Australian Water Recycling
Centre of Excellence

Project Report

Validation of maturation ponds in order to enhance safe and economical water recycling

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Australian Water Recycling Centre of Excellence

Griffith University, October 2015

Validation of maturation ponds in order to enhance safe and economical water recycling

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Validation of maturation ponds in order to enhance safe and economical water recycling

Applied Project

Final Report
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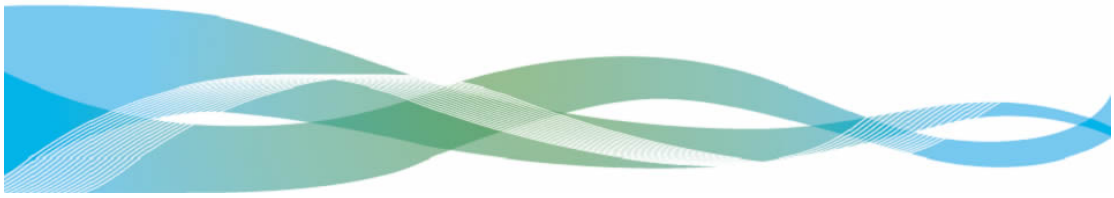

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EXECUTIVE SUMMARY

The overall aim of this research project is to provide regulators, decision makers, water managers and operators with information and tools to best operate and manage maturation ponds to protect public and environmental health and optimise uses of the treated water.

This project was funded by the Australian Water Recycling Centre of Excellence (AWRCE) and contributes to a broader research program on maturation ponds funded by the Queensland Science Funding Support (QSFS). The two projects are linked and the QSFS project will scope the levels of ecological and human health risks associated with a range of potential exposure scenarios associated with defined re-use options for the treated pond water, using data sourced from the AWRCE study.

The AWRCE project considered five ponds located in different geographical locations. Three ponds were located in Southeast Queensland (*i.e.,* Helidon, Forest Hill and Laidley) all operated by Queensland Urban Utilities and two in the Northern Territory (*i.e.,* Maningrida and Ngukurr), both operated by the Power and Water Corporation (PWC). Sampling was performed at each of these ponds, but to varying intensities for a number of reasons. Microcosm studies were also carried out using water from the Leanyer Sanderson pond (Northern Territory). Helidon was the most suitable for intensive ongoing investigation, due to its observed disinfection performance (necessary for model validation), proximity to the research team, and logistical support from the pond operators. Ngukurr was studied intensively for a limited duration (one week), due to logistical constraints related to its remote location and limited support for ongoing monitoring. The remaining ponds were excluded after preliminary assessments because of their design and operational issues. The research team will carry out further analysis of an alternative pond (proposed to be in Tamworth, New South Wales) as part of the QSFS project.

Four main categories of pathogens, bacteria, viruses, protozoan parasites, and helminths, may occur in faecally-contaminated wastewater (NRMMC *et al*., 2006). The Australian Guidelines for Water Recycling (AGWR, 2006) recommend characterisation of reference pathogens, which are representative of their respective microbial group (*i.e.,* bacteria, viruses and protozoa). The choice of reference pathogens should present a worst-case combination of factors such as: high concentrations, low removal during treatment, high pathogenicity and long survival in the environment.

Based on the review of literature and the findings of this study, the recommended target organisms for maturation ponds are:

- Bacteria – *E. coli* or enterococci
- Virus – Adenovirus
- Protozoa – both *Giardia* and *Cryptosporidium*

The AGWR validation guidelines recommend that ideally coliphage be measured as a surrogate for enteric viruses, however our findings indicated that MS2 phage (a commonly used coliphage) were extremely sensitive to light compared to the more resistant adenovirus (Mattle *et al*., 2015) and therefore may not be a suitable surrogate. It was also found that adenovirus was present and persisted when MS2 phage was not detected.

Microbial data have been collected from the Helidon pond since September 2013. Overall, there were 78 sampling events for *E. coli*, enterococci and *Campylobacter* and lesser numbers for Adenovirus (10) *Cryptosporidium* (13) and *Giardia* (13).

Analysis of the data has revealed that Helidon maturation pond, which is a baffled pond, can typically remove 2.00 log units of *E. coli* (*i.e.,* 99% removal rate) and 1.80 log units of enterococci (*i.e.,* 98% removal rate). Enterococci removal has shown a wider range of variation than *E. coli* removal, such that enterococci removal can be as high as 3.77 log units compared with 3.14 log units for *E. coli*. Human pathogenic *Campylobacters* were not detected. Preliminary results for *Giardia* in the Helidon pond indicated a 2.50 mean log removal. *Cryptosporidium* was only detected at very low levels in comparison to *Giardia*, and therefore *Giardia* was considered a better indicator of protozoa removal for this pond.

Microbial data were collected from one of the five ponds at the Ngukurr treatment plant in June 2014. Overall there were 5 sampling events for *E. coli*, enterococci and *Salmonella*, and a single sampling event for Adenovirus (1) *Cryptosporidium* (1) and *Giardia* (1).

Analysis based on Ngukurr data showed that there is 0.5 log units removal of *E. coli* (*i.e.,* 70% removal rate) or 0.5 log units of enterococci (*i.e.,* 69% removal rate). Ngukurr operational monitoring data (19 monthly data) obtained from PWC has shown 2.6 log units removal of *E. coli* for the whole treatment pond system (five ponds in series).

The log-removal efficiency for faecal indicators at Helidon pond was observed to be significantly higher than that for the Ngukurr pond. It is noted that for both Helidon and Ngukurr maturation ponds, the enterococci removal rate is slightly lower than the *E. coli* removal rate. This is likely to be due to gram-negative bacteria like *E. coli*, being more sensitive to direct sunlight and endogenous inactivation than gram-positive bacteria like enterococci, which are more sensitive to exogenous photo-oxidation.

The data were also used to calculate decay rate coefficients for the various pathogens and surrogates. Decay rate coefficients for *E. coli* obtained from microcosm studies were found to be similar at Helidon (Queensland) pond and Leanyer Sanderson (Northern Territory), in the range of 0.4 to 0.5 log units. This was also similar to the MIKE 3 Ecolab default value.

An assessment of the chemicals in the pond effluent showed that pond treatment removes limited amounts of emerging organic contaminants. It is considered that the small population size at Helidon contributes only low input levels of most compounds. Interestingly, all high-risk contaminants identified exiting the ponds are known to exhibit estrogenic properties. However, concentrations were generally below limits set out in the Standards for quality of recycled water supplied to augment a supply of drinking water (Queensland Government, 2015). No similar criteria exist for protection of aquatic wildlife or ecosystems, and the risk prioritisation flagged 9 compounds above levels expected to cause adverse biological outcomes in aquatic animals based on published “Predicted No-Effect Concentrations”.

The performance of the Helidon pond was modelled using the software package MIKE 3 (Danish Hydraulic Institute - DHI). This software uses the Finite Difference Method (FDM) to solve the governing equations subjected to boundary and initial conditions in the spatial and temporal domains. The modelling activities included four major components: 1) collecting modelling input information; 2) setting up and calibration of the model; 3) validation; and then 4) running various scenarios to evaluate pond dynamics and performance under different conditions. The MIKE 3 model has been found to be capable of simulating overall pond hydrodynamics as well as pathogen removal (represented by *E. coli* removal in this project).

The important findings of the study were:

1 From microcosm studies, light was found to be the most important factor in decay processes, with predation having only a minor impact;
2 There was efficient removal of *E. coli* at the Helidon pond, which typically has a 14-day detention time, but less removal at the Ngukurr pond which has only a 3-day detention time. The removal efficiency is impacted by the fact that Helidon is a baffled pond and Ngukurr is not and by their different sizes (length and width) and loading conditions;
3 *E. coli* was found to exhibit two phase decay kinetics, based on the microcosm studies. The implications of this on pond performance and the modelling require further investigation;
4 Decay rate coefficients for enterococci were higher than for *E. coli* based on the microcosm studies. This indicates that log removal should be higher in the pond for enterococci than for *E. coli*. However, observed removal was higher for *E. coli* than for enterococci in the Helidon pond. This may be due to lack of clarity of the water (inhibiting sunlight penetration) causing the suppression of exogenous photo-oxidation in the studied pond which was reported to be critical for inactivation of enterococci.
5 Quantitative Polymerase Chain Reaction (qPCR), while cost and time effective, was found to be useful for confirmation of detection of bacterial pathogens such as *Campylobacter jejuni* and *Salmonella enterica,* whereas culture-based tests could lead to false-positives results. qPCR was shown to be an alternative to culture-based method for enumeration of *E. coli* and enterococci in the pond. qPCR was used to quantify adenovirus concentrations in the influent of maturation ponds. However, for the effluent

samples the lower limit of quantification of 430 gene copies / 100 ml was found to be insufficient to report low viral concentrations that were equal to or below of this number.

6 An empirical rule of thumb exists that suggests a minimum of 20 sampling events with duplicate samples should be used. The statistical modelling undertaken as part of this project supports this rule of thumb - a minimum of 20 sampling events with duplicate samples is recommended. This modelling involved developing a hierarchical model to characterise the microbial concentrations based on validation monitoring sample data. The model characterises the relationships between the number of sampling events, the number of replicate samples (i.e., number of samples taken for each sampling event at a specific sampling point), and the level of precision that could be achieved (e.g., a confidence level) for microbial validation monitoring.

7 Numerical simulation results by MIKE 3 were found to effectively reflect observed performance at the Helidon pond for *E. coli* removal and provided a useful tool for investigating pond performance for different conditions. The numerical study has shown:

- Pond shape, more specifically the length-to-width (L/W) ratio, has a significant influence on pond treatment performance. Generally, the higher the *L/W* ratio, the better the pond performance.
- The optimal maturation pond depth is suggested to be between 0.8 m and 1.2 m.
- Ponds with small L/W ratios (< 5) would likely benefit significantly from the addition of well-designed baffles.
- The importance of inlet and outlet structures to maturation pond hydrodynamic and treatment efficiency is not as significant compared with that of the pond depth or pond length-to-width ratio.
- For un-baffled ponds, parallel wind (in relation to the length of the pond) encourages short-circuiting in the pond, whereas orthogonal wind reduces short circuiting and enhances circulation and vertical mixing. Oblique wind also poses positive effects on pond performance. Therefore, it is highly beneficial if ponds are oriented orthogonal to prevailing wind directions.

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Glossary

AGWR	Australian Guidelines for Water Recycling
AWRCE	Australian Water Recycling Centre of Excellence
CFD	computational fluid dynamics
CFU	colony forming units
DHI	Danish Hydraulic Institute
DO	Dissolved oxygen
EC	electrical conductivity
EDC	Endocrine Disrupting Compound
FDM	Finite Difference Method
HRT	Hydraulic Retention Time
LRV	Log reduction value
MIKE 3	MIKE 3 Hydrodynamic Flow Model
NATA	National Association of Testing Authorities
NOM	Natural organic matter
pH	A numeric scale ranging from 0-14 to specify acidity (0) or alkalinity (14)
PNEC	Predicted No-Effect Concentration
PPCP	Pharmaceutical and Personal Care Product
PWC	Power and Water Corporation, Northern Territory
QMRA	Quantitative microbial risk assessment
qPCR	quantitative Polymerase Chain Reaction
QSFS	Queensland Science Funding Support
QUU	Queensland Urban Utilities
SPE	Solid phase extraction
SS	Suspended solids
STP	Sewage Treatment Plant
TDS	Total dissolved solids
TrOC	Trace organic contaminant
TSS	Total suspended solids
UV	Ultraviolet (light / radiation)
WET	Whole effluent testing
WSP	Waste Stabilisation Pond
WWTP	Wastewater Treatment Plant

1 Introduction

The overall aim of this research program is to provide regulators, decision makers, water managers and operators with information and tools to best operate and manage maturation ponds to protect public and environmental health and optimise uses of the treated water. This project was funded by the Australian Water Recycling Centre of Excellence (AWRCE) and contributes to a research program on Maturation Ponds (Technology, Treatment and Training) which has been funded by the Queensland Science Funding Support (QSFS).

Specific research objectives addressed in this project include:

- To elucidate key factors and mechanisms involved in pathogen die-off within treatment ponds;
- To characterise fate and effects of chemicals of concern in wastewater treatment ponds;
- To prepare a rational, knowledge-based, validated model to predict disinfection and treatment capability of different pond designs using principles from microbial ecology;
- To characterise variability in log removal of specific micro-organisms; and
- To develop a methodology for validation of pond treatment efficacy.

The focus of the project is on pathogen removal and removal of chemicals of concern, and not treatment efficiency related to organics, suspended solids or nutrient removal.

Further work will be carried out as part of the QSFS project to scope the levels of ecological and human health risks associated with a range of potential exposure scenarios associated with defined re-use options for the treated pond water.

2 Background

In a treatment system, maturation ponds (normally 0.9 to 1.5 m deep) are primarily used for pathogen removal. Maturation ponds usually follow facultative ponds, which are deeper ponds (normally 1.5 to 2 m deep) that utilise both anaerobic and aerobic processes.

Maturation ponds/lagoons are used worldwide as secondary or tertiary steps for wastewater treatment, and in some cases, as the only treatment. In Australia, hundreds of municipal treatment systems rely on pond technology (facultative and maturation ponds) as a disinfection process, particularly in smaller towns and rural areas. Larger treatment plants also sometimes rely on maturation ponds for additional disinfection (polishing) purposes. An audit of sewage treatment plants in Australia conducted as part of the QSFS project showed over 600 plants incorporating pond treatment as part or whole of the treatment process.

The treated wastewater is usually held in maturation ponds for several days to weeks before being recycled or released into the environment. To assess the treatment efficacy of these systems, most studies have previously relied on faecal indicator organisms to predict the presence and die-off patterns of human pathogens (Maïoga *et al*., 2009; Reinoso *et al*., 2011; Ansa *et al*., 2012). Through previous studies, ponds have been reported as being quite effective at removing pathogens (helminths, protozoa, bacteria and viruses), albeit with varying degrees of specificity (Mara, 2004; Shilton, 2005; Von Sperling, 2007). However, comprehensive assessment of operational ponds in Australia has not been carried out, and this represents an important gap in the literature.

Previous modelling efforts assessing the disinfection capacity of maturation ponds have been based on either measurements of indicator organisms, or at most a single nominal pathogen (Maïoga *et al*., 2009; Reinoso *et al*., 2011; Ansa *et al*., 2012), or theoretical die-off rates (von Sperling, 1999). Additionally, most attempts to model maturation ponds have not sought to marry hydraulic and physicochemical modelling with measurements of pathogens and disinfection efficacy (Nameche and Vasel, 1998; Wood *et al*., 1998; Esen and Al-Shayji, 1999; Medri and Medri, 2002; (Brissaud *et al*., 2003; Brissaud *et al*., 2005; Bracho *et al*., 2006). When they have, the applications have been limited (Sah *et al*., 2011; Alvarado *et al*., 2012).

As such, a need for an integrative framework for evaluating pond performance was identified. The overarching goal of the current project was to draw on different types of disciplinary knowledge (hydrodynamic, microbiological, ecotoxological and risk assessment) data, and modelling techniques to investigate maturation pond performance. This approach was widely supported by the water utilities, regulators and researchers consulted, since the information would add significantly to our understanding of the potential health risks associated with the reuse of pond-treated waste water, as well as possible adverse environmental impacts.

3 Work Carried Out

3.1 Fieldwork

This original AWRCE project proposed to intensively study a remote Northern Territory pond. This added to the QSFS project, which included two other ponds from different climatic zones.

Based on results obtained from an initial visit to the Northern Territory, the selected pond at Maningrida was found to be overloaded and unsuitable for further modelling and assessment. An alternative remote Northern Territory pond was selected at Ngukurr, sampling undertaken, results analysed and data used to carry out validation of the hydrodynamic model. Constraints imposed on the project in accessing and sampling this second site limited further sampling.

Three sites were investigated in South East Queensland, these being at Helidon, Laidley and Forest Hill. The Helidon pond was ultimately selected for detailed and ongoing studies. Sampling at Helidon was carried out over a 12-month period to cover all seasons.

For the third climatic zone, a site in Tamworth NSW has been selected and additional study work for this site will be carried out as part of the QSFS project.

3.1.1 Ngukurr Pond

The Ngukurr STP is operated by the Northern Territory Power and Water Corporation (PWC). It consists of a primary pond followed by four maturation ponds in series. The final maturation pond is an evaporation pond that empties in the dry season but discharges to a local billabong during the wet season. Sampling work and computer modelling has been carried out for the first maturation pond.

3.1.2 Helidon Pond

The Helidon WWTP is operated by Queensland Urban Utilities (QUU). It consists of an initial screen, a primary facultative pond and a maturation pond followed by two wetland cells and three parallel reed beds. The maturation pond is a baffled pond, approximately 60 m x 35 m, with a nominal 12-20 days retention time based on the average inflow and total pond volume. Average daily flow is 60 kL/d. Effluent from the reed beds is chlorinated and then pumped to a local farm dam where it is reused for agricultural irrigation. Sampling work and computer modelling has been carried out for the maturation pond.

3.2 Modelling

Numerical modelling using MIKE 3, developed by Danish Hydraulic Institute (DHI), was conducted as part of this project, with the aim of investigating pond hydrodynamics, as well as performance in terms of pathogen removal.

3.2.1 Modelling basics

The software package used was MIKE 3. This particular software was an attractive option because of the following features, which allow for the development of a generic three-dimensional waste stabilisation pond (WSP) model (Scientific Documentation, MIKE 3 Flow Model, Hydrodynamic Module, 2014):

1) applying a non-hydrostatic engine to simulate unsteady three-dimensional flows;

2) taking into account density variation, bathymetry and external forces such as wind and temperature; and
3) incorporating hydrodynamic, transport and ecological sub-modules to account for the physical, chemical and biological processes of WSPs.

The model uses the Finite Difference Method (FDM) to solve the 3D governing equations subjected to boundary and initial conditions in the spatial and temporal domains.

The input information includes:

a) Pond geometric information: including pond bathymetry data, pond geometry dimensions, any baffles and their locations;
b) Pond influence information: inflow rate, inflow velocity and inflow direction, influence temperature;
c) Pond inlet and outlet information: exact locations of the inlet and outlet (depth as well as horizontal locations in relation to the pond);
d) Environmental data: cloudiness, air temperature, wind direction and speed, humidity, precipitation, solar radiation;
e) Influence bacteria concentration; and
f) Bacteria die-off equations and the die-off rate coefficient.

The main outputs from the modelling are:

a) The hydrodynamic information - flow velocities, surface elevation;
b) Water temperature; and
c) Bacterial concentration at various locations in the pond, as well as at the outlet.

3.2.2 Modelling formulation

The mathematical formulations of the maturation pond modelling are expressed as (Scientific Documentation, MIKE 3 Flow Model, Hydrodynamic Module, 2012):

$$\frac{1}{\rho c_s^2}\frac{\partial P}{\partial t}+\frac{\partial u_j}{\partial x_j}=S \tag{1}$$

$$\frac{\partial u_i}{\partial t}+\frac{\partial\left(u_i u_j\right)}{\partial x_j}+2\Omega_{ij}u_j=-\frac{1}{\rho}\frac{\partial P}{\partial x_i}+g_i+\frac{\partial}{\partial x_j}\left(\nu_T\left(\frac{\partial u_i}{\partial x_j}+\frac{\partial u_j}{\partial x_i}\right)-\frac{2}{3}\delta_{ij}k\right)+u_i S \tag{2}$$

$$\frac{\partial C}{\partial t}+\frac{\partial}{\partial x}(Cu)+\frac{\partial}{\partial y}(Cv)+\frac{\partial}{\partial z}(Cw)=D_x\frac{\partial^2 C}{\partial x^2}+D_y\frac{\partial^2 C}{\partial y^2}+D_z\frac{\partial^2 C}{\partial z^2}-KC \tag{3}$$

Equation (1) is the mass equation, in which ρ is the density of the fluid; c_s is the speed of sound in water; u_j represent the velocity components u, v and w when j varies from 1 to 3; x_j denote the spatial variables x, y, z and t is the temporal variable; P is the fluid pressure; S refers to the source and sink terms.

Equation (2) is the Reynolds-averaged Navier-Stokes equations, in which Ω_{ij} represents the Coriolis tensor; g_i stands for the gravitational acceleration; ν_T denotes the turbulent eddy viscosity; δ_{ij} is the Kronecker's delta (δ_{ij} = 1 if $i = j$ and δ_{ij} = 0 if $i \neq j$) and k is the turbulent kinetic energy. In equations (1) and (2), subscript j is a summation index, meaning that j-subscripted terms are to be summed over when j varies from 1 to 3; subscript i is a free index, resulting in three equations in the x, y and z directions respectively when i runs from 1 to 3.

Equation (3) describes the transport of pollutant concentration and the pathogen die-off processes, in which $D_j = D_x$, D_y and D_z are the dispersion coefficients in the x, y and z directions. The very last term in Equation (3), KC, describes the die-off mechanism, in which K is the die-off rate coefficient. It is essentially a time-dependent variable and is written as:

$$K = K_0 \cdot k_I{}^{I} \cdot k_T{}^{(T-20)} \tag{4}$$

In Equation (4):

K_0 (/day): decay rate coefficient at 20°C, with a salinity of 0‰, and darkness;

k_I, k_T: light coefficient and temperature coefficient, respectively.

I and *T* denote, light intensity (Kw/m^2) and temperature (°C).

K_I quantifies the die-off of *E. coli* due to light intensity. It is a calibrated parameter. The light intensity was calculated at each model cell location in the pond at each model time-step based on the meteorological data and using Beer's law. The light attenuation was calculated by specifying a light extinction factor, which is a calibrated parameter based on data from field measurements. The temperature coefficient k_T is also a calibrated parameter in the model and the temperature of pond water is calculated using the heat exchange module in MIKE 3.

3.2.3 Modelling calibration and validation

Model calibration and validation was carried out for the Ngukurr and Helidon ponds. In each instance the models were calibrated and validated by:

- Empirical solutions for wind-driven circulation within a pond;
- Field collected temperature profile data;
- Dye tracking data; and
- *E. coli* concentration data.

An outline of the Helidon pond validation work is discussed following. Further information on the Ngukurr pond validation work can be found in the supporting document.

For the Helidon pond, the intensive field studies provided a relatively detailed dataset for modelling calibration and validation. Figure 3.1 shows a diagram of the layout of the Helidon pond, which is approximately 60m x 35m.

The MIKE 3 model and its associated ECOLAB toolbox have been proven to be capable of representing pond hydrodynamic properties in the pond, as well as bacterial inactivation occurring in the pond.

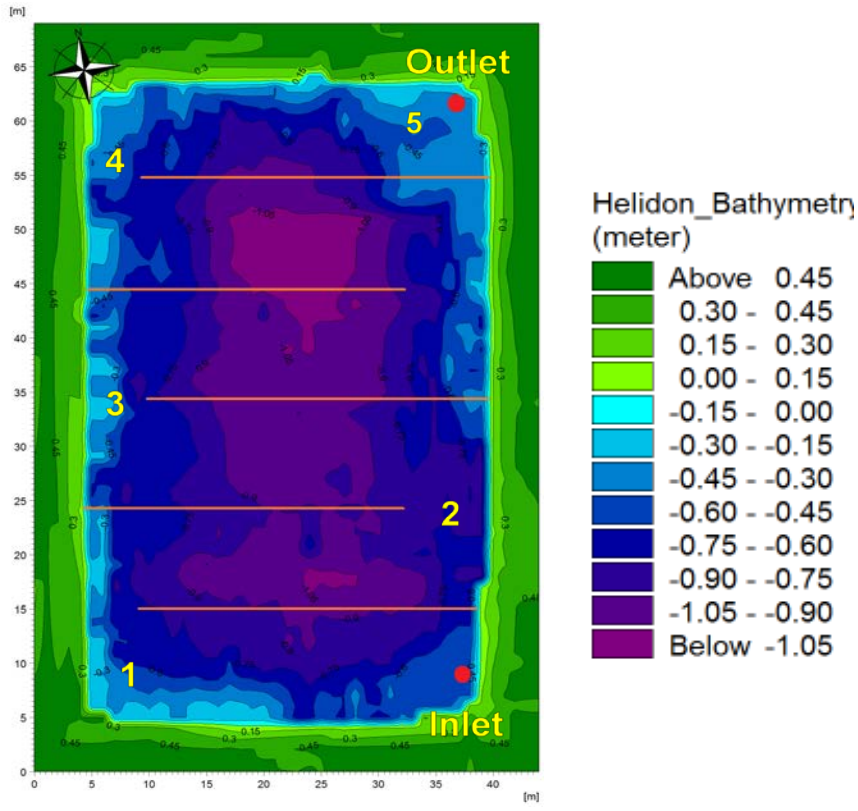

Figure 3.1: Diagram of Helidon maturation pond showing bathymetry, location of baffles (red horizontal lines) and five numbered sampling points.

Convergence test and modelling validation using a wind-driven set-up case

To examine the performance of the model in terms of its convergence behaviour and validity, the wind driven set-up in a pond was chosen as a benchmark case, as the solution of this problem is readily available. Winds with a constant velocity of 5 m/s were applied to the surface of a pond. In total, 9 different grid schemes with different mesh resolutions were tested, aimed at determining the optimal mesh resolution for both horizontal and vertical directions. The wind set-up Δh and corresponding relative error e_R in relation the empirical result were tested. A horizontal grid size of 1 m (in length) by 1 m (in width) with 7 layers (in the vertical direction) was suggested as the best mesh resolution. The flow velocity component in the x direction (pond central section in the longitudinal direction) was also compared with empirical expressions. It was shown that results from MIKE 3 agreed well with the empirical formulations, demonstrating the credibility of the proposed model.

Validation using temperature profile data

A bathymetric survey of the Helidon pond (conducted by the research team using a specially developed survey system) conducted in 2013, found the water depth in the pond averaged 1 metre.

Thermistor chains, consisting of up to 5 equally spaced HOBO sensors (20 cm apart) were placed in the pond to record the temperature structure of the water column as a function of time and space. The chains were positioned in the pond on January 9, 2014.

Comparison between the measured and the MIKE 3 simulated temperature data at the water surface is shown in Figure 3.2. To quantify the accuracy of the modelling results, a relative difference between the modelled results and the field data is represented by the root mean square error, RMSE = 1.01°C, as opposed to an averaged value of the measured data which is 27.04 °C. This resulted in only 3.74% difference between the measured and the calculated temperature data. An R squared value of 0.87 was obtained, which again demonstrates a high level of accuracy of the numerical simulation.

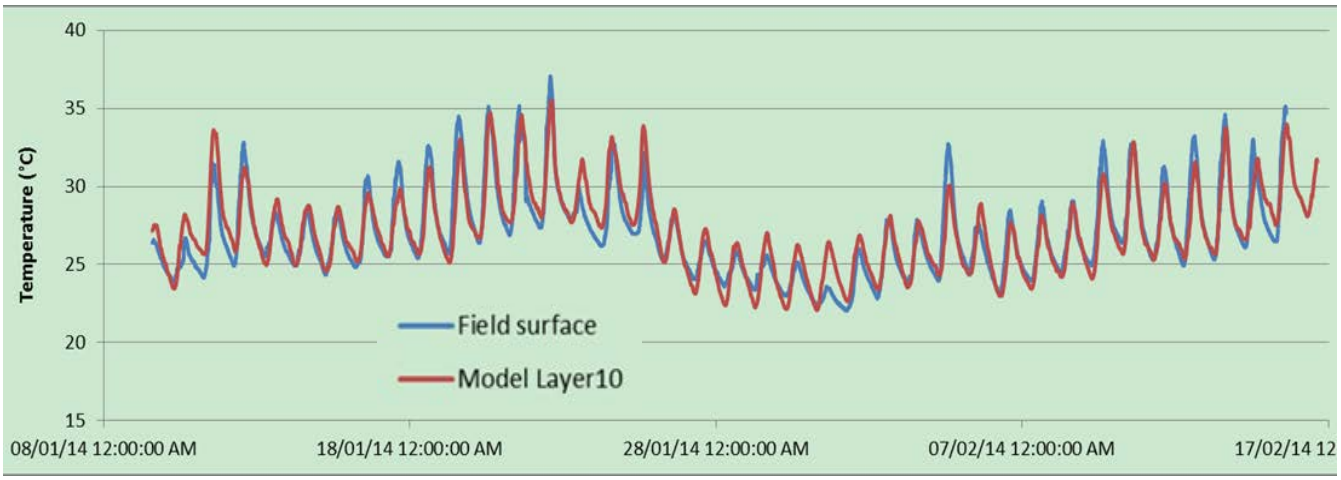

Figure 3.2: Comparison of temperature between measured and simulated results from Jan 09, 2014 to 16 Feb, 2014 at water surface.

A comparison between the modelled and the measured results along the water depth of stratified and de-stratified cases is demonstrated in Figure 3.3. The red stars in the plot denote measured data, the blue lines represent modelled results. These two figures confirm a high level of consistency between the modelled and the measured data.

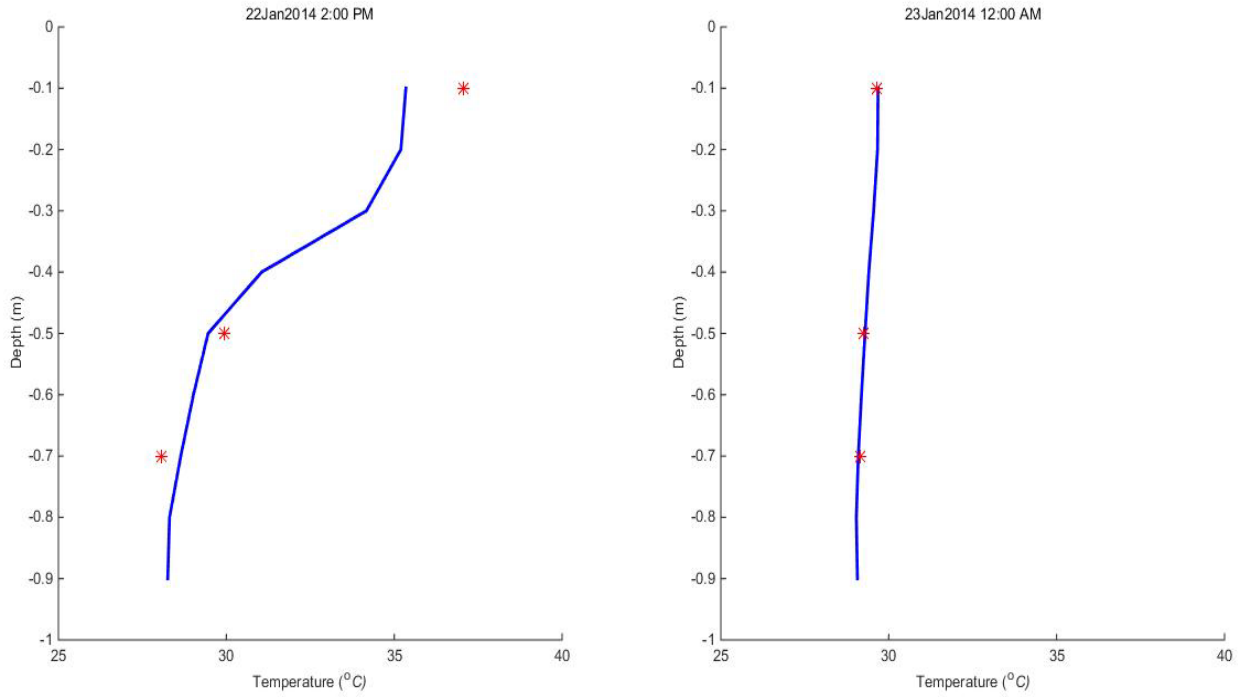

Figure 3.3: Temperature profile along water depth at Jan 22nd 2014 2:00 pm (Stratified) and 23 Jan 2014 12:00 am (non-stratified). The red stars represent measured temperature from HOBO temperature sensors; the blue lines represent the simulated results.

Validation using dye concentration data

Further validation of the model was conducted by comparing simulated concentration data with field-collected data. In the field, a dye solution was injected at the inlet to the Helidon pond. A small raft, carrying a submerged fluorometer and self-recording GPS unit, was used to measure the dye concentration distribution in the pond. The dye concentration is a function of the elapsed time since dye injection, and the location of the fluorometer in the pond at a particular time. Figure 3.4 presents an example of the data generated in a dye injection (tracer) study. The fluorometer was set at a depth of 44 cm with the boat track commencing at 3:57 pm. The colour on the boat track denotes the concentration level of dye at specific locations.

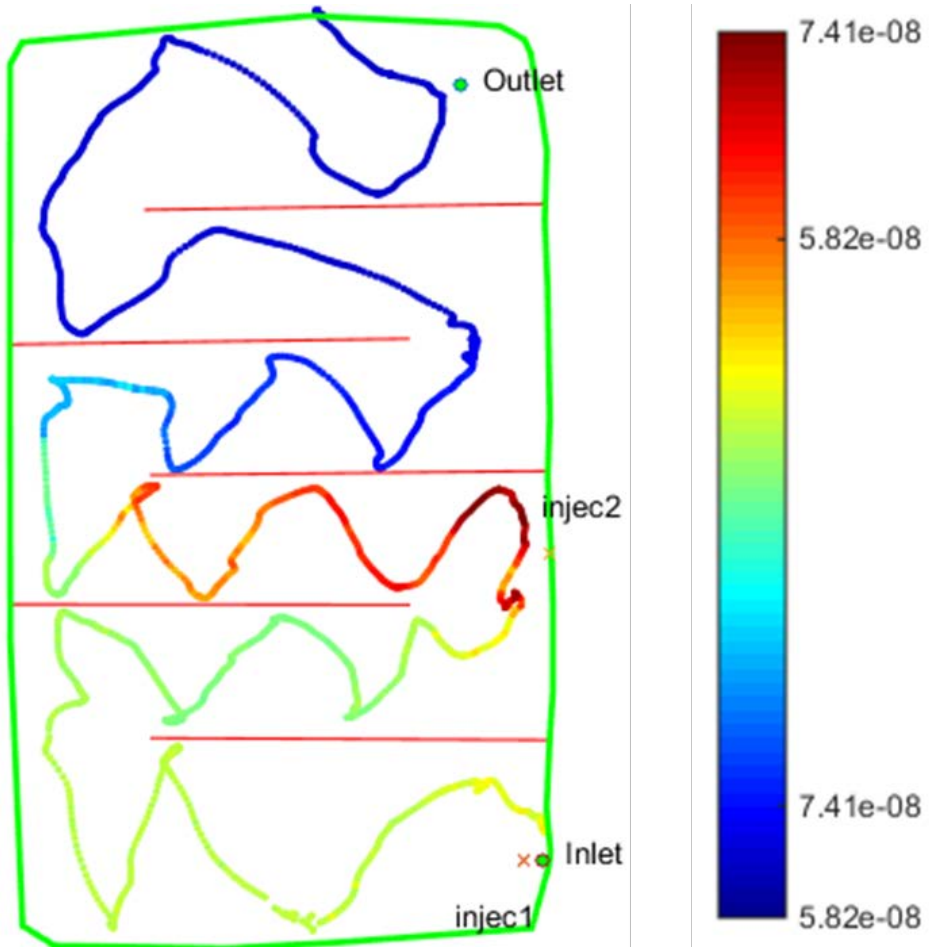

Figure 3.4: Dye concentration map at 03:57 pm from fluorometer at D = 44 cm below water surface, with 10 ml dye injection @ inlet at 9:20 am and 20 ml dye injection at location injec2 at 10:39 am, field measured data. The path is the track taken by the boat carrying the fluorometer.

For qualitative comparison, dye concentration data from both the field and the modelled results were normalised. Figure 3.5 shows a comparison between the recorded dye concentration in the field and the modelled results using a dimensionless dataset. For this case, 40 ml of dye was injected into the Helidon maturation pond at the inlet at 14:16 pm on 6 March 2015, and the concentration was mapped at 14:51-15:01 pm at a depth of 6 cm. The non-dimensional concentration was plotted against the modelled data in Figure 3.5. Due to the sensitivity issue when the fluorometer responses to the dye concentration in the field, the data seems scattered in Figure 3.5. However, the overall trend indicates a reasonably satisfactory level of correlation between the modelled and the captured field data.

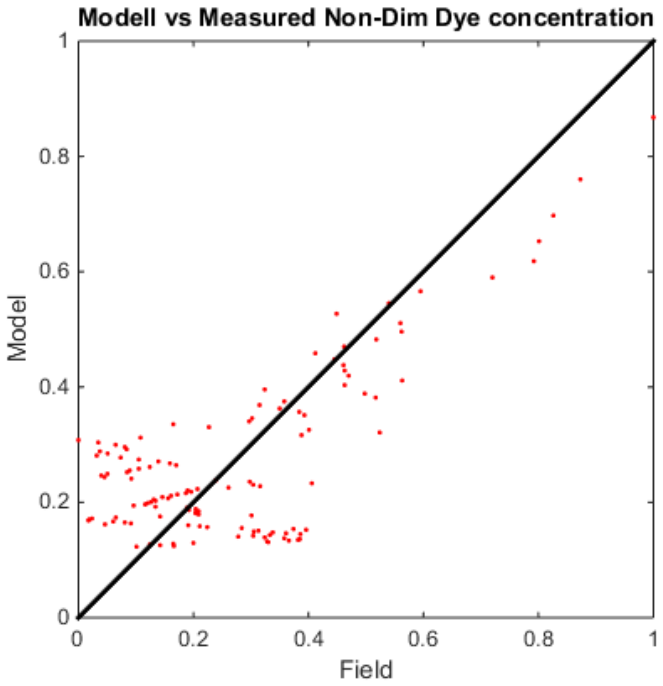

Figure 3.5: Comparison of non-dimensionalised dye concentration from modelled and field data at 14:51-15:01 pm at D = 6 cm below the water surface, with 40 ml dye injection @ inlet at 14:16 on 06 March 2015.

Validation using E. coli concentration data

Water sampling within the pond (including the inlet and outlet) provided *E. coli* concentration data for comparison with the modelling results. Figure 3.6 shows the *E. coli* die-off at various locations in the pond (see Figure 3.1 for locations). The blue diamonds represent field sampling data. The purple crosses show the modelling results using ECOLAB toolbox in which the die-off mechanism of *E. coli* is expressed using die-off equations related to temperature and light. Triangles denote the modelling results using an average die-off rate coefficient in the pond from the microbiological chamber study. Apart from only one sampling location, the modelling results and the simulated results match very well.

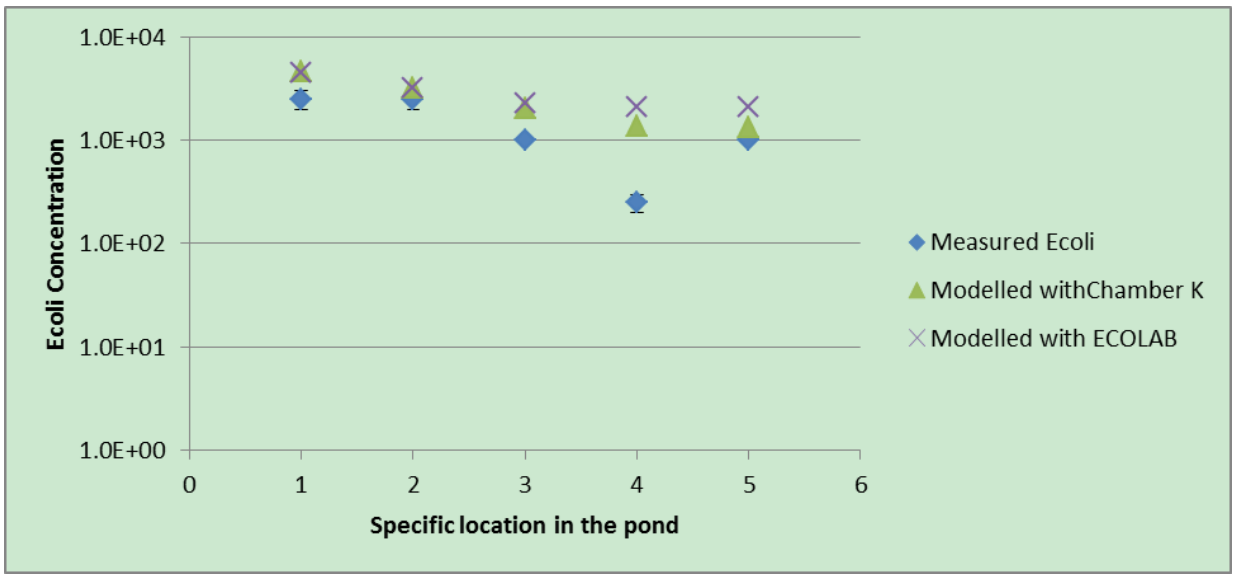

Figure 3.6: Comparison between measured and modelled *E. coli* concentration (CFU/100 ml) at Helidon maturation pond. See Figure 3.1 for location of sampling points. (Data taken from daytime sampling in the intensive sampling period 12-16 May, 2014).

Comparisons of *E. coli* concentration were also undertaken between modelling results and field sampling results at the outlet. Data were collected for the Helidon maturation pond from February to March, 2015. Water samples were collected using on-site refrigerated auto-samplers every eight hours for consecutive days, interrupted by a cyclone period from 19th Feb to 21st Feb, 2015. Figure 3.7 shows a comparison between modelled and measured *E. coli* results. The die-off coefficient was calculated using equation (4) in Section 3.2.2. It depends on pond water temperature and the light intensity at any location in the pond at any time. For the Helidon pond, the die-off rate coefficient is ranging from 0.32-1.06 per day. Comparison of the results shows a good correlation between the modelled and the (mean of the) measured *E. coli* levels - demonstrating the credibility of the model in predicting *E. coli* die-off. The range in fluctuations of the *E. coli* is not fully understood at this stage and certainly warrants further investigation, however the mean trends remain the same.

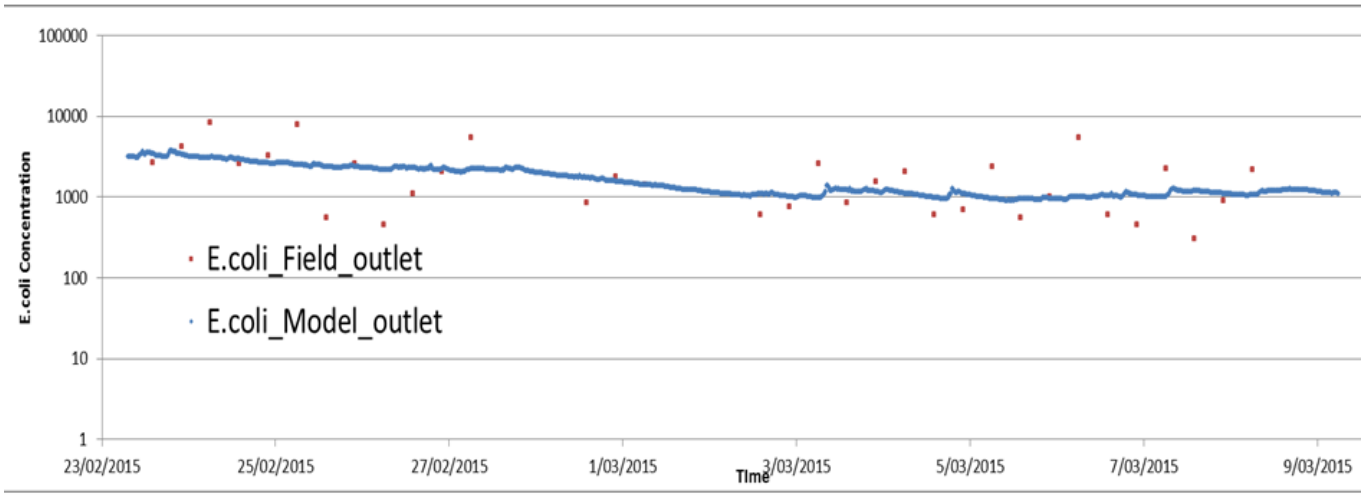

Figure 3.7: Comparison of *E. coli* concentration (CFU/100 ml) at the outlet between field data and modelled results (the red dots denote field *E. coli* concentration data at the outlet measured from 23rd Feb to 8th March 2015. The blue line represents modelling results).

3.2.4 Modelling results for retention time

Simulated tracer analysis was conducted using the model to examine the retention time distribution of the maturation pond. Retention time in the pond is mainly affected by the hydrodynamic regime in the pond, which is closely related to the inflow rate and meteorological conditions such as wind impacting upon pond hydrodynamics. As both the inflow rate and the meteorological conditions are constantly changing, the retention time will change according to the prevailing conditions.

The retention time for the Helidon pond was determined using the MIKE 3 model using five simulated tracers that were injected at the pond inlet. Under normal weather conditions at Helidon, it was found that it takes an average of 14 days from the time of injection at the inlet for the peak outflow to appear at the outlet. However, for an extreme event such as the cyclone that occurred over the period 18th Feb to 21st Feb, 2015, the retention time reduced to approximately 8 days. This reduction in detention time was due to the high inflows from the heavy rainfall leading to a significant inflow rates and subsequently a lower retention time.

The calculation of the residence time of the maturation pond is one of the major advantages of the MIKE 3 numerical model. Once calibrated and validated, the MIKE 3 model will allow rapid analysis of a range of conditions that would be difficult to simulate in the field. In addition, it avoids the labour intensive work required for the field experimentation. It is also noted that it is very difficult to derive a concentration–time curve from an experimental study, whilst it is readily extracted from the modelling results.

3.2.5 Transferability of modelling technique

The advantage of using a three-dimensional model is that it takes into account the variability of flow in both the vertical and horizontal planes. In a typical pond, the horizontal flow is rarely uniform, and in the vertical plane, stratification occurs. This stratification needs to be addressed to account for the effect of temperature and light on pathogen die-off.

The three dimensional model can be readily adapted to other sites. The following information is required:

- Pond bathymetry and geometry;
- Pond baffle arrangements;
- Pond inlet and outlet locations and depths;
- Pond daily flow and other pertinent operational details;
- Local meteorological conditions.

Numerically, a convergence test in terms of the number of elements or nodes used in the model should be conducted to ensure an efficient simulation. The grid resolution can be quite different for different cases. Experience from the current project suggests that for a pond site with a dimension of 60 m (length) by 30 m (width) by 1 m (depth), a horizontal grid size of 1 m (length) by 1 m (width) is sufficient and a grid size of 0.1 m in the depth direction is recommended. The chosen time step for MIKE 3 simulation is based on the knowledge of flow velocity and grid size to ensure the numerical stability of the simulation. The setting-up of the Helidon maturation pond model required a time interval of a maximum 5 seconds for the simulation. Any time interval greater than 5 seconds may have compromised numerical stability.

Pond performance can be modelled for any given time period, *i.e.,* yearly, seasonally, weekly or daily, depending on the requirement of pond operators and water authorities, as long as the datasets are sufficient for modelling input and validation purposes.

4 Validation of Pathogen Removal of Maturation Pond(s)

Waste stabilisation pond (WSP) systems are designed to treat wastewater. The primary function of maturation ponds within such systems is pathogen removal (Mara, 2004; Von Sperling, 2007; Maynard *et al*., 1999). The validation guidelines below were derived from the study of single maturation ponds within waste stabilisation pond systems in several locations across Australia. The guidelines are structured according to the template provided by the National Validation Protocol Development Group (an AWRCE managed and funded project). This chapter represents the initial phase of developing this guideline and requires further work and the input of regulators before the guideline can be finalised.

4.1 Mechanisms of pathogen removal and factors influencing pond performance

Pathogen log removals within WSPs vary considerably (Mara 2003). Similarly, the mechanisms of pathogen removal vary depending on the design, configuration, number and operation of ponds within a WSP system. Several studies have indicated a 1 to 6 log reduction in pathogen concentrations (*i.e.,* 90.0000 – 99.9999% reduction/removal; WHO, 2006; NRMMC *et al*., 2006) and others describe much narrower ranges of removal, such as the work of von Sperling (2007) where 3-6 log units were described for bacteria and between 2-4 log units for viruses. Relatively little information exists in describing the efficacy of pathogen removal from operational maturation ponds in Australia.

The removal or inactivation of pathogens in ponds involves complex interactions between numerous physical, chemical and bio-chemical processes that are controlled by a variety of factors. These include but are not limited to retention time, temperature, sunlight, dissolved

oxygen, pH, algal and microalgae populations, predation and starvation, and attachments to suspended particles (Pearson, 2003; Bolton *et al*., 2010). Davies-Colley (2005) provides a summary of the factors proposed to cause or influence pathogen reduction in WSPs, along with the likely mechanisms associated with each (Table 4.1). It is difficult to distinguish between the specific roles of these individual factors in pathogen reduction due to their complex interactions.

Table 4.1: Factors that have been proposed to cause or influence disinfection (pathogen removals) in WSPs (Source: Davies-Colley, 2005).

Factor where active	Likely mechanism(s)	Micro-organisms affected[1]	Ponds where active[2]
Temperature	Affects rates of removal processes	B, V, P, H	A, F, M
Hydraulic Retention Time	Affects extent of removal (time for operation)	B, V, P, H	A, F, M
Algal toxins	Algal exudates are toxic to certain bacteria	Mainly B	F, M
Sedimentation	Settlement of infectious agent (e.g, ova, cysts) OR settlement of aggregated solids including the infectious agent attached	H, P P, H, (B, V)	A, F, M A, F, M
'Biological disinfection'	Ingestion by higher organisms (protozoans)	B, V, P	F, M
Sunlight	DNA damage by solar UVB radiation and photo-oxidation (DO-sensitive) (range of wavelengths)	B[3], V, P B, P, V	F, M F, M

1. Microorganism: B – bacteria, V – viruses, P – protozoan parasites, H – helminth worms.
2. Ponds: A – anaerobic, F – facultative, M – maturation.
3. Most of the DNA damage to bacteria by UV-B radiation is repaired, and the lethal effect is related mainly to overwhelming of the repair capacity.

It is reported that sunlight-induced inactivation and algal activity are the main mechanisms driving the pathogen removal in both facultative and maturation ponds (Mara and Horan, 2003; Shilton, 2005a). Consistent with this, Pearson (2003) argues that light, pH and oxygen are important in controlling the removal of most faecal bacteria and at least some virus in ponds, more so than starvation and predation. Sedimentation is also mentioned as an important mechanism with regard to the removal of helminth eggs and protozoan cysts from WSPs (Reinoso *et al*., 2011). The sections below discuss sunlight induced inactivation, sedimentation and predation as three key mechanisms for pathogen removal in WSPs.

4.1.1 Sunlight induced inactivation

Ultraviolet (UV) light has been shown to inactivate 99% of all microorganisms. It has been demonstrated that about half of all faecal coliforms in WSP effluent can be damaged by UVB light (280-320 nm) (Sinton *et al*., 2002).

There are three main mechanisms of disinfection by sunlight exposure (Davies-Colley *et al*., 1999). The first involves direct damage to cellular DNA when UVB radiation is absorbed by the cell, inhibiting DNA replication and transcription (Krebs *et al*., 2014). This mechanism is independent of pH and dissolved oxygen (DO). The second mechanism involves photo-oxidation by reactive oxygen species (ROS), where endogenous photo-sensitisers absorb UVB radiation and damage the internal structures of microorganisms. This mechanism seems to be independent of pH (Davies-Colley *et al*., 1999). The third mechanism of disinfection by sunlight exposure also involves photo-oxidation by reactive oxygen species, but via exogenous photo-sensitisers (*e.g.,* humic acids) that absorb a wide range of wavelengths causing damage to external structures of

microorganisms. This mechanism is both DO and pH dependent (Curtis *et al*., 1992; Davies-Colley *et al*., 1999). A summary of these three mechanisms of sunlight-induced inactivation is presented in Table 4.2 below.

Table 4.2: Features of the three main mechanisms of sunlight inactivation causing pathogen removals in WSPs (source: Davis-Colley *et al*., 2000).

Mechanism	Contributing wavelengths (nm)	Absorbed by	Primary target	Oxygen dependence	pH dependence	Micro-organisms affected
Direct DNA/RNA damage	UV-B 280-320	Nucleic acids	Nucleic acids	No	No	bacteria, viruses, protozoa, cysts
Endogenous photo-oxidation damage (primarily to DNA/RNA, proteins)	UV-B +UV-A 280-400	Nucleic acids	Nucleic acids	Yes	No	bacteria
Exogenous photo-oxidatation (proteins, nucleic acids)	UV-B UV-A PAR 280-700	Natural organic matter (eg humics, acids, algae)	Cell membranes Capsid proteins	Yes	Some bacteria (incl. *E. coli*)	bacteria, viruses

Within a maturation pond, a range of factors influence sunlight-induced inactivation. The water is often characterised by high turbidity (*i.e.,* TSS up to 100 mg/L) and high concentrations of algae (*e.g.,* 500 - 2,000 µg/L chlorophyll *a*) (Mara, 2003). As a result, UV light only penetrates several centimetres into the water column due to high absorbance by the natural organic matter (NOM), algae and other particles present in wastewater (Curtis *et al*., 1992). Thus, the first direct mechanism of UV light on pathogen inactivation appears to be limited to the surface layer in highly turbid waters (as a rule of thumb, ≤5cm).

Although algae inhibit UV penetration through increasing the turbidity, they also raise the pH and DO levels, which contribute to the photo-oxidation-based mechanisms. In the case of exogenous photo-oxidation, NOM in pond water can absorb sunlight, and this also results in the production of ROS that inactivate both viruses and cellular microorganisms. Photo-oxidation also contributes to pathogen inactivation under dark conditions. In addition, these sunlight-induced inactivation mechanisms are influenced by the intensity and duration of sunlight exposure and impacts vary for different microorganisms (Davies-Colley *et al*., 1999).

The exact nature of sunlight damage to viruses is less clear than for bacteria. Virus inactivation by UVB damage is influenced not only by nucleic acid type (RNA or DNA), but also genome length and morphology. It is also well established that viruses exhibit high adsorption to suspended particles, which inhibits UV disinfection. The uncertainty around mechanisms of sunlight inactivation for viruses represents an important area requiring research, and this is particularly relevant with respect to maturation ponds. The role of direct and indirect UV damage to microbial cells involving ROS has been summarised by Silverman *et al* (2013) in Figure 4.1. Fisher and Nelson (2015) point out that it has been known for decades that Fenton-like reactions are important in *E. coli* photo-activation (Cooper, 1989). For a full explanation of how the Fenton-like reactions can work internal in *E. coli* the reader is referred to Fisher and Nelson (2015).

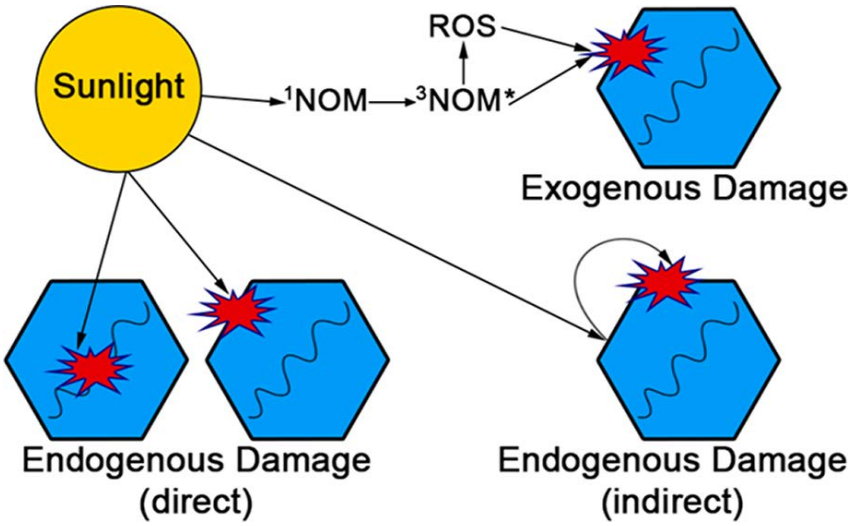

Figure 4.1: Schematic of the theory of direct and indirect microbial inactivation involving sunlight irradiation and reactive oxygen species (Silverman *et al.,* 2013).

Based on Tables 4.1 and 4.2 above, a few important conclusions can be drawn about the roles of some influencing factors played in the pathogen removal mechanisms.

- **Temperature:** Temperature is itself only lethal to micro-organisms (thermal shock) at high values above around 45°C, and therefore, temperature should be regarded as a secondary factor influencing the rate of action of primary factors (Davies-Colley, 2005).
- **Hydraulic Retention Time (HRT):** HRT controls the time available for primary inactivation or pathogen removal mechanisms to take effect within WSPs, and should thus be regarded, like temperature, as a secondary factor (Maynard *et al*., 1999). The highly inconsistent and sometimes poor removal of indicator bacteria in WSPs is partly due to short-circuiting (Mara, 2004), where influent wastewater is conveyed rapidly to the pond outlet with minimal time for 'primary' removal mechanisms such as sunlight, sedimentation, or predation to operate (Davies-Colley, 2005).
- **Oxygen**: Mechanisms 2 and 3 presented in Table 4.2 are both photo-oxidation reactions that rely on the presence of dissolved oxygen (DO) (Davis-Colley *et al*., 2000). In WSPs, DO is highly variable over time (with diurnal changes driven by algal metabolism) and space (with strong vertical gradients from the sunlit euphotic zone to the virtually dark hypolimnion) (Shilton, 2005a; Mara, 2004). Studies (*e.g.,* (Curtis *et al*., 1992) have shown that oxygen concentrations in the pond do not reach toxic concentrations which will kill pathogenic micro-organisms. It is the interaction of oxygen and sunlight produced radicals that are capable of damaging cell membranes and genetic material.
- **pH**: pH, like DO, varies in WSPs on a diurnal cycle (Shilton, 2005a). Experiments with exposure of faecal indicator bacteria (in the dark) to elevated pH have shown that pH alone is not toxic except at extreme low values not normally encountered in WSPs (Curtis *et al*., 1992). However, at a given level of sunlight exposure (Watts/m^2), faecal coliform die-off increases significantly with increasing pH above a threshold of 8.5 (Davis-Colley *et al*. 2000).
- **Natural Organic Matter (NOM):** Mechanism 3 presented in Table 4.2 involves photo-oxidation of external structures by photo-oxidising species produced in the external medium of the WSP water. Natural organic matter has two important considerations in WSPs: 1) reduces light absorption in WSPs; and 2) are an important photosensitiser for pathogen removal (Curtis *et al*., 1992), as detailed above.

4.1.2 Sedimentation

Sedimentation is an important mechanism for the removal of those pathogens which can either settle on their own accord or attach to settleable particles. The degree to which pathogens adsorb onto settleable particles is influenced by both environmental conditions and the surface

characteristics of the pathogens themselves through their influence on electrostatic, hydrophobic, and steric interactions (Templeton *et al*., 2008). This mechanism is considered to be important for both helminth eggs and protozoan cysts within WSPs, though recent studies have hypothesized that sunlight and physicochemical conditions of water could have a higher impact on cyst removal than previously thought (Reinoso *et al*., 2011). Removal rates of Ascaris eggs was demonstrated to be as high as 1 m per hour in a study by Ayres *et al*. (1992), however the eggs retained viability in sludge for up to a year. In comparison, *Cryptosporidium* oocyst and *Giardia* cyst settling velocities were reported to be approximately 0.25 m per hour by Robertson *et al*. (1999), although other studies found that the attachment of oocysts to suspended particles influenced actual velocities making them comparable with values for helminth eggs (Brooks *et al*., 2004). Such oocyst aggregates are strongly negatively charged at neutral pH (Ongerth and Pecoraro, 1996), and therefore do not adsorb well to soil particles or natural clays, and could travel free in water (Dai and Boll, 2003).

The degree to which viruses adsorb to settleable particles is particle-size dependent. Earlier work by LaBelle and Gerba, (1979) reported up to 99% adsorption to sediments for eukaryotic viruses such as poliovirus, coxsackevirus, echovirus and rotavirus, while Gantzer *et al*., (2001) reported variable adsorption values for bacteriophages ranging from 61 – 78 % However, more recent work in WSPs by Templeton (2008) and Symonds *et al*., (2014) focused more on the influence of particle size on virus adsorption to sediments. They showed that only a small proportion of viruses were associated with settleable particles (>180µm), whereas a higher proportion were associated with suspended particles (between 0.45 µm and 180 µm). These suspended particles include smaller particles, unicellular algae or bacterial flocs that will not settle.

The attachment of viruses to suspended particles may result in them being shielded from direct UV inactivation, slowing the disinfection process in maturation ponds (Symonds *et al*., 2014; Templeton *et al*., 2008). They conclude that sedimentation is likely not the primary mechanism for virus removal in maturation ponds.

4.1.3 Predation

Predation, as a mechanism of pathogen removal in WSPs, has received comparatively little attention in the literature and is limited primarily to the removal of bacteria and protozoans. For pathogenic bacteria, grazing by protozoa, bacteriophage infections, and predation by other bacteria may work to control their abundance in WSPs. Korajkic *et al*. (2013) suggests that competition between bacteria may have a greater influence on bacterial inactivation than predation.

Direct correlation between predation and temperature have been found in a variety of environments, with increased grazing rates on Gram-negative bacteria (e.g. *E. coli*) and lower rates on Gram-negative bacteria at high temperatures (Byappanahalli *et al*. 2012). Free-living ciliates and rotifers have been shown to ingest *Cryptosporidium parvum* oocysts in laboratory-based experiments, indicating that this may be an important removal mechanism in constructed wetlands or WSPs (Stott *et al*., 2001, 2003). It is not certain, however, whether ingested oocysts remained viable (Fayer *et al*., 2000). Predation and natural mortality were suggested as likely factors in the removal of parasites in a facultative pond (free living protozoa are obligate aerobes), whereas sunlight and physicochemical conditions were key factors in anaerobic and maturation ponds (Reinoso *et al*., 2011). The overall contribution of predation on pathogen removal in WSPs remains unclear.

4.2 Target pathogens

Four main categories of pathogens, bacteria, viruses, protozoan parasites, and helminths, may occur in faecally-contaminated wastewater (NRMMC *et al*., 2006). Table 4.3 summarises those pathogenic micro-organisms that may be present in sewage of faecal origin and the illnesses related to these pathogens. The determination of the target pathogens for measuring the maturation pond disinfection performance should best represent the removal of the pathogens listed in Table 4.3.

Table 4.3: Categories of pathogenic micro-organism that may be present in sewage and other organic wastes of faecal origin (adapted from Bitton, 2005).

Category of Pathogens	**Example organisms**	**Illness(es) caused**
Bacteria	*Salmonella* spp.	Typhoid fever, salmonellosis
	Shigella spp.	Bacterial dysentery
	Vibrio cholera	Cholera
	Escherichia coli (pathogenic strains)	Diarrhoea
	Campylobacter spp.	Campylobacteriosis
	Yersinia enterocolitica	Acute gastroenteritis
	Leptospira	Leptospirosis
Viruses	Poliovirus	Paralysis, meningitis
	Enteroviruses	Meningitis, respiratory infection
	Hepatitis A virus	Hepatitis
	Norwalk types virus	Gastroenteritis
	Rotavirus	Gastroenteritis and dysentery
	Adenoviruses	Respiratory diseases, Acute conjunctivitis
Protozoan parasites	*Giardia* spp.	Giardiasis
	Cryptosporidium spp.	Cryptosporidiosis
	Entamoeba spp.	Amoebic dysentery
Worm parasites (helminths)	Tapeworms (*e.g., Taenia* spp.)	Parasitism (range of symptoms)
	Roundworms (*e.g.,* Acaris spp.)	
	Trichuris trichiura	
	Hookworms (*e.g., Necator americanus*)	

The Australian Guidelines for Water Recycling (AGWR) (2006) recommend the characterisation of reference pathogens, which are representative of their respective microbial group (*i.e.,* bacteria, viruses and protozoa). The choice of reference pathogens should present a worst-case combination of factors such as: high concentrations, low removal during treatment, high pathogenicity and protracted survival in the environment.

Table 4.4: Reference pathogens and log reductions required for recycled water from treated sewage as prescribed in the 2006 Australian Guidelines for Water Recycling (AGWR).

Pathogen type	Reference pathogen	Log reduction required for irrigating food crops
Bacteria	*Campylobacter*	4.2-5.0
Viruses	Adenovirus	
	Rotavirus	5.3-6.1
Protozoa and helminths	*Cryptosporidium parvum*	4.0-4.8

(Source: Natural Resource Management Ministerial Council, 2006).

In recognition of the difficulty of measuring each of these, Table 5.2 in the AGWR – *Examples of validation monitoring for health risks* - states: '*at the very least should include E. coli, and ideally include coliphage and clostridial spores and may include some pathogens.',* and from Table 5.3: '*If microbial indicators are not present at high enough concentrations to reliably validate the log reductions, seeded organisms, such as F-RNA coliphages or Bacillus subtilis bacteria are used as seeds*.'

4.2.1 Bacteria

E. coli has been recommended as the best surrogate for assessing the treatment efficiency of maturation ponds for removing pathogens (Mara and Horan, 2003; Blumenthal *et al*.. 2000). *E. coli* is also the preferred indicator because measurement is affordable, fast, sensitive, specific and relatively easy to perform, but recent studies reported poor correlation between faecal indicator bacteria and enteric pathogens in water (Ahmed *et al*., 2012). In some studies, the bacterial pathogens *Salmonella* spp. and *Campylobacter jejuni* are completely removed at the primary stages of WSPs (Oragui *et al*., 1987; Mara, 2000), thus have a significantly higher die-off rate than *E.coli*. Therefore *E.coli* is a more conservative choice for the presence of faecal material which potentially may indicate human pathogens in maturation ponds.

4.2.2 Viruses

Adenovirus has been reported as constantly present in raw sewage in Australia (Mena and Gerba, 2009). Rotaviruses have a relatively high infectivity compared with other waterborne viruses and a dose-response model has been established based on human feeding experiment data while no such data are available for adenovirus. It was also found that prevalence of rotavirus and adenovirus in sewage could be similar. However, rotaviruses have historically been difficult to measure *in situ*. Therefore, this rationale is given in the AGWR (2006) for the recommendation that on-site adenovirus data are to be collected and then apply the rotavirus dose response relationship for health risk assessment.

Surrogates are important as most existing pathogen detection methods underestimate true abundance in the environment due to technical difficulties in efficiently recovering and detecting the microorganisms in such samples (Haas *et al*., 2014). Appropriate surrogates may therefore provide a robust indication of actual removal. The AGWR (2006) validation guidelines recommend that ideally coliphage be measured as a surrogate for enteric viruses. However, our findings indicated that MS2 phage (a commonly used coliphage) were extremely sensitive to light compared to the more resistant adenovirus (Mattle *et al*., 2015) and therefore may not be a suitable surrogate. It was also found that adenovirus was present and persisted when MS2 phage was not detected.

There are interesting recent developments with regards to the measurement of enteric viruses (using both qPCR and culture based methods). Potentially suitable surrogates such as pepper mild mottle virus (Symonds *et al.*, 2014; USEPA, 2012) have been identified as an alternative to coliphages, which have commonly been used as a surrogate to determine removal of enteric virus in wastewater treatment systems (Verbyla and Mihelcic, 2015).

4.2.3 Protozoan parasites and helminths

The removal mechanisms of pathogenic parasites in WSPs are very different to those for bacteria and virus removal. Therefore, the removal of parasites needs to be monitored separately (Mara and Bos, 2010).

The AGWR recommend both *Giardia lamblia* and *Cryptosporidium parvum* as the target pathogens for assessing the removal performance of the protozoan parasites and as the surrogate for assessing helminth removal in ponds. However, if only one can be measured the guidelines recommend *Cryptosporidium* due to higher resistance to treatment processes. Our findings suggest that *Giardia* may be more prevalent than *Cryptosporidium* in the maturation ponds investigated, therefore the absence of *Cryptosporidium* may not ensure the absence of *Giardia*.

4.2.4 Recommended Target Organisms for Maturation Pond Validation

- Bacteria – *E. coli* or enterococci
- Virus – Adenovirus
- Protozoa – both *Giardia* spp and *Cryptosporidium* spp

4.3 Operational monitoring parameters

Operational monitoring refers to the routine measurements performed to assess the continued operational efficiency or compliance testing of the maturation pond system, and is an important component of ensuring pathogen removal performance. Operational monitoring should be facilitated by simple measurements that can be taken easily and quickly. It provides advance warning that a system may be deteriorating to the point where effluent quality is no longer meeting the discharge criterion or the designed reuse requirement. Operational monitoring is often carried out to measure the performance of a pond or overall treatment system rather than for a single pond. Operational monitoring of pond systems should also take into account the end uses of the effluent. Typically, pond treatment systems would produce an effluent quality suitable for agricultural or pasture irrigation with some restrictions on the use where there is potential for public access to irrigation areas or where irrigation is used for growing food crops. For example, the median *E. coli* (cfu/100 mL) concentrations are required to be less than 1,000 for class C recycled water and less than 10,000 for class D recycled water according to Queensland Water Recycling Guidelines (2005). Class C recycled water is recommended for irrigation of crops such as sugar cane and grapes for wine production; or pasture/fodder for dairy animals with a withholding period of five days. Class D recycled water is recommended for irrigation of non-food crops such as turf, cotton, wholesale nurseries with controlled access to protect the health of workers. Details of the specific requirements in this regard can be found in State Codes and Guidelines and the Australian Guidelines for Water Recycling (2006). Minimum monitoring requirements will also be set by the State Environmental Protection Agency. Typically, the monitoring parameters for pond treatment systems would be:

- *E. coli*
- Suspended Solids (SS)
- Total Dissolved Solids (TDS)
- pH

E. coli, and SS are particularly relevant to measuring the pathogen removal performance. SS may be used as an indicator of treatment effectiveness as suspended solids contribute to poor water quality by providing a carrier for pollutants such as heavy metals and pathogens (Shilton, 2005b; WHO, 2006). Operational monitoring samples should be collected and tested on a weekly basis or as required for compliance by the Environmental Protection Agency.

4.4 Validation methodology

Validation monitoring is used to determine whether the maturation pond system in question is capable of adequately maintaining effluent water quality within the bounds required to achieve health and environmental target criteria as specified in the critical limits section. A full evaluation of the performance of a WSP system is a time-consuming and expensive process, and it requires experienced personnel to obtain and interpret the data. Readers are referred to Chapter 15 of Mara (2004), Chapter 10 of Frank *et al*. (2014), and Pearson *et al*. (1987) for better information regarding validation of WSP systems. The following bullet points are intended to provide specific recommendations for validation methodology for a maturation pond within an established WSP system.

- **Basic information** about the WSP system and the maturation pond should be collected and stored in a format suitable for subsequent analysis. The information which needs to be collected includes (but is not limited to):
  - any WSP design information in terms of flow rate
  - maturation pond length, width, and depth measurements and location of inlet and outlet pipes
  - types of sewage received by pond
  - treated effluent quality requirement and/or intended discharge destination or reuse option.

- **Actual measurement information:**
  - flow rate
  - maturation pond size and water volume
  - sludge depth and thus impact on hydraulic retention time
  - local temperature
  - rainfall, wind speed, wind direction, solar radiation
  - evaporation rate
  - any historical operational monitoring data of the WSP system.

- A correct **functional flow chart** for the full treatment chain of the WSP system should be established in which the role of the studied maturation pond is identified.

- **Water samples and flow rate measurements** should be taken over a one-week period for both the hottest and coldest periods of the year, presumably corresponding to the best and the worst pond performance seasons in Australia. In each of the two sampling weeks (*i.e.,* one sampling week for the hottest period and one sampling week for the coldest period), two days are intensive sampling days on which samples and flow rate measurement are taken at the frequency of once every eight hours (*i.e.,* three times a day). One of the two intensive sampling days should be either on Saturday or on Sunday and the second day should be at least two days apart. The rest of the five days are normal sampling days on which samples and flow rate measurements are taken once every day preferably in the morning to avoid sampling during the hottest time period of the day. In each sampling event, duplicate (preferably triplicate) water samples should be taken at both the inlet and outlet points of the monitoring maturation pond. This is the minimum requirement for capturing the possible diurnal, weekly, and seasonal variation patterns.

- The **Hydraulic Retention Time** (HRT) should be investigated to provide information for determining a reasonable flow regime for the subsequent analysis of pathogen removal. The theoretical HRT, which is the pond volume divided by the average flow rate, is only suitable for plug flow conditions. The actual HRT could be significantly shorter depending on the pond design and forcing conditions, and therefore it may be best assessed through tracer and/or appropriately calibrated numerical studies.

The above bullet point steps should provide a good starting point for the application of the advanced validation methods. It is the users' option to use more advanced technology such as

computational fluid dynamics (CFD) **simulation models** (Shilton and Harrison, 2003) which may be employed to assist in checking pond detention time or the effect of pond improvements. Water samples from different locations of the maturation pond may be required with the advanced CFD simulation model validation approach. More detailed requirements on data collection and analysis are provided in the following sections.

4.5 Methodology for collection and analysis of data

Samples can be collected by auto-samplers (if applicable), or manually (grab) in 500 mL sterilized sample bottles directly from the inlet pipe of the influent and near the entrance of the outlet pipe. Water samples should be collected at 10-20 cm depth below the water surface, but care needs to be taken to ensure the sludge at the bottom of the pond is not disturbed. Target pathogens or surrogates (or other site specific monitoring requirements) concentrations should be monitored for both inlet and outlet locations of the pond. SS, TDS, and pH may be monitored for the outlet samples only to assess treated water quality, but inlet measurements will also add valuable information about the overall treatment efficacy of the system.

A summary of the methodology employed for indicator and pathogen analyses is presented in Table 4.5. Full methodology is detailed in the Material and Methods subsection.

It is noted that qPCR has been nominated in Table 4.5 as an alternative method of measurement. qPCR is increasingly accepted as a quantitative method for microbial contaminants in water. This is based on numerous studies that have compared qPCR and culture based methods (Botes *et al*., 2013). It is recommended that, prior to using qPCR in preference to culture-based methods, the advice of state health regulators is sought. qPCR data may not be able to be used in QMRA as they do not indicate infectivity and may not be suitable for use in dose response models.

Table 4.5: Standard (traditional) and alternative (molecular) methods applied in evaluation of maturation pond performance in the current study.

Target microorganism	Traditional method	Molecular method
E. coli	Modified mTEC agar (BD)	*uid*A, qPCR (Frahm and Obst, 2003)
Enterococcus spp	mEI agar (BD)	23S rRNA, qPCR USEPA 1611
Salmonella enterica	XLD agar (Merck)	*ttrn*R, qPCR (Jakočiūnė *et al*., 2014) *gyr*B, qPCR (Leo *et al*., 2013)
Campylobacter jejuni	mCCDA agar (OXOID)	*map*A, qPCR (Inglis and Kalischuk, 2004) *VS*1, qPCR (Matthews, 2010)
adenovirus (all serotypes)	A549 cell line, MP568 in-house protocol at ALS	Hexon site, qPCR (Heim *et al*., 2003)
F+ RNA coliphage	Double agar plaque assay Single layer assay, APHA 2012	RT-qPCR, this study
Cryptosporidium parvus and C. hominis, Giardia lamblia	GC Combo (IDEXX) Microscopy USEPA 1623	Challenging due to low abundance of oocysts and cysts in water and detection limit of qPCR

4.6 Materials and methods

4.6.1 Sample collection

Physico-chemical characteristics (*i.e.,* temperature, dissolved oxygen, pH, conductivity and turbidity) should be measured using a multi-parameter probe (*e.g.,* YSI probe Hydrolab DS-5, Hach Environmental). Nutrients concentrations should be analysed in samples from inflow and outflow of the pond according to standard guideline methods (APHA, 1992). Samples for bacterial and phage analyses must be collected in sterile 500 ml Pyrex (polypropylene) bottles, and immediately transferred to the analytical laboratory on ice for processing (*i.e.,* within 8 hours of collection). Samples for detection of protozoa and adenovirus should be collected in separate 10 L polypropylene containers.

4.6.2 Bacterial isolation and enumeration

Most bacterial enumeration in water should utilise a NATA registered method. Generally, the accepted methods for total coliforms, *Escherichia coli*, enterococci, *Salmonella* spp. and *Campylobacter* spp., should be enumerated using membrane filtration techniques. Wastewater samples should be mixed thoroughly, serially diluted where applicable and passed through 0.45 µm sterile mixed cellulose ester membrane filters (Advantec, Japan) using a manifold filtration unit (*e.g.,* CombiSart®; Sartorius, Gottingen, Germany). The following methods are suggestions that may be used. National Association of Testing Authorities (NATA) accredited methods must be used where available.

- **For enumeration of *Escherichia coli*,** filters are placed on modified mTEC agar plates (BD, Sparks, AR, USA) and incubated at 35°C for 2 hours followed by an additional incubation at 44.5°C for 24 hours. Single magenta colonies are then quantified and reported as *E. coli* numbers.
- **For enumeration of enterococci**, filters are transferred onto mEI agar plates (BD) supplemented with 0.024% (w/v) of nalidixic acid (Sigma-Aldrich, St Louis, MO, USA) and plates incubated at 41.5°C for 24 hours. Blue colonies are regarded as enterococci.
- **Enumeration of total coliforms** can be performed by transferring filters onto coliform agar plates (*e.g.,* Chromocult®; Merck, Darmstadt, Germany) and incubated at 35°C for 24 hours. The sum of salmon to red colonies and dark blue colonies are reported as total coliforms.
- ***Salmonella* spp**. are best enumerated using the mini MPN technique (Fravalo *et al*., 2003), using Rappoport-Vasiliadis liquid media (Oxoid, Hampshire, UK).
- **For enumeration of *Campylobacter***, filters are placed on Campylobacter mCCDA agar (Oxoid, Hampshire, UK) with selective supplement SR0155 (Oxoid). Plates are incubated at 43°C for 48 hours under microaerofilic conditions using a Campygen system (Oxoid). All colonies are counted as thermophilic Campylobacters, which include human pathogens *C. jejuni* and *C. coli*.

4.6.3 F+ RNA coliphage enumeration

Male-specific (F+) RNA coliphages should be enumerated according to APHA 9224B double-agar-layer method APHA, 1992). Briefly, an overnight host strain *E. coli* (ATCC 700891) is grown for 4 hours and kept on ice to avoid losing pili. Tubes for assays should be filled with 3 mL of soft 0.7% trypton agar (BD) containing ampicillin and streptomycin and 100 µL of the host strain, and kept at 47°C in a water bath. To this mixture, 1 mL of wastewater sample is added. The contents of the tube should be mixed gently and poured onto plates with 1% (w/v) trypton agar supplemented with antibiotics. After the top agar layer solidifies, plates are incubated at 36°C overnight. Plaque counts are reported as PFU/100 mL. A diluted stock of MS2 phage should be used as control.

4.6.4 Viral concentration with PEG

Viral particles in 1L wastewater samples should be precipitated in 8% PEG6000 with 1% Tween 20 (Sigma-Aldrich) and 0.5% 1 M $CaCl_2$. After an overnight incubation at 4°C, samples are centrifuged at 10,000 g for 30 min at 4°C. Pellets are washed with 5 mL of PBS and eluted for an hour with periodic vortexing. The final 10 mL is extracted with an equal volume of chloroform and stored at -

80^{o} C. Two hundred microliters of the eluted sample is used for DNA extraction (*e.g.,* using a DNeasy Blood and Tissue Kit; Qiagen).

4.6.5 *Cryptosporidium* and *Giardia* enumeration

Cryptosporidium oocyst and *Giardia* cyst enumeration is a NATA registered assay and a NATA approved laboratory should be used where possible. The USEPA method 1623 is usually the method of choice that is NATA approved, however variations on this method can be accepted. A suggested method that closely follows the USEPA 1623 method follows.

A vial of ColorSeed (BTF, Sydney, Australia) is stained with TexasRed and used as an internal control by spiking into 1L of sample. Samples are then filtered through Filta-Max membranes units (IDEXX, Westbrook, ME, USA) according to the manufacture guidelines. Routine elution on Filta-Max manual wash station (IDEXX) and immune magnetic separation (IMS) concentration steps are to be conducted according to the manufacture protocols using Dynabeads® GC Combo Kit (IDEXX) which contained monoclonal antibodies specifically binding to oocysts. The final 50 µL of IMS concentrate is fixed on a slide, stained with DAPI and EasyStain (BTF) containing FITC-labelled antibodies and visualised by epifluorescent microscopy (Nikon, Japan). Hypothetical counts of oocysts are calculated based on recoveries of ColorSeed (BTF).

4.6.6 The use of non-conventional molecular genetic techniques - quantitative Polymerase chain reaction (qPCR)

DNA extraction and recovery

Between 10 and 100 mL of wastewater are filtered onto 47 mm 0.45 µm mixed cellulose ester filters (Advantec, Japan) and stored at -20°C until DNA extraction. Filters are aseptically cut and placed into 2 mL microcentrifuge tubes for nucleic acid extraction steps following the manufacturer's instructions. Several nucleic acids extraction kits were tested through the project, including PowerWater DNA and PowerSoil RNA/DNA (MoBio, Carlsbad, CA, USA), FastDNA SPIN kit for Soil (MP Biomedicals, Santa Ana, CA), MasterPure Complete DNA and RNA (Epicentre, Madison, WI, USA), DNeasy Blood and Tissue Kit and QIAamp DNA stool mini kit (Qiagen, Dusseldorf, Germany). The FastDNA SPIN kit for Soil demonstrated the best recoveries values for enterococci and *E. coli* and these should be used to isolate genomic DNA from all environmental samples. An initial cell disruption step for this kit is conducted in a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK, USA) for 60 sec.

Preparation of stocks for qPCR calibration curves

E. coli (ATCC 15766)*, Enterococcus faecalis* (ATCC 19433) and *Salmonella enterica* serovar Tythimirium (isolated and identified in our laboratory for the project) are enriched overnight into Brain Heart Infusion (Oxoid) broth. *C. jejuni* (NCTC 11168) is enriched in Preston media containing defibrinated horse blood in Hungate tubes under microaerophilic conditions. Cells are then harvested by centrifugation at 3000 g for 10 min, washed with PBS and extracted with the FastDNA soil kit (MP Biomedicals). DNA concentrations are assessed by BioPhotometer plus (Eppendorf, Hamburg, Germany) with 1 mm Hellma TrayCell microcell (Hellma Analytics, Germany). Serial dilutions of DNA stocks re performed to obtain 10^5 to 10^1 genome copies.

Plasmid construct for adenovirus detection

A 130 bp PCR product of an Adenoviral DNA extract was ligated into pGEM® T-Easy Vector (Promega, Madison, USA) overnight at room temperature. Mix a 10 µL of ligated to transformed into competent XL1-blue cells on ice, incubate for 40 min in LB broth (BD, Sparks, AR, USA) and plated on LB agar with ampicillin containing X-gal and IPTG (Merck, Darmstadt), white colonies were picked up and screened with hexon gene primers. Colonies with plasmids of 130 bp insert are then enriched in LB broth (BD) and plasmid purified with QIAprep Spin miniprep kit (Qiagen). Purified stock of plasmid pAdV (3145 bp) is then used for generation of qPCR standard curves for adenovirus enumeration.

PCR primers and TaqMan probes

qPCR assays are used to enumerate the following organisms: *E. coli*, *E. faecalis*, *S. enterica*, *C. jejuni* and adenovirus. All primers and probes can selected from previously published data except for the probe for *gyr*B for *S. enterica* enumeration.

To design the probe for *S. enterica* the online PrimerQuest tool available at IDT web-site (http://www.idtdna.com/primerquest/home/index) can be used. The specificity newly designed probes need to be verified by performing a BLAST search of Genbank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers and probes can be purchased from Macrogen (Seoul, Korea) or from IDT (San Diego, CA). Internal DNA amplification controls in qPCR must be used to ensure that a detection of DNA in the sample is real and not a result of a false positive or false negative. An example that may be employed is the - phagemid pM13mp18 (Usachev and Agranovski, 2012).

4.6.7 Details of the reaction mix and cycle of amplification for Real-time qPCR

As an example of the protocol for a real-time PCR reaction, the following may be used. A 25 µL of qPCR assay contains: 12.5 µL of 2× GoTaq Probe qPCR Master Mix (Promega), forward, reverse primers and hybridization probe for target gene and 0.4 µM of forward, 0.4 µM of reverse primer and 0.2 µM of probe for internal amplification control (IAC), 10^3 copies of IAC, DNAse-RNAse free water, and 5 µL of DNA template. Real-time PCR reactions may be executed in CFX96 thermal cycler (Bio-Rad, Hercules, CA) (any real-time capable thermal cycler can be used).

Temperature profile

- initial polymerase activation at 95°C for 10 min
- followed by 40 cycles at 95°C for 10 s, 60°C for 1 min
- For every analysis, new standard curve can be produced from serial dilutions 10^5 to 10^1 gene copies in triplicate reactions. Efficiency of calibration curves should be within 90-105%
- Reactions for unknown samples should be run in duplicates.

Confirmation of Campylobacter jejuni colonies using qPCR

Grey colonies from mCCDA agar plate can tested for the identification of *C. jejuni* using the following procedure. DNA is extracted according protocol described previously (Denis *et al*., 1999) and 5 µL of template added in molecular assay that is specific for *C. jejuni* (Yang *et al*., 2003). DNA extracts of the colonies, which provide positive signal in qPCR is regarded as *C. jejuni.* DNA extract from *C. jejuni* (NCTC 11168) may be used as positive control.

4.6.8 Presentation of data from real-time qPCR

All data testing results (whether based on the water samples collected for validation monitoring or for operational monitoring) should include all information regarding the water sample collection and be recorded in a format that is suitable for data analysis. All relevant information should be kept permanently for future reference.

For example, *E. coli* concentration should be expressed as number of colony-forming units (cfu) per 100 mL or number of genome copies per 100 mL; coliphage concentration should be expressed as number of plaque-forming units (pfu) per 100 mL or number of genome copies per 100 mL; SS measurement units are in mg per litre; TDS in mg per litre or EC (electrical conductivity) in µS per cm. Based on all the collected data, median values should be calculated for *E. coli* and coliphage concentrations, and SS and TDS measurements; the maximum and minimum should be calculated for pH values. The resulting monitoring parameter values are compared against the critical limits as specified in the next section for decision making. *E. coli* and coliphage concentration data are used for determination of the log reduction value (LRV) and the methodology details are given in section 4.9.

4.7 Determination of Critical Limits

The Australian Guidelines for Water Recycling (AGWR, 2006) states that "For preventive measures identified as critical control points, critical limits (which can be quantitative or qualitative)

must also be defined and validated. A critical limit is a prescribed tolerance that distinguishes acceptable from unacceptable performance. When a process that represents a critical control point is operating within critical limits, performance in terms of hazard removal is regarded as being acceptable. However, deviation from a critical limit represents loss of control of a process and indicates that there may be an unacceptable health or environmental risk. Corrective actions should be instituted immediately to resume control of the process, and the health or environmental regulator may need to be notified."

When intended for land applications (a major pond effluent reuse option), it is recommended that the target pathogen and operational monitoring parameter critical limits should be established according to end use, method of application of the effluent to land, control measures adopted for the effluent application and licencing requirements.

Further details of the specific requirements in regard to pathogen or surrogate critical limits can be found in State Codes and Guidelines and the Australian Guidelines for Water Recycling (AGWR, 2006). Minimum requirements may also be set by the State Environmental Protection Agency.

4.8 Methodology to determine LRVs

A simpler approach for calculating LRV is recommended by the AGWR (2006) using the following formula (NRMMC *et al*., 2006).

LRV = average ($\log_{10}$(inlet concentrations)) – average ($\log_{10}$(outlet concentrations)).

A more robust method of calculating the LRV is by the Monte Carlo simulation approach. For this method the inlet and outlet microbial concentrations in maturation ponds are characterised as a probabilistic distribution of all possible values. For example, the inlet concentration data may be best characterised by a log-normal distribution (Limpert *et al*., 2001) and the outlet concentration data is best fitted by another log-normal distribution. The resulting LRV is therefore expressed as a probabilistic distribution of all possible log reduction values. This analysis is best to carry out using a Monte Carlo simulation approach using computer software such as @Risk (Palisade Corporation, 2014). The mean LRV can be used to represent a typical condition or the lower 5th percentile to represent a worst-case scenario for any subsequent health risk assessment. The verified LRV is used as a measure of the target pathogen reduction efficacy of the maturation pond.

Note that the AGWR (2006) method yields a single value estimation for LRV. However, the AGWR (2006) method is much easier to implement. LRV results obtained from AGWR (2006) method are best used for a fast assessment of the pond pathogen removal performance. For the health risk assessment purposes, however, it is recommended to employ the Monte Carlo simulation approach for a more informative estimation of LRVs.

As a rule of thumb, 20 sampling events each with duplicate samples are required for a reliable estimation of LRV distribution. As pointed out in section 4.4, the data should be representative of all typical operational conditions such as low and high flow rate, varying wind direction (or worst case) and speed, and hot and cold seasons.

4.9 Re-validation

Re-evaluation or additional onsite validation testing is required if one of the following situations transpires: (1) operational monitoring data analysis indicates that a key parameter such as *E. coli* concentration has exceeded the critical limit, or a deviation is detected that is trending to exceed the limit based on a 12-month time series plot; or (2) there are design modifications or major operational procedural changes to the maturation pond.

In the event that situation 1 occurs, the responsible operator should first promptly notify the regulator. In-house process experts should be consulted in order to identify the cause(s) of the problem. An assessment of the risks to public health due to the quality of recycled pond effluent exceeding the critical limit should be carried out and actions should be taken accordingly. After the process or operational problem has been rectified, a one-week validation-monitoring program (details see section 4.4) should be performed to verify the rectification result. In the event that situation 2 occurs, full validation as described in section 4.4 should be implemented.

5 Log Removal of Pathogens in Helidon Maturation Pond

5.1 Number of samples

Microbial data have been collected from Helidon pond since September 2013. Table 5.1 provides a summary of the number of sampling events for each of the five selected indicator organisms. Prior to 28 January 2015, sampling events occurred roughly once every two weeks to once per month. Duplicate samples were taken for some of the sampling events up to January 2015 and most sampling events for the intensive sampling period from 28 January 2015 to 10 March 2015. For the intensive sampling period (28 January – 10 March, 2015) at Helidon there were 30 days on which samples for testing *E. coli* and *Campylobacter* were taken (three sampling events each day, *i.e.,* every 8 hours), and four sampling events for testing Adenovirus and five sampling events for *Giardia* were completed.

Table 5.1: Summary of sampling events in which indicator organism and reference pathogen were collected.

	E. coli	**enterococci**	***Campylobacter***	**Adenovirus**	***Giardia***
Number of sampling events before 28 Jan 2015	10	10	7	5	4
Number of sampling events during 28 Jan -10 Mar 2015	78	78	78	5	5

The research activities have identified certain data quality concerns regarding the *Campylobacter* testing results. In addition there are less than 20 data points available for Adenovirus and *Giardia* which has implications on the reliability of the LRV estimates. Consequently LRV estimates have only been provided for *E. coli* and enterococci data for the Helidon pond.

Pond performance could have a significant seasonal variation, so the extended period that data has been collected from Helidon pond should span this seasonal variation pattern (if any) and provide accurate estimates of the range of performance. Data collected before the intensive sampling period is somehow different from those collected during the period. For example, even though the number of samples taken during 28 January – 10 March, 2015 was several times more than those taken during the period September 2013 to January 2015, the latter data points are likely more representative of the seasonal variation patterns. For this reason, it was decided to randomly select 16 data points from those 78 points of *E. coli* and enterococci collected during the intensive sampling period and combine these with those 10 data points that were collected before January 2015 to form a single data set for calculating the LRVs. The numeric summary of these three data sets are displayed in Table 5.2.

Table 5.2: Numeric summaries of data sets used for calculation of LRVs.

cfu/100mL		**minimum**	**mean**	**median**	**maximum**
E. coli	Inlet (26 data points)	6,000	170,290	98,000	1,450,000
	Outlet (25 data points)	100	1,383	890	8,000
enterococci	Inlet (26 data points)	930	854,324	146,000	15,300,000
	Outlet (25 data points)	60	6,850	1,695	47,500

5.2 Method of assessment

Two methods have been applied for calculating the log reduction values (LRV):

- Method 1 follows the AGWR formula: LRV = average ($\log_{10}$ (inlet concentrations)) – average ($\log_{10}$ (outlet concentrations)); whereas
- Method 2 utilises Monte Carlo simulation to estimate the LRV which is a two-step process. This involves (1) fitting a hierarchical statistical probability distribution model to the inlet and outlet concentration data, respectively using the statistical software R (R Development Core Team, 2014); and (2) deriving the distribution of the differences between the $\log_{10}$ transformed inlet concentrations and the $\log_{10}$ transformed outlet concentrations by Monte Carlo simulation. The Monte Carlo simulation is performed using the risk assessment software @Risk by Palisade Corporation.

Essentially, Method 1 provides a geometric mean estimate for LRV. Method 1 is simple to apply but is unable to describe the variation in LRVs. In contrast, Method 2 estimates the LRVs through a probability distribution rather than a single summary statistic, *e.g.,* mean or median. Although Method 2 requires more effort in data analysis and is more demanding computationally, it provides more information on LRVs allowing us to characterize the pond performance using different assessment criteria. For example, we may use the mean LRV to represent a typical condition or the lower 5th percentile to represent a worst scenario in the subsequent health risk assessment analysis. Further benefit can be gained with Method 2 in that using the hierarchical model to characterise the microbial concentrations enables us to investigate the relative importance of the number of sampling events versus the number of replicate samples within each sampling event on the accuracy of the estimated LRVs.

5.3 Results

The estimated LRV results using both methods are presented in Table 5.3. The results show that *E. coli* and enterococci have similar LRVs, both showing about 2 log units reduction. *E. coli* has a slightly higher estimated mean LRV, 2.01 versus 1.76 by Method 2 (mean LRV 2.00 versus 1.80 by method 1). Enterococci has a relatively wider variation range (-0.43, 3.77) as compared to *E. coli* (0.83, 3.14). The AGWR recommended method (Method 1) appears to produce slightly higher LRV values than the Monte Carlo method (Method 2).

It is commonly accepted that WSPs can generally achieve 1 to 6 log reduction in pathogen concentrations (*i.e.,* 90 – 99.9999% reduction/removal) (WHO, 2006, NRMMC *et al*., 2006) for the overall treatment process. In this analysis, the Helidon pond has been verified as achieving 2 log units reduction for *E. coli* and 1.76 log units reduction for enterococci over an 18-month period. It is therefore concluded that Helidon maturation pond functions normally in removing faecal indicators.

Table 5.3: Summary of estimated LRVs for *E. coli* and enterococci based on data sets detailed in Table 5.2.

Distribution of estimated LRVs		**5th percentile**	**mean**	**median**	**95th percentile**
E. coli	Method 1	NA	2.00	NA	NA
	Method 2	0.83	2.01	2.02	3.14
enterococci	Method 1	NA	1.80	NA	NA
	Method 2	-0.43	1.76	1.82	3.77

NA=Not Applicable

5.4 Determination of Number of Sampling Events and Replicate Samples

For validation monitoring of pond performance, it is a recognised practice that microbial data (*e.g.,* inlet and outlet concentrations) utilise replicate samples taken over a range of operating conditions. The variations between data points (*e.g.,* the measurements of organism concentrations) can be attributed to:

1) Variation between sampling events due to different operating conditions (the upper level); and
2) Variation between replicate samples within each sampling event due to random fluctuation (lower level).

For this study a lognormal-gamma-Poisson hierarchical model was proposed for the characterisation of the interrelationship between the number of sampling events, replicate samples (*i.e.,* number of samples taken for each sampling event), and the achievable precision of estimation (*e.g.,* a confidence level) for microbial validation monitoring.

At the sampling event level, a lognormal distribution was used to characterize the distribution of the expected concentrations of replicate samples taken from each sampling event. Conditional upon the expected concentration, replicate samples within each sampling event were assumed to follow a negative binomial distribution expressed as a marginal distribution of Poisson process conditioned on a gamma-distributed Poisson parameter. The graphical representation of the lognormal-gamma-Poisson model is shown in Figure 5.1 with a directed acyclic graph (DAG) (Lunn *et al*., 2013). The OpenBUGS (Spiegelhalter *et al*., 2007) code of this lognormal-gamma-Poisson hierarchical model ('the hierarchical model') is detailed in Figure 5.2. A hierarchical model is featured by modelling observable outcomes conditional on certain parameters, which themselves are given a probabilistic specification in terms of further parameters known as hyper-parameters (Gelman *et al*., 2014). In a Bayesian statistics setting, the parameters of the prior distribution are often referred to as hyper-parameters. Therefore, the microbial concentrations are characterised by a hierarchical model with m, tau, and b as the hyper-parameters which are to be estimated for subsequent statistical inference. A Bayesian approach can be followed for the model parameter estimation.

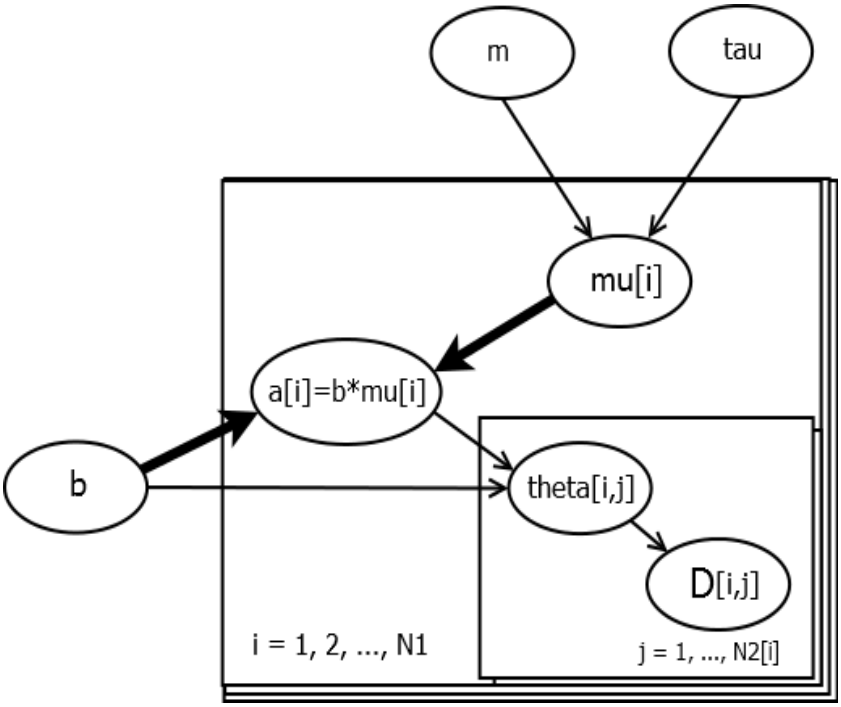

Figure 5.1: The directed acyclic graph (DAG) representation of the lognormal-gamma-Poisson hierarchical model. The model is completely defined by the hyper-parameters m, tau, and b.

```
model
      {
 for( i in 1:N1)
    { mu[i] ~ dlnorm(m,tau)
      a[i] <- b*mu[i]

  for(j in 1:N2[i])
 { theta[k[i]+j] ~ dgamma(a[i],b)
   D[k[i]+j] ~ dpois(theta[k[i]+j]) }}

 m ~ dunif(0.1,25)
 tau ~ dunif(0.01, 20)
 b ~ dunif(0.0000001,5)

            }
```

Figure 5.2: OpenBUGS code for the lognormal-gamma-Poisson hierarchical model corresponding to the DAG representation of the model presented in Figure 5.1.

Figure 5.3 shows that the level of confidence in the *E. coli* log removal performance at Helidon pond is very good, with 92% confidence level that the true mean LRV is within the range (1.76, 2.26) based on the collected data (25 sampling events with duplicate samples in most sampling events). Even if the sampling events drop to 20, we can see from Figure 5.3 that the confidence level drops only slightly: approximately from 92% to 89%.

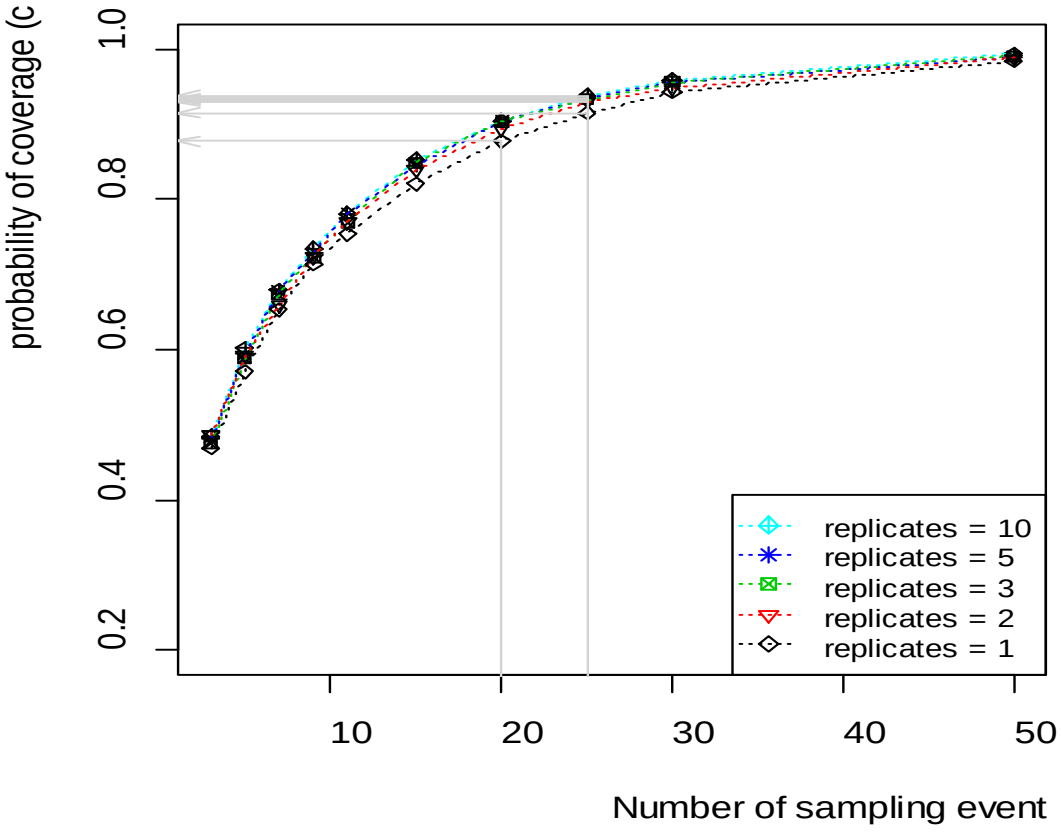

Figure 5.3: Confidence level of *E. coli* LRV estimates in Helidon Pond: true mean LRV range (1.76, 2.26), *i.e.,* true mean LRV +/- 0.25. 'replicates = 1' stands for single sample taken in the sampling event; 'replicates = 2' for duplicate samples; 'replicates = 3' for triplicate samples; and so on.

The confidence level for log removal analysis for enterococci, as shown in Figure 5.4, shows a different result to *E. coli*. Because the enterococci concentration distributions have much wider variation than the *E. coli* concentration distributions, this results in a wider variation range in LRV distribution. This explains why enterococci LRV results have a lower confidence level given the same specified precision range. For example, comparing Figure 5.3 with Figure 5.4 (a), given the

specified LRV precision range as +/- 0.25, *E. coli* has a confidence level 92% (Figure 5.1) while enterococci can only achieve 0.59 in the confidence level (Figure 5.4 (a)). However, we do have a 90% level of confidence that the mean enterococci LRV is between the range (1.26, 2.26) as shown in Figure 5.4(b).

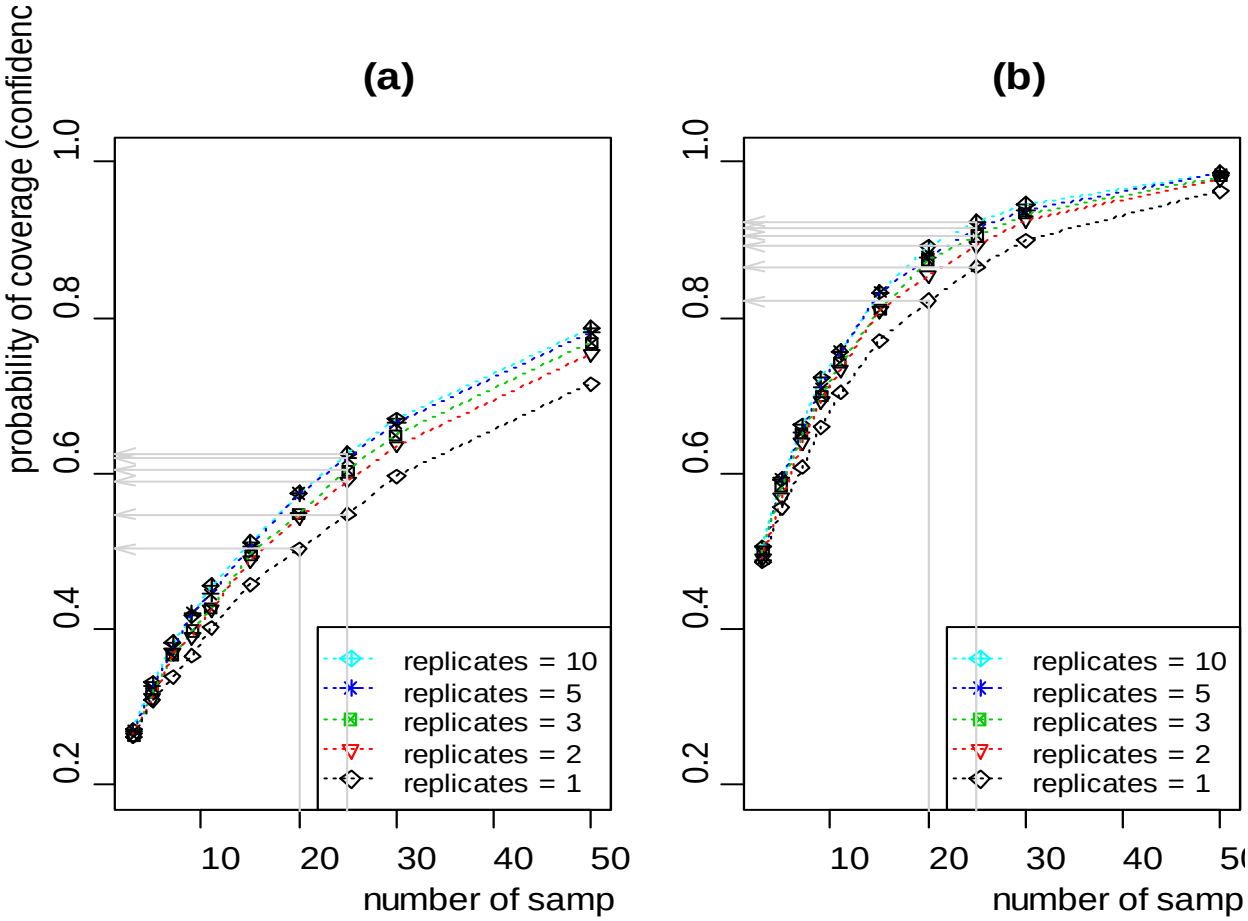

Figure 5.4: Confidence level for Helidon Pond enterococci log removal values (LRV): (a) true mean LRV +/- 0.25 (1.51, 2.01); (b) true mean LRV +/- 0.5 (1.26, 2.26). 'replicates = 1' stands for single sample taken in the sampling event; 'replicates = 2' for duplicate samples; 'replicates = 3' for triplicate samples; and so on.

It is concluded that:

1. A precision of +/- 0.25 would mean a possible error of 12.5% for 2 log removal, whereas a precision of +/- 0.5 would mean a 25% possible error. A precision of +/- 0.25 is thus considered a preferred target.
2. The analysis results strongly support the empirical rule of thumb that a minimum of 20 sampling events is necessary and the sampling points should be representative of the temporal variations.
3. For validation monitoring of pond performance, it is strongly recommended that replicate samples are collected for each sampling event. However, the analysis showed that there is little advantage in increasing the number of replicates beyond three and that the duplicate samples in each sampling event provide the biggest marginal gain in confidence level.
4. In addition to the above conclusions, which counted for the statistical analysis results only, it is noted that with only duplicate samples, one runs the risk of only having 1 data point should there be an issue with one of the replicates.

6 Chemical and Eco-toxicological Assessment

6.1 Background

Municipal wastewater contains complex mixtures of contaminants, but most wastewater treatment systems, including ponds, were never designed for the removal of broad classes of chemicals (Ratola *et al*., 2012, Rojas *et al*., 2013). As such, there is a global need for research aimed at: 1) identifying specific compounds or contaminant classes representing the greatest threats to aquatic biota; and 2) explore how efficiently maturation ponds remove contaminants (and eliminate toxicological risks) from domestic wastewater. Once these questions have been addressed, efforts can then be directed at engineering operational improvements to maturation ponds that will increase the effectiveness of ponds for removing broad chemical classes.

6.2 Chemical screening

Chemicals of interest in domestic wastewater include various Endocrine Disrupting Compounds (EDCs), Pharmaceutical and Personal Care Products (PPCPs), herbicides and pesticides, and metals. Methods for chemical analysis of trace organic contaminants have been well established, including (Liquid or Gas) Chromatography tandem Mass Spectrometry (LC-MS/MS and GS-MS/MS) for organics, High Resolution Mass Spectrometry (HRMS), Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) or Mass Spectrometry (ICP-MS) for metals, and Ion Chromatography (IC) for inorganics. When contaminant concentrations are low, Solid Phase Extraction (SPE) can often be used to enrich samples to detectable levels, but this focuses on organic compounds and ignores risks posed by metals and inorganics. Additional drawbacks of chemical analysis are that: 1) analytes must be selected prior to analysis, therefore unknown compounds or biologically active transformation products may not be detected; and 2) chemical analysis does not account for mixture toxicity.

Investigating toxicity while considering possible mixture effects generally involves Whole-Effluent Testing (WET), and/or the application of *in vitro* bio-analytical tools (Ra *et al*., 2007; Escher and Leusch 2012). Ideally, comprehensive assessment will involve multiple lines of evidence to fully account for the multiple stressors known to be present in wastewater.

6.3 Whole-Effluent Testing

Because of the shortcomings of chemical analysis, and the broader ecological concerns associated with exposure of wildlife to wastewater contaminants, it is often beneficial to perform exposures with relevant (*e.g.,* native) aquatic organisms. Traditionally, animal testing has focused on survival, development and reproduction as toxicological endpoints, as these hold clear implications for individual animals and ecosystems (Hood, 2005; Stadler, 2011). However, there is a growing interest in monitoring tools that are fast and sensitive to a wide range of compounds and indicate potential effects on survival, growth and fitness. One such approach that is gaining a great deal of research attention is the assessment of behavioural responses and indicators of functional performance (*e.g.,* swimming abilities), since these offer both time and financial improvements over traditional tests (Melvin and Wilson, 2013).

In vitro bioassays are also becoming increasingly important to assess the potential for toxicity or endocrine activity in sewage, treated effluent and drinking water (Escher and Leusch, 2012). Such assays are based at the molecular or cellular level, and facilitate the analysis of biologically active compounds in various water samples. Many different *in vitro* tests exist and more are continually being developed (Poulsen *et al*., 2011), including those assessing non-specific toxicity and a large number specifically for identification of endocrine activity in environmental waters (GWRC, 2006; 2012). *In vitro* tests can be highly sensitive, and responses are often well correlated with whole organism effects (Sonneveld *et al*., 2011; Sonneveld *et al*., 2006). With the complexity of wastewater, *in vitro* bioassays can provide a useful mechanistic link between chemical analysis and biological effects in whole organisms.

6.4 Prioritization of high-risk compounds

The first step in evaluating risks posed by specific trace organic contaminants (TrOCs) in aquatic receiving environments (including various EDCs, PPCPs, pesticides and herbicides) is to effectively prioritize important contaminants in wastewater based on existing quality science and precautionary principles. The US Water Environment Research Foundation (WERF) recently proposed and applied different approaches to prioritise TrOCs based on risk or potential risk to aquatic biota (WERF, 2011). The approach applies three levels of assessment to prioritize research efforts at sites posing the greatest threats to aquatic animals and ecosystem health:

1) primary chemical screening
2) site-specific screening, and
3) high-risk site diagnostics.

Primary chemical screening (1) was performed through detailed review of the primary literature surrounding the occurrence and toxicity of TrOCs. The site-specific screening approach (2) was therefore applied at all sites investigated through the ponds project. Subsequent research will be able to follow on the results of these activities by performing high-risk site diagnostics (3).

Primary screening essentially culminated in broad screening for 116 contaminants comprising the Queensland Health Forensic and Scientific Services (QHFSS) analytical suites for Endocrine Disrupting Chemicals (EDCs), Pharmaceutical and Personal Care Products (PPCPs) and pesticides and herbicides (see Appendix 3 in Supporting Documents). Using this information collected from the various ponds, a risk quotient (RQ) was calculated wherever possible for each of the compounds on the list, according to the formula:

$$\text{Risk Quotient (RQ)} = \frac{\text{Measured Environmental Concentration (MEC)}}{\text{Predicted No Effect Concentration (PNEC)}}$$

where the MEC is the highest concentration measured in the pond, and PNEC is the concentration below which no adverse effect is expected based on existing toxicity data with aquatic organisms. Existing PNECs are based on species sensitivity distributions (SSDs) when the availability of data permits, or alternatively the PNEC can be based on the most sensitive endpoint identified in the literature with an adequate uncertainty (assessment) factor applied. Once all RQs were calculated, these were ranked from lowest to highest and assigned a 'relative risk' in line with previously published conventions (*e.g.,* Sanchez-Avila *et al*., 2012; Cristale *et al*., 2013), where $RQ < 1$ indicates no risk, $1 \leq RQ < 10$ represents a low risk, $10 \leq RQ < 100$ signifies a high risk, and $RQ \geq 100$ indicates that an adverse environmental outcome is expected.

Applying these methods to the ponds studied through the ponds project, and specifically the intensively studied Helidon pond, several high-risk compounds were identified as continually present in treated wastewater (see Supporting Document). These include the broad-spectrum antimicrobial agent triclosan (TCS), the selective serotonin re-uptake inhibitor fluoxetine (FLX), and nonylphenol (NP). Triclosan is used in a large number of consumer products and thus frequently occurs in municipal wastewater (Murray *et al*., 2010). Because of its prevalence in the environment and potential for eliciting adverse effects on aquatic wildlife (Orvos *et al*., 2002; Dann and Hontela, 2011), TCS has frequently been flagged as a priority pollutant (Murray *et al*., 2010). Fluoxetine (also known as Prozac) has been a focal contaminant of concern for decades (Brooks *et al*., 2003), due to its poor removal from wastewater and range of toxicological effects on aquatic wildlife (Mennigen *et al*., 2009; Guler and Ford, 2010; Mennigen *et al*., 2010; Winder *et al*., 2012). Nonylphenol is commonly used in a variety of pesticides and consumer products, and is therefore commonly found in the aquatic environment. Importantly, each of these compounds has been shown to exhibit estrogenic activity and subsequently elicit a range of adverse toxicological outcomes in wildlife (Lech *et al*., 1996; Kinnberg *et al*., 2000; Fent *et al*., 2006; Lister *et al*., 2009; Routledge and Sumpter, 1997; Laws *et al*., 2000; Gee *et al*., 2008).

In conclusion, our assessment of chemicals in the Helidon pond suggests that pond treatment removes limited amounts of emerging organic contaminants. Risk prioritisation identified all high-risk contaminants exiting the ponds as known estrogenic compounds. Concentrations were generally below limits set out in the Standards for quality of recycled water supplied to augment a supply of drinking water (Schedule 3B Public Health Regulation 2005 – Standards for quality of

recycled water supplied to augment a supply of drinking water). Unfortunately, similar water quality standards are absent (for most compounds) for the protection of aquatic wildlife or ecosystem health, and our prioritization identified 9 compounds above levels expected to cause adverse biological outcomes in aquatic animals based on published “Predicted No-Effect Concentrations” (see supporting document). Fluoxetine, Nonylphenol and Triclosan were identified as high-risk at each sampling event at the Helidon pond, meaning these compounds are important to target for improved removal if the goal is to mitigate potential negative outcomes on aquatic biota. Importantly, chemical risk will differ between sites and therefore site-specific risk prioritization is necessary.

7 Factors Affecting Pond Performance

7.1 Factors Considered

The successfully calibrated three dimensional model for the Helidon pond was used to model the impact on *E. coli* removal of a number of different hypothetical scenarios in order to determine what factors had the biggest impact on disinfection performance. These scenarios considered included:

- Pond length to width ratio
- Pond depth
- Baffles
- Inlet-outlet configuration
- Wind affects

For each of these scenarios, the pond performance in reducing *E. coli* concentrations was compared. A relatively long period of simulation, from December 16, 2013 to March 18, 2014, was chosen to cover the equivalent of 6 retention times and the period for which there was significant atmospheric forcing data available. This long simulation period minimised the influence of the initial *E. coli* levels in the pond.

Each pond variant was subjected to identical influent concentrations and inflow rates and identical meteorological condition over the selected time period, including air temperature, wind condition, relative humidity, solar radiation, and precipitation.

The results of the analysis are discussed in more detail in the following section.

7.2 Pond Length-to-Width Ratio

Pond shape, more specifically the length-to-width (L/W) ratio has a significant influence on pond treatment performance. For this scenario, a series of ponds with different length to width ratios, L/W = 0.625, 1, 1.6, 2.5, 5 and 10, were analysed. A pond depth of 0.8 meters was assumed with no baffles.

The results, shown in Figure 7.1, reveal that the higher the L/W ratio, the better the pond performance. For example, a pond with a L/W ratio of 10 has a hydraulic efficiency 10 times greater than that of a pond with a L/W ratio of 0.625, and the *E. coli* removal efficiency is increased by 2 log units, from nearly zero to almost 2 log units. This is the result of the flow path created by pond shape, the hydraulic flow pattern and subsequently the hydraulic retention time.

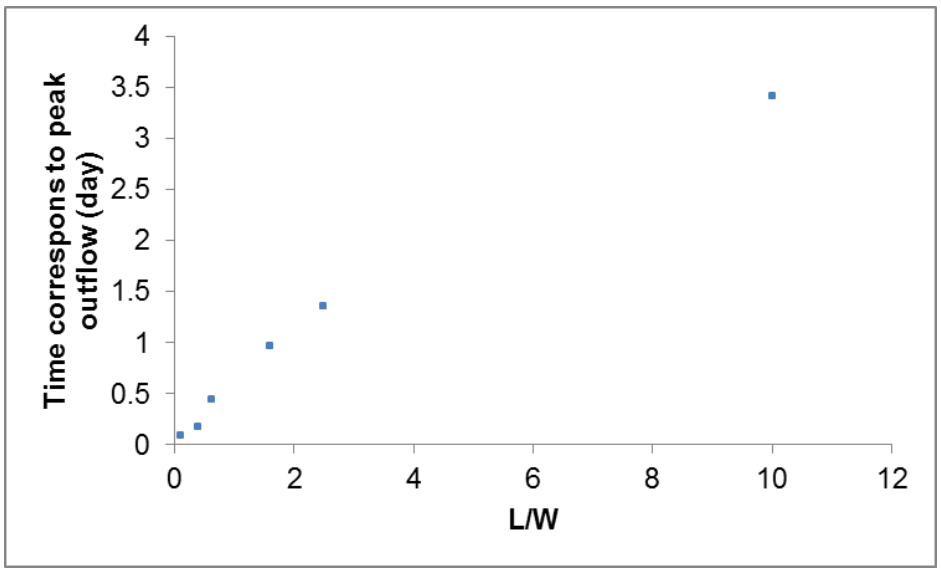

Figure 7.1: Retention time analysis for ponds with varying length-to-width ratio (L/W).

7.3 Pond Depth

Pond depth is another parameter affecting the maturation pond treatment efficiency. A series of hypothetical ponds with different depths, 0.4 m, 0.8 m, 1.2 m, 1.6 m and 2.0 m were examined. The hypothetical ponds were designed to be un-baffled and have a length of 50 meters and a width of 20 meters. Figure 7.2 shows the modelled results, and it can be seen that the most gain in pond performance was achieved when the pond depth was increased from 0.4 to 0.8 m with only a minor gain by increasing the depth from 1.6 to 2.0 m. The removal of *E. coli* at the outlet was increased by approximately 0.5 log unit by increasing the depth of ponds from 0.4 m to 2.0 m. The optimal maturation pond depth is suggested as between 0.8-1.2 m, which is in keeping with the suggestions of the Waste Stabilisation Pond Design Manual (Power and Water Corporation, 2011).

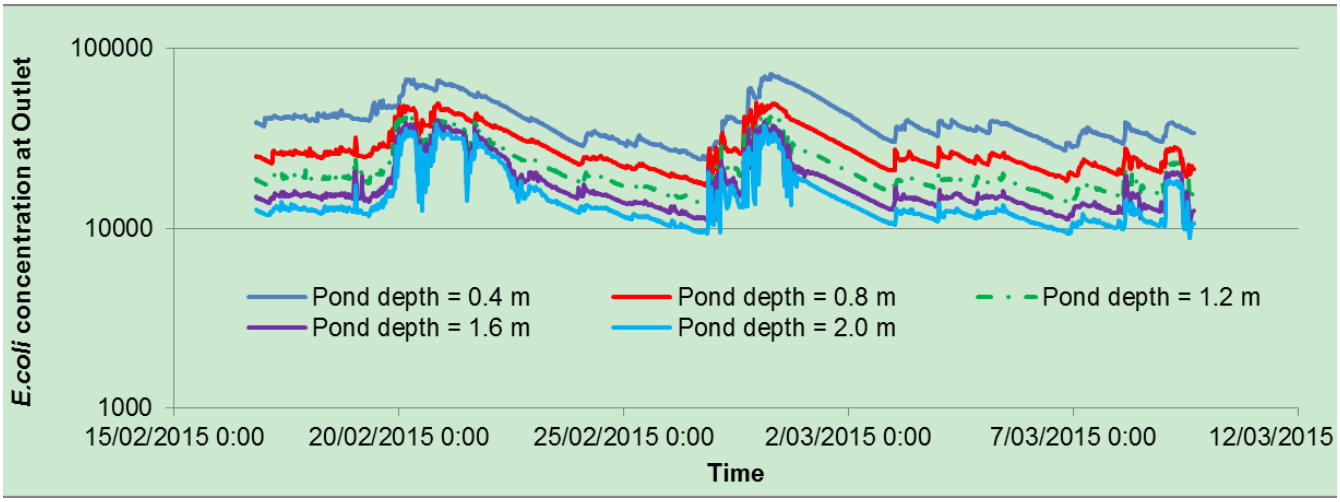

Figure 7.2: Pond depth effects on *E. coli* concentration (cfu/100 ml) at pond outlet (the lines from top to bottom correspond to, respectively, depth = 0.4 m, 0.8 m, 1.2 m, 1.6 m, and 2.0 m).

7.4 Baffles

Maturation pond treatment efficiency can be improved by installing baffles (Mara, 2009). Shilton and Mara (2005) also stressed that “there is certainly large potential for significant cost optimisation to be achieved by the intelligent incorporation of baffles in facultative and maturation ponds in tropical countries”.

Results from this study showed that for a rectangular maturation pond with a typical length-to-width ratio being 2.5:1 and using alternating staggered baffle arrangements (see Figure 7.3), the best hydraulic performance can be achieved by: a ratio of baffle length (Lb) to the width of the pond (W) Lb/W = 70%; and with a ratio of baffle spacing (Δb) to the length of the pond (L) Δb/L = 1/7. This is because the channelling effect reduces stagnant zones and the flow regime is maintained more like a plug flow regime. Importantly, the degree of short-circuiting is significantly lessened, and essentially removed.

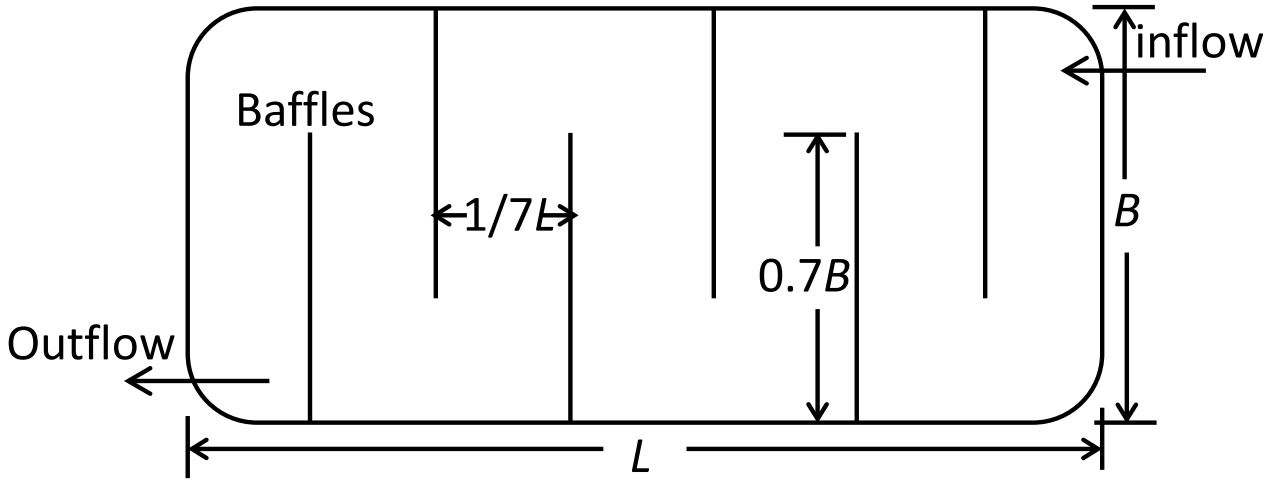

Figure 7.3: Plan view of pond with suggested baffle arrangement (after Mara, 2009).

Table 7.1: Baffle design layout.

Case	Layout	L_b/W	Δ_b/L
1		70%	1/7
2		50%	1/5
3		50%	1/7
4		70%	1/5

For ease of comparison, hydraulic measures of both the non-baffled pond and the four baffled ponds shown in Table 7.1 are tabulated in Table 7.2. It can be seen that baffled ponds show an overall increase in the hydraulic efficiency compared with the non-baffled pond. All temporal factors t_{16}, t_p and t_m have increased due to the channelling effect from baffles. The volume of stagnant zones is reduced and the flow regime is more like plug flow as shown in the decreased d and $\overline{\theta}_{pf}$ and the increased N. The degree of short-circuiting is lessened as noticed in the increasing S value. Overall, the hydraulic efficiency is improved from 0.31 to 0.50~0.62 compared to the non-baffled pond.

Table 7.2: Hydrodynamic measures of baffled ponds.

Case	t_{16}	t_p	t_m	t_{85}	σ^2	V_d	$\overline{\theta}_{pf}$	d	N	S	λ
No-baffle	0.31	0.31	0.85	1.77	0.89	0.15	0.59	1.23	1.11	0.31	0.31
1	0.62	0.62	1.05	1.55	0.40	0.01	0.27	0.36	2.53	0.62	0.62
2	0.49	0.49	0.99	1.58	0.62	0.01	0.39	0.64	1.61	0.49	0.49
3	0.50	0.50	1.05	1.77	0.61	0.01	0.38	0.55	1.64	0.50	0.50
4	0.49	0.49	0.99	1.60	0.61	0.01	0.40	0.63	1.64	0.49	0.49

However, with non-rectangular pond geometric configurations, the retrofitting baffling design may indeed be different. Ponds with small L/W ratios (< 5) benefits significantly from the baffling effect. On the other hand, ponds with larger L/W ratios (> 5) don't necessarily benefit from retrofitting baffles. Therefore, it is suggested that the pond be designed with reasonable L/W ratios (L/W <5) combined with appropriate number of baffles to achieve expected treatment performance.

7.5 Inlet-Outlet Configuration

The importance of inlet and outlet structures to maturation pond hydrodynamic and treatment efficiency is not as significant compared with that of pond depth or pond length-to-width ratio. In total, six cases as shown in Figure 7.4 were examined in this project. It was found that the six cases can be grouped into two categories according to their hydraulic performance:

(1) cases 1, 4 and 5 in which the inlet and outlet are positioned at various levels along pond depth but horizontally diagonally opposite to each other; and
(2) cases 2, 3 and 6 where inlet and outlet are also positioned at various depths but horizontally right opposite to each other.

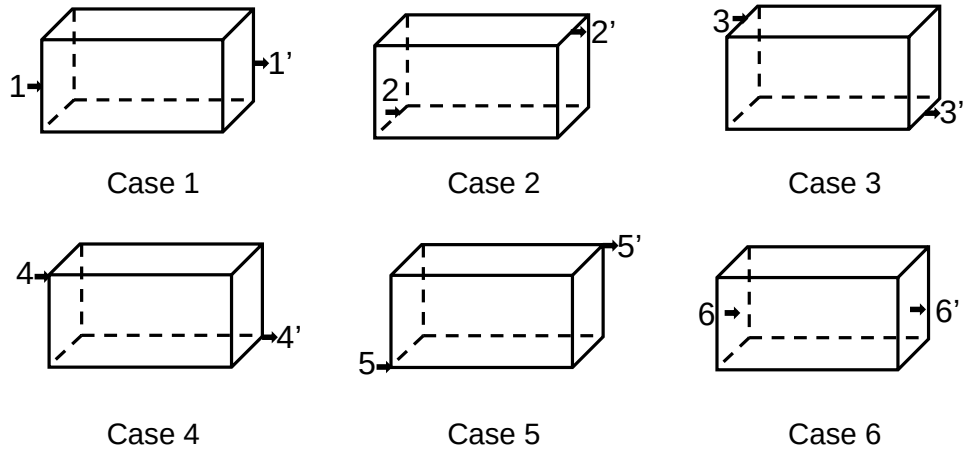

Figure 7.4: Inlet and outlet positioning scheme.

Hydraulic measures are quantified for the six cases and are tabulated in Table 7.3 below. Here in:

t_{16}, t_p (Persson, 2000): represent the time for the passage of the 16th percentile of the tracer through the outlet and of the peak flow, respectively.

Mean residence time t_m (Fogler, 1992): calculated as the first moment of the $E(t)$ function. It measures the average time tracer particles spend in a pond before exit:

$$t_m = \int_0^\infty tE(t)dt \qquad (1)$$

Variance σ^2 (Fogler, 1992): an indication of the 'spread' of $E(t)$ curve. The greater the value is, the greater the distribution's spread is:

$$\sigma^2 = \int_0^\infty \left(t - t_m\right)^2 E\left(t\right) dt \quad (2)$$

Dead space parameter V_d (Mangelso and Watters, 1972): a measure of the amount of pond volume where mixing is less than desirable:

$$V_d = 1 - t_m / T_{theor} \quad (3)$$

Deviation from plug-flow parameter $\overline{\theta}_{pf}$ (Watters *et al.*, 1973): $\overline{\theta}_{pf} \rightarrow 0$ if the flow approaches ideal plug flow:

$$\overline{\theta}_{pf} = T_{theor} - \int_0^{T_{theor}} tE\left(t\right) dt \Big/ \int_0^{T_{theor}} E\left(t\right) dt \quad (4)$$

Dispersion number d (Thackston *et al.*, 1987): $d = 0$ suggests the plug flow condition and $d \rightarrow \infty$ indicates the completely mixed flow condition:

$$d = \sigma^2 / t_m{}^2 \quad (5)$$

Number of CSTRs (continuously-stirred reaction tank) in series N (Fogler, 1992): $N = 1$ for continuously stirred flow and $N \rightarrow \infty$ if the flow approaches plug flow:

$$N = \left(T_{theor} / \sigma\right)^2 \quad (6)$$

Short-circuiting quotient S (Persson, 2000): smaller S values correspond to severer short circuiting:

$$S = t_{16} / T_{theor} \quad (7)$$

Hydraulic efficiency λ (Persson *et al.*, 1999): a measure of hydraulic conditions of a pond, and is defined as the ratio of the time corresponding to peak outflow at the outlet over the theoretical retention time:

$$\lambda = t_p / T_{theor} \quad (8)$$

Group 1 presents a hydraulic efficiency of 0.27 as opposed to 0.13 for group 2. The hydraulic efficiency value ranges from 0 to 1, representing low to high hydraulic performance. This suggests that for un-baffled ponds, a diagonally positioned inlet-outlet arrangement leads to better pond performance. The vertical variation of the inlet/outlet position does not impose much influence on the pond performance. This is because the long retention times within the maturation pond overshadow the inlet mixing conditions.

Table 7.3: Hydrodynamic measures of ponds with varying inlet/outlet locations.

Case	Inlet-outlet	t_{16}	t_p	t_m	t_{85}	σ^2	V_d	$\overline{\theta}_{pf}$	d	N	S	λ
1	1-1'	0.26	0.27	0.65	0.95	0.69	0.35	0.64	1.63	1.46	0.26	0.27
2	2-2'	0.13	0.13	0.78	1.95	1.11	0.22	0.77	1.85	0.90	0.13	0.13
3	3-3'	0.12	0.12	0.78	1.95	1.13	0.22	0.77	1.87	0.89	0.12	0.12
4	4-4'	0.25	0.26	0.70	1.28	0.77	0.30	0.64	1.55	1.30	0.25	0.26
5	5-5'	0.26	0.27	0.61	0.86	0.60	0.39	0.64	1.63	1.68	0.26	0.27
6	6-6'	0.13	0.13	0.74	1.79	0.91	0.26	0.76	1.65	1.01	0.13	0.13

7.6 Wind Affects

A generally established belief has prevailed that wind has a positive impact on pond performance as a result of wind-induced aeration and mixing. However, results from this study show that, for un-baffled ponds, it appears wind can encourage short-circuiting, and thus reduce pond performance (see also Brissaud *et al*., 2000; Brissaud *et al*., 2003; Lloyd *et al*., 2003; Li *et al* 2014).

For un-baffled ponds, this study found that parallel wind shown in Figure 7.5 (in relation to pond longitudinal layout) encourages short-circuiting in the pond, whereas orthogonal wind, as seen in Figure 7.6, enhances circulation and vertical mixing in the pond. Oblique wind also poses positive effects on pond performance. Therefore, it is highly beneficial if ponds are oriented orthogonal to prevailing wind directions. Constructing ponds with inlet/outlet orientated in alignment with the direction of prevailing wind is not recommended as this enhances short circuiting, hence reducing pond treatment performance.

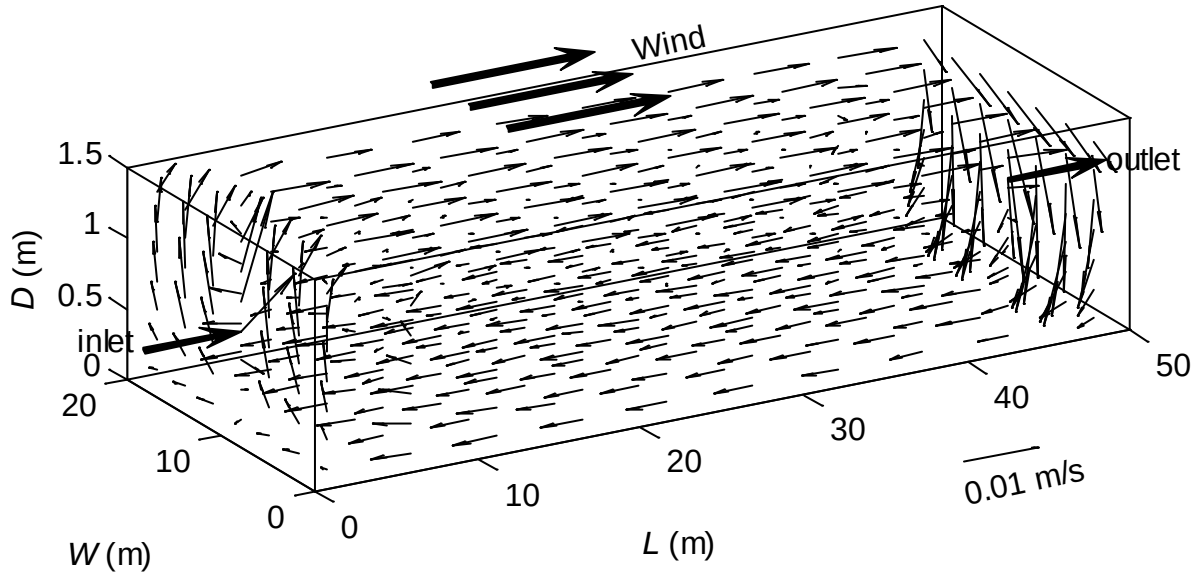

Figure 7.5: Velocity field showing pond water flow when inlet and outlet orientated is in alignment with the direction of prevailing wind (Not-recommended). The thin arrows indicate the water flow direction, while the fat arrows indicate the wind and inflow directions.

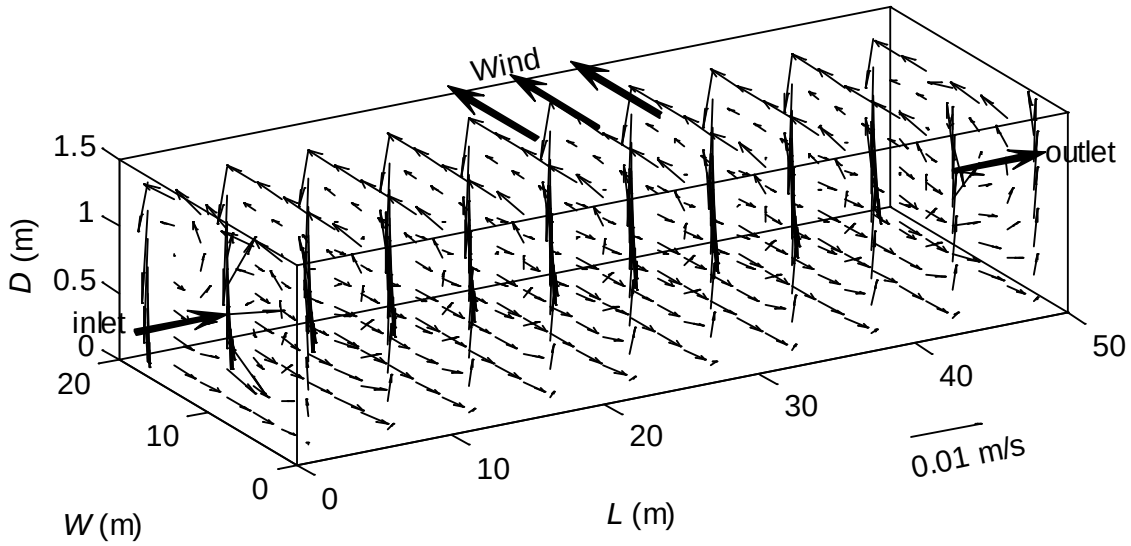

Figure 7.6: Velocity field showing pond water flow when prevailing wind is orthogonal to pond orientation (Recommended). The thin arrows indicate the water flow direction, while the fat arrows indicate the wind and inflow directions.

To quantify the comparison of the effects of different wind directions on pond residence, cases with wind blowing over the surface of the pond from θ_W = 0°, 45°, 90°, 135° and 180° in relation to the inflow direction were studied. By examining velocity vectors of the wastewater flow in ponds when subjected to wind with different directions, the selected wind cases can be grouped into three categories, *i.e.,* parallel to the inflow direction, θ_W = 0° and 180°; oblique to the inflow direction, θ_W = 45° and 135°; and orthogonal to the inflow direction, θ_W = 90°. In the study, the inlet and outlet are positioned along the longitudinal direction of the pond, a major circulation pattern is formed in the longitudinal direction when θ_W = 0°. On the other hand, the circulation is in the transverse direction when θ_W = 90°. This essentially creates a relatively thorough mixing in the pond, which subsequently affects the treatment efficiency of the pond. When θ_W = 45°, water at the pond surface predominantly follows the wind, whereas the velocity vector of the water flow at the bottom layer presents opposing directions.

Table 7.4 lists hydraulic indicators for all cases and for comparison purposes, those of a no wind case are also tabulated. By comparing the hydraulic efficiency λ with the no wind case, it can be concluded that parallel wind has an adverse effect on pond hydraulic efficiency, whereas the other two cases have positive effects, with orthogonal wind being the preferable scenario (λ close to 1). More specifically, all temporal indicators t_{16}, t_p and t_m are rather small for the parallel case in comparison to the orthogonal case. In the parallel case, a certain portion of wastewater short circuits to the outlet and does not reside in the pond for a sufficient period of time. On the other hand, in the orthogonal case, wastewater follows a path that is much longer than the direct inlet-to-outlet distance, thus increasing the detention time. By reading $\overline{\theta}_{pf}$, the flow regime in a pond subject to orthogonal wind is analogous to plug flow. The relatively big values of S and small values of d when θ_W = 90° also confirmed this information.

Table 7.4: Hydrodynamic measures of ponds with varying wind conditions.

Case	t_{16}	t_{peak}	t_{mean}	t_{85}	σ^2	V_d	$\overline{\theta}_{pf}$	d	N	S	λ
Parallel	0.04	0.03	0.45	0.93	0.49	0.55	0.80	2.44	2.03	0.04	0.03
Oblique	0.36	0.43	0.91	1.52	0.38	0.09	0.45	0.46	2.64	0.36	0.43
Orthogonal	0.82	0.90	0.94	1.06	0.01	0.06	0.12	0.02	67.6	0.82	0.90
No wind	0.13	0.13	0.74	1.79	0.91	0.26	0.76	1.65	1.01	0.13	0.13

By examining variance σ^2 and the dispersion number N, it is clear that for the orthogonal case, wastewater is adequately recirculated and mixed in the pond under the effects of orthogonal wind and that all water particles exit the pond at roughly the same time. The higher values of N for θ_W = 90° also indicates that the flow regime is approaching that of plug flow. The hydrodynamic performance of the pond with oblique wind lies in between the parallel and the orthogonal scenarios. This can be explained by the flow pattern shown in Figure 7.7(a) and (b), in which wastewater circulates with a certain angle to the inlet-outlet direction.

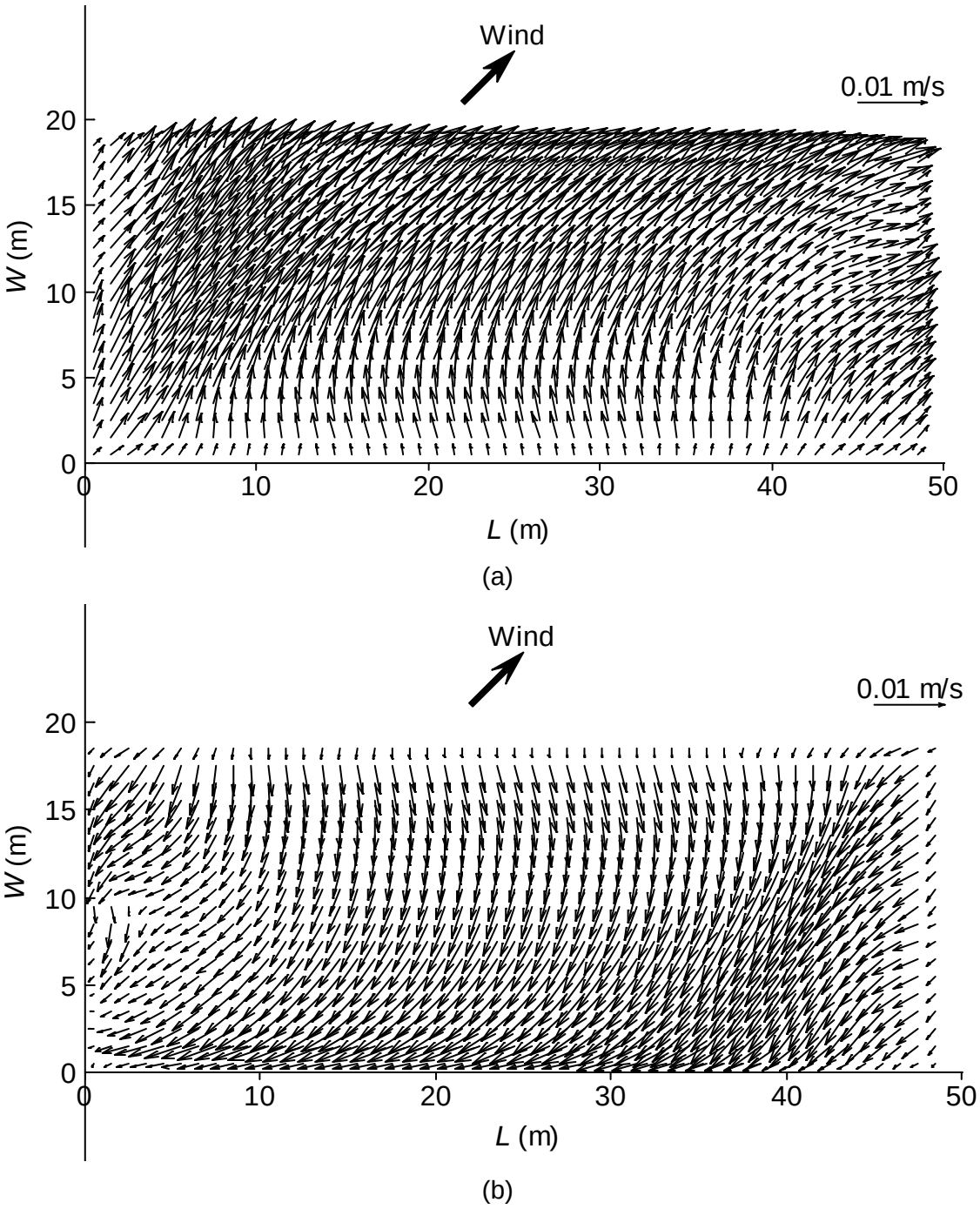

Figure 7.7: Velocity vectors of wastewater flow within the pond under oblique wind condition: (a) at the pond surface level and (b) at the pond bottom level.

Modelling the Helidon pond site with no wind conditions showed the pond performance was improved compared to the actual performance on site with actual measured wind conditions. For example the Helidon maturation pond showed an increase of 0.5 log unit *E. coli* removal under no wind conditions as compared to that measured on site with wind. This implies that applying wind breaks at the Helidon pond site will benefit pond operators in terms of *E. coli* removal.

Mara and Pearson (1998) found that wind may encourage short-circuiting and therefore reduces pond performance. This study found pond performance was case specific. The effect of wind on baffled ponds is not as significant as that on non-baffled ponds, as the baffles break up the lateral and longitudinal flow fields and generate a more plug-like flow.

8 Fieldwork Overview

8.1 Sampling and Testing

Over the period of January 2013 to March 2015, the pond project team visited five pond wastewater treatment systems/plants. These five pond systems were at: Maningrida (PWC, NT); Laidley (QUU, Qld); Forest Hill (QUU, Qld); Helidon (QUU, Qld); and Ngukurr (PWC, NT). Of these five pond-based wastewater treatment systems only maturation ponds in the Helidon treatment plant and Ngukurr treatment plant were found to be suitable for this study. One field trip was made to Ngukurr pond in June 2014 but multiple field trips were made to Helidon pond through September 2013 to March 2015.

The Australian Guidelines for Water Recycling (AGWR, 2006) recommend that indicator organisms (*e.g.,* faecal coliforms, *E. coli*, or enterococci) and reference pathogens (*Campylobacter*, *adenovirus*, and *Giardia*) data should be collected for assessment of microbial risks related to recycled water. AGWR also recommended that, for microbial monitoring, a statistically valid number of samples are required to cover different operational conditions. From the research done for this study, it was determined that at least 20 data points (ideally with replicate samples) should be obtained in order to provide a reliable estimate of the results. This number of sampling events has thus been targeted for each of the four organisms: *E. coli*, *Campylobacter*, adenovirus, and *Giardia* for a reasonable QMRA.

To date, sufficient *E. coli* and enterococci data have been collected from the Helidon maturation pond for the purpose of evaluation of indicator organism removal performance. With regard to pathogens, the following resource constraints and technical difficulties were encountered:

- *Campylobacter* - more than 100 samples were collected via an auto-sampler and processed. However some spurious results led to further investigation which revealed the standard media was unsuitable due to overgrowth of *Acinetobacter spp*. A new chromogenic media which can distinguish *Acinetobacter spp*. is in the process of being trialled. It should be noted that qPCR revealed no *campylobacter* in either the inlet or outlet of the maturation pond.
- Adenovirus – 44 qPCR test results are available from 22 sampling events (11 from the inlet, 11 from the outlet). However, while this method has some advantages, the samples provided (with recovery around 30%) insufficient data in ponds with low incoming virus concentrations. With the low limit of quantification of 430 genome copies/100 mL it was sometimes impossible to enumerate adenovirus in effluent (and therefore difficult to calculate log removal of the virus) to the standard required for QMRA. Conventional cell culture method was not used due to equipment and permit constraints. This work is being repeated as part of the ongoing QSFS project.
- *Giardia lamblia* (USEPA 1623) – 48 test results were taken from 24 sampling events (12 from the inlet, 12 from the outlet). The variability of results has been found to be quite low. Based on these samples, preliminary calculations indicate a log removal of 2.5. Further investigations into the impact of recovery rates on QMRA, and the need for additional samples will be carried out as part of the QSFS project.
- *Cryptosporidium parvum* (USEPA 1623) - 48 test results were taken from 24 sampling events (12 from the inlet, 12 from the outlet. Very few presumptive oocysts (0 or 1) were found at either the inlet or outlet and it was therefore determined that this was not a useful indicator in the case of the Helidon pond. *Giardia* was considered to be a more useful indicator for protozoa for the Helidon pond.

During the June 2014 field trip to Ngukurr ponds, samples for analysis for *E. coli* and enterococci were also collected in five sampling events (each with triplicate samples) from the first of the three maturation ponds in operation at this site. Details of these data sets can be found in the support documents.

8.2 Characterising Log Removal Efficiency

Both the AGWR method and the Monte Carlo simulation method were used to estimate the LRVs for Helidon data. Only the AGWR method was used to calculate LRV for Ngukurr as data were not adequate for characterising LRV with a probability distribution.

8.2.1 Helidon

The analysis has revealed that the Helidon maturation pond can typically remove 2 log units of *E. coli* (*i.e.,* 99% removal rate) and 1.8 log units of enterococci (*i.e.,* 98% removal rate). The enterococci removal has shown a wider range of variation than the *E. coli* removal such that enterococci removal can be as high as 3.77 log units compared with 3.14 log units for *E. coli*. Based on a proposed hierarchical probabilistic model (details in the support document), it is estimated that we have more than 90% confidence that the true mean LRV is within the interval (1.75, 2.25) for *E. coli*; whereas in contrast, we have only a slightly less than 60% confidence to say that the true mean LRV for enterococci is within the interval (1.55, 2.05). We may claim a 90% confidence with a LRV range (1.3, 2.3) for enterococci removal.

8.2.2 Ngukurr

Analysis based on Ngukurr data showed that there was 0.528 log units removal of *E. coli* (*i.e.,* 70% removal rate) and 0.508 log units of enterococci (*i.e.,* 69% removal rate). Ngukurr operational monitoring data (19 monthly data) obtained from PWC has shown a 2.6 log units removal of *E. coli* for the whole treatment pond system (five ponds in series).

8.2.3 Discussion of Findings

The log-removal efficiency for faecal indicators at the Helidon pond was observed to be significantly higher than that for the Ngukurr pond. It is noted that for both Helidon and Ngukurr maturation ponds, the enterococci removal rate is slightly lower than the *E. coli* removal rate and there is a plausible microbiological explanation for this phenomenon. In contrast however, enterococci demonstrated higher inactivation rate than *E. coli* under the similar environmental conditions which were found in our microcosm studies and noted by others (Kadir and Nelson, 2014). Gram-positive bacteria like enterococci are found to be more sensitive to exogenous photo-oxidation than Gram-negative bacteria like *E. coli* which are more sensitive to direct sunlight and endogenous inactivation. In maturation ponds, exogenous mechanisms can be impacted widely by other characteristics of pond water (e.g. seasonal variations in concentrations of ROS and NOM), which might explain a higher variation in LRV for enterococci compare to *E. coli* in same samples.

Von Sperling (1999) investigated 33 facultative and maturation ponds in Brazil, and reported that the faecal coliform removal rate was between 0.34 to 3 log units (*i.e.,* 53.4% to 99.9% removal rate). It is commonly accepted that WSPs can generally achieve 1 to 6 log reduction in pathogen concentrations (*i.e.,* 90 – 99.9999% reduction/removal) (WHO, 2006; NRMMC *et al*., 2006). Considering that maturation ponds form only one link in the full pond treatment chain in both Helidon and Ngukurr treatment plants, the above field data analysis results indicate that both pond treatment systems performed normally within the reported pathogen removal performance range in literature.

8.3 Applicability of 3D Modelling

The three-dimensional modelling procedure and its associated field work activities have been proven to be applicable and valid to evaluate treatment performance of maturation ponds. The MIKE 3 model was verified as being capable of simulating pond hydrodynamics as well as pathogen removal (represented by *E. coli* removal in this project). An outline of the modelling framework is illustrated in Figure 8.1.

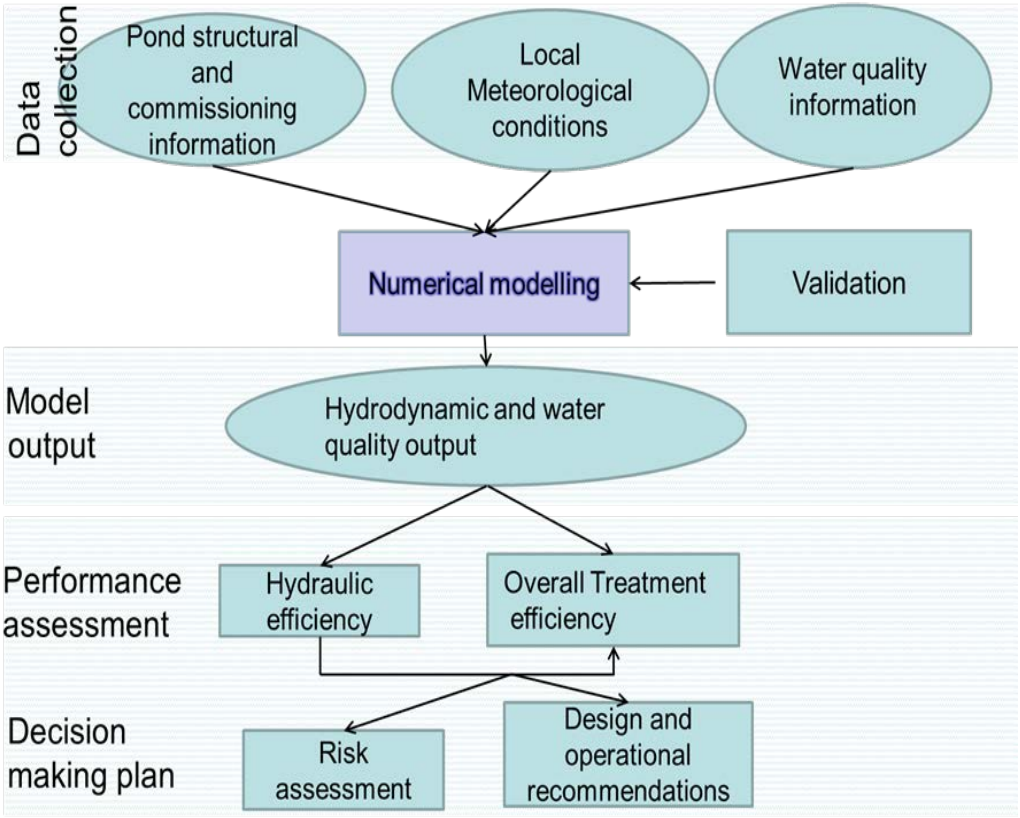

Figure 8.1: Pond project modelling framework.

The required information and the data sources is summarised in Table 8.1. This informs pond operators or water authorities as to what information to gather for pond design or performance evaluation.

Table 8.1: Information for modelling maturation pond performance.

Data type	Parameters	Source
Pond structural information	Pond geographical location; orientation; length; width; average depth; inlet and outlet locations; baffling information including length, depth, number and location	From existing pond or design
Meteorological data	Air temperature, relative humidity; wind speed, wind direction; precipitation; solar radiation	From BOM
Influent and effluent information	Inflow rate (design); influent velocity; indicators and pathogen concentration at the influent and the effluent	From pond operational information
Microbiological information	Decay rate coefficient: a bulk decay rate coefficient of 0.5 per day, or the equation using: $k = k_0 \times k_I{}^{I} \times k_T^{T-20}$ with k_0 = 0.2-0.4; k_I = 6; and k_T = 1.05 for a performance evaluation to find out the log removal	From project experience, in Queensland

9 Key Findings and Further Work

9.1 Key Findings

1. Baffles in ponds, by effectively altering the pond length-to-width ratio, can substantially improve the retention time and therefore pond performance.
2. Wind may contribute negatively to *E. coli* removal performance, depending on pond orientation, baffles and inlet-outlet arrangement.
3. Numerical results by MIKE 3 were found to effectively reflect observed performance at the Helidon pond for *E. coli* removal. The MIKE 3 model is a useful tool for monitoring and managing maturation pond performance, and this model is therefore recommended for ongoing use for design and retrofitting of maturation ponds.
4. Decay rate coefficients for *E. coli* obtained from microcosm studies were found to be similar at Helidon (Queensland) and Leanyer Sanderson (Northern Territory), in the range of 0.4 to 0.5. This was also similar to the MIKE 3 Ecolab default value.
5. From microcosm studies, light was found to be the most important factor in decay processes, with predation having only a minor impact.
6. *E. coli* was found to exhibit two phase decay kinetics, based on the microcosm studies. The implication of this finding for modelling, and if a two-phase die-off is always the pattern observed for *E. coli* (and other organisms) in WSPs, requires further investigation. The difficulty with two-phase modelling is the determination of when the second phase commences.
7. Decay rate coefficients for enterococci were higher than for *E. coli* based on the microcosm studies. This indicates that log removal should be higher in the pond for enterococci than for *E. coli*. However, observed removal was higher for *E. coli* than for enterococci in Helidon pond. This brings into question the use of microcosm studies for the measurement of the decay rates for enterococci in maturation ponds.
8. Observed mean log removals for single maturation ponds were found to be 0.5 and 2.0 for *E. coli*, and 0.5 and 1.8 for enterococci in Ngukurr and Helidon, respectively.
9. Preliminary results for *Giardia* in the Helidon pond indicated a 2.5 mean log removal. *Cryptosporidium* was only detected at very low levels in comparison to *Giardia*, and therefore *Giardia* was considered a better indicator of protozoa removal in this instance. Work for protozoa to better determine *Cryptosporidium* removal rates compared to *Giardia* is continuing.
10. qPCR, while cost- and time-effective, was found to be useful only for quantifying adenovirus concentrations in the influent of maturation ponds. However, for the effluent samples the lower limit of quantification of 430 gene copies/ 100 ml was found to be insufficient to report low viral concentrations that were equal or below of this number.
11. For future validation studies, a hierarchical model was proposed for characterisation of microbial concentrations based on validation monitoring sample data. Analysis results from this model support the empirical rule of thumb suggesting a minimum of 20 sampling events with duplicate samples.
12. Pond treatment removes limited amounts of emerging organic contaminants, however the small population size at Helidon contributed only low input levels of these contaminants
13. Risk prioritisation for chemical toxins frequently identified high risk compounds exiting the ponds as known estrogenic compounds.
14. Concentrations of chemicals of concern were generally below limits set out in the Standards for quality of recycled water supplied to augment a supply of drinking water. (Schedule 3B Public Health Regulation, 2005). However, risk prioritization identified 9 compounds above levels expected to cause adverse biological outcomes in aquatic animals based on published “Predicted No-Effect Concentrations”.

9.2 Future work as part of the QSFS project

9.2.1 Adenovirus and *Cryptosporidium/Giardia* Sampling and Testing

Further adenovirus sampling will be undertaken at the Helidon site and testing undertaken using the culture method. This work is scheduled to be carried out in the next few months. Findings will be incorporated into the QSFS report and may be used to update the NatVal documents at a later date.

9.2.2 NSW Pond

It is proposed to carry out the final sampling and testing work at a pond operated by the Tamworth Regional Council. Tamworth Regional Council staff will carry out the sampling work and the study team will complete the relevant site work for the hydrodynamic study and collecting relevant data for the model validation. The results for this pond will be reported on in the QSFS project at a later date.

9.2.3 QMRA and Health Risk Assessment

- Continue both power and sensitivity analysis of the hierarchical statistical models for the determination of an optimal sampling scheme (*i.e.,* an 'optimal' combination of the number of sampling points, in terms of both number of sampling events and replicate samples within each sampling event, and the estimation precision) for pond performance validation monitoring.
- Development of a simple decision-support tool for health risk assessment for the selection of suitable pond effluent reuse options. This research is based on the QMRA model framework established in the AWRCE project which is implemented using @Risk (a commercial risk assessment computer software package). The next stage of research will focus primarily on two aspects: (1) exposure assessment with regard to specified irrigation reuse options; and (2) determination of the health-based targets (*e.g.,* the tolerable probability of infection per person per year) using as much Australian epidemiological data as possible instead of relying primarily on the WHO data.
- Validate the health risk assessment tool with empirical data collected from the Helidon pond, more specifically with *E. coli*, adenovirus, and *Giardia* data. If cell cultured adenovirus is not available for the analysis, the minimum goal is to complete the validation analysis using *E. coli* and *Giardia* data.

9.3 Future work outside of the scope of this study or the QSFS project

- Determination of decay rate coefficients for target pathogens and indicators for other pond systems
- Investigation of the impact of sedimentation on *E. coli* decay
- Investigation of the impact of *E. coli* two phase decay kinetics on the model findings
- Investigation of the impact of re-suspension of bottom sediments on *E. coli* decay
- Investigation into improvements in enumeration techniques for pathogens and/or suitable surrogates
- Application of the conditional probability approach to investigate the association between the removal of faecal indicators and the removal of pathogens in WSPs (rather than the regression approach)
- Further investigation into characterising the impact, benefits, problems and control of algae and cyanobacteria in WSPs.

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