Investigating Environmental and Health Risks of Greywater use in New Zealand

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Abstract

Many countries, including New Zealand, are investigating alternative water management practices to address increasing demands on freshwater supply. One such practice is the diversion and reuse of household greywater for irrigation. Greywater is a complex mixture containing contaminants such as microbes and household chemicals. These contaminants may present an environmental and public health risk, but this has never been characterised in a New Zealand context.

This thesis aims to reduce this knowledge gap by characterising the fate and effects of a representative chemical contaminant, the antimicrobial triclosan (TCS); and the microbial indicator, *E. coli*, in three soils. It also investigated public attitude towards the fate of household products in the environment.

In Chapter 4, microbial biomass was used to determine an EC_{50} for TCS in one soil type (silty clay loam: EC_{50} = 803 ppm). This determined the loading rate of TCS for the lysimeter study in Chapter 5, where triplicate cores of 3 soil types were irrigated with greywater treatments (good/bad quality) or a freshwater control. Leachate samples throughout the study and soil samples from three horizons at the end of three months irrigation were analysed for TCS and *E. coli.* The results indicate that regardless of soil type, *E. coli* and TCS leached from the lysimeters posing a risk for groundwater contamination. *Escherichia coli* levels in the leachate were as high as 4.71 x 10^6 CFU/100ml for the GQGW treatments (Lincoln soil) and 6.97 x 10^7CFU/100ml in the BQGW treatment (Gisborne soil). Triclosan concentrations between 0.03ppb and 3.17ppb were measured in the leachate from the GQGW treatment and 0.03ppb - 42.3ppb for the 10ppm TCS treatments. Soils with high clay content had even larger potential for leaching through preferential flow as the average levels of *E. coli* found in the leachate from the BQGWD were at least on log_{10} lower than the average found in the BQGW leachate (Gisborne & Katikati). In contrast the levels of *E. coli* detected in the Lincoln soil were similar for both treatments. The effects of TCS on soil health parameters in the top horizon were also investigated, but were not found to be significant at concentrations used in this study.

To address the source of greywater contamination, i.e. use of household products, I engaged with school children to investigate if awareness of household-contaminants will support behaviour change with respect to what products are used (Chapter 6). With my scientific guidance, the children successfully designed and implemented a greywater experiment and presented their results at a local hui.

The results from this study provide New Zealand specific, scientifically-robust information on potential environmental and public health risks associated with domestic greywater reuse for soil irrigation.

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Contents

Abstract i

Acknowledgements iii

List of Figures viii

List of Tables xi

1. Introduction and Aims 1

1.1 Introduction 1

1.2 Aims 3

1.3 Research approach 3

2. Literature review 6

2.1 What is greywater? 6

2.2 Greywater reuse drivers 7

2.2.1 Water shortages 8

2.2.2 Surplus water 8

2.3 Risks and benefits associated with greywater reuse 9

2.3.1 Risks 9

2.3.2 Benefits 10

2.4 Greywater composition 10

2.4.1 Microbiological quality 10

2.4.2 Nutrients in greywater 12

2.4.3 Chemicals in greywater 12

2.5 Triclosan (TCS), Chemical properties 13

2.5.1 Fate of TCS in soil 14

2.5.2 Effects of TCS in soil 16

3. Methods 18

3.1 Moisture content, dry matter content and water holding capacity 18

3.1.1. Moisture and dry matter content: 18

3.1.2 Water-holding capacity (WHC): 19

3.2 pH 19

3.3. Substrate induced respiration 19

3.4 Sulphatase enzyme activity 20

3.5 Microbial biomass 21

3.6 Microbial metabolic quotient 22

3.7 Triclosan analysis 22

3.7.1 Extraction from soil 22
3.7.2 Soil sample concentration and derivatisation 26
3.7.3 Extraction from leachate 26
3.7.4 Leachate sample concentration and derivatisation 27
3.7.5 Analysis of Triclosan residues by Gas Chromatography Mass-Spectrometry 29
3.8. Molecular analysis of *Escherichia coli* 30
3.8.1. Microbial DNA extraction from leachate 30
3.8.2 Microbial DNA extraction from soil 31
3.8.3. Quantitative Polymerase Chain Reaction (qPCR) 31
4. Determining the EC_{50} of triclosan (TCS) in a silty clay loam 33
4.1 Introduction 33
4.2 Materials and methods 35
4.2.1 Soil sampling and initial characterisation 35
4.2.2 Triclosan addition to soil 35
4.2.3 Analysis 36
4.2.4 Statistics 37
4.3 Results 38
4.3.1 Soil sampling and initial characterisation 38
4.3.2 Substrate Induced Respiration 38
4.3.3 Microbial biomass 38
4.3.4 Microbial metabolic quotient (qCO_2) 39
4.3.5 Sulphatase enzyme activity 40
4.3.6 EC_{50} determinations 40
4.4 Discussion 42
4.5 Conclusion 44
5. Lysimeter study 45
5.1 Introduction 45
5.2 Materials and methods 47
5.2.1 Source of soils 47
5.2.2 Lysimeter facility 48
5.2.3 Irrigation volumes and for lysimeters. 49
5.2.4 Composition of greywater treatments. 50
5.2.5 Experimental procedure 51
5.2.6 *Escherichia coli* preparation and enumeration. 52

5.2.7 *Escherichia coli* survival 53
5.3 Analytical methods 54
5.3.1 Substrate induced respiration, sulphatase, biomass, triclosan analysis and *Escherichia coli* quantification 54
5.3.2 Statistics 54
5.4 Results 54
5.4.1 *Escherichia coli* survival in soil and greywater 54
5.4.2 Lysimeters 55
5.4.3 Soil health indicators 65
5.5 Discussion 72
5.5.1 *Escherichia coli* in soil 72
5.5.2 *Escherichia coli* in leachate 74
5.5.3 Triclosan in soil 76
5.5.4 Triclosan in leachate 77
5.5.5 Soil health indicators 79
5.6 Conclusion 80
6. Education intervention 83
6.1 Introduction 83
6.1.1 The school. 84
6.1.2 Student engagement 84
6.1.3 Greywater experiment Introduction 85
6.2. Aim 86
6.3. Materials and method 86
6.3.1. Student engagement 86
6.3.2 Greywater experimental design 87
6.3.3. Hui 90
6.3.4. Student worksheet and survey 92
6.4. Results 93
6.4.1. Student involvement 93
6.4.2. Experimental results 93
6.5. Discussion 94
6.5.1 Student engagement 94
6.5.2 Greywater experiment 95
6.5.3 Hui 95

6.5.4 Student worksheet and survey 96
6.6 Summarising conclusion 96
6.7 Acknowledgements 98
7. General discussion 99
8. Conclusions 102
9. References 103
10. Appendix ..... A
10.1 Presentation school children gave at the hui ..... A
10.2 Worksheet and survey filled out by school children with guidance from Donna Andrews ..... H
Worksheet: I
Survey: ..... O

List of Figures

Figure 1: New Zealand rainfall 1971 – 2000 (Source: NIWA)..... 7
Figure 2: Chemical structure of triclosan (source: www.niehs.nih.gov)..... 13
Figure 3: Biodegradation and biotransformation of TCS in soil..... 15
Figure 4: The conversion of the colourless substrate, potassium p-nitrophenyl sulphate, to the yellow p-nitrophenol compound by the sulphatase enzyme. 20
Figure 5: The derivatisation process of TCS with MTBSTFA before analysis 29
Figure 6: Substrate induced respiration (SIR) at investigated TCS levels. The brackets on the graph indicate subsets of data where a statistically significant ($p<0.05$) change in respiration in relation to time was observed. 38
Figure 7: Microbial biomass at investigated TCS levels on day 17. Asterisk indicates difference of statistical significance ($p<0.05$) from the control calculated using Tukey's HSD test. 39
Figure 8: Microbial metabolic quotient at investigated TCS levels on day 17. The asterisk indicates differences of statistical significance ($p<0.05$) from the control calculated using Tukey's HSD test. 40
Figure 9: Sulphatase activity at investigated TCS concentrations on day 17. The asterisk indicates statistical significance from the control calculated using Tukey's HSD test..... 40
Figure 10: EC_{50} determined with sulphatase activity at investigated TCS levels. 41
Figure 11: EC_{50} determined with microbial biomass at investigated TCS levels..... 41
Figure 12: Sample sites, clock-wise from top left; Gisborne, Lincoln, Katikati 47
Figure 13: The excavation process of the lysimeters used in this study..... 49
Figure 14: Left: Lysimeters positioned in the facility. Right: the compartment at the bottom of the funnel in which the lysimeters sits is large enough to just accommodate sample collection containers. 49
Figure 15: Copies of the uidA gene in E. coli detected in each layer of the Gisborne soil indicated for all 4 treatments. Error bars were calculated using standard deviation. The graph's y-axis begins at the average detection limit for all datasets analysed. 56
Figure 16: Copies of the uidA gene in E. coli detected in each layer of the Katikati soil indicated for all 4 treatments. Error bars were calculated using standard deviation. The graph's y-axis begins at the average detection limit for all datasets analysed. 56
Figure 17: Copies of the uidA gene in E. coli detected in each layer of the Lincoln soil indicated for all 4 treatments. Error bars were calculated using standard deviation. The graph's y-axis begins at the average detection limit for all datasets analysed. 57
Figure 18: Copies of the uidA gene in E. coli detected in fortnightly leachate composites of the Gisborne soil. 58
Figure 19: Copies of the uidA gene in E. coli detected in fortnightly leachate composites of the Katikati soil..... 59
Figure 20: Copies of the uidA gene in E. coli detected in fortnightly leachate composites of the Lincoln soil..... 59
Figure 21: Concentration triclosan detected in each horizon of the Gisborne soil indicated for all 4 treatments. Error bars were calculated using standard deviation. 60
Figure 22: Concentration triclosan detected in each horizon of the Katikati soil indicated for all 4 treatments. Error bars were calculated using standard deviation. 61

Figure 23: Concentration triclosan detected in each horizon of the Lincoln soil indicated for all 4 treatments. Error bars were calculated using standard deviation. 61
Figure 24: Concentration TCS detected in fortnightly leachate composites of the Gisborne soil. 62
Figure 25: Concentration TCS detected in fortnightly leachate composites of the Katikati soil. 63
Figure 26: Concentration TCS detected in fortnightly leachate composites of the Lincoln soil. 64
Figure 27: Concentration Met-TCS detected in fortnightly leachate composites of the Lincoln soil. 64
Figure 28: Fortnightly rainfall for Mana Island. Mana Island was the closest weather station to where the experiment was conducted (Data supplied by Metservice). 65
Figure 29: Substrate induced respiration for the 0-5cm horizon of the Gisborne soil cores. Error bars were constructed using standard deviation. 66
Figure 30: Substrate induced respiration for the 0-5cm horizon of the Katikati soil cores. Error bars were constructed using standard deviation. 66
Figure 31: Substrate induced respiration for the 0-5cm horizon of the Lincoln soil cores. Error bars were constructed using standard deviation. 67
Figure 32: Microbial biomass for the 0-5cm horizon of the Gisborne soil cores. Error bars were constructed using standard deviation. 67
Figure 33: Microbial biomass for the 0-5cm horizon of the Katikati soil cores. Error bars were constructed using standard deviation. 68
Figure 34: Microbial biomass for the 0-5cm horizon of the Lincoln soil cores. Error bars were constructed using standard deviation. 68
Figure 35: Sulphatase activity for the 0-5cm horizon of the Gisborne soil cores. Error bars were constructed using standard deviation. 69
Figure 36: Sulphatase activity for the 0-5cm horizon of the Katikati soil cores. Error bars were constructed using standard deviation. 69
Figure 37: Sulphatase activity for the 0-5cm Horizon of the Lincoln soil cores. Error bars were constructed using standard deviation. 70
Figure 38: Microbial metabolic quotient for the 0-5cm horizon of the Gisborne soil cores. Error bars were constructed using standard deviation. 70
Figure 39: Microbial metabolic quotient for the 0-5cm horizon of the Katikati soil cores. Error bars were constructed using standard deviation. 71
Figure 40: Microbial metabolic quotient for the 0-5cm horizon of the Lincoln soil cores. Error bars were constructed using standard deviation. 71
Figure 41: Flow patterns created after 3 additions of greywater at the recommended rate for that soil type, and marking the infiltration front as soon as the irrigation stopped, and 5mins after. This was done for all 3 soils. 74
Figure 42: The 9 kōhūhū plants chosen for the experiment. Plants were grouped in 3 treatments with 3 replicates for each treatment. 88
Figure 43: The plants were irrigated twice each weekday for 7 weeks. 89
Figure 44: Measurements of stem height, assessment of appearance and leaf count were recorded twice weekly 90
Figure 45: The result boards containing the observational data and conclusions made by the students. These were presented at the hui to the community along with a PowerPoint presentation. 91

Figure 46: One of the students asked to represent her class and give the presentation containing the results and conclusions at the hui. 92

List of Tables

Table 1: Greywater quality (Sources: Western Australia department of health (2005). Code of Practise for Reuse of Greywater in Western Australia, and Australian National Guidelines for Water Recycling (2006)) 6
Table 2: Sequences of the primers used in the method optimisation for qPCR analysis 32
Table 3: The EC_{50} values for sulphatase and biomass as determined by the 50% decline in activity of the soil microbial community. The EC_{20} is a concentration of TCS (ppm) where 20% of the community's activity is affected. The R2 refers to figures 10 and 11. 41
Table 4: Physical properties of the 3 soils chosen for the experiment 50
Table 5: Composition of greywater used for lysimeter study (See footnotes1 & 2 for references) 51
Table 6: The survival of E. coli in soil after addition of greywater containing 10ppm triclosan. 55
Table 7: Constituents of synthetic greywater. *Selection of specific brands does not represent an endorsement by this research project. 89

1. Introduction and Aims

1.1 Introduction

Managing municipal water resources and minimising the impacts of wastewater discharge are critical elements of sustainable modern communities. The separation of greywater streams, specifically those from bathroom sinks, showers and laundry, from blackwater streams originating from toilets and kitchen sinks, is emerging as a potential water management tool (Casanova, Gerba, & Karpiscak, 2001; Chaillou, Gérente, Andrès, & Wolbert, 2011; Donner et al., 2010; Eriksson, Auffarth, Henze, & Ledin, 2002). There is an increase in the usage of greywater for irrigation especially in countries with arid regions, such as China, Australia and the south-western United States (Harrow & Baker, 2010; Waller & Kookana, 2009). In New Zealand, where water shortages are not typically an issue, greywater diversion is practiced as a means of excess water disposal, with little or no knowledge regarding environmental impact (Cass, Beecroft, & Lowe, 2012).

Current regional guidelines generally propose the use of diverted greywater for sub-surface irrigation of land, and restrict the use of greywater for irrigation to the property from which it originated (e.g. Kapiti Coast District Council). The reasoning behind such restrictions is that greywater contains an extremely variable and complex mixture of microbes and chemicals, potentially at high concentrations (Casanova et al., 2001; Gross, Kaplan, & Baker, 2007). These compounds can be considered to be a contaminant if present in the environment at high concentrations. Therefore, while greywater diversion is potentially beneficial from a water conservation and sustainability point of view, its use may be a high-risk activity with potential detrimental impacts on the environment and public health.

While greywater management is extensively practiced around the world, there is little NZ experience or scientific information, in particular regarding the impacts of biological and chemical contaminants of greywater on soils, groundwater and public health. There are no national guidelines or model health risk assessments for greywater use; hence the design of greywater application systems is difficult and inconsistent across the country.

Greywater contains a range of contaminants, including metals such as Zinc and Copper, nutrients such as phosphates and nitrates, and, micro-organisms such as *Escherichia coli* (Eriksson et al. (2002).

Untreated greywater also contains other organic contaminants including pharmaceuticals, fragrance fixing agents, preservatives and antimicrobial chemicals (Donner et al., 2010; Eriksson, Auffarth, Eilersen, Henze, & Ledin, 2003). These compounds are of concern as the practice of greywater reuse could possible introduce them into the environment.

One such a compound is triclosan (TCS; 5-chloro-2-[2, 4-dichlorophenoxy]-phenol) (Harrow, Felker, & Baker, 2011).According to Harrow and Baker (2010), TCS is the most commonly used antibacterial compound in the United States. In New Zealand, such antibacterial compounds are found in many common personal care products including toothpastes, hand washes and in sports clothing (McMurry, Oethinger, & Levy, 1998). Once these compounds enter a domestic greywater stream where the water is reused, there is a direct route for TCS to the receiving environment.

Not a lot is known about how triclosan affects microbial communities in soil when greywater containing the compound is used for irrigation (Harrow & Baker, 2010), and due to the compounds high affinity for organic matter (Butler, Whelan, Ritz, Sakrabani, & van Egmond, 2011) the unsafe irrigation of greywater could result in the accumulation of TCS in receiving soils.

Studies have shown that microbial function such as respiration, community composition and enzyme activity in soil is affected by the presence of TCS (Ali, Arshad, Zahir, & Jamil, 2011; McMurry et al., 1998; Waller & Kookana, 2009). Federle, Kaiser, and Nuck (2002) also found that TCS affects basic microbial functions analysing the effect of TCS on nitrifying bacteria by measuring bacterial respiration rates.

Gaining a greater understanding of the impacts of greywater diversion and disposal practices on the environment, particularly focusing on the long-term implications for soil, groundwater and public health, is essential for sustainable management of greywater reuse in New Zealand.

1.2 Aims

1.2.1 Determination of the EC_{50}[1] for triclosan in soil to inform the dosing rate for the lysimeter experiment.

1.2.2 Firstly, investigating the partitioning of TCS and *E. coli* through the soil profile of 3 different soils, and what the potential was for TCS and *E. coli* to leach through the soil profile, and secondly, investigating the effect potentially accumulated TCS has on soil health parameters.

1.2.3 Raising awareness to support behavioural change in a community (regarding the fate and effects of household chemicals in the environment) through the engagement with primary-level students while providing them with an authentic scientific experience.

1.3 Research approach

Greywater can be a valuable resource regardless of the drivers that motivate its reuse. Its reuse by application to soil however needs to be done with care, taking into account the public health risks and possible long term effects on soils. These effects have not yet been characterised in New Zealand soils. The research in this thesis was carried out to investigate the effect of TCS found in greywater on the receiving soil environment. The knowledge gained from this study will aide in the construction of consistent general greywater irrigation guidelines.

A combination of soil microbiology, biochemistry, molecular biology and analytical chemistry techniques were employed in this study with the aim of gaining more information on the fate and effects of greywater contaminants. A representative organic contaminant (TCS) and an indicator microbial contaminant (*E. coli*) were investigated.

The first phase of the study explored how the soil microbial community reacted to TCS exposure over time and the effects that TCS had on soil health parameters such as; soil bacterial respiration, enzyme activity and a stress biomarker; metabolic quotient. These indicators of soil health are used routinely in soil ecology for monitoring the impacts of land treatment, such as the application of sewage biosolids to soils (Speir, van Schaik, Hunter, Ryburn, & Percival, 2007).

[1] The EC_{50} of a contaminant is the concentration at which at least 50% of a specific microbial function is affected. It is the point half way between the baseline and the maximum of a microbial response after exposure to the toxin. It is a measure of toxic potency.

In the second phase of the study, the fate and effect of TCS and *E. coli* irrigated onto soil cores (lysimeters) with greywater were investigated. This aimed to understand the partitioning of TCS through the soil profile of 3 different soils, and what the potential was for TCS and *E. coli* to leach through the soil profile. The potential public health risk of pathogen exposure was explored by including various concentration of E. *coli* in the treatments.

There have been numerous studies of the fate of microorganisms in the waste water treatment process; however there is a knowledge gap on the survival of pathogens like *E. coli* in soil after irrigation with greywater. It is neither practical nor economical to continually monitor greywater for the presence of pathogens. It would therefore be valuable to gain an indication on the fate of a model organism such as *E. coli* in the soil environment after introduction to the soil environment by the irrigation of greywater. *Escherichia coli* is a practical indicator organism as it has a high abundance in comparison to other pathogens that might be introduced to soil. It is expected to behave in the same way as other pathogens due to similarities in life processes and it is easy and cost-effective to detect.

In chapter 3, the major methods employed in this study are presented. Chapter 4 investigates the effect of TCS on a silty clay loam soil. This chapter included an analysis of the SIR, sulphatase, microbial metabolic quotient and how the SIR for this soil changed over a period of 20days after initial exposure to various concentrations of TCS in a dose-response experiment.

The lysimeter study where the fate and effect of TCS was investigated in the soil environment is described in Chapter 5. The chapter includes the experimental protocols employed, results and a discussion on the observations made. The analysis performed in this study included the movements of TCS through the soil profile by measuring TCS in 3 different soil horizons, and the leaching of TCS by analysis of the TCS content of weekly collected leachate samples. Soil health parameters such as microbial biomass, sulphatase activity and SIR[2] were measured for the top 5cm of the soil cores. The movement of *E. coli* through the soil profile was also monitored by quantification of *E. coli* by qPCR[3]. This analysis was performed for the 3 soil horizons as well as the leachate.

Chapter 6 discusses the involvement of the scientific community at primary level education in order to raise awareness of the effects that greywater constituents might have on the receiving environment. The engagement involved helping the student formulate a scientific hypothesis, how to design an experiment and draw conclusions from observations. The

[2] Substrate induced respiration.
[3] Quantitative polymerase chain reaction

students learned about what contaminants might be in greywater while relating the knowledge gain back to the curriculum. The aim of the engagement was to promote consciousness of what is put down the drain.

The final discussion and future recommendations are presented in chapter 7, and Chapter 8 contains the final conclusions.

2. Literature review

2.1 What is greywater?

Greywater is defined as the water originating from wash basins, showers and laundry effluent in a household (Chaillou et al., 2011) and includes all untreated household waste water except for toilet water (McCormack, 2011). It typically contains high concentrations of soaps, oils and detergents from personal care products such as body-washes, laundry detergent, and hair care products (Gross et al., 2005). The composition of greywater varies within a household, depending on where it originates from. Greywater from laundry will contain lint, laundry detergents, sodium, and other nutrients such as nitrates and phosphates. Greywater from baths and showers will contain hair, toothpaste, body fat, oils, and faecal coliforms such as *Escherichia coli.* See Table 1 for chemical and nutrient composition. The composition and volume of greywater varies greatly between households (Casanova et al., 2001; Eriksson, Andersen, Madsen, & Ledin, 2009; Jefferson, Palmer, Jeffrey, Stuetz, & Judd, 2004). This is not surprising as the composition mainly depends on the number of inhabitants, their habits and age.

Table 1: Greywater quality (Sources: Western Australia department of health (2005). Code of Practise for Reuse of Greywater in Western Australia, and Australian National Guidelines for Water Recycling (2006))

Comparison of Greywater Quality		
Parameter	Greywater	
	Range	Mean
Escherichia coli/Thermotolerant coliforms/100ml	10^1 - 10^7	No value
Suspended solids (mg/L)	2 - 1500	99
BOD (mg/L)	6 - 620	430
Nitrate (mg/L)	<0.1 - 4.9	No value
Ammonia (mg/L)	0.06 - 25.4	2.4
TKN (mg/L)	5.0 - 25.4	12
Total Phosphorus (mg/L)	0.04 - 42	15
pH	5.0 - 10.0	8.1
Sulphate (mg/L)	7.9 - 110	35
Conductivity (mS/cm)	325 - 1140	600
Hardness (mg/L)	15 - 55	45
Sodium (mg/L)	29 - 230	70

The average greywater production in industrialised countries average between 100-150L of greywater per person per day (Friedler, 2004).

2.2 Greywater reuse drivers

New Zealand is a country of dramatically different climatic regions with climates varying in the North from warm sub-tropical, to a cool temperate climate in the South. There is also significant differences in annual rainfall in New Zealand with the west coast of the South Island receiving more than 6000mm of rain per year (Mackintosh, 2001).In contrast to this, areas less than 100km from the west coast experience rainfalls as low as 400mm annually (Mackintosh, 2001).There is a clear spatial difference in rainfall (Fig. 1).

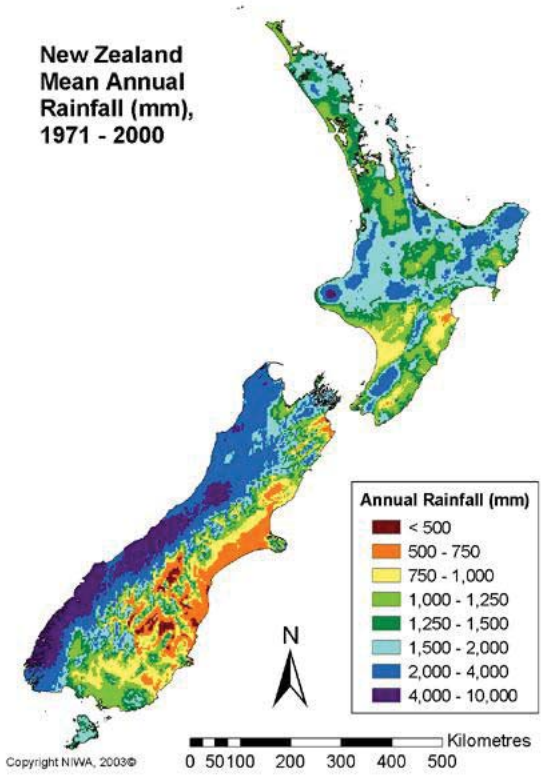

Figure 1: New Zealand rainfall 1971 – 2000 (Source: NIWA)

In the North and central areas of the country, the rainfall is predominantly spread out throughout the year with a dry period during the summer. In the South Island, winter gets

least rainfall (Mackintosh, 2001). This indicates that there are spatial as well as temporal differences in national rainfall patterns.

With soil in New Zealand being such an important resource, it is important to remember that the country's soil can be as variable is its climate. Soils are classified using various properties of the soils (McLaren & Cameron, 1996). These properties might include the chemical and physical properties such as soil texture, structure and history. Water holding capacity (WHC) is related to the soil texture and composition as it is dependent on the size of the pores in the soil (McLaren & Cameron, 1996). WHC determines how much water the soil is able to retain. Soil function is related to the properties and moisture content of soil and is thus influenced by the amount of water it receives.

2.2.1 Water shortages

Where soils with low WHC occur in a low rainfall region, there might routinely be soil water deficits (SWD) causing water shortages. These water shortages could become more pronounced by a changing climate, urbanisation and the cultivation of an increased awareness to conserve water (Harrow et al., 2011). With modernisation, household water budgets in both industrialising and industrialised nations are on the increase, and fresh water resources for domestic use are becoming all the scarcer. Along with other options such as the use of rainwater from collection tanks, the reuse of greywater as a resource reduces the requirements for potable water in a household. There is an increase in the usage of greywater for irrigation especially in countries with arid regions, such as China, Australia and the south-western United States (Harrow et al., 2011; Waller & Kookana, 2009).

2.2.2 Surplus water

In New Zealand, where water shortages are not typically an issue, greywater diversion is often practiced as a means of excess water disposal (Siggins et al., 2013). The practice can assist in alleviating the strain on the receiving environment after waste water treatment, as well as on waste water treatment infrastructure. The practice could be beneficial for reticulated as well as unarticulated areas. Siggins et al. (2013) found that the treatment efficacy and life of old and poor-functioning septic tanks could be improved by diverting the greywater fraction of the household waste water. The greywater diversion in this study was done with a manufactured

Watersmart® greywater diversion system. The system makes use of sub-surface irrigation of the greywater onto soil.

An increased awareness of the need to conserve potable water also drives greywater reuse. This is, however, frequently done without proper knowledge of greywater constituents, its environmental fate and effects and the potential public health risks associated with irrigating greywater without proper knowledge of how to do so responsibly (Cass et al., 2012).

2.3 Risks and benefits associated with greywater reuse

2.3.1 Risks

There are potential health and environmental risks associated with the reuse of greywater. These risks arise when a reuse system functions poorly, or is overloaded. The risk is mainly attributable to the over irrigation of soil to beyond its capacity. This results in waterlogging of the soil and ponding of greywater on the surface (Cass et al., 2012). The greywater could then potentially damage soil systems, bring people into contact with pathogens such as *E*. *coli*, and the untreated greywater might make its way to receiving water ways.

In a public survey conducted in (Cass et al., 2012), 42 households were interviewed on their opinions regarding water conservation, environmental health risks, regulations, and greywater promotions and information associated with greywater reuse. The perceived risks associated with greywater reuse as identified in the survey include the following:

• Health concerns, particularly from households with a higher risk of faecal content in their greywater;
• Odour (including due to hydrogen sulphide);
• Pets bring potential contaminants into the house after being in contact with the greywater applied outside;
• Contamination of neighbouring properties and potential complaints;
• Soil contamination;
• What will happen when someone takes over a greywater system in their new home and are not interested in its management;
• Long term impacts on the soil;
• The difficulty of managing the soaps and fat content of the greywater;

• The Regional Council will make changes that will negate greywater systems that have been installed;
• NZ greywater systems require more precision than Australian systems;
• Less water in the reticulated system will cause blockages;
• The nutrients from the greywater area are an important part of the solid waste breakdown therefore inappropriate to remove it;
• Sodium will damage soils to which greywater is applied; and
• Phosphorus
(Results taken directly from report by Cass et al. (2012))

2.3.2 Benefits

It is clear that there are positive and negative aspects to the reuse of greywater. Some of the positives include that the reuse of greywater will reduce the demand for the use of potable water (McCormack, 2011). There could also be significant benefits for municipal waste plants. The volume of discharge water would be greatly reduced if greywater diversion/reuse were to be employed on a large enough scale. The nutrients occurring in greywater will not reach coastal waters like 60% of New Zealand sewage (McCormack, 2011).

The reuse and subsequent irrigation of greywater also hold benefits for the garden. It will relieve water stress in plants if the soil is at a soil-water deficit, and nutrients from the greywater will have positive effects on plant nutrition. It thus has positive economic implications if the practise is employed in an area where water is metered for example Kapiti Coast.

The load on old and failing septic tanks may also be alleviated, allowing the septic tank to operate more efficiently (Siggins et al., 2013).

2.4 Greywater composition

2.4.1 Microbiological quality

The presence of faecal coliforms is routinely used as a general indicator of the microbial quality of water (Glassmeyer et al., 2005). The detection of faecal coliforms (FCs) in water indicates a potential health risk to humans. In greywater, the abundance of FCs are relatively low, however, the levels might rise to those typically found in blackwater as a result of washing nappies and clothing that might have been contaminated with faeces or vomit (Birks, Colbourne, & Hobson, 2004; Birks & Hills, 2007).

In the majority of New Zealand households where greywater diversion or reuse is practised, greywater is irrigated onto soil without being treated. Several studies have shown that untreated greywater may contain faecal contamination by the presence of indicator organisms such as *E. coli* (Birks & Hills, 2007; Casanova et al., 2001; Eriksson et al., 2009). A study conducted by Birks and Hills (2007) characterised the greywater from a block of flats with a dual-reticulation system. That study confirmed that levels of *E. coli* as high as 10^7 CFU/100ml could be found in a "real" source of greywater. There is thus a potential to increase the number of faecal coliforms in soil by irrigation of untreated greywater (Negahban-Azar, Sharvelle, Stromberger, Olson, & Roesner, 2012). It has been shown that microbes can survive in soil for long periods of time. *E. coli* has been reported to survive for weeks and under the ideal conditions (Mawdsley, Bardgett, Merry, Pain, & Theodorou, 1995). X. Jiang, Morgan, and Doyle (2002) found that *E. coli* was able to survive in soil amended with manure for longer than 200 days. The survival of *Salmonella typhimurium* have been illustrated to reach beyond 70 days in an experiment conducted by Turpin, Maycroft, Rowlands, and Wellington (1993) where the bacteria was cultured in constructed microcosms at 22°C and at 15% moisture content.

Negahban-Azar et al. (2012) measured the number of faecal indicator organisms in soil that has been irrigated with greywater for 30yrs and compared it to soil on the same section that had been irrigated with fresh water. The study investigated soil from a selection of states, and in one soil from Texas, *E. coli* levels of 543 CFU/g soil were detected in the top 15cm of the soil horizon. More interesting is the detection of *E. coli* levels up to 1093 CFU/g soil in the 30-100cm layer. *E. coli* levels found in the areas where freshwater was irrigated ranged from between 136 CFU/g soil and 160 CFU/g soil for the 0-15cm and 30-1400cm layers respectively.

When numbers of *E. coli* are elevated in soil, there is an increased risk for *E. coli* to leach through the soil profile and be carried off site. A study conducted by Jiang et al. (2010) have shown that some soils are prone to preferential flow and that indicates that there is potential for *E. coli* to leach through the soil profile and potentially impairing groundwater quality. Pang et al. (2008) conducted a study where the transport of microbes was modelled through the soil profile of ten undisturbed soils. Microbial breakthrough curves were assessed for the lysimeters and it was concluded that rapid breakthrough of microbes in the leachate in structured soils with low moisture content could be as a result of bypass flow. The conclusions of that study support the hypothesis that transport of microbes through the soil profile by preferential flow is mediated by a compromised integrity of the soil's macroporosity.

Birks and Hills (2007) argued that the presence of *E. coli* in greywater does not infer the presence of pathogens, however if pathogens are present in greywater, their movement through the soil could be compared to the movement of *E. coli*. Monitoring the movement of *E. coli* thus provides an easy and relatively economical way of assessing the health risk associated with the irrigation of untreated greywater into soil.

2.4.2 Nutrients in greywater

Amongst other nutrients, greywater contains phosphorus and nitrogen-containing compounds such as nitrates and ammonia arising from the use of detergents such as washing powder, shampoo, and other personal care products (Rodda, Salukazana, Jackson, & Smith, 2011). These nutrients might be beneficial for plants and could potentially reduce the use of commercial fertilisers.

2.4.3 Chemicals in greywater

According to Eriksson et al. (2002), greywater contains a range of contaminants, including metals such as Zinc and Copper. It also contains other organic contaminants. These include pharmaceuticals, fragrance fixing agents, preservatives and antimicrobial chemicals (Donner et al., 2010; Eriksson et al., 2003). These compounds are of concern as the practice of untreated greywater reuse could directly introduce them into the environment.

One such a compound of concern is triclosan (TCS; 5-chloro-2-[2, 4-dichlorophenoxy]-phenol) (Harrow et al., 2011).According to Harrow and Baker (2010), TCS is the most commonly used antibacterial compound in the United States. In New Zealand, such antibacterial compounds are found in many common personal care products including toothpastes, hand washes and in sports clothing (McMurry et al., 1998). Once these compounds enter a domestic greywater stream where the water is reused, there is a direct route for TCS to the receiving environment.

With the use of 2 supermarket products (e.g. toothpaste and mouth wash) containing TCS, used at realistic amounts, it is possible that approximately 450mg of TCS could enter the environment from a single household's greywater over a period of 10 years.

2.5 Triclosan (TCS), Chemical properties

Triclosan is a white crystalline powder with a bulk density of 1.55 x 10^3 kg/m^3 and a solubility of 0.0010g/L in water at 20°C (TOXNET, 2004). However, TCS has an increased solubility of more than 100g/L in organic solvents such as acetone. Triclosan is also known as Irgasan.

Triclosan is a polycyclic organic compound (fig.2) that was introduced and used for its antibacterial and antifungal properties in the 1960s (Ledder, Gilbert, Willis, & McBain, 2006).

Its effectiveness against bacteria such as *E. coli* is presumably due to fatty acid synthesis, and cell lysis at higher concentrations (McMurry et al., 1998).Triclosan may also influence the transcription of genes related to lipid, carbohydrate and amino acid metabolism (Reiss, Lewis, & Griffin, 2009).

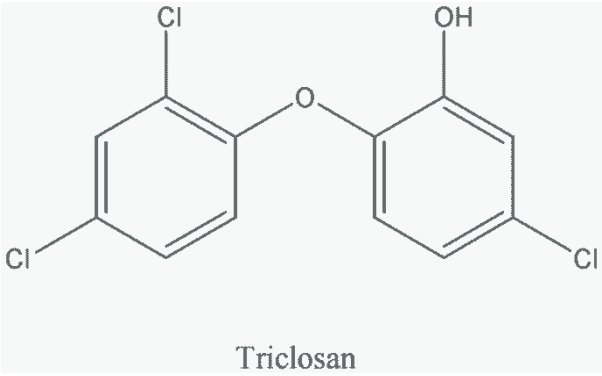

Figure 2: Chemical structure of triclosan (source: www.niehs.nih.gov)

Recent studies have also suggested that there is a link between TCS resistance in microbial communities and antibiotic resistance in bacteria (McMurry et al., 1998). In contrast, Ledder et al. (2006) concluded that bacterial colonies exposed to TCS did not undergo a significant increase in resistance to a range of antibiotics, including tetracycline, ciprofloxacin, nalidixic acid and chloramphenicol. Not a lot is known about how triclosan affects microbial communities in soil when greywater containing the compound is used for irrigation (Harrow & Baker, 2010).

Triclosan has a dissociation constant (pKa-value) of 8.14 according to Butler et al. (2011). It is therefore a weak acid, more stable in the protonated form. The effects of soil pH could therefore affect the mobility of TCS due to its ionisable nature. The deprotonation of TCS at high pH to produce the phenolate-ion, a negatively charged particle, will facilitate the compound's transport through the soil profile due to repelling effects of some organic colloids

and clay particles. This indicates that TCS might not strongly associate with soil particles. In contrast, Agyin-Birikorang, Miller, and O'Connor (2010) concluded that TCS desorption is much slower than the desorption in a retention-release study. This indicates that triclosan does form strong associations with organic matter. In addition, Butler et al. (2011) noted that TCS would have a high affinity for organic matter due to its log K_{OW} of 4.78 (log octanol:water partition coefficient). This could result in the accumulation of TCS in receiving soils. In that study it was found that the addition of TCS to soil does have an effect on the substrate induced respiration (SIR) of soil microbial communities. This indicates the bioavailability of TCS in the soil environment.

2.5.1 Fate of TCS in soil

There are many factors affecting the fate of TCS in soil such as the soil composition, climatic factors, and other abiotic factors such as soil moisture. Triclosan could be transported through the soil profile to enter groundwater reserves, or it could be degraded or transformed by soil microbes to other compounds. Another form of degradation is by sunlight. The variety and composition of microbial communities in soil could impact on the amounts and types of biodegradation products formed.

Abiotic factors also the fate of TCS in soil (Butler, Whelan, Sakrabani, & van Egmond, 2012). These include the soil temperature, moisture content and the type of land-use.

2.5.1.1 Transport through soil

Butler, Whelan, Sakrabani, et al. (2012) found that traces of TCS leaches through the soil profile over time despite its strong affinity for adsorption to soil particles. Soil texture and composition also affects the fate of TCS. In that study it was indicated that TCS leaches through the soil profile and that the soil texture could influence the fate of the compound's partitioning. It was indicated that TCS more strongly associates with clay soils when compared to sandy soils and loamy sand soil.

Yet Kwon and Xia (2012) found no leaching of TCS through the soil horizon. In that study soil columns containing sandy soil were amended with a layer of soil which had been spiked with TCS to a concentration of 760mg/kg TCS per dry weight of soil. The columns were subsequently irrigated with water. After 101 days of irrigation, the soil horisons and leachate from the cores were analysed for TCS. They concluded that their inability to detect TCS in the

leachate was due to high sorption to soil and that TCS had limited transport through the soil profile.

The soil columns used for this study were not intact, but instead repacked and can therefore not be compared to studies using intact soil cores. Kwon and Xia (2012) did, however, find that there was leaching of TCS in soil columns amended with biosolids containing TCS, bolstering the theory the TCS adsorbs to organic particulates.

Due to TCS's affinity for organic compounds, particulate matter and the variability of soil characteristics of New Zealand soils, it follows that TCS bioavailability and transport within the soil profile would vary between soils.

2.5.1.2 Degradation

Triclosan is available to bacteria for uptake and is readily degraded or transformed to products that might be even more persistent in the environment that TCS itself (Gangadharan Puthiya Veetil, Vijaya Nadaraja, Bhasi, Khan, & Bhaskaran, 2012). Typically the breakdown products in soil include 2, 4-dichlorphenol (2, 4-DCP) and 4-chlorocatechol (Fig 3).

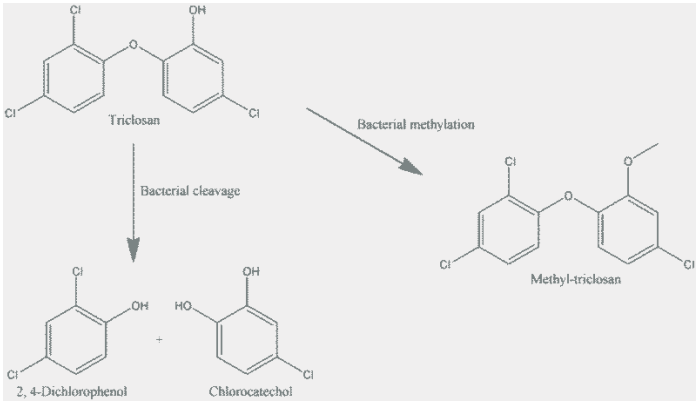

Figure 3: Biodegradation and biotransformation of TCS in soil.

Triclosan is also photolytically degraded to 2, 4-DCP and 2, 8-dichlorodibenzo-*p*-dioxin (2,8-DCDD) (Latch, Packer, Arnold, & McNeill, 2003). 2, 4-DCP in a liquid form and 2,8-DCDD is absorbed through the skin, and excessive exposure might have serious health complications and could even result in death (Latch et al., 2005).

2.5.2 Effects of TCS in soil

2.5.2.1 Microbial respiration

Microbial function in soil is affected by the presence of TCS. Ali et al. (2011) noted a statistically significant effect of TCS on soil microbial respiration by measuring a decrease ^{14}C-glucose mineralisation with increased TCS concentration. Butler et al. (2011); McMurry et al. (1998) and Waller and Kookana (2009) concluded the same.

The effect however, that Waller and Kookana (2009) observed was not as dramatic as the effects reported by Butler et al. (2011) where much higher concentrations were used. Using higher concentrations of TCS might be a better approach to describe the effects of TCS accumulation in soil and impacts on soil microbial communities. It has also been demonstrated by Federle et al. (2002) that TCS affects basic microbial functions analysing the effect of TCS on nitrifying bacteria by measuring bacterial respiration rates.

2.5.2.2 Microbial community composition

It is also possible that the composition of soil microbial communities is altered after exposure to TCS. Harrow et al. (2011) demonstrated that soil microbial communities and bacterial numbers changed in soils irrigated with greywater containing TCS. However McNamara and Krzmarzick (2013) found that the composition of a soil microbial community remains intact. That study was conducted using minute amounts of TCS and didn't provide for the concentrations of TCS residues in soil after accumulation.

2.5.2.3 Enzyme activity

Soil enzyme activity could be used to assess the health of soil microbial communities (Winding, Hund-Rinke, & Rutgers, 2005). Liu, Ying, Yang, and Zhou (2009) demonstrated how the presence of TCS in soil affected the soil enzyme activity. In this study it was concluded that phosphatase activity was significantly inhibited by concentrations of TCS between 0.1mg/kg dry weight of soil and 50mg/kg dry weight of soil, although the soil microbial community recovered in the later stages of a 23 day incubation process. In contrast Waller and Kookana (2009) measured the effect of TCS on four enzymes. Only one enzyme, β-glucosidase responded negatively to the addition of TCS.

2.5.2.4 Microbial stress

Soil microbial communities show signs of microbial stress when exposed to TCS. Park, Zhang, Ogunyoku, Young, and Scow (2013) observed an increase in microbial stress indicators when soil microbes were exposed to soils and biosolids amended with TCS. The increase in microbial stress biomarker used (cy17/precursor ratio) correlated positively with the increase in TCS concentration. This particular stress indicator was chosen as bacteria responding to stress from the environment react by transforming a *cis*-double bond in the cell membrane in order to achieve greater cell membrane stability. The indicator was measured by extraction cell membrane phospholipids and measuring by gas chromatography.

The effect of TCS might be amplified in the presence of co-contaminants. A study conducted by Horswell et al. (2014) investigated possible effects that co-contaminants to TCS, such as copper (Cu) and zinc (Zn), might have on the soil microbial community. It this study, various amounts of Zn, and Cu were added to soil. Various amounts of TCS were also added to investigate the environmental effects of a complex cocktail of chemicals on a soil eco-system. It was concluded that the presence of toxic metals such as Zn and Cu enhance the persistence of TCS in the soil environment (Horswell et al., 2014). The chemical cocktail had a synergistic effect on soil health indicators.

3. Methods

3.1 Moisture content, dry matter content and water holding capacity

The moisture, dry matter content and water holding capacity for each soil was determined from the bulk soil collected at each sample site.

3.1.1. Moisture and dry matter content:

Field moist soil was sieved to < 2mm in order to break up soil aggregates, remove vegetation and debris and homogenise the samples. For each soil type, triplicate subsamples containing a recorded weight of no less than 10g of soil, were weighed into porcelain crucibles. The samples were dried at 105°C for 24hrs to evaporate all moisture. Samples were allowed to cool to room temperature in a desiccator containing blue silica gel, and the dry weights were recorded to 3 decimal places.

The dry matter and moisture content were calculated as below:

Fresh weight (g) = (crucible + fresh soil) — crucible (g)

Dry weight (g) = (crucible + dry soil) — crucible (g)

$$\text{Soil Dry matter (DM \%)} = \frac{\text{Dry weight of soil (g)}}{\text{Fresh weight of soil (g)}} \times 100$$

Soil Moisture content (MC%) = 100 – DM

3.1.2 Water-holding capacity (WHC):

WHC is the maximum potential volume of water held by 100g of soil.

Eleven plastic funnels with a diameter between 7cm and 10cm were prepared by placing 0.25g (± 0.05g) cotton wool in the top of the stem. The cotton wool was then loosely compacted with a narrow spatula. The bottom ends of the funnels were plugged with Blu-tack® to prevent leakage and the funnels were then placed in 25ml measuring cylinders.

Soil samples (25g ± 0.05g) were weighed in triplicate and placed in the funnels. The test also included two blank funnels that contained everything except soil.

Exactly 25ml of water was poured into each funnel. The Blu-tack® plugs were removed 30min later to allow the filtrate to drip through into the measuring cylinders. The volume of water retained by 100g of soil was calculated as follows:

WHC = 4x (volume collected from blanks – volume collected from samples) + MC

3.2 pH

Four grams (±0.05g) of moist soil was weighed into 50mL glass beakers and 10ml deionised water was added. The mixture was stirred with a glass rod, capped and left overnight to equilibrate. The pH was measured on a Thermo-Orion Model 310 pH meter by glass electrode.

3.3. Substrate induced respiration

Substrate induced respiration (SIR) was performed according to Degens and Harris (1997), and Campbell, Chapman, Cameron, Davidson, and Potts (2003). The apparatus used for soil loading and the trays containing the reaction wells in this analysis was produced by Macaulay Enterprises Limited and the method suggested by the manufacturer followed. The method entailed the capture of bacterial carbon dioxide (CO_2) produced after the addition of an appropriate substrate (glucose in this case) and measurement of the absorbance of a microtitre plate filled with pH sensitive agar. The CO_2 was absorbed into the agar and altered the pH in each well and a colour change from pink to yellow was observed. The difference between the initial absorbance of the well and the absorbance after 6h indicated the amount of CO_2 produced during the test time period.

3.4 Sulphatase enzyme activity

The sulphatase enzyme is an important indicator of soil biological processes, soil fertility and therefore soil health. Soil sulphatase activity was measured using the *p*-nitrophenyl colorimetric method employed by Speir (1984) and Tabatabai (1970) with slight modifications. Briefly, the method involves the addition of potassium *p*-nitrophenol sulphate as a substrate to the soil sample. Enzyme substrate (0.5ml) was added to 0.5g of each soil sample and the reaction was allowed to continue for 4hrs.

During that time the substrate was converted to *p*-nitrophenol, by the activity of the sulphatase enzyme. *P*-nitrophenol is a yellow coloured complex in the presence of a base (NaOH) and is quantifiable by spectrophotometry (Fig 4). The quantity of *p*-nitrophenol can then be directly related to sulphatase enzyme activity.

Calcium chloride ($CaCl_2$) was added before the base to prevent the dispersion of clay particles in the soil as the organic material extracted by NaOH might have interfered with the colorimetric analysis of the yellow compound. Dispersed clay particles also hindered the filtration of the extract.

Prior to spectrophotometric analysis, samples from the Lincoln soil samples were diluted 1:50 with Milli-Q water and the absorbance analysed at 400nm on a Varian Cary 50 spectrophotometer.

Sulphatase activity of the 0-5cm fraction of soils from the lysimeter study (Chapter 4) was diluted 1:4 with deionised water and the absorbance analysed on a FLUOstar OPTIMA spectrophotometer by BMG LABTECH at 405nm.

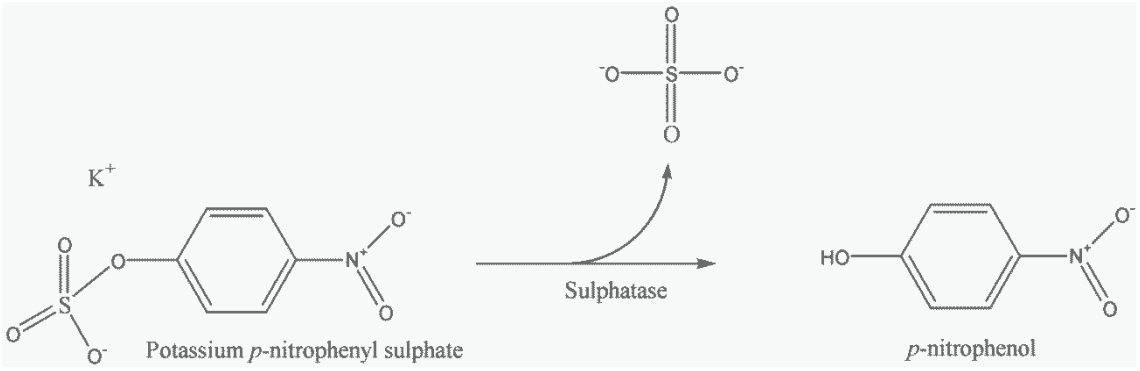

Figure 4: The conversion of the colourless substrate, potassium p-nitrophenyl sulphate, to the yellow p-nitrophenol compound by the sulphatase enzyme.

3.5 Microbial biomass

Two replicates of soil were sieved to <2mm and adjusted to 60% WHC. Triplicate sub-samples of each sieved soil (2g ± 0.002g) were placed into 20ml glass screw-top vials. Two sets of triplicate blanks containing no soil were also prepared. The total organic carbon (TOC) of one set of triplicate samples and blanks was extracted immediately and the other set was fumigated for 24hrs prior to TOC extraction. The fumigation with dichloromethane (DCM) caused the death of soil microbes. Consequently the carbon-contents of the cells became available for extraction.

TOC was extracted as follows: 8ml of 0.5M potassium sulphate (K_2SO_4) was added to each sample. The screw cap was tightly secured and the vial was shaken for 30min on an Orbit Shaker (Labline) at 3500 revolutions per minute (RPM). The resulting extract was filtered (Whatman No. 1 filter paper placed in 5cm diameter funnels) into sterile 50ml centrifuge tubes. Opaque samples were filtered a second time. The resulting filtrate was transferred to 15ml polypropylene Falcon centrifuge tubes and diluted 1:3 with deionised water.

The fumigation method was adapted from Vance, Brookes, and Jenkinson (1987). Samples to be fumigated were placed in a desiccator containing wet paper towel under the bottom of the grate. The paper towel aided in the regulation of the humidity in the fumigation chamber. A 25ml vial containing soda lime (mixture of chemicals containing KOH, NaOH, $Ca(OH)_2$) was added. A 50ml glass beaker containing approximately 5 boiling stones and no less than 30ml of chloroform ($CHCl_3$) was placed in the middle of the desiccator between the samples. The lid of the desiccator was secured using petroleum jelly and a rubber seal ring before a vacuum was applied to evacuate the desiccator chamber. The chloroform was allowed to boil for 2min under vacuum, after which the vacuum was turned off and the valve at the top of the desiccator was closed. The assembly was left to fumigate in the dark for 24hrs at 25°C before TOC extraction.

After extraction, all extracts were sent to the Soil and Physical Sciences Department at Lincoln University for analysis of TOC on a Shimadzu analyser (TOC-5000A).

Calculation of microbial biomass:

$$\text{Total Organic Carbon (mg / kg soil)} = \frac{[\text{TOC (mg/L)} - \text{blank (mg/L)}] \times 0.008\text{L extract}}{0.002\text{kg soil}}$$

$$\text{Microbial biomass (mg microbial C / kg soil)} = \text{TOC (Fumigated)} - \text{TOC (Unfumigated)}$$

3.6 Microbial metabolic quotient

The microbial metabolic quotient was determined according to Anderson and Domsch (1986) by calculating the quotient between substrate induced respiration and the microbial biomass, i.e. substrate induced respiration / microbial biomass. This is expressed as µg CO_2-carbon / mg microbial-C / hour.

3.7 Triclosan analysis

3.7.1 Extraction from soil

After the destructive harvesting and subsequent subsampling of the 0-5cm, 5-10cm, and 10-30cm horizons of the soil lysimeters (Chapter 5) at the end of the experiment, the resulting subsamples sieved <2mm stored at -20°C until extraction (completed at Plant and Food Research in Ruakura, Hamilton). The Method development and optimisation for TCS extraction and analysis in both soil- and leachate-samples, was done by Dr. Grant Northcott (Northcott Research Associates, Hamilton).

3.7.1.1 Accelerated solvent extraction (ASE)

Accelerated solvent extraction provided an efficient and rapid way to extract TCS from the soil samples before solid phase extraction, elution and derivatisation of TCS before analysis by gas chromatography mass-spectrometry.

The samples were extracted in 22ml stainless steel extraction cells. Paper filters used to retain the sample and filter the sample solvent extracts were pre-cleaned by soxhlet extraction

(approximately 5hrs at 6 cycles/hour) using a solvent mixture of dichloromethane/acetone (1:1 v/v). Following solvent extraction the filter papers were dried overnight at room temperature.

Two filter papers were loaded into the barrel of an extraction cell and compacted into the base of the extraction cell. A level spatula full of Hydromatrix (a beaded form of diatomaceous earth) was added onto the top of the paper filters to allow for further filtration.

The dry weight equivalent of 5g of soil was weighed and combined with 3.5g of hydromatrix in a plastic weighing tray. Hydromatrix was used to assist the drying of the soil sample and increase the friability of soil, and therefore the penetration of solvent into the sample matrix. The soil-hydromatrix mixture was ground to disperse and break up soil aggregates. The ground mixture was transferred to the 22ml stainless steel extraction cell, compacted and the dead-space in the barrel of the extraction cell filled with Ottawa sand which had been pre-extracted with DCM. A spike recovery standard containing TCS-d3 (triclosan with three deuterium atoms), Me-TCS-d3 (methyltriclosan, with three deuterium atoms) and bisphenol-C in acetone was added to the sample. A cellulose filter was placed on top of the sample and the top pressure cap firmly screwed into place. The cell unit was then placed in the extraction module where it was extracted with a 1:1 solution of acetone and Milli-Q water. Each batch of extracted samples included a method blank comprised of 3.5g Hydromatrix, and Ottawa sand, and a control soil sample spiked with the previously described deuterated recovery standard and a mix of the target analytes in acetone. The mix of target analytes contained triclosan and the microbial degradation products methyl triclosan, 2, 4 dichlorophenol, and 4-chlorocatechol.

The soil samples contained within the stainless steel extraction cells were extracted using a Dionex ASE200. The soil samples were extracted with a mixed solution of acetone-water (1:1 v/v) at a temperature of 100°C and pressure of 1500psi. The samples were statically extracted for a period of 5min after which solvent extract was transferred into a 60ml glass vial along with 12ml of acetone-water used to dynamically flush and rinse the contents of the extraction cell.

The extracts collected in the 60ml glass vials were concentrated using a Zymark TurboVap sample concentrator at 35°C under a flow of nitrogen gas in order to partition the target analytes into the aqueous phase. The sample extracts were concentrated until all the acetone had evaporated or less than half the original sample volume remained. A volume of 40ml of phosphate buffer (prepared by dissolving 14g of KH_2PO_4 and 20g of K_2HPO_2 in 2L of Milli-Q

water) adjusted to pH7 with 6M KOH was added to each vial and mixed before solid phase extraction.

3.7.1.2 Solid phase extraction (SPE)

3.7.1.2.1 Cartridge conditioning

Strata-X 33u polymeric reverse phase solid phase extraction cartridges (6ml, 500mg supplied by Phenomenex NZ Ltd) were pre-conditioned prior to extracting the target compounds from the phosphate buffer sample extracts. The SPE cartridges were positioned in an IST solid phase vacuum extraction (SPE) manifold with the taps closed to prevent flow through the cartridges. An aliquot of 5ml DCM:MeOH (95:5%) was added to each cartridge, the taps opened, and the solvent mix allowed to flow through the SPE cartridges under gravity and into the lower glass collection tank. When half the solution had passed through the SPE cartridges, the taps were closed for 2min to allow the solvent mixture to permeate and soak into the SPE adsorbent bed. Following this equilibration period the taps were opened and the solvent mix drained through the SPE cartridges. This rinsing procedure was repeated and vacuum applied to remove residual solvent and dry the cartridges.

Methanol (5ml) was added to the SPE cartridges and vacuum applied to initialise flow through the cartridge. As soon as flow was initiated the vacuum was turned off and the solvent allowed to flow under gravity. As soon as half the methanol had eluted through the SPE cartridges the taps were closed to allow the methanol to soak into the adsorbent bed. After 2min the taps were reopened and the methanol was allowed to drain through the cartridges until it was level with the top of the SPE adsorbent bed, at which point the taps were closed. Another 5ml of methanol was added to the SPE cartridges and this procedure repeated.

Two individual 5ml aliquots of Milli-Q water were added in the same way as the methanol to ensure complete removal of the solvent without exposing the column bed to air. The last 5ml aliquot of water was left sitting on top of the cartridge bed.

Strata Florisil SPE cartridges (6ml, 1 gram, supplied by Phenomenex NZ Ltd) were mounted on a separate SPE manifold and with a 3cm layer of anhydrous Na_2SO_4 added to the top of the florosil bed. The Florisil SPE cartridges were rinsed with 5ml of acetone. When approximately half the acetone had passed through the column, the taps were closed to allow the cartridges to equilibrate. The taps were opened after 2min and the solvent allowed to drip under gravity

until it reached the top of the column bed. The process was repeated and as soon as all the acetone was drained off, full vacuum was applied to remove all traces of acetone.

3.7.1.2.2 Cartridge loading

Teflon end caps were placed in the top opening of the Strata-X SPE cartridges and Teflon transfer tubes were connected to each SPE-cartridge with the other end of the tubes placed into the bottom of the corresponding sample vial. The taps of the SPE manifold were opened and a slight vacuum applied to initialise and maintain flow from the sample vials into the SPE cartridges at a flow rate of approximately one drop per second. Flow was discontinued when the sample had passed through the SPE cartridge. The sample vials were washed individually three times with Milli-Q water, ensuring all sides were rinsed, to ensure quantitative recovery of the sample. The water rinses were similarly passed through the Strata-X SPE cartridges. After rinsing the SPE cartridges were dried under full vacuum for at least 10min to ensure removal of residual water. The glass sample vials were turned upside down to allow them to dry.

3.7.1.2.3 Elution of cartridges and primary clean-up

The Strata-X SPE cartridges containing the samples were connected to the tops of the Florisil cartridges on the SPE manifold using Teflon SPE connector plugs. Amber glass EPA sample collection vials (40ml) were placed in a rack within the collection tank under the corresponding SPE cartridges. The dried sample vials were rinsed with 2 aliquots of 5ml DCM:MeOH (95:5%) and the solvent added to the corresponding Strata-X SPE cartridges. The flow of solvent was initiated by applying a light vacuum to initialise flow. The flow of solvent through the combined SPE cartridges was set to one drop per second. A further 25ml of the DCM:MeOH (95:5%) solvent mix was passed through the SPE cartridges to elute the target analytes from the Strata-X SPE cartridge, onto the Florisil SPE cartridge where soil matrix components were retained, and into the collection vials. When the elution solvent had passed through the combined SPE cartridges, vacuum was briefly applied to recover residual solvent. The amber sample collection vials were removed from the SPE manifold, capped, and stored at 4°C until sample concentration.

3.7.2 Soil sample concentration and derivatisation

The sample extracts were concentrated under a stream of nitrogen gas using a TECHNE® sample concentrator supplied by Total Lab Systems Ltd. The heating plate of the sample concentrator was heated to 30°C and the solvent evaporated under a constant nitrogen stream until approximately 500µl of DCM:MeOH extract remained in the vials. A note was made of any samples that completely evaporated and 500µl DCM:MeOH was added to resolubilise the sample. The concentrated sample extracts were transferred to 3ml conical base glass Reacti-vials with 3 consecutive washes of approximately 750µl DCM. Before the sample extracts were concentrated again, an aliquot of internal standard solution containing 2, 6-*di*bromo-4-methylphenol and deuterated bisphenol-A (BPA-d_{16}) was added to the sample vials. The samples were concentrated using the TECHNE® sample concentrator until nearly dry. An aliquot of 50µL of ethyl acetate was added to each sample and the sides of the capped vials rinsed by gently rotating them. A 30µL aliquot of the derivatisation reagent, *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA, SIGMA-ALDRICH®, >97%) was added to the sample vials and the reaction initiated by vortex-mixing the vials for 3 seconds. The sample reaction mix was left to react at room temperature for 30min and the volume adjusted to 1ml with ethyl acetate. The contents of the Reacti-vials were mixed by vortex and the ethyl acetate sample solutions transferred to 2ml glass GC vials using glass Pasteur pipettes. The GC vials were firmly capped and stored at 4°C until analysis by gas chromatography mass-spectrometry. Calibration standards containing the target compounds, surrogate recovery compounds and internal standards were similarly prepared with each individual batch of derivatised sample extracts. For analysis of triclosan and its degradation products, please refer to section 3.7.5 Analysis of Triclosan residues by Gas Chromatography Mass-Spectrometry.

3.7.3 Extraction from leachate

Weekly samples of soil lysimeter leachate (Chapter 5) were taken and 5ml of leachate was transferred into 15ml glass vials. A 2ml aliquot of dichloromethane (DCM) was added to each vial, the vials firmly capped, and vigorously shaken to initiate the extraction of TCS from the aqueous leachate sample, inhibit microbial activity, and stabilise TCS residues present in the leachate. The samples were stored in the dark at 4°C until analysis.

Fortnightly composites of leachate for each lysimeter were prepared from weekly samples. Where there was only one week's sample, that sample was used as representative for the fortnightly composite.

Before the final extraction the composite samples were spiked with a spike recovery standard containing TCS-d_3 (triclosan with three deuterium atoms), Me-TCS-d_3 (methyltriclosan, with three deuterium atoms) and bisphenol-C in acetone. The leachate samples were vortexed to enhance the extraction of organic chemicals into the DCM previously added to the sample after initial subsampling. The vials of sample and solvent were stood upright to allow the DCM to separate to the bottom of the vial. After extraction some samples formed a persistent water-DCM emulsion which prevented the DCM and aqueous leachate sample from separating. These samples were subjected to two minutes ultra-sonication in a sonication bath to break and disperse the emulsion and effect the separation of the DCM layer so it could be recovered from the aqueous leachate sample.

The separated solvent layer was carefully removed from the vial using a glass Pasteur pipette and transferred to a column of anhydrous sodium sulphate (Na_2SO_4) to remove any residual water from the DCM extract. The dried DCM eluting from the drying column was collected in a 22mL glass scintillation vial. Another 2ml aliquot of DCM was added to the leachate solution, the extraction repeated, and the DCM extract recovered and passed through the same Na_2SO_4 drying column and collected in the same glass scintillation vial. This procedure was repeated a total of three times to ensure quantitative recovery of any triclosan residues in the aqueous leachate samples. The resulting composite dried DCM sample extracts were capped in scintillation vials and stored at 4°C until sample concentration.

3.7.4 Leachate sample concentration and derivatisation

The DCM sample extracts were concentrated under a stream of nitrogen gas using a TECHNE® sample concentrator supplied by Total Lab Systems Ltd. This was done in order to reduce the total sample volume before the sample could be transferred and derivatised. The bases of the vials were heated to 30°C under a constant nitrogen stream until approximately 250µL of DCM extract remained in the vials. A note was made of any samples that completely evaporated and 250µL acetone was added to resolubilise the sample. The concentrated sample extracts were transferred to 2mL glass gas chromatography vials (GC-vials) with 3 consecutive washes of DCM; one wash of 500µl, and two of 250µl.

The sample extracts in the GC vials were then evaporated under nitrogen gas until they were just dry. As soon as the DCM extract was fully evaporated, 100µl ethyl acetate was added to the vial with a positive displacement pipette. The vials were gently rotated to rinse the walls of the vial and resolubilise the organic compounds extracted from the leachate solutions. The GC

vials of concentrated sample extracts in ethyl acetate were capped and stored at 4ºC until derivatisation and analysis.

Immediately before the sample extracts were derivatised an aliquot of internal standard solution containing 2, 6-*di*bromo-4-methylphenol and deuterated bisphenol-A (BPA-d_{16}) was added to the sample vials.

A 30µl aliquot of the derivatisation reagent, *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) (SIGMA-ALDRICH®, >97%) was added to the sample vials and the reaction initiated by vortex-mixing the vials for 3 seconds. The sample reaction mix was left to react at room temperature for 30min, the volume adjusted to a final volume of 500µl with ethyl acetate, and the vials firmly capped and stored at 4°C until analysis by gas chromatography mass-spectrometry. Calibration standards containing the target compounds, surrogate recovery compounds and internal standards were similarly derivatised with the batches of sample extracts.

The silylation reagent MTBSTFA was used in favour of *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) as the later silylation reagent is more reactive with atmospheric moisture and requires heating to produce alkyl silyl ethers of phenols such as triclosan.

Derivatisation takes place by the displacement of a hydrogen from the phenolic functional group of triclosan, and the formation of a bond between silicon and the oxygen. The Butyldimethylsilyl-group forms an ether with triclosan. See figure 5 for a diagrammatic representation of the process.

In its underivatised free phenolic form TCS is relatively involatile and is unsuitable for analysis by gas chromatography (GC). Derivatisation of TCS to form corresponding alkyl silyl ethers produces a volatile ether that can be analysed by GC. Furthermore, the formation of alkyl silyl ethers is advantageous for mass spectrometric detection as the cleavage and loss of the alkyl-silyl functional group produces a characteristic mass fragment that is readily detectable. The derivatisation allows for less interaction with the column and shorter retention times of triclosan.

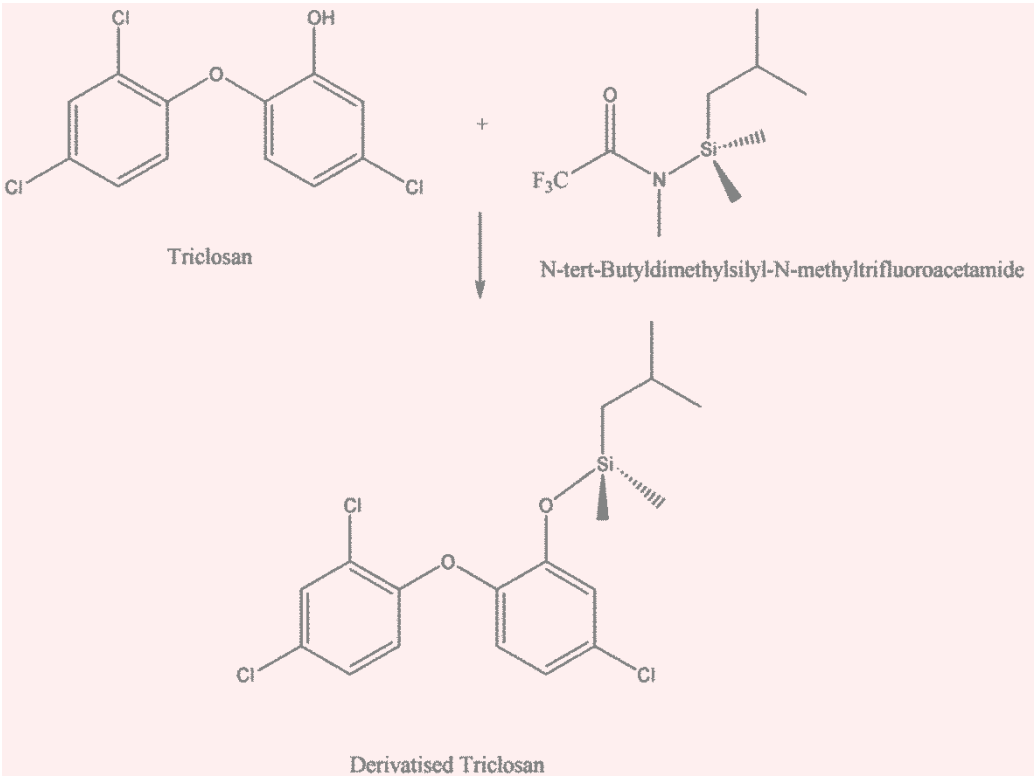

Figure 5: The derivatisation process of TCS with MTBSTFA before analysis

3.7.5 Analysis of Triclosan residues by Gas Chromatography Mass-Spectrometry.

The resulting trimethylsilyl-ethers of the target compounds triclosan, 2,4-dichlorophenol, 4-chlorocatechol and methyltriclosan; recovery compounds triclosan-d3 and methyltriclosan-d3; and the internal standards 2,6-dibromo-4-methylphenol and Bisphenol-A-d16 were analysed by high resolution gas chromatography mass-spectrometry using an Agilent 6890N gas chromatograph (GC) coupled to an Agilent 5975A inert XL mass spectrometer (MS) and CTC autosampler. A 1µl volume of derivatised sample extract was injected into an Agilent split/splitless injector at 280°C with a splitless time of 45secs and the components separated on an Agilent DB5-MS capillary column (30m length; 0.25µm film thickness; 0.25mm ID) using a constant flow rate of helium (1ml/min). The column was held at 90°C for 1.5min, increased at 20 °C/min to 150°C, with a second increase of 8°C/min to 234°C, followed by a 6°C/min increase to 280°C, and finally increasing at 20°C/min to 330°C with a 5.5min hold. The mass spectrometer interface temperature was maintained at 280°C, and ion source and quadrupole at 230°C and 150°C respectively. Compound specific mass fragments were obtained using single ion monitoring and were used in combination with retention time matching for positive identification. The concentration of compounds was determined by extracting compound specific mass ions and comparing the relative abundance of the three mass ions against those

obtained from the calibration standards. Quantification of target analytes was completed by internal standard quantitation using Agilent MSD Enhanced Chemstation quantitation software.

3.8. Molecular analysis of *Escherichia coli*

Escherichia coli is commonly studied as an indicator of pathogenic bacteria in environmental samples, including greywater (Birks et al, 2004; Birks et al, 2007). In Chapter 5, an environmental isolate (source: KSC culture collection) *E. coli*, provided by the Reference laboratory at ESR, was added to the synthetic greywater used to irrigate the lysimeters. Leachate samples collected from each lysimeter throughout the trial, and soil samples collected at the end of the trial, were analysed by qPCR in an attempt to identify the fate of the *E. coli* applied to the lysimeters.

The qPCR assay targeted the *uidA gene,* which encodes the β-glucuronidase enzyme, and is typically present in *E. coli*, including our environmental strain. The reaction was carried out as described by Silkie, Tolcher, and Nelson (2008).

3.8.1. Microbial DNA extraction from leachate

The protocol for DNA extraction from leachate was adapted from Noble, Blackwood, Griffith, McGee, and Weisberg (2010) by Dr Alma Siggins, ESR.

Greywater weekly leachate samples were stored at 4°C and were filtered aseptically within 24hrs of sampling. Nitrocellulose filters (0.45µm; ø 47mm; Millipore) were pre-wet with phosphate buffered saline (PBS). The sample (100ml) was filtered, and the filter paper was rinsed with PBS and filtered to dryness. Filters were stored at 20°C prior to extraction.

A sterile scalpel blade was used to aseptically cut up the filter and with sterile forceps it was transferred to a 2ml screw-cap vial containing 0.3g of pre-sterilised zirconium silica beads and 1ml of 1X TE buffer (Tris-HCl and EDTA;Invitrogen). The samples were shaken in two cycles on a FastPrep-24™ (MP Biomedicals) at 6m/s for 30sec, and incubated in an ice-block for 1min between cycles. The resulting sample was centrifuged at 12 000g for 1min and 300µl of the supernatant was transferred to a sterile 1.5ml Eppendorf tube. The supernatant was centrifuged at 12 000g for 5min and 250µl of the supernatant was transferred to a sterile 1.5ml tube for storage at -20°C before analysis. Fortnightly composites were constructed from

two consecutive weekly samples, by combining a 50µl aliquot of each into a single sample. Where there were not samples from both weeks to composite (Birks, Colbourne, & Hobson, 2004; Birks & Hills, 2007) the remaining weekly sample was used in the place of the composite.

3.8.2 Microbial DNA extraction from soil

DNA extraction from soil samples were carried out using the PowerSoil DNA Isolation kit (MOBIO Laboratories Inc.) with the exceptions that 0.3g (± 0.002g) soil was used and the final elution volume was 50µl, in TE buffer. This kit was chosen as it contains a humic substance removal step in order to remove interferences with downstream analyses. The resulting DNA extracts were stored at -20°C prior to further analysis. Samples were shipped on ice to ESR's Christchurch site for quantitative PCR analysis targeting the *E. coli uidA gene*.

3.8.3. Quantitative Polymerase Chain Reaction (qPCR)

Optimisation of the method for quantitative PCR of *E. coli*, and subsequent analysis were performed by Dr Aynsley Hickson, Susan Lin and Paula Scholes (ESR, Christchurch), based on the method described by Silkie et al. (2008). This method used primers that targeted the *uidA gene,* which encodes for β-glucuronidase.

Briefly, duplicate DNA samples were analysed with the primers uidA4-F (1286) and *uidA4*-R (1376) and the probe u*idA4*-P (Table 2). The probe fluorophore was changed from FAM-TAMRA to a FAM-black-hole quencher (BHQ) probe which allowed for greater sensitivity at lower copy number.

The 20µl qPCR reaction included 10µl of 2X PerfeCTa® qPCR ToughMix™ (Quanta Biosciences®), 500nM of each of the *uidA* primers, 250nM of the *uidA* probe, 0.2 mg/ml BSA, and 2 µl of template DNA extract.

Amplification was carried out on a Roche LightCycler® 480 using a three-step thermal cycling protocol consisting of a 5min initial denaturation step at 95°C, followed by 45 cycles of denaturation at 95°C for 20secs, annealing at 65°C for 60sec and elongation at 72°C for 20sec.

Quantitative standard curves were constructed using standard plasmids containing the *uidA gene* from the *E. coli*-KSC strain (environmental isolate) used in the lysimeter study. Plasmids contained the amplicon generated from two primers, UAL754 and UAR2105 (C. Lee, Kim,

Hwang, O'Flaherty, & Hwang, 2009) (Table 2), and the resulting plasmid was determined to be 5307kb. The mass concentration of the plasmid was measured in duplicate using a Qubit system (Invitrogen) and converted into its copy concentration as described by (C. Lee et al., 2009). A 10-fold serial dilution series (10^{-5}-10^{-10} copies μl^{-1}) was generated and analysed by qPCR in duplicate.

The threshold cycle (Ct) values determined were plotted against the logarithm of their input copy concentrations. The *uidA gene* copy concentrations was estimated against the corresponding standard curves within the linear range (R^2 = 0.989).

Table 2: Sequences of the primers used in the method optimisation for qPCR analysis

Primer	Sequence
UAL 754	5'-AAAACGGCAAGAAAAAGCAG-3'
UAR 2105	5'-TGTTTGCCTCCCTGCTGCGG-3'
uidA4-F (1286)	5'-CGGAAGCAACGCGTAAACTC-3'
uidA4-R (1376)	5'-TGAGCGTCGCAGAACATTACA-3'
uidA-P	5'-6FAM-CGCGTCCGATCACCTGCGTC-BHQ-3'

4. Determining the EC_{50} of triclosan (TCS) in a silty clay loam

4.1 Introduction

There are benefits to the reuse of greywater such as relieving the load on treatment infrastructure and reducing the demand for potable water (McCormack, 2011). However, the greywater diverted from the main waste water stream does not go through the same treatment process. Instead of relying on industrial processes to degrade potentially harmful chemicals, we then depend on the receiving environment, for example soil, for the treatment and degradation process of organic chemicals found in greywater (D. G. Lee, Cho, & Chu, 2013; Ying, Yu, & Kookana, 2007). In soil, degradation primarily relies on biological processes such as the biodegradation of emerging organic contaminants by microorganisms. One such emerging contaminant is TCS.

Triclosan is a broad spectrum antimicrobial agent used in a wide variety of personal care products. A major pathway for the movement of organic contaminants such as triclosan to the environment is through the land application of wastes such as greywater. Pharmaceutical compounds such as TCS are designed to alter both the biochemical and physiological functions of biological systems in humans and animals, however, the possible impacts of long-term exposure of soil microbes to, and accumulation of compounds such as triclosan remain unknown.

Triclosan disrupts or negatively impacts the microbial life-cycle (Park et al., 2013). For example, TCS is found in some brands of plaque control toothpastes and mouthwashes where it has a bactericidal effect. It follows that the TCS entering the environment via household greywater would have a negative impact on the microbial communities and ecosystems present in the receiving environment (Harrow & Baker, 2010)(Ali et al., 2011; Butler et al., 2011; Horswell et al., 2014; Liu et al., 2009; Waller & Kookana, 2009).

Previous studies have reported the effects of TCS on the microbial community in soil. For example, a dose-response study investigated the impacts of concentrations of TCS ranging from 10ppm to 1000ppm, on substrate induced respiration (SIR)(Butler et al., 2011). That study found that TCS concentrations of 1000ppm resulted in 50 to 70% inhibition of the respiration rates of the soil microbial community, with lower levels of inhibition reported at lower TCS concentrations. No attempt was made in that study to determine an EC_{50} value for SIR, or from any other microbial biomarker, in response to TCS contamination in soil (where

EC_{50} is the concentration of contaminant at which at least 50% of the microbial population's activity is affected). To determine an EC_{50} value, the TCS concentration range must span the EC_{50} value, ideally resulting in total inhibition of the activity in question. As Butler et al. (2011) observed 50% inhibition at 1000ppm TCS, a significantly higher TCS concentration is required to determine an EC_{50} value.

The aim of this chapter was to select a broader range of TCS concentrations than previous studies, which would potentially allow us to determine an EC_{50} value for TCS in soil.

In this chapter a dose response experimental protocol was used and the toxicity of TCS using SIR (substrate induced respiration) with glucose as a substrate was monitored. The activity of the sulphatase enzyme and microbial biomass was also monitored.

SIR is a measure of the amount of substrate (glucose for the purposes of this study) converted by a microbial community in a given timeframe, a measure of microbial health.

Microbial biomass is often used as a bioindicator for environmental changes (Moscatelli, Lagomarsino, Marinari, De Angelis, & Grego, 2005). He, Yang, and Stoffella (2005) concluded that microbial biomass and function are sensitive enough to be used to assess soil contamination or the health of the soil environment, even though that study investigated the impacts of trace elements on the soil environment. There is a distinct gap in the literature for the use of microbial biomass as bioindicator for TCS in soil. Microbial biomass, has however been used for the indication of toxicity in soil by Horswell et al. (2014). Sulphatase activity was also used for the determination of an EC_{50} in that study; however, it was used to determine the toxicity of Cu in the presence of various concentrations of TCS. In a study conducted by Speir et al. (2007) an increase in a toxic compound, zinc (Zn), has been indicated to be negatively correlated with sulphatase activity.

Microbial biomass was determined to indicate amount of microbial carbon in soil. Under stress conditions it is expected to see a decline in microbial biomass. As SIR gives an indication of the respiration rate of a microbial community and biomass provides information on its size, the quotient of SIR and biomass provides the specific microbial respiration. Put differently, the metabolic quotient is a measure microbial stress, measured by the rate of substrate conversion by a given size microbial biomass unit.

In a study conducted by Pavan Fernandes, Bettiol, and Cerri (2005) the microbial metabolic quotient (qCO_2) in soil was measured after the addition of sewage sludge to soil. It was

interpreted as “metabolic efficiency” as it is a measure of “*the energy necessary to maintain metabolic activity in relation to the energy necessary for synthesizing biomass*” (Pavan Fernandes et al., 2005). It follows that a higher qCO_2 would indicate an elevated stress level in the soil.

The aim of this study was to determine an EC_{50} value for TCS in soil, and to use this value to identify a suitable TCS loading rate for the subsequent lysimeter study (Chapter 5). In the lysimeter study, greywater containing TCS was irrigated onto soil cores for a period of 3 months and the fate and effects of TCS in the soil environment measured. Identification of the optimal TCS range for use in the lysimeter study would allow us to ensure that a quantifiable amount and effect of TCS on the soil microbial community could be readily detected.

In the lysimeter study 3 soils will be used; a sandy loam from Katikati, fine sandy loam from Gisborne, and a silty clay loam from Lincoln (Christchurch). Only the Lincoln soil was selected for this study. It had the highest clay and silt content and it was suspected that the TCS applied to the soil would have a lower bioavailability (Ali et al., 2011) although Waller and Kookana (2009) found TCS respiration inhibition in clay soils, but not in sandy soil. This potentially provided a scenario where the effect of the TCS on the microbial community would be the lowest. A more pronounced response to TCS in the other 2 soils could then be expected.

4.2 Materials and methods

4.2.1 Soil sampling and initial characterisation

Approximately 30kg of soil was sampled from an agricultural ground near Lincoln, Christchurch, transported to ESR, Porirua, and stored at 4°C. Initial tests were carried out by Massey University to characterise the soil.

The sample was diluted 1:10 with distilled water and the pH determined using a *Thermo-ORION* pH meter.

4.2.2 Triclosan addition to soil

As the solubility of TCS in water is extremely low at10ppm (EU-SCCP, 2009). Triclosan is typically solubilised in an organic solvent prior to addition to soil. The solubility of TCS in acetone is much higher at 10,000ppm (EU-SCCP, 2009). This was used in our study, and a

concentration range that did not exceed this value was selected. The method used for spiking the soils with TCS was taken from Brinch, Ekelund, and Jacobsen (2002) where a small amount of soil was spiked with acetone containing the desired amounts of TCS. The acetone was evaporated overnight and the rest of the soil mixed through thoroughly before the experiment commenced.

50g of soil was used to construct glass jar microcosms at a range of TCS concentrations. These were prepared as follows: The dry weight of the soil was determined as described in Chapter 2 and this value was used make a stock-solution of TCS to result in a final concentration of 7500ppm per dry weight of soil once added. Serial dilutions of this stock solution were prepared in acetone resulting in final TCS concentrations of 5000ppm, 2500ppm, 1500ppm, 1000ppm and 500ppm (per dry weight of soil). A 0ppm sample, containing acetone but not TCS was used as a control.

The soil was adjusted to 60% water holding capacity (WHC). Each microcosm was weighed and the weight recorded in order to replace moisture that would be lost in the following steps. One millilitre of the relevant acetone-TCS solution was added to a quarter of the soil sample (approximately 12.5g soil), and the acetone was allowed to evaporate at ambient temperature from the microcosms for 24 hours. After 24 hours of evaporation, the amount of moisture lost was determined by subtracting pre- and post-evaporation sample masses the microcosms. Tap water was added to re-adjust the soil's WHC to 60%. The rest of the soil was added and mixed with the spiked soil in 3 separate additions of equal mass (12.5g), mixing thoroughly in between as described by Brinch et al. (2002).

4.2.3 Analysis

Substrate induced respiration, microbial biomass, sulphatase activity and the microbial metabolic quotient were analysed and calculated according to the descriptions found in chapter 2, Methods sections. SIR was measured on days 0, 6, 10, 17 and 20 in order to assess the patterns in respiration as the microbial community recovers after TCS exposure. On day 17, sulphatase activity was measured. On the same day, the microbial biomass was assessed by measuring the difference in extracted carbon between a subsample that has been fumigated with DCM, and a subsample that has not been fumigated (see section 3.5 in the Methods chapter for further information). The difference in the extracted carbon from each subsample was attributed to microbial carbon and thus indicative of microbial biomass. The microbial

biomass was measured in order to relate the data to the SIR by determining the microbial metabolic quotient (qCO_2).

4.2.4 Statistics

Microsoft Excel 2010 was used for all statistical analysis.

A linear regression analysis of the SIR results was performed. The change in values over time was considered to be statistically significant when $p < 0.05$, calculated from the slope of the regression lines.

One-way ANOVA was performed for all other data sets. Standard errors indicating upper and lower limits were calculated (= std. dev. /√n; where n = number of replicates).

Post-hoc analysis was performed using a Tukey HSD pairwise comparison of the means.

Soil biological properties, sulphatase and microbial biomass were related to total TCS concentration using the sigmoidal dose response equation developed by CSIRO, Australia ((Barnes, Correl, & Stevens, 2003) and (Smolders, Buekers, Oliver, & McLaughlin, 2004)), based on the model developed by Haanstra, Doelman, and Voshaar (1985). The equation is:

$$Y = \frac{C}{1 + e^{(-H(\log X - \log EC50))}}$$

Y is the biological activity; *C* is the calculated maximum value of *Y*; *X* refers to the metal concentration; EC_{50} is the TCS concentration at which the biological activity is inhibited by at least 50%; and *H* refers to the Hill slope. The mean values for data from replicates were calculated prior to this analysis.

4.3 Results

4.3.1 Soil sampling and initial characterisation

Initial characterisation of the soil determined it to be a silty clay loam (35% sand; 40% silt; 25% clay) with a pH of 4.5.

4.3.2 Substrate Induced Respiration

Regression analysis showed that microbial respiration increased from day 0 to day 20 in all treatments (Fig. 6). A one-way Anova comparing respiration at each time point indicated that SIR at day 20 was signifcantly reduced in the 500, 5000 and 7500 ppm TCS spiked soils when compared to the control samples.The difference in SIR exhibited by the control soil between day 0 and day 20 at 0ppm was the largest of all of the soil microcosms.

Generally at all TCS concentrations, a decline in respiration from days 0-6 was observed, followed by successive increases at each sampling point. Some exceptions to this were noted, e.g. 1500ppm increased from days 0-6 and 1000ppm decreased from day 10-17 (Fig 6).

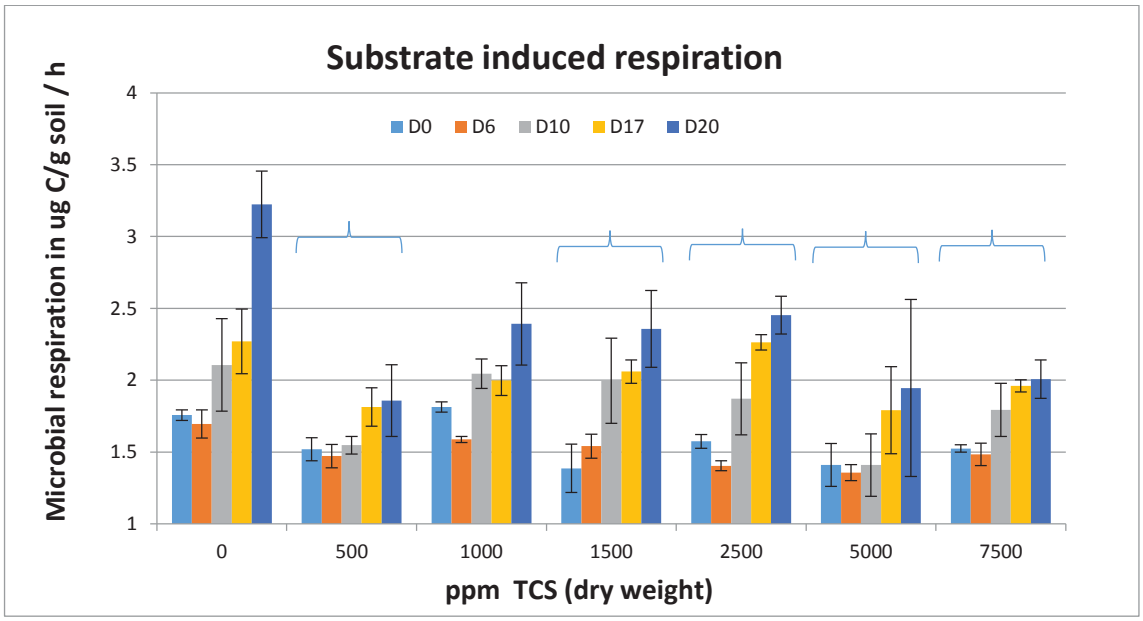

Figure 6: Substrate induced respiration (SIR) at investigated TCS levels. The brackets on the graph indicate subsets of data where a statistically significant ($p<0.05$) change in respiration in relation to time was observed.

4.3.3 Microbial biomass

The microbial biomass decreased with increasing TCS concentraion (Fig. 7) with the sample exposed to 7500ppm TCS containing approximately 90% less microbial biomass than the

control, 0ppm sample (Fig. 7). A significant ($p<0.05$) decline in biomass compared to the control was observed at TCS concentrations ≥ 500ppm (Fig. 7).

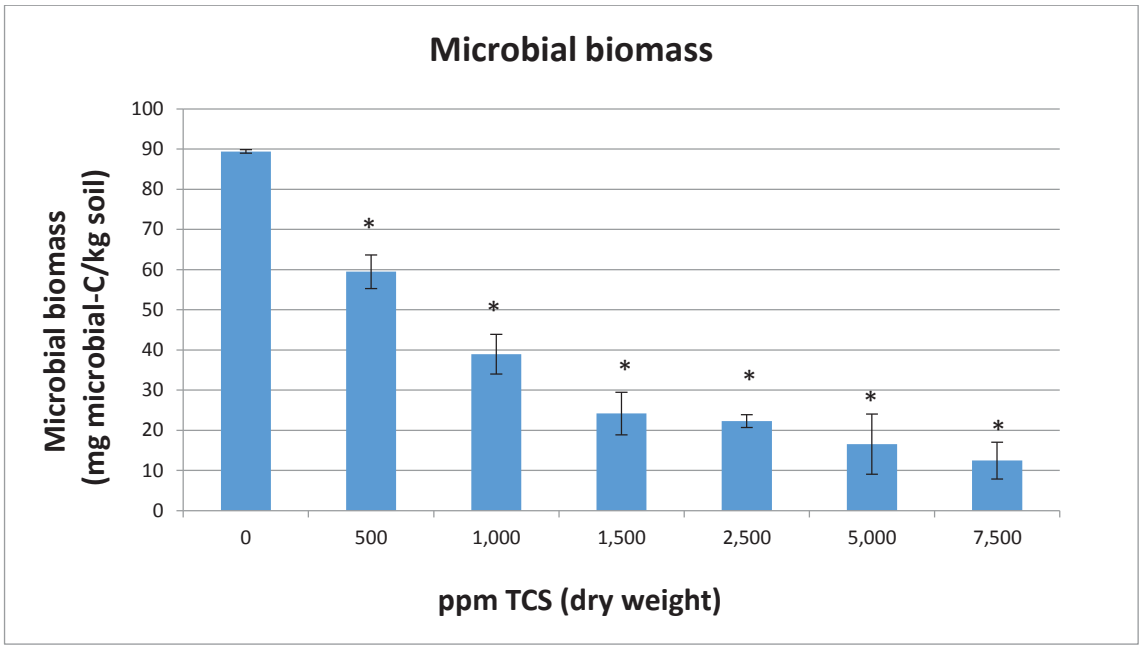

Figure 7: Microbial biomass at investigated TCS levels on day 17. Asterisk indicates difference of statistical significance ($p<0.05$) from the control calculated using Tukey's HSD test.

4.3.4 Microbial metabolic quotient (qCO_2)

The increase in qCO2 was directly related to TCS concentration, thus indicating a TCS mediated increase in microbial stress (Fig. 8). This increase, compared to the control, was only significant ($p<0.05$) in the 7500ppm TCS soils (Fig. 8).

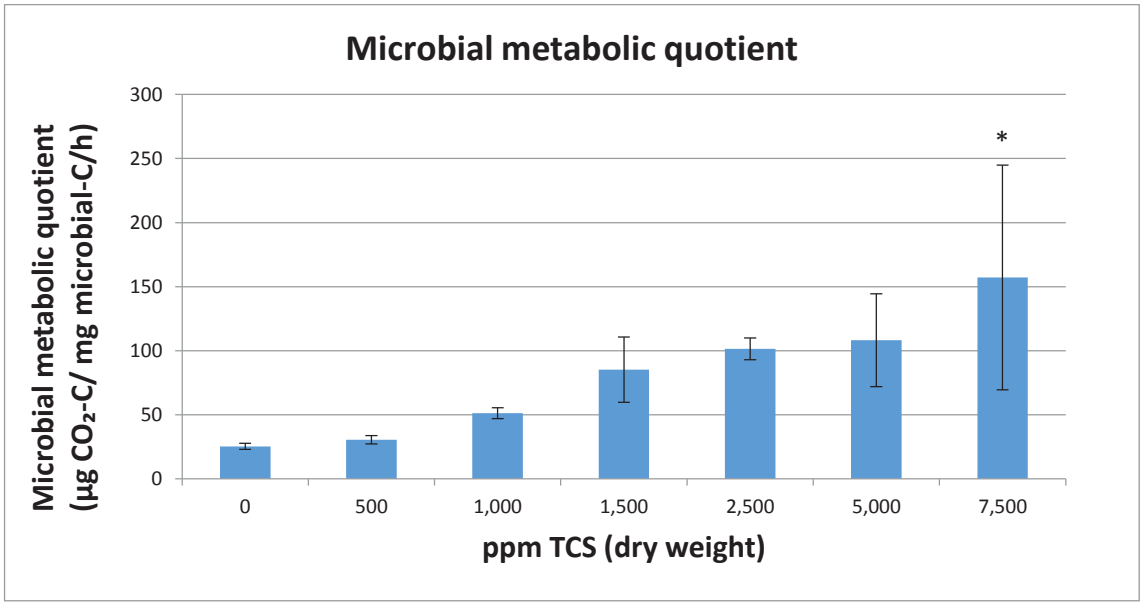

Figure 8: Microbial metabolic quotient at investigated TCS levels on day 17. The asterisk indicates differences of statistical significance ($p<0.05$) from the control calculated using Tukey's HSD test.

4.3.5 Sulphatase enzyme activity

On day 17, sulphatase activity was inversely related to TCS concentration (Fig. 9). At the three highest TCS concentrations (2500ppm, 5000ppm and 7500ppm) sulphatase activity was significantly reduced compared to the control sample (0ppm; $p < 0.05$; Fig. 9).

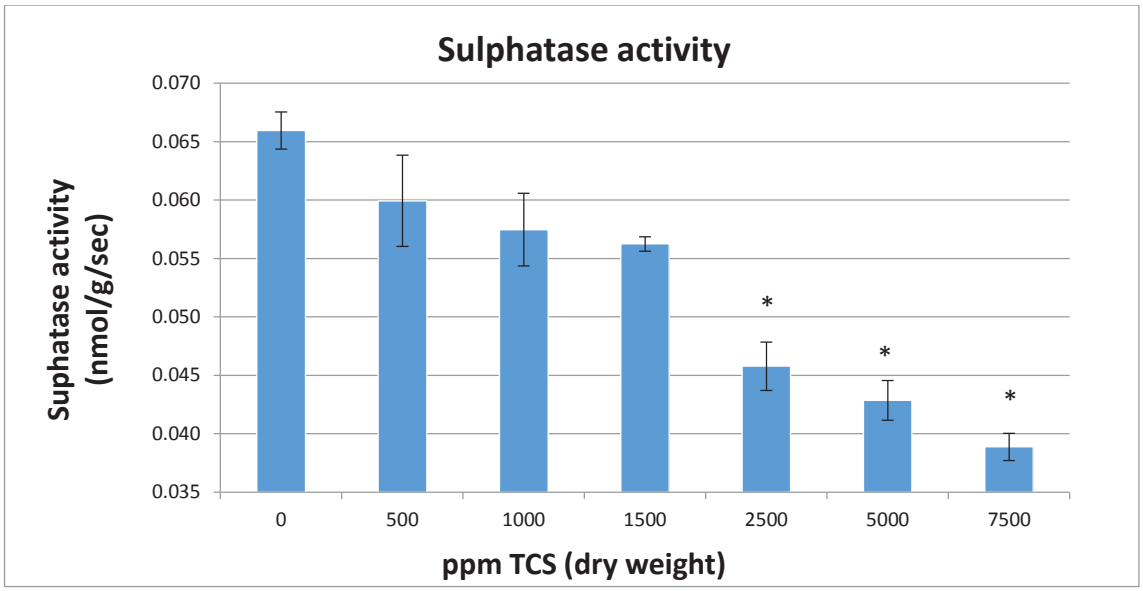

Figure 9: Sulphatase activity at investigated TCS concentrations on day 17. The asterisk indicates statistical significance from the control calculated using Tukey's HSD test.

4.3.6 EC_{50} determinations

Effective concentrations of TCS that caused a 50% decline in activity (EC_{50}) were calculated for sulphatase activity and microbial biomass, as previously described in Chapter 2. EC_{20} values, i.e. concentrations of TCS that caused a 20% decline in the measured activity, were also calculated for these parameters. Results of both EC_{50} and EC_{20} calculations are shown in Table 3 and Figures 10 and 11.

Table 3: The EC_{50} values for sulphatase and biomass as determined by the 50% decline in activity of the soil microbial community. The EC_{20} is a concentration of TCS (ppm) where 20% of the community's activity is affected. The R2 refers to figures 10 and 11.

Property	EC_{50}	R^2	Lower 95%	Upper 95%	EC_{20}	Lower 95%	Upper 95%
Sulphatase	11326	0.9921	6331	20262	1737	769	3927
Microbial Biomass	803	0.9955	489	1319	195	70	546

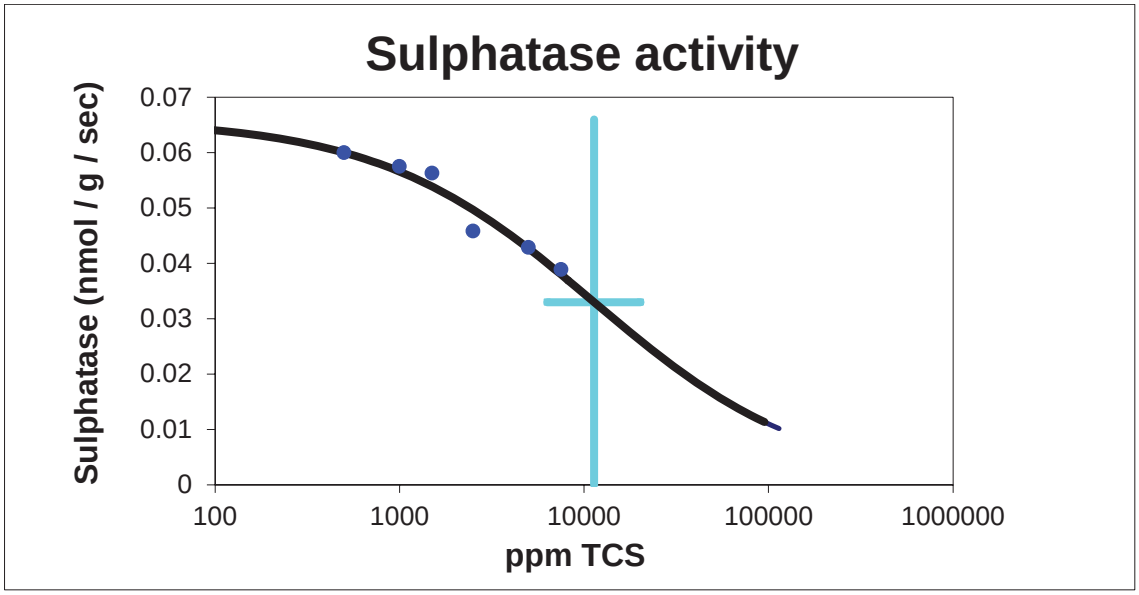

Figure 10: EC_{50} determined with sulphatase activity at investigated TCS levels.

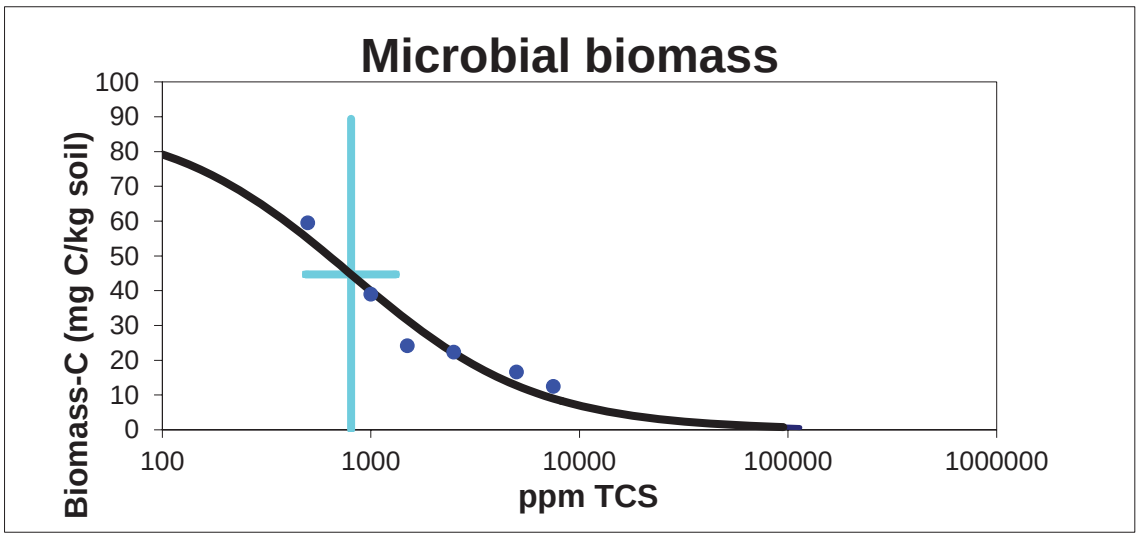

Figure 11: EC_{50} determined with microbial biomass at investigated TCS levels.

4.4 Discussion

In this study TCS inhibited substrate induced respiration when added to soils at concentrations above 5000ppm. In a previous study Butler et al. (2011), found that in some soil types TCS caused a stimulation in respiration at lower concentrations (10-100 ppm). However in a sandy loam soil, similar to the soil type used in our study, Butler et al. (2011) found that TCS reduced SIR to below control levels at concentrations > 500ppm. In this study, SIR was not used to determine the EC_{50} as there wasn't sufficient inhibition and therefore the data did not fit the model for EC_{50} determination well. This could be due to the fact that even though the microbial community has been compromised the inhibitory effect of TCS was being masked by added nutrients from dead microbes

Triclosan negatively impacted microbial biomass as microbial biomass decreased as TCS concentrations increased. This provides evidence of TCS's cidal properties.

A microbial community under stress has a higher respiration rate per microbial biomass unit than a healthy microbial community and this effect was observed with the calculation of the qCO_2. There was a positive corelation between the increase in TCS concentration and microbial metabolic quotient (Fig. 8). The change in microbial metabolic quotient between the control and experimental samples were only significant ($p<0.05$) for the 7500ppm TCS treatment. This indicates that microbial biomass decreased, but did not necessarily show signs of stress until encountering very high levels of TCS. Butler, Whelan, Ritz, Sakrabani, and van Egmond (2012), also found that the addition of TCS to soil, in nominally increasing concentrations, appeared to be linked to the bacterial stress levels. This is also in accordance with the results found in Pavan Fernandes et al. (2005), even though in that study the environmental impacts of the addition of sewage sludge was assessed. Microbial biomass and qCO_2 proved to be sensitive enough an indicator to measure toxicity (He et al., 2005).

Sulphatse activity was also shown to be linked to TCS concentration with impacts on sulphatase activity being significant only in higher TCS concentrations, 2500ppm-7500ppm. This suggests that sulphatase may be a less sensitive soil health indicator than microbial biomass for TCS.

In order to fit our model for EC_{50} determination, the concentrations of the contaminant being examined (TCS) needed to be of a sufficient concentration as to show close to 100% inhibition of the measured activity. As can be seen from the microbial biomass-EC_{50}, 7500ppm was a concentration sufficient for almost complete die-off (86.05%) of the microbial population. In

contrast, the concentrations of TCS used were not large enough for the complete inhibition of sulphatase activity and therefore the 95% confidence intervals are very large.

EC_{50} and EC_{20}'s were calculated for TCS using microbial biomass and sulphatase as indicators of soil health and function. The EC_{50} and EC_{20}'s values for biomass carbon were 803 ppm TCS and 195 ppm respectively (Table 3); considerably lower than the values for sulphatase (EC_{50} = 11326ppm, EC_{20} = 1737ppm). These results suggest that the numbers of soil microbes are significantly ($P<0.05$) impacted by the presence of TCS, perhaps not surprising as TCS is a generic anti-microbial. However, the investigated soil function parameter, sulphatase activity, was not as sensitive to TCS amendment as microbial biomass. To address this knowledge gap a molecular community profiling method such as DGGE (Denaturing Gradient Gel Electrophoresis), or TRFLP (Terminal Restriction Fragment Length Polymorphism) might be employed. This allows for the monitoring of certain microbial groups and may give insight into how the community structure changes over time after addition of TCS.

The response of the soil microbial community to concentrations of TCS at 195ppm is of concern. A household using a TCS-containing toothpaste and mouthwash could be responsible for 450mg TCS entering the environment via greywater disposal over a period of 10 years. Lozano, Rice, Ramirez, and Torrents (2010) observed a half-life for TCS in soil of 107 days. Even if 50% breakdown over the 90 days experimetnal time for the lysimeter study is assumed, the accumulated TCS will be close to 225mg. Butler, Whelan, Sakrabani, et al. (2012) found that the bulk of the TCS in sewage applied to land remains in the top 5 cm of soil. Thus assuming a greywater receiving area of 10m x 10m, the total accumulated amount of TCS could approach 450ppm (dry weight soil with a bulk density of 1:1). This value exceeds the EC_{20} value of 195ppm determined by our study. The movement of TCS through the soil profile with greywater application could possibly differ from what was found by Butler, Whelan, Ritz, et al. (2012).

4.5 Conclusion

Triclosan has a negative effect on soil microbes at the concentrations investigated in this study by impacting on soil biomass and function. This study showed that an increase in TCS concentration can induce stress in the microbial community at concentrations as low as 195ppm (EC_{20} for biomass).

The two EC_{50}'s calculated for TCS indicated that sulphatase activity and microbial biomass have different sensitivities to TCS (11326ppm and 803ppm respectively). Microbial biomass was the most sensitive soil health indicator in this study, thus the EC_{50} of 803ppm was used to inform the dosing rate of TCS for the lysimeter study that followed. This allowed me to ensure that the dosing rate of TCS in the soil cores from the lysimeter study would approach the EC's determined in this experiment (733ppm TCs in soil if greywater with a TCS concentration of 10ppm were to be irrigated onto the lysimeters for the experimental time).

Substrate induced respiration levels for all treatments had an upward trend over time e.g. the 7500ppm treatment increased from 1.53ug C/g soil/h to 2.01ug C/g soil/h from day 0 to day 20 i.e. the results from our study indicated that organic contaminats such as TCS are metabolised in the receiving soil environment from .However, because of the bacteriocidal effects of TCS, the soil microbial community could become severely stressed. If there are continuous inputs of TCS into the environment, for example from applications of greywater, it is possible that TCS could accumulate to concentrations that may affect the soil microbial population in a receiving environment. Triclosan is readily degraded in the environment, thus it is important that disposal/re-use of wastes that might contain organic contaminants such as TCS are regulated to avoid any potential environmental impacts.

5. Lysimeter study

5.1 Introduction

The quality and composition of greywater is highly variable between households (Casanova et al., 2001; Eriksson et al., 2009) and there is relatively little scientific information available about the environmental impacts, long term effects and potential health risks associated with the reuse of greywater. Previous studies conducted have aimed to describe the fate and effects of the long term use of greywater on the soil environment (Al-Hamaiedeh & Bino, 2010; Rodda et al., 2011; Travis, Wiel-Shafran, Weisbrod, Adar, & Gross, 2010; Wiel-Shafran, Ronen, Weisbrod, Adar, & Gross, 2006), however comparatively few studies have been conducted on the irrigation of greywater onto New Zealand soils.

Greywater contains a range of contaminants such as microbiological contaminants and emerging organic contaminants. Microbiological contaminants can be the same as for sewage effluent and can include causative agents of enteric illness such as *Salmonella* spp. and Campylobacter spp. (Casanova et al., 2001; Eriksson et al., 2009; Eriksson et al., 2002) .*Escherichia coli* are used as an indicator of microbiological quality and have been found and at concentrations between 10^1 – 10^7 CFU/100ml (Birks et al., 2004; Birks & Hills, 2007) high end of the concentration spectrum is similar to levels typically observed in blackwater. Friedler (2004) measured concentrations of faecal coliforms as high as 4 x 10^6 CFU/100ml in bath- and shower water.

Organic and inorganic contaminants in greywater arise from the use of personal care products and may contain compounds such as heavy metals (e.g. copper and zinc) and emerging organic contaminants such as triclosan (TCS) (Chen et al., 2013; Heidler, Sapkota, & Halden, 2006). Triclosan has been found at concentrations between 0.6µg/L (Eriksson et al., 2003) and 16.6µg/L (Almqvist & Hanæus, 2006) in greywater.

Previous studies have indicated that TCS can impact the health of the receiving soil environment (Butler et al., 2011; Butler, Whelan, Ritz, et al., 2012; Butler, Whelan, Sakrabani, et al., 2012; Harrow et al., 2011). Of further concern would be the potential for TCS to move through the soil profile and contaminate ground and surface waters. Previous studies have investigated the persistence (Al-Rajab, Sabourin, Scott, Lapen, & Topp, 2009) and movement of TCS through the soil profile (Butler, Whelan, Sakrabani, et al., 2012; Kwon & Xia, 2012), however the conclusions were inconsistent. Kwon and Xia (2012) observed no movement of

TCS through a repacked soil column where-as Butler, Whelan, Sakrabani, et al. (2012) observed movement of TCS through the soil profile that had been excavated *in-situ*. In both studies comparable amounts of approximately 1mg/kg of TCS per dry weight soil was used. These concentrations were however not indicative of the fate and effects of TCS in soil where the compound accumulated after successive irrigations.

Previous studies have indicated that the fate of TCS in the soil environment is affected by various factors including the soil texture (Agyin-Birikorang et al., 2010; Butler, Whelan, Ritz, et al., 2012; Butler, Whelan, Sakrabani, et al., 2012; Waller & Kookana, 2009), moisture content and the amounts of potential co-contaminants present in the soil (Horswell et al., 2014). Other factors such as soil-pH and rainfall also affect the movement of TCS through the soil profile (Agyin-Birikorang et al., 2010).

The movement of microbial contaminants through the soil profile also holds potential public health risks as they can leach from greywater applied to soil into groundwater reserves or contaminate receiving waterways (Eriksson et al., 2002). As for chemical contaminants, the fate and effects of microbial contaminants is also affected by soil texture. Pang et al. (2008) investigated the transport of microbes through soil cores, and found that when preferential flow takes place, breakthrough of *E. coli* happens sooner than for soils with intact structure. The potential for soil to crack is related to the texture (McLeod, Aislabie, Ryburn, & McGill, 2008).S. Jiang et al. (2010) also demonstrated that soils with higher clay content have a greater risk of microbial leaching by applying dairy shed effluent to undisturbed lysimeters and analysing leachate over 124 days.

There are no national guidelines for the management of greywater. In practice, Australian/New Zealand Standard "On-site domestic wastewater management" (2012) AS/NZS 1547: 2012 is often used to guide best practice for greywater management (AS/NZS1547:2012; personal communication. Dr Jacqui Horswell, ESR). However, AS/NZS1547 states:

"Although this standard covers the subsurface irrigation of greywater after primary or secondary treatment, it does not cover greywater reuse by direct application onto land" (AS/NZS1547, 2014, pp. 6).

Thus, there is a need for information on the fate and effects of rates of contaminants in untreated greywater on New Zealand soils.

With the variability of greywater and the difference in soil properties of the receiving environment, there is a need to characterise the fate and effects of both chemical and microbiological contaminants in New Zealand soils.

In light if the above, the aim to this study was two-fold; firstly to characterise the movement of a representative chemical contaminant, TCS; and the microbial indicator *E. coli* through the soil profile after irrigation with greywater. The second aim was to determine the effects TCS had on soil health indicators in the top 5cm of the soil lysimeters after it had been introduced through continuous surface irrigation with greywater.

5.2 Materials and methods

5.2.1 Source of soils

Three New Zealand soils were identified and used for the research (Fig 12). One soil was taken from an agricultural plot in Lincoln (Templeton), and two domestic soils were used; one soil from Gisborne and another from Katikati. The Gisborne soil was taken from on open plot in a new residential area in Wainui, southeast of Gisborne CBD .The Katikati soil was collected from a residential property in close proximity to a vegetable garden. None of the soils have been reported to be exposed to TCS in the past, however, the full history of the soils are unknown.

Figure 12: Sample sites, clock-wise from top left; Gisborne, Lincoln, Katikati

Trenches were dug to allow for a starting point in the lysimeter excavation and intact soil cores were dug out of the ground *in-situ*, making use of polypropylene buckets with the bottoms removed. The soil cores were sculpted out of the ground while gently pressing down the buckets around the excavated edges. This process was continued until a soil core of at least 30cm in depth was contained in the bucket (Fig 13). The soil cores were lifted out of the ground and the bases cut flush with the edge of the containers that held them. Melted petroleum jelly was poured around the sides on the inside of the buckets to seal the soil core and prevent preferential flow around the soil core-casing interface. The lysimeters were sealed in cling-film and transported to the lysimeter facility at ESR in Wellington.

The soils were chosen from 3 different sites to compare how different soils react to the exposure to TCS and how TCS and *E. coli* are transported through the soil profile. This would then indicate the suitability of the soil for greywater irrigation and also potentially distinguish a high risk soil from a low risk soil.

5.2.2 Lysimeter facility

The existing ESR lysimeter facility was modified to accommodate the size of the newly dug lysimeters. The modifications involved the installation of a wooden platform on which the lysimeters sat in large 30cm diameter funnels. The funnel-ends protruded through a whole drilled through the platform above either 500ml or 1L SCHOTT® bottles for leachate sample collection. The bottoms of the lysimeters were fitted with mesh before being lowered into the funnels, to prevent large aggregates of soil falling through the funnel into the sample being collected, so compromising the integrity of the soil core structure. Once the lysimeter, mesh and funnel were in place, the barrel of the lysimeter was secured by filling in the space between the lysimeter and the bay with gravel. Drip irrigators were made from 250ml plastic bottles and adjustable taps, and calibrated to irrigate at a rate of 1.6L/h (Fig 14). This irrigation rate was chosen to mimic the effect of John Deer HYDRO PC drip irrigation line.

Figure 13: The excavation process of the lysimeters used in this study

Figure 14: Left: Lysimeters positioned in the facility. Right: the compartment at the bottom of the funnel in which the lysimeters sits is large enough to just accommodate sample collection containers.

5.2.3 Irrigation volumes and for lysimeters.

The soils had different textures and pH's which was expected to affect the movement of TCS trough the soil profile (Table 4). The texture analysis was carried out by Dr D.J Horne at Massey University, Palmerston North, using an approximate method known as 'soil texture by feel'.

Table 4: Physical properties of the 3 soils chosen for the experiment

Soil name	Soil texture	% Sand	% Silt	% Clay	pH	WHC (ml water held at 100% WHC per 100g oven dried soil)
Katikati	Sandy loams	70	15	15	5.78	98.1
Gisborne	Fine sandy loam	70	15	15	5.91	44.1
Lincoln	Silt loam / Silty clay loam	35	40	25	4.49	47.6

As the soils had different textures the irrigation rates differed as well. The greywater application rates were adapted from Table M1 of AS/NZS1547:-2012, which recommends maximum irrigation rates based on soil texture that should be applied to soil over a 24 hour period without causing soil leaching. Taking into account the surface area of the receiving lysimeters, irrigation rates were calculated to be 123mL greywater twice daily for the Gisborne and Katikati soil and 86ml greywater twice daily for the Lincoln soil.

5.2.4 Composition of greywater treatments.

The EC_{50} data obtained in Chapter 4 was used in determining the TCS concentration of the synthetic greywater applied to the lysimeters. Therefore, the cumulative TCS loading of 733ppm in the top 5cm of the soil cores was likely to be sufficient to detect a quantifiable change in the soil microbial community. This would bring the accumulated TCS in the soil close to the EC_{50} of 803ppm determined from the microbial biomass (Chapter 4, section 4.3.6). The accumulative loading of 733ppm TCS was calculated on the basis that an average of volume of 200mL greywater was to be irrigated onto the soil cores, at a concentration of 10mg/L, the maximum solubility of TCS in water. The experiment had 3 different treatments of greywater and a control consisting of tap water. Two of the greywater treatments consisted of the application of a bad- and a good quality greywater through the flow-rated drip irrigation device. The fourth treatment was the application of bad quality greywater (BQGW) without the use of an irrigation device as one quantitative transfer of the irrigation volume to simulate flood irrigation. The greywater's preparation was based on Jefferson, Burgess, Pichon,

Harkness, and Judd (2001). The preparation was adapted from a formula developed by the UK water industry (Table 5).

Table 5: Composition of greywater used for lysimeter study (See footnotes1 & 2 for references)

Ingredient	"Good quality greywater" Quantity per litre	"Bad quality greywater" Quantity per litre
Soap	0.005 g	0.05 g
Shampoo	0.011 ml	0.11 ml
Vegetable oil	0.001 ml	0.01 ml
Laundry powder	0.0028 ml	0.028 g
Tertiary Effluent	0.24 ml	2.4 ml
E.coli [4]	1 x 10^2 CFU/100ml	1 x 10^8 CFU/100ml
TCS[5]	5.9 ppb	10 ppm

Lysimeters were planted with rye grass to ensure differences in root exudates do not affect the metabolism of TCS. Niemeyer et al. (2012) observed a positive correlation between vegetation coverage and soil health parameters such as microbial metabolic quotient (qCO2) and substrate induced respiration (SIR). Differences in coverage could mask the effect the TCS was having on the soil microbial communities.

5.2.5 Experimental procedure

The lysimeters were irrigated twice daily for 90 days to simulate the normal routine of a household. The leachate was collected weekly and prepared for analysis within 24hrs of collection (Chapter 3, sections 3.7.3. and 3.8.1). Fresh synthetic greywater was made every day from a concentrate prepared at the beginning of the experiment. Triclosan and *E. coli* were added within 10mins of irrigation to limit the amount of time that *E. coli* was exposed to TCS

[4] *Escherichia coli* added immediately before irrigation. Values derived from GW/Septic tank study where *E.coli* was measured. This is the upper and lower values

[5] The lower value of TCS was taken from Almqvist&Hanæus, 2005. Higher value based on 10 year accumulation of TCS in soil and an assumed 50 % breakdown. The value was calculated for 3 person household assuming 234L greywater (Kapiti coast district council) production per day, and irrigation of 200ml per day per lysimeter.

outside the experimental environment. The *E. coli* stock was enumerated every week to ensure the correct amount was added every day. TCS stock was prepared in acetone and spiked into the respective greywater treatments after *E. coli* was added.

After the 3 months of irrigation, the lysimeters were destructively sampled by removing the polypropylene casing around the soil core. The petroleum jelly was carefully removed to prevent accidental cross-contamination between soil horizons and the lysimeters were sectioned into 3 soil horizons: 0-5cm, 5-10cm, and 10-30cm, similar to Butler, Whelan, Sakrabani, et al. (2012). Horizons were sieved <2mm to remove debris and organic material.

5.2.6 *Escherichia coli* preparation and enumeration.

Three tubes containing 10ml Luria-Bertani broth (LB-broth) was inoculated with the glycerol stock containing the environmental isolate of *E. coli* and grown overnight on a shaker-incubator set at 180rpm and 37°C. A vial with sufficient growth was identified and the contents streaked with a dilution-method on an LB-agar plate which was incubated at 37°C overnight. A single colony was selected from the growth on the agar plate and suspended in LB broth to ensure a monoclonal *E. coli* stock. The vial was cultured overnight at 180rpm and 37°C. If sufficient growth occurred (cloudy suspension) the contents of the vial was centrifuged at 2500rpm in 50ml polypropylene centrifuge tubes for 1hr and resuspended in 3ml of Ringers reagent. The suspension was aseptically transferred to 2 x 1.5ml Eppendorf® tubes and centrifuged at 14 000rpm for 1min. The supernatant was discarded; the pellets resuspended on a total of 3ml Ringers and combined. Enough *E. coli* stock was cultured to last the duration of the experiment. This was to ensure consistency in the genome of the *E. coli* applied every day and to restrict large genetic variation in the *E. coli* applied with each treatment.

For enumeration of *E. coli*, 10 x Eppendorf tubes containing 900µl of Ringers reagent were prepared and set up in a laminar flow cabinet. To the first tube, 100µl of *E. coli* stock solution was added. With serial dilutions, the concentrate was diluted down to 10^{-10}. LB-Agar plates were quartered with a vivid marker and 10µl of solution from each dilution was dotted in triplicate on each quarter. The plates were incubated at 37°C overnight and the colonies counted the following day.

5.2.7 *Escherichia coli* survival

Before the *E. coli* stock could be added to the greywater and irrigated onto soil it was necessary to assess the survival rate of *E. coli* in soil and in greywater.

5.2.7.1 Survival in soil and greywater

An *E. coli* stock was prepared and enumerated as in Chapter 3, section 3.2.4. Field moist soil was sieved <2mm and 200g were weighed for each soil. A synthetic greywater was prepared according to the formula in section 5.2.4 containing 10ppm TCS. The *E. coli* was spiked into the greywater to a final concentration 10^8 CFU/100ml. A soil-type dependent volume of this greywater (18µl for Gisborne and Katikati, and 12µl for the Lincoln soil) was added to each soil immediately after the *E. coli* was added. The soils were mixed thoroughly and incubated at room temperature. After 2 hours a subsample of 10g was taken from each soil and added to 90ml PBS (phosphate buffered saline) along with approximately 1 teaspoon of 3mm glass beads. The mixture was agitated at 200rpm for 1hr on an orbital shaker. A serial dilution was made in triplicate from the saline extracts for each soil type in order to obtain 5 dilutions. An aliquot was taken from the triplicate diluted samples (1ml) and transferred to vials containing Lauryl Tryptose (LT) Broth (Difco) with inverted tubes for gas collection. The samples were incubated at 35°C ± 0.5°C in a water bath for 24hrs. Positive tubes (air bubbles in the inverted tubes) were inoculated with a loop into vials containing EC-broth + MUG (DIFCO) and incubated at 44°C ± 0.2°C. Negative LT tubes were allowed to incubate for a further 24 hours. EC-broth + MUG tubes fluorescing under UV light were marked positive. Subsequent samples were taken on day 2 and day 5 from the soil stock and prepared in the same way. Enumeration was done with the 3-tube MPN (Oblinger & Koburger, 1975).

5.2.7.2 Survival in greywater.

Escherichia coli stock was prepared and enumerated as in section 5.2.4. Synthetic greywater was prepared as for the lysimeter experiment, also containing 10ppm TCS. The *E. coli* was spiked into the greywater and aliquots of 1ml of greywater were diluted into 9ml of PBS in the same way the soil extracts were diluted. The test was carried out in triplicate and also contained 5 serial dilutions. The rest of the protocol was followed as in section 5.2.5.1.

5.3 Analytical methods

5.3.1 Substrate induced respiration, sulphatase, biomass, triclosan analysis and *Escherichia coli* quantification

The 0 – 5cm fraction of soil from each lysimeter was separated into two samples. One fraction was stored at 4ºC for SIR, sulphatase and microbial biomass was analysed (see chapter 3 for methods), and the second fraction was stored at - 20ºC for extraction and analysis of microbial DNA and TCS (See Chapter 3 for methods).Quantitative PCR analysis of *E. coli* was carried out by targeting the *uidA gene* which encodes for the beta-glucuronidase enzyme. As only one copy of *uidA* is found in an *E. coli* cell, detected and quantified copy numbers of the gene are used interchangeably with numbers of *E. coli* in this chapter, as described by Bej, DiCesare, Haff, and Atlas (1991).

5.3.2 Statistics

Microsoft Excel 2010 was used for all statistical analysis.

One-way ANOVA was performed for all data sets. Standard deviations indicating upper and lower limits were calculated and post-hoc analysis was performed using a Tukey HSD pairwise comparison of the means to identify significant differences between treatments.

5.4 Results

5.4.1 *Escherichia coli* survival in soil and greywater

The recovered *E. coli* numbers from soil after the addition of greywater containing 10ppm TCS to soil is presented in Table 6. The survival of *E. coli* in the Lincoln and Katikati soil seemed to be stable whereas a decline was observed over time in the Gisborne soil. Due to the large confidence intervals of the 3-tube MPN method, there is no statistical significance ($p>0.05$) between recoveries for each day.

Table 6: The survival of E. coli in soil after addition of greywater containing 10ppm triclosan

Soil	Sample day	Added CFU/1g	Recovered (MPN/1 g)	Lower 95% CI	Upper 95% CI
Gisborne	2 Hours	90	15	3.7	42
	Day2	90	9.2	1.4	38
	Day5	90	7.4	1.3	20
Lincoln	2 Hours	60	<3	0	9.5
	Day2	60	3.6	0.17	18
	Day5	60	3.6	0.17	18
Katikati	2 Hours	90	3.6	0.17	18
	Day2	90	3.6	0.17	18
	Day5	90	3.6	0.17	18

5.4.2 Lysimeters

5.4.2.1 Escherichia coli survival in soil at different depths

The numbers of *E. coli* detected in each soil horizon in each soil type are shown in Figures 15-17. For the Gisborne soils, in the 0-5cm soil horizon, more *E. coli* was detected in the bad quality greywater (BQGQ) and bad quality greywater dump (BQGWD) than in the control and good quality greywater (GQGW). However due to the large variability in data sets caused by a number of 'non-detects' in the qPCR analysis the results are not statistically significant ($p>0.05$) (Fig 15). The same 'trends' can be observed with higher *E. coli* in the BQGW treatment in the 5-10cm horizon compared to the other treatments for that layer (Fig 15). This, however, was also not statistically significant ($p>0.05$).

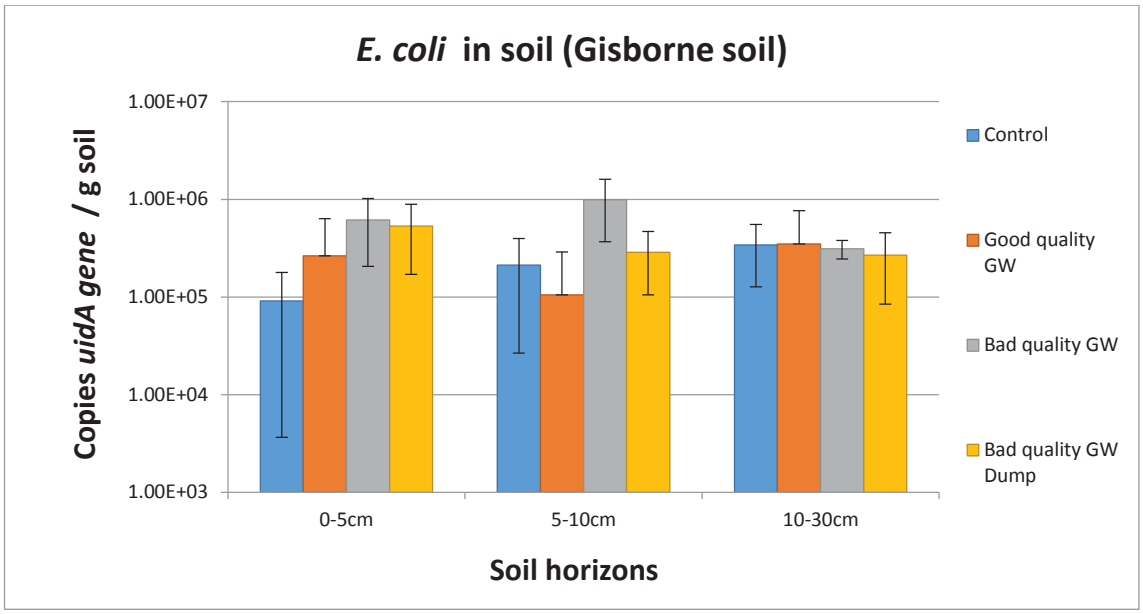

Figure 15: Copies of the uidA gene in E. coli detected in each layer of the Gisborne soil indicated for all 4 treatments. Error bars were calculated using standard deviation. The graph's y-axis begins at the average detection limit for all datasets analysed.

In the Katikati soils high background *E. coli* in the control treatments resulted in no significance differences between the controls and the greywater treatments. In some cases the numbers of *E. coli* detected in the controls were similar to levels detected in the bad quality greywater treatments (Fig 16).

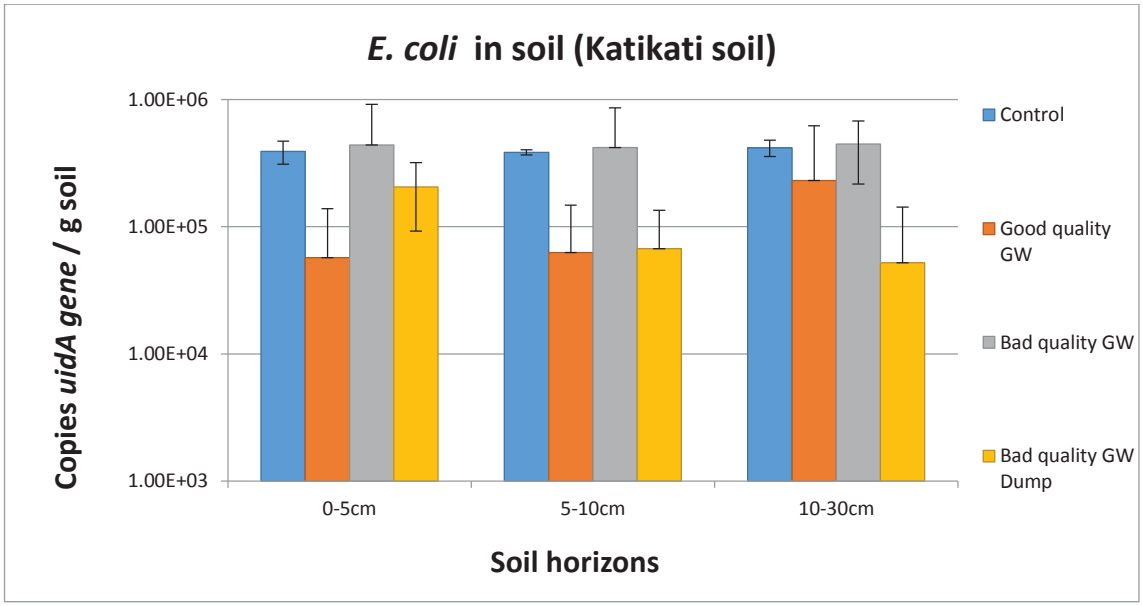

Figure 16: Copies of the uidA gene in E. coli detected in each layer of the Katikati soil indicated for all 4 treatments. Error bars were calculated using standard deviation. The graph's y-axis begins at the average detection limit for all datasets analysed.

In the Lincoln soil there is no significant difference (p>0.05) between the levels of *E. coli* detected in each soil horizon (Fig 6). There was also no difference between the numbers of *E. coli* between treatments, except for the control treatment, in which there was no copies of the *uidA-gene* detected in any of the control treatment's field replicates. A single factor ANOVA, however, found the trend not to be statistically significant. This is likely due once again to high variability in the replicates as *E. coli* was not detected in two field replicates (BQGQ 0-5cm, and BQGW 10-30cm) causing large standard errors with the detection limit (1.17 x 10^3 copies/g soil).

The data suggest that there is no *E. coli* build-up in the Lincoln soil, as the concentration of *E. coli* in each layer was similar, regardless of treatment (Fig 6). The levels detected (1.21 x 10^5 copies/g soil – 2.47 x 10^5 copies/g soil) in each layer were less than the maximum amount added throughout the experimental timeline (1.11 x 10^6 copies/g soil) (assuming an even distribution of *E. coli* through the soil core).

In the Lincoln soil there is no statistical difference (p>0.05) between the GQGW, BQGW and BQGWD treatments.

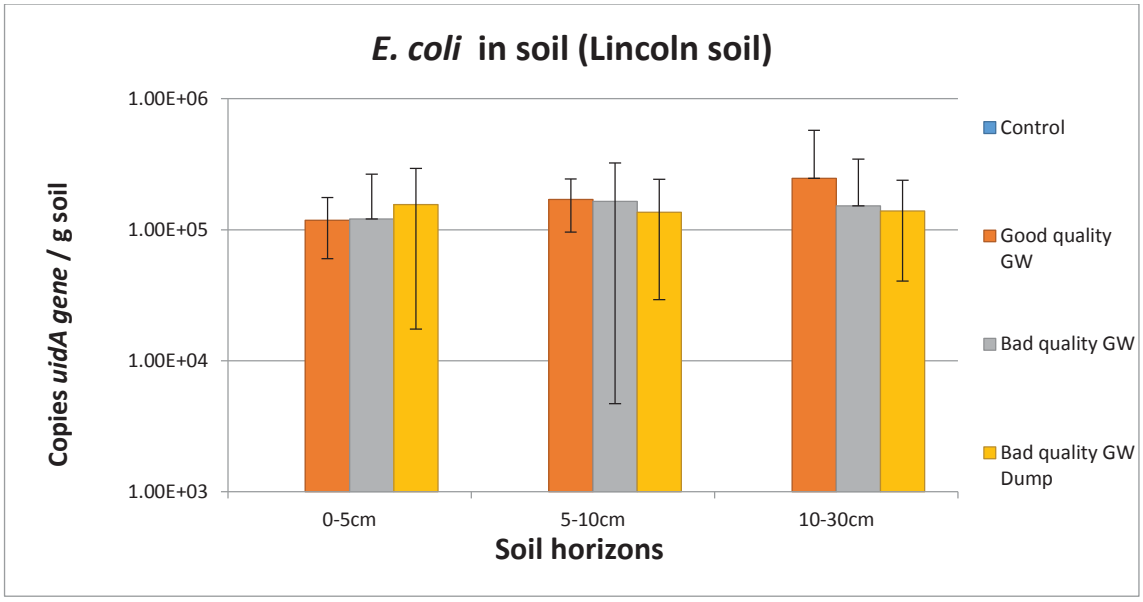

Figure 17: Copies of the uidA gene in E. coli detected in each layer of the Lincoln soil indicated for all 4 treatments. Error bars were calculated using standard deviation. The graph's y-axis begins at the average detection limit for all datasets analysed.

In general there is a trend of higher accumulation of *E. coli* in the BQGW than the BQGWD for both Gisborne and the Katikati soil (Fig 15 & 16).This, however is not the case in the Lincoln soil.

In the Gisborne and Katikati soils the levels *E. coli* detected in the control treatment were similar to levels detected for bad quality greywater (BQGW) (Fig 15 & 16).

5.4.2.2 Escherichia coli in leachate

The numbers of *E. coli* detected in each leachate in each soil type are shown in Figures 18-20. For the Gisborne soil the levels of *E. coli* detected in the leachate were consistently higher for the BQGW treatment compared to the control, GQGW and the BQGWD. The same trend could be observed for the first 8 weeks of irrigation in the Katikati soil. After 8 weeks, the levels of the *uidA-gene* detected decreased and fell below levels observed for the BQGWD treatment in the Katikati soil.

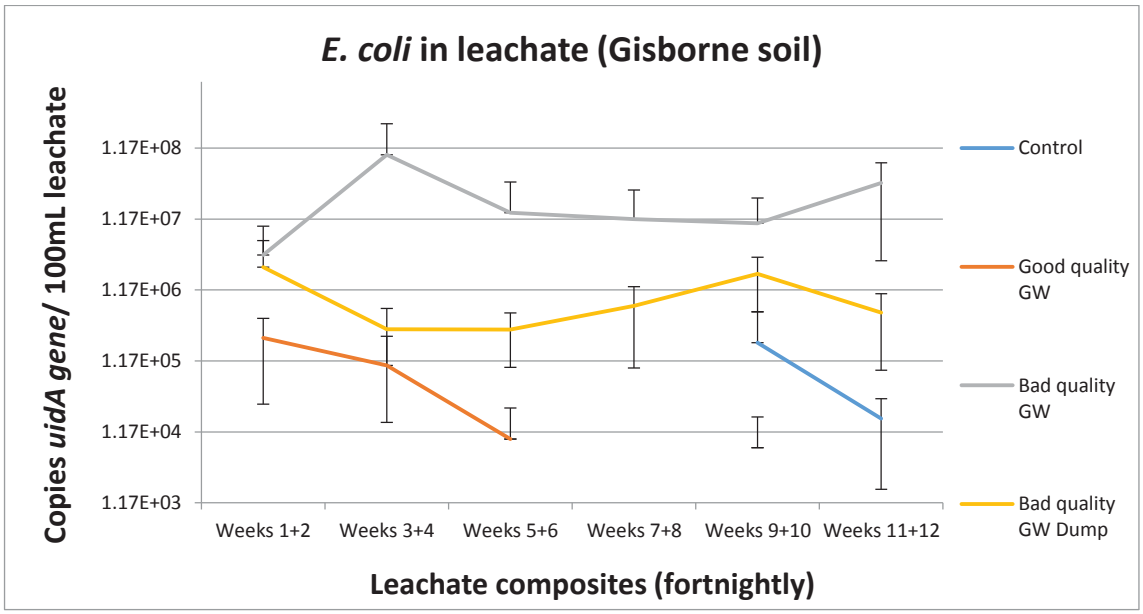

Figure 18: Copies of the uidA gene in E. coli detected in fortnightly leachate composites of the Gisborne soil.

Generally there are higher levels *of E. coli* in leachate for BQGW than for BQGWD treatment in all composites for the Gisborne soil (Fig 18). This is similar to the trend observed in the *E. coli* detected in the Gisborne soil cores.

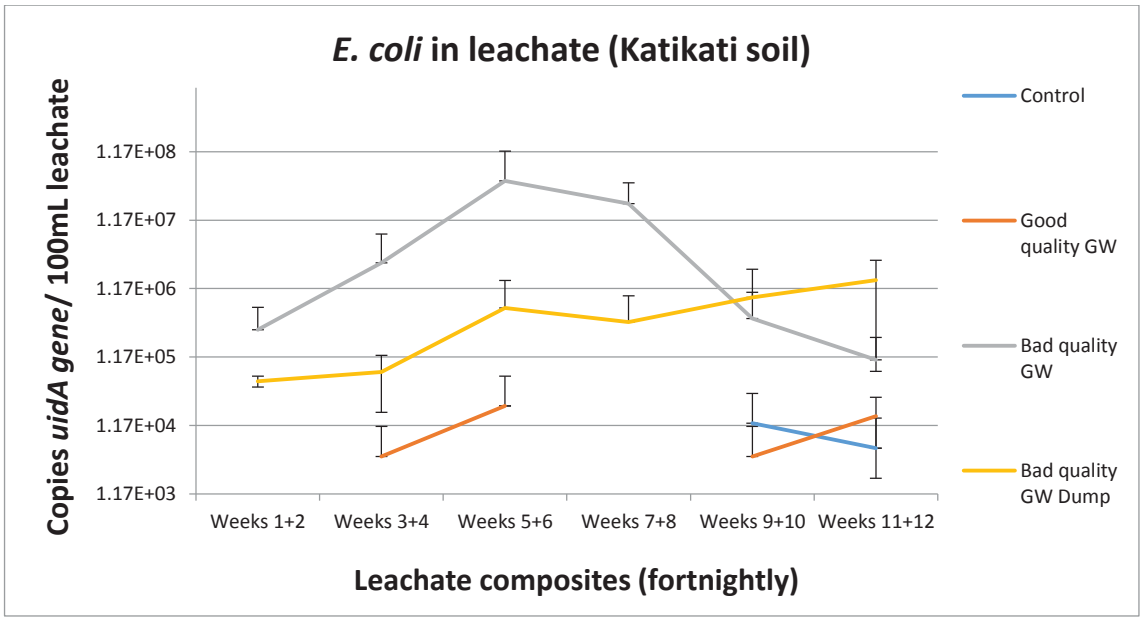

Figure 19: Copies of the uidA gene in E. coli detected in fortnightly leachate composites of the Katikati soil.

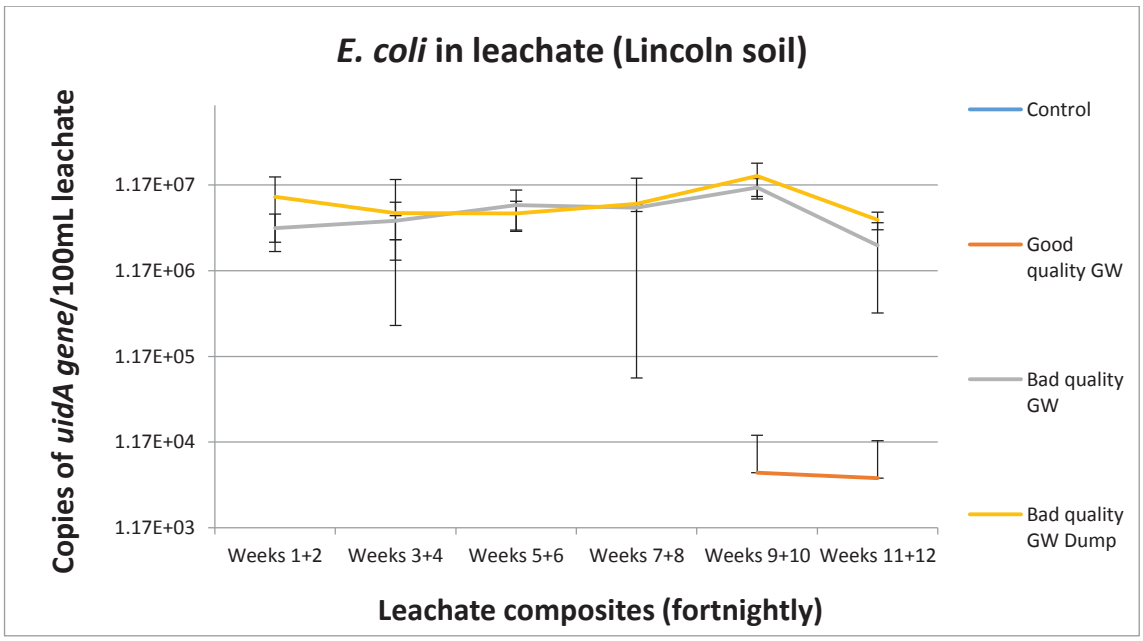

Figure 20: Copies of the uidA gene in E. coli detected in fortnightly leachate composites of the Lincoln soil.

The levels of *E. coli* in the BQGWD were similar to the levels found in the control and GQGW treatments, except for the Lincoln soil (Fig 20) where the BQGW and BQGWD followed the same trend at similar levels.

There was not much difference between the *E. coli* levels detected from control and GQGW.

5.4.2.3 Triclosan in soil

The concentrations of TCS detected in each soil horizon in each soil type are shown in Figures 21-23. The detection limit for the triclosan analysis in soil was 5ppb and all samples below that threshold were not reported.

In all 3 soils, low levels (0-33.7 ppb) of TCS were detected in all soil horizons for the GQGW treatment (Figures 21-23).

In the Gisborne soil (Fig 21), the concentrations of TCS for the BQGWD treatment was significantly lower ($p<0.05$) than the concentration of TCS for the BQGW treatment in the 10-30cm horizon and the concentration of the BQGW was significantly higher ($p<0.05$) than the GQGW at the same depth. However, in general there was no difference between the BQGW and BQGWD treatments.

In the 5-10cm horizon the TCS concentration measured for the BQGW treatment was significantly higher than for the GQGW treatment (Fig 21).

There is a downward trend in the concentrations for both the BQGW and the BQGQD treatments with increasing depth in the Gisborne soil (Fig 21).

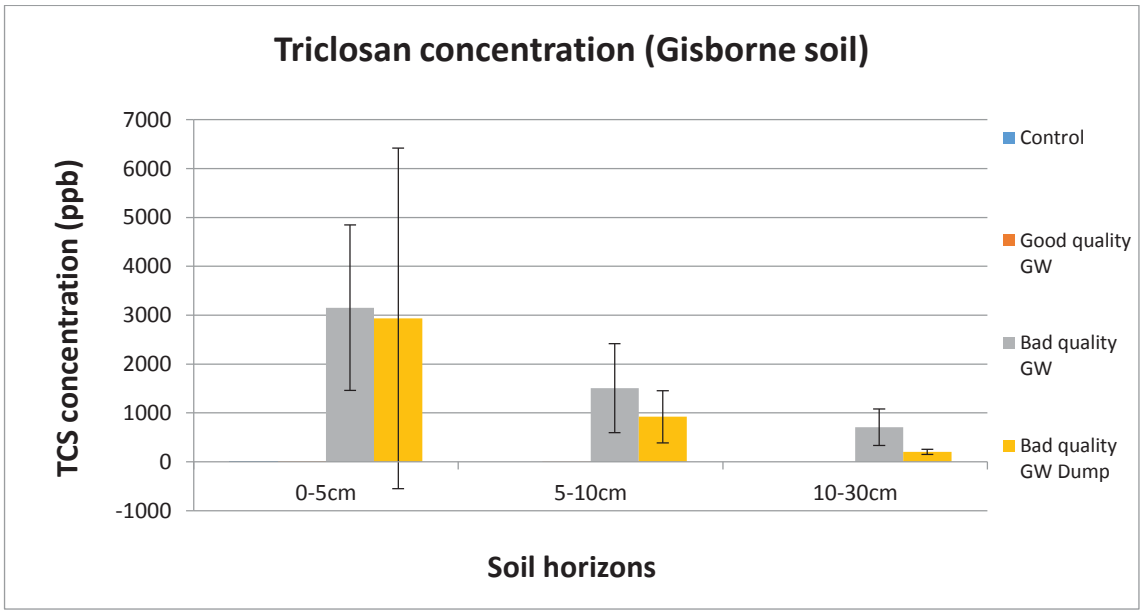

Figure 21: Concentration triclosan detected in each horizon of the Gisborne soil indicated for all 4 treatments. Error bars were calculated using standard deviation.

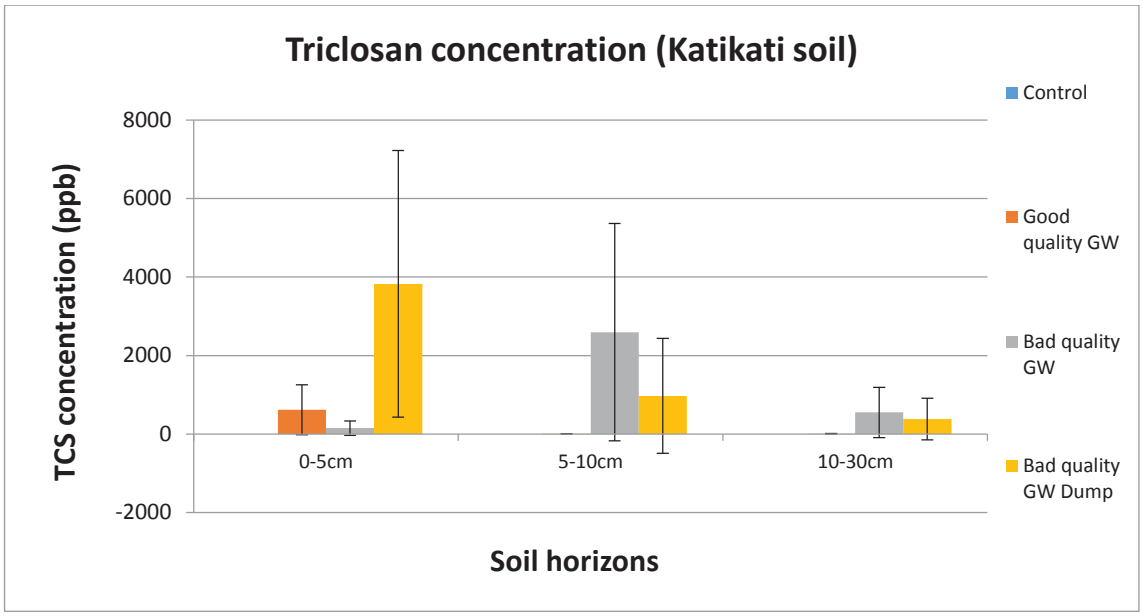

Figure 22: Concentration triclosan detected in each horizon of the Katikati soil indicated for all 4 treatments. Error bars were calculated using standard deviation.

There was a downward trend in TCS concentration through the Katikati soil profile for the BQGWD treatment (Fig 22). Except for the 0-5cm profile, TCS levels for the BQGWD treatment appeared to be less than for the BQGW treatment. Due to the high variably in the replicates for each treatment and the consequent large standard deviations, there was no significant difference ($p>0.05$) between the different greywater treatments.

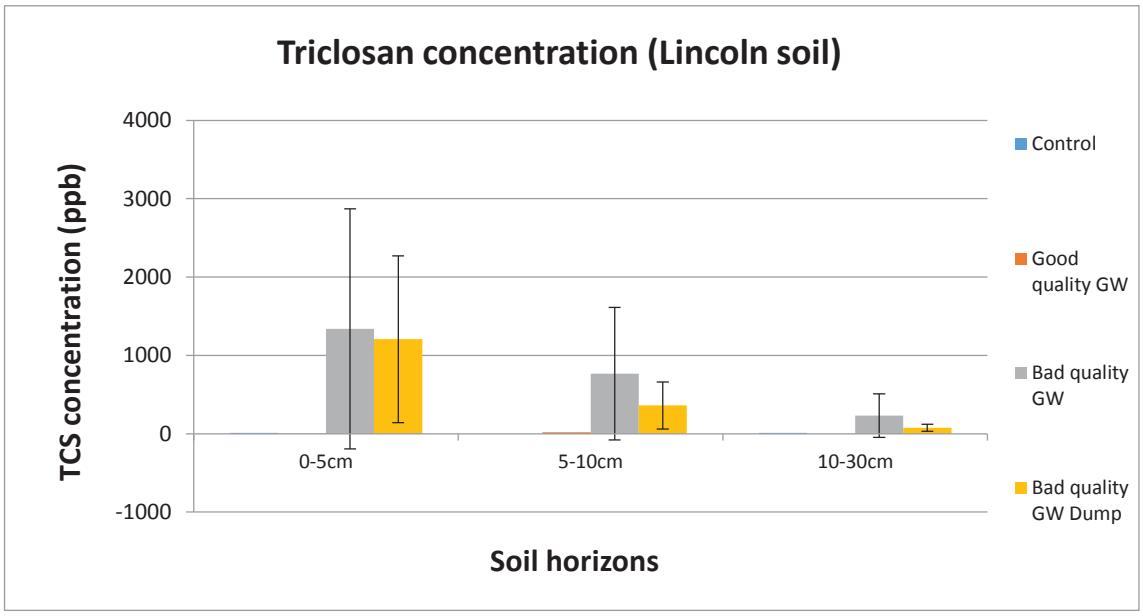

Figure 23: Concentration triclosan detected in each horizon of the Lincoln soil indicated for all 4 treatments. Error bars were calculated using standard deviation.

As with the Gisborne and Katikati soils, there is a downward trend in concentrations of TCS detected through the soil layers for the BQGW and BQGWD in the Lincoln soil (Fig 23). For all 3 soils the concentrations of TCS for the BQGWD appears to be lower than that of the BQGW treatment.

5.4.2.4 Triclosan in leachate

The concentration of TCS detected in the leachate from each soil type is shown in Figures 24-26.

Low levels of TCS were detected in the control samples (0.2-2.8ppb) in all the leachates from all the soils.

In the Gisborne soil, initially low levels of TCS leached from week 0 to week 10 (Fig 24). There was an increase in the concentration of TCS leaching in the BQGW treatment during weeks 11 to 12 (17.5ppb). Generally, higher levels of TCS were detected for this treatment through the course of the experiment than was detected for any other treatment (Figures 24&25).

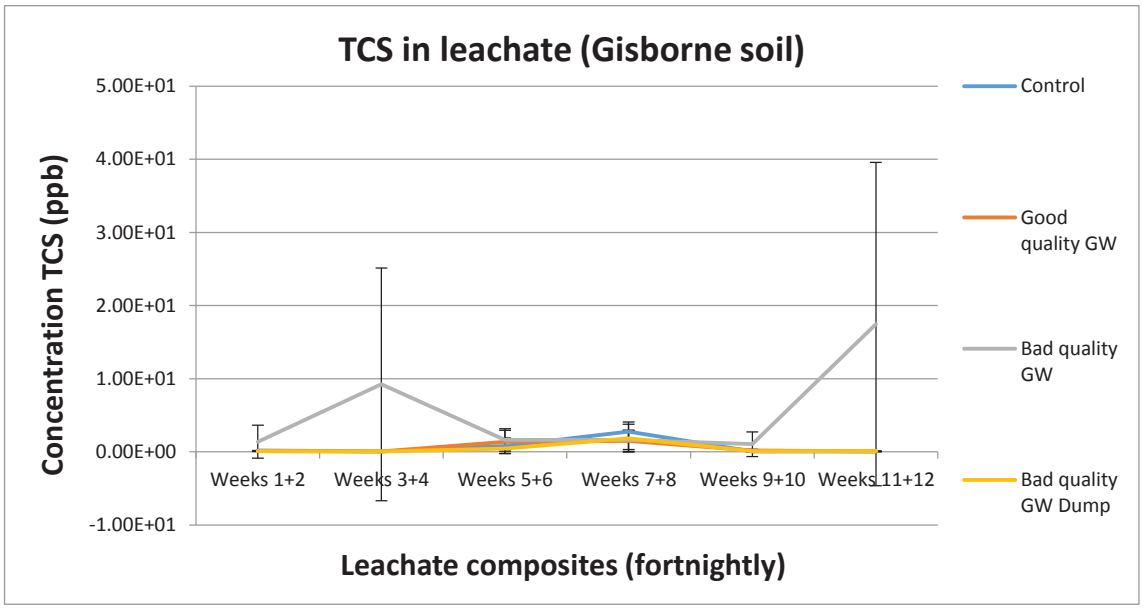

Figure 24: Concentration TCS detected in fortnightly leachate composites of the Gisborne soil.

In the Katikati soil, the concentration of TCS in the leachate from the BQGW was consistently higher than for all other treatments, including the BQGWD throughout the 12 weeks of sampling (Fig 25). There was a spike in TCS leaching (8.3ppb) at weeks 5 and 6 for the BQGW treatment.

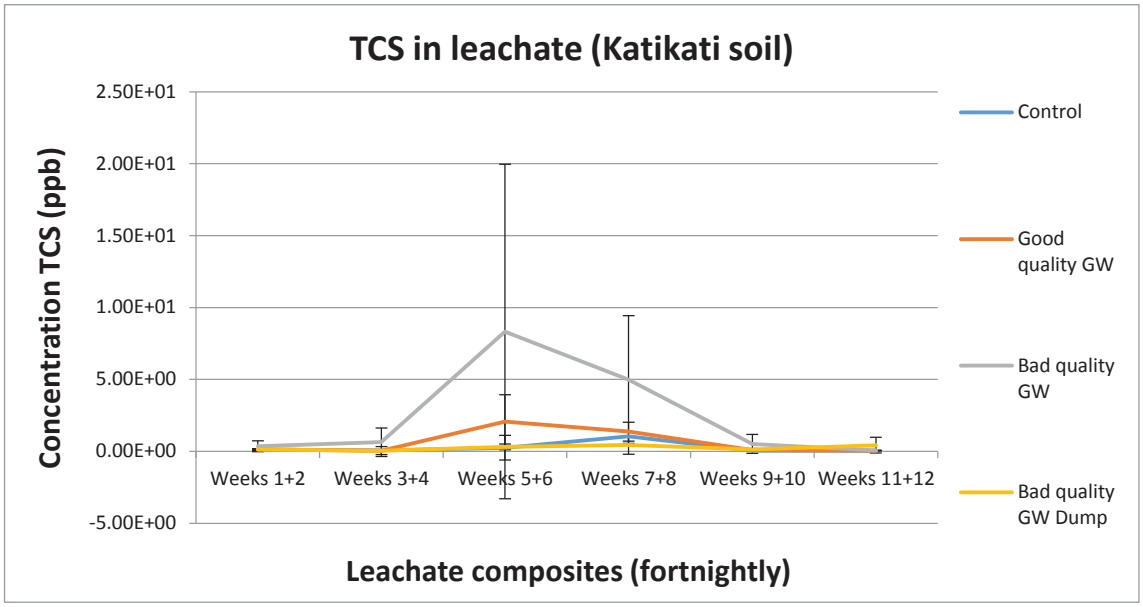

Figure 25: Concentration TCS detected in fortnightly leachate composites of the Katikati soil.

In the Lincoln soil, in general, TCS concentrations peak between weeks 5 and 8 (Fig 26) in all the treatments. In this soil type, similar amounts of TCS were detected in the control leachates as in the greywater treated soils, however the BQGW dump had the highest concentrations of TCS during the peak (weeks 5-8).

Low levels of TCS were detected during weeks 11 and 12 for all soils in all treatments, except for the BQGW treatment in the Gisborne soil which increased to 17.5ppb (Fig 24).

In all soils, the primary product detected was TCS as opposed to Met-TCS (Fig 27) (TCS, Met-TCS, 2, 4-dichlorophenol and 4-chlorocatechol was measured in soil, see Appendix for details). The concentrations of 2, 4-dichlorophenol and 4-clorocatechol (microbial breakdown products) were mostly undetected.

There was notably less TCS detected in the Lincoln soil. This is not surprising as the recommended irrigation rates for that soil types were lower, thus resulting in less TCS applied.

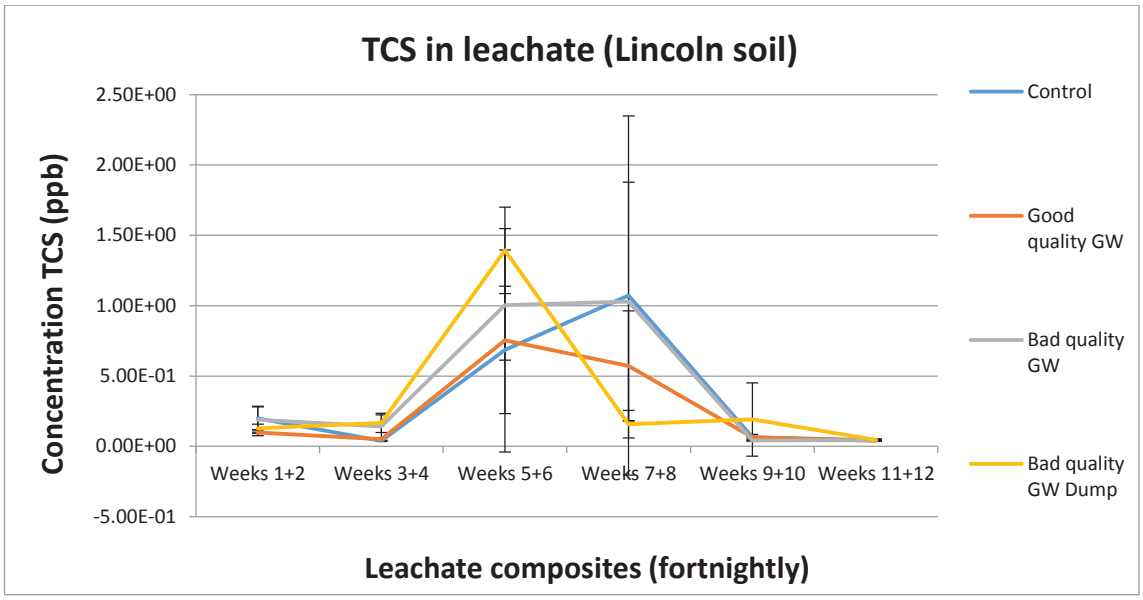

Figure 26: Concentration TCS detected in fortnightly leachate composites of the Lincoln soil.

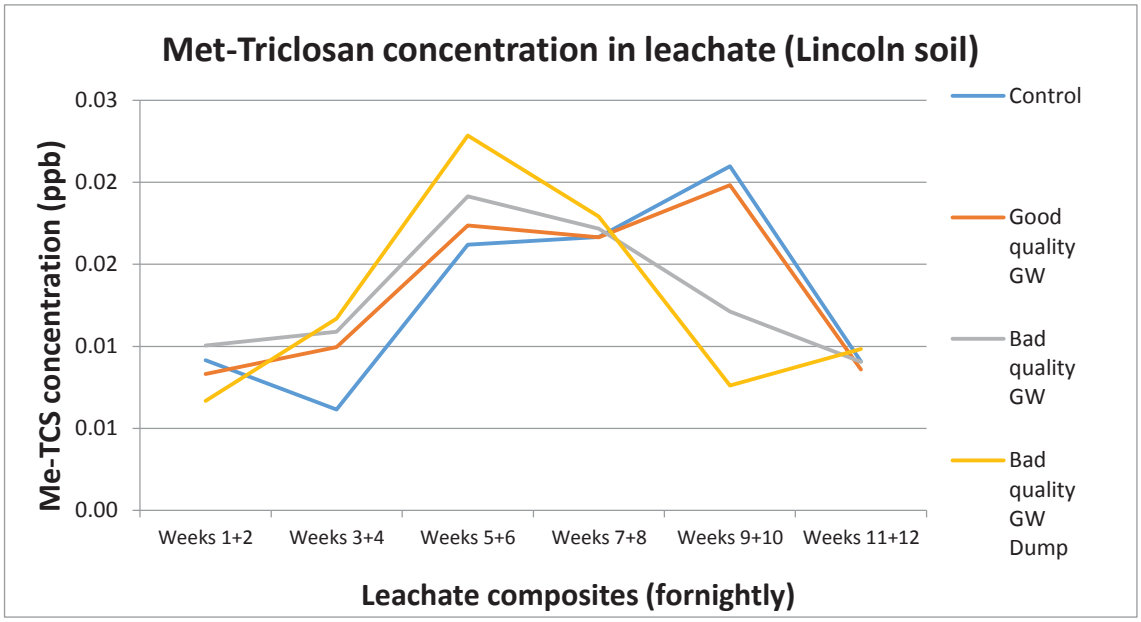

Figure 27: Concentration Met-TCS detected in fortnightly leachate composites of the Lincoln soil.

Figure 28 illustrates the total rainfall for the experimental period. Total precipitation declined between weeks 3 and 7. A sharp increase in precipitation was observed between weeks 8 and 10, followed by a drier period at the end of the experiment.

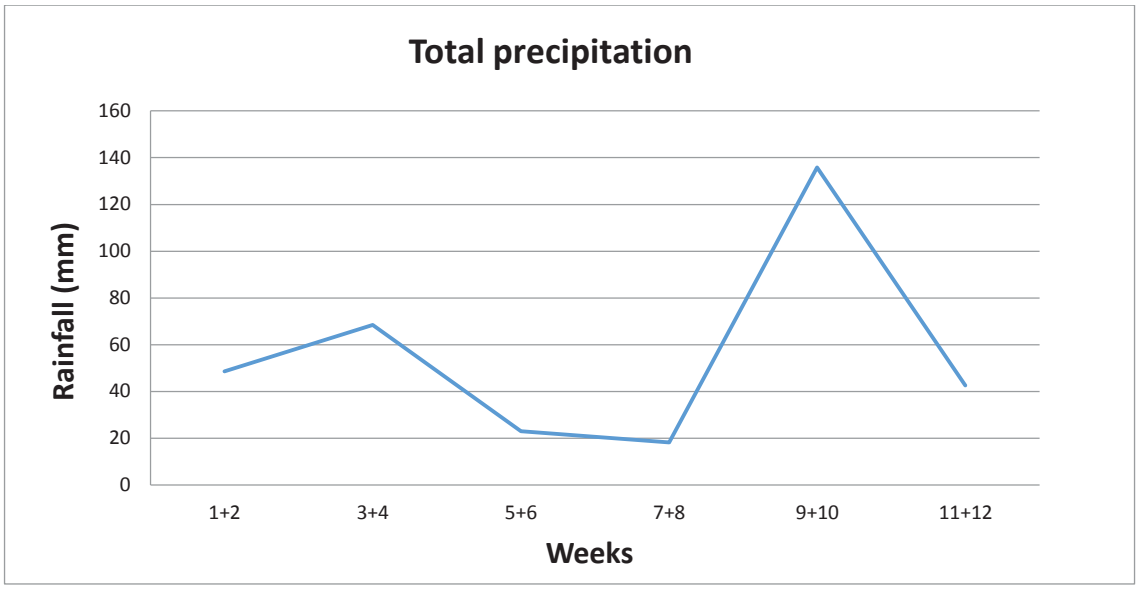

Figure 28: Fortnightly rainfall for Mana Island. Mana Island was the closest weather station to where the experiment was conducted (Data supplied by Metservice).

5.4.3 Soil health indicators

5.4.3.1 Substrate induced respiration

The SIR rates for the 0-5cm soil horizon in each soil type are shown in Figures 29-31.

There were no differences in substrate induced respiration between treatments for the top 5 cm of the soil cores in the Gisborne and Lincoln soils (Fig 29 & Fig 31).

The SIR in the control treatment in the Katikati soil was significantly lower ($p<0.05$) than for the GQGW treatment and the SIR for the soil from BQGWD treatment was significantly lower ($p<0.05$) than the GQGW (Fig 30). The SIR for the control treatment and the BQGW dump were similar.

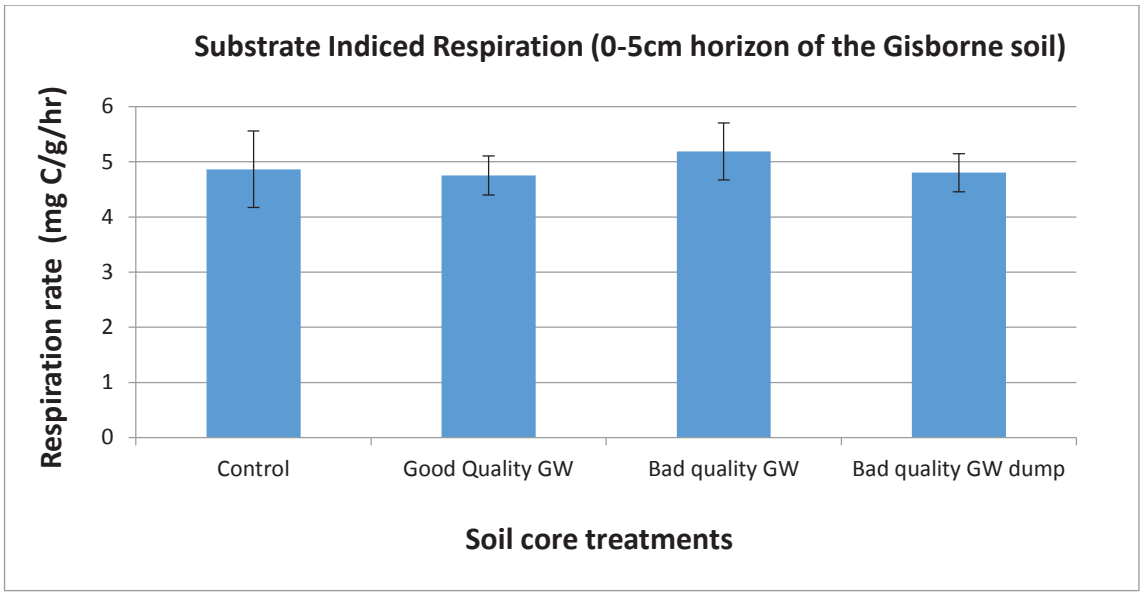

Figure 29: Substrate induced respiration for the 0-5cm horizon of the Gisborne soil cores. Error bars were constructed using standard deviation.

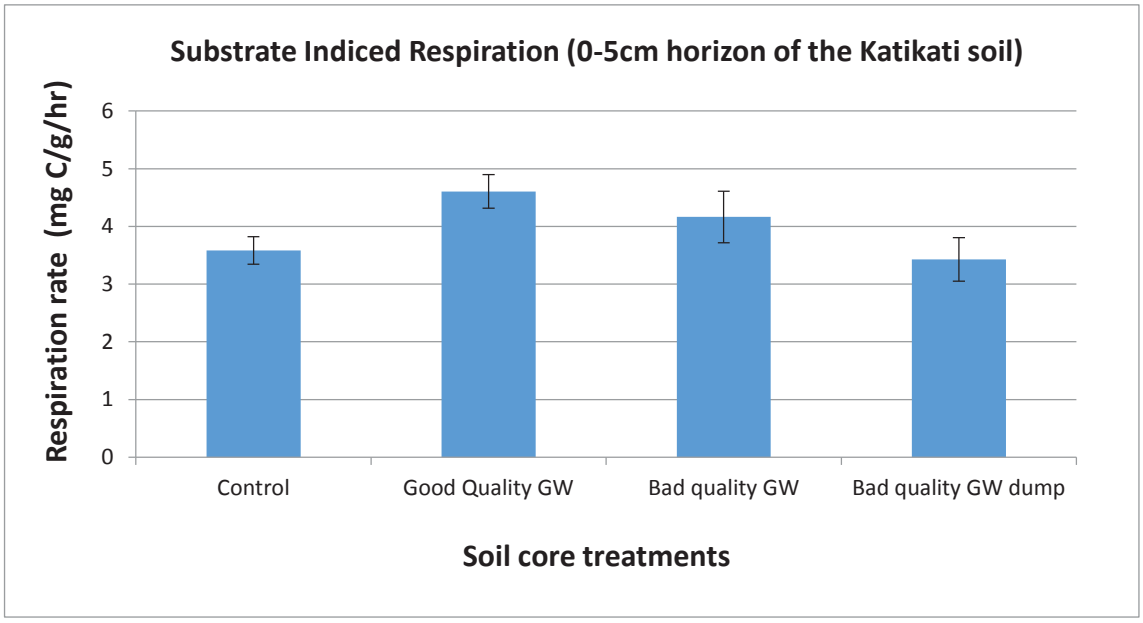

Figure 30: Substrate induced respiration for the 0-5cm horizon of the Katikati soil cores. Error bars were constructed using standard deviation.

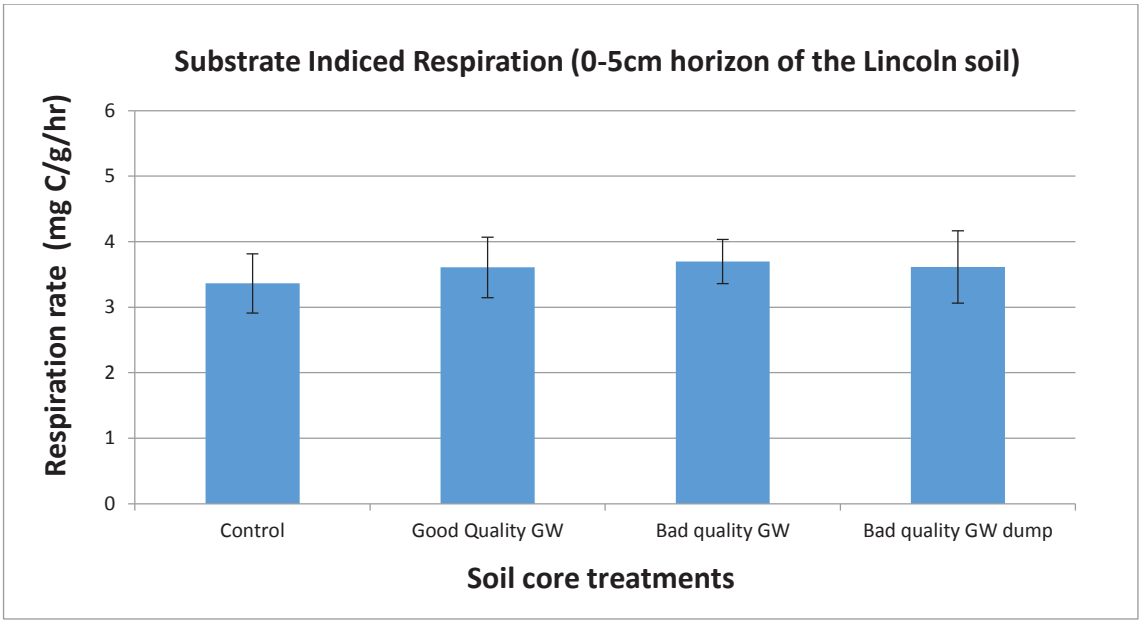

Figure 31: Substrate induced respiration for the 0-5cm horizon of the Lincoln soil cores. Error bars were constructed using standard deviation.

5.4.3.2 Biomass

The microbial biomass for the 0-5cm soil horizon in each soil type is shown in Figures 32 - 34. There were no significant ($p<0,05$) differences in microbial biomass between treatments for the top 5cm layer of the soil cores of all 3 soils (Figures 32 - 34).

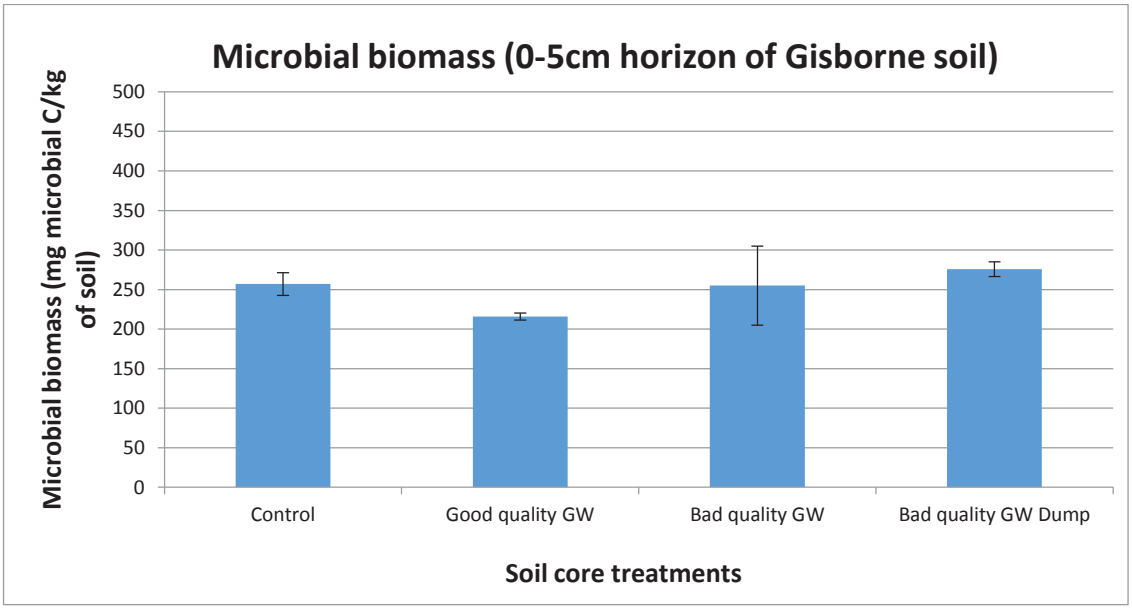

Figure 32: Microbial biomass for the 0-5cm horizon of the Gisborne soil cores. Error bars were constructed using standard deviation.

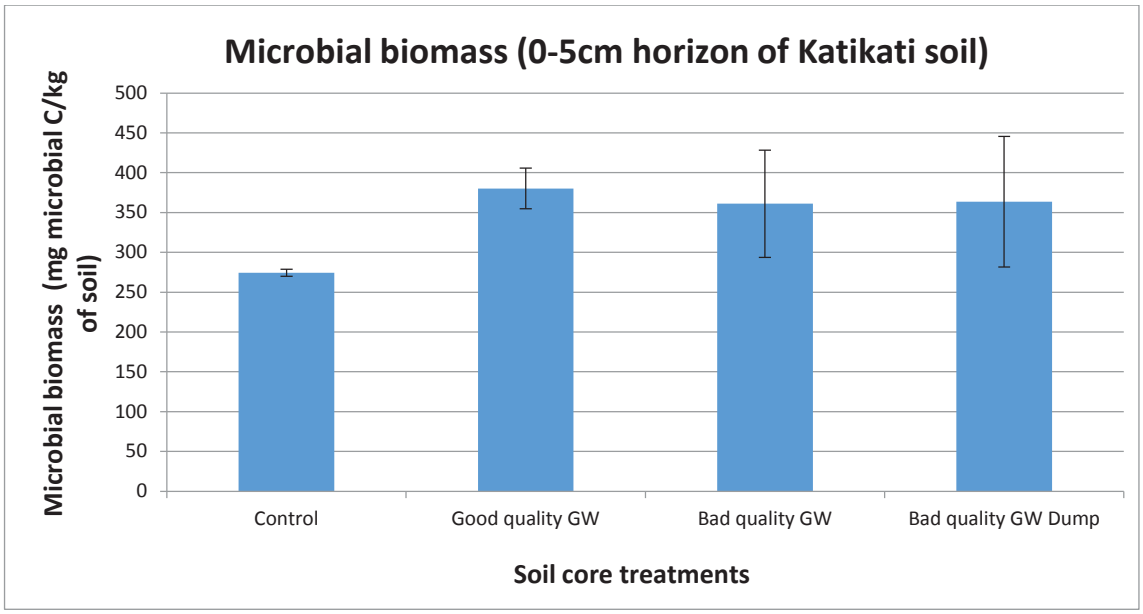

Figure 33: Microbial biomass for the 0-5cm horizon of the Katikati soil cores. Error bars were constructed using standard deviation.

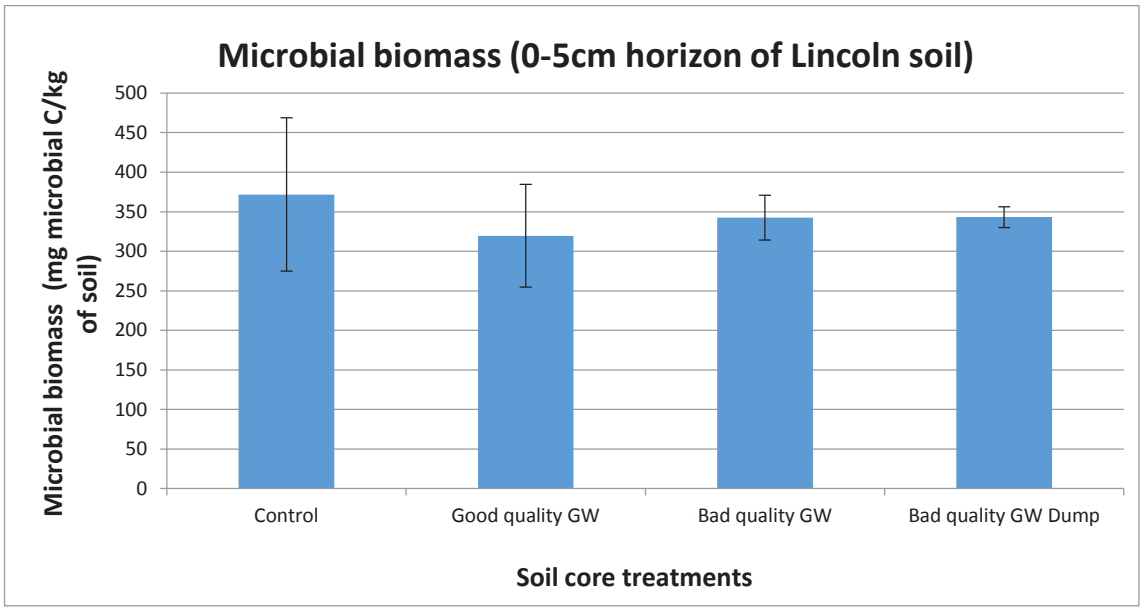

Figure 34: Microbial biomass for the 0-5cm horizon of the Lincoln soil cores. Error bars were constructed using standard deviation.

5.4.3.3 Sulphatase

The sulphatase activity for the 0-5cm soil horizon in each soil type is shown in Figures 35 - 37. There were no significant differences (P>0.05) in the sulphatase activity between treatments for the 0-5cm layer in all 3 soils (Figures 35 - 37). Note the different scales of the y-axis.

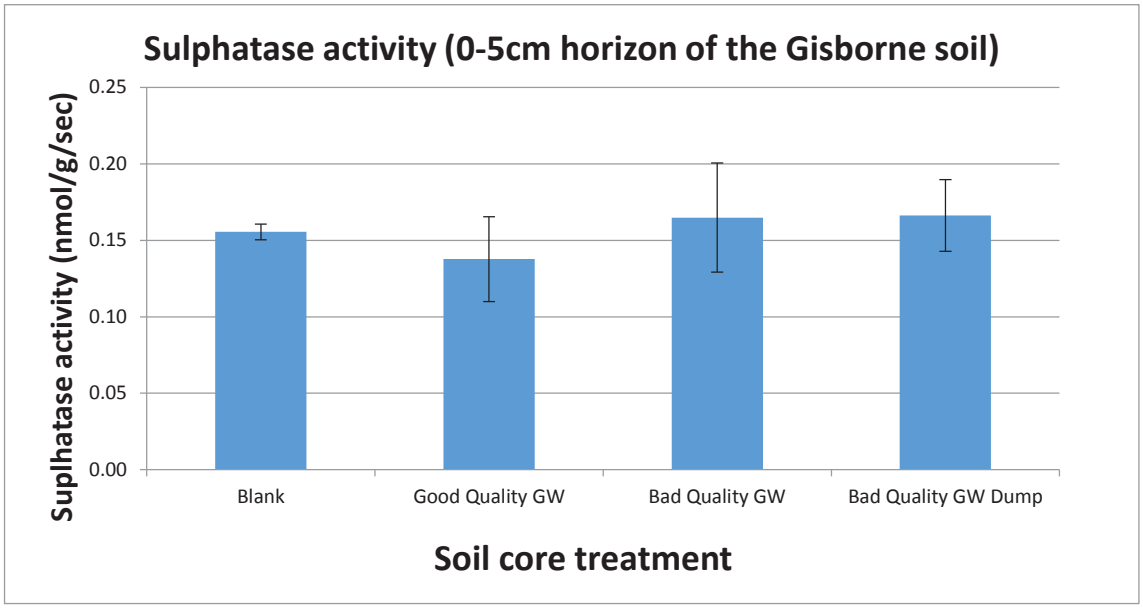

Figure 35: Sulphatase activity for the 0-5cm horizon of the Gisborne soil cores. Error bars were constructed using standard deviation.

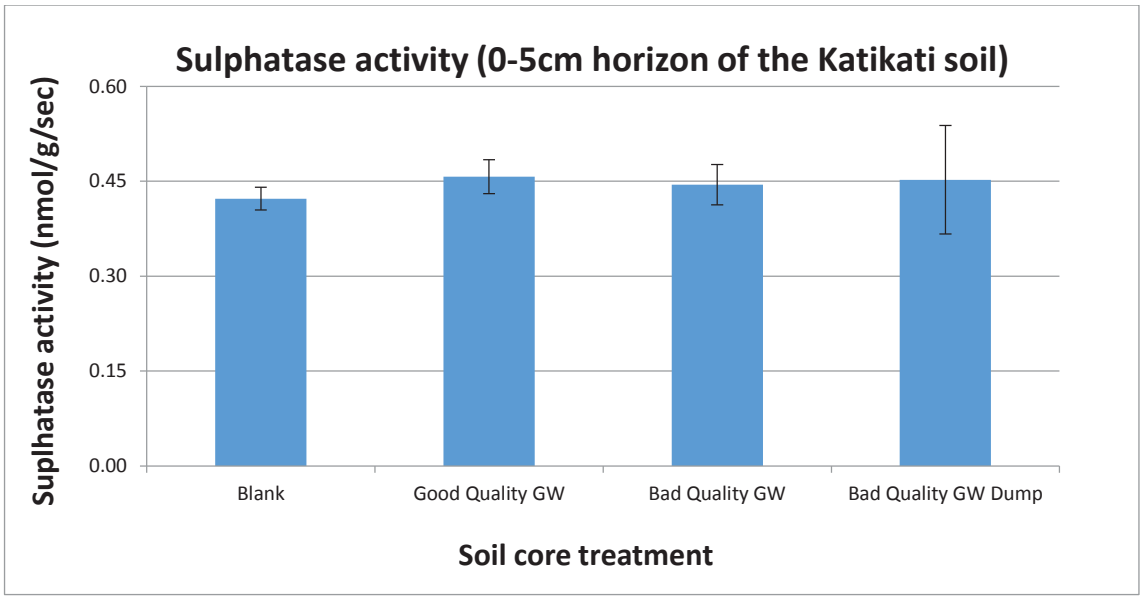

Figure 36: Sulphatase activity for the 0-5cm horizon of the Katikati soil cores. Error bars were constructed using standard deviation.

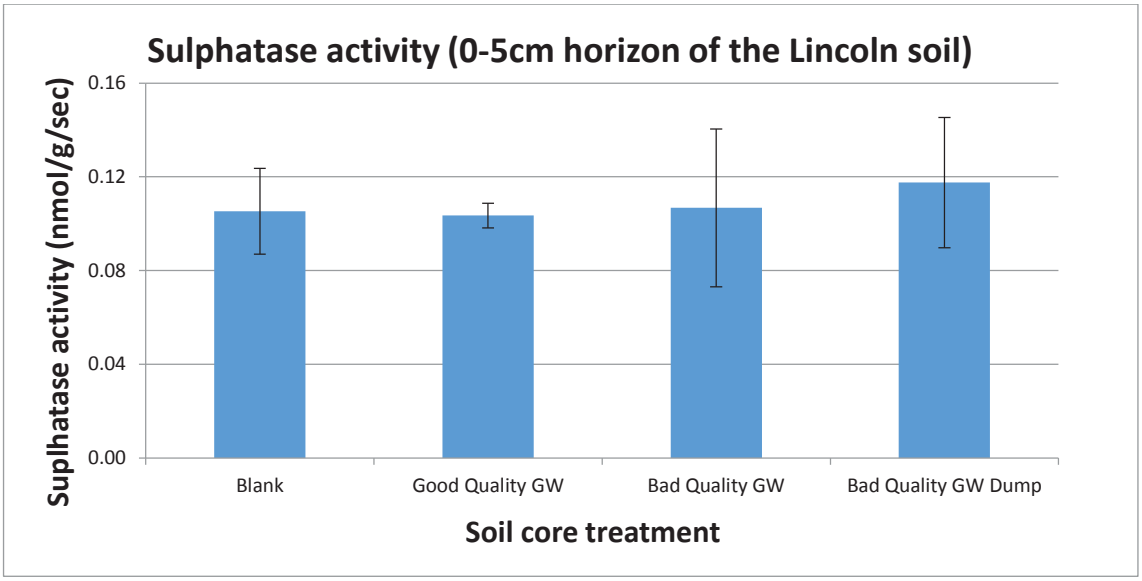

Figure 37: Sulphatase activity for the 0-5cm Horizon of the Lincoln soil cores. Error bars were constructed using standard deviation.

5.4.3.4 Microbial metabolic quotient

The microbial metabolic quotient for the 0-5cm soil horizon in each soil type is shown in Figures 38 - 40. There were no significant differences ($p>0.05$) in the microbial metabolic quotient between treatments for the 0-5cm layer in all 3 soils (Figures 38 - 40).

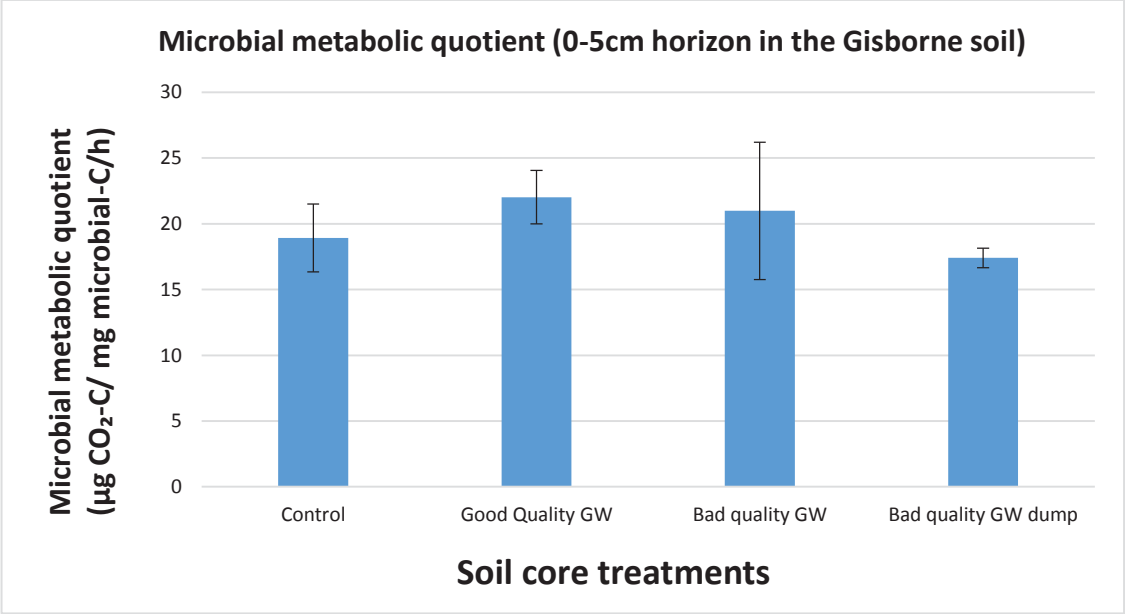

Figure 38: Microbial metabolic quotient for the 0-5cm horizon of the Gisborne soil cores. Error bars were constructed using standard deviation.

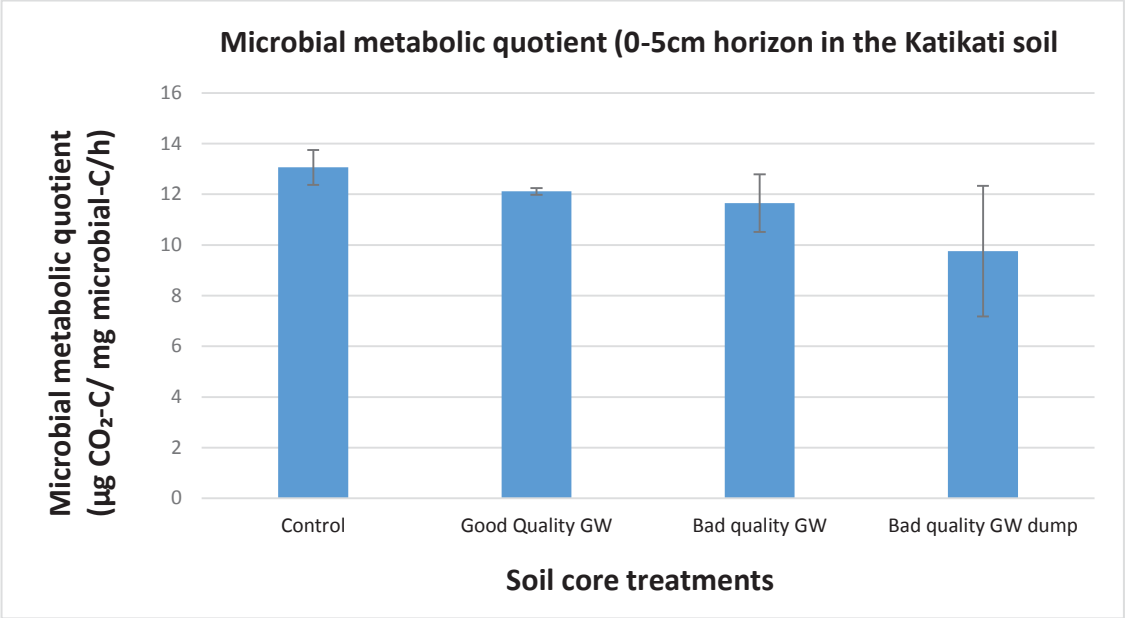

Figure 39: Microbial metabolic quotient for the 0-5cm horizon of the Katikati soil cores. Error bars were constructed using standard deviation.

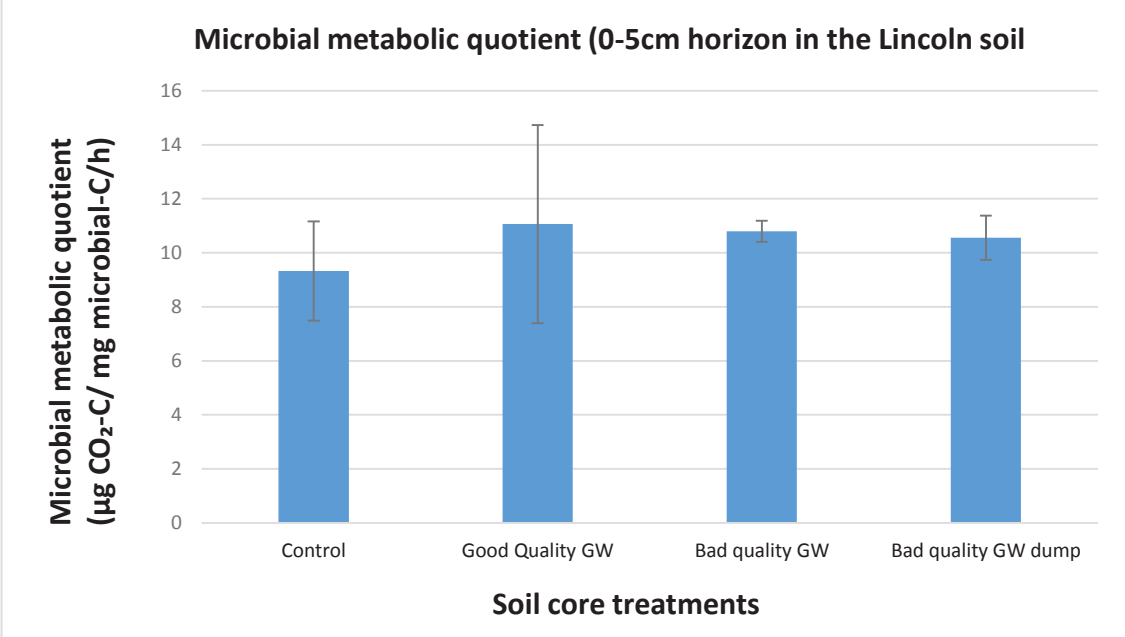

Figure 40: Microbial metabolic quotient for the 0-5cm horizon of the Lincoln soil cores. Error bars were constructed using standard deviation.

5.5 Discussion

5.5.1 *Escherichia coli* in soil

5.5.1.1 Gisborne soil

In general there is less retention of *E. coli* in the lower layers of the Gisborne soil cores, compared to the upper level (0-5cm) (Fig 15) suggesting that there might be a build-up of microbes in the top layers of soil irrigated with greywater containing *E. coli*. The Gisborne soil is a fine sandy loam with a clay base. The lysimeters were excavated to 30cm in depth and the base consisted of at least 5cm clay. The 0-5cm layer contains more organic matter due to rye-grass cover and therefore it is more likely that *E. coli* will bind and accumulate in the upper soil layers. Smith and Hegazy (2006) conducted an investigation where the retention of *E. coli* was related to the soil organic content. It was found that the adsorption and subsequent retention of *E. coli* was increased proportionally with an increase in soil organic material.

The organic matter for each soil layer was not analysed due to time and financial constraints.

5.5.1.2 Katikati soil

The Katikati soil was excavated from a private section in an urban setting. The excavation site was close to a vegetable garden and the inhabitants of the house had pets roaming freely around the garden. Initial qPCR characterisation of the soil before placement in the lysimeter facility and irrigation did not detect *E. coli* in the native soil. However, at harvest *E. coli* was detected in all control samples. A possible explanation for the high *E.coli* levels detected in the control treatments (Fig 16) might be due to environmental influences in the lysimeter facility. The lysimeters were placed in close proximity to where a flock of Pukeko resides and their droppings frequently had to be removed from the surfaces of the soil cores. A qPCR analysis could have been carried out to determine if the *E. coli* from the control samples were avian of origin. The differences in concentration of *E. coli* between treatments detected in the Katikati soil were however not significantly different from one another ($p>0.05$).

5.5.1.3 Lincoln soil

Compared to the control treatment, there were relatively high levels of the *uidA gene* detected for the GQGW, BQGW and BQGWD treatments in the Lincoln soil. The difference proved not to be statistically significant ($p>0.05$). This is due to *E. coli* not being detected in two other field replicates (one field replicate for BQGQ 0-5cm, and one field replicate for BQGW 10-30cm) causing large variability and large error bars.

The trend that the levels of *E. coli* detected were similar between treatments and soil depths suggests that there is little retention of *E. coli* in the Lincoln soil. The Lincoln soil was a silty clay loam with a 25% fraction of clay. This might be due to the clay being better at filtering and retaining *E. coli*. Naclerio, Nerone, Bucci, Allocca, and Celico (2009) concluded that the clay fraction in soil plays a significant part in the adsorption and consequent retention of *E. coli* in the soil profile. In that study *E. coli* was added to the top of 2 sets of soil cores, one set containing a clay fraction. Soil samples were tested after the experimental period. The average percentage retention of *E. coli* in the soil containing the clay faction was 0.11% and the retention in the soil without clay was 0.04%.

The small difference between the GQGW and BQGW treatments in the Lincoln soil could indicate that the irrigated *E. coli* leaches through the soil profile by preferential flow instead of adsorbing to the clay particles as predicted by Naclerio et al. (2009). Clay soils usually contain dense structural units and are at risk of cracking, facilitating bypass flow. Insufficient retention along with preferential flow of greywater through soil implicates a potential risk of groundwater contamination.

The lower levels of *E. coli* detected for the GQGW treatment compared to the BQGW treatment in the Gisborne and Katikati soils are not surprising as the total *E, coli* loading between the treatments were different. Total *E. coli* loading for the GQGW was approximately 2.21×10^4 CFU and 2.21×10^{10} CFU for the BQGW treatment. There was no such difference apparent for the Lincoln soil. This indicates that there is less risk of accumulation of *E. coli* in the sandy loam soils if irrigated with greywater containing low levels of *E. coli*. According to Eriksson et al. (2003), household greywater can contain 1×10^2 CFU/100ml to 2.8×10^3 CFU/100ml. These concentrations are similar to concentrations measured by Siggins et al. (2013). However greywater could contain up to 2.51×10^7 CFU/100ml in elevated levels (Chaillou et al., 2011).

From the data it is possible that *E. coli* could potentially leach through the soil profile and contaminate groundwater if greywater containing high concentrations of *E. coli* is reused on sandy loam soils.

5.5.2 *Escherichia coli* in leachate

Escherichia coli were found in the leachate from the BQGW treatments in all soil types. This suggests that there is risk of ground and surface water contamination by microbial contaminants if greywater reuse is not appropriately managed.

The higher levels of *E. coli* in the leachate from the BQGW compared to the BQGWD in the Gisborne and Katikati soils might be due to efficiency of *E. coli* retention with a longer residence time of greywater in the soil cores. The lysimeters were irrigated with a constructed devise calibrated to deliver the treatment at 1.6L/h. It is possible that infiltration patterns between the "dump" treatments and the irrigation treatments were different. A preliminary study to assess the infiltration patters of drip irrigation at 1.6L/h on all 3 soils indicated that with consecutive irrigations there was a preferential flow pattern created. This could allow the irrigated sample accelerated transport through the soil profile *via* a hydrological pathway and thus result in higher concentrations of *E. coli* in the leachate (Fig 41).

The preferential flow patterns described here are not the same as preferential flow created by discrepancies in soil integrity e.g. cracks in the soil core.

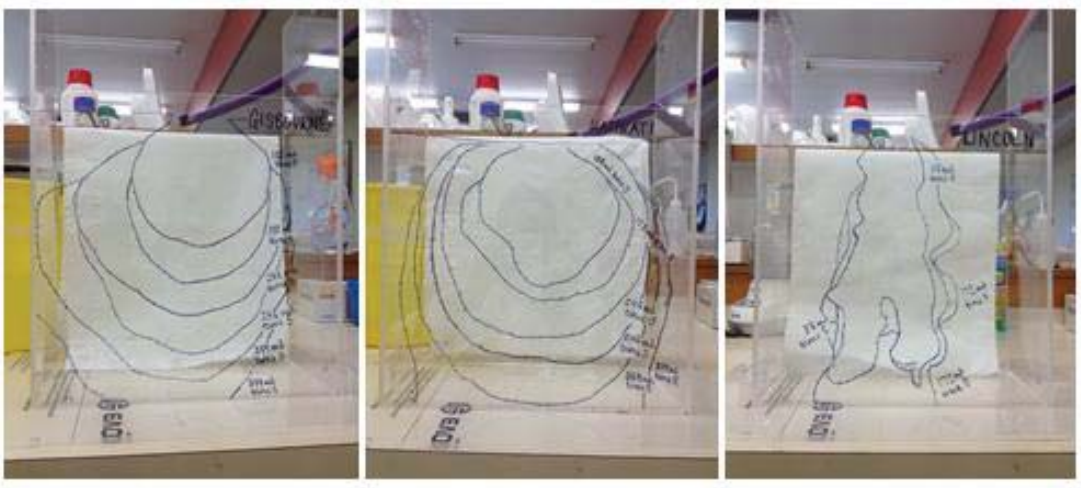

Figure 41: Flow patterns created after 3 additions of greywater at the recommended rate for that soil type, and marking the infiltration front as soon as the irrigation stopped, and 5mins after. This was done for all 3 soils.

With the “dump” treatment the surface of the lysimeters was flooded with greywater and the treatment mostly covered the surface of the soil core before it filtered down the soil profile. Provided there was no preferential flow down the sides of the lysimeters, this would have allowed for a slower infiltration rate allowing a longer residence time in the soil’s top layers before leaching. Smith and Hegazy (2006) concluded that the effect the soil organic matter has on the adsorption of *E. coli* is related to the hydraulic loading rate, and consequently how much water moves down the soil profile in a given time. In that study, the impact that soil organic matter had on adsorption of *E. coli* was the largest for lower hydraulic loadings (5cm/h and 12cm/h). That indicates that adsorption of *E. coli* would be larger with lower irrigation rates. This might seem counter-intuitive as the “dump” treatment in this study was included to illustrate the effect of flood-irrigation, which could be compared to the higher hydraulic loading from the study that Smith and Hegazy (2006) conducted. However the volumes irrigated (123ml twice a day for Gisborne and Katikati soils, and 86ml twice a day for the Lincoln soil) were within the absorptive capacity specific to the soil types (AS/NZS1547:2012-Table M1)

The similar levels of *E. coli* in the leachate for the BQGW and BQGWD treatments detected in the Lincoln soil (clay loam) supports the assumption made in 5.5.1.3 that bypass flow might have taken place in the Lincoln soil cores. For this treatment the “dump” irrigation would not have filtered steadily through the soil profile as with the Gisborne and Katikati soils, but instead followed flow paths where water velocity was at its highest, resulting in higher levels of *E. coli* in the leachate. A study conducted by Aislabie, McLeod, Ryburn, McGill, and Thornburrow (2011) investigated a clay loam soil and a sandy loam soil for potential bypass flow and microbial transport. A 25mm depth of dairy shed effluent was irrigated at 5mm/h three times a year and the leachates were collected and analysed for *E. coli*. Levels of *E. coli* between 4.3×10^3 CFU/100ml and 2.4×10^6 CFU/100ml were detected in the leachate for the clay loam, and no *E. coli* were detected in the sandy loam. It was concluded that soils high in clay content have a risk for preferential flow. The results from that investigation support the assumption that preferential flow might have occurred during this lysimeter study in the Lincoln soils. Thus caution should be applied if greywater is to be irrigated on soils with high clay contents.

An increase in rainfall relates to the decrease in concentration of *E. coli* in the weekly composites. It is also follows that with higher rainfall, more *E. coli* would leach, however his could not confirmed as not all the leachate was collected. The fortnightly composite sample

provided discrete sampling points and merely suggests higher leaching with increased precipitation.

The leachates from the control and GQGW treatments contained similar levels of *E. coli*. There is limited literature available on the leaching after the application of greywater containing low levels of *E. coli*, however these results suggest that there would be minimal leaching of *E. coli* if its concentration in greywater is comparable to what is normally found in households (Eriksson et al., 2003). If good management practices are used and greywater is diverted when there are individuals in the house that are ill, or when nappies are being washed, there is a relatively low risk of *E. coli* leaching down the soil profile.

This study suggests that it remains important to practice sub-surface irrigation to prevent ponding of greywater on the soil surface, subsequently exposing pets and children to microbial contaminants.

5.5.3 Triclosan in soil

There is a trend that TCS decreases with each horizon in each of the 3 soils. This decrease in TCS concentration might be due to a number of factors such as conversion and degradation, or that the bulk of the TCS was adsorbed in the upper layers of the soil cores. In order to accurately predict the fate of TCS it was necessary to do a mass balance of the compound and its metabolites. Due to time and financial constraints, this was unable to be undertaken.

An important factor in a transport model is the retention/release characteristics of an organic compound. Agyin-Birikorang et al. (2010) conducted a study where the partition coefficient of TCs was investigated in soil, and in soil containing biosolids. The partition coefficient for soil alone was determined to be *log* K_d 2.25±0.26 and for soil and biosolids, *log* K_d 3.76±0.39 thus indicating a higher affinity of the compound for biosolids. The *log* K_{OC} is an organic matter corrected value for the *log* K_d and was similar for both treatments (4.26±0.31). This indicates that TCS tends to partition into matrices with higher organic content. It is then expected that the mobility of adsorbed TCS would be negatively affected resulting in restriction of movement through the soil profile. Thus, most TCS would absorb to the top 0-5cm of the soils in this study where there is higher organic matter.

Xia et al. (2010) investigated the TCS concentrations in various soil horizons of plots that had been receiving biosolids application for 33yrs. Four depths of the soil cores collected were analysed for TCS concentration. It was found that cumulative loading of TCS were more

pronounced in the top 15 cm of the soil and less pronounced in the 15-30 cm layer, however the top 15cm mostly consisted of biosolids material. This supports the conclusion from Agyin-Birikorang et al. (2010) and Butler, Whelan, Sakrabani, et al. (2012). In that study the fate of TCS was investigated in soil after biosolids application by measurement of TCS in 3 soil horizons (0-10cm; 10-20cm; 20-30cm). It was determined that after 1 year, TCS and its major degradation product, Met-TCS (methyl triclosan) was mainly found the top 10cm of soils.

In the study Xia et al. (2010) conducted, it was found that with heavy application of biosolids containing TCS, 49-65% of TCS applied was detected 30-120cm horizon. This suggests that with the application of high concentrations of TCS there is an associated risk of the compound leaching. The loading rate of the BQGW was indicative of the accumulative loading of soil with high concentrations TCS in greywater.

In this study, TCS degradation was regarded as minimal because very small amounts of the degradation products were detected in the soils. Triclosan degradation is mediated by soil microbes and the rate of degradation is limited by various abiotic factors. Degradation mainly takes place under aerobic conditions and during wet winter months (the time this lysimeter experiment was conducted) when the soil cores were at field capacity for most of the time, minimal degradation would have taken place. Low temperatures also impede the degradation of TCS in soil (Butler, Whelan, Sakrabani, et al., 2012). This is possibly due to anaerobic conditions in the soil structure created by an excess of soil moisture preventing oxygen diffusion (Butler, Whelan, Sakrabani, et al., 2012). Butler, Whelan, Sakrabani, et al. (2012) also noted a relatively low degradation rate during wet winter periods. Triclosan is at risk of leaching in winter months due to heavy hydraulic loading if it is not degraded by soil microbes.

With the continuous application of TCS through greywater irrigation, the soil microbial community might be overwhelmed, resulting in a build-up of TCS in the soil.

5.5.4 Triclosan in leachate

In general the BQGW treatments resulted in significant amounts of TCS leaching through the soil profile. This indicates that TCS is able to move through the soil profile and potentially contaminate ground and surface waters. Thus, best practice for greywater reuse should include the reduction of use of potentially eco-toxic chemicals such as TCS in household products.

In both the Katikati and Gisborne soils the TCS levels detected in the BQGW treatment were consistently higher than the BQGWD. This is possibly due to the slower infiltration effect for the "dump" treatment described in 5.5.1.3. A longer residence time would allow more efficient binding of TCS to soil particles. In some rural communities greywater is land applied via a hose pipe from the bathroom window. The BQGWD treatment was designed to replicate this situation. Results suggest that this form of application is no higher risk in terms of risks to ground and surface water than more managed greywater systems where greywater is applied by sub-surface irrigation.

The TCS concentrations for all soils and treatments were generally low during the last weeks of sampling. This could indicate adaption of the soil microbial community and an enhanced ability to degrade TCS after prolonged TCS exposure. In a study conducted by Butler et al. (2011) it was indicated by measuring basal respiration rates that after initial TCS exposure, soil microbial communities recovered after 7 days where basal respiration rates for the control and the treatments containing TCS were similar. The study was conducted on 3 soil types (clay, loamy sand, sandy loam). It was also noted that in the clay soil and the loamy sand, respiration rates exceeded that of the control on day 14. This could mean a stimulation in the soil microbial community and potentially that the microbes were using it as a food source, thus facilitating the breakdown of the TCS itself.

As a similar trend was observed throughout all 3 soils, it is reasonable to assume that abiotic influences could also be responsible for the drop in TCS concentrations. The decrease might have been due to high levels of precipitation, thus considerably diluting the sample collected.

As in the soil, the primary product detected in the leachate was TCS, and not Met-TCS. The microbial breakdown products of TCS; 2, 4-dichlorophenol and 4-chlorocatechol, were not detected in the leachate possibly due to the nature of the method of analysis. ; 2, 4-dichlorophenol and 4-chlorocatechol are volatile compounds (#), and as the analytical protocol for the sample preparation of the leachate involved sample concentration by evaporation of extraction solvent that contained the target analytes, it is possible that the compounds evaporated with the solvent.

Butler, Whelan, Sakrabani, et al. (2012) found that conversion of TCS to breakdown products mainly takes place in soils with lower moisture content. If TCS is not degraded by soil microbes, it has the potential to accumulate, overwhelm the soil microbial community and impede subsequent breakdown. There is also a risk that the accumulated TCS be transported off site and continue its antimicrobial action in the receiving environment. The diversion and irrigation

of greywater containing TCS during winter months is thus a high risk activity. Irrigation during the summer when the soil microbial community is more active means less of an accumulation risk. This will not only ensure maximum breakdown of TCS but could also supplement the soil water deficit.

Low concentrations of TCS were detected in the control treatment of the Gisborne soil; this result might be explained by drift during irrigation events. The techniques applied to analyse for TCS in the leachate was also very sensitive and slight cross contamination in laboratory equipment could have had an effect.

5.5.5 Soil health indicators

In this study, the addition of greywater of varying qualities had no impact on SIR, biomass or metabolic quotient. This suggests that the levels of TCS accumulated in the soil were not high enough to have any significant effect on soil health indicators. Triclosan levels measured in the 0-5cm horizon ranged from <0.012ppm in the control sample, to 3.83ppm dry weight in the BQGWD treatment. The accumulated values of TCS obtained from this study corresponds to the values reported by Negahban-Azar, Sharvelle, Stromberger, Olson, and Roesner (2012) after assessing the TCS concentration in soils that had received greywater irrigation over 30yrs.

5.5.5.1 Substrate induced respiration (SIR)

The application of different qualities of greywater to the soil cores did not have any significant effect on SIR; this is likely due to the low levels of TCS that accumulated. In other studies where soil microbial communities were exposed to higher concentrations of TCS, e.g., impacts on soil microbial respiration have been recorded. Both Liu et al. (2009) and Butler et al. (2011) found that TCS concentrations higher that 10ppm significantly inhibit SIR. In the same study Liu et al. (2009) also assessed the effect of 0.1ppm and 1 ppm TCs on soil microbial respiration and concluded that there was no significant differences between those treatments and the control treatment which contained no TCS. The concentrations of TCS found in the soil at the end of a 3 month greywater irrigation period ranged from <0.012ppm - 3.83ppm, similar to those used by Liu et al. (2009). In another study Waller and Kookana (2009) assessed the effects of various concentrations of TCS (0, 1, 5, 10, 50, and 100ppm) on substrate induced nitrification. In that study it was noted that for concentrations lower than 5ppm there was no significant difference.

5.5.5.2 Biomass and microbial metabolic quotient

Changes in the microbial metabolic quotient can be used as an indicator to assess microbial stress with changes in the soil environment. The parameter is determined by calculating the specific respiration per unit of microbial biomass. It indicates the rate at which a specific unit of microbial biomass respires. The literature available on the effects of TCS on microbial metabolic quotient is rather scarce; however other parameters have been employed to measure microbial stress in relation to TCS concentration. Butler, Whelan, Ritz, et al. (2012) investigated the effect of various TCS concentrations (0, 10, 100, 500 and 1000ppm) on the cyclo: mono-unsaturated PLFA marker (CMR) 3 difference soils (loamy sand, sandy loam and clay). In the loamy sand and clay soils there was a significant stress response for the soil amended with 10ppm TCS compared to the control. There was no significant response at that concentration for the sandy loam. It is not surprising from this lysimeter study that the levels as low as 3.83ppm had no effect on metabolic quotient, considering both the Gisborne and Katikati soils were sandy loam soils.

5.5.5.3 Sulphatase

Sulphatase activity was not impacted by the application of greywater. As found in Chapter 4, sulphatase activity was not sensitive, even at concentrations as high as 7500ppm TCS. Only at TCS concentrations higher than 1500ppm was there a significant difference from the control treatment which didn't contain any TCS.

5.6 Conclusion

The qPCR analysis of the *uidA gene* in *E. coli* indicates that there is a higher risk of *E. coli* accumulation in soils with higher clay content. In order to limit health risks associated with greywater irrigation it is therefore necessary to practice sub-surface irrigation where children and pets cannot be exposed to faecal coliforms or potential pathogens. Irrigation of greywater containing lower concentrations of *E. coli*, for example approximately 1x 10^2 CFU/100ml, onto soil with a higher sand content appears to be a safer activity as there appears to be less build-up of *E. coli* resulting in a smaller potential for leaching.

From the BQGW treatment data it is clear that there is a risk of *E. coli* leaching, regardless of soil type, when greywater containing elevated levels of *E. coli* is diverted and irrigated. Generally concentrations higher than 1 x 10^6 CFU/100ml were detected for leachates from all soils receiving the BQGW treatment for most of the experimental time however up to as 2.82 x

10^8 CFU/100ml were detected in some field replicates. This stresses the need to refrain from greywater irrigation when members of the household is ill or when high risk activities such as washing of nappies and being engaged in.

In general there were small difference (although not significant) in the *E. coli* leaching between the BQGW and the BQGWD for the Gisborne and Katikati soils (a difference of approximately one $\log_{10}$). This however was not the case in the Lincoln soil where suspected preferential flow took place and there was little difference between the *E. coli* concentration of the BQGW and BQGWD leachates (2.31 x 10^6 CFU/100ml and 4.59 x 10^6 CFU/100ml at their lowest concentrations respectively). Flood irrigation of greywater might lead to ponding on the surface of soil, potentially exposing individuals to pathogens. Even though it appears to assist in the attenuation of large concentrations of *E. coli* in some soil types, it remains a high risk activity.

In some communities flood irrigation is practiced by directly diverting laundry water onto lawns. Depending on the soil type, this might not be high risk. Irrigation should however still be done where contact with the soil is minimised, such as in garden beds where there are no food items being grown. Depending on the origin of the greywater, this might negate having to purchase expensive greywater diversion systems.

There appeared to be no build-up of TCS in any of the soils where the GWGQ was irrigated, however up to 3.9ppb TCS was found in the leachate. The TCS concentration in the GQGW was 5.9ppb, comparable to literature values of concentrations frequently found in household greywater (Eriksson et al., 2003; Palmquist & Hanæus, 2005). This indicates that although there is little risk of accumulation in soil, irrigating greywater containing TCS, even at levels routinely observed, could be transported to groundwater causing environmental contamination. Thus, best practice for greywater reuse should include the reduction of use of potentially eco-toxic chemicals such as TCS in household products.

After irrigation of soil cores from 3 different origins it is clear that the accumulated TCS in the top 5cm had no significant effect on soil health indicators (3.83ppm at the highest for the BQGWD treatment). Average values of accumulated TCS observed for each treatment were <0.01ppm for the control, 0.21ppm for the GQGW, and 1.6 and 2.7ppm for BQGW and BQGWD treatments respectively. Although the irrigation with these treatments did not affect soil health indicators, TCS accumulation in soil can far surpass the accumulated values observed from this study.

These conclusions reinforce the need for behavioural change in the household. Individuals need to be aware of how they contribute to the composition of their wastewater and of the potential environmental and health risks associated with its reuse. This can be accomplished using tools such as education interventions as described in Chapter 6, and engagement between the local communities and scientists.

6. Education intervention

6.1 Introduction

One of the major challenges of greywater re-use is that it contains a complex mixture of chemicals used in households. Household products such as cleaners and body care products can contain over 2,500 different chemicals (The National Institute of Health 2004). These include metals, and new emerging contaminants such as triclosan. In septic tank and municipal treatment systems many organic chemicals are degraded to some extent by microbial action, but this does not happen in a greywater system where the 'untreated' water is released directly into the soil environment with potential negative impacts. Thus, there is the potential for 'antimicrobials' such as triclosan to continue their 'antimicrobial' action on the soil ecosystem. Many of the emerging organic contaminants are very difficult to reduce by legislative means and require community 'buy-in' and behavioural change to reduce their use. The primary aim of this chapter was to explore ways to go "up-the-pipe" to prevent pollutants or contaminants being put in into the system rather than an "end-of-the-pipe" approach.

When untreated greywater is reused for irrigation, the contaminants originating from the personal care products enter the environment. Results from Chapter 5 indicate that with irrigation of good quality greywater there is no build-up of chemicals such as TCS in soil when applied at concentrations commonly found in household greywater (5.9 ppb, (Harrow & Baker, 2010)), however TCS does leach through the soil profile and potentially can make its way into groundwater reserves. There is thus a potential for the contaminants to be carried off-site where they can adversely impact the environment. A recommendation could be to stop using potentially eco-toxic compounds in the household; however this requires a behavioural change which can only be achieved through community engagement and education.

This chapter focused on education to raise awareness and support behaviour change by encouraging students to be change instigators within their whanau. One of the key messages of the project was to equip the students with knowledge and cultivate an awareness of what goes down the drain. The educational setting was utilised to anchor the behaviour change aims of the project within established learning mechanisms (e.g. the NZCEA "Nature of science") and additionally it can be argued that this setting also provides a captive and receptive audience. A further aim of the project was the development of the education intervention and to develop tools/techniques for the 'new curriculum' approach for science education (Bull, Gibert, Barwick, Hipkins, & Baker, 2010)This approach situates students'

science learning in an authentic 'real world' context of genuine relevance to the community/society, bringing them into connection with scientists and supporting them to achieve and share real outcomes as a result of their learning work. Bull et al. (2010) proposed this approach to science education has increased benefits for students wishing to pursue careers in science, and more importantly, increase the "science citizenship" i.e. the skill the general community require to take part in societal discussion on 21st century issues such as discussions on environmental pollution and climate change.

6.1.1 The school.

Tirohanga School in Mokai is a rural school in an area with a high Māori population. The school has 22 students aging from 5 to 12, divided into 2 classes. The school provides a strong learning environment under the guidance of engaging teachers who foster a learning approach based on critical thinking whilst developing an array of skills through creatively designed learning activities. As part of their curriculum the students engaged in a module where activities such as the exploring the function of a septic tank, the ecology in an wetland system, fresh water as taonga and a greywater experiment were the main focuses. The engagement with the school was enabled by the principle, Donna Andrews. Donna assisted in creating learning activities and facilitated the greywater experiment by making the material suitable for students of a younger age group.

6.1.2 Student engagement

The engagement involved 2 visits to the school, one before the experiment commenced, and one visit after the experiment terminated in order to help interpret results which was presented at a hui the following day. Throughout the duration of the experiment there was on-going contact with Donna and the student through various means including electronic- and social media (e.g. email and Facebook).

On the first visit, the scientific process of research was introduced to the students by guiding them in the design of an experiment involving the effect of contaminants from greywater on a garden. The students listened to a presentation on where fresh water comes from and why it is valuable. During the presentation they also learned where the greywater fraction in a home has its origins in order to cultivate an understanding of what household greywater might contain. They were engaged in activities to identify products used in the house that might

make its way into household greywater and were also directed by suggestion to reflect upon the possible uses for greywater, its reuse drivers and potential negative impacts it might have on the environment. The students were also presented with scenarios where their household habits could negatively impact the environment e.g. when contaminants originating from household waste water move off-site.

During the second visit just before the hui, the students were assisted in interpreting their results and drawing a conclusion from the data collected. The experiment was presented at the hui the following day by the students themselves in the form of a PowerPoint presentation.

6.1.3 Greywater experiment Introduction

The case-study community in Mokai is located in a rural area with no access to a reticulated wastewater treatment system. Consequently all properties treat their own wastewater on-site, typically in the form of a traditional primary treatment septic tank system. This is a common practice in rural New Zealand, and there are an estimated 270,000 properties operating septic tanks throughout the country (MfE, 2008). Issues such as; undersized or poorly located soakage fields, minor system damage and insufficient maintenance means that up to 50% of these septic tank systems are estimated to be performing sub-optimally (MfE, 2008). There are health and environmental risks involved with failing wastewater treatment systems as a result of surface ponding and excessive leaching of partially treated waste, respectively. A study by Siggins et al. (2013) has illustrated that the diversion of greywater can improve the function of old and failing septic tanks. This would improve the quality of treated effluent and reduce any associated public or environmental health risks. It is also a cost-effective alternative to replacement of an existing treatment system, which would be an expensive process for homeowners. The diversion of greywater from a septic tank for this purpose has been regularly reported by homeowners, although Siggins *et al.,* 2013 were the first to investigate the hypothesis that it could have a beneficial impact.

The composition of household greywater can be extremely variable and may contain a diverse mixture of metals, microbes, salts, nutrients and organic contaminants. The composition of greywater, and therefore the concentrations of contaminants that it contains, is directly related to the products used in a house-hold (Casanova et al., 2001; Eriksson et al., 2009; Jefferson et al., 2004). In small concentrations, nutrients might have a positive effect on plant growth when irrigated onto soil, however after accumulation to higher concentrations,

nutrients, salts and metals and organic contaminants might have a negative effect on the soil environment (Rodda et al., 2011; Wiel-Shafran et al., 2006).

6.2. Aim

The aim of the experiment was three-fold:

1. To encourage children and their families to consider the fate and impacts of the components of their wastewater by going "up-the-pipe" to prevent pollutants or contaminants being put in into the system.
2. To give children 'authentic' scientific experience by mentoring primary school children in the design and execution of a scientific experiment.
3. To allow the students to participate in a wider research program and contribute to an already existing body of knowledge.

The research questions developed by the students included the following:

- Could soil be overloaded with nutrients?
- How does irrigation of greywater affect plant growth?
- Is the use of greywater beneficial to the garden?

6.3. Materials and method

6.3.1. Student engagement

A class of 10 students were chosen between the ages of 9 and 11yrs to conduct the greywater experiment. There were 2 groups of 3 students, and one group of 4 students chosen, each group to take care of a specific treatment for the greywater experiment. They were selected on the basis of where they were already seated. During their initial engagement the students were presented with a potential experimental layout which included an introduction to science philosophy. The need for replication was discussed along with a potential set of parameters that they could monitor. It was decided that they would monitor the stem heights, leaf number, visual appearance and size of the root mass. A friend would then check the results to ensure that the record is accurate.

After all the data was collected during the course of the experiment, the students presented it visually on information boards. They used creative techniques to make the posters interesting

and colourful. The posters contained all their data collected, conclusions and pictures taken during the course of the engagement. The data was represented on bar graphs which the students created themselves in Excel.

The on-going contact with the students and teacher was achieved by using a Facebook page to share photos and arrange a timeline. The Facebook page was set up between the scientist and the teacher, and this provided the students access to the page at school. The page was designed to be an open forum for discussion of results.

After the presentation of their results and what they have learned during the course of the engagement, the students were given a worksheet and survey to fill out. The worksheet contained hypothetical results for the greywater experiment and questions on the data to encourage the student to employ critical thinking skills. The survey contained questions on how much the students enjoyed doing the experiment and what they had learned conducting it.

6.3.2 Greywater experimental design

The native species kōhūhū *(Pittosporum tenuifolium ssp. Colensoi*) was used for this experiment. A total of fifteen plants were supplied by Mark Ross (Whenua.biz), of which 9 were selected for the experiment based on similarity in appearance, and the remaining 6 were set aside to be planted on the school grounds to remind the children of their experiment and the knowledge gained from it.

Plants were potted in individual containers, and irrigated with three different treatments, with three replicate plants per treatment (Fig. 42). One treatment was a “bad quality” greywater (BQG), which represented the potential accumulation of sodium in soil over a 10 year period from daily irrigation. The second treatment was a “good quality” greywater (GQG), which was a 1:10 dilution of the BQG. The third treatment was clean tap water to act as a control.

Figure 42: The 9 kōhūhū plants chosen for the experiment. Plants were grouped in 3 treatments with 3 replicates for each treatment.

Greywater (15ml) (or tap water for the control treatment) was irrigated onto the soil around the plants twice every day, except weekends and school holidays (Fig. 43). As the pots were 25cm in diameter, this equalled an irrigation rate of 0.6 mm/day, which was well below the maximum rate of 4mm/day (AS-NZS1547:2012, table M1). Students manually irrigated the plants twice daily as opposed to using an automated system that could irrigate continually over a 24 hour period. Therefore the daily irrigation rate applied to the pots was much lower than the potential maximum rate, so as to prevent overloading the soil, resulting in leaching from the containers. It was hoped that this would still be a sufficient rate to observe differences in the plants between the treatments.

Figure 43: The plants were irrigated twice each weekday for 7 weeks.

A synthetic concentrated greywater stock was made at ESR according to (Jefferson et al., 2001) and provided to the school (Table 7). It was stored at 4ºC for the duration of the experiment. Each day a fresh stock of greywater was made from the concentrate.

*Table 7: Constituents of synthetic greywater. *Selection of specific brands does not represent an endorsement by this research project.*

Ingredient	BQG (per litre)	GQG (per litre)
Soap (Lux)*	0.05g	0.005g
Shampoo (Schwartskopf)*	0.11ml	0.011ml
Vegetable oil (Homebrand sunflower oil)*	0.01ml	0.001ml
Laundry Powder (Cold power with SARD Oxy plus)*	0.025g	0.0025g
Sodium Chloride	305g	30.5g

Figure 44: Measurements of stem height, assessment of appearance and leaf count were recorded twice weekly

6.3.3. Hui

A hui was held on Wednesday the 14th of August 2013 at the Mokai Marae near Taupo where the students had the opportunity to present their results and conclusions of the experiment they conducted with the greywater. In attendance were residents from the area, other prominent members of the community and local- and central government representatives. The focus was on the communication of science to a non-specialist community.

The students were allowed to explore various ways of representing their experiments and data at the hui and they decided to make result boards for exhibition and present their findings on PowerPoint to the community. See figures 45, and 46. Two students were chosen by Donna to deliver the oral presentation. The students themselves were responsible for the design and production of the result boards.

Their presentation involved an introduction by me where a brief background to the engagement was given. The scope of the experiment and the context of the engagement were also presented. The students were then allowed to present their finding of the experiment themselves. They elaborated on what they had learned about the water cycle, where

greywater comes from, what it contains and how it can affect the environment. The emphasis of the presentation was on the safe use of greywater and the experimental process they had followed. The presentation also contained the data they collected and the conclusions they had made.

Figure 45: The result boards containing the observational data and conclusions made by the students. These were presented at the hui to the community along with a PowerPoint presentation.

Figure 46: One of the students asked to represent her class and give the presentation containing the results and conclusions at the hui.

6.3.4. Student worksheet and survey

The students were provided with a worksheet and survey after the engagement in order to instil an understanding of the principles of making deductions and conclusions from results. The survey served to provide information on how the students enjoyed the engagement and what they had gained from the experience.

In the worksheet, the students received a fictitious data set based on the experiment that they had conducted. The same parameters that the students assessed in the greywater experiment were presented in the data set. The worksheet also contained graphs to make assessing the data easier. There were, however, anomalies in the data which the students had to identify.

In the survey at the end of the worksheet the students were presented with the opportunity to comment on their experience. They were asked how they would approach a future experiment and what research questions they would like to formulate.

See appendix for full presentations made by the students.

6.4. Results

6.4.1. Student involvement

From the initial visit the students were enthusiastic to learn about science and greywater. They worked well together to execute the experiment and construct the results boards for presentation at the hui.

The experiment was a success in many ways as the students learned the value of record keeping and peer review. Some of the results data sets were lost as one of the students entered data into a computer and forgot to save before closing the program. The students, however, diligently irrigated their experiments every day and took great care in trying to record the results as accurately as they could. They found that measuring the stem-height, and visual inspection of the plants' general colour and health easy, however counting the leaves proved to a little more challenging. Along with the inspection of the root-stock at the end of the experiment, the parameters assessed were very visual and also helped the students learn about basic plant biology. By making use of various methods of presenting their results the students' learning outcomes encompassed various parts of the curriculum such as art, maths, design and literacy.

6.4.2. Experimental results

The students compiled a presentation of their results, and they presented them to the hui with assistance from Morkel Zaayman (Appendix). A summary of results is outlined as follows:

6.4.2.1 Leaf number

Leaf numbers of each plant at the beginning and the end of the experiment were counted. The greywater treated plants demonstrated an average leaf gain of 9 and 8 leaves for good quality greywater and bad quality greywater, respectively, while no change in leaf numbers was observed for the control plants.

6.4.2.2 Stem Height

The average stem-height increased in all treatments with the largest increase in stem height observed for the GQG treatment. The average increase for the control treatment was 1.45cm compared to 2.15cm for GQG and 0.1cm for BQG.

6.4.2.3 Appearance

Plants exposed to all treatments indicated similar changes in appearance throughout the experimental timeline. Initially plants for all of the treatments were classified as “green”. The appearance progressed to light green and then finally to green with brown spots. Most of the plants seemed to appear green with brown spots approximately 4 weeks after the start date. The plants irrigated with the BQG were only classified as green with brown spots a week before the experiment ended.

6.4.2.4 Root mass

The largest root mass increase was observed in the control plants, with almost no root-mass increase in the other experimental treatments. It should be noted that the balance available for use by the school only provided measurements to 1 decimal place.

6.5. Discussion

6.5.1 Student engagement

During this study the students learned that greywater in the house comes from bathtubs, hand basins and laundry water. They also learned that greywater contains nutrients, salts and other chemicals that come from the products used in the house. They came up with examples of negative impacts that these chemicals might have on the environment and that they need to be conscious of their own habits regarding what goes down the drain. It was hoped that the students, equipped with an improved understanding of environmental impacts, would be the instigators of behavioural change in the home.

During the initial visit the students became acquainted with science philosophy. They learned about hypothesis formulation and forming research questions. The students themselves came up with the research questions mentioned in section 6.2. They were guided and learned about the scientific process of setting up an experiment in such a way that it answers the research questions formulated and how to decide what parameters to monitor. The parameters selected for measurement were within their capability and in general they found the observation stage easy. The necessity of replication was discussed in class and why it is important to replicate results. The students all agreed that the variability in the plants made it necessary to have 3 replicates for each treatment in the experiment. Having a friend check the results before they were noted down proved to be a valuable tool as quite often the observations between individuals were different e.g. counting the leaves remaining on the

plant. Good scientific practises like accurate and diligent record keeping were also reinforced. They also made conclusions from their results with the aid of a scientist. There was a clear progression in the level of participation and understating of the scientific process from the students.

During the engagement the students grew more acquainted with electronic resources such as word processing software and calculation tool such as MSWord and Excel. They learned about social media such as Facebook and the utility it has as a tool to communicate and facilitate a scientific study. The activities that the students were engaged in related back to the curriculum. They understood the value of accurate measurement, calculation and they were challenged with designing their own results boards. Here they learned about design and had the opportunity for creative expression. The students were continuously involved in an open conversation with the scientist and teacher. This was a valuable tool to develop oral skills and build self-confidence.

6.5.2 Greywater experiment

The students decided that plant growth seemed unaffected with the irrigation of greywater. They used the increase in leaf number and increased stem height with the irrigation of greywater compared to the irrigation with tap water as evidence for their conclusion. Although the appearance of the leaves changed, the normal growth function of the plants seemed to be unaffected during the experimental timeline.

The knowledge gained from the experiment is inconsequential compared to the skills and abilities acquired by designing and being actively involved in a scientific study. Through this activity the students developed critical and ethical skills or “science literacy” (Gilbert, Bulte, & Pilot, 2011).

6.5.3 Hui

The students delivering the oral presentation executed it with self-confidence, taking pride in the experience of being part of a larger research project. The presentation was well received by the community and their contribution to the engagement was acknowledged. They learned about working in a team and advanced their collaborative skills while making the result boards in their individual groups. Allowing the students to design and make their own result boards

and presentations was an innovative way to relate two divergent disciplines, science and art, to each other.

Enabling science at a primary school level and allowing them to present the outcomes to the community proved to be a novel tool for communicating science to non-specialist communities. The students presented their work at a level familiar to the audience and therefore were successful in communicating one of the key messages for the project; to develop an awareness of what they put down the drain.

The hui provided the students with a sense of science citizenship.

6.5.4 Student worksheet and survey

The students collectively filled out the worksheet provided and from their answers it is apparent that they learned from the greywater experiment. They were able to apply critical thinking skills and assess a fictitious data set. They were generally able to answer detailed scientific questions and they showed an understanding for the need to do so by being able to point out trends, and indicating irregularities in the data. They were able to relate observations to the treatments applied in the experiment which helped them make conclusions.

In the survey the students showed enthusiasm for science and indicated that they enjoyed the experimental process and learning how science helps address issues in real life situations. There was an understanding of the content of the experiment and they particularly enjoyed being able to creatively display their results. They were also inspired to do an experiment in the same format as the greywater experiment.

The engagement caused them to stretch their imagination. They showed interest in exploring other scientific areas such as how climate change affects the weather and how a volcano works, through enquiry. The engagement and activities seems to have stimulated further discussion.

6.6 Summarising conclusion

The Tirohanga School engagement was developed on the premise that awareness of household-contaminants will support behaviour change with respect to what products are used. It made complex science accessible and meaningful to inspire behaviour change. Anecdotal evidence suggests that the students were instigating behavioural changes at home

by making suggestions on the use of alternative products that are more environmentally friendly. They were planning on making home-made cleaning products and selling it at a local market day.

The engagement took into account the role that local communities and Maōri play in conjunction with biophysical research by enabling the use of science at a grassroots level and stimulating an interest from a young age.

Through on-going contact and engagement with the students they learned where various contaminants in greywater come from, various ways of introducing them into the environment, and what potential effects they might have.

Feedback received from the students indicated that they enjoyed being part of the Hui and the experience of being part of a scientific program involving research with a field of experts. They particularly enjoyed the experimental process where they were responsible for the irrigation of the plants and also gaining the knowledge that greywater could be applied as resource. They also learned that the quantity and nature of the products used has the potential to be introduced into the environment and may have a negative effect on soil and the life it sustains.

The students found that science is a fun discipline that can yield exciting outcomes and that it could be used as a tool to help alter an outlook on matters of environmental concern.

The students gained knowledge on data collection and recording, comparing their results and making conclusions from their observations. They also learned to explore different media for the representation of the results to a wider audience. This activity relates to the “Nature of science” standard in the NZ curriculum by providing the students with the opportunity to “learn how scientists carry out investigations, how science ideas are communicated and to make links between scientific knowledge and everyday decisions and actions”. It also relates to the “Key competencies and capabilities for living and lifelong learning” of “thinking” and “relating to others”.

With the focus on raising awareness to support behavioural change in a community, the engagement provided the students with an authentic scientific experience.

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7. General discussion

The reuse of domestic greywater is of growing interest, both in New Zealand and worldwide. In some water short regions of New Zealand, such as Gisborne, Central Otago and the Kapiti Coast, greywater reuse for irrigation is permitted and encouraged. In water-rich regions, the diversion of greywater is commonly practiced to relieve the pressure on wastewater treatment infrastructure. Untreated greywater also contains other organic contaminants including pharmaceuticals, fragrance fixing agents, preservatives and antimicrobial chemicals (Donner et al., 2010; Eriksson et al., 2003). These compounds are of concern as the practice of greywater reuse could possible introduce them into the environment.

There are significant knowledge gaps regarding the impact of greywater on the receiving environment (soil and groundwater), particularly in a New Zealand specific context, with respect to our soil types and vegetation. Also, there is a lack of information as to how greywater impacts the soil microbial community that is essential for plant growth.

Firstly the aim was to determine the EC_{50} for TCS in soil by using a dose-response model in a microcosm experiment. The parameters measured included SIR, sulphatase and microbial biomass. The EC_{50} of 803ppm TCS calculated from that study informed the dosing rate for the lysimeters study where greywater containing TCS and *E. coli* was introduced to intact soil lysimeters through continuous irrigation. The second aim was two-fold; firstly to investigate the partitioning of *E. coli* and TCS in the soil profile in 3 different soils, and secondly to determine the effects TCS had on soil health indicators in the top 5cm of the soil lysimeters after it had been introduced through continuous irrigation with greywater.

The research in this thesis aimed to fill this knowledge gap by characterising the fate and effects of a representative chemical contaminant, TCS; and the microbial indicator, *E. coli* in three New Zealand soils. Previous studies have not attempted to derive an EC_{50} for TCS in soil.

The effects of TCS on a clay loam soil was assessed in Chapter 4, making use of a dose response model and measuring microbial respiration, sulphatase activity and a stress-biomarker, microbial metabolic quotient. The results from this study found that TCS can induce microbial stress in the soil environment at concentrations as low as 195ppm resulting in a 20% loss of soil function. In a home garden this may not be an issue. However, previous research has indicated that the breakdown of TCS can be reduced in the presence of co-contaminants such as copper (Horswell et al., 2014). Greywater also contains a variety of co-contaminants such as

detergents, dyes, bleaches, UV-filters, Enzymes (Eriksson et al., 2002) thus with continuing inputs of TCS through greywater reuse it is possible that TCS could build up to concentrations that seriously impacts soil function. An EC_{50} of 803ppm TCS (dry weight) was derived using microbial biomass as an indicator. If microbial communities in a soil (receiving regular inputs of greywater containing TCS) become severely stressed and overwhelmed, the subsequent breakdown of the ecotoxic compounds like TCS might be limited and soil function could be seriously impacted. It would be interesting to repeat the dose-response study and include a number of soils with different textures to assess the impact of soil type on the effects of TCS on the microbial community. A retention-release experiment (K_d) would give an indication of the bioavailability of TCS in each soil and the determination of the K_{OC} would correct for the effects that organic matter might have on the bioavailability of TCS in that particular soil. In this study due to limitations of time, only 3 soil health indicators were measured, and some were more sensitive to the impacts of TCS than others. The inclusion of sulphatase analysis would be unnecessary as in Chapter 4 it was found that sulphatase activity was not a sensitive indicator for the effects of TCS on the Lincoln soil. Future research should include a broad-scale analysis of soil characteristics including texture, soil organic matter and pH. A more in-depth investigation into impacts on the soil microbial community using molecular tools such as terminal restriction fragment length polymorphism (TRFLP) analysis would possibly allow the determination of which members of the community are impacted in a TCS-rich environment.

In the lysimeter study in Chapter 5, the fate and effects of TCS and the microbial indicator *E. coli* were investigated in soil cores from 3 locations (Gisborne, Katikati and Lincoln). The soils were a fine sandy loam, a sandy loam and silty clay loam respectively. This study showed that that soil with higher clay content are at risk of *E. coli* accumulation when irrigated with greywater containing elevated levels of *E. coli* (10^8 CFU/100ml). It was also found that soils with higher clay content have the potential to leach *E. coli* through bypass flow and potentially contaminate ground and surface water. *E. coli* was detected in the leachates for all 3 soils receiving greywater with high levels of *E. coli* (10^8CFU/100ml) (bad quality greywater). These results can help provide information for best management practice for greywater reuse such as stressing the need to divert greywater back to mains or the septic tank when high risk activities such as washing nappies are practised. It was interesting to observe attenuation for the *E. coli* levels from the BQGWD treatment in the Gisborne and Katikati soils, compared to the BQGW. This suggests that the practice of flood irrigation of greywater might be suitable to some soil types, negating the need to purchase expensive greywater diversion equipment. This makes the practise of greywater irrigation to alleviate the load on a septic tank an option for

rural communities. Flood irrigation, however needs to be practised with care as during drier months, soils with high clay content might crack and facilitate the rapid movement of *E. coli* down the soil profile and into groundwater.

Triclosan was found in the leachate of soils receiving the GQGW and although the results suggest that TCS does not build up in the soil, it does have the potential for environmental contamination, even at levels routinely observed in the household.

The accumulated levels of triclosan in the top 5cm of the soil cores after the 3 month irrigation period ranged from 0.21ppm in the soil irrigated with more dilute greywater treatment, to 2.66pm in soil receiving a bad quality greywater. The reason for the minimal build-up could possibly be heavy precipitation accelerating the movement of TCS through the soil profile.

A mass balance for TCS could not be done as the total volume of leachate was not collected. It would be interesting to know what the exact fate of TCS was in each soil core. Future work could include a similar study under more controlled conditions. It should include the collection of the total volumes of leachate in order to obtain a mass balance between the TCS added and the TCS in the leachate. An analysis for the major breakdown and conversion products (methyl triclosan, 2, 4-dichlorophenol, and 4-chlorocatechol) in both the soil and leachate would give an indication on how the microbial community in each soil degrades TCS added through greywater irrigation. A radio-labelled-TCS compound could be used in conjunction with microautoradiography fluorescent in situ hybridization (MAR-FISH) to determine which microbes are responsible for the degradation.

8. Conclusions

In general, New Zealand climatic conditions would ensure enough rainfall to prevent greywater chemical components from accumulating to concentrations that impact soil function or plant health; however, chemicals such as TCS and microbial contaminants could potentially be transported via leaching through the soil profile and may cause environmental contamination. The risk of environmental contamination highlights the need for regulation of eco-toxic compounds like TCS in personal care products. Many of the emerging contaminants are difficult to reduce by legislative means and the reduction of their use requires behavioural changes on a community based level. Public engagement and education by the scientific community have proven to be a useful tool to encourage changes in behaviour in local communities and so reduce their overall contribution of emerging organic contaminants to their household waste water. However behavioural change is incredibly difficult to measure. Future work could include social science studies to measure this.

With respect to public health risks from microbial contaminants, practises to reduce heavy contamination of GW are important, such as sub-surface irrigation and diversion of GW when members of the household are ill or when high risk activities such as washing of nappies are being undertaken.

Potentially, the knowledge generated from this study could inform the science behind New Zealand guidelines for the diversion and irrigation of greywater onto soil. These guidelines should take into account chemical composition of household greywater and the promotion of safe-practise recommendations to reduce the risk for public health.

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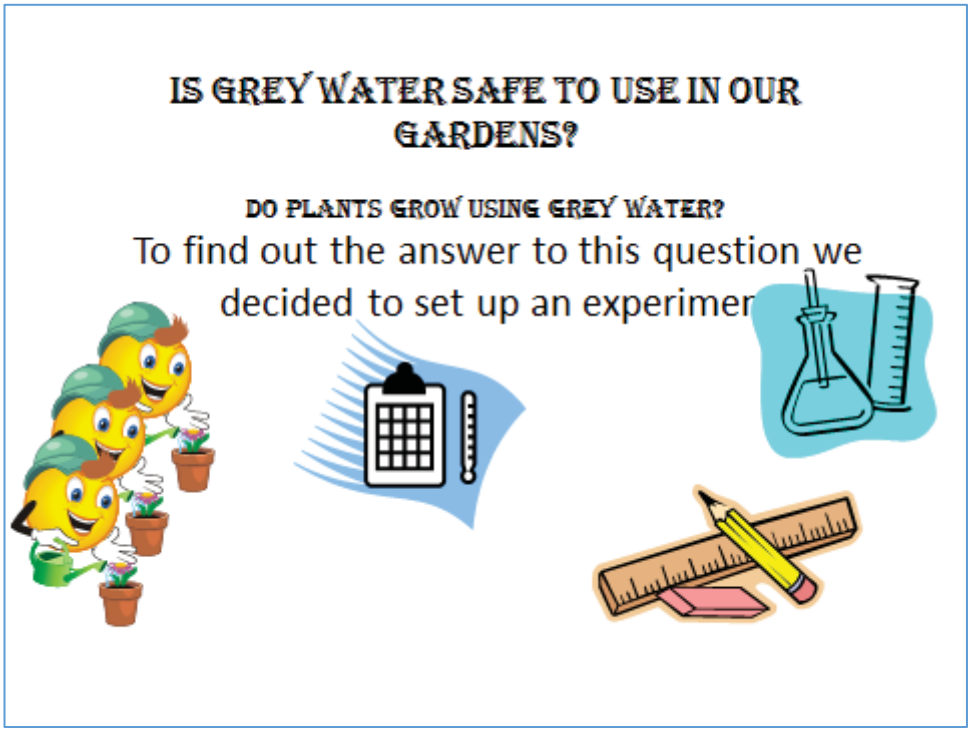
10. Appendix

10.1 Presentation school children gave at the hui



How is our experiment going to work?

How are plants affected by grey water? QUESTION

Water plants with grey water TEST

How do the plants change in relation to the control? OBSERVE

What is the outcome? CONCLUSION

What do we need?

9 native plants (Whariki-Pitto Sporum)

Rulers

Record results

Control Plant

What are we going to do?

Make up 3 solutions of different strengths of grey water

- Mix 1ml of concentrate with 99mls of water – this makes up "bad grey water"
- 1 part bad grey water added to 9 parts water – this makes up "good grey water"
- Lastly container of straight tap water

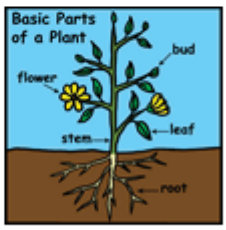
Three groups set up using /good grey water, bad grey water and tap water

Each day, groups will feed their plants with the various solutions, observe any changes in the plants and record

Every Monday and Thursday, we will measure the stems, count amount of leaves and note any change in the colour of the leaves. Record data

How is this data going to be represented?

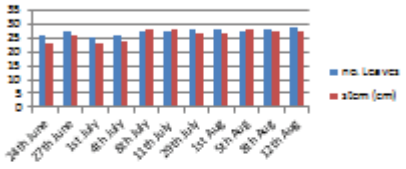
- For each group we have used bar graphs to represent data relating to stem length and number of leaves. We have used a table to represent data of physical changes in the plants.



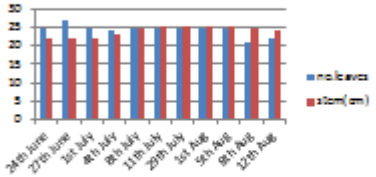
Group 1: Tap Water

(no report for plant 2)

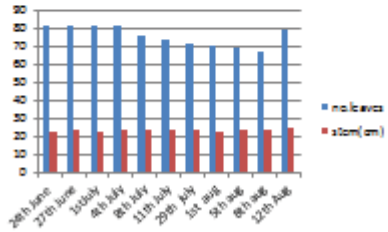
Plant 1



Plant 3



Plant 2



Report- Plant 1 (Rangikateau)			
Dates	24th June	12th August	Variance
Stem length	20.4cm	25.3cm	Growth of 4.9cm
No. Leaves	26	29	Gain 3 leaves

Report- Plant 3 (Amber)			
Dates	24th June	12th August	Variance
Stem length	22cm	25	Growth of 3cm
No. Leaves	24cm	22	Loss of 3 leaves

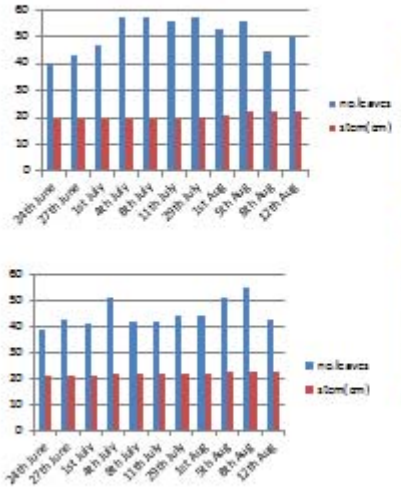
Physical appearance of leaves

(No data for plant 2)

Dates	Green	Green with brown spots	Light green	No changes
24th June	✓			
27th June	✓			
1st July	✓			
4th July	✓			
8th July	✓			
11th July			✓	
15th July			✓	
18th July		✓		
22nd July		✓		
25th July		✓		
29th July		✓		
1st Aug		✓		
5th Aug		✓		
8th Aug		✓		
12th Aug		✓		

Dates	Green	Green with brown spots	Light green	No changes
24th June	✓			
27th June	✓			
1st July	✓			
4th July	✓			
8th July	✓			
11th July	✓			
15th July				✓
18th July				✓
22nd July		✓		
25th July		✓		
29th July		✓	With spots	
1st Aug				✓
5th Aug				✓
8th Aug				✓
12th Aug				✓

Group 2: Good Grey Water



Report- Plant 3 (Quinton)			
Dates	24th June	12th August	Variance
Stem length	19cm	22cm	Growth of 3cm
No. Leaves	40	50	Gain 10 leaves

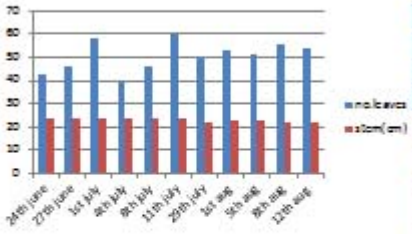
Report- Plant 1 (Green&Taylor)			
Dates	24th June	12th August	Variance
Stem length	21cm	22.5cm	Growth of cm
No. Leaves	39	47	Gain of 8 leaves

Physical appearance of leaves

Dates	Green	Green with brown spots	Light green	No changes
24th June			✓	
27th June			✓	
1st July	✓			
4th July	✓			
8th July	✓			
11th July		✓		
15th July		✓		
18th July		✓		
22nd July		✓		
25th July		✓		
29th July		✓		
1st Aug		✓		
5th Aug		✓		
8th Aug		✓		
12th Aug				✓

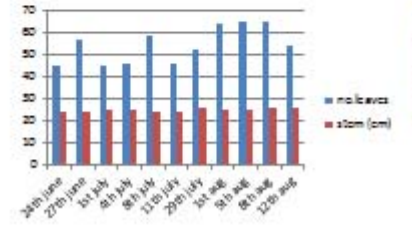
Dates	Green	Green with brown spots	Light green	No changes
24th June	✓			
27th June	✓			
1st July	✓			
4th July			✓	
8th July			✓	
11th July				✓
15th July				✓
18th July				✓
22nd July				✓
25th July		✓		
29th July		✓		
1st Aug		✓		
5th Aug		✓		
8th Aug		✓		
12th Aug				✓

Group 3: Bad Grey Water



Report- Plant 1 (Zac)			
Dates	24th June	12th August	Variance
Stem length	24cm	25cm	Growth of 1cm
No. Leaves	45	54	Gain 9 leaves

Report- Plant 2 (Michaela)			
Dates	24th June	12th August	Variance
Stem length	24cm	22cm	Loss of 2cm
No. Leaves	43	50	Gain of 7 leaves



Report- Plant 1 (Grant/Taylor)			
Dates	24th June	12th August	Variance
Stem length	21cm	22.5cm	Growth of cm
No. Leaves	39	47	Gain of 8 leaves

Physical appearance of leaves

Dates	Green	Green with brown spots	Light green	No changes
24th June	✓			
27th June	✓			
1st July	✓			
4th July	✓			
8th July			✓	
11th July			✓	
15th July			✓	
18th July	✓			
22nd July			✓	
25th July	✓			
29th July	✓			
1st Aug	✓		✓	
5th Aug			✓	
8th Aug		✓		
12th Aug		✓		

Lost data from Zac and Kruze

Weighing mass of plant roots

	Control Group 1: Root weight	Good Grey Water Group 2: Root weight	Bad Grey Water Group3: Root weight
Plant 1	0 grams(Rangi)	1gram (Zac)	0 gram (Taylor/Groor)
Plant 2	½ gram(Tash)	0 gram (Michaela)	1/2gram (Jessica)
Plant 3	1gram (Amber)	0 gram Kruse	0 gram (Quinton)

Conclusion to original question: Do plants grow using grey water?
Yes they do. As our data shows, leaf numbers increased, stem growth was observed and while there was some discolouration in the leaf colour (brown spots) leaves continued grow.

10.2 Worksheet and survey filled out by school children with guidance from Donna Andrews

Remember... with science you can have SO MUCH FUN ;-)

What effect does greywater have on a garden plant when used to water it?

Worksheet:

Hello everyone. I conducted the same experiment as you and here are my results. Please look at them carefully and answer the questions that follow. Look at the following table to answer the questions below.

	Treatment	Replicate	Stem height (cm)	Number of leaves	Leaf colour
Day 0	Control (water only)	Plant 1	30	32	Bright green
		Plant 2	34	36	Bright green
		Plant 3	31	37	Bright green
		Average	**31.7**	**35**	
	Good Greywater	Plant 1	37	36	Bright green
		Plant 2	35	36	Bright green
		Plant 3	36	39	Bright green
		Average	**36.0**	**37**	
	Bad Greywater	Plant 1	35	35	Bright green
		Plant 2	34	37	Bright green
		Plant 3	32	39	Bright green
		Average	**33.7**	**37**	

(The average is all the numbers of the plants of a specific treatment added together, and then divided by the number of samples in that treatment. This gives us an overall indication of how the plants reacted to the treatment)

1) What do you notice about the stem heights for day 0? Are they the same or different? ___Different_____

2) What do you notice about the number of leaves? Are they the same or different___Different, but while a number are different for both height and leaves the numbers range between 30-40 therefore not a great variance.

3) Is this important because all of our plants need to be the same before the experiment starts? Or could all the plants be different? Plants can be different. The recording is the important factor.

	Treatment	Replicate	Stem height (cm)	Number of leaves	Leaf colour	Root Mass (g)
Day 48	Control (water only)	Plant 1	46	50	Bright green	4
		Plant 2	48	53	Bright green	4
		Plant 3	46	53	Bright green	3
		Average	**46.7**	**52**		**3.7**
	Good Greywater	Plant 1	59	61	Bright green	4
		Plant 2	61	69	Bright green	3
		Plant 3	63	68	Bright green	4
		Average	**61.0**	**66**		**3.7**
	Bad Greywater	Plant 1	35	35	Bright green	2
		Plant 2	34	37	Bright green	2
		Plant 3	32	84	Bright green	3
		Average	**33.7**	**52**		**2.3**

4) Look at the table and graph for day 48 above. Write down the average stem heights for each treatment: Control-__46.7_, Good GW-___61.0_, Bad GW-_33.7

5) Which treatment's plants grew the highest? ___ Good grey water

6) Which treatment's plants grew the most? Do you mean Day 0 minus day 48 – if so you need to state this or the question seems very similar to number 5.

__ Between the 0-48 days (good water)

7) Write down the number of leaves for each treatment: Control-_50, 53, 53__, Good GW-__61, 69, 68___, Bad GW-___35, 37, 84_____

8) Write down what each of treatment's roots weighed at the end of the experiment. Control-__4, 4, 3___, Good GW-__4, 3, 4__, Bad GW-___2, 2, 3____

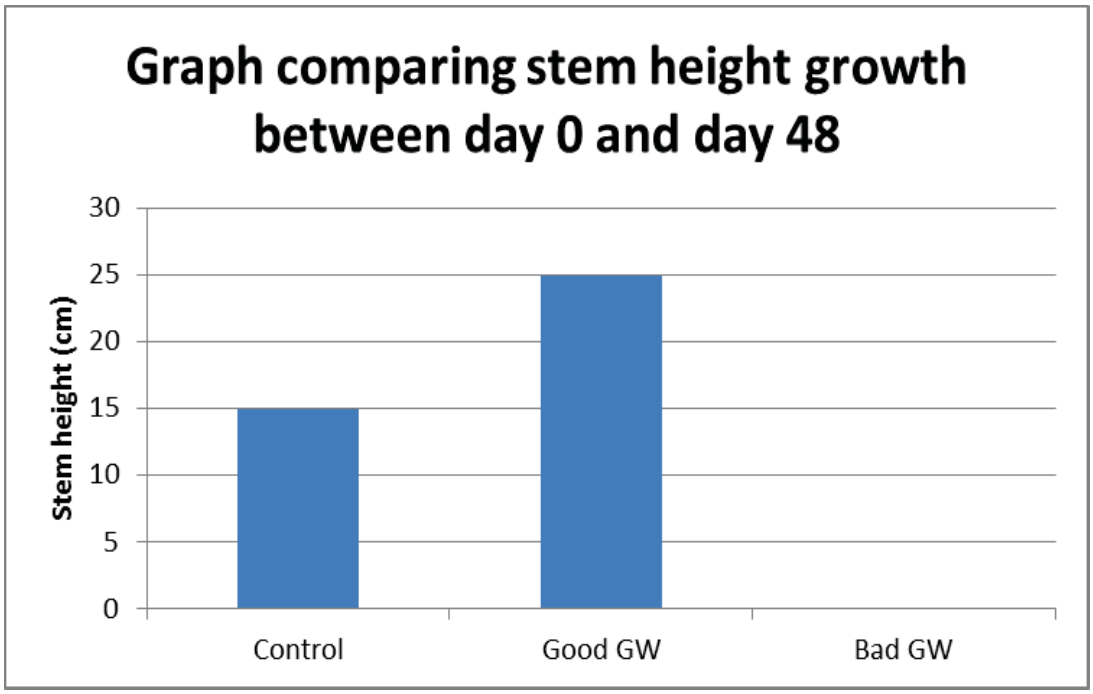
9) Looking at the average number of leaves, what do you notice? Is the number of leaves for the "Water Only" treatment and the ad quality greywater the same of different? _Different__

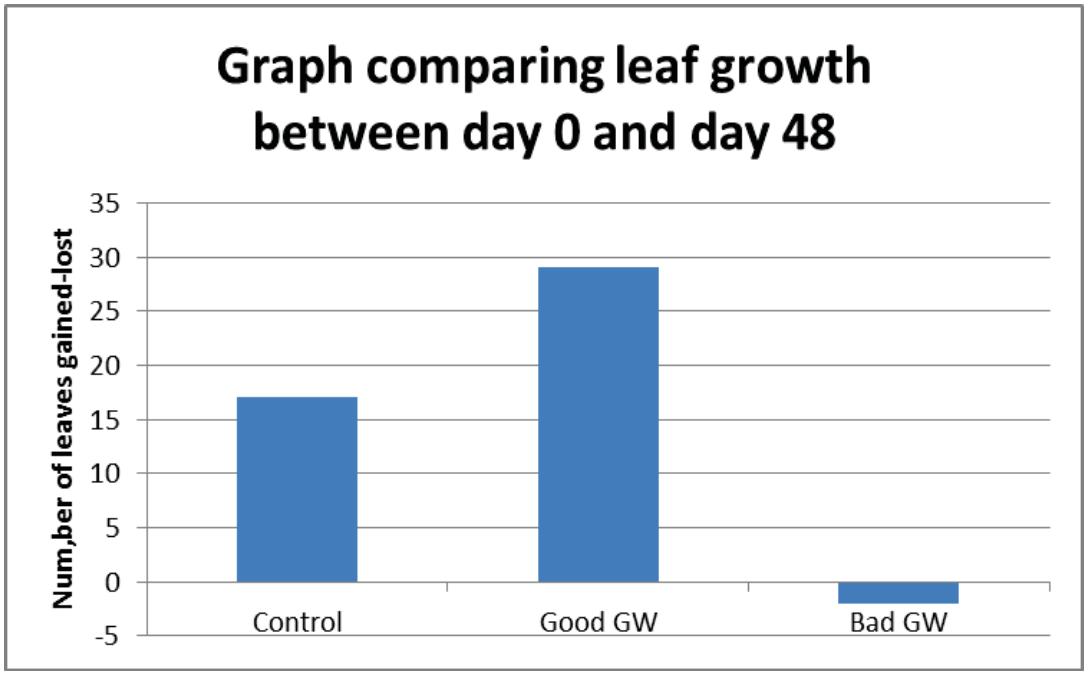
10) Look carefully at the values for the number of leaves in the bad GW treatment that the average is determined from. What do you notice? _____2 very low, 1 high___

11) Now look at the corrected graph below this question, do you notice that the values for the average leaf numbers have changed? This is the correct value; the other was entered by mistake. Can you tell me why it is important to write down your observations carefully? _____So that the information that is gathered is accurate so you can give an explanation as to "why"___

	Treatment	Replicate	Stem height (cm)	Number of leaves	Leaf colour	Root Mass (g)
Day 48	Control (water only)	Replicate 1	46	50	Bright green	4
		Replicate 2	48	53	Bright green	4
		Replicate 3	46	53	Bright green	3
		Average	**46.7**	**52**		**3.7**
	Good Greywater	Plant 1	59	61	Bright green	4
		Plant 2	61	69	Bright green	3
		Plant 3	63	68	Bright green	4
		Average	**61.0**	**66**		**3.7**
	Bad Greywater	Plant 1	35	35	Bright green	2
		Plant 2	34	37	Bright green	2
		Plant 3	32	33	Bright green	3
		Average	**33.7**	**35**		**2.3**

The following questions are about the graph where we compare our different treatments. Remember we measured how many leaves the plants had, and how tall it was in the beginning. We are now interested in seeing not how tall it became, but by **how much** it grew, and not how many leaves it has, but **how many more** leaves it grew.





This is calculated by subtracting the initial reading from the one I made later. An example follows:

Control number of leaves = 35 on day 0

Control number of leaves = 52 on day 48

Control number of leaves increased by (52 - 35) = 17

Good greywater number of leaves = ____ on day 0

Good greywater number of leaves = ____ on day 48

Good greywater number of leaves increased by (66 - 37) = _29___

Bad greywater number of leaves = ____ on day 0

Bad greywater number of leaves = ____ on day 48

Bad greywater number of leaves increased by (35 - 37) = ____

12) Based on your calculations above, which treatment do you think was bad for the plant? The "Water only", bad quality greywater, or the good quality greywater? ___Bad water__

13) Why did you choose that answer?

_____

14) What other change in the plant's overall appearance did we also measure that can confirm your conclusion above? ___Weighed the roots of the plants_____

15) What happened with the plants' leaves that received the bad quality greywater? _____Had brown spots___

16) Why did we use 3 plants in each experiment? ___each person could have control over the plant___

Survey:

a) Did you enjoy the experiment and the process of learning how an experiment works?

____Yes_____

b) What was your favourite part? ___Measuring, doing the display boards

_____

c) Did you understand the experiment? __yes___

d) Thinking back on your own experiment that I helped you set up, is there anything you would have done differently? No

Would you try to keep better track of you samples to avoid mixing them up? ___We didn't mix them up___

Would you use a timetable to remind you when to water the plants?__We did use a timetable

Would you use a friend to double-check that your observations are right? ___We had someone else checking our work__

Would you write down your results in a workbook and make sure they don't get lost?__we wrote down or results on our timetable

Other: Please tell me what it is.

_____

_____

_____

_____

e) Would you like to do an experiment of your own again? _____Yes

f) What would the experiment be about? ____* Volcanoes – how do they erupt, * Global warming – how does it affect our weather, * How do engines work in different modes of transport___

g) How many replicates would you use? __3 x 3 groups__

h) How long will the experiment run? ___As long as it takes 8-10 weeks____

i) What results do you expect to observe? Would you for example expect to see your samples die, or would they stay the same? Would they the ones receiving a treatment do better than the control? (You can measure all kinds of changes in your samples. This might include colour, smell, overall health, weight…)_____* they would die

*change colour – leaves/loose leaves

*grow or not grow