 1 and 2 and EQ1 wells were largely dominated by the family Comamonadaceae, while the others had greater bacterial diversity. Groundwater in the wells were consistent with groundwater microbial communities, with no indication of pathogens, faecal or surface water microorganisms.

Compared with the well samples, there was a 10-fold increase in read count in the filter samples with a change in the bacterial community. The filter, headworks and reticulation samples contained fewer taxa at both the family (Figure 2) and genus level. These microorganisms were consistent with organisms enriched in the manganese filters. Post-groundwater sources, *Nitrospira* was the dominant genus in all samples and was the dominant member of the Nitrospiraceae family (Figure 2). *Nitrospira* is a complete ammonia-oxidising (Commamox) bacterium that converts ammonia to nitrate in a single-step (Daims et al., 2015). *Nitrospira* is usually present in oligotrophic waters with low ammonia concentrations such as groundwater conditions.

Throughout the network from the filter to the reticulation sites, samples were dominated by *Nitrospira* and ammonia-oxidising *Nitrosomonas* (Figure 2). When

these two genera were omitted from the analysis, there was a wide diversity of organisms present but the community composition remained consistent from the filters onwards through the distribution system. Pathogens were not identified in the water samples from the filter, headworks and reticulation network.

PERMANOVA analysis showed that the date of sampling had a variance contribution of 1.4%, which was not statistically significant. In comparison, bacterial community variability in samples was explained by the sample type and sample origin (47.9% and 45.1%, respectively, p-value <0.001). Groundwater wells were less similar to each other; however, there was high continuity between filter, headworks/transfer line and reticulated water samples as expected from the similar bacterial communities observed post-Manganese filtration (Figure 3). This similarity highlights the impact the manganese filters have on the bacterial community, which was highly diverse between the different groundwater sources in wells before passing through the manganese filters (Figure 2).

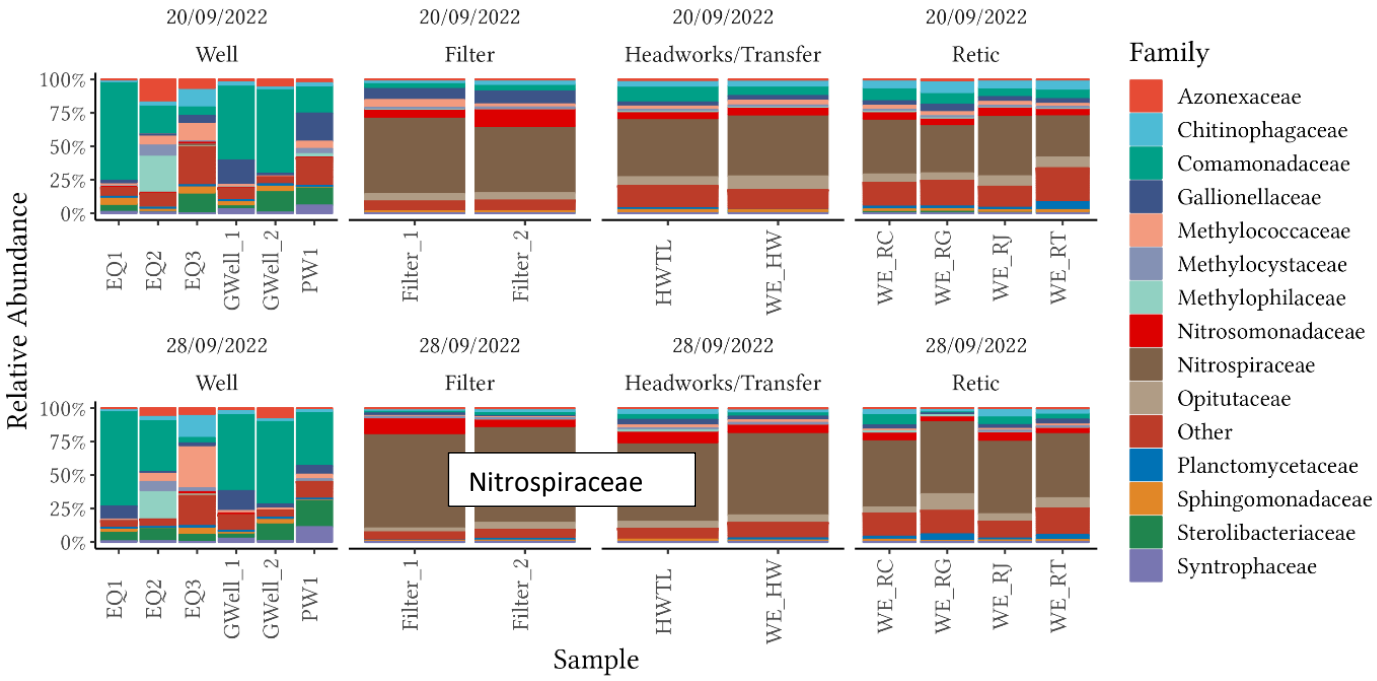

Figure 2: Water Supply with manganese filters a) bacterial read counts; b) bacterial family relative abundance for Case Study 1. Samples were collected from the water supply on the two sampling dates of 20th and 28th of September in 2022.

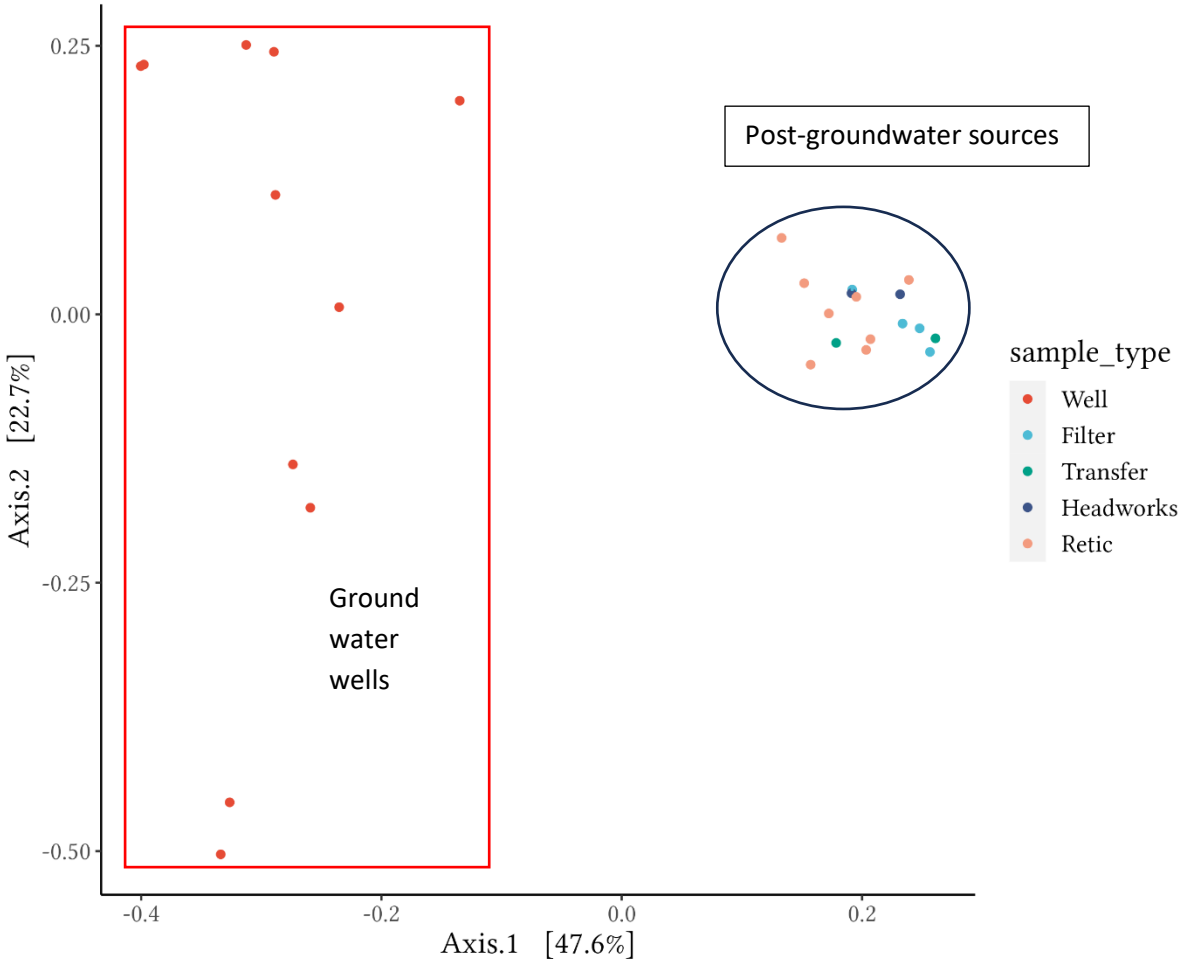

Figure 3: Bray–Curtis Principal Coordinates Analysis of well and reticulation samples showing the diversity of the bacterial community in the wells compared with the similarity of the bacterial communities sampled from the filters onwards through the distribution system.

2.1.4 CONCLUSION

Groundwater in the wells were consistent with groundwater microbial communities, and there was no indication of pathogens, faecal or surface water microorganisms in any of the groundwater or network samples. Compared with the well samples, there was a 10-fold increase in read count in the filter samples with a change in microbial community reflective of organisms enriched in the manganese filters. The filter, headworks and reticulation samples contained fewer taxa at both the family and genus taxa levels showing a notable reduction in community diversity compared to source waters. This similarity highlights the impact the manganese filters have on the bacterial community, which was highly diverse between the different groundwater sources in the wells prior to the manganese filters.

2.2 CASE STUDY 2: DECTECTION OF IRON AND MANGANESE IN A WATER SUPPLY

2.2.1 BACKGROUND

In March 2023, Total Coliforms (TC) were detected in a drinking water scheme. Chlorination was initiated at the treatment plant and black metallic flecks of material were subsequently identified in the drinking water. Laboratory analyses revealed the flecks to be high in iron and manganese in these reticulation samples. The black metallic flecks may have come from reactions between chlorine and the pipes in the distribution system, or chlorine reactions with the iron and manganese in solution, producing compounds that precipitated out as metallic flecks (Allard et al., 2013; IARC, 1991). Historically, there have been iron-associated bacteria recorded in the system, and there have been elevated iron levels recorded, but consistently low manganese levels in the water.

2.2.2 ANALYSIS

Amplicon metagenomic analysis was conducted on water samples to determine what role bacteria had in the formation of the black flecks, and the potential origins of Total Coliforms (TCs) that triggered chlorination within the treatment plant.

Water samples were collected from a water treatment plant (Figure 4) on 5/03/2023 and 6/03/2023 (Appendix B). At the time of sampling low levels of TC were detected in raw water tanks and pre-filter and pre-UV samples in the samples taken on 6/03/2023. Nucleic acids were extracted and metagenomic analysis performed targeting the 16S rRNA genes using Oxford Nanopore Technology (ONT). Figure 1 presents the workflow pipeline for processing sequences and assigning bacterial taxonomies to each read; including using an expectation-maximization algorithm for potentially pathogenic species (Curry et al., 2022).

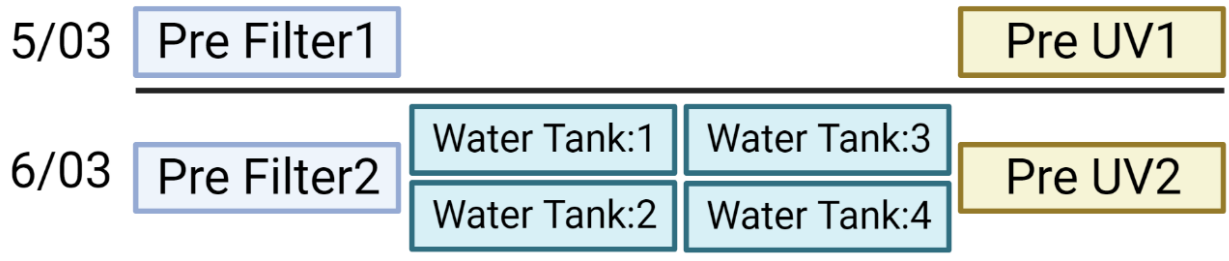

Figure 4: Sampling Locations at the rural water supply for Case Study 2

2.2.3 RESULTS

Between 52,000 and 72,000 reads were obtained from each sample with a relatively consistent level of reads between samples (Figure 5). All samples contained sufficient reads for subsequent interpretation.

Between sampling days, the detected bacterial families were relatively consistent, with no substantive deviations in the bacterial communities (Figure 5). Although Gemmataceae (phylum: Planctomycetes) dominated all samples, the Raw Water Tanks 3 and 4 had greater proportions of these aquatic bacteria, which are often identified in water supplies associated with filtration systems (Lautenschlager et al., 2014). Potential pathogens or faecally-associated bacteria were not identified in samples.

The metagenomic analysis in this study identified a number of bacteria that could contribute to the appearance of black flecks. These included the identification of *Gallionella*, *Sideroxydans* and *Hyphomicrobium*. For instance, *Gallionella capsiferriformans* has been reported to concentrate organic iron compounds (Ridgway et al., 1981). In addition, manganese oxidising bacteria may sequester manganese in biofilms that could be disrupted and release the manganese into the water during chlorination (Piazza et al., 2019). Members of the family Gallionellaceae can carry out both roles, such as the genus *Sideroxydans*, which was identified in all samples. Manganese oxidation is a ubiquitous process among many bacteria (Ghiorse, 1984; Nealson, 2006).

Total coliforms are a large group of bacteria, many of which belong to the Enterobacteriaceae family (e.g., *Escherichia coli*) which are prevalent in the intestinal gut of mammals. Total coliforms are defined as Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with the production of gas and acid within 48 hours at 35°C (APHA, 1998). Eleven non-coliform genera were identified in the bacterial communities which have the capability to produce the enzyme used for confirmation of total coliforms by the Colilert test. However, the proprietary media from Colilert utilises a defined substrate medium that supports the growth of total coliforms and *E. coli*, largely excluding the growth of non-coliform bacteria (Rompré et al. 2002). Further investigation of total coliforms detected by Colilert is an important next-step for the delivery of safe drinking water, and metagenomic analysis has provided the ability to determine whether total coliform detections are indicative of pathogens in a water supply.

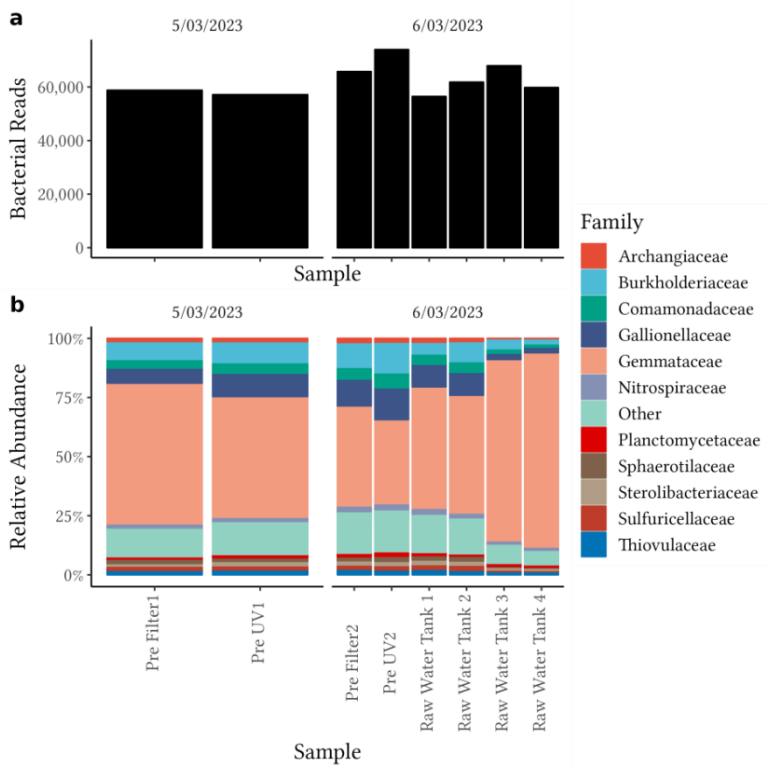

Figure 5: Relative abundance of identified microbial families across samples in Case Study 2. a) total bacterial read counts; b) relative abundance of bacterial families. Those families with relative abundances >1% are listed as "Other'.

2.2.3 CONCLUSIONS

A consistent bacterial community was identified in all samples and sites: pre-filter, water tanks and pre-UV treatment. Detection of total coliforms by Colilert was not associated with metagenomic identification of members of the coliform bacteria, which are the target of the Colilert testing media. Furthermore, potential bacterial pathogens were not identified in the drinking water network. Bacteria identified were of environmental origin, and no bacteria of faecal origin or surface water ingress were evident. Bacteria that could have contributed to the appearance of metallic black flecks were identified across samples supporting the hypothesis that bacteria (e.g., the identified genera of *Gallionella* and *Sideroxydans*) can sequester and concentrate iron and manganese in their cells, and chlorine can cause these elements to precipitate resulting in metallic flecks in the water post-chlorination.

2.3 CASE STUDY 3: BACTERIAL COMMUNITIES WITH AND WITH OUT CHLORINATION

2.3.1 BACKGROUND

Routine sampling of network locations in a town municipal supply detected total coliforms (TC) in a number of samples. No *E. coli* were detected. These sampling locations had previously not had any TC detections for many months. It was

hypothesised that contamination could be due to a 'slug' of biofilm being pushed into the reticulation network from the opening of a pressure release valve (PRV) located upstream of where the water was sampled.

2.3.2 ANALYSIS

Samples were collected from 2 pump stations and 10 reticulation locations (Table 1). The pump station samples were unchlorinated. Chlorination was initiated immediately prior to sample collection for the reticulation sites but only three of the network sites had FAC >0.1 mg/L at the time of sampling (Table 1). Five of the network locations had total coliforms detected, albeit at lower levels than seen in earlier routine sampling events.

Amplicon metagenomic analysis was conducted on the water samples to determine the potential origins of Total Coliforms (TCs) that triggered chlorination within the treatment plant. Nucleic acids were extracted from water samples and metagenomic analysis performed targeting the 16S rRNA genes and analysed using an Oxford Nanopore Technology (ONT). Figure 1 presents the workflow pipeline for processing sequences and assigning bacterial taxonomies to each read; including using an expectation-maximization algorithm for potentially pathogenic species (Curry et al., 2022). Additional analysis for potential pathogens utilised a full metagenomic approach to confirm species identification (Brumfield et al., 2020).

Table 1: Samples collected from 2 pump stations and 10 reticulation sites in Case Study 3

Sample Number	Site	Time	Total Coliforms (MPN/100 mL)	Free Available Chlorine (mg/L)	Read counts	Potential Pathogens
CMB220538	Pump 1	14.10	<1	NA	1,400	
CMB220539	Pump 2	14.58	<1	NA	11,036	
CMB220536	Retic A	13.53	<1	0.82	431	
CMB220533	Retic B	14.24	<1	0.72	336	
CMB220534	Retic C	14.41	<1	0.68	95	
CMB220528	Retic D	12.12	250	<0.10	1,274,051	
CMB220529	Retic E	12.43	140	<0.10	1,190,670	
CMB220530	Retic F	12.24	350	<0.10	1,067,940	
CMB220537	Retic G	10.35	55	<0.10	467,442	*
CMB220535	Retic H	13.00	8	<0.10	187,052	
CMB220531	Retic I	15.31	<1	<0.10	67,507	
CMB220532	Retic J	15.15	<1	<0.10	109,037	

**Listeria innocua*- confirmed by full metagenomic (shotgun) sequencing

2.3.3 RESULTS

Total coliforms were identified in five of the seven reticulation samples that contained <0.10 mg/L free available chlorine (FAC) (Table 1). These five

samples (Retic D-H) also had a range of 187,000 to 1.2 million read counts of bacteria in each sample (Table 1). In comparison, the samples from Reticulation sites A-C that had FAC ranging between 0.68-0.82 mg/L contained the lowest number of read counts. Read counts do not correlate directly to numbers of bacteria in a sample but provide an indication of the concentration of bacteria. Chlorination of the network, where there was a residual of FAC, appeared to reduce the read counts of bacterial communities to low levels compared to the higher read counts in other parts of the reticulation system where FAC was <0.10 mg/L. The few microorganisms detected in these samples (Retic A-C) with a residual FAC may not be in a viable state and were environmental bacterial taxa such as the members of the family Comamonadaceae (Figure 6).

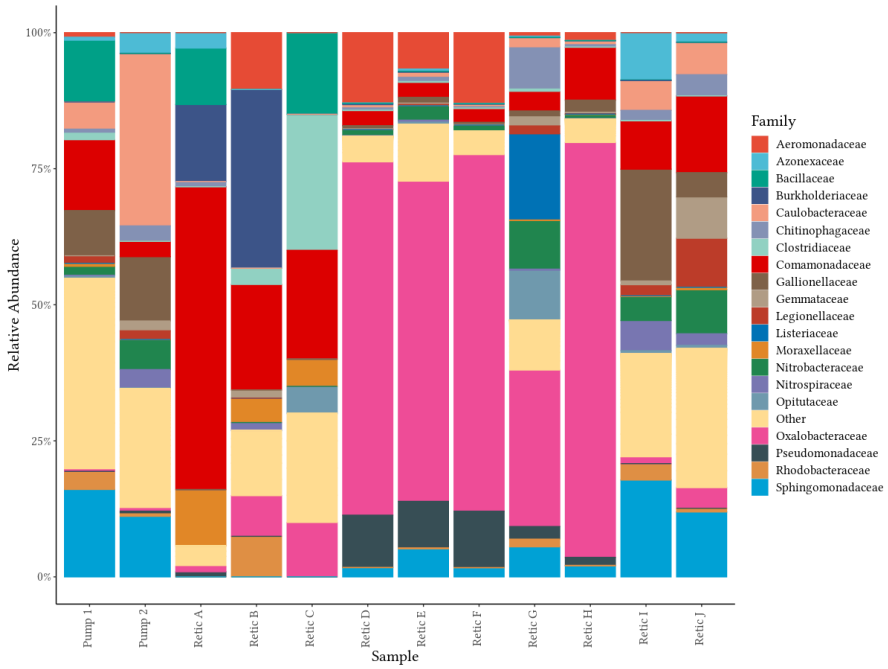

Figure 6: Sampling Locations in the municipal water supply from Case Study 3; Relative abundance of each family of bacteria identified at >1% abundance. "Other" category contains bacteria that did not reach the 1% threshold for abundance.

A member of the Listeriaceae family was identified at site Retic G where TC was identified in conjunction with <0.10 FAC. Further analysis by advanced full metagenomic approaches suggests these *Listeria* are likely to be *L. innocua*, a biofilm producing soil-borne species with low pathogenicity risk (Perni et al., 2006; Perrin et al., 2003; Orsi & Wiedmann, 2016). *L. innocua* is common in urban environments. While this could indicate sample contamination, it more likely points to a localised source of bacteria associated with pipes at this location and may support the proposal of a biofilm 'slug' released during opening of a pressure release valve. Removal of the *Listeria* from the Retic G sample suggests a metagenomic profile, similar to the other sites (Retic D-F and H) where total coliforms were detected (Figure 6). In addition, members of the Legionellaceae family detected in the reticulated system were identified as

species not regarded as pathogenic to humans (Figure 6).These samples with total coliforms were dominated by the Oxalobacteraceae family whose members are found in diverse environmental habitats like water, soil, and plant-associated, with some members adapted to low nutrient environments (Baldani et al. 2014).

Retic samples I and J shared a similar bacterial community profile to the two pump station bacterial communities and FAC >0.10 mg/L with read counts <110,000 (Figure 4). These two sites were on separate lines independent of the other reticulated sites.

2.3.4 CONCLUSIONS FOR CASE STUDY 3

While this is only a snapshot of the water network of samples taken during implementation of chlorination it does suggest that the increased total coliforms are associated with environmental biofilm-related taxa that have become established within the pipes. Chlorination, where there is residual FAC, appears to reduce this microbial community.

CONCLUSIONS

Initial studies have shown the value of metagenomic analysis to better understand and manage microbial health risks affecting drinking water. Specifically, metagenomic analysis of drinking water samples in distribution supplies has shown the potential to provide timely information to:

- Evaluate the implications of detection of total coliforms and *E. coli* in drinking water.
- Identify the ingress of faecal contamination sources into the water supply.
- Confirm the potential detection of (faecal and non-faecal) pathogens in a water supply.
- Identify changes in the bacterial community within the treatment plant, especially post-treatment processes, e.g., manganese filters. In addition, monitoring of bacterial communities could potentially identify disturbance events such as climate impacts.
- Identify bacteria in a water supply that have the potential to contribute to precipitation of iron and manganese post-chlorination.

Although Colilert™ (IDEXX) detections of total coliforms can be generated by non-coliform bacteria, it is unlikely that the proprietary media formulation of Colilert™ would permit significant growth of these organisms. Total coliform detections, therefore, provide a sentinel, alerting further investigation is required to ensure non-detection of faecal contamination and potentially pathogenic microbes in a water supply.

ACKNOWLEDGEMENTS

ESR would like to acknowledge the drinking water treatment plant operators and staff who have worked with us to supply samples and information that allowed this metagenomic investigation to be conducted in their environment. The

research is funded by ESR through the Ministry of Business, Innovation and Employment's Strategic Science Investment Fund, to advance science and research capabilities for Aotearoa.

DISCLAIMER

This is a preliminary scientific research report on the microbial world identified in drinking water. This research has been carried out as part of an Institute of Environmental Science and Research (ESR) project to explore and test novel approaches for examining bacteria within water environments using genomic tools. Due to the novel and experimental aspects of this research, any analysis and interpretations should be taken as preliminary only. Findings through this method may require additional validation or explanation.

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NOMENCALTURE

Bp	base pairs
DNA	Deoxyribonucleic acid
ONT	Oxford Nanopore Technology sequencing platform
PRV	pressure release valve
TC	Total Coliforms

APPENDICES

Appendix B: Samples received for analysis for Case Study 1: The bacterial community associated with manganese filters.

	Sample Number	Site	Date Collected	Read Count
Wells	CMB220624	Well1	20/09/22	38,245
	CMB220660		28/09/22	17,531
	CMB220625	Well 2	20/09/22	123,345
	CMB220661		28/09/22	80,205
	CMB220626	EQ1	20/09/22	56,959
	CMB220662		28/09/22	12,204
	CMB220627	EQ2	20/09/22	9,211
	CMB220663		28/09/22	4,598
	CMB220628	EQ3	20/09/22	1,182
	CMB220664		28/09/22	10,062
	CMB220629	PW1	20/09/22	16,709
	CMB220665		28/09/22	55,131
Filters	CMB220630	Filter 1 outlet	20/09/22	629,302
	CMB220666		28/09/22	408,106
	CMB220631	Filter 2 outlet	20/09/22	473,464
	CMB220667		28/09/22	66,230
Headworks	CMB220632	Transfer line	20/09/22	131,856
	CMB220673		28/09/22	432,593
	CMB220633	Headworks 2	20/09/22	204,357
	CMB220668		28/09/22	579,478
Reticulation Sites	CMB220634	Retic A	20/09/22	26,023
	CMB220669		28/09/22	406,326
	CMB220635	Retic B	20/09/22	1,979,555
	CMB220670		28/09/22	436,755
	CMB220636	Retic C	20/09/22	8,424
	CMB220671		28/09/22	434,001
	CMB220637	Retic D	20/09/22	282,258
	CMB220672		28/09/22	422,761

Appendix B: Samples received for analysis for Case Study 2: Detection of iron and manganese in a water supply.

	Sample Number	Site	Date Collected	Total Coliforms (MPN/100 mL)	Volume Filtered
Pre Filter	CMB230182	Pre Filter1	5/03/2023	0	800
	CMB230183	Pre Filter2	6/03/2023	12	1000
Pre UV	CMB230192	Pre UV1	5/03/2023	0	800
	CMB230193	Pre UV2	6/03/2023	5	1000
Raw Water Tank	CMB230188	Raw Water Tank 1	6/03/2023	2	1000
	CMB230189	Raw Water Tank 2	6/03/2023	3	1000
	CMB230190	Raw Water Tank 3	6/03/2023	11	1000