Australian Water Recycling
Centre of Excellence

Project Report

National Validation Framework for Water Recycling: Overview of Priority Research

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Australian Water Recycling Centre of Excellence

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National Validation Framework for Water Recycling: Overview of Priority Research

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Partners

CSIRO	University of Queensland	SA Water
Curtin University	Victoria University	SouthEast Water
Griffith University	WaterFutures	Sydney Water Corporation
University of New South Wales	Melbourne Water	Water Corporation

About the Australian Water Recycling Centre of Excellence

The mission of the Australian Water Recycling Centre of Excellence is to enhance management and use of water recycling through industry partnerships, build capacity and capability within the recycled water industry, and promote water recycling as a socially, environmentally and economically sustainable option for future water security.

The Australian Government has provided $20 million to the Centre through its National Urban Water and Desalination Plan to support applied research and development projects which meet water recycling challenges for Australia's irrigation, urban development, food processing, heavy industry and water utility sectors. This funding has levered an additional $20 million investment from more than 80 private and public organisations, in Australia and overseas.

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Introduction

Goal Two of the Australian Water Recycling Centre of Excellence (AWRCoE) is that "A national validation framework for water recycling be established". In a first phase, the structure and essential components of a national framework for validation of recycled water systems were identified and a pathway for implementation was recommended which included two core components:

- The creation of a Protocol Development Group (PDG) as the centre piece of the proposed national validation framework and responsible for the development of nationally accepted guidelines for the validation of water recycling technologies, and
- The implementation of a research program aimed at addressing priority knowledge gaps identified as barriers to a national validation framework and transferring outcomes of the research to nationally endorsed guidelines and validation protocols.

The research program referred to as NatVal Priority Research Program was delivered from September 2013 to December 2015 with a total cash and in-kind value of approximately $6.3 million.

This program-level report provides an overview of the research conducted and describes how the five subprojects under this priority research program contributed individually and collaboratively to the objectives mentioned above. This report is supplemented by five executive summaries (in appendices) providing further details on each subproject and associated research outcomes. In addition, a number of standalone technical reports and publications have been released by the subproject research teams which are available on the AWRCoE website.

Gap analysis and program scoping

The initial scoping report delivered by Water Quality Research Australia on behalf of AWRCoE entitled "*NatVal Road Map Report - The road map to a national validation framework for water recycling schemes*" (Muston & Halliwell 2011) included a systematic analysis of knowledge gaps which could be considered as barriers to implementation of the recommended framework.

These gaps covered a range of areas such as governance and policies, risk assessment and treatment technologies. The research needed to fill gaps associated with technology and risk assessment was also identified as summarised in Table 6 adapted from Muston and Halliwell (2011).

Based on this gap analysis and taking into considerations time and resource factors, the AWRCoE Project Advisory Committee (PAC) for the NatVal project developed a list a priority research projects as follows:

- Validation of membrane bioreactors (MBR);
- Integrity monitoring of Reverse Osmosis (RO) membranes for virus rejection;
- Validation of biological systems;
- Validation of ozone processes;
- Development of an integrated testing strategy in a multiple barrier approach;
- Standardisation of methods for pathogen (including virus) isolation, culture, detection and enumeration; and
- Methods for quantitative microbial risk assessment in source water characterisation.

Following this initial process, Melbourne Water was able to make its existing research data on ozone disinfection available and the validation of ozone disinfection was no longer considered a research need. Proposals were sought from research providers and following a PAC review and Research Advisory Committee (RAC) endorsement process, the list of research projects was finalised as:

- Validation of MBR;
- Validation of RO and Nano-Filtration (NF) membranes;
- Validation of Activated Sludge Treatment (AST) processes;
- Development of an integrated testing strategy in a multiple barrier approach; and
- Standardisation of methods for pathogen (including virus) isolation, culture, detection and enumeration.

Program description

The program was organised in five independent subprojects, with three of the five subprojects (SP1, SP2 and SP3) dedicated to the development of validation protocols for specific treatment processes and two subprojects (SP4 and SP5) focusing on research which can support the validation of treatment processes generally (Figure 1).

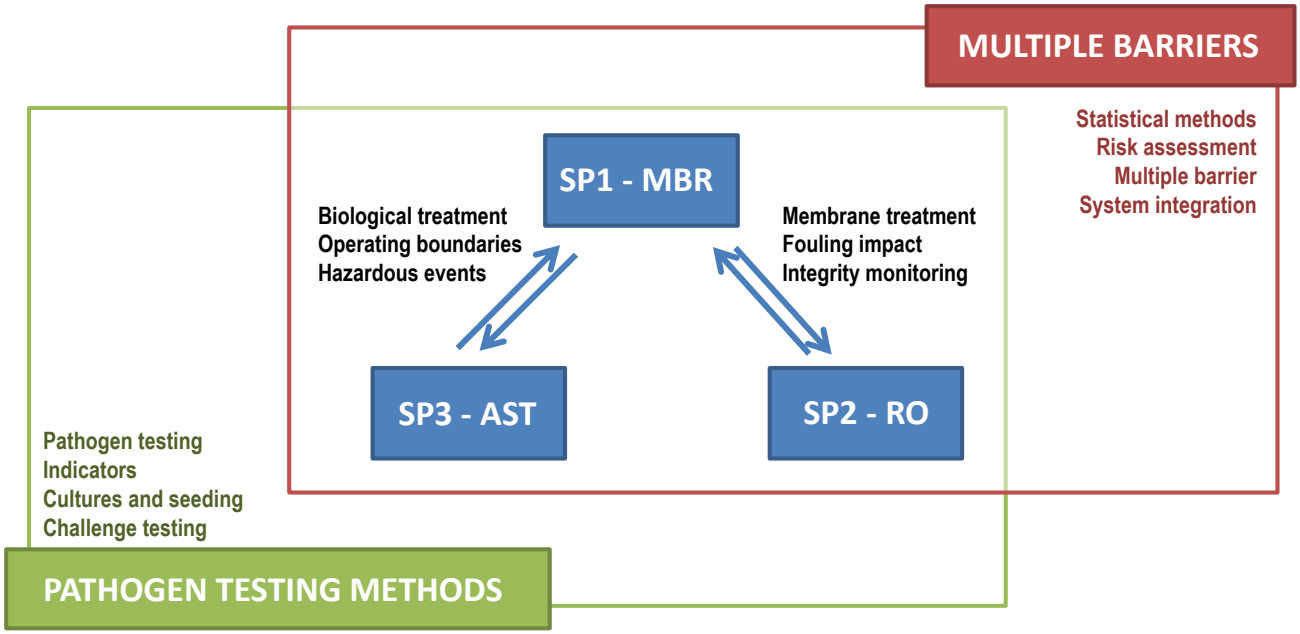


Figure 1. Overview of NatVal Priority Research Program.

Figure 2 describes governance arrangements for the NatVal Priority Research Program underpinned by the following elements:

- The **Project Advisory Committee** (PAC), composed of international and national experts, met on a three- to six-monthly basis and focused on the overall NatVal research program and the effective integration of its subprojects. The PAC reviewed progress and milestone reports, attended key workshops, contributed via its designated members to each of the subproject advisory working groups and provided recommendations to the AWRCoE Research Advisory Committee (RAC). The PAC convenor is a member of the RAC.
- The **Subproject Advisory Working Groups** (SAWGs) were composed of one PAC member (subproject champion), the Program Manager, representatives from industry partners and independent experts as required. SAWGs operated strictly in an advisory role, assisting the Program Manager in the assessment of subproject progress and providing guidance to research teams.
- The **Program Manager** coordinated the delivery of the program (including budgeting, progress tracking, milestone reviews and reporting) and managed day-to-day interaction with subproject leaders. The Program Manager was the main point of interface with the Protocol Development Group.

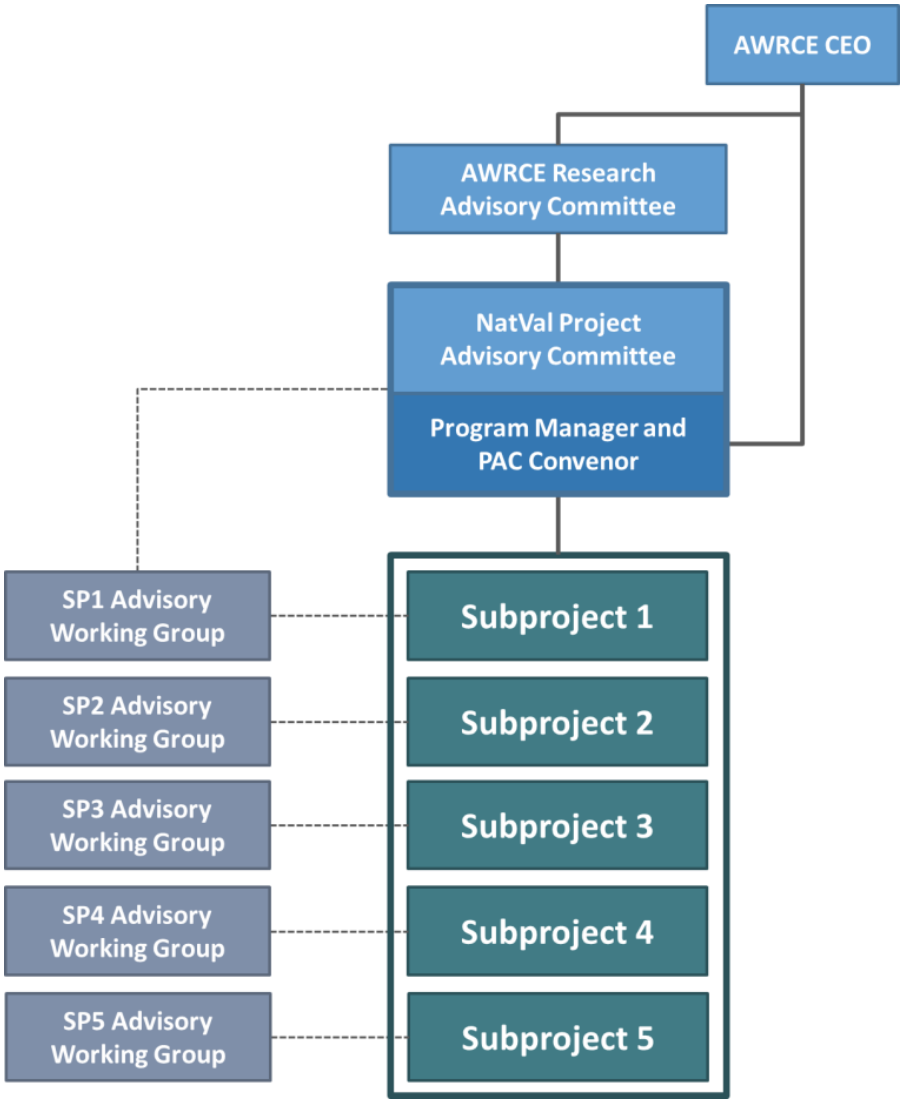


Figure 2. NatVal Priority Research Program governance framework.

Participants to the program included a number of research institutions and utilities across a number of states as summarised in Table 1. The overall program budget was approximately $6.3 million including $3.4 million as in-kind.

Table 1. NatVal Priority Research Program participants.

Universities / Research Providers	Utilities / Private Sector
CSIRO	Melbourne Water
Curtin University	SA Water
Griffith University	SouthEast Water
National Measurement Institute	Sydney Water Corporation
University of New South Wales	Water Corporation
University of Queensland	
Victoria University	
WaterFutures	

Industry engagement and adoption strategy

In addition to the industry partners directly involved in subprojects, each research team conducted specific engagement with industry as required by their research activities. This included consultation with utilities, manufacturers and regulators and direct technical engagement through site visits and requests for operator input.

At the AWRCoE and NatVal program level, a number of stakeholder consultation initiatives have been undertaken to support the development of the National Validation Framework (referred to as *WaterVal*), including:

- Initial roadshows and workshops to identify the key requirements and benefits of a national validation framework;
- Specific workshops involving all relevant sectors (regulators, utilities, designers, manufacturers, operators and researchers) to develop recommendations for the design of the framework and to identify knowledge gaps and focus areas for research; and
- Engagement of industry partners through their representation on various committees such as Subproject Advisory Working Groups, PAC and RAC.

To ensure effective adoption of research outputs and to facilitate the translation process, milestones were aligned across subprojects and focused on outcomes as follows:

- Milestone 1 – Contract execution
- Milestone 2 – Literature reviews and preliminary draft validation protocols (where applicable)
- Milestone 3 – Interim report of experimental research activities
- Milestone 4 – Final report on all experimental research and final draft validation protocols (where applicable)
- Milestone 5 – Final project report and close-out.

The three subprojects focusing on specific treatment technologies were tasked with developing draft validation protocols and to achieve further consistency, a validation protocol template (AWRCoE 2015) was developed by the PDG and made available to the researchers at the start of the program. As described in Table 2, it includes nine steps against which researchers were able to map their research outputs and to some extent structure their reports. For example, the literature review was split across elements 1 to 4 whereas the monitoring and validation research outputs could be structured around steps 4 to 7.

Table 2. Validation protocol template steps

Step	Description
1	Identification of the mechanisms of pathogen removal by the treatment process unit
2	Identification of target pathogens and/or surrogates that are the subject of the validation study
3	Identification of factors that affect the efficacy of the treatment process unit in reducing the target pathogen
4	Identification of operational monitoring parameters that can be measured continually and are related to the reduction of the target pathogen
5	Identification of the validation method to demonstrate the capability of the treatment process unit
6	Description of a method to collect and analyse data to formulate evidence-based conclusions
7	Description of a method to determine the critical limits, as well as an operational monitoring and control strategy
8	Description of a method to determine the LRV for each pathogen group in each specific treatment process unit performing within defined critical limits
9	Provision of a means for revalidation or additional onsite validation where proposed modifications are inconsistent with the previous validation test conditions.

Researchers and the PDG were able to formally engage at two combined workshops and researchers were invited to contribute to PDG discussions on specific protocols on an ad-hoc basis. In combination with the use of the validation protocol template, this approach allowed a relatively seamless translation of research outputs into consistent protocols (Table 3) which are now integrated within *WaterVal*. This optimised research output translation process is not limited to these projects but can also be applied to future research on treatment technologies.

Table 3. Translation of NatVal Priority Research Program outputs into validation protocols.

Technology	Research inputs
Membrane Bioreactor	The MBR technology is considered in many decentralised systems and despite the understanding that high LRVs can be achieved, proponents and manufacturers have experienced difficulties in demonstrating that the system integrity could be continuously monitored. Subproject 1 provided the underlying evidence for a national validation protocol and delivered: • A comprehensive review of log reduction data from literature and operational sites to establish default LRVs for MBR; • An evidence based operating envelope and monitoring program dealing with hazardous events and under which the risk to public health can be managed consistently; and • A promising validation method relying on the online monitoring of permeate turbidity for which further evidence will be required.
Reverse Osmosis and Nano-Filtration	High pressure membrane filtration is known to deliver very high removal of pathogens however, based on current integrity monitoring techniques, only much lower removal credits can be granted by regulators. There is a lack of consistency between jurisdictions about the potential impact of operating conditions on surrogates and about the type and frequency of integrity monitoring considered acceptable. Subproject 2 provided the underlying evidence for a national validation protocol and delivered: • A comprehensive literature review of log reduction data, hazardous events and integrity monitoring techniques; • A thorough assessment of the impact of operating conditions on the suitability of surrogates and indicators leading to the definition of conservative integrity testing conditions; and • A method to obtain approval for up to 4-log removal credits based on a range of surrogates and best practice integrity monitoring strategies.
Ozone Disinfection	Ozone disinfection is not in widespread use for recycled water in Australia but the availability of a validation protocol would facilitate adoption in specific applications. While this process is well known in drinking water applications, the translation to recycled water applications required new evidence and a specific strategy. The research data provided by Melbourne Water allowed the design of a national validation protocol focusing on defining the conditions under which USEPA disinfection tables can apply and how temperature correction is to be applied.

Program outcomes

Subproject 1 overview – National validation guidelines for MBR

An executive summary for subproject 1 can be found in Appendix 2.

Challenges

- Lack of removal performance and operational monitoring data able to be directly compared
- No direct or indirect continuous integrity monitoring strategy available
- No clear evidence on the impact of hazardous events on MBR removal performance
- Guidelines only available in Victoria.

Outcomes

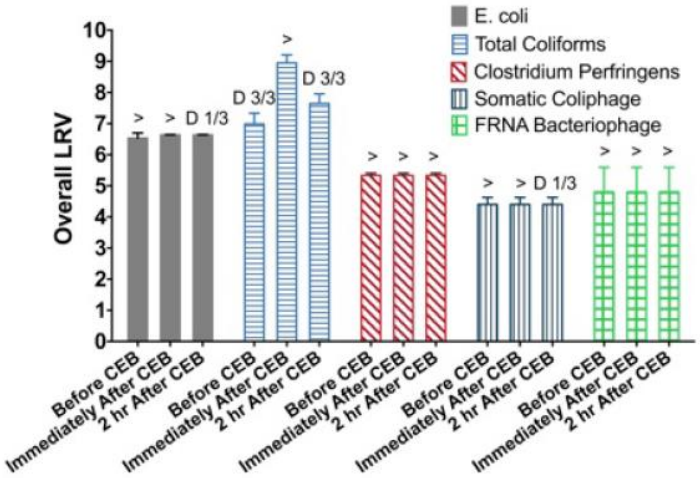
- A critical review of current literature on LRV achieved by MBR (more than 1000 LRV data points obtained) and validation reports/guidelines was conducted.
- A sampling campaign took place with a total of 180 visits to 11 different full scale MBRs in order to create a database of MBR performance and operation.
- Bayesian Belief Networks, created and trained on the data collected, were used to identify significant influencing factors. Operation under the following conditions was confirmed to lead to a higher likelihood of a poor LRV: low HRT, high permeability, high permeate turbidity and low MLSS. These conditions were used to define a conservative operational envelope for validation testing.
- Probability density functions (PDF) were fit to all data collected from literature and data from site sampling to establish default LRVs and a corresponding operating envelope. The 5th percentile of resulting LRV PDFs were collated and the most conservative values for viruses, bacteria and protozoa were rounded down to form the basis of default LRVs, summarised in Table 4.

Table 4. Tier 1 default LRV for each type of pathogen.

Pathogen type	Default LRV
Viruses	1.5
Protozoa	2
Bacteria	4

- Consequences of hazardous events were scoped in detail including chemical cleaning and membrane ageing due to their perceived impact on pathogen removal. An overview matrix of process failures from pilot testing and full-scale site investigation was provided which also considered recovery times. For 0.04 µm hollow fibre membranes operating at low to moderate flux (6 – 25 L/(m^{2}h)), intensive clean in place (CIP) and regular chemically enhanced backwash did not reduce LRV below typically observed process variability (5th percentile).

Figure 3. LRV before and after CEB with NaOCl. '>' indicates permeate concentrations below LOD. Fractions indicate the number of permeate trials at or above LOD.



- Findings were translated into a draft validation protocol consistent with the nine-step template provided by the *WaterVal* Protocol Development Group. The protocol includes proposed tiers (default values, commissioning validation or indirect continuous integrity monitoring) for the validation of MBRs.

Detailed research report available at www.australianwaterrecycling.com.au/LiteratureRetrieve.aspx?ID=152451

Subproject 2 overview – National validation guidelines for RO/NF

An executive summary for subproject 2 can be found in Appendix 3.

Challenges

- Lack of evidence on impact of operating conditions on the LRV of pathogen surrogates and on measures of integrity
- Lack of consistency on the type/frequency of integrity monitoring required to achieve LRVs.

Outcomes

- A critical review of current literature was conducted to consider the removal mechanisms by RO/NF membranes, monitoring techniques and correlations with virus surrogates.
- The benefits, limitations and LRVs achievable using a range of surrogates or techniques were reviewed, in continuous or pulsed mode. These included Electrical Conductivity (EC), Total Organic Carbon (TOC), Dissolved Organic Matter (DOM), Sulfate, fluorescent dyes (Rhodamine WT, Uranine and Trasar™) and S::CAN™.
- The impact of operating factors on the rejection of surrogates was assessed to ensure that the selected surrogates are not better rejected than viruses (conservative approach) but also to select the most appropriate conditions to conduct validation testing.
- The rejections of MS2 phage, R-WT, DOM, sulphate and EC were studied as a function of cross-flow velocity, permeate flux, recovery, membrane types, feed temperature, pH and ion strength within the operating range determined by membrane manufacturers, as summarised in Table 5. Overall, the removal of MS2 phage was not significantly influenced by typical changes in operating conditions and membrane types, LRVs being higher than 4-log under all conditions. Only the solutes (sulfate and EC) were significantly impacted by changes in operating conditions.

Table 5. Impact of changes in operating conditions on the rejection of surrogates.

Operating conditions	Rejection				
	MS2 phage	R-WT	DOM	Sulphate	EC
↗ Permeate flux	→	→	→	↗	↗
↗ Cross-flow velocity	→	→	Membrane dependent	↗	↗
↗ Recovery	→	↘	↗	↗	↗
pH ↗ from 3 to 5	→	→	→	↗	↗
pH ↗ from 5 to 8	→	↗	→	→	↗
pH ↗ from 8 to 10	N/A	→	→	↘	↘
↗ Temperature	→	↘	↘	↗	↘

↗↘ : increase or decrease → : no impact N/A : not applicable

- Research on the impact of ageing on the ability of membranes to remove viruses showed that the reduction in conductivity removal and permeability decline would likely trigger membrane replacement well before experiencing significant reductions in LRV (LRV > 4-log at 80% EC removal).
- Spiked salt removal was demonstrated as a conservative procedure for confirmation of MS2 LRV in ageing membranes and a correlation of MS2 and NaCl LRV values was obtained at different levels of ageing.
- Findings were translated into a draft validation protocol consistent with the nine-step template provided by the *WaterVal* Protocol Development Group, providing a pathway for the validation of LRVs of up to 4-log.

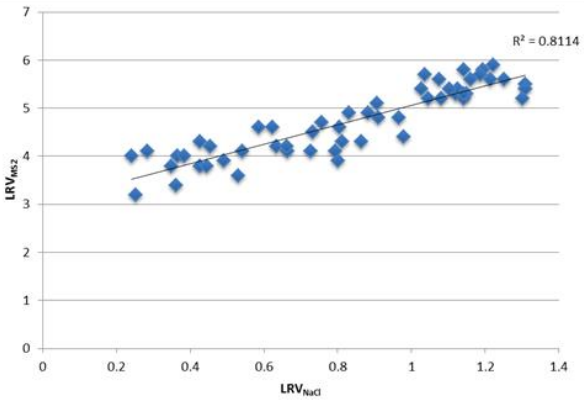


Figure 4. Correlation between LRV_{NaCl} and LRV_{MS2} for RO membranes tested at different degree of ageing during four cycling experiments.

Detailed research report available at www.australianwaterrecycling.com.au/LiteratureRetrieve.aspx?ID=152187

Subproject 3 overview: National validation guidelines for ASP

An executive summary for subproject 3 can be found in Appendix 4.

Challenges

- Lack of removal performance and operational monitoring data able to be directly compared
- Lack of evidence on impact of operating parameters on the LRV of pathogens and surrogates
- No guideline for the validation of the activated sludge process (ASP).

Outcomes

- The subproject included a comprehensive literature review to identify pathogen removal mechanisms and factors which may influence such removal. In general, pathogen reduction during the activated sludge process is driven by three mechanisms; (i) adsorption to suspended solids followed by settling of sludge flocs; (ii) natural decay of pathogens due to environmental stress; and (iii) predation by other organisms such as protozoa.
- The pathogen and indicator microorganism removal efficiency varies according to the treatment process type, retention time, O_2 concentration, pH, temperature, biological flora present in activated sludge, and the efficiency in removing suspended solids. Also, large scale activated sludge treatment processes are influenced by a range of physical and chemical factors including the level of aeration, mixing and seasonal temperature variations.
- The study involved sampling three activated sludge treatment plants, Oxley Creek (sub-tropical), Beenyup (mediterranean), Boneo (cool temperate) and a trickling filter plant, Rosny (mild temperate oceanic) representing different geographical regions and population sizes. The performance of each plant was assessed by measuring LRVs and collecting a range of physicochemical parameters, both from historical records and during the current study.

It was demonstrated that the ASP plants could consistently achieve *E. coli* removal with LRV geometric means ranging from 2.5 to 3.4 $\log_{10}$ (Figure 5). Virus LRVs were of comparable magnitude to those measured for *E. coli* but were site-specific for all three viruses tested.

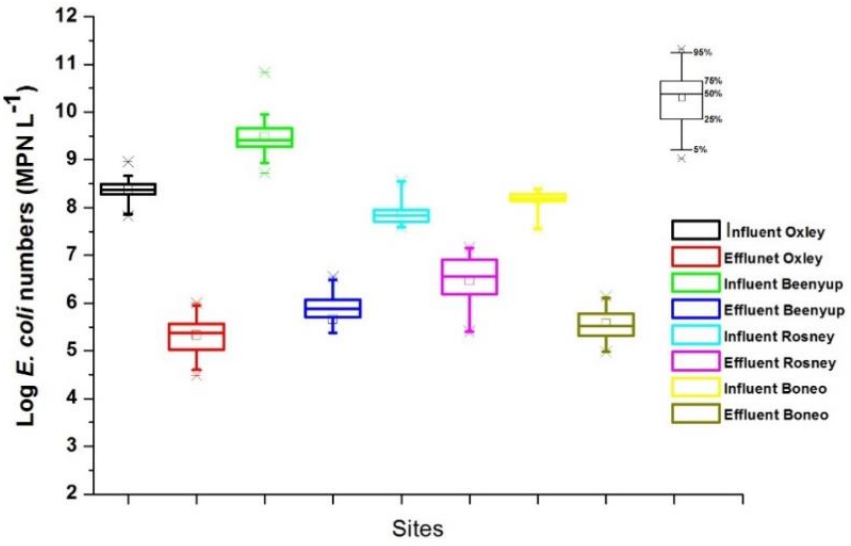


Figure 5. Comparative distribution of *E. coli* in influent and effluent across all four sites.

- Human adenovirus was consistently detected in both influent (10^6 to 10^8 L^{-1}) and effluent samples (10^3 to 10^5 L^{-1}). The LRVs determined in the ASP WWTPs had a geometric means from 2.1 to 2.7 $\log_{10}$ indicating that adenovirus can be used as a conservative indicator.
- Principal component analysis and Bayesian belief network models were used to identify potential correlations between physicochemical parameters and microbiological removal however no clear correlation or relationship could be demonstrated. The physicochemical parameters monitored as well as the frequency of data collection varied across the treatment plants making it difficult to perform a direct comparison between treatment plants.

Detailed research report available at:
www.australianwaterrecycling.com.au/ literature 154072/Development of Validation Protocol for Activated Sludge Process

Subproject 4 overview: Comprehensive Bayesian recycled water validation

An executive summary for subproject 4 can be found in Appendix 5.

Challenges

- Lack of a framework to provide consistency in the approach taken to validate an overall "system" in addition to the validation of its individual components
- Compounding of multiple conservative assumptions leads to the requirement for additional treatment steps and adding cost to recycled water schemes.

Outcomes

- A review of risk management tools applicable to water treatment led to the identification of key principles for a multiple barrier validation framework. Such framework should clarify the relationship between contaminants, indicators and surrogates and ensure the quantification of risk and treatment effectiveness is transparent and readily auditable.
- Validation approaches for recycled water schemes tend to consider each process individually without quantifying the benefits of synergies and multiple barrier reliability. Multiple conservative assumptions are compounded, potentially leading to an over-investment in treatment steps. Probabilistic analysis, using conventional Monte Carlo assessment or Bayesian Networks (BNs) provides alternative means of combining LRVs from multiple barriers.
- Where LRVs are known to be uncertain, current techniques adopt lower-range values such as 5th percentile values. Using a Monte Carlo simulation to combine barriers rather than the current approach based on summing individual LRVs can provide benefits, especially in cases where LRV distributions are quite broad (Figure 6). The same level of conservatism (e.g., the use of a 5th percentile LRV value) can be maintained regardless of the number of barriers.

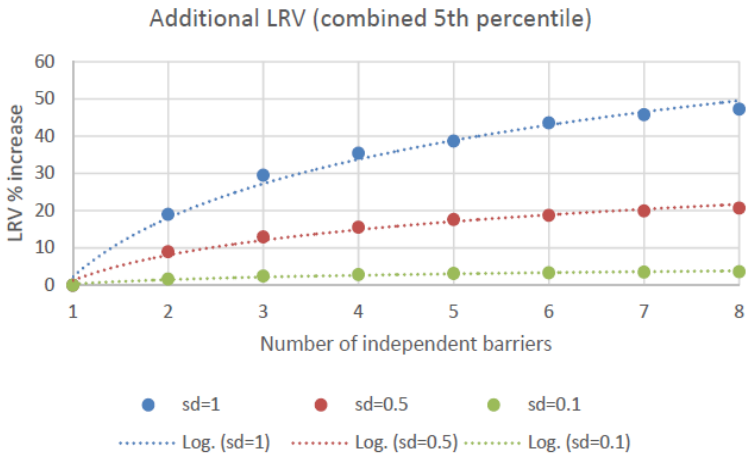


Figure 6. Relative increase in attributable LRV for a combined multiple barrier 5th percentile, compared to summed individual barrier 5th percentiles

- BNs were assessed as a platform for recycled water treatment validation, developing a Bayesian validation framework which is consistent with risk-based management principles. These networks capture beliefs about a system in a concise form and relationships between variables can be unambiguously defined and therefore audited. Relationships can also be 'learned', combining historical data and expert opinion with new information generated through validation testing. BNs can accommodate the equivalent of Monte Carlo simulations for quantitative risk assessment as well as the consideration of discrete hazardous events within different risk exposure scenarios.
- The concepts of "naïve" and "semi naïve" BNs was also introduced for water recycling systems. This proved to be of significant value for identifying the predictive capability of various operational parameters in complex systems where relationships between what can be measured and pathogen LRVs are often not well defined.
- A number of examples or case studies were considered to demonstrate the range of applications of BNs and simulate the validation of a variety of treatment trains, including SA Water's Bolivar Sewage Treatment Plant, Melbourne Water's Eastern Treatment Plant and SA Water's Glenelg Water Recycling Plant.

Detailed research report available at:
www.australianwaterrecycling.com.au/_literature_153253/Comprehensive_Bayesian_Recycled_Water_Validation

Subproject 5 overview: Methods for pathogen isolation, culture, detection and enumeration

An executive summary for subproject 5 can be found in Appendix 6.

Challenges

- Various methods are used for isolation, culture and detection of reference pathogens, which makes comparison of data difficult
- Methods are in some cases limited in application due to highly variable results
- Lack of consistency on virus indicators and surrogates.

Outcomes

- A literature review was conducted focusing on indicators and surrogates for viruses, bacteria and protozoa, leading to specific recommendations for water recycling applications.
- Shortly before the commencement of this project, Keegan *et al*. (2012) reported method improvements for virus concentration and culture and the focus of virus method improvement was to verify the reports of Keegan *et al*. (2012).
- Virus recoveries were inconsistent for both viruses used and for different concentration techniques (direct PEG or filtration + PEG) suggesting that a processing step was causing a loss of spiked virus (or infectivity). Overall, the direct PEG method gave better recoveries for both adenovirus and coxsackievirus, although recovery rates were lower than reported by Keegan *et al*. (2012) for samples collected from the same locations. Future work should focus on using a wider range of primary and secondary effluents.
- In terms of *Cryptosporidium*, recovery methods based on initial sample dilution followed by concentration using either calcium carbonate precipitation or filtration were compared in primary and secondary effluent. Recoveries were very similar in primary effluent but the calcium carbonate flocculation method performed consistently better in secondary effluent. The infectivity of oocysts recovered appeared to vary, and in primary effluent, the infectivity was higher for oocysts recovered by direct centrifugation and filtration. Given its simplicity, the performance of the direct centrifugation method is still adequate for oocyst concentration, but including a recovery control is essential to identify changes in recovery performance.

A single round of inter-laboratory comparison was conducted for virus and *Cryptosporidium* analyses. The results for the primary effluent samples for both viruses (Figure 7) and *Cryptosporidium* were comparable across the different laboratories, particularly for adenovirus detection by PCR. There was greater variation between the results from the different laboratories for secondary effluent, most likely due to differences in assay detection limit, low levels of virus present and also potentially due to differences in recovery rate.

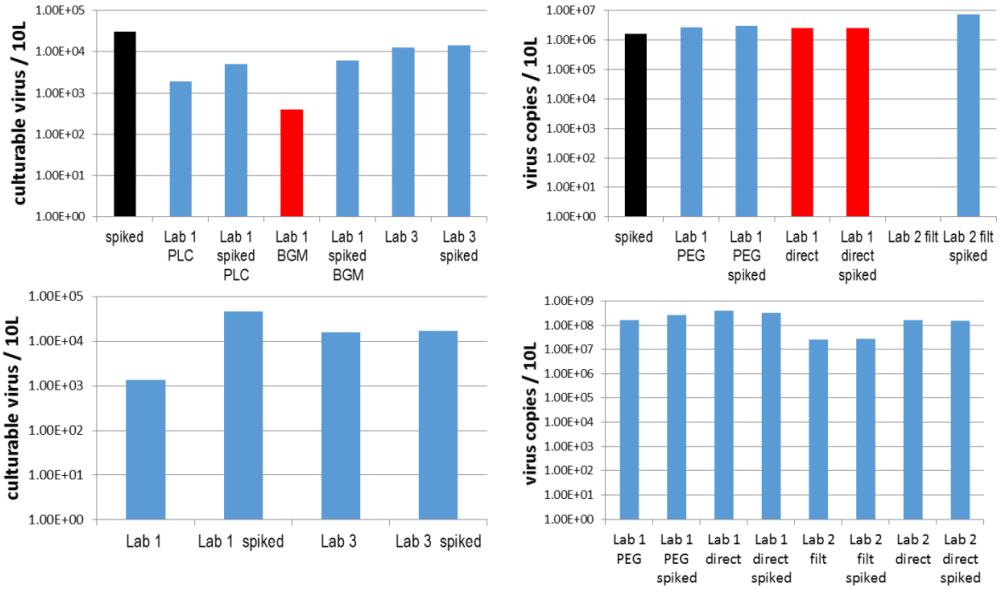


Figure 7. Virus comparison data in primary effluent, for enterovirus by culture (top left panel) and PCR (top right panel) and for adenovirus by culture (bottom left panel) and PCR (bottom right panel).

The black column indicates the number of viruses / 10 L spiked into the sample, the red column indicates a below detection limit result.

Detailed research report available at www.australianwaterrecycling.com.au/LiteratureRetrieve.aspx?ID=153271

Collaborations between subprojects

As discussed above and summarised in Figure 1, three of the five subprojects (SP1, SP2 and SP3) were dedicated to the development of validation protocols for specific treatment processes, whereas two subprojects (SP4 and SP5) were focusing on research which can support the validation of treatment processes generally. All subprojects shared some technical or research elements and the program aimed to align approaches facilitate the direct exchange of data.

Three program level workshops were organised at project inception (July 2013), at the start of the experimental research program (April 2014) and approximately two-third of the way through the said program (February 2015). Subproject leaders were encouraged to identify key interface points and links between their respective research activities. Specific areas which led to significant collaboration include:

- Identification of adequate pathogen indicators and surrogates for specific treatment processes.

  The literature review conducted by SP5 was expanded to include the consideration of viral indicators for MBR. Several meetings took place between the SP1 and SP5 project teams and content expert PAC members before a consensus was reached on the microorganisms which were to be sampled and tested as part SP1 field campaigns.

- Use of Bayes Nets to identify key influencing factors and relevant operating parameters for specific treatment technologies.

  MBR features a large number of potentially interrelated factors that could contribute to LRV. As a collaboration between SP1 and SP4, a Bayesian network was constructed and successfully implemented to assess the impact of these factors on indicator LRV. The MBR Bayesian network was trained on over 100 site visits worth of data. Node connections were informed through an iterative process, incorporating expert knowledge workshops and automated structure learning.

  Similarly, the teams explored the application of BNs to pathogen LRV estimation from data collected from full-scale activated sludge systems as part of SP3. The data provided included influent/effluent pathogen concentrations from four activated sludge sewage treatment plants. Based on the complex biological nature of these systems, it was apparent that a naïve/semi naïve BN approach was more suitable than a causal model, however clear links between operating conditions and LRV could not be established.

- Review and comparison of methods for the concentration and enumeration of viruses and protozoa.

  A number of subprojects involved the concentration and enumeration of target pathogens and indicator microorganisms to characterise the LRV of various technologies and configurations. At an early stage in the program, the microorganism analysis techniques proposed to be applied were reviewed by project leaders and content-expert PAC members, helping to ensure best practice was applied and potentially allowing results to be compared between projects (eg. source water concentrations).

Conclusions and updated gap analysis

The technology and risk management gaps identified in "*NatVal Road Map Report - The road map to a national validation framework for water recycling schemes*" (Muston & Halliwell, 2011) led to the design of the NatVal Priority Research Program. Table 6 summarises the systematic assessment of how these gaps were addressed by the research conducted under this program (this does not cover gaps in areas such as governance and policies).

The key areas which remain significant gaps and potential barriers to further adoption of a national validation framework were identified as follows:

- **Quantification of microorganisms in raw and treated water**

  The research conducted under this program demonstrated the complexity associated with the concentration and enumeration of viruses and protozoa, not only in primary effluent but also in treated water. It also highlighted issues with spiking of indicator microorganisms with the evidence pointing towards a different behaviour between spiked and indigenous organisms. This reinforces the need to develop certified standards (to confirm the performance of the analytical techniques) and reference methods which can be implemented in a consistent manner across multiple laboratories.

- **Validation of activated sludge processes (secondary treatment)**

  The research program outcomes emphasised the complex relationships between environmental factors, operating parameters and pathogen removal performance. It also highlighted the variability between sites, designs and operating environment. Nevertheless, there is an increasing body of knowledge confirming that significant LRV can be achieved for a range of pathogens through activated sludge treatment. Further research is required to identify key influencing factors and to establish standard operating envelopes and critical control points.

- **Chemicals**

  Principles applied to the research under this program, including the process of translation of research outcomes into validation guidelines and recommendations, are not specific only to the validation of pathogen removal. A similar approach could be followed to establish validation protocols for the removal of chemicals by a range of processes such as RO/NF.

- **Packed beds**

  Filtration and adsorption systems such as dual-media filtration or biologically activated carbon are commonplace within the industry, both in recycled and drinking water treatment applications. Specific research aimed at collating historical information and generating new data on pathogen removal performance and integrity monitoring could provide the basis for the development of validation protocols for such treatment technologies. These protocols would be consistent with accepted risk management methods currently applied across both the recycled and drinking water industries.

Table 6. Knowledge gap areas for which further research was recommended.

Knowledge gaps	Initiative	Addressed	Comments
In-situ verification and monitoring and national validation guidelines for MBRs	NatVal Subproject 1: National Validation Guidelines for Membrane Bioreactors	YES	The research project outputs have led to the development and release of a validation protocol for MBR endorsed by the *WaterVal* Protocol Development Group
In-situ verification and monitoring and national validation guidelines for high-pressure membrane systems Evaluation of full potential of LRVs for RO membranes Effect of high pressure membrane ageing on integrity for chemicals and pathogens Effect of catastrophic failures such as o-rings	NatVal Subproject 2: National Validation Guidelines for Reverse Osmosis and Nano-filtration Membranes	YES	The research project outputs as well as research outputs from the WateReuse Research Foundation project WRRF-13-02 have led to the development of a validation protocol for RO currently being reviewed by the *WaterVal* Protocol Development Group
National validation guidelines for ozone disinfection	*WaterVal* Protocol Development Group	YES	Prior research by Melbourne Water was analysed and translated into a validation protocol endorsed by the *WaterVal* Protocol Development Group
Development of an integrated testing strategy in a multi-barrier approach Development of a standardised approach for integrating "hazardous event" conditions into treatment performance assessment	NatVal Subproject 4: Comprehensive validation strategies for water recycling systems	YES	The research project addressed the issue of compounding conservativeness and provided a framework (Bayesian Nets) for comprehensive assessment of multi-barrier treatment performance including hazardous events. Adoption by industry may present a challenge as the method recommended is a departure from traditional approaches.
Standardised methodology for virus isolation, culture and detection for recycled waters Comparison of the use of molecular and culture-based techniques for validation of water treatment barriers Use of laboratory-grown versus indigenous strains of microbial organisms Improvements to and standardisation of protocols for the concentration of pathogens	NatVal Subproject 5: Methods for pathogen isolation, culture, detection and enumeration	PARTIAL	The research project included some activities to address all identified gaps but results were not conclusive enough to consider these gaps addressed. Further research is required, especially with regard to the concentration of pathogens and the behaviour of indigenous versus laboratory-grown organisms. Such research should be linked to the development of standards which is identified as a separate gap.

Knowledge gaps	Initiative	Addressed	Comments
In-situ verification and monitoring and national validation guidelines for biological wastewater systems Biological process surrogates and indicators	NatVal Subproject 3: National Validation Guidelines for Activated Sludge Treatment	PARTIAL	The research project was focused on activated sludge treatment. Outputs did not directly lead to the development of a validation protocol. Gaps remain in the identification of suitable physico-chemical surrogate parameters that can be linked to pathogen reduction performance. A similar WateReuse Research Foundation project has started in 2015 and the combined results of this and the NatVal project may help address this gap.
In-situ verification and monitoring and national validation guidelines for low-pressure membrane systems Correlation of LRV with pressure decay on new and aged membranes to assess long-term removal of viruses		NO	The use of Direct Integrity Testing is currently recognised in a number of (jurisdiction specific) validation guidelines. The development of a national validation protocol is under consideration by *WaterVal*
Suitable chemical indicators for various treatment processes Enhanced surrogate measures for chemical treatment performance Management and risk assessment of transformation products		NO	The focus of this phase of research was on pathogens. Chemicals may be considered in a second phase.
Efficacy and prediction of pathogen removal in packed beds and adsorption systems		NO	This remains a gap which is of significance to the industry and may be considered under *WaterVal*
Needs assessment and development of priority reference standards for validation of water recycling schemes		NO	This remains a gap which is of significance to the industry and may be considered under *WaterVal* in conjunction with further research on pathogen concentration and quantification methods
Benchmarking of water quality and of different technologies using bioanalytical tools		NO	The use of bioanalytical tools will require further demonstration before it can be considered by regulators.
Characterisation of water source (focusing on stormwater)		NO	
Need for more cost-effective methods for assessing UV RED using actinometric methods		NO	This gap is very technology-specific and best addressed at manufacturer or utility level
Evaluation of particle counters for on-line monitoring of membrane performance		NO	This gap is very technology-specific and best addressed at manufacturer or utility level

Appendix 1 - Program outputs

Table 7 provides a summary of the program outputs. It does not include manuscripts under preparation.

Table 7. Overview of program outputs.

Subproject	Selected outputs
1 – MBR	**Technical reports** Subproject report including executive summary, literature review, research report, draft MBR validation protocol and MBR default values report. **Peer-reviewed journal articles** Trinh T, Branch A, Hambly AC, Carvajal G, Coleman HM, Stuetz RM, Drewes JE, Le-Clech P, Khan SJ, 2014, 'Hazardous events in membrane bioreactors - Part 1: Impacts on key operational and bulk water quality parameters', *Journal of Membrane Science*, vol. 497, pp. 494 – 503. Branch AD, Trinh T, Zhou B, Leslie G, Le-Clech, 2015, 'Chemical cleaning in membrane bioreactors: Implications for accreditation in water recycling', *Australian Water Association: Water Journal*, vol. 42, no. 4, pp. 60 – 64. Branch A, Trinh T, Carvajal G, Leslie G, Coleman HM, Stuetz RM, Drewes JE, Khan SJ, Le-Clech P, 2016, 'Hazardous events in membrane bioreactors - Part 3: Impacts on microorganism log removal efficiencies', *Journal of Membrane Science*, vol. 497, pp. 514 – 523. **Conference papers** Branch A; Leslie G; Le-Clech P, 2014, 'The current state of under validation of MBR in Australia', Australian Water Association AWA (ed.), presented at *Ozwater 2014*, Brisbane, 29 April - 1 May 2014. Branch A; Trinh T; Zhou B; Leslie G; Le-Clech P, 2015, 'Chemical cleaning in Membrane Bioreactors: Implications for accreditation in water recycling', in Australian Water Association AWA (ed.), presented at *Ozwater 2015*, Adelaide, 12 - 14 May 2015.
2 – RO/NF	**Technical reports** Subproject report including executive summary, literature review, research report and draft RO/NF validation protocol. **Peer-reviewed journal articles** Pype M.-L., Lawrence M.G., Keller J. and Gernjak W. (2016) Reverse osmosis integrity monitoring in water reuse: The challenge to verify virus removal - A review. *Water Research*, *98* 384-395. doi:10.1016/j.watres.2016.04.040. **Conference papers** Pype M-L, Cran M, Le-Clech P, Gray S, Leslie G, Busetti F, Arrigan DMW and Gernjak W, 2014, 'Developing Australian national guidelines to validate reverse osmosis processes in water recycling', International Water Association IWA, Korea, 2014.

Subproject	Selected outputs
3 – AST	**Technical reports** Subproject report including executive summary, literature review, research report and draft AST validation protocol. **Peer-reviewed journal articles** Ahmed W, Sidhu JP, Smith K, Beale DJ, Gyawali P, Toze S, 2015, 'Distributions of fecal markers in wastewater from different climatic zones for human fecal pollution tracking in Australian surface waters', *Applied Environmental Microbiology*, vol. 82, no. 4, pp. 1316 – 23. **Conference papers** Sidhu JP, Ahmed W, Smith K, Palmer A, Hodgers L, Wylie J, Nichlos C, Low J, Toze S, 2015, 'Comparative removal of enteric virus during activated sludge process', International Water Association IWA, presented at *IWA World Water Congress 2014*, Lisbon, 21 – 26 September 2014.
4 – Multiple Barriers	**Technical reports** Subproject report including executive summary, research report, case studies and Bayes primer report. **Peer-reviewed journal articles** Carvajal GE, Roser DJ, Sisson SA, Keegan A, Kahn SJ, 2015, 'Modelling pathogen $\log_{10}$ reduction values achieved by activated sludge treatment using naive and semi-naive Bayes network models', *Water Research*, vol. 85, pp. 304 – 15. **Conference papers** Carvajal GE, Roser DJ, Sisson SA, Kahn SJ, 2015, 'Multivariate analysis of activated sludge pathogen removal through Bayesian network modelling', in Australian Water Association AWA (ed.), presented at *Ozwater 2015*, Adelaide, 12 - 14 May 2015.
5 – Methods	**Technical reports** Subproject report including executive summary, literature review, research report and inter-laboratory study.

Appendix 2 – NatVal subproject 1: National validation guidelines for membrane bioreactors

Subproject 1 (SP1) has critically assessed the current state of membrane bioreactor (MBR) validation in Australia and conducted a significant research program in order to propose a streamlined and appropriate validation protocol. A successful validation protocol will ensure that a process can and will continually meet log removal value (LRV) requirements for pathogens. SP1 focused on determination of the relationship between MBR operational parameters and LRV in order to highlight the key influencing parameters. In addition, online monitoring options were evaluated for their capacity to correlate with and provide continual assurance pathogen reduction performance. Furthermore, hazardous events that were perceived to compromise the removal efficiency of pathogens, such as chemical cleaning, were investigated with respect to their impact on overall pathogen reduction. The findings of this project have underpinned the development of a national validation protocol for MBR.

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Subproject 1 Research Report

Branch A & Le-Clech P 2015, *National Validation Guidelines for Water Recycling: Membrane Bioreactors*, Australian Water Recycling Centre of Excellence, Brisbane.

ISBN: 978-1-922202-67-3

www.australianwaterrecycling.com.au/LiteratureRetrieve.aspx?ID=152451

Introduction

This subproject was initiated to develop appropriate validation guidelines for MBRs in Australia. The uncertainty as to whether an MBR can be accredited to the required LRV presents significant financial risk for suppliers and designers. In at least one instance, no efforts were made to validate a MBR due to fears that overall scheme delivery would be slowed. Instead, an ultrafiltration unit was placed after the MBR, essentially introducing 100% membrane redundancy and resulting in an increase CAPEX and in energy consumption estimated at 30%.

Only one state based validation guideline developed by the Victorian Department of Health exists for MBR in Australia. The industry perspective is that this guideline is very conservative and potentially difficult to implement, especially for smaller schemes. Regardless, the fact that only one state based guideline exists is evidence that there is insufficient guidance in other states, which has led to inconsistent and case by case assessment of MBRs in Australia.

From the regulators perspective, there is significant uncertainty about the effect and significance of operational parameters for MBR pathogen reduction performance, and on the capacity of online monitoring options to correlate with LRV. There is still limited available data on the suitability of surrogates used for performance monitoring with respect to target pathogens.

The overall aim of this subproject was to develop validation protocols for MBRs in water recycling schemes. In order to achieve this, multiple objectives were identified:

- Objective 1. Collect data from literature, existing validation reports/guidelines and sampling activities in order to identify the LRV applicable to MBR and the mechanisms responsible, identify significant factors that influence LRV and to establish the current practice for MBR validation in Australia.
- Objective 2. Perform multivariate analysis, including the use of Bayesian Belief Networks, to isolate the complex relationships between operational parameters and determine factors that significantly influence LRV.
- Objective 3. Assess the potential for online monitoring to correlate with LRV in order to provide continual assurance.
- Objective 4. Document and quantify the impact of various hazardous events that could lead to diminished LRV in MBRs including integrity failure and shock loading as well as events that occur during operation such as ageing of membranes and chemical cleaning.
- Objective 5. Translate evidence based conclusions from research outputs, as well as the perspectives gained from a review of current practice into appropriate validation guidelines for MBR, consistent with the 9-step validation protocol template as developed by the *WaterVal* Protocol Development Group.

The team conducted a critical review of current literature on LRV achieved by MBR (more than 1000 LRV data points obtained), validation reports/guidelines, and a sampling campaign with a total of 180 visits to 11 different full scale MBRs in order to create a database of MBR performance and operation. Bayesian Belief Networks, created and trained on the data collected, were used to identify significant influencing factors. The new data obtained, combined with the assessment of current validation practice, was used to populate the 9-step validation protocol template provided by the Water Protocol Development Group.

Outputs of this research have been used in the development of proposed validation guidelines for MBRs. This subproject summary is supported by a detailed research report (Branch and Le-Clech, 2015).

Review of MBR literature on LRV and Online Monitoring

Published scientific literature was evaluated in order to identify the mechanisms and expected performance of pathogen removal in MBRs as well as potential online monitoring strategies.

Pathogen reduction mechanisms in MBR include: 1) size exclusion by the clean membrane, 2) adsorption to suspended solids (MLSS) increasing the effective particle size and removal in waste activated sludge, 3) exclusion by the fouling layer and 4) biological predation. The principal removal mechanism will vary depending on the pathogen concerned.

For pathogens larger than the membrane pore size, typically 0.04 – 0.4 µm in MBR, size exclusion is the predominant mechanism. For viruses, which size (typically < 0.1 µm) is in the order of the membrane pore size, rejection by MBR is greater than that expected of a brand new membrane alone, due to the dynamic fouling layer and a strong tendency to adsorb to MLSS. For this reason, there is limited evidence of significant differences in virus removal due to pore size in full scale MBRs.

It is not typical for all pathogens to accumulate within the bioreactor of an MBR after being rejected by the membrane for two reasons: (1) biological predation will occur to some extent and (2) overall accumulation can be limited through sludge wasting (ie proportional to solids retention time).

Turbidity is the most convenient online monitoring technique to infer membrane integrity and hence pathogen removal in MBR. Turbidity measures light (or laser) scatter at 90°, proportional to the amount of suspended solids in a solution. An MBR contains a significant amount of MLSS adjacent the membrane (2,000 – 14,000 mg/L). As a result, significant loss of membrane integrity should result in spikes in turbidity due to transfer of detectable quantities of SS. At this point, corrective actions such as diversion of product water could take place automatically to protect against loss of containment of pathogens.

Direct membrane integrity testing techniques, such as pressure decay testing (PDT), are not favoured in MBR due to the difficulty in maintaining control PDT in the harsh operating environment, the limitation to specific membrane configurations (certain hollow fibre and tubular, not flat sheet) and the lack of correlation between PDT and LRV in MBR; due to the action of mechanisms other than pure size exclusion.

Even though more than 1000 LRV data points has been reported in over 30 published papers for MBR in the last 20 years, the corresponding operational data is not often reported or provided in a consistent manner. As a result, no correlations or identification of statistically significant operating parameters could be made directly from literature alone.

Review of Current Validation Practices

Key elements were evaluated from the Victorian validation guidelines (VDoH 2013) and also from two validation reports, two recycled water quality management plans and one set of validation testing results.

Turbidity was the chosen monitoring technology in all situations. In one case, an attempt was made to correlate turbidity with MLSS and achievable LRV. Operating parameters were documented in most reports, however analysis of their influence on LRV was limited or non-existent.

Default or indicative values for LRV in MBR were claimed based on direct microfiltration listed in the Australian Guidelines for Water Recycling Phase 1 2006, Table 3.8, for two of five sites. No indicative value is listed for MBR in VDoH 2013, although there is one for activated sludge alone.

Three of the five sites conducted challenge testing and the indicators used for virus, bacteria and protozoa were predominantly somatic coliphages (FRNA bacteriophage at 1 site), *Escherichia coli* and *Clostridium perfringens*, respectively. These indicators were consistent with surrogates listed in VDoH 2013. However, no attempt was made to correlate the use of these indicators with target pathogens, enteroviruses and *Cryptosporidium*. Sampling frequency and period was less than that recommended in VDoH 2013 and different in all cases, with the total number of sampling events varying between 14 and 30 over a period of 7 to 14 weeks. The VDoH 2013 recommends analysis of 3 different fouling conditions, at 3 points in the filtration cycle for 6 consecutive cycles each on non-consecutive days, spread over extreme seasonal periods unless a worst case period can be justified. This equates to a minimum of 54 samples taken over a year. One site did not need to conduct challenge testing as it provided literature for performance of the membranes and had historical challenge test data on the activated sludge plant that was upgraded.

Sampling and Analysis of Full Scale Site Data

No adequate data set, containing both microorganism removal and operational parameters, was available to allow correlation and determination of influencing factors on LRV. MBR removal mechanisms are complex and interdependent, leading to difficulties when applying simplistic modelling approaches.

Indicator LRV data for viruses (somatic coliphage, FRNA bacteriophage), bacteria (*E. coli*) and protozoan (*C. perfringens*) was collected alongside a shortlist of operational and monitoring parameters during a sampling campaign across 11 full scale MBRs for a total of 180 site visits. Bayesian belief networks were constructed to elucidate significant relationships and determine influencing parameters.

Based on a preliminary analysis, operation under the following conditions was confirmed to lead to a higher likelihood of a poor LRV: low HRT, high permeability, high permeate turbidity and low MLSS. These conditions were used to define an operational envelope for validation testing.

Consequences of Hazardous events on MBR LRV

Consideration of hazardous events and likely monitoring/control strategies is important for on-going validation of MBR systems and the potential consequences of hazardous events were scoped in detail. Chemical cleaning and membrane ageing were included as hazardous events due to their perceived impact on pathogen removal by the membrane.

An overview matrix of process failures from pilot testing and full scale site investigation was provided which also considered recovery times.

To date, chemical cleaning has been assessed at 3 full-scale sites. For 0.04 µm hollow fibre membranes operating at low to moderate flux (6 - 25L/(m^2h)) intensive clean in place (CIP) and regular chemically enhanced backwash did not reduce LRV below typically observed process variability (5^{th} percentile) as shown in Figure 8 and Figure 9. However, when 0.4 µm flat sheet membranes operated at high flux (30 L/(m^2h)) underwent intensive CIP with NaOCl and Oxalic acid, a significant reduction in LRV occurred. Permeability change before and after cleaning was negligible for hollow fibre membranes, but increased 5 fold upon cleaning flat sheet membranes. A significant change in permeability from nominal conditions is considered to be a site specific indicator that membrane rejection may have reduced.

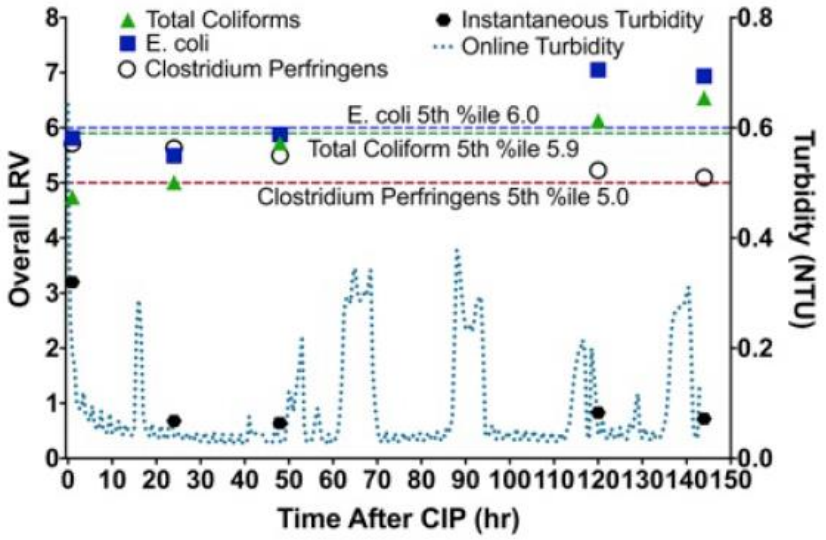

Figure 8. LRV for total coliforms, E. coli and Clostridium perfringens and turbidity for 5 days following a CIP

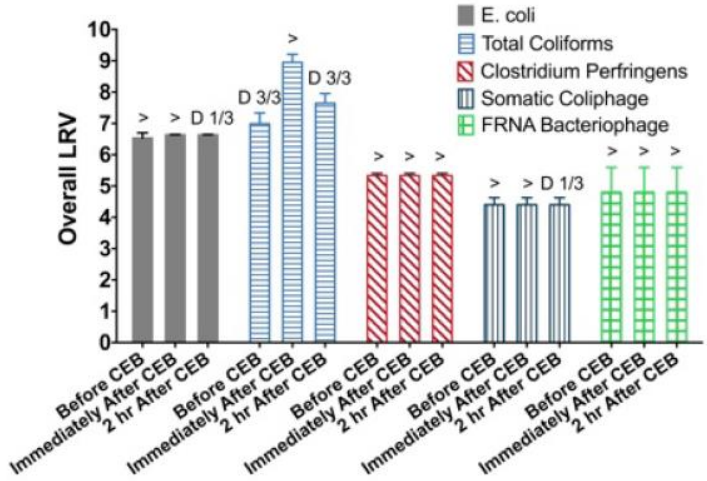

Figure 9. LRV before and after CEB with NaOCl. '>' indicates permeate concentrations below LOD. Fractions indicate the number of permeate trials at or above LOD

Membrane performance after 10 years was not significantly different to LRV documented for the same plant at 5 years operation. After membrane replacement, size exclusion improved resulting in an increase in retention of larger microorganisms. However, the new highly permeable membranes appeared to have lower virus rejection in situ than older fouled membranes. LRV for all indicators in situ was > 3.5 before and after replacement.

Pilot scale assessment of hazardous events demonstrated that plugging and shielding of damage to hollow fibre membranes could occur rapidly (< 15 min) and result in recovery of LRV to nominal values. Plugs were reversible and could be removed during backflush. High NaCl intrusion reduced virus rejection, believed to be due to dispersion of particles from sludge, but recovered within 2 days, upon washout from the reactor. Most other chemical shock loads induced severe fouling, that may have mitigated excess breakthrough as a result of reduced activated sludge performance.

Establishment of default LRV values for MBR

A default or indicative LRV could be used to provide a basis for conservative accreditation of MBR systems where extensive validation testing is not considered feasible.

Probability density functions (PDF) were fit to all data collected from literature (n > 1000 LRVs) and data from site sampling (example in Figure 10). In addition, an operating envelope was established based on the same sites and sources. Also, the results from sampling of 2 sites to a total of 8 samples for *Cryptosporidium*, *Giardia*, enteroviruses, reoviruses and adenoviruses were reported (all > 4 LRV).

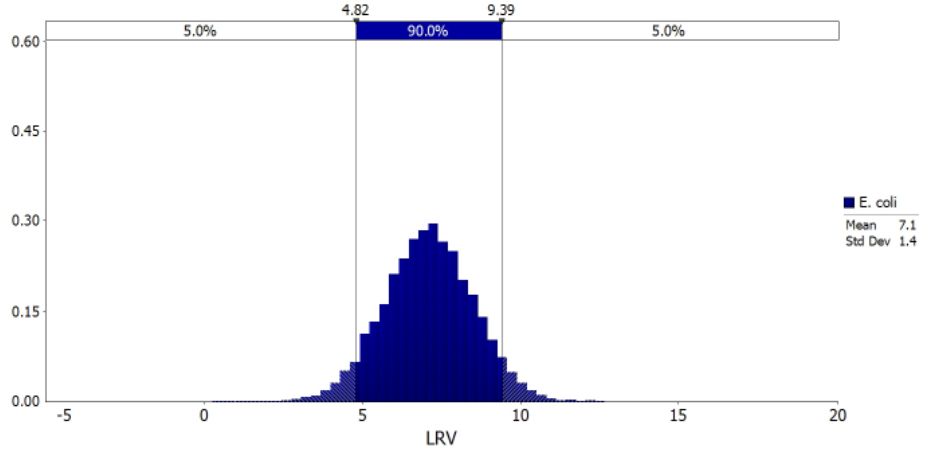

Figure 10. E. coli LRV probability density function from site visits and validation reports

The 5th percentile of resulting LRV PDFs were collated and the most conservative sets of viruses, bacteria and protozoa were rounded down to form the basis of default LRV. The following values were proposed as conservative indicative LRVs for MBR:

- Virus: 1.5
- Bacteria: 4.0
- Protozoa 2.0

The 95*th* percentile of permeate turbidity for the corresponding operating envelope was 0.4 NTU. Hence, as long as permeate turbidity remains less than 0.4 NTU and MBRs are operated within the range of conditions specified in Table 8 of Appendix C, LRVs are likely to remain above default values.

Proposal of a Validation Protocol

The findings of this research were translated into a draft validation protocol consistent with the template provided by the PDG.

The proposed validation protocol is based extensively on the existing VDoH 2013 guidelines however, some alterations were made including a reduction in sampling requirements, consideration of eligibility for pre-validation and listing significant influencing parameters as a result of Bayesian analysis.

The reduction in sampling requirement was justified by recommending samples should only be taken under the most conservative conditions, i.e. highest permeability (lowest fouling). The LRV determined during challenge testing should represent the worst case expected during operation.

Appendix 3 – NatVal subproject 2: National validation guidelines for reverse osmosis and nanofiltration membranes

This subproject aimed to create a framework based on literature review, operational experience from stakeholders, experimental results, scientific knowledge and manufacturer software to develop validation and verification monitoring protocols for the rejection of pathogens (in particular viruses) using online monitoring and challenge testing techniques for RO/NF. The full research report compiles all experimental data produced and describes how this translated into a validation protocol for RO/NF.

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Subproject 2 Research Report

Pype M-L, Alvarez de Eulate E, Antony A, Arrigan D, Busetti F, Le-Clech P & Gernjak W 2015, *National Validation Guidelines for Water Recycling: Reverse Osmosis Membranes*, Australian Water Recycling Centre of Excellence, Brisbane.

ISBN: 978-1-922202-31-4

www.australianwaterrecycling.com.au/LiteratureRetrieve.aspx?ID=152187

Introduction

Validation and monitoring of treatment by high pressure membranes (reverse osmosis - RO, and nanofiltration - NF) is critical to ensure the risk to public health associated with pathogens is adequately managed. To-date, there is no accepted Australian or international validation protocol for this type of membranes, despite conventional monitoring techniques such as electrical conductivity (EC; only for RO), total organic carbon (TOC) or sulphate rejection having been used for this purpose albeit based on ad-hoc approval by regulators. An agreed validation protocol establishing a correlation between log removal value (LRV) and indirect continuous online monitoring would provide confidence to recycled water treatment plant operators and project developers. In particular, the ability to accredit LRV of three and above could reduce the investment costs and simplify treatment process trains by removing unnecessary treatment barriers.

The team conducted a literature review, considering the three types of removal mechanisms by RO/NF membranes and focusing on those involved in virus removal. The review also covered monitoring techniques and correlations with virus surrogates (MS2 phage) based on removal data gathered from the literature.

An experimental study was conducted to assess the impact of operating conditions on the rejection of surrogates in order to establish conservative operating conditions under which testing should be conducted. The team also considered new integrity monitoring techniques based on the spiking (continuous or pulse) of surrogates that can be monitored online or semi-continuously. Finally, the impact of fouling/ageing cycles on the rejection of MS2 phage and EC was systematically assessed.

Two studies were conducted in parallel on (i) the development of a new electrochemical sensor for online measurement of sulphate and (ii) testing the commercially available sensor S::CAN in full-scale.

Literature review

High pressure membrane filtration is a very effective physical barrier to remove inorganic and organic contaminants including pathogens such as viruses. Viruses are the smallest pathogens and the ones found in wastewater can be as small as 24 nm. High pressure membranes are using three different types of removal mechanisms: size exclusion, charge repulsion and adsorption/diffusion. The main removal mechanism for viruses is size exclusion, and charge repulsion improves their removal. Membrane studies generally used MS2 phage as virus model due to its characteristics being similar to enteric viruses (size and surface charge). The advantages of this surrogate are the possibility to culture in high quantity and the fact that it is harmless to human health. However, its quantification is time consuming (24h) and not practical in full-scale application. Hence, it is advantageous to find a non-biological surrogate to avoid the risk involved in performing challenge test with native viruses. Moreover, the use of non-biological surrogate allows online or near online measurement which is currently not possible with live organism such as bacteriophages.

The aim of the project was not to develop a new surrogate or a new monitoring technique, but to provide all the information necessary to support a validation framework. Several surrogates and membrane integrity monitoring are found in the literature and have been described previously. Electrical conductivity, TOC and sulphate are already used in full-scale to monitor the integrity of RO membranes. Dissolved organic matter is another surrogate naturally present in feed water gaining interest. From the literature data, a correlation study has been conducted in order to determine the best potential surrogate for MS2 phage. This correlation study proved that R-WT is a good substitute to MS2 phage in contrast to EC. Sulphate and DOM have the potential to be used to validate the RO/NF process up to 3 LRV, which is lower than MS2 phage but higher than EC. Thus, they are of high interest in the context of NF/RO validation and have been selected for further research.

Rhodamine WT (R-WT)

Rhodamine WT is a non-reactive dye chemical approved by the USEPA for use in drinking water (Zornes *et al*., 2010). It has a molecular weight (MW) of 487 g mol-1 and a pKa of 5.1. Thus, this marker should be well removed by high pressure membranes due to its larger size than the membrane cavities (size exclusion mechanism) and its negative charge at a typical feedwater pH (charge repulsion mechanism). For these reasons, and also due to its low cost and ease to quantify by fluorescence, R-WT is considered an appropriate non-microbiological alternative to MS2 phage. Its rejection by RO membrane ranged from 2.8 - 4 LRV.

Pulsed marker technique

This technique is a deviation of the R-WT monitoring technique. A high concentration of dye is pulse-spiked in the feed side and monitored online by fluorescence detection in the permeate side. This technique permits validating RO membrane for 3.3 – 4.3 LRV using uranine (Surawanvijit *et al*., 2015). Uranine is also a non-reactive, non-toxic tracer dye (Smart and Laidlaw, 1977; Behrens *et al*., 2001) having a MW of 332 g mol-1, which is lower than R-WT.

TRASAR™

TRASAR™ (Nalco company) is a fluorescent tracer dye attached to an antiscalant and it is also gaining interest (Kelle Zeiher *et al*., 2003; Portillo, 2015). The cities of San Diego (California, USA) and Big Spring (Texas, USA) conducted a study comparing MS2 phage and TRASAR™ integrity monitoring techniques (MWH, 2007; Steinle-Darling *et al*., 2015). TRASAR™ was dosed continuously as pure chemical to the RO feed and the permeate concentration was determined using a portable microprocessor-based analyser (TRASAR™ Pen Fluorometer, Nalco). Under this condition, the TRASAR™ marker achieved more than 4 LRV.

Total organic carbon (TOC)

Total organic carbon measurement is one of the current online techniques used in full-scale to monitor RO membranes but it can only be used to validate LRVs typically below 3 due to the limited rejection of organics by the RO process (Adham *et al*., 1998; Kitis *et al*., 2003; Kumar *et al*., 2007). It can also be argued that TOC rejection varies during operation as it is a function of the organic composition. Nevertheless, TOC compounds are smaller than viruses by at least an order of magnitude and thus TOC will generally be more conservative than virus measurement.

Electrical conductivity (EC)

Electrical conductivity is one of the current online techniques used to monitor the integrity of the RO process. It measures all the ions present in the feed and permeate water. This technique can currently validate this process for 1.4 - 2 LRV (Zornes *et al*., 2010).

Sulphate

Sulphate (SO42-) is already used in some plants to monitor the integrity of RO membranes and is measured offline by ion chromatography. The advantage of sulphate is its natural presence in feed water which can be used to validate LRV of up to 3-log (Kruithof *et al*., 2001a). In the case of low sulphate feed concentration, MgSO4 can be spiked into feed water. The full research report refers to research conducted within this project to develop a new online sulphate sensor using electrochemical techniques. Different tests have been carried using a commercially available ionophore which binds to sulphate ions. The ionophore helps to transfer sulphate from one phase (the RO feed or permeate) into the sensor phase. A sulphate sensor prototype was developed with a limit of detection (LOD) of 0.6 µM using this commercially available ionophore combined to a pre-concentration step. Selectivity studies for a range of anions (PO43-, H2PO4-, SO32-, NO3-, CH3COO-, OH-, Cl- and SCN-) were carried out which showed potential interferences by the ions PO43-, NO3-, OH-, Cl-. To date, chloride ions interfere in the sulphate measurement, but this might be reduced using electrolysis to remove chloride, and the use of new, improved ionophores.

Dissolved organic matter (DOM)

Dissolved organic matter (DOM) is a heterogeneous mixture of aromatic and aliphatic hydrocarbon structures containing different functional groups. In the last decade, the use of excitation-emission matrix fluorescence (EEM) has been widely studied to analyse DOM in aquatic samples (Chen *et al*., 2003; Leenheer and Croue, 2003; Her *et al*., 2008; Singh *et al*., 2009; Hambly *et al*., 2010; Peiris *et al*., 2010a; Peiris *et al*., 2010b). Recently, two research groups demonstrated the feasibility to monitor the integrity of RO process using DOM rejection analysed by EEM (Singh *et al*., 2012; Pype *et al*., 2013). With this technique, it is feasible to obtain 1.9 – 2.7 log credit.

S::CAN

S::CAN is a commercially available UV/visible spectrometer sensor able to monitor different water quality parameters including TOC, EC, turbidity and total suspended solids (TSS) as well as specific groups of

organic contaminants. This sensor is of interest in the context of validation as it is able to measure up to eight parameters simultaneously, which could support the online monitoring of RO/NF integrity. Thus in the context of the NatVal project, this sensor was tested to measure specific operational parameters including TOC, R-WT and some organic contaminants and indicator compounds such as metolachlor, trifluralin, metformin, carbamazepine and N-Nitrosodimethylamine in RO water. The findings of this study are briefly summarised as follows:

- Due to the S::CAN low sensitivity and selectivity, it was not possible to directly measure organic contaminants at concentration limits described within drinking water guidelines, except for the pharmaceutical carbamazepine. S::CAN was only able to demonstrate 3 LRV for R-WT under standard challenge testing conditions and an online fluorescence probe would be better suited to demonstrate 4 LRV of R-WT.
- TOC monitoring using S::CAN was compared to a more conventional online Sievers TOC analyser. It was difficult to correlate the results from these two instruments as the varying offsets between the two trends could either be a function of the instrument, substrate or calibration issues.

Impact of operating conditions

The rejection of surrogates depends on their intrinsic properties, but also on operating conditions (e.g. feed pressure, cross-flow velocity, etc.), the type of membranes and feed water quality (Antony *et al*., 2012). It is important to understand the impact of these factors on the rejection of surrogates to ensure that the selected surrogates are not better rejected than viruses (conservative approach) but also to select the most appropriate conditions to conduct validation testing.

The rejections of MS2 phage, R-WT, DOM, sulphate and EC were studied as a function of cross-flow velocity, permeate flux, recovery, membrane types, feed temperature, pH and ion strength within the operating range determined by membrane manufacturers. The benchmark conditions were at permeate flux 20 $L \cdot m^2 \cdot h$, cross-flow velocity 0.1 $m \cdot s^{-1}$, 22 ± 0.5^0C and pH 7 using a flat-sheet cross-flow bench-scale filtration system with concentrate and permeate recirculation. The recovery experiment was conduct with a single 4" spiral wound module membrane.

Table 8 summarises the results of this study. Overall, the removal of MS2 phage was not influenced by changes in operating conditions and membrane types. Under all conditions, the LRV was higher than 4, which is the maximum LRV Australian regulators will credit to a single process (NRMMC *et al*., 2008). In general, only the solutes (sulphate and EC) were significantly impacted by changes in operating conditions.

Table 8. Impact of changes in operating conditions on the rejection of surrogates.

Operating conditions	Rejection				
	MS2 phage	R-WT	DOM	Sulphate	EC
↗ Permeate flux	→	→	→	↗	↗
↗ Cross-flow velocity	→	→	Membrane dependent	↗	↗
↗ Recovery	→	↘	↗	↗	↗
pH ↗ from 3 to 5	→	→	→	↗	↗
pH ↗ from 5 to 8	→	↗	→	→	↗
pH ↗ from 8 to 10	N/A	→	→	↘	↘
↗ Temperature	→	↘	↘	↗	↘

↗↘ : increase or decrease → : no impact N/A : not applicable

Influencing factors of RO membrane performance - ageing

Although membrane ageing is known to change the physicochemical properties of the membrane active layer, the virus rejection efficiency was observed to remain consistent, under certain conditions. In a controlled lab experiment, LRV_{MS2} for virgin RO membranes was > 6.2-log with salt rejection of 97% (2000 ppm NaCl); aged membranes, featuring salt rejection as low as 80%, consistently resulted on LRV_{MS2} values greater than 4-log. Also, industrially aged membranes of 2 - 5 years, tested in this study,

were still a resilient barrier for MS2 sized particles, LRV_{MS2} always greater than 4.6-log. For the industrially aged membranes tested, when compared with aged virgin membranes, a higher LRV_{MS2} was observed at equivalent conductivity removal, but permeability was lower, suggesting development of an irreversible fouling layer. The irreversible fouling layer may assist with preservation of virus rejection, however, the reduction in conductivity removal and permeability decline would likely trigger membrane replacement, prior to significant reductions in LRV. Therefore, the potential risk of membranes losing their integrity as a mechanical barrier to pathogens (considering the smallest virus size is 24 nm) due to ageing is considered to be marginal.

Spiked salt rejection for integrity monitoring

Spiked salt conductivity is a simple test employed as quality assurance testing for RO membrane integrity, recommended by manufacturers. Commonly this test involves spiking 2000 ppm NaCl for a brackish water RO membrane or $MgSO_4$ for a NF membrane and challenge testing at an applied pressure of 7 – 15 bar. The type of salts, concentration and operating pressures may vary depending on manufacturers. Performance of spiked salt testing on a regular basis during operation would enable the comparison of current against benchmarked performance. While salt removal is not in principle directly equivalent to the rejection of MS2 or other pathogens of concern, it can be used as a conservative indicator of the state of the membrane. Spiked salt rejection can be especially useful when feedwater conductivity is low (i.e. the LRV_{EC} able to be demonstrated is limited by the sensitivity of permeate conductivity meters).

For pristine and aged RO membranes tested in this study, LRV_{NaCl} was up to 4 times lower than the corresponding LRV_{MS2} and correlated well. Given the significantly smaller size of NaCl (the hydrated size of Na^+ is 0.36 nm and Cl^- is 0.33 nm compared with the diameter of MS2 - 26 nm) and the correlation observed in this study, spiked salt rejection can be considered as a highly conservative procedure for confirmation of LRV_{MS2} in ageing membranes. A correlation of LRV_{MS2} and LRV_{NaCl} values obtained for RO membrane tested at different levels of ageing during four different cyclic ageing experiments is presented in Figure 11.

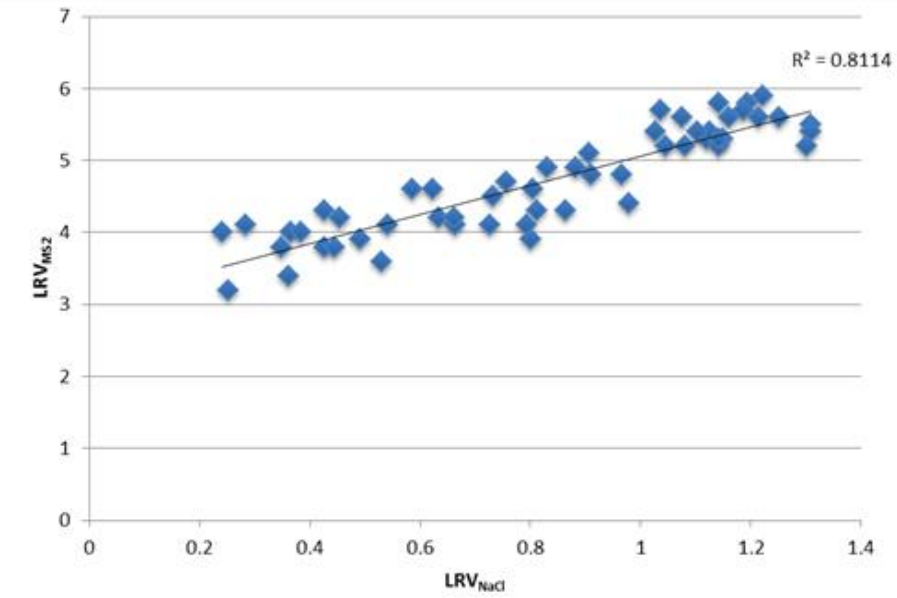

Figure 11. Correlation between LRV_{NaCl} and LRV_{MS2} for RO membranes tested at different degree of ageing during four cycling experiments.

Conclusions for the validation of RO/NF membranes

In the context of challenge testing, the research led to the following conclusions for the two surrogates which are generally used for challenge testing:

- MS2: operating conditions do not significantly influence its removal;
- R-WT: changes in operating conditions can impact its rejection and conditions giving the lowest $LRV_{R\text{-}WT}$ should be selected during challenge testing as follows:
  - Low pH
  - high temperature
  - high permeate recovery.

In the context of operational monitoring, the following parameters should be continuously monitored:

- Permeate flux
- Cross-flow velocity
- Recovery
- pH
- Temperature

In the context of integrity monitoring, several surrogates can be used depending on the LRVs the RO process is validated for:

- EC (LRV between 1 and 1.5-log; online measurement)
- TOC (LRV ≤ 2-log; online measurement)
- DOM (LRV ≤ 2-log; offline measurement)
- Sulphate (LRV ≤ 3-log; offline measurement)
- R-WT or similar fluorescent dye (LRV ≤ 4-log; online or offline measurement).

The LRV of these indicators can be limited by the detection limit of the analytical instrument or their concentration in feedwater. Thus, indicator spiking such as sulphate or salt in the RO feed can increase the resulting LRV being demonstrated.

In order to validate LRVs above 3 and up to 4, specialised dyes such as R-WT and TRASAR™ can be used, spiked continuously or as a pulse. This approach can introduce a significant cost and more studies are needed to assess the long-term impact on membranes.

Operating conditions may change significantly over time and revalidation may be required depending on the surrogate used to conduct the initial process validation. As an example, a revalidation may not be necessary when using MS2 phage as a surrogate based on its rejection not being significantly impacted by changes to operating conditions provided the process remains within the operating range defined by membrane manufacturers.

Appendix 4 – NatVal subproject 3: National validation guidelines for activated sludge processes

Reclaimed water from treated municipal wastewater is increasingly considered as viable and sustainable option to alleviate water shortages in Australia. Biological systems can form the major treatment component of a water-recycling scheme, particularly for small-scale schemes, or from an initial treatment stage within larger, multi-barrier scheme. High quality recycled water could be used for a variety of direct or indirect potable reuse, agricultural irrigation, managed aquifer recharge, industrial use, recreational use and environmental enhancement.

The activated sludge process (ASP) is the most commonly used wastewater treatment option in Australia and around the world (Carducci and Verani, 2013; NRMMC, 2006; Tandukar *et al*., 2007). The primary objective is removal of bio-degradable organic matter and suspended solids. Therefore, performance of the wastewater treatment plant (WWTP) is generally measured on the basis of chemical parameters such as BOD, COD and nutrient removal (Carducci and Verani, 2013). To date, there has been limited information documenting how well ASP remove pathogens especially under Australian conditions.

The overall objective of the project was to collect scientific data on enteric pathogen removal and its relationship with physicochemical parameters frequently recorded at Australian wastewater treatment plants.

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Subproject 3 Research Report

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www.australianwaterrecycling.com.au/_literature_154072/Development_of_Validation_Protocol_for_Activated_Sludge_Process

Introduction

Decreasing rainfall, frequent drought and population growth in urban environments along with an overall desire to achieve greater water sustainability have increased the demand for alternative water sources such as recycled water. This has resulted in an increased attention on the types of contaminants in wastewater and the need to protect the health of the public while implementing these water sustainability initiatives. One important means of safeguarding appropriate health standards is to ensure that contaminants are removed to appropriate levels in the treated water. This means that there needs to be the correct controls and monitoring of the treatment processes to continually meet the determined treatment requirements. One of the important initial steps is to be able to accurately validate what removal capacity a treatment process can achieve when it is operating optimally, and what conditions can cause failure in the established removal capacity.

Many water recycling schemes use wastewater treatment plants (WWTPs) as a common treatment process. WWTPs have traditionally been designed and operated to maximise the removal of nutrients and suspended solids from municipal wastewater to prevent environmental contamination. These WWTPs are now frequently required to produce high quality water (alone or in conjunction with other treatment steps) that can be recycled for a variety of direct or indirect potable reuse, agricultural irrigation, managed aquifer recharge, industrial use, recreational use and environmental enhancement. A large majority of these WWTPs utilise the activated sludge process (ASP) as a major treatment process.

Due to the inherent complexity of the activated sludge process, to date data on the level of contaminant removal has been sparse and conflicting. In addition, differences in the design of the ASP process, the types of contaminants studied, along with variations in sampling and detection methodologies have made it difficult to gain an accurate understanding of the treatment capability of ASPs. This lack of adequate data precludes the development of adequate validation steps that can assist in establishing appropriate removal credits.

The potential public health risk associated with recycled water predominantly originates from the potential presence of enteric viruses and protozoan parasites due to their high infectivity and low dose. These pathogens are also recognised to have high environmental resistance and are commonly found in higher numbers in untreated municipal wastewater than in other environmental sources. The presence of viruses in treated water used for recycling may vary according to the type of treatment process, population size, geographical location and prevalence of disease in the community. This makes it difficult to generalise what and how much treatment a WWTP must achieve (Gerba *et al*., 2013). This means that any assessment of the treatment capacity of an ASP within a wastewater treatment train needs to be assessed on an individual basis, taking into account the common microbial constituents present in that wastewater, and how well the ASP performs under local conditions.

The overall aim of this project was to collect data on pathogen removal in activated sludge plants that could be used in the development of a validation protocol to provide a standardised format for validating ASP plants in different regions across Australia. The secondary aim was to attempt to determine if there were relationships between the measured microbial log removal values and frequently recorded (and/or easily measured) physicochemical parameters. The identification of relationships would enable, through appropriate operational monitoring and verification, the demonstration that appropriate pathogen log removals were being achieved in these biological systems. It was also hoped that such relationships could also indicate when an ASP was not operating to specifications and therefore when pathogen removal could be impacted.

Literature review

The subproject included a comprehensive literature review to identify pathogen removal mechanisms and factors which may influence such removal.

In general, pathogen reduction during the activated sludge process is driven by three mechanisms: (i) adsorption to suspended solids followed by settling of sludge flocs; (ii) natural decay of pathogens due to environmental stress; and (iii) predation by other organisms such as protozoa. The first two factors play a major role whereas predation, while less significant, contributes towards removal of bacterial and protozoan pathogens, and viral pathogens to a lesser extent, from wastewater matrices (Chabaud *et al*., 2006; Gerba *et al*., 1978; Glass and O'brien, 1980; Kim and Unno, 1996; Medema *et al*., 1998; Stadterman *et al*., 1995). The principal removal mechanism is expected to vary with the type of pathogen in question, and depending on plant operational conditions.

The degree of removal of pathogens during activated sludge treatment is influenced by a variety of plant operational variables and conditions, which can vary between treatment plants and which may often

deviate from the ideal parameters. The pathogen and indicator microorganism removal efficiency varies according to the treatment process type, retention time, O_2 concentration, pH, temperature, biological flora present in activated sludge, and the efficiency in removing suspended solids. Also, large scale activated sludge treatment process is influenced by a range of physical and chemical factors including the level of aeration, mixing and seasonal temperature variations. These factors are linked with the removal of pathogens and can be monitored during the activated sludge process.

Measurement of pathogen LRVs at activated sludge treatment plants

The study involved sampling three activated sludge treatment plants, Oxley Creek (sub-tropical), Beenyup (mediterranean), Boneo (cool temperate) and a trickling filter plant, Rosny (mild temperate oceanic). These WWTPs represented different geographical regions of Australia and different population sizes. The selected treatment plants also varied in design and operating conditions. The performance of each plant was assessed by measuring LRVs and collecting a range of physicochemical parameters, both from historical records and during the current study. The historical records provided information on the stability of the plant operation and were used to demonstrate that the plant was operating to specifications during the time when microbial LRVs were assessed.

The study of microbial removal efficiencies at each plant was done using selected microorganisms from the three major pathogen groups of concern in Australia, namely bacteria, viruses and protozoa. The bacteria were represented by *E. coli* as this bacterium is the most commonly used microbial indicator and has been used historically to inform the quality of treated effluent. It was also assumed that removal efficiencies for *E. coli* would be representative of other bacterial species. Three DNA viruses (adenovirus, polyomavirus and the *Microviridae* coliphage) were tested as potential viral surrogates. Adenovirus and polyomavirus were selected as it has been previously suggested that these viruses could potentially be suitable as representative indicator pathogens. *Microviridae* was tested because somatic coliphages had often been used to represent enteric viruses until recent advances in molecular technologies improved the detection capabilities for enteric viruses. Finally, *Cryptosporidium* was chosen as the representative protozoan pathogen due to its known resistance to environmental pressures and chlorination.

The results found that *E. coli* numbers were fairly constant in the influent throughout the year at all the WWTPs ranging from 7 to 9 $\log_{10}$ L^{-1}. Effluent *E. coli* numbers were also constant in the effluent from each of the WWTPs, with mean values from 5.3 to 5.9 $\log_{10}$ L^{-1}. When these influent and effluent numbers were used to calculate LRVs, it was demonstrated that the ASP plants could consistently achieve *E. coli* removal with LRV geometric means ranging from 2.5 to 3.4 $\log_{10}$ (Figure 12).

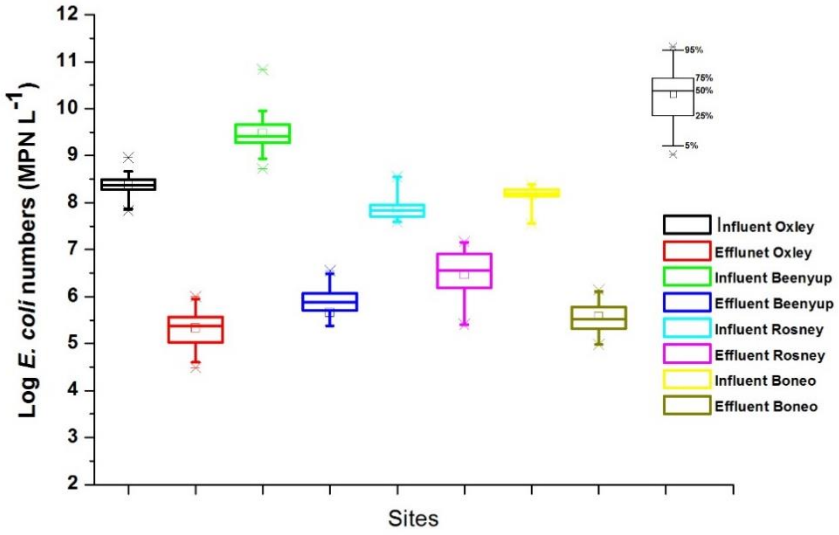

Figure 12. Comparative distribution of *E. coli* in influent and effluent across all four sites.

The virus data from all four WWTPs (Figure 13) suggest that human adenovirus was consistently present in detectable numbers in both influent (10^6 to 10^8 L^{-1}) and effluent samples (10^3 to 10^5 L^{-1}). The LRVs determined for adenovirus in the ASP WWTPs had geometric means from 2.1 to 2.7 $\log_{10}$ indicating that adenovirus is indeed suitable for use as a conservative viral surrogate in a validation protocol. LRVs were of comparable magnitude to LRVs measured for *E. coli*, however the site-specificity for all three viruses meant that validation would need to be undertaken for each individual WWTP in order to determine appropriate virus log removal credits.

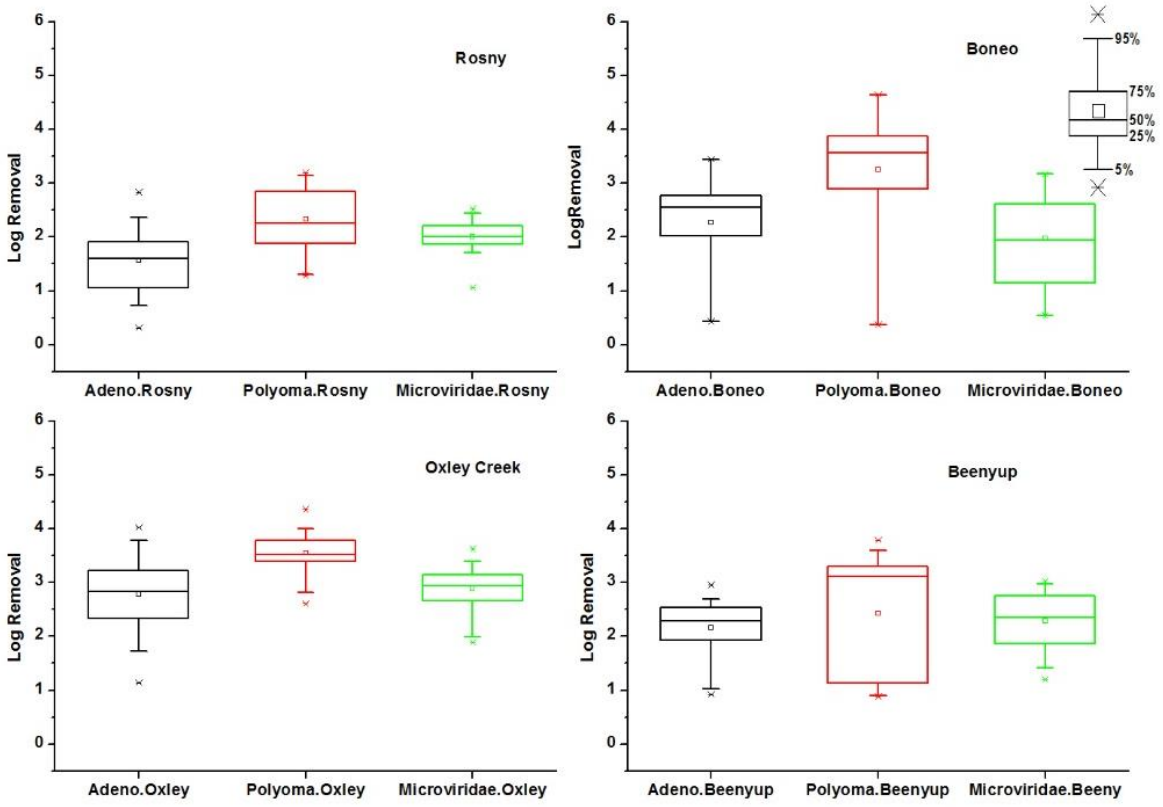

Figure 13. LRVs distribution for adenovirus, polyomavirus and Microviridae across all four sites.

The initial attempts to detect *Cryptosporidium parvum* oocysts was found to provide numbers that were inconsistent in the influent of all WWTPs. Further research determined that this was caused by a low detection limit and issues associated with the recovery of oocysts from raw influent. Changes to the detection methodology including the volume of sample tested (decreased from 30 mL to 15 mL) and using *Cryptosporidium* sp. genus specific primers rather than *C. parvum* species specific primers provided results in the case of Beenyup and Boneo WWTPs. These two WWTPs respectively presented average *Cryptosporidium* sp. numbers in influent of 4.1 and 4.5 $\log_{10}$ L^{-1} and in effluent of 1.4 and 0.7 $\log_{10}$ L^{-1}. The calculated mean LRVs were 2.8 $\log_{10}$ for Beenyup WWTP and 3.8 $\log_{10}$ for Boneo WWTP. These initial removal rates are similar to those determined for viruses, however these were only preliminary conclusions based on limited data. More testing would be needed from these and other WWTPs in order for more accurate conclusions to be reached on the ability of activated sludge plants to remove *Cryptosporidium* from wastewater.

Impact of sampling strategy

The impact of using a paired sampling strategy (collecting the sample influent and delaying the collection of a matching effluent sample by the equivalent of the hydraulic retention time of the plant) versus simultaneous or random sampling was assessed at Oxley Creek WWTP. Samples were collected in triplicates (random samples 40x3; HRT samples 20 x3) and LRVs obtained based on the two sampling methods were compared to identify any statistically significant difference. The data analysis showed no statistically significant difference in LRVs (t test, $P > 0.05$) for adenovirus, polyomavirus and *Microviridae*. Paired sample collection requires the calculation of HRT at time of sampling, a parameter which may often vary as a function of plant inflow, which is itself influenced by precipitation and seasonal variation such as surge in population during the holiday season for smaller treatment plants. Therefore, the collection of simultaneous or random samples was considered appropriate for the determination of LRVs as part of the validation of ASP.

It was also considered important to determine the ideal number of samples required for validation purposes. The results suggested that the analysis of 10 samples was not sufficient to capture variations in LRVs while the mean and geometric means of 20, 30, and 40 samples were statistically similar and therefore, little additional benefit was obtained by collecting more than 20 samples. This result is consistent with literature data on representative sample sizes for validation purposes.

Impact of operating conditions on pathogen LRVs

The physicochemical parameters monitored in the influent and effluent as well as the frequency of data collection varied across the treatment plants. This made it difficult to perform a direct comparison between treatment plants. The subsequent findings indicated that the design of the plant was as important (if not more of an influence) than the geographical location of the plant. In fact, the Rosny trickling filter plant was so different that ultimately the results from this WWTP were not used for direct comparison with the three ASP WWTPs and was examined on its own as an example of the trickling filter technology.

Despite the differences noted between the WWTPs, the analysis of physicochemical parameters (temperature, pH, DO, BOD, COD) showed little variation within an individual treatment plant indicating that all plants were operating under stable conditions. The average effluent temperature at Oxley Creek treatment plant was the highest (26.9±3.0 °C) and Rosny the lowest (18.5±3.5 °C) and this significant difference ($P < 0.05$) in operational temperature between the two plants reflected the influence of ambient climatic conditions. Sludge parameters such as sludge retention time (SRT) and mixed liquor suspended solids (MLSS) varied in response to plant design and operation, seasonal variations in wastewater inflows, and ambient temperature.

The data on calculated microbial LRVs and measured physicochemical parameters at each plant were compared using Principal Component Analysis (PCA) to identify any potential correlations between physicochemical parameters and microbiological removal. No strong correlations or relationships could be identified. Future improvements in sensing technology and the testing of a large number of WWTPs over a longer time period may lead to the identification of significant links, allowing the monitoring of specific physicochemical parameters to be used to demonstrate pathogen removal.

Through a collaboration with the UNSW Water Research Centre, Bayesian Belief Network models were also used to investigate potential relationships between operating conditions, monitoring parameters and microbiological removal, and assess the capacity of these models to predict ASP performance. Similar to the PCA analysis, there were limited links found between the microbial LRVs and the physicochemical parameters using the Bayesian Network modelling. The Bayesian Network analysis, however, did find potential links of low LRVs being closely associated with high concentrations of reduced nitrogen, and higher LRVs associated with much lower than average NH_4^+-N and TN concentrations. This suggests that, while these physicochemical parameters may still not be directly correlated to pathogen removal, they may be able to be associated with monitoring that demonstrates that ASP processes are performing adequately.

This study has found that activated sludge plants are able to reduce the numbers of bacteria, viruses and protozoan by 2 $\log_{10}$ or more. No seasonal impacts were observed, but design and geographical locations do have an influence on the overall efficiency of the WWTPs ability to remove microorganisms. No direct links between physiochemical parameters and microbial LRVs were identified, however, further research and data collection from a wider number of WWTPs may assist in potentially identifying suitable linkages. The additional information will also be important to further demonstrate that the LRVs of surrogates such as adenovirus can also represent the removal of other microbial pathogens, in particular RNA viruses such as norovirus and reoviruses.

Appendix 5 – NatVal subproject 4: Comprehensive Bayesian recycled water validation

Subproject 4 was tasked with identifying a framework which could provide some consistency in the approach taken for various treatment processes and a means of validating an overall "system" in addition to the validation of its individual components.

After careful consideration of a wide variety of risk assessment and risk management tools, the use of Bayesian Nets (BNs) was identified as a means of collating information describing system performance, as well as producing validation conclusions through the formalized description of cause-effect relationships that define treatment process mechanisms and observational data. There are a large number of software packages available for constructing and analysing BNs and a number of them were reviewed and presented various advantages and disadvantages. For the sake of simplicity and consistency, the vast majority of work conducted in this project was developed using Netica™ by Norsys Software.

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Subproject 4 Research Report

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www.australianwaterrecycling.com.au/_literature_153253/Comprehensive_Bayesian_Recycled_Water_Validation

Introduction

Validation approaches for recycled water schemes tend to consider each process one step at a time and do not sufficiently integrate between process steps to quantify the benefits of synergies and multiple barrier reliability. As a result, multiple conservative assumptions are often compounded, leading to a requirement for additional treatment steps and adding cost to recycled water schemes. The purpose of the research is to build on previous work undertaken on chemical and microbial reduction to validate the multi-barrier approach using whole-of-process-train reliability engineering.

The specific aims and objectives adopted for this work were as follows:

- Review available risk assessment methods for implementation in the validation of water recycling processes and projects,
- Provide recommendations for the collection and incorporation of suitable chemical, microbial, or surrogate data in performance assessment/validation activities. This will specifically address factors such as the appropriate duration of validation testing,
- Identify key characteristics of a framework to apply to the validation of water recycling unit processes to ensure consistency of data collection, statistical evaluation, and performance assessment,
- Develop a rigorous basis for the incorporation of potential hazardous events (i.e., non-ideal operational conditions) and performance failures in the validation process,
- Provide case studies of appropriate risk assessment methods for the validation of a specific water recycling process, and
- Provide recommendation of practical approaches for combining the individual validation of unit processes to achieve the overall validation of multi-barrier water recycling projects.

Performance validation is a key step in managing risks associated with water recycling projects. As a component of risk management, formalised validation guidance should be consistent with current best practices for risk management. Current risk management standards and guidelines provide an array of at least 31 diverse risk assessment and risk management tools (IEC/ISO, 2009, ISO, 2009, Standards Australia and Standards New Zealand, 2009, Standards Australia and Standards New Zealand, 2013). These tools are listed below in Table 9. A review of the literature indicated all of these tools were applicable to water recycling, though a number had only seen moderate application in the water supply and treatment industry so far.

In addition to this list, the team considered emerging best practices such as Quantitative Microbial Risk Assessment which is central to the Australian Recycled water guidelines and is being rolled out as best practice in USA water management as well (U.S. Environmental Protection Agency and U.S. Department of Agriculture, 2012).

Table 9. ISO 31010 risk management tools.

Tool Class	Tool Code	Tools and Techniques
Supporting	B01	Brainstorming
	B02	Structured or semi-structured interviews
	B03	Delphi
	B09	Structure « What if? » (SWIFT)
	B20	Human reliability analysis
Look up	B04	Check-lists
	B05	Primary hazard analysis
Function Analysis	B06	Hazard and operability studies (HAZOP)
	B07	Hazard Analysis and Critical Control Points (HACCP)
	B13	Failure mode effect analysis
	B22	Reliability centred maintenance
	B23	Sneak circuit analysis
	B27	FN curves
Scenario analyses	B08	Environmental risk assessment
	B10	Scenario analysis
	B11	Business impact analysis
	B12	Root cause analysis
	B14	Fault tree analysis
	B15	Event tree analysis

Tool Class	Tool Code	Tools and Techniques
	B16	Cause and consequence analysis
	B17	Cause-and-effect analysis
	B19	Decision tree
	B28	Risk indices
	B29	Consequence/probability matrix
	B31	Multi-criteria decision analysis (MCDA)
Controls assessment	B18	Layer protection analysis (LOPA)
	B21	Bow tie analysis
Statistical Methods	B24	Markov analysis
	B25	Monte Carlo simulation
	B26	Bayesian statistics and Bayes Nets
	B30	Cost/benefit analysis

Note: Some tools were not allocated to classes in ISO 31010. So the allocations shown include our suggestions based on what the tools are used for.

The team concluded that the framework and tools proposed should:

- Incorporate or allow for the use of all or the large majority of the 31 ISO 31010 risk management tools;
- Include or clarify the relationship between primary contaminants, indicators and surrogates so that recycled water validation testing assessments could be cost effective and the data underpinning this credible and auditable; and
- Ensure the quantification of risk, and treatment train and individual process effectiveness is transparent, standardised, readily auditable and straightforward to understand for regulators as well as technical specialists.

Provisionally the team formed the opinion that Monte Carlo risk assessment methods (Haas *et al*., 1999, Haas and Eisenberg, 2001) and Bayesian Networks (BNs) (Korb and Nicholson, 2011, Kragt, 2009) might significantly contribute to the solution to these broad problems as well as providing operational methodologies. This view was based on earlier experience of risk assessment application to recycled water (Roser *et al*., 2006, Khan and Roser, 2007, Khan *et al*., 2007) and an earlier effort to apply BNs to recycled water (Donald *et al*., 2009, Donald *et al*., 2010, Cook *et al*., 2013).

Accordingly, the methodology and case studies conducted in this project seek to demonstrate how the use of BNs (incorporating Monte Carlo-type probabilistic assessment) can support the validation of water recycling system by providing a means of collating information describing system performance, as well as producing validation conclusions through the formalised description of cause-effect relationships that define treatment process mechanisms and observational data. There are a large number of software packages available for constructing and analysing BNs. A number of them were reviewed and all had various advantages and disadvantages. For the sake of simplicity and consistency, the vast majority of work presented was developed using Netica™ by Norsys Software.

Assessment of Compounding Conservativeness of LRV Attribution for Increasing Numbers of Treatment Barriers

The project explored the effect of compounding conservative assumptions and demonstrated how probabilistic analysis (which may be achieved by conventional Monte Carlo assessment or by the use of BNs) can provide an alternative means of summing LRV credits from multiple barriers.

Where LRVs are known to be variable or uncertain, current techniques tend to adopt lower-range values such as 5th percentile values. If LRVs attributed to multiple barriers are treated in this way and then summed, the final 'multiple barrier' LRV is increasingly conservative, depending on the variability of each barrier and the number of barriers summed. A consequence of this increasing conservatism for increasing numbers of barriers is that, in some cases, additional LRVs could be attributed to multiple barrier systems while maintaining the same level of conservatism that would be required for systems with fewer independent barriers.

The degree to which this could be achieved is dependent upon the number of sequential independent barriers, as well as the relative variability (e.g., LRV standard deviation) of the individual barriers. For barriers with very tight LRV distributions (in this case s.d.=0.1), the advantage achieved by using a Monte Carlo simulation to combine the barriers compared to summing the individual 5th percentiles is minimal,

as indicated in Figure 14, whereas for broad distributions (in this case, s.d.=1), the advantage can be more significant.

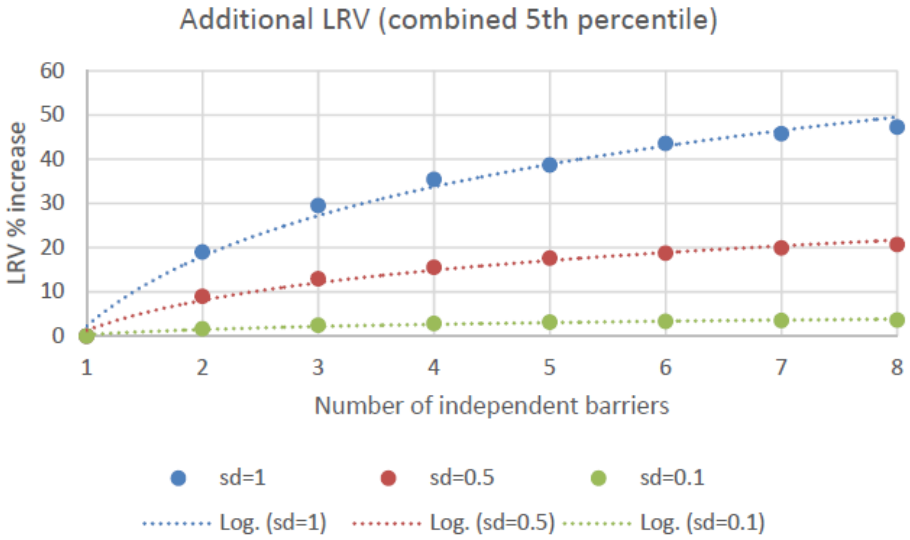

Figure 14. Relative increase in attributable LRV for a combined multiple barrier 5th percentile, compared to summed individual barrier 5th percentiles.

The potential advantage is that the same level of conservatism (e.g., the use of a 5th percentile LRV value) can be maintained regardless of the number of barriers summed.

Bayesian nets

Introduction to Bayesian Nets

Bayesian Nets (BNs) are acyclic Cause=>Effect based computer models where variables ('Nodes') are linked to one another by unidirectional 'Arcs' (Figure 15). Typical Directed Acyclic Graph (DAG) format is a 'boxes and arrows' flow diagram or network. Unlike DAGs created for HACCP analysis using graphic programs, links between Nodes must be mathematically defined, logically coherent and possess data integrity.

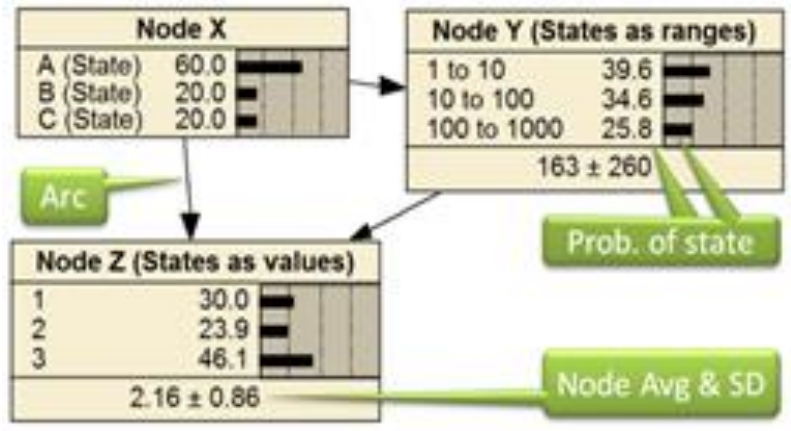

Figure 15. Bayesian net representation.

BN Nodes can represent most discrete concepts and variables that might interest a water engineer or scientist e.g. water quality, treatment options, expert opinions, true/false. Each Node takes on one or more different 'States' e.g. categories, values, value ranges; with probabilities summing to 1.0. BN software can display Node data in the form of probabilities and miniature bar graphs. When a Node represents a quantitative variable, its average and standard deviation is also displayed. BNs exploit Bayes Theorem (equation 1); the set theory based rule that '*a priori*' data (Ai /historical probability estimates) can be combined with '*a posteriori*' (Bj /new) data to improve '*a priori*' estimate accuracy. As an example, an operator has data on filter failure from local tests (new evidence) and from the manufacturer (old priors). Bayes Theorem describes how to combine the two data sets and obtain a better (new posterior) failure likelihood estimate.

$$P(A_i|B_j) = \frac{P(A_i)\,P(B_j|A_i)}{\sum_i P(A_i)\,P(B_j|A_i)}$$ (equation 1)

Where $P(A_i|B_j)$ = the conditional (posterior) probability of each *A* i^{th} event given each *B* j^{th} event; $P(A_i)\,P(B_j|A_i)$ = the product of that (prior) i^{th} *A* probability and the conditional probability of each *B* j^{th} event given the *A* i^{th} event ; $\sum_i P(A_i)\,P(B_j|A_i)$ = the sum of the products of each (prior) *A* i^{th} probability and the conditional probability of each *B* j^{th} event given each *A* i^{th} event.

BNs make possible model conceptualisation, definition, probability calculations and exploration in a single platform. Bayes theorem, together with graphical control elements in modern BN software, allows the impacts and implications of changing Nodes or Node states to be rapidly and interactively assessed e.g. exploration of diverse '*What-If?*' risk exposure and management scenarios. BNs are somewhat analogous to spreadsheets. They are a general computer modelling platform which can be used for many similar tasks. Nodes, like spreadsheet cells, can reference one another, and Node states can depend conditionally on other linked cells. BN data may be inputted as single values, be calculated using algorithms, transformed using formulae or imported as large data sets e.g. CSV files.

However the BN programming structure sets them apart. Instead of traditional linear computer code and functions, the Node heart involves defining BN relationships (arcs) between independent variables ('parent' Nodes) and dependent variables ('child' Nodes) in the form of probability value matrices known as contingency tables. These tables define how the probability of a 'child' Node taking on a particular state, value or range, depends on the state/value/range of each 'parent'. BN software ensures that Arcs are logically consistent in a manner analogous to relational databases enforcing referential integrity. The probability table based structure allows BNs to be a more flexible and intuitive modelling tools than spreadsheets.

Application to recycled water systems

BNs can provide a common platform for most recycled water treatment validation activities. BNs allow the use of either machine optimisation techniques and or expert beliefs of how system components and variables are inter-related to define and construct quantitative relationship networks reflecting the physical recycled water processes and systems. Within these networks which capture and communicate beliefs about a recycled water system in a concise form, the relationships between variables are unambiguously defined and can therefore be audited. These relationships can also be 'learned', effectively combining historical/supporting data and expert opinion with new information for example generated through validation testing.

Validating a water treatment system involves collecting a range of data sets and combining these to infer how the system will operate and reduce public health risk to a predictable degree (LRVs) provided standard operational conditions are maintained. Historically such inference process has been conducted using common sense and expert opinion with different quantitative data sets used to varying degrees for decision support (the application of consequence x likelihood matrices to draw conclusions about risk is an example). BNs can offer a conceptual/theoretical framework to support the inference process by providing mathematical rules by which most data and expert opinions can be combined (e.g. scientific literature, validation testing results) and used to infer and ask questions about system behaviour as a whole (or large parts thereof) to an extent not possible otherwise. This process termed 'Bayesian Inference' is explained by Ellison (1996) using ecosystems for illustration. Directly linking *prior* data and new evidence to generate new *posterior* risk estimates and comparing these with acceptable risk via Bayesian inference underpins the concept of 'Bayesian validation' discussed in subsequent sections.

In terms of risk assessment and supporting decision making, BNs can accommodate the equivalent of Monte Carlo simulations for quantitative risk assessment as well as the consideration of discrete hazardous events within different risk exposure scenarios. These networks offer the option of 'backcasting', for example by defining set goals such as tolerable risk targets and assessing what treatment performance is required to achieve these. It is also possible to link wider considerations (downstream exposure risks, influence of external barriers such as access control and management options) to the overall performance of the treatment system being modelled.

The proposed framework is consistent with many existing practices and much of what is proposed should already be undertaken guided by Australian Guidelines for Water Recycling (AGWR), HACCP and risk management principles. The framework is designed not to replace risk-based management, but to reframe it slightly in respect to terminology and how operators and managers think about validation so as to be able to exploit Bayesian inference related concepts, methods and tools. The full research report describes in detail a series of principles for the Bayesian validation of recycled water systems which have been applied in several cases studies discussed in the following sections.

Process validation with large data sets - Naïve & semi-naïve versus causal BNs

An approach based on the concepts of “naïve” and “semi naïve” BNs proved to be of significant value for identifying the (combined) predictive capability of various operational and monitoring parameters. Complex systems (in this case, activated sludge) are difficult to model since knowledge of cause-effect relationships between what can be measured (e.g. monitoring parameters) and what is desired to be known (e.g. pathogen LRVs) are often not well defined. Naïve and semi naïve BNs differ from ‘causal’ BNs in that they do not begin with a fully defined understanding of the system. Instead, procedures are used to ‘learn’ predictive relationships among the available data.

A clear stepwise procedure for doing this was developed and described in detail in the full research report and associated peer reviewed publication (Carvajal *et al*. 2015). Outcomes were compared to findings using a causal BN based on expert understanding of system cause-effect. The development of Naïve and semi naïve BNs was aided by the use the general data mining software WEKA.

Naïve Bayes (NB) models are non-causal BN models commonly used for classification problems (Kjræulff and Madsen 2008). They often provide good accuracy, while offering simplicity and efficiency. Their construction employs a range of objective rules and tests, which address modelling traps with causal BNs including the use of inappropriate variables, modeller bias and over-fitting. By definition, the structure of a NB model always employs a “class node” which is the only parent of each other node (attribute nodes), all of which are conditionally independent given the class node. LRVs are a ‘logical class’ node.

In the case of the related Semi-naïve Bayes (SNB) models, the independence assumption is relaxed by allowing some arcs between the attribute nodes as well as the class node using link selection rules not necessarily involving a choice by the investigator.

Work on this involved analysis of the activated sludge pathogen reduction dataset developed and published by Flapper *et al*. (2012) based on a pilot study. The latter looked at factors controlling *Cryptosporidium* and *Giardia* reduction. Approximately 98 measurements were taken under varying operating conditions and a range of water quality measures and microbial indicators were concurrently measured.

The conclusions from this work included the following. NB and SNB models can be used to predict and manage pathogen reductions. The methodology developed in this study is objective, systematic and applicable to the analysis of water treatment processes more generally. Though the study identified operational parameters potentially useful for the prediction of *C. parvum* removal efficiency, modelling *G. lamblia* suggested that its removal by activated sludge is not sufficiently understood and cannot yet be quantified based on removal of microbial indicators, even though assignment of average reduction credits of ≥1-$\log_{10}$ is still reasonable judging by the raw LRV probability density function.

Our non-causal models also provided a reference and starting point for BN modelling by identifying those variables most likely to be useful when constructing causal models with the minimum number of nodes. The SNB models provide an objective way of estimating the maximum accuracy that is possible with a causal Bayes model. The models were relatively easy to understand which should assist uptake by non-experts in BNs interested in other nonstandard treatment approaches. Finally, the method here can reduce potential disagreements between model developers about what form BNs should take.

Case studies

A number of examples or case studies were considered to demonstrate the range of applications of BNs, by simulating the validation of a variety of treatment trains, including:

Best practice development of the BNs was generally straightforward as water treatment systems are inherently well defined in terms of purpose and structure. Aspects that were investigated include:

- Network conceptualisation and model parameterisation;
- Model evaluation, for example in terms of accuracy and prediction reliability;
- Gathering summary statistics pertinent to LRV calculation and crediting;
- Scenario analysis;
- Use of semi-naïve BNs compared to causal BNs;
- Bayesian validation

As expected the BN showed concisely, in a HACCP-style flow chart (technically a network):

- The summary concentration and removal statistics;
- LRVs for different processes and process combinations and how they were derived;
- The spread of the concentration data and LRV estimates;
- The impact of data censorship;
- The causal relationships believed to apply based on system knowledge.

SA Water's Bolivar Sewage Treatment Plant (STP) and supply of feed water to a recycled water plant

The primary barriers to microbial risks are the activated sludge plant (ASP), the STP lagoon system, a coagulation and filtration system and chlorination. A whole of system BN was developed (Figure 16) which was suitable for undertaking a range of validation tasks.

This case study is an example of "whole of system validation", in which the overall system performance is characterised in terms of log reduction values (LRVs) for the multiple barrier system. This example shows how a BN can be constructed based on an existing understand of cause-and-effect relationships. It directly reflects the way the system was designed and is assumed to operate. Individual water treatment barriers are constructed with a particular design performance, which is subsequently cross-matched with validation data. The design and validation values can be assessed in combination or separately. Various scenarios (including combinations of scenarios) can be quickly tested to determine whether validation objectives can be assumed to have been met under each one.

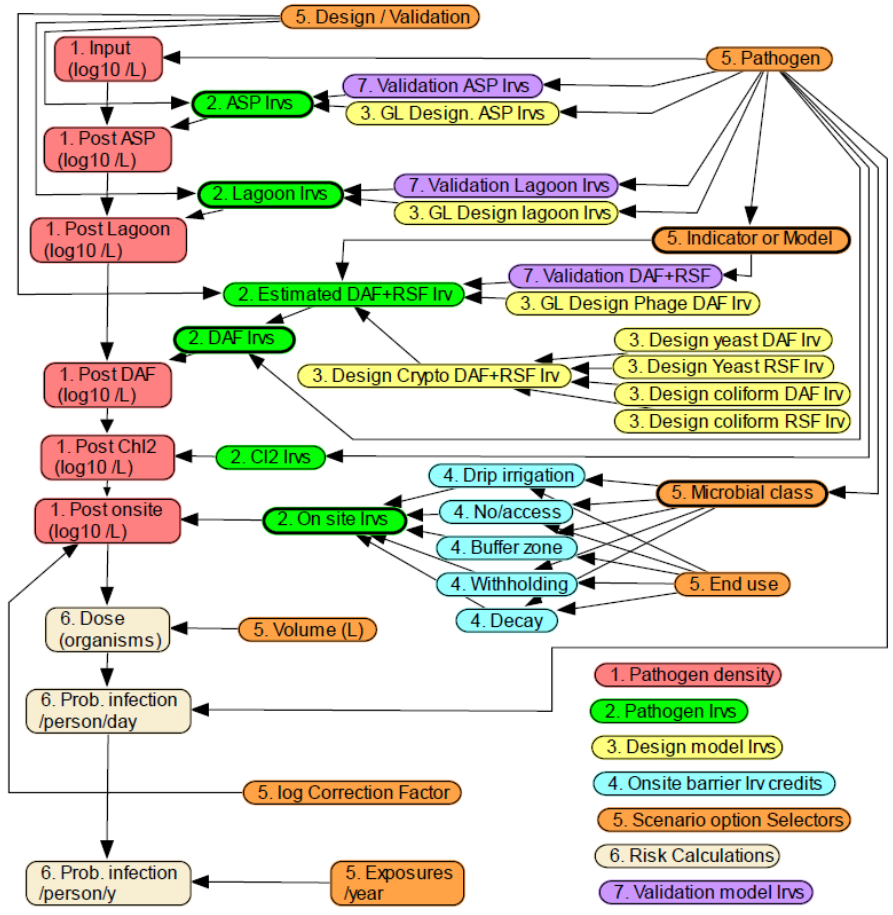

Figure 16. Bayesian network of bolivar recycled water system.

Melbourne Water's Eastern Treatment plant (ETP) - First case study

This water recycling plant treats secondary effluent with key barriers being pre-ozonation biological media filtration, ozone disinfection, UV disinfection and chlorination.

The first of two case studies undertaken in collaboration with Melbourne Water was based on the reanalysis of a historical dataset containing 1757 microbial measurement records for *E. coli*, somatic coliphage and *C. perfringens* (measured at 6 locations between October 2013 and September 2015)

along with physicochemical data. This large dataset was used to illustrate real world Bayesian validation, exploring changes in treatment performance over time and providing an example of how operating parameters might be related to microbial concentrations or LRVs.

The outcomes demonstrate the use of these data for:

- Initially constructing causal BNs for the three central analytes which characterise treatment effectiveness as LRVs;
- Comparing the result of calculating LRVs using a BN vs. learning from primary data tables;
- Assessing model accuracy and hence prediction reliability;
- The use of semi-naïve BNs compared to causal BNs;
- Gathering summary statistics pertinent to LRV calculation and crediting;
- Improving understanding of system structure and function using Netica's Sensitivity (to findings) analysis tool;
- Bayesian Validation;
- The use of WEKA in data mining especially large on line data sets.

Melbourne Water's Eastern Treatment plant (ETP) - Second case study

In a second phase, acknowledging the limitations of the historical data (sampling timing and matching, unavailability of several physico-chemical parameters), an experimental monitoring program was designed to address these validation issues and uncertainties. Specific aims included the collection of new microbial concentration and removal data concurrently with a full range of physicochemical parameters, the estimation of microbial LRVs and comparison with previous estimates, gathering information on whether pre-ozonation and biofiltration should be considered as two individual processes or as a single unit process.

The experimental campaign confirmed the insights and estimates obtained as part of the initial validation assessment using ETP historical data and indicated that the establishment of LRV credits for the treatment process (pre-ozonation and biofiltration) was feasible. This work demonstrated that variance in LRVs over the short-term were comparable to those which had previously been observed over a longer term. The performance of pre-ozonation alone for disinfection was observed to be highly variable, but much more consistent results were achieved by considering the pre-ozonation and biofiltration units as a single treatment step.

Activated Sludge LRV data analysis and estimation

This case study explored the application of BNs to pathogen LRV estimation from data collected from full scale activated sludge systems by CSIRO Land and Water as part of subproject 3 (National validation guidelines for activated sludge treatment). The data provided included influent/effluent pathogen concentrations for four activated sludge sewage treatment plants. Due to the complex biological nature of these systems, it was apparent that a naïve/semi naïve BN approach would be more suitable than a causal model, which would require explicit assumptions of cause-effect relationships between operational/monitoring parameters and achieved LRVs. This work demonstrated that significant bacterial and viral LRVs could be achieved, but a high variation in performance was also observed. Temperature and nitrogen concentrations were determined to be effective predictive parameters for LRV performance.

SA Water Glenelg water recycling plant

In June 2015 SA Water undertook to 'revalidate' its water recycling plant at Glenelg. This system is designed to treat chlorinated activated sludge secondary effluent through ultrafiltration (UF) membranes prior to reuse. The revalidation involved directly measuring the reduction in feedwater of seeded MS2 bacteriophage numbers, under typical membrane pressure and other operating conditions. For the purposes of this BN analysis, SA Water also measured the reduction in total coliform numbers and concurrently a range of physico-chemical parameters as grab samples and on-line parameters.

For comparison, both causal and semi-naïve models were developed, which yielded similar predictions for pathogen LRVs. The causal model was constructed based on the assumption that the different experiments, units, time-step and replicate measurements could independently influence inlet and outlet bacteriophage concentrations, and hence LRVs, in different ways and extents. Many of the statistics and observations generated from this study could have been alternatively generated using conventional means. However, BNs allowed the whole LRV picture to be captured in one platform and in a clear graphic format which was helpful for communication, discussion and decision support with regard to achieving concurrence on LRV credits.

Appendix 6 – NatVal subproject 5: Methods for pathogen isolation, culture, detection and enumeration

A number of issues exist with the enumeration of reference pathogens used to demonstrate treatment system performance as specified in the Australian Guidelines for Water Recycling (AGWR). The reference pathogens include human enteric viruses (in particular adenovirus and rotavirus), *Campylobacter* and *Cryptosporidium*. Various methods are used for isolation, culture and detection of reference pathogens, which makes comparison of data difficult. The methods are in some cases limited in application (such as *Campylobacter*) due to highly variable results and may be improved through application of new technologies.

The project aimed to deliver improved methods for the detection and enumeration of reference pathogens that are relevant for the development of validation protocols for individual treatment processes under investigation within other NatVal subprojects. The final research report includes a literature review that summarises the current status of methods for use in wastewater matrices, as a starting point for any method improvement.

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Subproject 5 Partners

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Subproject 5 Research Report

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http://www.australianwaterrecycling.com.au/LiteratureRetrieve.aspx?ID=153271

Introduction

Water scarcity is driving increased reuse of alternative water sources, such as wastewater and storm water. The Australian Guidelines for Water Recycling (AGWR) provides a framework for the safe use of these alternative water sources. Key elements within the framework requires characterisation of the hazards in the water, such as pathogens, and the use of effective barriers to remove or control the hazards to reduce risk to end users or the environment an acceptable level. Not all utilities, particularly those in small regional locations, have the resources for detailed characterisation of hazards or measurement of the performance of treatment barriers. In the absence of such information, the AGRW provides default values for the numbers of pathogens in sewage (Table 10).

Table 10. Indicative numbers of pathogens in sewage (AGWR 2008).

Organism	Numbers in sewage (per litre)
Bacteria	
Escherichia coli (indicators)	10^5–10^{10}
Pathogenic *E. coli*	Low
Enterococci (indicators)	10^6–10^7
Shigella	10^1–10^4
Campylobacter	10^2–10^5
Salmonella	10^3–10^5
Clostridium perfringens (indicator)	10^5–10^6
Viruses[a]	
Enteroviruses	10^2–10^6
Adenoviruses	10^1–10^4
Noroviruses	10^1–10^4
Rotaviruses	10^2–10^5
Somatic coliphages (indicators)	10^6–10^9
F–RNA coliphages (indicators)	10^5–10^7
Protozoa and helminths	
Cryptosporidium	0–10^4
Giardia	10^2–10^5
Helminth ova	0–10^4

a Colony-forming units for bacteria, plaque-forming units for bacteria, oocysts for *Cryptosporidium* and cysts for *Giardia*

Similarly, the AGWR also provides indicative removal values for the performance of different steps in the wastewater treatment train (

Table 11). A limitation of the values in the AGWR is that they represent ranges of pathogen numbers or treatment performance from a wide variety of locations and process designs, which may vary in terms of pathogen challenges or treatment effectiveness. Site-specific pathogen data can allow better system design to meet treatment requirements for the production of safe and fit for purpose reuse water. In addition, validation of treatment process performance will ensure that the treatment train is effective at achieving the desired level of treatment with appropriate safety factors, avoiding either under-treating the water or excessive operational or capital costs associated with over-treating the water. A difficulty with the validation of wastewater treatment processes is the lack of any national standard protocols, which are required to ensure that the validation approach is reliable and that it is consistently applied. The development of such protocols is a key element of other subprojects within the NatVal project.

A more fundamental issue that underlies process validation is the selection of appropriate representative pathogens or surrogates for use in validation studies and the availability of suitable methods for their enumeration. The latter is particularly critical because there are known limitations with the methods used to detect some reference pathogens, including human enteric viruses (in particular adenovirus and rotavirus), *Campylobacter* and *Cryptosporidium*. Various methods are used for isolation, culture and detection of these reference pathogens, which makes comparison of data difficult. The methods are in some cases limited in application (such as *Campylobacter*) due to highly variable results and may be improved through application of new technologies.

Table 11. Indicative performance of different treatment processes for the removal of pathogens in wastewater (AGWR 2008).

	Indicative log reductions[a]							
Treatment	*Escherichia coli*	Bacterial pathogens (including *Campylobacter*)	Viruses (including adenoviruses, rotaviruses and enteroviruses)	Phage	*Giardia*	*Cryptosporidium*	*Clostridium perfringens*	Helminths
Primary treatment	0–0.5	0–0.5	0–0.1	N/A	0.5–1.0	0–0.5	0–0.5	0–2.0
Secondary treatment	1.0–3.0	1.0–3.0	0.5–2.0	0.5–2.5	0.5–1.5	0.5–1.0	0.5–1.0	0–2.0
Dual media filtration with coagulation	0–1.0	0–1.0	0.5–3.0	1.0–4.0	1.0–3.0	1.5–2.5	0–1.0	2.0–3.0
Membrane filtration	3.5–>6.0	3.5–>6.0	2.5–>6.0	3–>6.0	>6.0	>6.0	>6.0	>6.0
Reverse osmosis	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0
Lagoon storage	1.0–5.0	1.0–5.0	1.0–4.0	1.0–4.0	3.0–4.0	1.0–3.5	N/A	1.5–>3.0
Chlorination	2.0–6.0	2.0–6.0	1.0–3.0	0–2.5	0.5–1.5	0–0.5	1.0–2.0	0–1.0
Ozonation	2.0–6.0	2.0–6.0	3.0–6.0	2.0–6.0	N/A	N/A	0–0.5	N/A
UV light	2.0–>4.0	2.0–>4.0	>1.0 adenovirus >3.0 enterovirus, hepatitis A	3.0–6.0	>3.0	>3.0	N/A	N/A
Wetlands — surface flow	1.5–2.5	1.0	N/A	1.5–2.0	0.5–1.5	0.5–1.0	1.5	0–2.0
Wetlands — subsurface flow	0.5–3.0	1.0–3.0	N/A	1.5–2.0	1.5–2.0	0.5–1.0	1.0–3.0	N/A

N/A = not available; UV = ultraviolet
a Reductions depend on specific features of the process, including detention times, pore size, filter depths, disinfectant

In the case of *Cryptosporidium*, well-validated methods are available for the concentration and enumeration of oocysts from surface and potable waters. However, the relatively poor quality of raw and primary treated wastewaters has been problematic for *Cryptosporidium* enumeration, often limiting the sample volume (20 mL to 250 mL) that can be processed and adversely affecting the accuracy of enumeration of pathogen loads. Direct concentration of small volumes or primary effluent is currently by centrifugation followed by oocyst purification using immunomagnetic separation (IMS). Possible alternatives include resuspension of smaller volumes of raw and primary treated sewage in large volumes of water prior to concentration, allowing dispersion of particulates and dilution of the fats and oils. Homogenization of samples is also a possible option to disaggregate particulates and expose particle-bound oocysts for more even recovery. Any improvements in the method will not compromise additional analyses such as oocyst infectivity measurement or genotyping using molecular methods.

A current key issue faced by the water industry is validation of virus removal for the production of reuse water as required by the AGWR. The use of a single standardised method (also measuring infectivity in the case of disinfection process validation) would allow direct comparison of results between schemes. The challenges related to virus enumeration include sample transport and storage, virus concentration and recovery and virus detection. Some viruses may be sensitive to storage / transport, even at 4°C. Recovery and detection can both be affected by the matrix, which can reduce recovery efficiency and also affect downstream detection by culture-based or molecular methods by interfering with binding to host cells or inhibiting the reactions used to detect the viruses. Improved recovery and detection methods will provide better process performance data and ultimately provide better data for incorporation into future revisions of the AGWR. Further work is required to improve the recovery of viruses in raw and primary treated wastewaters and determine any factors that influence the detection of infectious viruses by cell culture. Sample stability also needs to be reviewed for samples that require transport to interstate laboratories for analysis.

Campylobacter is a key reference bacterial pathogen that is considered in the production of reuse water. The standard method for enumeration of *Campylobacter* uses a combination of membrane filtration and

MPN enumeration, culture in semi selective (Preston's) enrichment broth and, subculturing into enrichment agar or broth. Confirmation of *Campylobacter* species is complex, using Gram staining and biochemical markers (APHA, 9260G). There are inherent problems with this methodology due to matrix effects (especially filter blockage) and also by the use of MPN, which can have large uncertainties within the count estimate. Selective chromogenic agar offers an alternative for the enumeration of *Campylobacter*. Although still requiring membrane filtration, the detection and enumeration of *Campylobacter* species is simplified by the allowing colony counts. The applicability of chromogenic agars was originally going to be considered within this project. However, during the start-up phase of this project key technical issues were raised regarding the detection of *Campylobacter*, particularly in relation to oxidative stress and the inability of culture techniques to detect *Campylobacter* cells that are in a viable but non-culturable state. Due to these issues the proposed *Campylobacter* method development was abandoned.

The project aims were as follows:

- The initial aim of this project was to undertake literature review of pathogens and surrogates in order to identify any appropriate pathogen-surrogate pairs that may be of use in other sub-projects within NatVal 2.2. In addition, a review was conducted of the available methods for viruses and *Cryptosporidium*;
- Following completion of the literature review, an additional aim was to provide guidance regarding appropriate protocols for surrogates/pathogens suitable for validation activities across particular WWTP processes and source waters destined for re-use;
- The main aim of this project was to develop improved methods for the isolation, culture, detection and enumeration of reference pathogens (eg *Cryptosporidium*, adenovirus) in wastewater matrices; and
- Following completion of any method improvement, the final aim of this project was to undertake inter-laboratory trials to confirm the wider use of methods across Australian laboratories.

Literature Review Outcomes

The focus of the literature review was on virus surrogates, given that viruses are the most problematic in terms of detection techniques and that there is a real need for virus surrogates for the validation of physical removal processes such as membrane filtration. The selection of a representative bacterial pathogen and surrogate was relatively straightforward and so this was not considered in great detail. *Escherichia coli* fulfilled the criteria required for both representative pathogen and indicator (cost, presence, ease of detection, behaviour) and was recommended for use in validation studies. One consideration for using *E. coli* or more broadly using coliforms is the nature of the matrix and environmental conditions, bearing in mind that under favourable conditions of temperature and nutrients these faecal organisms can propagate in the environment, which would confound any validation study. There was limited literature available on surrogates for enteric protozoa. In effect there is no ideal surrogate for *Cryptosporidium*. Spores of sulphite reducing clostridia appear to be conservative indicators for *Cryptosporidium* and *Giardia* removal, but if the pathogen numbers are high enough it would be better to used *Cryptosporidium* oocysts to directly measure process performance, especially since a surrogate provides no information on inactivation of *Cryptosporidium*. Recent advances in methods now allow enumeration of total and infectious oocysts, making it possible to measure the effects of treatment processes on *Cryptosporidium* infectivity.

Of the virus pathogens, adenovirus presents benefits as an indicator due to its prevalence and the relative simplicity of the analytical method, especially for PCR-based detection. However, its relatively large size (60 – 80 nm) means that it might not be a good indicator for processes that rely on size exclusion, such as filtration. In addition, adenovirus is not suitable as a representative virus for UV disinfection on account of its high UV resistance compared with other enteric viruses. Poliovirus has appropriate properties in terms of size and the ability to measure both presence and infectivity. However, with the live vaccine no longer used it can no longer be detected in wastewater and so can no longer be used as an indicator. Enteroviruses are in the correct size range but their presence is strongly seasonal, mostly in summer and autumn. As a result, this group of viruses is at low levels or is not detected in wastewater samples during the other seasons, meaning that enteroviruses can only be used as process indicators in particular seasons. Similarly, norovirus is mostly observed in winter and such a seasonal pattern prevents further use as an indicator. In the case of norovirus, there is no readily available infectivity assay, so it is only of use for validation of processes that use physical removal. Given the absence of a more suitable representative pathogen, adenovirus would be the best option as a process indicator using indigenous human pathogenic virus.

During project workshops somatic coliphages were raised as a possible virus surrogate. The method for somatic phage detection is technically simpler than for F-RNA. The hosts for somatic phage are easier to prepare and so the assay is less likely to fail, especially in the hands of a novice user. However, some somatic phage are very large. For example, T4 is a somatic phage, the head is about 100nm, tail an extra 300 nm, so they are potentially poor surrogates for filtration validation. Additional basic research would need to be done to identify the best candidate species of somatic coliphage. Reoviruses, which are mammalian viruses, could be potential candidates as surrogates for human enteric viruses, but further research would be required to evaluate this. F-RNA phage appear to me*et al*l the necessary requirements in terms of size, prevalence all year round and ability to measure presence and infectivity with reliable methods. A limitation of F-RNA is that their numbers can be low in some water types (eg. secondary effluent or lagoon effluent), but F-RNA such as MS2 can be readily produced by culture methods and spiked into test water for treatment performance validation trials.

The shortlist of representative pathogens and indicators for measuring wastewater treatment performance identified from the literature review is presented in Table 12.

Table 12. Summary of representative pathogens and indicators for wastewater.

Representative Pathogen	Indicator
Virus **By cell culture:** **adenovirus** **reovirus** **enterovirus** **rotavirus** **HAV** **By PCR:** **norovirus** **adenovirus** **enterovirus**	Virus By culture: F-specific coliphages By PCR: Polyoma viruses
Bacteria ***E. coli***	Bacteria Faecal streptococci / enterococci *E. coli* Total coliforms
Protozoa **Cryptosporidium**	Protozoa Sulphite-reducing clostridia

Methods Review

Shortly before the commencement of this project Keegan *et al*. (2012) reported method improvements for virus concentration and culture. In particular, direct precipitation using PEG was reported to give better virus recovery compared with ultrafiltration followed by PEG precipitation. This same study also identified cell lines that appeared to support better virus growth and resulted in more sensitive virus detection. Based on the available information, the focus of virus method improvement was to verify the reports of Keegan *et al*. (2012). In terms of *Cryptosporidium*, shortly before the commencement of this project some method improvements for concentration of *Cryptosporidium* from primary effluent were made available by the AWQC NATA accredited laboratory. This method is based on dilution of the primary effluent and concentration using calcium carbonate precipitation. The recovery data suggested that this method would be ideal for analysis of primary effluents. Since the calcium carbonate method is not in widespread use, the same approach (sample dilution followed by concentration) was applied to a filtration technique for *Cryptosporidium* oocyst concentration. Another major method improvement for *Cryptosporidium* was the publication of an integrated assay to provide oocyst counts and infectivity data. The new oocyst concentration method and integrated assay were selected for evaluation within this project.

Virus Methods

The comparison of the different cell lines for supporting virus infection was largely consistent with the findings of Keegan *et al*. (2012). The PLC cell line supported growth of the adenovirus and enterovirus strains/species tested (Figure 17). The BGM cell line supported enterovirus infection but was a poor host for adenoviruses, although low level of infection by adenovirus was detected.

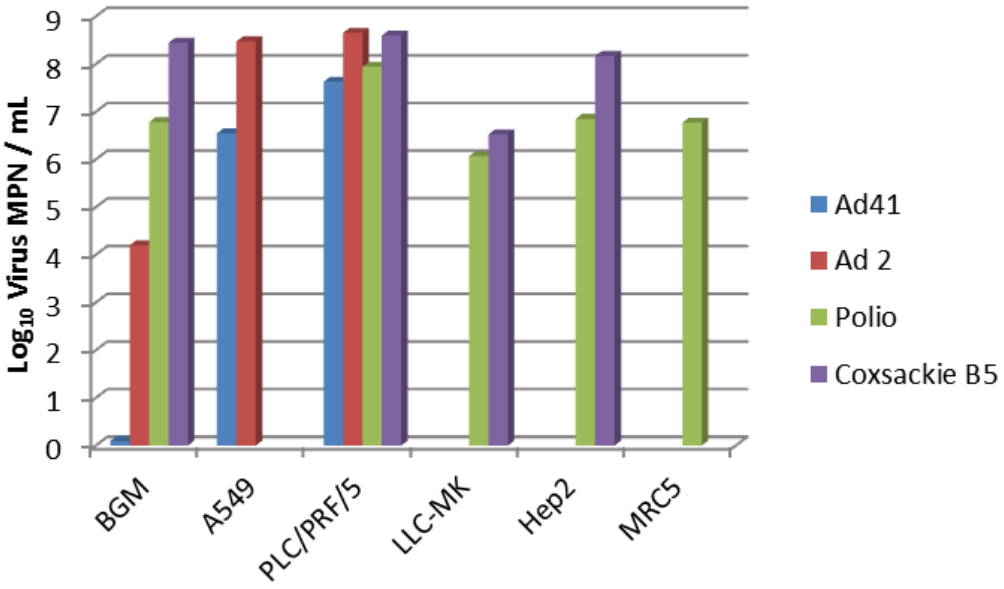

Figure 17. Comparison of virus counts (MPN / mL) for different viruses cultured with different host cell lines.

The addition of Ca2+ as an infectivity supplement had no benefit and was detrimental at higher doses, causing a dose-dependent reduction in infectivity, with the impact larger for adenovirus compared with coxsackie virus (Figure 18).

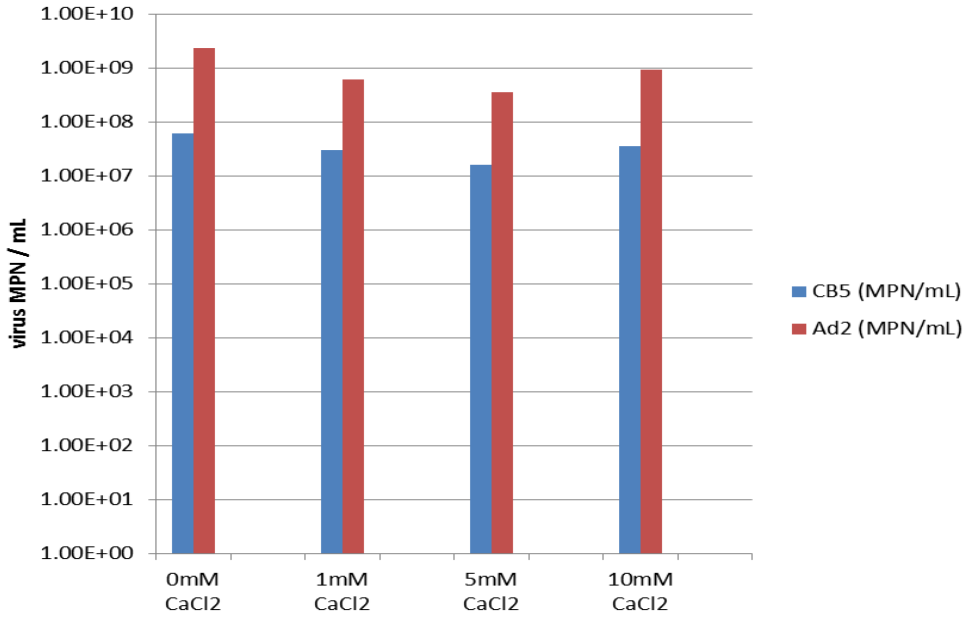

Figure 18. Comparison of virus counts (MPN / mL) for different concentrations of calcium chloride used as a supplement for cell culture.

Virus recoveries were inconsistent for both of the viruses used and for the different concentration techniques (direct PEG or filtration + PEG). The adenovirus results in particular may have been impacted by the presence of indigenous viruses (especially adenoviruses) and appeared to be worse for infectivity compared with PCR. The detection of indigenous viruses and apparent loss of the spiked Ad 2 could indicate a specific process impact affecting the Ad 2 but not the indigenous viruses. An example of the adenovirus recovery data (detection by PCR) is given in Figure 19.

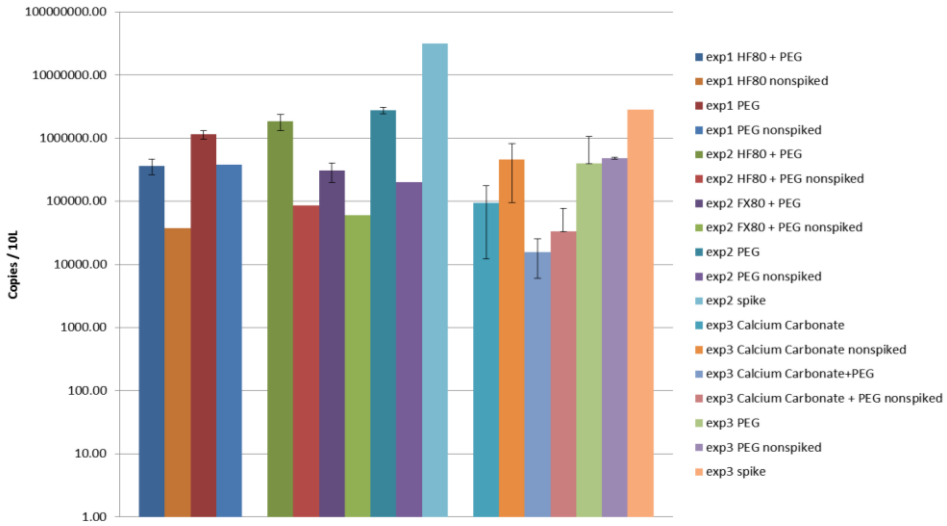

Figure 19. Comparison of Ad 2 numbers recovered by different concentration methods in spiked and un-spiked Bolivar secondary effluent (error bars indicate %CV).

The direct analysis of spiked samples (no virus concentration prior to cell culture or PCR) suggests that the Ad 2 were not impacted by the sample matrix and that the viruses were stable in the secondary effluent for up to 168 hours (Figure 20). This suggests that a step in the processing was responsible for the loss of the spiked virus (or loss of infectivity). A further consideration for interpreting the cell culture results is that the PLC cell line was used for all MPN analyses, this cell line supports a wide range of viruses and so detection in the un-spiked samples could be due to the presence of adenovirus or enterovirus. To differentiate this post-cell culture PCR analysis would be required to identify the virus causing the detected infection. The PCR analysis of the un-spiked sample concentrates suggested that indigenous adenovirus was more frequently detected, with indigenous enteroviruses detected by RT-PCR in only 1 batch of samples processed.

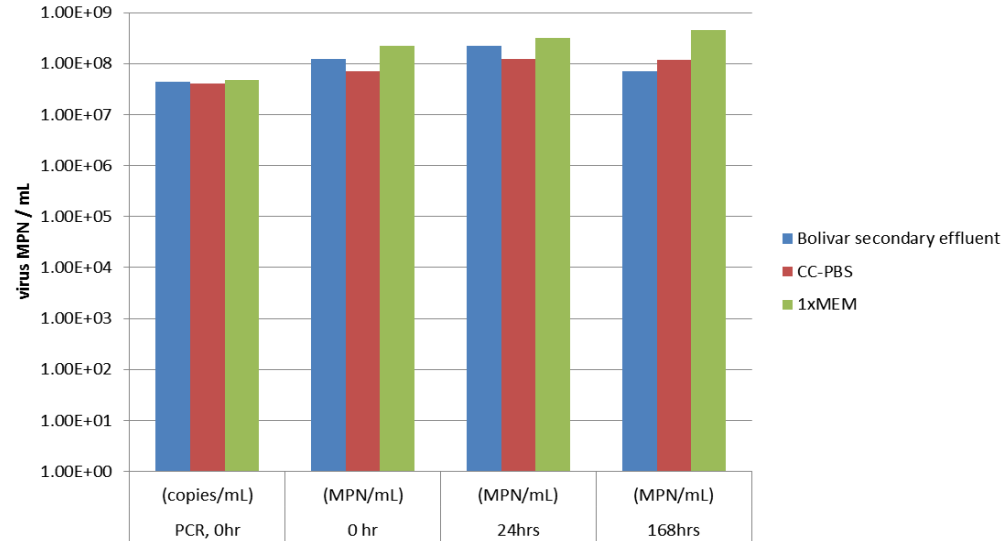

Figure 20. Comparison of virus numbers after spiking into secondary effluent, buffer or cell culture medium and storage at 4°C.

Overall, the direct PEG method gave better recoveries for both Ad 2 and CB5, although the recovery rates were not as good as that reported by Keegan *et al*. (2012) for samples collected from the same locations. The recovery rates for Ad 2 in primary effluent were better (although still variable) compared with secondary effluent, which is counter to what would normally be expected (based on previous results from Keegan *et al*. (2012) and earlier AWQC monitoring). It is possible that factors affecting the secondary effluent quality have greatly affected some of the testing conducted in this project. Future work should focus on evaluating the concentration methods using a wider range of primary and secondary effluents. The use of a surrogate such as suitably modified nanoparticles would greatly assist with method development, allowing the use of simpler enumeration methods and also eliminating any interferences from indigenous viruses, which can complicate enumeration.

Cryptosporidium methods

The comparison of methods for processing primary effluent found similar recovery rates for all 3 methods for the first 2 rounds of testing, though in the third round both direct centrifugation and filtration of diluted primary effluent appeared to perform significantly worse. The cause for this variation is unclear, but it is possible that this could coincide with a bad batch of IMS beads (that were recalled by the manufacturer after this work was completed). For secondary effluent, the calcium carbonate flocculation method was consistently better than Envirochek filtration. Although the recoveries were lower, the oocyst counts (which incorporate recovery rate and % sample processed) were generally higher for direct centrifugation and filtration compared with flocculation. The infectivity of the oocysts recovered by the different methods appeared to vary. In the case of primary effluent, the infectivity of oocysts recovered also appeared to be higher in the oocysts recovered by direct centrifugation and filtration, although in some cases the variation between replicates was large for some of the samples because of the small number of oocysts applied to cell culture. It is possible that the calcium carbonate, which has a higher recovery rate, is better at recovering all oocysts, whereas centrifugation or filtration may be selective for healthier oocysts. If this is the case, it could account for the higher infective fraction for these samples compared with calcium carbonate. Of interest, the infectivity of the oocysts in the primary and secondary effluent was different to that observed for Glenelg WWTP (Brendon King, pers. comm.), with higher infectivity observed in the Bolivar primary effluent and lower infectivity observed in the Bolivar secondary effluent. Given the relative simplicity of direct centrifugation, the method performance is adequate for oocyst concentration, but the inclusion of a recovery control is essential to identify any changes in recovery performance.

Inter-laboratory comparison

Due to time constraints, it was only possible to conduct a single round of inter-laboratory comparison for virus and *Cryptosporidium* analyses. The results for the primary effluent samples for both viruses (Figure 21) and *Cryptosporidium* (Figure 22) were comparable across the different laboratories, particularly for adenovirus detection by PCR. There was greater variation between the results from the different laboratories for secondary effluent, most likely due to differences in assay detection limit, low levels of virus present and also potentially due to differences in recovery rate. The latter is less likely considering that the participating laboratories used similar filtration methods for secondary effluent. The coxsackie CB5 spiked into some of the samples did not appear to persist and there also appeared to be enterovirus within the un-spiked samples at comparable levels to the CB5 used as a spike. These factors made calculation of a recovery rate difficult and it is unclear if the result was due to poor recovery of spiked virus or other factors affecting the stability or culturability of spiked CB5.

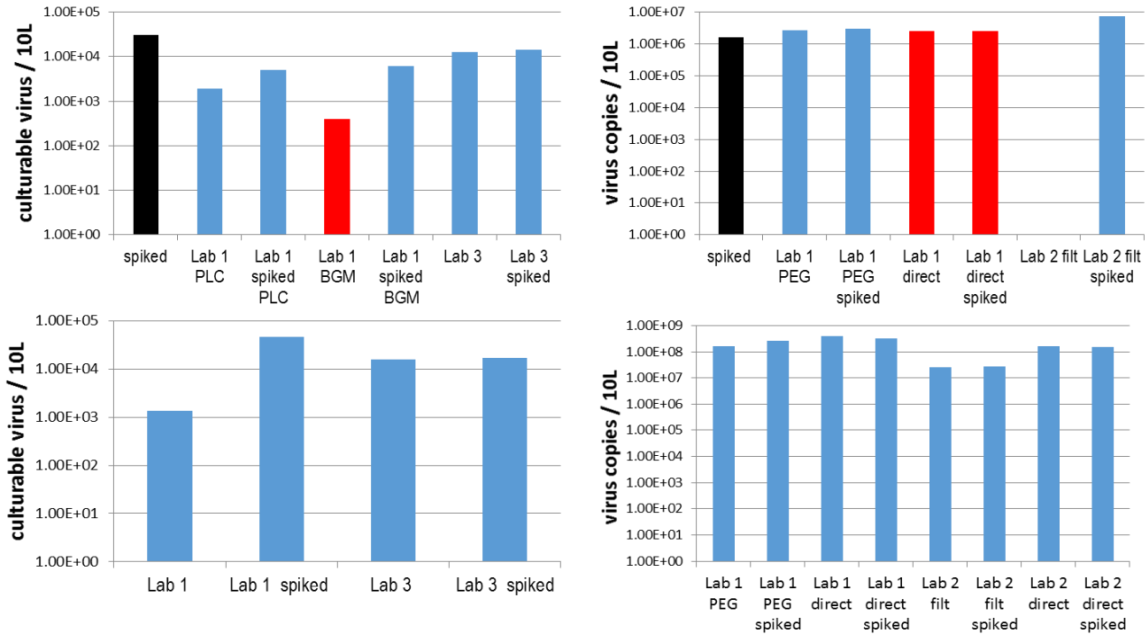

Figure 21. Summary of virus comparison data in primary effluent, for enterovirus by culture (top left panel) and PCR (top right panel) and for adenovirus by culture (bottom left panel) and PCR (bottom right panel). PLC indicates viruses detected by MPN culture on PLC/PRF/5 cells, BGM indicates viruses detected by plaque assay using BGM cells. The black column indicates the number of viruses / 10 L spiked into the sample, the red column indicates a below detection limit result.

For primary effluent, the number of culturable viruses versus virus genomes detected by PCR was 2 $\log_{10}$ lower for enterovirus and up to 4 $\log_{10}$ lower for adenovirus (Figure 21). A similar difference was observed for adenovirus but not for enterovirus in secondary effluent. The detection of indigenous adenovirus in the primary and secondary effluent samples used for the inter-laboratory comparison was similar to the results obtained during the initial method evaluation. In the earlier trials, PCR detected 10^4 – 10^5 adenovirus copies / 10 L and $<10^2$ – 10^3 culturable adenovirus / 10 L in un-spiked secondary effluent samples and 10^5 – 10^6 adenovirus copies / 10 L and 10^2 – 10^4 culturable adenovirus / 10 L in un-spiked primary effluent. Culturable enterovirus were not detected in the secondary effluent samples used for the initial method evaluation, although enterovirus were detected by RT-PCR (10^3-10^5 copies / 10 L), unlike in the case of the samples used for the inter-laboratory trial.

The PCR method for adenovirus appears to be suitable for directly measuring virus numbers without the need for sample concentration, provided the virus numbers are above 1 x 10^4 / 10 L. Compared with the direct detection of viruses, sample concentration appeared to result in the loss of 1 – 2 $\log_{10}$ of adenovirus. Direct RT-PCR detection of enterovirus was not successful and was only successful for primary effluent concentrates.

Based on the limited data available, direct PCR detection of adenoviruses (without any sample concentration) is recommended as a useful and cost effective option for measuring physical removal in both primary and secondary effluents. If sample concentration is required, then PEG precipitation of primary effluent samples allowed detection of both enterovirus and adenovirus for cell culture and RT-PCR. The results for secondary effluent concentration were equivocal for the comparison of PEG versus ultrafiltration + PEG, although both methods appeared to provide better performance than ultrafiltration + molecular weight cut-off filters. A key consideration for future method development is improvement of assay sensitivity, which at the moment can only be achieved by analysing a larger proportion of the sample concentrate, which adds to the assay cost.

While the recovery rates for *Cryptosporidium* in primary effluent were similar between Lab1 and Lab3 methods, there was a large difference in recovery rate for secondary effluent, with the filtration-based method (Lab3) achieving less than 5% recovery rate (Figure 22). No oocysts were detected in the Lab1 samples but small numbers (1 – 4 oocysts) were detected in the Lab3 samples. Allowing for the low recovery rate, this resulted in high apparent counts for oocysts in primary effluent (50 – 100 oocysts / 10 L), similar in magnitude to the oocyst numbers in primary effluent.

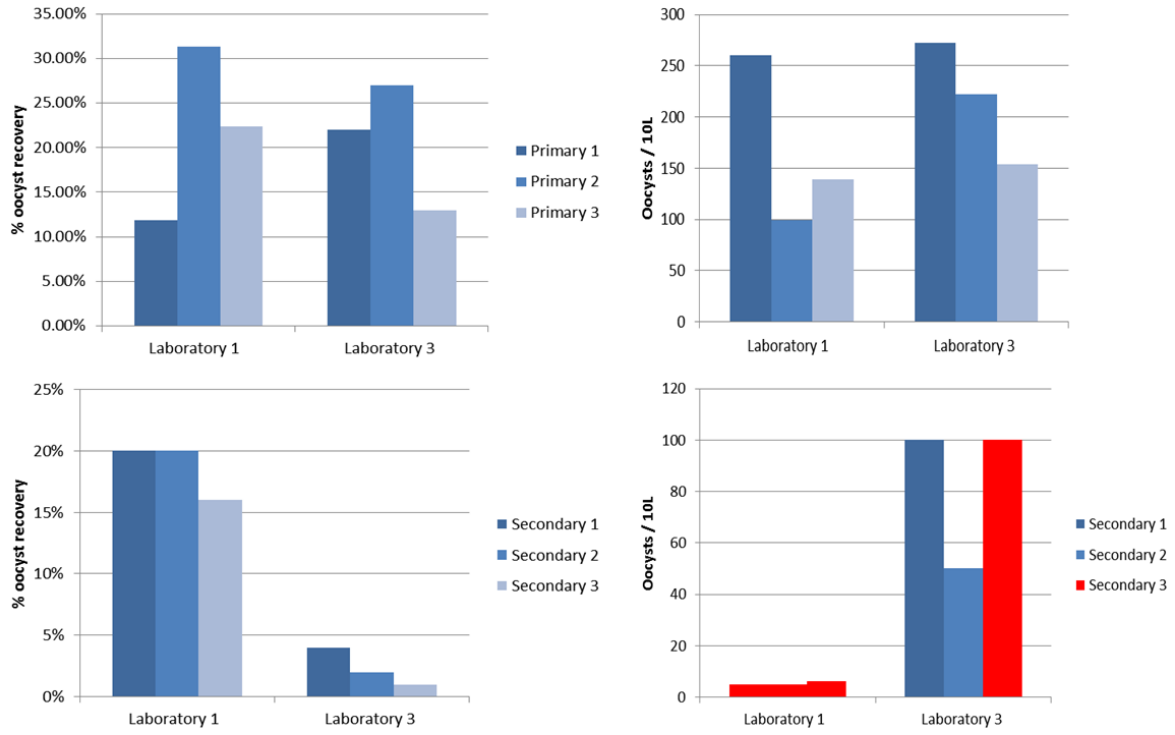

Figure 22. Summary of *Cryptosporidium* comparison data for primary effluent recovery rates (top left panel), primary effluent oocyst counts (top right panel), secondary effluent recovery rates (bottom left panel) and secondary effluent counts (bottom right panel). The solid bars in red represent results below detection limit.

Recommendations

The literature review was used to recommend surrogates or pathogens for use in treatment validation studies in other sub-projects of NatVal2. An obvious next step is to ground truth those selections by conducting comparative trials of the relevant pathogen-surrogate pairs. Such data may already be available from other sub-projects within the NatVal2 project and should be able to inform the design of any future surrogate validation studies.

The virus method development work that was conducted in this project suggested that some cell lines were better for detection of enterovirus and adenovirus compared with others in current use. However, not all of the cell lines behaved as anticipated with real wastewater sample concentrates. Future work should compare the performance of the different cell lines for virus detection using real wastewater samples. Many of these cell lines can host multiple virus species, so any such study would need to incorporate PCR to allow for detection of specific viruses such as enterovirus or adenovirus. A cell culture / MPN assay using PCR as the virus detection endpoint is a potential assay format for such a study to more rigorously field test the cell lines used for virus culture.

The *Cryptosporidium* method comparison suggested that the calcium carbonate method combined with IMS gave the highest recovery rates for secondary effluent, and this was confirmed in the inter-laboratory comparison. This method also performed consistently well for primary effluent samples. It appeared that the infectivity of the oocysts recovered by the calcium carbonate method was lower than that of oocysts recovered by other methods. The reasons for this need to be investigated in future work. One possibility is that the calcium carbonate method is better at recovery of both live and dead oocysts, whereas the other methods might have poorer recoveries because they selectively recover live oocysts, resulting in a higher infectious fraction in those concentrates. Spiking trials using fresh oocysts in primary and secondary effluent would address this question.

The inter-laboratory comparison of methods yielded very promising results. Future work needs to be done using the same and different wastewater locations to determine how reproducible and robust the methods are. Prior to any further studies the baseline numbers of virus need to be determined for each matrix so that appropriate spike levels can be used to allow determination of recovery rates. In addition, virus stability needs to be determined for each matrix. Stability experiments with adenovirus suggested that Ad 2 was stable when spiked into Bolivar secondary effluent, yet these spiked viruses could not be efficiently recovered using the PEG of filtration concentration methods. The possibility of matrix interference needs to be investigated – is there something in the Bolivar wastewater that is particularly challenging for virus recovery or is this a general issue for wastewater?

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