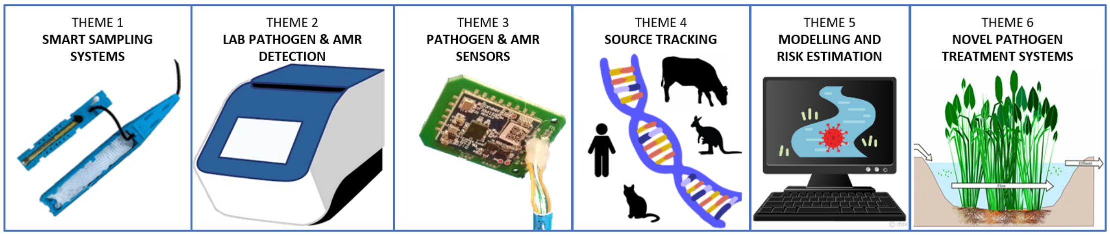
HEADING 1. HIGH-LEVEL RESEARCH PLAN, INCLUDING KNOWLEDGE MOBILIZATION

Aim. This PROGRAM will innovate, develop and validate novel sampling and sensing methods, rapid diagnostic tools, integrated models and treatment options for the surveillance, prediction and mitigation of waterborne pathogens via six research themes:



Deliverables. (1) smart sampling and sensing systems to detect temporal and spatial trends of pathogens in water systems, (2) rapid and near-real-time assays to detect pathogens and their sources, (3) tools/models that can provide early warning of, and mitigation options to limit, disease in our community, (4) treatment of pathogens to protect human health when water is used by humans and (5) training, standard operating procedures, reference materials and guidance manuals to ensure our outputs are useful and adopted by our partners.

Background, novelty and significance

Access to safe water and sanitation is a public health concern. The World Health Organization estimates a global mortality of 2.2 million per year due to waterborne diseases[1], resulting in $12 billion/year of economic loss worldwide[2]. While these numbers are mostly from the global south and marginalized communities[3], all nations including Australia face similar challenges when managing risks from waterborne pathogens[4]. Management of waterborne risks will intensify as we face challenges to water supplies and quality due to population growth and urbanization[5], our rapidly-changing climate[6], land-use alterations and aging infrastructure[7].

Waterborne disease is caused when we interact with water carrying pathogenic microorganisms, many sourced from the feces of animals and humans[8]. These microbes may also carry antimicrobial resistant genetic material (AMRs)[9] which can be transferred to others in environmental settings and reduce the effectiveness of treatment options[9]. Feces from domestic, wild and agricultural animals enter our water during rainfall events via stormwater runoff from our catchments[10]. Human feces is not always treated prior to release into our water systems, and raw human wastewater can enter via emergency overflow structures, leaking on-site septic systems or cross-connections between the sanitary and storm sewers[11]. The rich sources and transport pathways in catchments combined with the vast array of pathogens and AMRs that animals and humans excrete result in a complex problem that this PROGRAM aims to address through surveillance, modelling and mitigation.

Monitoring pathogens and AMRs in water is needed to understand and manage the risks of waterborne risk, and commonly required for regulatory purposes. It is also a critical step in Quantitative Microbial Risk Assessments (QMRA[12]) to understand hazards and exposure. Monitoring is essential for wastewater-based epidemiology (WBE)[13], whereby signals within wastewater are used to understand and manage the use of drugs[14] or the spread of community disease such as COVID-19[15].

Grab- or spot-sampling is a common first step for pathogen monitoring owing to its simplicity and ease of execution[16]. But grab-sampling can miss important events of interest[17] and may therefore underestimate health risks, such as peak pathogen-loading events entering a drinking water treatment plant or the shedding event from an individual being tracked within a catchment via WBE[15]. Autosamplers can take more frequent samples across periods of interest[16], however they are expensive, require highly trained staff to install and operate, come with significant installation risks, have high space and power requirements and are designed for small sample volumes. Such problems limit the use of autosamplers for multiple monitoring locations, which we know is important to manage risks in water systems adequately as pathogen types and levels can vary significantly between sites[18, 19].

Smart sampling systems are needed that can be easily and cost-effectively deployed at high spatial resolution yet can capture the temporal dynamics of pathogens in water systems. Theme 1 of this PROGRAM will build on our award-winning sampling technologies (see Significant Contributions, CV), which has been used globally to monitor pathogens in water[15, 20-23].

Once sampled, health agencies prefer traditional culture-based analytical methods for risk assessments as they ensure the pathogen is active/viable. These are, however, time-consuming and result in long delays in reporting (Turn Around Time [TAT] of up to 14 days). Molecular methods (such as qPCR) reduce labour costs, reduce TAT and are sensitive and specific, making them preferred for many applications (e.g., WBE). However, molecular assays need to be optimized for the low pathogen/AMR levels found in environmental water and the high inhibition that can occur following the laboratory concentration steps required to detect these low levels. Further, the viability of microorganisms is difficult to determine using molecular approaches[24-26], making them less applicable for assessing and managing risks. A compromise is to use molecular detection coupled with pre-enrichment culturing to ensure viability, such as our *Campylobacter* spp. assay which has a TAT of just 3 days (vs. 10 days)[27]. Culturing also enables further processing to better appreciate the potential to cause infection (such as our *in vitro* attachment, invasion and cytotoxicity assays[28, 29] or whole-genome sequencing[30], transcriptomics[30] and Multi-Locus Sequence Typing[31]). Such techniques are not standardized[30], so large methodological differences exist to determine viability and infectivity for risk assessment, making it difficult to transfer knowledge to other, perhaps more marginalized, communities.

Optimized and standardized pathogen, infectiousness and AMR detection methods in environmental systems are missing, in particular for use in risk assessments. Theme 2 of this PROGRAM builds upon our existing research to optimize pathogen and AMR detection techniques[82] and to determine pathogen viability and potential infectiousness[28,29].

More responsive TATs than those provided by qPCR are sometimes required for quick decision-making or implementation of targeted health actions, e.g., managing recreational waters by providing real time water quality information to swimmers[32]. Such applications have driven the development of spectro-photometric sensors[33], biosensors[34-36] and Lab-on-a-Chip technologies[37] that are either used manually at the site of sample collection (in-field) or positioned within the water for continuous sensing of pathogens (in situ). In-field detection systems[33, 36, 38] are advanced, however, their uptake in industry and in practice is modest, perhaps due to lack of independent comparisons and validation against traditional measurement methods. *In situ* and real-time monitoring sensors are much less advanced, being plagued by limit of detection issues, maintenance requirements (including requiring the continuous supply of media) and high costs[33-35, 37]. Another solution are software sensors (or soft-sensors), where algorithms and artificial intelligence combine more-easily-measurable water quality metrics or surrogates to predict a pathogen concentration[39, 40].

Field-based and in situ detection of pathogens is a useful surveillance tool for managing waterborne risks. Theme 3 of this PROGRAM will build on our sensing expertise[41-43] to (1) provide a platform for technology developers to test and validate products, (2) develop and validate in situ *soft-sensors for pathogen detection in water systems, and (3) conduct fundamental research to develop and test in situ pathogen and AMR sensors with reduced maintenance and costs.*

While the surveillance of pathogens is important to understand and manage risks, understanding pathogen and AMRs sources is required to determine where and how to mitigate (e.g., [44]). Microbial Source Tracking (MST) discerns pollution origins but specificity and accuracy issues have been noted especially when using just one marker[45, 46], hence newer evidence requires multiple microbial markers for source attribution[47, 48]. Our Next Generation Sequencing (NGS) source tracking methods are based on 16s rRNA amplicons, and generate fingerprints with thousands of potential markers[11, 49]. However, issues remain with sensitivity being limited by use of broad-spectrum primers that target all bacteria (i.e., not just those associated with fecal sources) and the fact that all NGS-based MST remains focused on bacterial genes, not viral or protozoan.

MST is a viable method for understanding sources/origins of pathogens, and a useful input into QMRA. With the advance of next-generation sequencing techniques, Theme 4 of this PROGRAM will build on our experience in MST[11, 49] to uncover and validate more specific and sensitive bacterial, protozoan and viral markers, for laboratory use, in field testing and in situ sensing.

With the diverse origins/sources of pathogens and AMRs combined with complex water dynamics, risk management options are often difficult to develop. This can result in spending taxpayer money without measurable reductions in human health risks (e.g., [44]). Integrated modelling tools should be used to help assess potential mitigation options[50], answering questions like: “Should we fix asset A or asset B?” and “Is the cost justified by the predicted reduction in human health risk?” Furthermore, integrated microbial models coupled to QMRA have shown potential to replace continuous monitoring efforts to provide real-time information about waterborne risks (e.g., [32]). Integrated models play an integral part of WBE, including trend projection[51]. However, modelling pathogens in water is difficult, suffering from a poor understanding of pathogen sources, fate and transport, compounded by uncertain data available for calibration and validation[52]. This limits our ability to (1) provide real time predictive information to the public about risks, (2) do cost-benefit analysis of mitigation options, and (3) prioritize mitigation efforts.

Models that integrate pathogen and AMRs sources, fate and transport together with QMRA are useful for communicating and managing public health risks and to inform mitigation strategies. Theme 5 of this PROGRAM will build on our pathogen modelling[53-55] and QMRA[12, 56, 57] expertise to co-develop models with local agencies to help protect public health.

While non-structural options for managing risks can be preferred (such models inform public when to swim[32]), physical intervention is sometimes required to treat water prior to human interaction. While traditional methods exist to remove pathogens from water, they rely on high energy inputs, require frequent maintenance or use chemicals that cause ecosystem and human health risks. Systems that are based on natural treatment processes (such as plant- or sand-based biofilters[58, 59] or wetlands[60]) operate passively (i.e., gravity-fed so no pumping) and hence have no need for human intervention during operation, thus requiring less energy, lower maintenance and are low cost. However, their adoption is hindered by their higher variability in performance and are hence not considered part of the pathogen treatment train by many organizations. However, our 13years of research demonstrated that, through careful design[58, 59, 61, 62] and real-time monitoring and control[63], these passive and natural treatment systems can deliver similarly high pathogen reductions as traditional treatment methods.

Low-energy, low-maintenance, no-chemical treatment systems that can inactivate pathogens are sustainable. Theme 6 of this PROGRAM will build on our expertise in water treatment[59, 61, 64, 65] to optimize the design and control of passive treatment systems to mitigate waterborne risks.

Research strategies and key activities

Leveraging the research expertise and experience of McCarthy, the core team, and collaborators, this collaborative and interdisciplinary proposal delivers its aim via six research themes. As demonstrated by our CVs, the team is capable and dedicated to work closely with end-users to positively impact society through the development of tools to manage waterborne risks.

THEME 1. SMART SAMPLING SYSTEMS

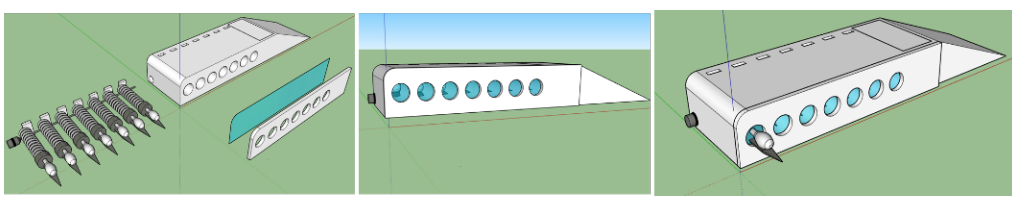
Theme 1 will develop sampling systems to detect pathogens and AMRs in water that are easy to use, low-cost and some of which will be open-source and open-hardware, enabling the adoption of these technologies in all parts of the globe. To deliver Theme 1, this PROGRAM will be leveraged for further support from federal and provincial water, environment and public health agencies, private, not-for-profit partners (see F4 and Supporting Section J).

RA1.1 Low-cost passive sampling technologies. Passive sampling presents a viable alternative to traditional sampling and involves the deployment of a device in a waterbody for a known time period, allowing for pollutants in the water to interact with the device[66-68]. Passive sampling is advantageous as its deployment is easy, cheap, rapid and usually does not require confined space entry permits.

Furthermore, the continuous exposure of the passive sampler to the water reduces sampling errors that exist when taking discrete or grab water samples. While passive samplers have been applied to detection of pathogens in water[69-72], including by the team[15, 20-23], significant research challenges remain, especially if they are to be used instead of water samples for risk management. As opposed to passive sampling for chemicals, passive sampling of pathogens presents challenges as they can rapidly grow and die, and DNA or RNA can be degraded. These conditions, together with dynamic flow and water quality (e.g., changing ionic strengths), can influence the reliability of passive sampling to estimate water pathogen concentrations because of non-linear association rates, reduced maximum association capacities and limited deployable durations. Interestingly, most microbial passive sampling studies do not bother to even report association rates, often set arbitrary deployment durations and do not include appropriate exposure or normalization controls. Through similar lab and field experiments employed for our novel, award-winning and internationally-recognized passive sampler[15, 20-23], RA1.1 will address these challenges for a variety of pathogens, water matrices and passive sampling materials, thus providing confidence in using this technique for surveillance of pathogens and AMRs.

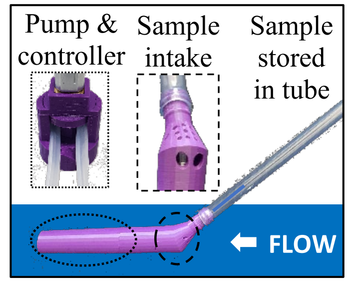
RA1.2 Novel passive sampling materials. While embracing the simplicity of passive sampling, we will explore new materials and coatings to improve the specificity of pathogen uptake by passive sampling, increase association rates/capacities and prolong the maximum duration of deployment. We will develop a suite of advanced materials and coating techniques for passive samplers (e.g., coating with antibodies[73], chitosan[74] and prebiotics[75] that selectively associate specific pathogens), while reducing DNA/RNA stripping or degradation. Batch, mesocosm and flow-through experiments will determine the potential of new materials. We will also determine the potential to use FTA™ cards as passive samplers, to simultaneously associate and extract DNA[76] and RNA[77], making the transition to qPCR seamless and without complicated and time-consuming protocols.

RA1.3 Activating passive samplers. Passive sampling is constrained by the limited duration of deployment and the inability to report the temporal variability of pathogens across events (i.e., they are time-integrative samplers and unable to separate what happens at the start, middle or end of a deployment period). Activating passive samplers to enable longer deployment durations could include housing multiple passive samplers in one unit and have time- or flow- based ejection of passive sampling materials into the water column (and retraction into stabilizing solutions), so that the maximum duration of exposure for each individual sampler is not exceeded. One example design is shown here:



To our knowledge, no attempts exist to make passive samplers smarter or more active for pathogen detection. As such, significant questions remain: what is the stability of microbes on the passive samplers? What types of solutions could be used to stabilize retracted passive sampling materials? What are the error rates of these devices? And how can we control for clogging and fouling? We will develop novel, activated passive samplers, overcoming the limitations in deployment durations of microbial passive sampling. With our partners, we will deploy these in various use cases, helping to further understand their potential and improve their design.

RA1.4 Novel water samplers. While passive sampling has many advantages, there is still a role for accurate time- or flow-based sampling (e.g., for risk assessments). However, as highlighted above, existing sampling solutions are expensive (CA$10k), not practical for high spatial resolution monitoring and not designed to collect the large water volumes required to detect the low pathogen concentrations commonly observed in water. The advent of low-cost sensors, 3D printing and IoT systems provide opportunities to develop and test a range of open-source sampling tools[78-80] but significant questions remain, including: What pumping volumes and clogging rates are experienced in different water matrices? What is the stability of the collected water in the sampler? And what uncertainties are introduced through automatic in-field concentration on the specific pathogen(s) of interest? We will develop low-cost automatic sampler systems that are open-source/open-hardware, easily available and easily produced with commonly-available materials, and that are physically scalable to fit within space-constrained environments while flexible enough to sample the volume of water required for accurate pathogen and AMRs detection. As initial work done for this PROGRAM, we developed an auto-sampler (inset) which costs just CA$40, sits in the water column, contains our novel 3D-printed peristaltic pump[80] to take water using time- or flow-based intervals and is connected to the cloud so it can be remotely activated. It has already been adapted to take discrete samples over events of interest. We will design other samplers, including those that can sample and concentrate large volumes of water in situ (e.g., through incorporating filtration devices, e.g., [81]). Preliminary work shows that such systems could be used for stormwater (>50L) and wastewater sampling (>10L) and have shown 50-80% recovery rates for bacteria and viruses.



THEME 2. LAB-BASED PATHOGEN AND AMR DETECTION

Theme 2 builds on our strengths in optimizing pathogen detection in water[82] and viability and potential infectiousness of waterborne pathogens[27-29] to deliver methods for rapid, sensitive and accurate surveillance of pathogens, their infectiousness and AMRs in complex water samples. We will work with our partners (see Supporting Sections F & J) to co-develop solutions for pathogen and AMR detection.

RA2.1 Optimization/standardization for environmental pathogen and AMR detection. Building on our expertise[27-29, 82], RA2.1 will develop, test and optimize concentration techniques for pathogens and AMRs from water matrices (drinking, storm, river and waste-waters). These will include hemoflow concentration steps *in situ* (links to RA1.4[81]) and in lab and IMS bead separation techniques. We will develop standard operating procedures that others can use to standardize concentration steps prior to molecular or culture assays. We will optimize the applicability of novel molecular analytical platforms to environmental water samples, as we did for the TaqMan Array Card (TAC) system[82] for pathogens and AMRs. Similar research could be planned for the Fluidigm[83] array system and future potential of photonic qPCR[84], ensuring they are optimized for concentrated water samples that may have high inhibition levels. Our successful culture-PCR assay for *Campylobacter* spp.[27] will be leveraged to develop coupled culture-PCR detection systems for current and emerging pathogens, reducing TAT while delivering isolates for validation of viability and for further investigation of infectiousness.

RA2.2 Viability and infectivity. An important step in using pathogen datasets for QMRA is determining the viability and the infectiousness of the microorganism. Existing molecular-based viability detection methods have their limitations[24-26]. However, the consensus is that a universal method cannot be developed and instead, the choice of method needs to be made alongside the knowledge of how cell death is likely to have occurred[25]. With myriad sources, treatment processes and prolonged environmental exposure, it is almost impossible to understand the pathways of microbial death in water systems. Furthermore, the complex matrix of the water often limits the ease of viability tests[30]. We will research and develop novel approaches to detect viable pathogens using rapid molecular approaches, including the use of biosensors[34] for this purpose (see RA3.4).

Determining the potential infectiousness of a microorganism is also essential; e.g., our work in Australia demonstrated high levels of *Salmonella* and *Campylobacter* in the Yarra River were not of concern to recreators as only a small percentage of the former were deemed infectious[28, 29]. Alternative and standardized methods to detect infectiousness are required. We will leverage our experience in understanding infectiousness[27] and develop *in vitro* attachment, invasion and cytotoxicity assays[28, 29], whole-genome sequencing[30], transcriptomics[30] and Multi-Locus Sequence Typing[31] methods to detect infectiousness, and apply these with our partners (Supporting J) to manage risks in water systems.

RA2.3 Sequencing methods for pathogen and AMR detection. NGS is an evolving method to detect specific pathogens and the presence of AMRs in water systems. We demonstrated the potential of NGS to determine the overall contamination levels in recreational water systems[85], identifying specific pathogens of concern from animal feces within water catchments[86] and identifying pathogen sources in recreational waters[11, 49]. However, challenges with using NGS for specific pathogen and AMR detection remain, including: standardization of NGS is developing but still in its infancy[30, 87], metagenomics (e.g., 16s rRNA amplicon sequencing) has limited ability to provide sufficient resolution for specific pathogen detection[30, 86], viability-HTS systems are possible but need further research[26, 88], and quantification from NGS is highly uncertain as it currently relies on relative measures[30, 87, 88]. These challenges limit the use of NGS technologies in risk assessment[87]. With our team and partners, we will address these challenges by standardizing NGS for pathogen and AMR detection in waters, explore the potential for viability-NGS methods beyond simple mRNA applications[30], develop more specific primers for targeted NGS, and develop and validate novel quantitation techniques (such as that proposed by [89]) to determine absolute abundance.

THEME 3. IN SITU SENSORS AND IN-FIELD PATHOGEN DETECTION

Novel methods for in field or *in situ* pathogen and AMR detection can reduce laboratory-associated costs, TAT and enable: (1) responsive public health actions via WBE about communicable diseases, (2) provide real-time information about the risks of swimming, and (3) tracking sources of pathogens and AMRs. Theme 3 will advance the science and improve the adoption of novel technologies for the real-time detection of pathogens and AMRs in water. We will work with private and public partners (Section J) to develop sensing solutions, optimizing these via field testing with water utility and health agencies.

RA3.1 Water flow and quality sensing. Detecting water flows and water quality parameters are necessary to contextualize pathogen and AMR detection (e.g., high pathogen concentrations during high river flows might be low risk because swimming during storm events is uncommon). Furthermore, the ease, accuracy and availability of flow and water quality sensors means that these can be used to inform more targeted pathogen monitoring or as surrogates for pathogens (see RA3.2). While flow and water quality sensors are available, their costs and maintenance requirements limit their use to just one (or a few) sampling location(s). Many applications require better spatial resolution, such as tracking illegal human wastewater inputs into the environment[42]. We will leverage our experience[41-43, 90] to develop accurate and low-cost technologies to monitor flow, physical and chemical water quality. We will continue to research non-contact sensing options for water flow (e.g., via radar sensing[90]) and water quality (e.g., via hyperspectral imaging[91] and cameras[92]) that reduce maintenance requirements and installation times, both of which are key considerations for spatially distributed sensing applications. We will also further the concept of self-powering sensing options[93] to reduce battery replacement costs.

RA3.2 Soft sensing for pathogen detection. *In situ*, real-time pathogen monitoring is still restricted to the laboratory or at such high cost it is impractical to implement at scale (see RA3.4). Therefore, the concept of soft-sensing has gained traction[39, 40]. This exploits easy-to-measure variables to predict hard-to-measure variables, such as pathogens or AMRs[94]. Soft sensing has progressed from simple linear and multivariate statistical models to more complex combination of many parameters/signals via artificial intelligence (AI)-based modelling methods[39, 40] (i.e., machine learning methods, such as fuzzy logic particle swarm optimization, genetic algorithms, artificial neural networks and support vector machines). AI models for water quality prediction have showed significant promise[39, 40, 95] yet major challenges exist in the adoption and transferability of these for water quality prediction. Indeed, a recent systematic review[40] revealed very few papers had soft-sensed microbes in water (e.g., [94]) and those that did had limited accuracy, and none sensed specific pathogens. We will leverage our water quality sensing expertise[41-43, 85] and our AI-modelling experience[96] to research and validate soft sensors to predict pathogens in water. We will use sensors developed and tested in RA3.1 and our optimized pathogen detection methods from Theme 2 to develop and validate software sensors.

RA3.3 In-field pathogen detection. Traditional lab devices for high-specified medical applications are now being extended for rapid and in-field monitoring of pathogens, especially in low-resource settings such as the global south due to the advantages of low cost, accessibility and rapid responses. We will focus on commercially available tools, conducting side-by-side comparisons with lab-based testing (Theme 2), and include some assay optimization (such as improving field extraction and assay mixtures). Four portable detection machines (Biomeme Franklin™, BioMolecular Systems Mic and Orvion Udetect BV, a Loop mediated isothermal amplification [LAMP] by Microsensdx and the Oxford Nanopore) have been identified for testing and validation, with more to be identified throughout the PROGRAM. Our preliminary research focused on the Biomeme system, demonstrating that its 5-minute, in-field DNA and RNA extraction efficiency was comparable to our 30+ minute in-lab extraction methods (efficiency tested via salmon testes and ability for the assay to cope with inhibitors). Our initial testing also showed that the Biomeme portable qPCR machine has slightly higher limits of detection than lab-based Biorad CFX96 (20 copies/rxn vs. 7). Such work and comparisons made it possible for Australian water utilities to use the device to detect sewer overflows and the subsequent efficiency of clean-up practices. Also, health departments considered the device to detect SARS-CoV-2 in wastewater in remote communities. Such outcomes are also envisaged for Australian utilities and health agencies.

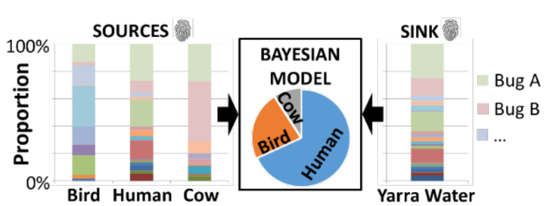
RA3.4 In situ, online pathogen sensors. Detecting pathogens *in situ* and (almost) in real-time is revolutionizing how we manage waterborne risks. There are commercially available pathogen detection sensors (e.g., vibrational spectroscopy or multi-angle light scattering sensors[33], flow cytometry sensors[97] and the ColiMinder[98, 99]). However, these are large, require mains electricity and expensive, limiting their widespread adoption. They also have issues, such as interference with other compounds[33], require continuous reagent supply[97-99] and are slow to report results (e.g. TAT of 30mins)[97-99].

Biosensing is a rapidly-evolving field[34, 36, 37], becoming a $31 billion market by 2024[100]. Biosensors use a signal transducer to detect the interaction of a target pathogen with a natural or synthetic recognition element which has been embedded into substrate material[34]. They have the potential to be a low-cost, rapid response, highly sensitive and specific pathogen detection tool[34]. However, several challenges remain[34-36]: (1) non-specific binding of non-target analytes to biorecognition elements, (2) low pathogen concentrations in water limit the sensitivity of biosensors, and (3) ineffective pre-treatment of the water to provide a favourable environment for the longevity of the biosensor.

We will develop and trial biosensors for online, real-time pathogen detection in multiple water matrices (drinking water, stormwater, agricultural water and wastewater). We will leverage our 3D printing expertise[15], nano-electronics expertise[41-43, 80] and impressive biosensing expertise[121-125] (and UoG's Electrochemical Technology Centre) to focus on optical- and electrochemical-based biosensors. We will meet the above challenges by progressing the development of the lab-on-a-chip concept[34-36] to pretreat and pre-concentrate water and incorporate multiplex biosensors[34] to improve sensitivity and specificity.

THEME 4. SOURCE TRACKING OF PATHOGENS AND AMRS

Theme 4 will develop novel, sensitive and accurate source-tracking methods using NGS technologies and SourceTracker (see inset)[49]. This process uses a Bayesian model to compare microbial fingerprints from a water (sink) to the fingerprints from animal and human fecal samples (sources) to discern the sources of feces in sink samples[11]. We will work with private and public collaborators (see Supporting Sections F4 and J) to explore the potential of these methods to help protect water resources from pathogens and AMRs. In particular, we will work with, e.g., AAFC to protect recreational waterways from fecal contamination.



A4.1 Source sample biobank. With our partners (such as OMAFRA, see F4), we will co-develop a biobank of source samples from human wastewaters and animal fecal scats that represent the major agricultural, domestic and wild animals in the region. These samples will form the backbone of the development of markers specific to individual sources of pathogens within our catchments. This biobank will accumulate ~ 250 samples/year, representing ~ 2,000 samples collected from ~ 25 animal species (i.e., 100 samples/specie). This could form a source tracking biobank for Ontario, and even Australia.

RA4.2 Stability of source fingerprints in environmental settings. Changes in source microbial community profiles creates uncertainty for the NGS-SourceTracker approach; indeed, if source community profiles change over time, the ability to detect that source accurately using this approach diminishes. We will explore the change in these community profiles using long-term field experiments to monitor scats from wild, agricultural and domestic animals.

RA4.3 Sink sample collection and preliminary source tracking using SourceTracker. With our partners, we will collect sink water samples from a range of environments that require pathogen and/or AMR source-tracking, sampled using the devices developed in Theme 1. Source samples will be processed using our carefully-designed 16s V3-V4 amplicon sequencing pipeline[11], and then used in SourceTracker with our validated process[49] to compare source and sink 16s community profiles for source attribution. As we have done previously, we will work with our partners and use this information to reduce human health risks (see Supporting Sections F4 and J for collaborators, such as OWC).

RA4.4 Bacteroides-specific fingerprinting, and new markers. Our preliminary work for this PROGRAM demonstrated that a carefully-constructed primer pair amplified a variable region of the *Bacteroides* spp. genome to generate fecal-specific fingerprints (as opposed to RA4.3, which looks at the full bacterial fingerprint). This allows us for the first time to conduct *Bacteroides*-specific NGS-based source-tracking, providing preliminary evidence of improved sensitivity for discerning fecal contamination sources (compared to our generic v3-v4 16s amplicon approach). To optimize and validate these methods, we will conduct laboratory spiking and field scale experiments similar to our previous work[11]. The source community profiles will be combined in random proportions to create artificial *in silic*o sink profiles that will be fed into SourceTracker to confirm the ability of the process to detect sources correctly. This will be repeated for thousands of scenarios. We will also create *in vitro* test samples by combining known proportions of raw scats, wastewater and PCR-grade water. The resultant *in vitro* community profiles are then used by SourceTracker to confirm its ability to detect those sources used to create this test sample. This will be repeated using many artificial *in vitro* sink samples.

We will use the biobank from RA4.1 and sequencing to uncover new bacterial source-tracking markers. Further, almost all previous studies only focus on the use of bacterial markers[101], with very few using markers designed to track viruses and protozoa known to cause the majority of risks in water systems[102]. We will develop and validate novel NGS-based source tracking markers for bacteria, protozoa and viruses. This will be the first known project where bacterial, protozoan and viral NGS-based source-tracking methods are used side-by-side to infer contamination sources, enabling fine-scale source attribution, and the first study to conduct Bacteroides-specific NGS-based source tracking.

A4.5 Rapid and distributed source tracking. Pending the above results, we aim to integrate specific source-tracking markers from RA4.4 into the biosensors developed in Theme 3. These sensors could be used to conduct source-tracking at a high spatial resolution, while capturing the temporal variability of source contributions. Such sensors are nonexistent, yet could help track and remediate illegal wastewater discharges, which are highly intermittent and come from unknown locations.

THEME 5. MODELS FOR REAL-TIME PREDICTION AND HYPOTHESIS/SCENARIO TESTING

While sampling, assays, sensing and source tracking should be optimized, these results will always represent either past, or at best current, trends in disease. Optimally, we seek real-time calibrated and integrated models that can predict the sources, fate and transport of pathogens and AMRs in systems and forecast the subsequent human health risks. Theme 5 will develop, calibrate and validate models and risk estimation tools. We work with public water utilities and public health agencies (see Section F4) to explore the needs of each sector and determine how modelling tools can help improve mitigation and reduce disease burden (and costs to the communities); e.g., working with PI Goodridge on WBE.

RA5.1 Understanding pathogen sources, fate and transport. Working with our partners, we will carefully select case studies where detailed investigations of pathogen sources, fate and transport will be conducted. These will include detailed monitoring efforts to measure transport processes, heavily relying on the sampling methods from Theme 1 and analytical methods in Theme 2. Pathogen survival experiments will be conducted similar to our previous research[103], while sources will be understood using the outputs from Theme 4. These datasets will be used to improve our understanding of pathogen behaviour to improve the PROGRAM team's existing models in this field[53-55].

RA5.2 Developing, testing and validating models for pathogen prediction. Building on our existing expertise in pathogen modelling in water[53-55], we will work with our partners and use datasets from RA5.1 to build coupled hydrodynamic-pathogen models. These models will predict the sources, fate and transport of pathogens through various water systems, including drinking water catchments, stormwater networks for recreational water impacts and wastewater systems for WBE.

RA5.3. Risk estimation, burden and mitigation. Following others[104] and leveraging our existing expertise[12, 56, 57], the models developed in RA5.2 will be coupled with a QMRA module that: (1) takes the predicted reference pathogen concentrations and combines these with exposure models to estimate pathogen doses[102, 105] and (2) uses peer-reviewed dose-response models to estimate the probabilities of infection, (3) converts these to probabilities of illnesses by following others[102] and estimates risks[106]. The outputs from of this risk model would ideally be used with severity statistics to provide burden of disease estimates, and hence provide economic burden estimates. This process would follow others[107] and use health costs from literature[108]. A mitigation module will be integrated into the model, allowing us to explore how various mitigation methods reduce risks and economic burden. With this module, our partner organizations can develop strong mitigation business cases, and ensure their investment results in desired outcomes.

RA5.3 Working with partners to implement models and to conduct hypothesis testing for mitigation. Dedicated sessions and workshops will engage our partners to develop multiple hypothesis testing scenarios in each of their case-studies. The PROGRAM team will run the models with the partners. The tools will generate data for building the business case of different mitigation scenarios; for example, the tools will estimate the reductions in contamination levels, risks and thus economic burden achieved through each mitigation scenario, which will be compared to the cost required to implement the intervention. Our objective is to transfer the knowledge of these models to our partners and provide training courses so our partners can own the model and use it for other prioritization tasks.

THEME 6. TREATMENT SYSTEMS FOR WATERBORNE PATHOGENS.

Treatment of water using low-cost, low-energy and low-maintenance systems, such as biofilters and wetlands, show promise for removing pathogens[58-62] (and likely AMRs too but these are not researched yet). Further polishing the effluent of such systems using advanced oxidation that do not rely on ongoing chemical or energy inputs will reduce costs and improve uptake of water recycling, especially in marginalized communities where these may be unavailable. Theme 6 will develop, optimize and implement treatment systems to remove pathogens and AMRs from various water matrices. With further funding from federal and provincial partners, we will co-develop solutions that are fit for purpose.

RA6.1 Design optimization for green infrastructure systems. Our team has conducted most of the research on pathogen removal in biofilters and wetlands systems[58-62], with only a few other studies reporting actual pathogen removal from these systems. All pathogen removal studies focused on temperate[58-62], tropical[109] or Mediterranean[110] climates, which are significantly different than those of Australia, thus may not be transferrable. Furthermore, most of the studies used plant species specific to each climate, again reducing our understanding of how Australian biofilters and wetlands may remove pathogens. No studies explored how these natural treatment systems remove AMRs. We will, for the first time, research and optimize biofilters and wetlands for pathogen and AMR reduction for Australian climates using native plant species and locally-available media. We will revisit our work on plants that produce antimicrobial root exudates[61, 62] to Australian species, and determine the impact these may have on pathogen reduction and on the microbial communities that are important to nitrogen removal[111], another important function of these systems to protect ecosystem health. We will also research the potential of our antimicrobial coatings for filtration media[75, 112] to inactivate pathogens through these treatment systems, and determine whether AMR may develop within such systems.

RA6.2 Real-time monitoring and control of green infrastructure systems. Our early experiments suggest that design optimization will not result in sufficient pathogen reduction to protect public health[59], and instead more emphasis must be placed on how the systems are operated. We demonstrated that large improvements in pathogen log reductions can be obtained in these treatment systems by implementing a small level of monitoring and real-time control, thus making them slightly more actively controlled. For example, we improved pathogen log reductions from just 1 to > 4 by placing and controlling a bypass valve at the inlet to a biofilter[63]. This early work provides the proof of concept that real-time monitoring and control of treatment systems can reduce waterborne disease risks. We will confirm these results are transferable to the field and to Australian contexts, demonstrating for the first time in such a climate that real-time control can improve pathogen removal. We will establish with our partners the first real-time controlled biofilters and wetlands in Ontario. These will be monitored using the techniques outlined in Themes 1-3. We will research the benefits and disadvantages of rule-based and model predictive control algorithms for pathogen reductions. We aim to test whether real-time control and monitoring can fundamentally improve the performance of treatment systems. To achieve this aim requires communication and technology transfer to be at the centre of this project, and hence we aim to create a series of reference materials, guidance manuals and models that will diffuse the insights from this research and accelerate uptake. The water industry is set for a boom in capital investment in water infrastructure to take advantage of innovative monitoring and control technologies, with Hexa Research predicting that the annual market in “smart water tech” will reach US$10 billion within 4 years[113]. Our project will help guide that investment and establish Australia as a world leader in smart water technologies.

RA6.3 Advanced treatment systems for water reuse and harvesting. Following our success in applying advanced oxidation to reuse and harvesting waters[64, 65, 114], we will explore the potential of dark photocatalytic oxidation[65] for continuous water treatment of water, and work with UoG’s Electrochemical Technology Centre to explore the potential for electrochemical oxidation of pathogens[114]. We demonstrated coupling of biofilters or wetlands with advanced oxidation[115] but further research is needed to optimize these technologies for field-scale treatment and to assess their potential for pathogen removal in Australian climates.