Modeling the decay of E.coli and Ascaris suum in wastewater irrigated vegetables: implications for microbial health risk reduction

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Abstract

Limited access to freshwater in developing countries has made the use of wastewater for irrigation a common practice in urban areas. Wastewater irrigated vegetables can pose significant health risks and lead to severe diseases to the consumers. Cessation of irrigation, ending irrigation a few days before harvesting, is considered one of the most important on-field measures to reduce pathogens on vegetables, but inadequate on-field inactivation rates of the pathogens are limiting the accuracy of risk assessments related to this practice. The existing values are few, uncertain and often modeled with first order inactivation models which may not be the most suitable model in health risk assessments.

The overall aim of this study was to contribute to models for health risk assessment associated with the consumption of wastewater irrigated crops. The study assessed the inactivation rates of *Escherichia coli* and *Ascaris* suum on lettuce and cabbage. The study also accounted for *E.coli* inactivation in the inner and outer parts of cabbage. The research was conducted on-field in Kumasi, Ghana where a significant proportion of urban farmers use wastewater for vegetable irrigation. Vegetables were spiked with wastewater and sampled to study the decay of *E. coli* for 11 days. The decay of *Ascaris suum* was studied over a 30-day period by spiking vegetables with a prepared solution containing *Ascaris suum* eggs. Control vegetables irrigated by the farmers were also sampled to examine the microbiological quality and effect of cessation of irrigation.

A major finding in this study was that inactivation of *E.coli* and *Ascaris suum* indicated the presence of minor and more resistant subpopulations. The decay value of the major subpopulation of *E.coli* was higher on lettuce (2.62 day^{-1}) than on cabbage (1.06 day^{-1}), suggesting that certain properties of the cabbage created a more favorable environment for *E.coli* survival. The decay values of *Ascaris suum*, on the other hand, were similar on lettuce (0.48 day^{-1} and 0.01 day^{-1}) and cabbage (0.44 day^{-1}), indicating that the proposed favorable environment on cabbage had less or no effect on *Ascaris suum*. Another important result in this study was that none of the survival curves of *E.coli* and *Ascaris suum* was best fitted with the log linear model, indicating that the classical first-order kinetics approach is inadequate in many cases. The testing of different models also revealed that large discrepancy can be found between the results of using classical first-order approach and other models. The big differences highlight the importance of testing different inactivation models for more accurate results in future health risk assessments.

Sammendrag

Begrenset tilgang på rent vann har gjort avløpsvann til et populært alternativ som irrigasjonsvann i utviklingsland. Avlinger som er vannet med avløpsvann kan føre til store helserisikoer og påføre forbrukerne alvorlige sykdommer. En av de viktigste metodene for å redusere sykdomsfremkallende organismer, eller patogener, på grønnsakene har vært å stanse irrigasjon av avlinger noen dager før de høstes. Det har imidlertid vist seg at unøyaktige inaktiverings-rater til patogenene begrenser påliteligheten til risiko vurderingene knyttet til dette. De eksisterende inaktiveringsverdiene er få, usikre og ofte kalkulert ved å bruke første ordens inaktiverings modeller, som ikke alltid er de mest passende modellene å bruke i helse risikovurderinger.

Den overordnede målsettingen for oppgaven var å bidra til modeller for helse-risikovurderinger knyttet til konsum av avløpsvann-irrigerte avlinger. Inaktiveringsrater av *Escherichia coli* and *Ascaris suum* på salat- og kålhoder ble beregnet, i tillegg til inaktivering av *E.coli* i indre og ytre del av kålhode. Forskningen ble utført i Kumasi, Ghana, hvor en betydelig andel av bønder i urbane strøk bruker avløpsvann til å irrigere grønnsaker. Salat og kål ble tilsatt avløpsvann og samlet inn fortløpende for å studere inaktiveringen av *E.coli* i løpet av 11 dager. Inaktiveringen av *Ascaris suum* ble studert over en 30-dager periode etter først å ha vannet grønnsaker med en løsning som inneholder *Ascaris suum* egg. Grønnsaker irrigert av bøndene ble også samlet inn som referanse for å analysere den mikrobiologiske kvaliteten og hvilken effekt opphøring av irrigasjon hadde.

Et viktig resultat i denne oppgaven var at inaktivering av *E.coli* og *Ascaris suum* indikerte tilstedeværelsen av en mindre og mer motstandsdyktig subpopulasjon. Inaktiveringsverdien av de store subpopulasjonene var høyere på salat (2,62 dag^{-1}) enn på kål (1,06 dag^{-1}), noe som tyder på at visse egenskaper ved kålhodet skapte et mer gunstig miljø for overlevelse av *E.coli*. Inaktiveringsverdiene av *Ascaris suum* var derimot lik på salat (0,48 dag-1 og 0,01 dag-1) og kål (0,44 dag-1). Dette tyder på at det forannevnte gunstige miljøet på kål hadde mindre eller ingen effekt på *Ascaris suum*. Et annet viktig resultat i dette studiet var at ingen av inaktiverings-kurvene til *E.coli* og *Ascaris suum* best lot seg beskrive ved å bruke log-lineær modell. Dette forteller at den klassiske tilnærmingen av første ordens kinetikk er utilstrekkelig i mange tilfeller. Testingen av ulike modeller viste også at det kan være stor forskjell mellom resultatene mellom bruk av en log-lineær og andre modeller. Dette

understreker viktigheten av å teste ulike inaktiveringsmodeller for mer nøyaktige resultater i fremtidige helse risikovurderinger.

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1. Introduction

Growing population, urbanization and degradation of freshwater resources around the world has made wastewater an attractive alternative source of water supply in urban agriculture. Despite containing pathogens, chemicals and other health hazards of public health significance, wastewater rarely receives adequate treatment before being reused in low income countries. Farmers therefore often have no other choice, but to use untreated or partly treated/diluted wastewater for irrigation.

In urban Ghana, the use of wastewater in agriculture is widely practiced (Keraita and Drechsel, 2008). Previous studies show that the irrigation water used by farmers contains high levels of microbial contaminants with significant health risks for farmers and consumers of wastewater irrigated lettuce salad (Keraita and Drechsel 2004; Seidu et al. 2008). However, simply banning the use of this water remains a major challenge as it contributes to farmers´ livelihoods and urban food security (Amoah 2008). Therefore interventions that contribute to health risk reduction without affecting farmers' access to water will be critical for sustaining the urban wastewater irrigation practice.

Cessation of irrigation is considered one of the most important on-field measures for reducing pathogens on crops (WHO 2006). Irrigation is stopped a few days before the crops are harvested so that unfavourable condition like heat, desiccation and sunlight can inactivate the microorganisms. This simple and low cost measure has proven to be efficient in reducing pathogens on wastewater-irrigated vegetables in urban Ghana; and thus have the potential to increase food safety significantly (Keriata et al. 2007)

Health risk reductions resulting from cessation of irrigation are modeled using Quantitative Microbial Risk Assessment (QMRA), where values of pathogen's occurrence, persistence and human dose-response are used to estimate health risks. However, the usefulness and accuracy of QMRA is dependent on the quality of available data, including data on inactivation rates of pathogens.

Since pathogen inactivation rates depend on several factors, like climate, type of crop, and method of irrigation, decay kinetics will be more accurate if measured in the field. Unfortunately, on-field inactivation rates are very limited and uncertain (Petterson and

Ashbolt 2001). Some of the inactivation rates are based on few data points and specific climatic conditions and can therefore be unsuitable in health risk assessment in other locations and on other crops.

In addition, some of the decay rates have been described by a first order inactivation model, which, as a previous study have shown (Petterson et al. 2001), may not be the most appropriate model to use in risk assessment (Petterson and Ashbolt 2006). Decay kinetics of pathogens can vary depending on several factors and have shown to often have non-linear behavior. Testing of different models before calculating the inactivation rates would therefore find a more precise estimate.

The accuracy of on-field data required in QMRA is dependent on finding indicator organisms appropriate for modeling of a particular pathogen. This is particularly important in developing countries where the capacity to assess pathogens is limited. Inactivation rates of only one indicator organism can be inadequate in health risk assessments as there is little correlation between the behavior of bacteria, virus and helminth eggs in the environment.

1.1 Aims of study

The overall aim of this study is to contribute to the modeling of the health risks associated with the consumption of wastewater irrigated crops. The specific objectives were to:

i) Assess the decay kinetics of indicator *Ascaris sum* and *E.coli* on lettuce and cabbage.

ii) Evaluate the accuracy of different decay models to identify the best-fit decay model.

iii) Assess the implication of the models for microbial health risk assessment.

2. Literature review

Ever since health risk was associated with wastewater irrigation, a wide range of studies has been conducted on the decay of microorganisms on vegetables. Studies have provided potential survival times for pathogens on crops which is essential in health risk assessments associated with the consumption of wastewater irrigated crops. When describing decay curves of pathogens, numerous factors need to be considered, including the choice of indicator organism(s), climate and other factors influencing the decay, and the model used to describe the curve.

The aim of this chapter is to provide an overview of these factors in literature, starting with the advantages and limitations of the indicator organisms used in this study. Further, the most important factors influencing the decay of microorganisms will be discussed, as well as the different effects these have on bacteria and helminth eggs. Finally the limitations and improvements of decay-kinetic models used to describe the inactivation curves will be presented. The literature review will, in this regard, give background information and reasons for several of the choices made during this study.

2.1 Indicator organisms

When monitoring and assessing the behavior of pathogens in the environment, indicator organisms are often used instead of the actual pathogens of concern. This is because pathogens can be hazardous to humans and often appear at low concentrations in natural environments, making them difficult and costly to detect. Indicator organisms are therefore chosen to simulate these hard-to-detect pathogens, making research on microbial behavior more reliable, faster and cost-effective.

However, indicator organisms have to meet several conditions in order to be reliable. Such conditions have been widely discussed (Payment and Franco 1993; Mara and Horan 2003; Hach 2000) and include;

- having the same origin as the pathogen it's representing
- always be present when (and only when) the pathogen is present
- exist in high enough numbers to be detected
- be equally persistent or more persistent than the pathogens it is representing
- be non-pathogenic

- be easy to measure in the laboratory

Based on these criteria, several organisms have been chosen frequently as indicators of microbial behavior. The following section provides insight into the strengths and limitations of the indicator organisms chosen in this study; *Escherichia coli* and *Ascaris suum*, as well as bacteriophages to emphasize the importance of including multiple indicator organisms in health risk assessments.

2.1.1 *Escherichia coli*

Total coliforms, fecal coliforms and *Escherichia coli* (*E. coli*) are the most conventionally used indicators for microbial quality of water. Coliforms are bacteria present in the digestive tracts of human and animals, and although also commonly found in the environment (Boyd and Boyd 1962; Goodrich et al. 1970), indicate the possible presence of entero-pathogens. If health risks are associated with the presence of total coliform, the sample should therefore also be tested for either fecal coliform or E. coli.

Fecal coliforms, a subgroup of total coliforms, appear in great quantities in the gut and faeces of warm-blooded animals and are considered a more accurate indication of animal or human waste than total coliforms (Mara and Horan 2003). They too, however, have been found to grow outside the intestinal tract of human and animals (Marino and Gannon 1991, Payment et al. 1993), and their accuracy as an indicator organism have been debated (Savichtcheva et al. 2007, McCarthy et al. 2008).

The major species in the fecal coliform group is *E. coli* (Winfield and Groisman 2003; Tyagi et al. 2006). It is the most used indicator of the coliform groups and is considered to be the best indicator of fecal contamination (Ashbolt et al. 2001). Still, various studies show that *E.coli* as an indication of pathogen behavior is not always accurate. This is specially the case in tropical and subtropical environments, where studies have shown that *E.coli* can survive for long periods of time and even regrow in water (Carrillo et al. 1985; Solo-Gabriele et al. 2000) and soils (Byappanahalli and Fujioka 2004; Desmarais et al. 2002). This can give results that are higher than what is expected from fecal impacts alone, and challenges the use of *E. coli* as a suitable indicator in such environments (Solo-Gabriele et al. 2000). Nevertheless, such regrowth is rare, and *E.coli* has often been found to relate with pathogenic microorganisms (Savichtcheva et al. 2007, Mons et al. 2009). It also has other advantages in that it is highly abundant in human and animal faces and that it can easily be detected.

2.1.2 Bacteriophages

Bacteriophages, or phages, are viruses that infect bacteria. They share many properties with human enteric viruses, such as composition, structure, morphology, size and mode of replication (Grabow 2001), and are therefore good indicators of enteric virus behavior. In addition, they are relatively persistent in the environment, generally have a high abundance in wastewater and excreta (Bosch 2007) and can be detected using simple and quick methods. Lastly, since they can only multiply in metabolically active host cells (Grabow 2001), they are not a threat to humans.

Because bacteriophages have a different morphology, resilience, and transport-behavior than bacteria, there is often little or no correlation between bacteriophages and coliforms in the environment (Hirotani et al. 2001; Endley et al. 2003; Espinosa et al. 2009; Gibson et al. 2011). Bacteriophages are therefore considered an important inclusion to faecal bacteria to better predict health risks of fecal contamination in the environment (Havelaar et al. 1991; WHO 2011). They also have some advantages to coliform bacteria in that they are obligate pathogens and cannot multiply in the environment as some coliforms (Tyagi et al. 2006). In addition, since animals and humans excrete different types of phages, bacteriophages make it possible to distinguish between animal and human contamination (Hsu et al. 1995; Grabow 2003).

2.1.3 Helminth eggs

The second group of microorganism used in this study is Helminth eggs, pluricellular pathogens with highly resistant biological structures. Their egg shell consists of a variable number of layers each providing mechanical resistance or protection from toxic compounds. They can remain viable for 1-2 months in crops and for many months in soil, fresh water, and sewage, making them the most resistant of all pathogen groups (Feachem et al., 1983, Brownell and Nelson 2006), and a good indicator of pathogen die-off.

In addition to being an indicator of other pathogens, helminth eggs itself can be highly pathogenic as penetration or ingestion of the eggs can lead to severe *helminthiases* (WHO 2012a). The eggs are contained in wastewater, sludge and excreta in variable amounts, depending on local health conditions, and have shown to be most abundant in developing countries, including Ghana (Schwartzbrod et al. 1989; Jimenez 2007). Two of the most common paths of infection are through direct contact with material containing the eggs, and

the ingestion of food crops polluted with wastewater, sludge or excreta (WHO 2012b). Studying the abundance and die-off rate of helminth eggs on crops is therefore very important and much used when assessing health risks associated with wastewater irrigation in developing countries (Stien and Schwartzbrod 1990; Hamouri et al. 1999; Amoah et al. 2005). *Ascaris* eggs, with their multilayered structure of chitin and lipid, are among the most resistant of the helminth eggs (Capizzi-Banas 2004; Brownell and Nelson 2006; Mara et al. 2010), and are therefore often used as the indicator organism when studying the survival of helminth eggs.

2.2 Factors affecting pathogen survival on crops

Depending on the quality and treatment of the wastewater used for irrigation, the aforementioned organisms and other feacal pathogens have the potential to reach the field. The ability of the organisms to survive, both in the treatment plant and in the field, is referred to as its persistence, and varies between different organisms. Most gastro-intestinal pathogens usually die off or lose their infectivity immediately after excretion, while the more resistant pathogens (e.g. helminth eggs) can survive for months, even years, after leaving their host. Based on data from numerous studies, Feachem et al. (1983) compiled the survival times for excreted pathogens on crops (Table 1).

In addition to micro-organisms natural ability to persist, studies have shown that their survival also depend on environmental conditions, like temperature, UV radiation, rainfall, crop type, and method of irrigation (Strauss 1985). These are all factors that can enhance the natural die-off of pathogens in water, crop and soil, and thus minimize health risks associated with wastewater irrigation. Some of the most discussed environmental influences are summarized by Strauss in 1985 (Table 2), and have been widely evaluated and discussed in literature.

Table 1: Survival times of selected excreted pathogens on crop surfaces at 20-30C^0

Pathogen	Survival time (days) on crops
Viruses	
Enteroviruses *	<60 but usually <15
Bacteria	
Faecal coliforms	<30 but usually <15
Salmonella spp	<30 but usually <15
Vibrio cholera	<5 but usually <2
Protozoa	
Entamoeba hystolytica cysts	<10 but usually <2
Helminths	
Ascaris lumbricoides eggs	<60 but usually <30
Hookworm larvae	<30 but usually <10
Taenia saginata eggs	<60 but usually <30
Trichuris trichiura eggs	<60 but usually <30

*** Includes polio-, echo-, and coxsackieviruses. Source: Feachem et al. (1983)**

Table 2: Factors affecting pathogen survival in the environment

Factor	Comment
Humidity/precipitation	Humid environments favour pathogen survival. Dry environments facilitate pathogen die-off. Rainfall can result in splashing of contaminated soil on crops.
Temperature	Most important factor in pathogen die-off. The impact of temperature varies for different pathogens. High temperatures lead to rapid die-off, normal temperatures lead to prolonged survival. Freezing temperatures can also cause pathogen die-off.
Acidity/alkalinity (pH)	Some viruses survive longer in more acid, i. e. lower pH soils, while alkaline soils are associated with more rapid die-off of viruses. Neutral to slightly alkaline soils favour bacterial survival.
Sunlight (UV radiation)	Direct sunlight leads to rapid pathogen inactivation through desiccation and exposure to UV radiation.
Foliage/plant type	Certain vegetables have sticky surfaces (e.g. zucchini) or can abrsorb pathogens from the environment (e.g. lettuce, sprouts) leading to prolonged pathogen survival. Root crops are more prone to contamination and facilitate pathogen survival.
Competition with native flora and fauna	Antagonistic effect from bacteria or algae may enhance die-off. Bacteria may be preyed upon by protozoa.

Source: Strauss (1985); modified by Drechel et al. (2010)

Temperature

Temperature is considered the most important factor in pathogen die-off where high or freezing temperatures lead to a more rapid decay (Strauss 1985). Reddy et al. (1981) found an inverse relationship between temperature and bacterial mortality (between 5 and 30°C), where

die-off rates increased approximately two times with a 10°C rise in temperature. Several studies carried out during the course of different seasons show that pathogens survive significantly longer during summer than winter (Van donsel 1967; Badawy et al. 1990; Sidhu et al. 2008). Badawy et al. (1990) for example, found that a 99% virus inactivation on turf grasses will take approximately twice as much time during the winter (16 to 24 hours) than the summer (8 to 10 hours).

Minimizing the influence of other potential factors like UV, moist and precipitation, Warnes and Keevil (2003) studied lettuce spiked with *Cryptosporidium* oocysts and incubated for 3 days at $20C^0$ and $4C^0$. They detected zero viable oocysts on the lettuce incubated at 20 C^0, but found 10% of the oocysts still viable from the lettuce incubated at $4C^0$.

The inactivation of *Ascaris* eggs is also temperature dependent and can die off in minutes by temperatures above 60°C, and survive for more than 1 year at 40°C (Feachem et al. 1980).

UV- radiation

In addition to the increase in temperature, the sun plays a vital role in pathogen die-off because of its solar radiation. Short-wavelength ultraviolet radiation (UV-C), in particular, is harmful to microorganisms as it deactivates their DNA and RNA, making the organisms unable to reproduce (Warson et al. 2007). The UV-dose (Ws/m2) required to inactivate the microorganisms varies based on the organisms persistence. For *E.coli*, the dose required for 90% inactivation (T90) is between 1300 and 3000 µWs/cm2 (Henze 2008) while *Ascaris suum* eggs would require a higher dose for inactivation as they have been found to be the most UV-resistant pathogen identified (Brownell and Nelson 2006).

Artificial UV-lamps has been used in a variety of applications, such as in disinfection of water, air and food. Arne Aiking and Frank Verheijen were granted an International Patent for their invention of using UV radiation to protect crops from pathogens as they grow in greenhouses (Aiking and Verheijen 2009). By using radiation intensity between 2 500 and 150 000 $\mu w/cm^2$ during 24 hours, they found that UV-irradiation are able to control pathogen growth without damaging the growth or yield of the plants.

In a study on the inactivation of microorganisms on grass surfaces, Sidhu et al. (2008) suggested that exposure to direct sunlight was one of the major factors affecting the inactivation during winter. The inactivation rates of the microorganisms was 2 to 3 fold faster in direct sunlight than in the shade where T90 for the bacteria varied from 6 to 11 hours in

direct sunlight and from 23 to 38 hours in shade. During the summer, however, no significant difference between the rate of decay in sunlight and shade was observed, indicating that other factors played a more significant role in the decay of the bacteria. Sidhu et al. proposed that it was the loss of water through rapid evaporation in the summertime that lead to the decay of pathogens in both sunlight and shadow.

Humidity

Pathogens thrive in humid environments, and their die off rate tends to increase with evaporation and desiccation (Reddy et. al 1981). During desiccation, loss of moisture causes the cells to become inactive or die because there is insufficient water for cellular reactions to proceed (Funke et al. 2007).

In their study on spinach leaves, Choi et al. (2011) found that when the leaves were stored at 25^0C for 120 hours, the microbial population decreased significantly at 43% relative humidity (RH), did not change at 85% RH, and increased significantly at 100% RH.

Like other factors affecting pathogen survival, the effect of desiccation varies greatly between different species. Dry surroundings can kill some organisms in one hour or less, while others can survive for months or even years (Funke et al. 2007). Viruses and endospores can be extremely resistant to desiccation. Yeager and O'Brien (1979) found that enteroviruses can survive in soil with moisture content under 10%. *Ascaris* eggs are also highly resistant to desiccation, and to be inactivated, moisture levels below 5% are needed (Feachem et al. 1983).

Rainfall

Because pathogens favor humid conditions, they tend to survive longer during times of high rainfall (Amoah et al. 2005; Keraita et al. 2007), and studies show that they generally have a much slower decay rate during the wet season than dry season. Keraita et al. (2007) found that the decay coefficient for thermotolerant coliforms on vegetables in Ghana was 0.66/day in the wet season compared to 1.49/day in the dry season. The levels of thermotolerant coliforms were also on average 2 log units per 100 g lettuce higher during the wet season than in the dry season.

In addition to the decreased die-off rate due to humidity, rainfall can also contaminate crops through splashing from soil. Because micro-organisms in soil are more protected from UV,

fluctuating temperatures, desiccation and other extreme conditions, they survive longer in soil (Feachem et al. 1983; Strauss 1985). When heavy rain falls onto the fields, soil can splash and thus transfer microbial organisms onto the crops. Keraita et al. (2007) recorded higher levels of thermotolerant coliforms on the outer leaves of lettuce that had more contact with soil than inner leaves, signifying the influence of contact with soil on contamination. In a recent study on the spreading of *E.coli*, Monaghan and Hutchison (2012) found that a large raindrop of 50 microliter volume can transfer *E. coli* up to 0.5 meters to the side and reach a height of 0.3 meters. The smaller the raindrops the less distance the pathogens were spread. Cevallos-Cevallos et al. (2012) did a similar study, but executed quite differently, on the spreading of *Salmonella*. They found that the dispersal followed a negative exponential model with a half distance of 3 cm at 110 mm/h. The results also showed that spreading enhanced when rain duration increased from 0 to 10 min, and that the dispersed *Salmonella* survived for 3 days on tomato leaflets.

Crop type

Die off rate of pathogens and the likelihood of contamination also depends on the type of crop (Melloul 2001; Armon et al. 2002; Islam 2004). Properties of certain crops such as hairy, sticky or rough surface can protect pathogens from UV-exposure, and also make some crops preserve more water (WHO 2006). Lettuce, for example, is shown to retain 10.8 ml of irrigation water, while cucumber only holds 0.36 ml (Shuval et al. 1997). In addition, lettuce is shown to retain more pathogens from irrigation water than bell peppers, which have a smoother surface than lettuce (Stine et al. 2005).

Pathogens in the soil also attract or attach more easily to the roots of certain crops, making vegetables' susceptibility to pathogens in soil different. After growing several vegetables in the same contaminated soil, Barak et al. (2008) found that radish, turnip, and broccoli had higher prevalence of *Salmonella* contamination as compared with lettuce, tomatoes, and carrots. Furthermore, they found different levels of contamination among tomato cultivars, showing that the presence of pathogens (and the associated health risks) can vary between varieties of the same crop.

Also, Warnes and Keevil (2003) observed different decay rates of *C. parvum* oocysts between different lettuce varieties. After being incubated for 4 days, less than 20% oocysts were viable on the smooth Romaine and Iceberg lettuce, while on the more rough Rav Baby Leaf and Red

Oak Leaf over 50% were still viable. This study suggested that it was the textured leaf of the latter that provided a protective area against desiccation of the oocysts.

Because of change in texture, pathogen survival also enhances by damage to the crops. Studies by Aruscavage et al. (2008), Khalil et al. (2010) and Harapas et al. (2010) show that *E. coli* is more persistent on injured tissue than uninjured tissue on vegetables of different kinds. Harapas et al. (2010) found that during one week, initial counts of about 5 log10 CFU/g decreased to fewer than 0.5 log10 CFU/g on the uninjured plants, compared to 4 log10 CFU/g or more on injured plants.

pH

Another important property that can retard or enhance growth of pathogens is the pH range in the environment. The pH of vegetables is usually near neutral which tend to favor the growth of most microorganism. Some vegetables, however, like fully ripe tomatoes, have a lower pH range which can limit the growth of some enteric pathogens (Buck et al. 2003). Although pathogens generally survive longer between pH of 4 and 10, their tolerance to lower or higher pH varies between the organisms. *Salmonella* is tolerant of acidic environments, and can survive in vegetables with a low pH, such as tomatoes. Fungi are even more acid-tolerant and can grow at pH values as low as 1.5 (Corlett and Brown 1980). Kyeongjin et a. (2012), found that although *norovirus* are more tolerant in slightly acidic (pH 4) or neutral (pH 7) conditions, it was still relatively resistant in strong acidic conditions (pH 2).

The die off rates of pathogens in alkaline soil also varies greatly between different organisms. While alkaline soils are associated with a more rapid die-off of viruses (Strauss 1985), bacteria have a longer survival time in alkaline soil than acid soil (Sjogren 1994). The pH in the soil also determines how easy pathogens adsorb to soil particles, with little adsorption at pH values above 8 and increased sorption at acidic or neutral pH (Toze, 1997).

Method and timing of irrigation

Apart from crop type and to some extent pH (by liming the soil), the aforementioned factors are uncontrollable influences of pathogen die-off on vegetables. The factors are related to unchangeable climatic conditions which can be beneficial or not beneficial. Irrigation practices, however, are controllable and by choosing the right irrigation method there are tremendous possibilities of reducing the undesirable effects of wastewater irrigation.

Different ways off irrigating results in different contamination levels on the crops. Sub-surface irrigation is generally associated with less contamination than surface irrigation because of higher water efficacy and less soil wetting and splashing of soil (Oron et. al 1990, 2001; Kouznetsov et al. 2004; Song et al. 2006).

Drip irrigation which allow water to drip slowly to the roots of plants, has shown to cause less contamination on crops than watering cans (Keraita et al 2007), furrow irrigation (Song et al. 2006) and sprinkler irrigation (Oron et al. 1992). However, drip irrigation is very expensive and tend to clog because of high turbidity levels in the wastewater (Capra and Scicolone 2007; Martijn and Redwood 2005). The choice of irrigation method in developing countries, which usually depends on cost and ability of the farmer to manage the system, thus often end on other methods.

Watering cans are the most popular irrigation method in urban Ghana (Keriata et al. 2002, 2007). Although they have low water efficacy and can lead to more contamination than other irrigation methods (Keraita et al 2007), they are accessible, manageable and very cheap, and therefore an obvious choice for many farmers in developing countries. When studying the effects of affordable changes in the use of watering cans, Keriata et al. (2007) found that simple changes like holding the water can closer to the ground and using an outflow cap can reduce soil splashing and crop contamination significantly.

Another affordable adjustment is changing the timing and frequency of irrigation which may be even more important than method of irrigation. Particularly cessation of irrigation is considered one of the most important on-field measurements to reduce pathogens (WHO 2006). Irrigation is then stopped a few days before the crops are harvested so unfavourable condition like heat, desiccation and sunlight can inactivate most of the microorganisms. This low-cost measure can reduce pathogens by approximately 1 log-unit per day, depending on climatic conditions. In warmer and hotter weather the die-off is approximately 2 log units per day, and in colder, more humid weather without much direct sunlight the die-off is approximately 0.5 log units per day (WHO 2006).

These inactivation rates are uncertain, however, due to the limited amount of data on such inactivation values (Petterson and Ashbolt 2001). Some of the values are based on few data points and specific climatic conditions and can therefore be difficult to apply in health risk assessment in other locations and on other crops. In addition, most of the studies have used a first order inactivation model to describe the inactivation rates, which (as explained in the

next section) may not be the most appropriate model to use in risk assessment (Petterson and Ashbolt 2006).

2.3 Decay kinetic equations

A number of models have been used to describe the inactivation of microorganisms in the environment. Models representing the nine most common survival curves (fig 3) for vegetative organisms are presented in this section including (i) the classical log-linear model, (ii) sigmoidal-like models with shoulder and/or tailing effects, (iii) concave and convex models, and (iv) biphasic models.

Log-linear models (shape I in fig 3)

One of the simplest and earliest inactivation models was proposed by Chick in 1908 who applied the theory of first order chemical reactions to the thermal destruction of microorganisms (Chick 1908). She proposed that there is a linear relationship between rate of inactivation (k) and contact time (t) with a disinfectant and suggested the following model:

$N = N_0 e^{-kt}$ (1) where N and N_0 are the number of organisms present at time t and zero

In terms of the base ten logarithm, the equation can also can be written as:

$log \frac{N(t)}{N_0} = -\frac{t}{D}\ (t \geq 0)$ (2) where D is the decimal reduction time $(\ln(10)\ /k)$

Subsequently, inactivation of microorganisms has generally been considered a first-order logarithmic process, and the chick model has been applied several times in risk assessments associated with wastewater irrigation on vegetables (Asano et al. 1992; Keraita et al. 2007). However, multiple studies since the 1960s have proven that inactivation rates, in fact, are not always constant and that microbial decay curves have been found in different shapes (fig 3). This has questioned the applicability of log linear model in risk assessment and has led to the development of new models that can assess non-linear survival curves more accurately.

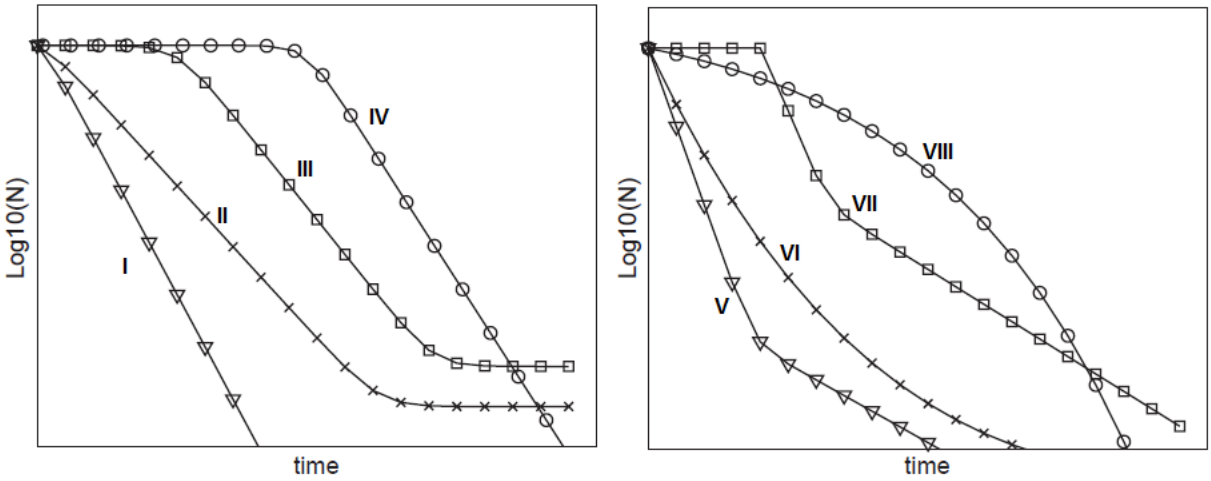

Figure 1 (Geeraerd et al. 2005): Commonly observed types of inactivation curves. Left plot: linear (shape I), linear with tailing (shape II), sigmoidal-like (shape III), linear with a preceding shoulder (shape IV). Right plot: biphasic (shape V), concave (shape VI), biphasic with a shoulder (shape VII), and convex (shape VIII).

Log-linear curves with shoulder and/or tailing (shape I, II, III, IV)

Common non-linear curves are linear curves with shoulder or tailing. A shoulder phase can be defined as an initial phase of an inactivation curve when the microbial population remains unchanged (fig 4). This stable period can have several explanations, like if the cells initially have a higher rate of synthesis than destruction, or if some components in the microorganisms (like proteins and fats) lead to a higher resistance (Geeraerd et al. 2000). Tailing is the lag phase at the end of the curve and implies a more resistant sub-population. Geeraerd et al. (2000) proposed a model that describes both these phases in a log-linear curve. The model was originally developed as two coupled differential equations where the first equation (1) models the tree phases (shoulder, log linear decay and tailing), and the second equation (2) describes the shoulder effect:

$$\frac{dN}{dt} = -kmaks \cdot N \cdot \left(\frac{1}{1+Cc}\right) \cdot \left(1 - \frac{Nres}{N}\right) \qquad (1)$$

$$\frac{dCc}{dt} = -kmaks \cdot Cc. \qquad (2)$$

Here k_{maks} is the specific inactivation rate [1/time unit], Cc is the physiological state of the cells, and N_{res} is the residual population density [cfu/mL].

In Equation (1), the first factor ($-k_{maks}N$) models the log-linear part of the inactivation curve. The second factor describes the shoulder effect where the value (1/(1+Cc)) approaches 0 at the beginning of the shoulder region, and 1 at the end of the shoulder region. The tailing effect is reflected in the third factor which implies the existence of a more-resistant sub-population (N_{res}). It is important to state that tailing in this model is considered for a population remaining constant in time (zero slope of tailing), as opposed to the tailing effect in biphasic models (a small slope of tailing) which will be explained later.

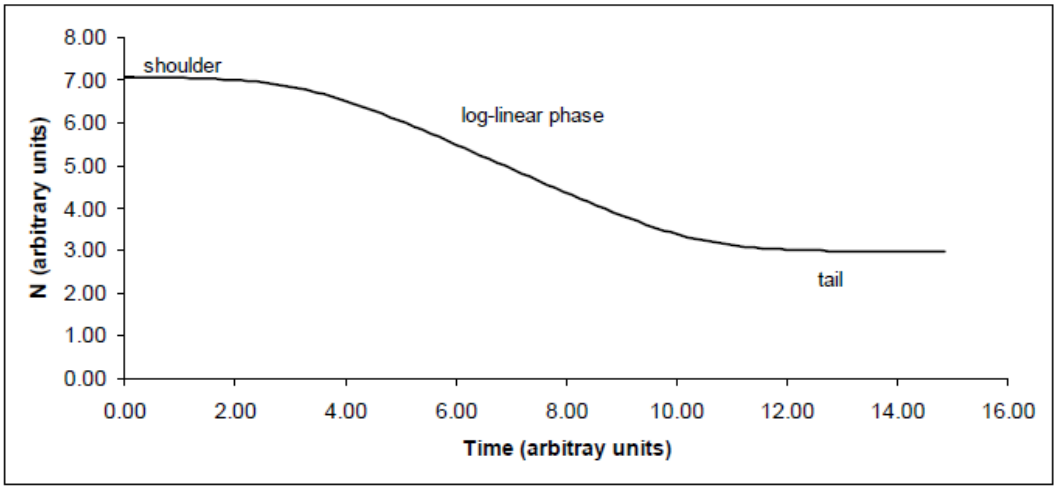

Figure 2 (Bevilacqua and Sinigaglia 2010): Inactivation kinetic with shoulder and tailing phases

Geeraerd et al. (2000) also derived an explicit solution of equation (1) and (2) which reads as follows;

$$N(t) = (N(0) - N_{res}) \cdot e^{-k_{max}t} \cdot \left(\frac{e^{k_{max}S_l}}{1+(e^{k_{max}S_l}-1)\cdot e^{-k_{max}t}}\right) + N_{res} \qquad (3)$$

Here $C_{c(0)}$ has been substituted by $e^{-k_{max}S_l} - 1$ and S is a parameter representing the shoulder.

Geereard's model has been successfully applied to represent survival curves of different microorganisms, like the inactivation pattern of *Botrytis cinerea* and *Monilia fructigena* on

strawberries and cherries during white light treatment (Marquenie et al. 2003), and the survival of *E.coli* K12 during mild temperature inactivation (Valdramidis et al. 2005).

Linear, convex and concave models (I, VI, VIII)

Another approach to model microbial inactivation is the Weibull model where survival curves are considered as a cumulative form for distribution of lethal events. The most used form of the Weibull equation is the one modified by Mafart et al. (2002), which is able to model log-linear, convex and concave curves. The model reads as follows:

$$log10(N) = \log(N(0)) - \left(\frac{t}{\delta}\right)^{\beta} \quad (4)$$

where δ is the time to reach a 1-log reduction in the population number, and β the shape parameter.
If β>1 convex curves are obtained, when β=1 linear survival curve are attained, while for β<1 concave curves are described (fig 5).

Van Boekel (2002) suggested that when β<1 the surviving cells have the capability to adapt to the applied stress at any point in the curve (hence the concave curve), whereas β>1 indicates that the remaining cells become increasingly damaged (hence the convex curve). As opposed to the other models described in this section, the shoulder is not included as a fitting parameter, but emerges from data fitting.

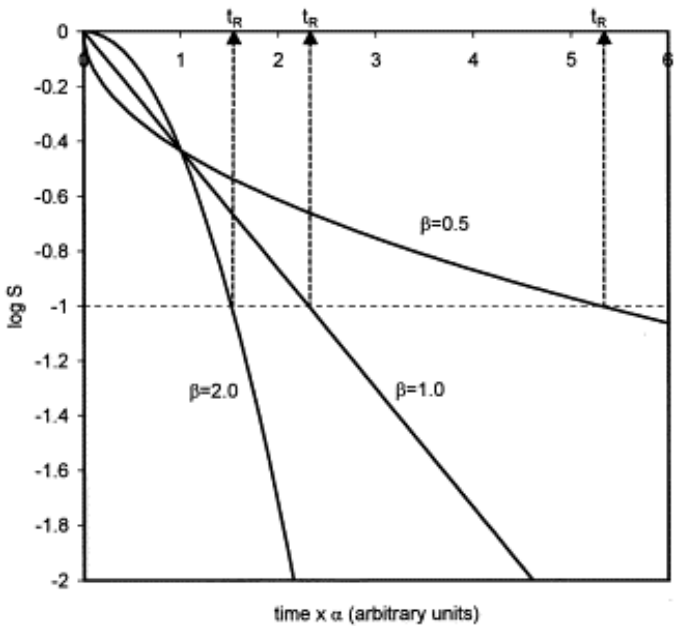

Figure 3 (Van Boekel 2002): The Weibull model simulating convex, linear and concave curves with shape parameter, $\boldsymbol{\beta}$.

A modified version of the Weibull model by Albert and Mafart (2005) introduces the residual population (N_{res}) as a fitting parameter and can thus describe the tailing effect in addition to concave and convex curves. The model can be written as follows:

$$log10(N) = log10\left[(N(0) - N_{res}) \cdot 10^{\left(-\left(\frac{t}{\delta}\right)^{\beta}\right)} + N_{res}\right] \quad (5)$$

The Weibull models has shown to be effective and a good fit in describing convex or concave survival curves with and without tailing for several microorganisms (Van Boekel 2002; Buzrul et al. 2005; Bialka et al. 2008; Izquier and Gómez-López 2011; Keklik et al. 2012).

Biphasic models (V, VII)

The last shape of inactivation kinetics is the biphasic trend where at least two sub-populations have different inactivation rates (fig 6). Such biphasic curves can be explained by weakness within the microbial population where some organisms are more resistant than others (Kamau et al., 1990; Ruiz et al. 2002). It can also imply organisms' different exposure to UV, desiccation and other external factors mentioned in the previous section. On vegetables, the

tailing part then models the pathogens inside the plant or behind the outer leaves, which are more protected and consequently have a slower decay rate (Ibekwe 2009; Erickson 2010).

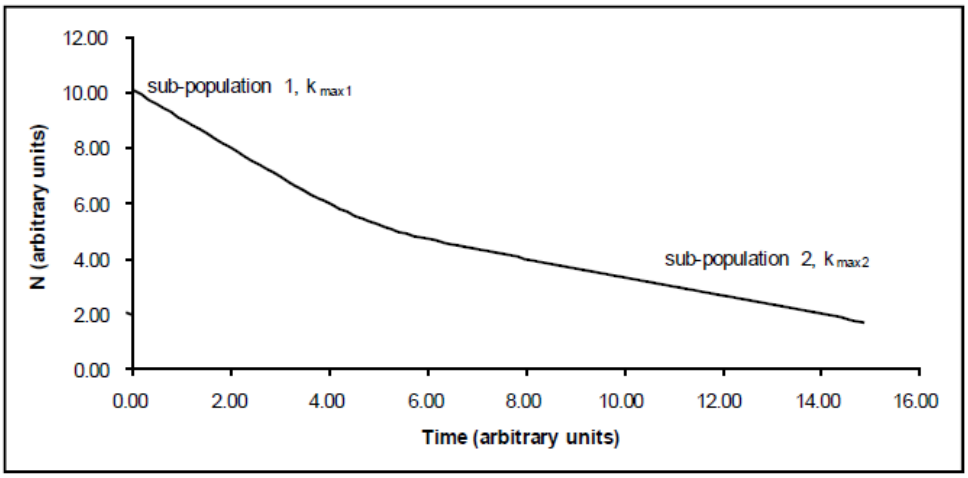

Figure 4 (Bevilacqua and Sinigaglia 2010): Biphasic model

Cerf (1977) proposed the following two-fraction model to describe biphasic linear curves (shape V)

$$log10(N) = log10\big(n(0)\big) + \log 10(f \cdot e^{-k_{max1}t} + (1-f) \cdot e^{-k_{max2}t}) \quad (6)$$

where *f* is the fraction of the initial population in a major subpopulation characterized by the death rate k_{max1}. (1-*f*) is the fraction of the initial population in a minor (and more resistant) subpopulation with an inactivation rate k_{max2}. In addition to biphasic curves, the model can describe linear curves with and without tailing (shape I and II).

Whiting (1993) proposed a biphasic logistic model that accounts for two subpopulations with a shoulder. The model involves the sum of two logistic models – one for each subpopulation:

$$log\left(\frac{N}{N_0}\right) = log\left[\frac{f(1+\exp(-k_1\lambda))}{1+\exp(k_1(t-\lambda))} + \frac{(1-f)(1+\exp(-k_2\lambda))}{(1+\exp(k_2(t-\lambda)))}\right] \quad (7)$$

The advantage of this model is that it can fit many shapes of inactivation curves (linear curves and curves with a shoulder and/or tail) and has proven successful for survival curves of certain microorganisms (Buchanan et al. 1994; Whiting et al. 1996; Ross et. al. 1998). However, the model also has weaknesses, as discussed by Xiong et al. (1999), including not being able to derive the first order kinetic model (when λ=0 and f=1). Xiong et al. (1999) proposed a model (equation (8)) which fits the four curves better than Whiting's model, but because of discontinuity from the shoulder phase to the linear phase, the model cannot cope with realistic temperature fluctuations and cannot be used for time-varying environmental conditions (Geeraerd et al. 2005).

$$\log\left(\frac{N}{N_0}\right) = \begin{cases} 0 & (0 \le t \le \lambda) \\ \log\left(f.exp\left(-k_1(t-\lambda)\right) + (1-f).exp\left(-k_2(t-\lambda)\right)\right) & (t > \lambda) \end{cases}$$

(8)

In order to describe biphasic curves with an initial shoulder (shape VII), Geeraerd et al. (2000) modified Cerf's model and set it up in differential equations. In dynamic conditions, the model reads as follows:

$$\frac{dN_1}{dt} = -k_{max1} \cdot N_1 \cdot \left(\frac{1}{1+C_c}\right)$$

$$\frac{dN_2}{dt} = -k_{max2} \cdot N_2 \cdot \left(\frac{1}{1+C_c}\right)$$

$$\frac{dC_c}{dt} = -k_{max1} \cdot C_c$$

(9)

In static conditions the model can be written:

$$log10(N) =$$

$$log10\big(N(0)\big) + log10 \times \left(\left(f \cdot e^{-k_{max1}t} + (1-f) \cdot e^{-k_{max2}t}\right) \cdot \frac{e^{k_{max1}S_1}}{1+\left(e^{k_{max1}S_1}-1\right) \cdot e^{k_{max1}t}}\right)$$

(10)

Here, as in Cerf's model, N is the total population and the sum of the two subpopulations N_1 and N_2, and the fraction f is defined as $N_1(0)/N(0)$. If the length of the shoulder, S_1, is 0 the model reduces in a natural way to Cerf's model (and is therefore also able to describe log-linear curves).

Despite the number of proposed models, none is able to fit all survival curves. Still, by applying several models before choosing the most fitting, all of the survival curves in fig 3 can be described.

2.4 Conclusions of literature review

A review of the literature shows that no single organism is able to fill all of the roles of what might be considered a perfect indicator of pathogen behavior. Studies show there is little correlation between the behavior of bacteria and helminth eggs in the environment, signifying the importance of using multiple indicators to provide a better overall quality of the vegetables in health risk assessments. *E. coli* was used as an indicator of bacterial behavior in this study as it is highly abundant in wastewater, can easily be detected and is considered the best indicator of fecal contamination. Because it is inadequate to solely rely on bacterial indicators for all feacally derived pathogens, the highly resistant *Ascaris suum* was chosen to indicate the inactivation of helminth eggs. They are abundant in wastewater in Ghana, the cause of severe diseases, and therefore important to include in health risk assessments. Numerous studies on the inactivation of these organisms in different climatic conditions, on different crops and with different method of irrigation show that the die-off rates, not only depends on the persistence of the organisms, but also on several environmental factors. Temperature which is considered the most important environmental influence of pathogen die-off was measured in this study along with precipitation and different types of crops (cabbage and lettuce). Due to protection from environmental factors like UV-radiation, desiccation and extreme temperatures, the pathogens inside the crops, or those protected by leaves, is expected to have a lower inactivation rate than the pathogens on the surface of the crops. Few studies have evaluated these different decay rates, however, and were chosen to be calculated in this study based on the possible measures in health risk reductions.

Despite the wide amount of research on the decay of microorganisms on vegetables, few inactivation rates have been provided. The inactivation values that do exist are therefore uncertain and may be inadequate to use in health risk assessments in other locations and environments. More studies on inactivation rates should be carried out to increase the certainty level of these values in risk assessments. In addition, most of the studies have used a first order inactivation model which is best fitted to describe linear curves. As multiple studies have shown since the 1960s, inactivation curves are in fact often not linear, but show a variety of shapes. Several models have therefore been proposed and modified to describe these curves more accurately and would be more appropriate to use in risk assessments. In this study three models were compared for each survival curve and the model best suited were chosen to describe the decay of the pathogens.

3. Methodology

3.1 Study site

The study was carried out in Kumasi - Ghana's second largest city located in the southern central part of the country. In Kumasi, as well as other major cities in Ghana, urban and peri-urban vegetable farming is a common practice. Two out of three households grow vegetables in their backyards for home consumption and many farmlands along the city's streams produce vegetables for the urban marked. The four main streams flowing through Kumasi city (Daban, Sisa, Wiwi and Subin) (fig 3.1), essentially a requirement for the adjacent farmers, are all polluted with wastewater due to poor infrastructure.

The research in this study was conducted on two farmlands surrounding Kwame Nkrumah University of Science & Technology (KNUST) located in urban Kumasi (6° 41′ 5.67″ N, 1° 34′ 13.87″ W). It's one of the major areas in the city where urban agriculture is practiced and the farmers grow various types of vegetables, including lettuce, cabbage, and spring onion. Lettuce (*Great Lakes 118*) and cabbage (*Brassica oleracea var capitata*) was chosen in this study as it is amongst the most consumed vegetables in urban Ghana. For irrigation, the farmers extract water from the Wiwi stream which runs through the farmlands and is polluted with effluent from KNUST wastewater treatment plant. The farmers irrigate once a day during rainy season and twice a day during dry season. They generally use watering cans for irrigation, except occasional use of pumping machines with showers.

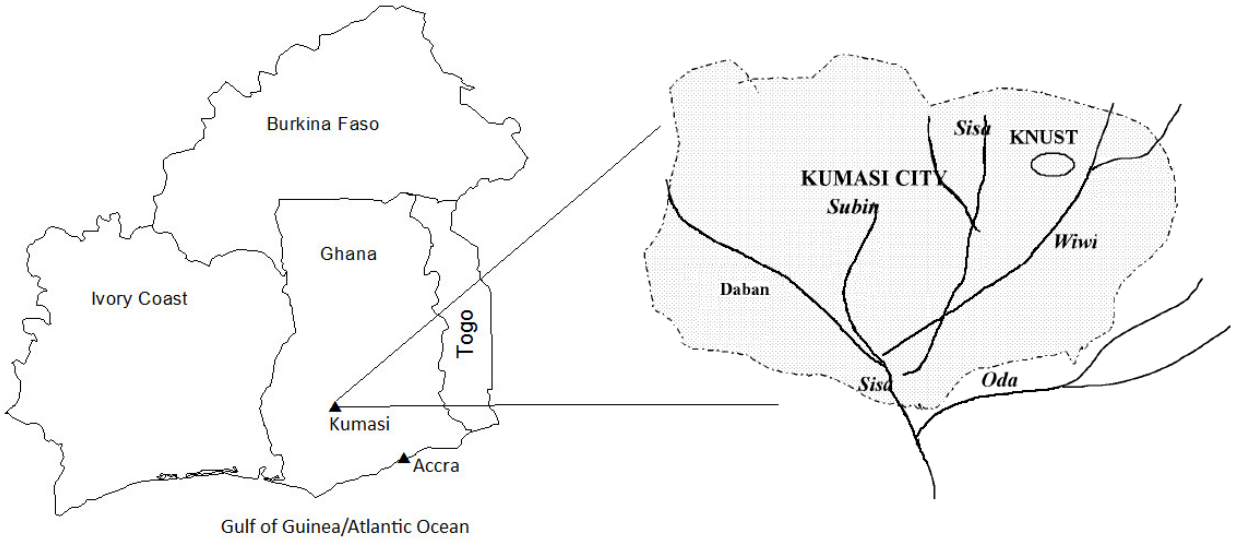

Figure 5: Map showing the four streams running through Kumasi and KNUST (area of study).

3.2 Measurement of temperature and rainfall on study site

Thermometer and rain catchments were set up on the study site the first day. The maximum and minimum temperature was measured every hour (from 6pm to 6am) and rain catchment after every rain fall.

3.3 Preparation of solution for spiking of vegetables

E.coli

The vegetables used for examining the decay of *E.coli* were spiked with wastewater collected from the inlet of KNUST treatment plant. The wastewater used for spiking contained $8.6 \cdot 10^6$ (MPN/100ml) *E.coli*.

Ascaris suum

Ascaris suum eggs used for spiking were recovered from female worms, collected from the intestines of infected pigs in a slaughterhouse. The worms were split longitudinally (mouth to anus) using a scalpel and pinned open to expose the internal contents (for picture of the dissection of the worm, see Appendix A). The uterus was then identified and carefully cut open in the anterior end, where the mature eggs are expected to be found. This section of the uterus was further cut into smaller pieces and macerated using a glass rod in a test tube containing water. The water was then sieved with a pore size that allows the eggs to pass through but prevented pieces of the uterus to pass. The final solution containing the eggs was diluted to a concentration of 150 eggs/100ml.

3.4 Sampling regime

The cabbages and lettuces were sampled from two separate, but adjacent farmlands and from 10 different beds, each bed containing approximately 40 vegetables.

Based on inactivation rates, the sampling regime was conducted differently on vegetables spiked with *E.coli* and vegetables spiked with *Ascaris suum* (table 3.1). Vegetables spiked with *Ascaris suum* which has a lower inactivation rate, was sampled every other day during a 30 days period starting from February 28th to March 28th, 2012. Vegetables spiked with *E.coli* which have a higher inactivation rate were sampled 9 days over an 11 day period (trial 1), starting from February 28th to March 09th. In addition to trial 1, a parallel study on *E.coli* was conducted over a five day period (trial 2) from March 4th to March 9th. This trial was

conducted to essentially compare the inactivation rates and increase the certainty of the values in trial 1.

Table 3: Period of sampling for vegetables spiked with *E.coli* (trial 1 and 2) and *Ascaris suum*.

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	….	28	29	30
Trial 1 (*E.coli* sampling)																		
Trial 2 (*E.coli* sampling)																		
***Ascaris suum* sampling**																		

Spiking and sampling in trial 1

On the first day in trial 1, 30 vegetables (15 cabbages and 15 lettuces) were each spiked with 75ml of raw wastewater for *E.coli* analysis. One lettuce and one cabbage were collected immediately after spiking, as well as every morning the following days. In addition, the same amount of control vegetables was collected.

Spiking and sampling in trial 2

40 more vegetables were spiked with wastewater (75ml) the first day in the second trial to perform a parallel study on the initial decay of *E.coli*. Two lettuces and two cabbages were collected every day the following days, as well as the same amount of control vegetables.

Spiking of *Ascaris suum* and sampling

For *Ascaris suum* analysis, 60 vegetables (30 lettuces and 30 cabbages) were each spiked with 75ml of solution containing *Ascaris* eggs. Subsequently one spiked lettuce and cabbage were sampled every other day along with one control of each vegetable.

All the vegetables were randomly collected with gloves and put into pre-labeled sterile bags (Stomacher® 400 classic, Seward, UK). They were then stored in cooling boxes and transported directly to the laboratory.

3.5 Microbial analysis

Once brought to the laboratory, the vegetables were tested for microorganisms. To examine the difference in content and decay of *E.coli* on the outer and inner part of cabbage, approximately 15% of the cabbages' outer layer were removed and examined separately.

3.5.1 *E.Coli*

E.coli populations were determined using Quanti-Tray®/2000 (IDEXX, Westbrook, USA) - a semi-automated quantification method based on the Standard Methods Most Probable Number (MPN) model. This method requires a 100ml coliform solution for examination, which was prepared slightly different in the two trials:

The vegetables were first weighed and cut in smaller pieces. 10g of each vegetable were randomly selected, mixed with 90 ml of distilled water and vortexed for 30 seconds. 1ml or 10ml (depending on the desired dilution) was transferred from the vortexed solution to an e-flask where it was mixed with 99ml or 90 ml of distilled water, making a total of 100ml, and ready for examination.

During the second trial, the whole vegetables were examined, rather than selecting 10g of each vegetable. After being weighed, the whole vegetable were then mixed with distilled water in a blender and blended for 30 seconds. Lettuce and the outer part of the cabbage were blended with 200ml of distilled water, while the inner part of the cabbage, which was heavier, was blended with 400ml of distilled water. The solution was subsequently mixed with distilled water to the desired dilution, making a total of 100ml.

The steps of the (Quanti-Tray®/2000) method proceeded as follows: A snap pack of Colisure® reagent was first poured into a 100ml coliform-solution. After allowing the reagent to dissolve, the solution was poured into a Quanti-Tray containing 49 large and 48 small wells. The tray was sealed in a Quanti-Tray Sealer which automatically distributes the reagent mixture into the separate wells. Finally the sealed tray was incubated with the well side facing down at 37°C for 24 hours. During incubation, total coliforms in the sample metabolize Colisure's nutrient-indicator CPRG (chlorophenyl red ß-D-galactopyranoside), turning the

colour of the solution from yellow to red/magenta. The number of red/magenta wells counted after incubation thus represents positive results for total coliform. During incubation, E. coli in the sample metabolize Colisure's nutrient-indicator MUG (4-methylumbelliferyl ß-D-glucuronide), turning the sample fluoresces. The number of red/magenta wells that fluoresce after incubation thus represents positive results for *E.coli*. To count the number of wells that has fluoresce, a 6-watt, 365 nm UV light was held within 5 inches of the tray. The number of wells that fluoresce is referred to the MPN table to obtain an *E. coli* MPN.

3.5.2 *Ascaris Suum*

Helminth eggs were isolated by using a combination of the sedimentation and flotation method, both of which are designed to separate helminth eggs from fecal debris in the sample. A combination of the two methods is believed to be most accurate, as they both carry benefits and limitations. The sedimentation technique leads to the recovery of all protozoa, oocysts, spores, eggs, and larvae, but the final solution can contain debris, making the sample more difficult to observe under microscope. The flotation method, on the other hand, permits the separation of helminth eggs and other heavier particles, and thus yields a cleaner solution. However, this method might not be as accurate, as some dense helminth eggs fail to be recovered in the flotation procedure. To ensure detection of all eggs in the sample, a combination of both methods is therefore believed to be most optimal.

Because of time limit and workload, the cabbages were examined as a whole, rather than looking at the outer and inner layer separately.

Sedimentation and flotation

The vegetables were first weighed and washed with 2l of distilled water - first dipped in a container with one liter of water, and then rinsed with an additional liter. The water was collected in a container and left over night, allowing the helminth eggs to sediment. The following day, the supernatant was sucked up with a pumping machine, leaving only the sediments and a small amount of water in the container. For further separation, the sediments were poured into 15ml test tubes (the container was rinsed two-three times to include all of the sediments) and centrifuged (CompactStar CS4, VWR) at 400g for 3 minutes (1500 rpm). The supernatant were once again discarded and sediments poured into one-liter containers. To facilitate floating of the eggs, 150ml of $ZnS0_4$ (zinc sulphate heptahydrate, GPR ^{TM}) was poured into each container and the solution divided in 15ml test tubes. The test tubes,

containing sediment and $ZnS0_4$, were centrifuged at 400g for 3 minutes (1500 rpm), making the debris in the solution sediment and the helminth eggs float. The supernatant (helminth eggs) was poured in to a separate container where it was diluted with 1 liter of distilled water to decrease the specific gravity. The solution was again allowed to settle overnight. The following day the supernatant was removed and the deposit was re-suspended by shaking and emptying into two 30ml tubes. To include all of the deposit, the container was rinsed two or three times with distilled water and poured into the centrifuge tubes. The tubes were centrifuged at 450g for 3 minutes (1600 rpm), the deposit regrouped into 50ml tubes and centrifuged once again at 450g for 3 minutes (1600 rpm). After centrifugation, the deposits were re-suspended in 15ml acid alcohol solution where 5ml of ethyl acetate was added. A cap was placed on the centrifuge tube and shaken vigorously for 30 seconds. To release pressure built up during shaking, the cap was removed occasionally to let gas out. After shaking, the solution was centrifuged one last time at 660g for 3 minutes (1900 rpm). At this point the Ethyl Acetate is believed to be in the top layer followed by a plug of fecal debris and fat, and the helminth eggs at the bottom of the tube. Finally, the supernatant was poured out to leave at most 1ml of liquid in the tubes, which was believed to be almost free from debris and ready for counting (clear enough to be observed under the microscope).

Counting

Counting of the eggs was made in a Sedgwick-Rafter counting chamber with a Leica® CM E Microscope. 1ml of the solution was added to a Sedgwick-Rafter cell with the help of a pipette. Each cell contained a grid subdivided into microliters, allowing the user to count the eggs more accurately. *Ascaris eggs* were separated from other helminth eggs by studying the morphology of the eggs.

Viability

The viability of the eggs was determined by ascertaining whether the eggs were fertile or not. This can be seen by studying the morphology of the eggs, as an infertile egg is more oval than the fertile.

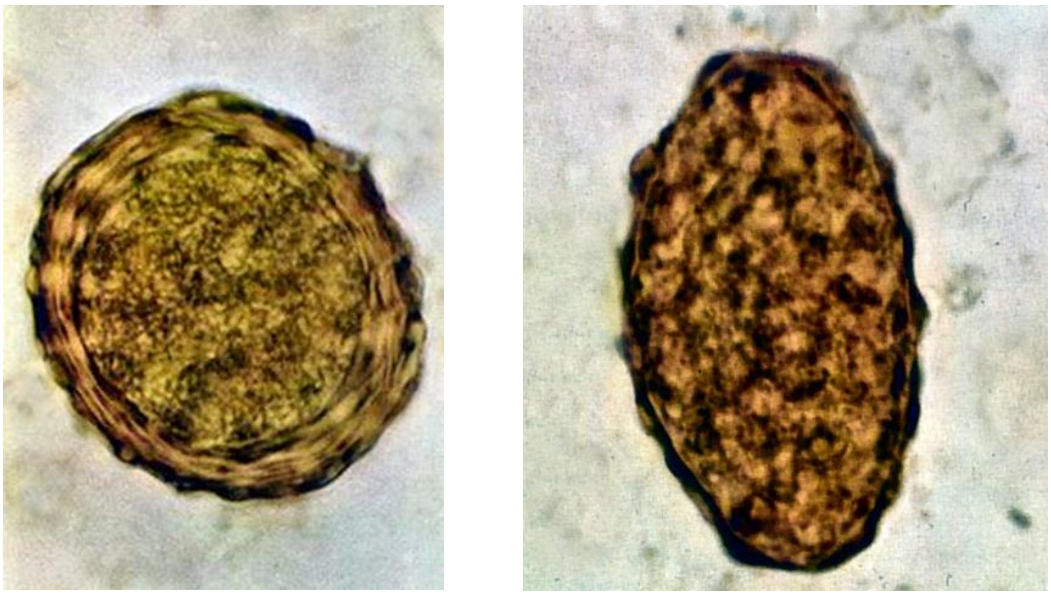

Figure 6: Fertile (left) and infertile (right) ***Ascaris egg***

3.5.3 Sterilization

To avoid cross-contamination, all the equipment that was used was sterilized subsequently through the examination. Test tubes and other containers that had microbial content were sterilized by autoclaving at 121^0C for 15 minutes. Cutting boards, knives and blenders were cleansed with ethanol and distilled water as they were not suitable for autoclaving.

3.6 Decay kinetic modeling

3.6.1 Inactivation values

To model the decay kinetics of organisms in this study, GInaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool), freeware add-in in Microsoft © Excel was used. GInaFiT is useful for testing different types of microbial survival models, allowing the user to find the most appropriate model to describe the inactivation curve. The ten models included in GInaFiT are believed to cover all known survivor curve shapes for vegetative microbial organism cells (illustrated and described in section 2.3).

In this study, three models in GInaFiT were tested for each survival curve. The inactivation value and the most appropriate model to describe the curve was chosen by comparing Mean Sum of Squared Errors, Root Mean Sum of Squared Errors, and R^2 of the different models. These measures demonstrate the difference between the predicted and true value, signifying

how well the model describes the curve. The Mean Squared Errors (MSE) represents the average of the squares of the errors, the error being the amount by which the predicted values differ from the measured value. A lower MSE value therefore indicates a better fit. R^2 is the square of the correlation between the model's predicted values and the actual values. It's bounded between 0 and 1, where a higher value indicates a better fit. If MSE equals 0, R^2 equals 1, and the model is a perfect fit.

3.6.2 Validation of the models

The best-fitted models were validated to evaluate the potential of their use in other health risk assessments. The models used on the spiked vegetables were then used to describe the decay on control vegetables and the predicted values were compared with the actual measured values. To determine the certainty of making predictions from the model, the coefficient of determination, R^2, was evaluated, which represents the proportion of the total variance explained by the model. In addition, the validation regression charts were examined to see the accuracy between the predicted and actual values.

4. Results

4.1 Inactivation of indicator organisms

4.1.1 *E.coli* inactivation

As shown in table 4.1, decay of *E.coli* on spiked lettuce was best described with the biphasic model, indicating the presence of a more resistant minor sub population. The inactivation rates, kmax1 and kmax2, were 2.62 day^{-1} and 0.22 day^{-1}.

Decay of *E.coli* on the outer part of cabbage was also biphasic and had inactivation rates of 1.06 day^{-1} and 0.53 day^{-1}. On the inner part of the cabbage, the decay was best described with log linear + shoulder model where kmax = 0.82 day^{-1}.

Table 4: Comparison of different models describing the decay of *E.coli* on lettuce and cabbage.

Decay of *E.coli*						
	F	Kmax1	Kmax2	Mean sum of squared error	Root mean of squared error	R^2
Lettuce						
Biphasic	0.99	2.62	0.22	0.0888	0.2979	0.9421
Log linear + tail	-	2.36	-	0.1168	0.3418	0.9129
Weibull + tail	-	1.56	-	0.1321	0.3634	0.9138
Outer cabbage						
Biphasic	0.89	1.06	0.53	0.0269	0.1640	0.9695
Weibull	-	0.75	-	0.0277	0.1664	0.9641
Log linear	-	0.53	-	0.0443	0.2104	0.9283
Inner cabbage						
Log linear + shoulder	-	0.82	-	0.0510	0.2257	0.9338
Weibull	-	0.15	-	0.0658	0.2565	0.9145
Log linear regression	-	0.55	-	0.0913	0.3022	0.8644

See appendix B for survival curves of *E.coli* described with the different models.

The parallel study conducted in trial two showed similar results as the initial decay in trial one (see appendix C for decay values for the five day trial), reinforcing the accuracy of the values found in trial one.

4.1.2 *Ascaris suum* inactivation

Decay of *Ascaris suum* on lettuce was best described with the biphasic model where kmax1 was 0.48 day^{-1} and kmax2 was 0.01 day^{-1}.

On cabbage the survival curve was best described with log linear + tail model where kmax was 0.44 day^{-1}.

Table 5: Comparison of different models describing the decay of *Ascaris suum* on lettuce and cabbage.

Ascaris suum						
	f	Kmax1	Kmax2	Mean sum of squared error	Root mean of squared error	R^2
Lettuce, spiked						
Biphasic	0.65	0.48	0.01	0.0012	0.0339	0.9233
Weibull	-	0.03	-	0.0021	0.0457	0.8558
Log linear	-	0.02	-	0.0048	0.0692	0.6563
Cabbage, spiked						
Log linear + tail	-	0.44	-	0.0042	0.0645	0.6777
Biphasic	0.68	0.47	0.00	0.0043	0.0656	0.6795
Log linear regression	-	0.02	-	0.0082	0.0908	0.3385

See appendix B for survival curves of *Ascaris suum* described with the different models.

4.2 Validation of the models

The best-fitted models were validated to see if they can be used to describe the decay of *E.coli* and *Ascaris suum* in other health risk assessment. R^2 (the proportion of the total variance explained by the model) were relatively high for the models, ranging between 0.63 and 0.99 (table 4.3). The survival curves described by log linear + tail and log linear + shoulder, were very accurate in describing the inactivation of *E.coli* and *Ascaris suum* (see Appendix D for validation regression charts).

Table 6: Coefficient of determination of the best-fitted models.

Organism and vegetable	Model	R^2
E.coli on lettuce	Biphasic	0.9506
E.coli on outer cabbage	Biphasic	0.8551
E.coli on inner cabbage	Log-linear + shoulder	0.9988
Ascaris suum on lettuce	Biphasic	0.6345
Ascaris suum on cabbage	Log linear + tail	0.8032

4.3 Weather conditions

During the time of sampling there were two minor and one major episode of rainfall. The average temperature was 30.7^0C (for further detail on measured rainfall and daily temperature see appendix E).

4.4 Health Risk Assessment

The initial concentration of *E.coli* on both lettuce and outer part of cabbage exceeded the recommended level for consumption, according to the ICSMF guidelines (ICMSF 1974) and the guidelines for microbiological quality of 'ready-to-eat' foods (Gilbert et al. 2000) of 100 *E.coli* per g vegetable (See Appendix F for daily values of *E.coli* on control vegetables in this study). After 4 and 9 days of cessation, however, *E.coli* on lettuce and cabbage, respectively, reached a tolerable level for consumption. The concentration of *E.coli* in the inner part of the cabbage also exceeded the recommended level for consumption after irrigation and reached a tolerable level after three days of cessation.

As opposed to *E.coli*, the level of *Ascaris suum* remained high on lettuce and cabbage and posed a significant health risk throughout the 30 day trial (see Appendix E for daily values of *Ascaris suum* on control vegetables in this study). Because longer irrigation cessation periods can adversely affect the productivity and freshness of vegetables other risk reduction measures is required to reduce the level of *Ascaris*. Such risk reductions include treatment of the wastewater running into the stream, method of irrigation, and post-harvest treatment. Studies have shown that washing or using a mild disinfectant post-harvest (Amoah et al. 2007) and irrigating with capped cans and from a lower height (Keriata et al 2007) can reduce the number of eggs significantly.

5. Discussion

5.1 Inactivation of indicator organisms

Inactivation of *E.coli* on lettuce and outer cabbage both indicated the presence of a minor and more resistant subpopulation of 1% and 11% of the total population respectively. Despite similar survival curves, the decay value of the major subpopulation was higher on lettuce (2.62 day^{-1}), than on cabbage (1.06 day^{-1}), suggesting that certain properties of the cabbage created a more favorable environment for *E.coli* survival. Several authors suggest that solar radiation is the dominant factor in coliforms inactivation, and has a substantial influence on the decay than temperature (Oron et al., 2000; Sinton et al., 2002; Zdragas et al., 2002; Manios et al. 2006). The character of cabbage with tightly packed leaves could therefore be one reason for the lower inactivation rate as it creates more protection from UV radiation than the more exposed leaves on lettuce (fig. 5.1). The tightly packed leaves may also create a more humid environment and protect the inner part from desiccation. Such favorable areas can also explain the bigger subpopulation on cabbage that have more areas for protection than lettuce as well as the lower inactivation rate in the inner part of the cabbage (0.82 day^{-1}).

The decay rates for *Ascaris suum*, on the other hand, were similar on lettuce (0.48 day^{-1} and 0.01 day^{-1}) and cabbage (0.44 day^{-1}), indicating that the proposed favorable environment on cabbage had less or no effect on *Ascaris suum*. In contrast to *E. coli*, *Ascaris* eggs are highly resistant to UV radiation (Brownell and Nelson 2006), which could explain why the effect of different solar exposure is smaller. Temperature, which is suggested to be a more dominant factor for their inactivation (Moe and Izurieta 2003), is considered equal in lettuce and cabbage in this study, and can explain the similar inactivation rates on the vegetables.

The best-fit models of *Ascaris suum* on lettuce and cabbage also indicated the presence of more resistant subpopulations. Both inactivation rates were lower than *E.coli* as was expected because of their highly resistant structure and results from previous research (Feachem et al. 1983). The decay rate of *Ascaris suum* of 0.4 day^{-1} on lettuce in this study corresponded well with the finding made in an earlier study (Keraita et al. 2007).

Figure 7: Cultivars of cabbage (*Brassica oleracea var capitata*) and lettuce (*Great Lakes 118*) used in this study.

5.2 Modeling of survival curves

This study has demonstrated that modeling of the die-off of microbial organisms based on the assumption that the rate of die-off is constant over time may not always be accurate. This may lead to inaccuracies in the calculated k value, as inactivation often has shown to have non-linear behavior (Hiatt 1964; Humpheson et al. 1997; Juneja et al. 2004). In fact, none of the survival curves of *E.coli* and *Ascaris suum* in this study was best fitted with the log linear model, indicating that the classical first-order kinetics approach is inadequate in many cases. More importantly, the testing of different models revealed that large discrepancy can be found between the classical first-order approach and other models. For example, cabbage spiked with *Ascaris suum* with log-liner + tail, showed an inactivation rate of 0.44 day^{-1}, while the same curve modeled with log linear model resulted in an inactivation rate of 0.02 day^{-1}. As shown in table 4.1 in the result chapter, substantial differences were also found between different modeling approaches to *E.coli.*

In addition to the disparities between the models in this study, a previous study showed a much greater initial inactivation rate of viruses than what was predicted from earlier research by considering the tailing-off phenomena in the survival curve (Petterson et al. 2001). In their study, Petterson et al. (2001) found evidence for biphasic inactivation of viruses on lettuce, signifying the presence of a persistent subpopulation. The results showed that the initial decay value in their study (2.48 day^{-1}) was much greater than previously log-linear calculated k-values of virus on lettuce, which has been estimated to be 0.69 day^{-1}, 0.3 day^{-1}, 0.8 day^{-1}, 0.6 day^{-1} (summed up by Petterson and Ashbolt 2006).

The slow inactivation rates of the more persistent sub-populations can have significant implication for health risk assessment. Petterson and Ashbolt (2001) have shown that by not considering the possible presence of persistent sub-populations, predicted infection rates are significantly underestimated. This study and that of Petterson and Ashbolt (2001) have clearly demonstrated that non-linearity should always be accounted for in risk assessment and different models should be compared before calculating the k value.

5.3 Health risk assessment

The study has demonstrated that significant health risk reduction can be achieved through cessation of irrigation for pathogens that can be modeled with *E. coli*. However, significant health risk still remained for *Ascaris* related infection even after 30 days of irrigation cessation. This shows that risk assessment in developing countries based on pathogenic bacteria may significantly masquerade actual risk levels with respect to parasitic organisms. It further indicates that health risk reduction cannot be solely achieved through cessation of irrigation. Studies have shown that washing or using a mild disinfectant post-harvest (Amoah et al. 2007) and irrigating with capped cans and from a lower height (Keriata et al 2007) can reduce the number of eggs significantly. For cabbage, removal of the outer layer is also shown to significantly reduce the level of pathogens on cabbage (Minhas et al. 2006), as supported in this study.

Another concern is the quality of the lettuce being sold to the local markets. The farmers revealed that lettuce is commonly washed in irrigation water to retain its physical quality and attractiveness before being sold to markets nearby. The main reasons for washing the lettuce were to remove attached soil particles and earthworms, to reduce the weight of the lettuce and make it more attractive. The microbial content of the lettuce is consequently increased to the initial contamination level, increasing the health risks significantly.

5.4 Limitations of results

Because of workload and limited amount of time, a small amount of samples were collected in this study. Damage or different structure on only a few vegetables could affect the inactivation of the organisms and subsequently the resulting inactivation value. For more accurate inactivation values, a larger sample size is preferred.

6. Conclusion

This study and previous research show that not only the inactivation rate, but also the survival curve can depend on several factors like pathogen of concern, climatic conditions and type of crop. This makes it difficult to choose specific models to describe inactivation of pathogens in other health risk assessments. The discrepancy of results by different models in this study demonstrates the importance of comparing several models before calculating the inactivation value. The different shapes of survival curves, including the possible presence of persistence sub-populations, can have implications for the calculated health risk and should be accounted for in future health risk assessments.

The study further shows that cessation of irrigation was successful in reducing the concentration of *E.coli* on lettuce, and to some extent, on cabbage, to a safe level for consumption. However, reaching a tolerable level for *Ascaris suum*, required more days, which can affect the productivity and freshness of the vegetables. To ensure safe consumption without affecting the value of the vegetables, other measurements are therefore needed. The removal of outermost leaves of cabbage which are more prone to contamination from irrigation water has good potential of reducing *E.coli* contamination level on cabbage.

To measure health risk reduction related to cessation of irrigation, accurate on-field inactivation rates are imperative and multiple indicator organisms should be included for a better overall assessment of the risks.

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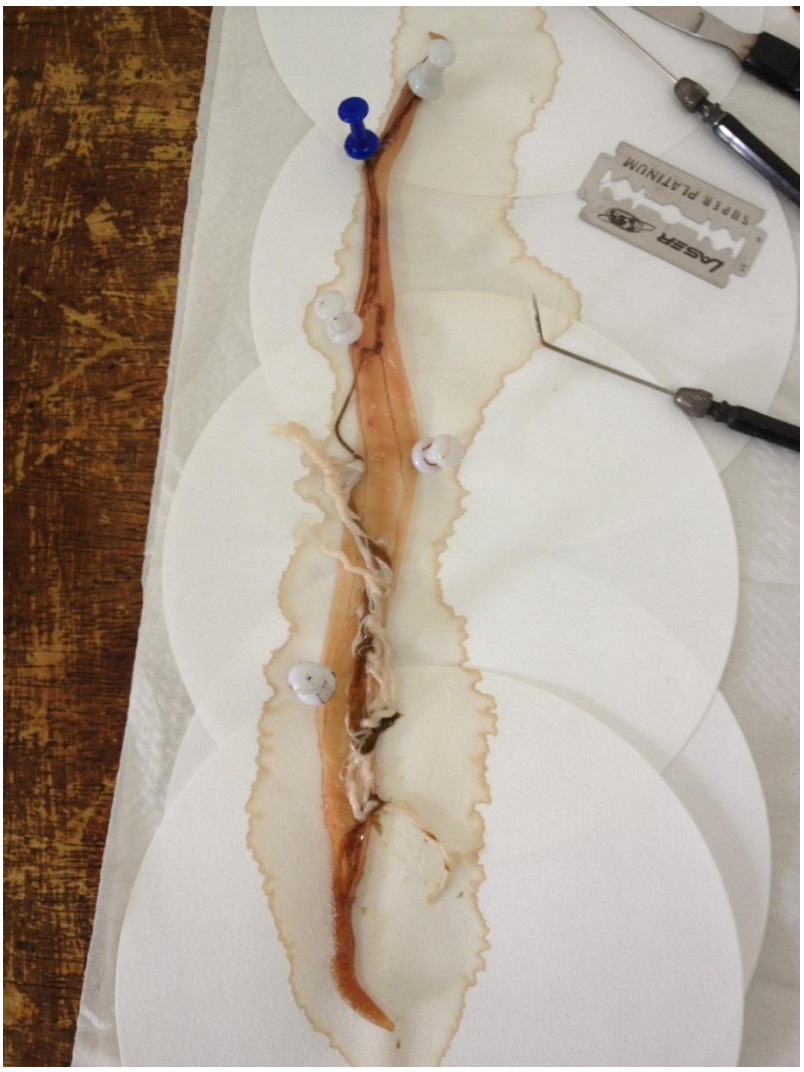
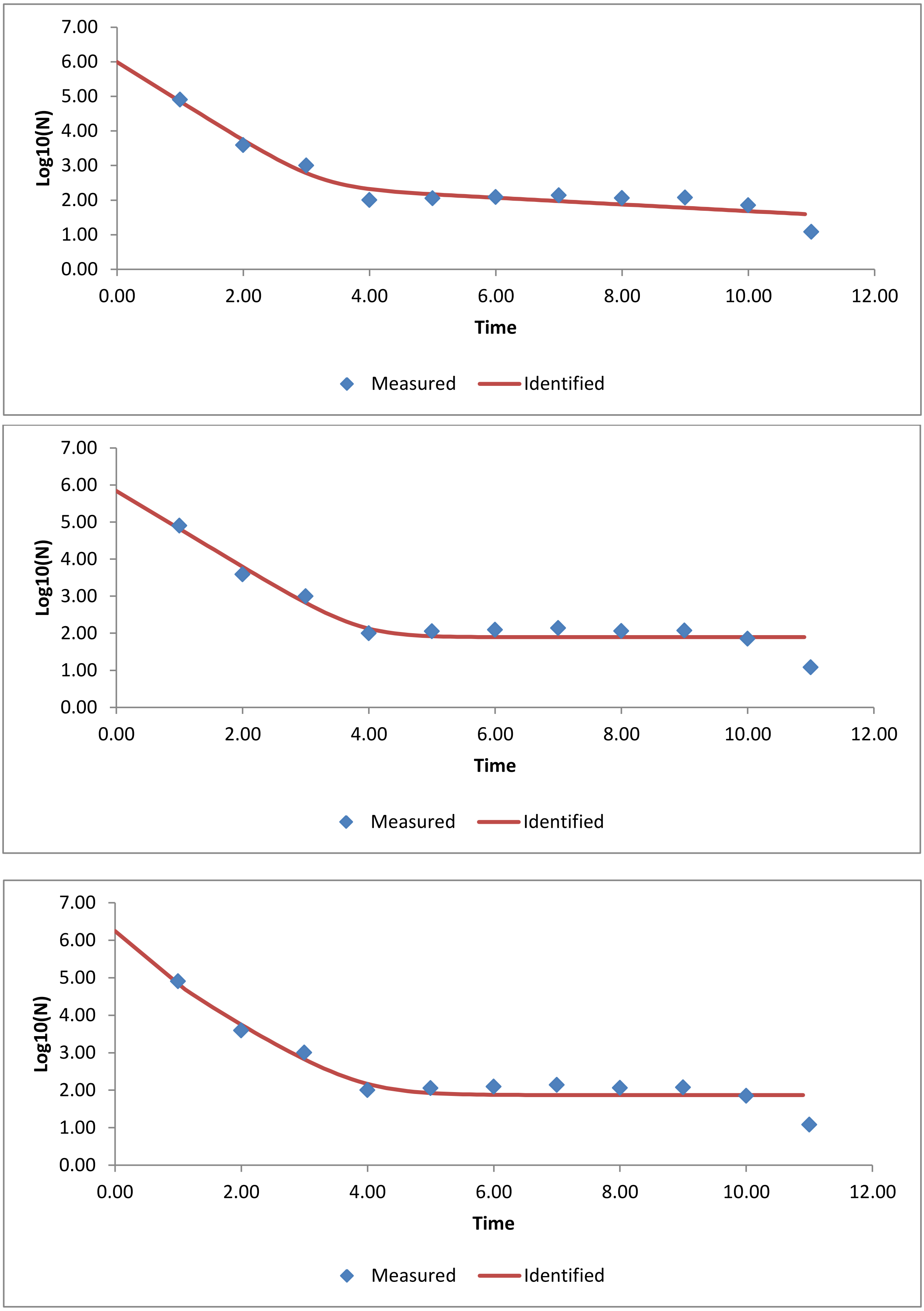
Appendix A – Dissection of worm containing helminth eggs

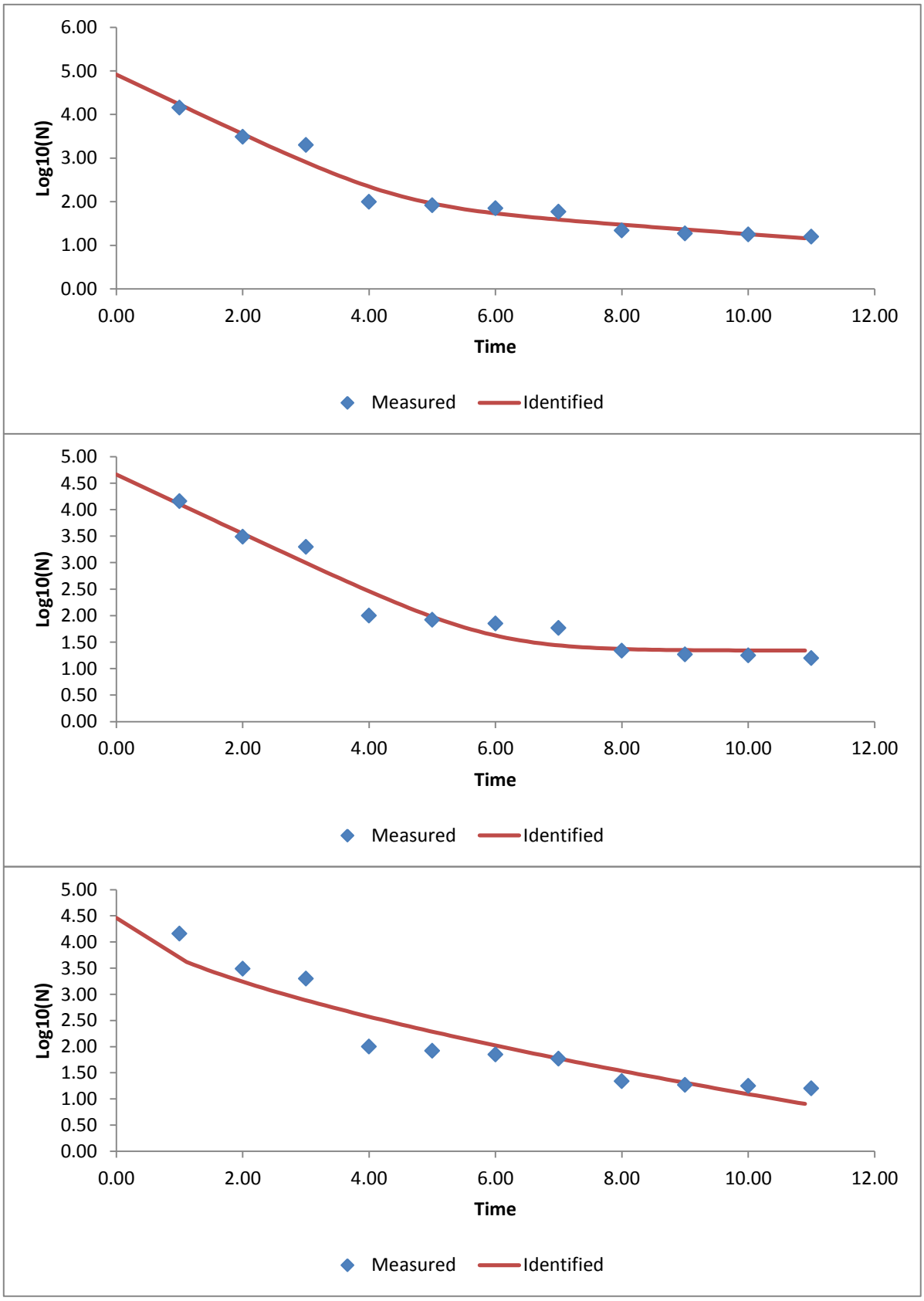


Appendix B - Decay curves of *E.coli* and *Ascaris suum*

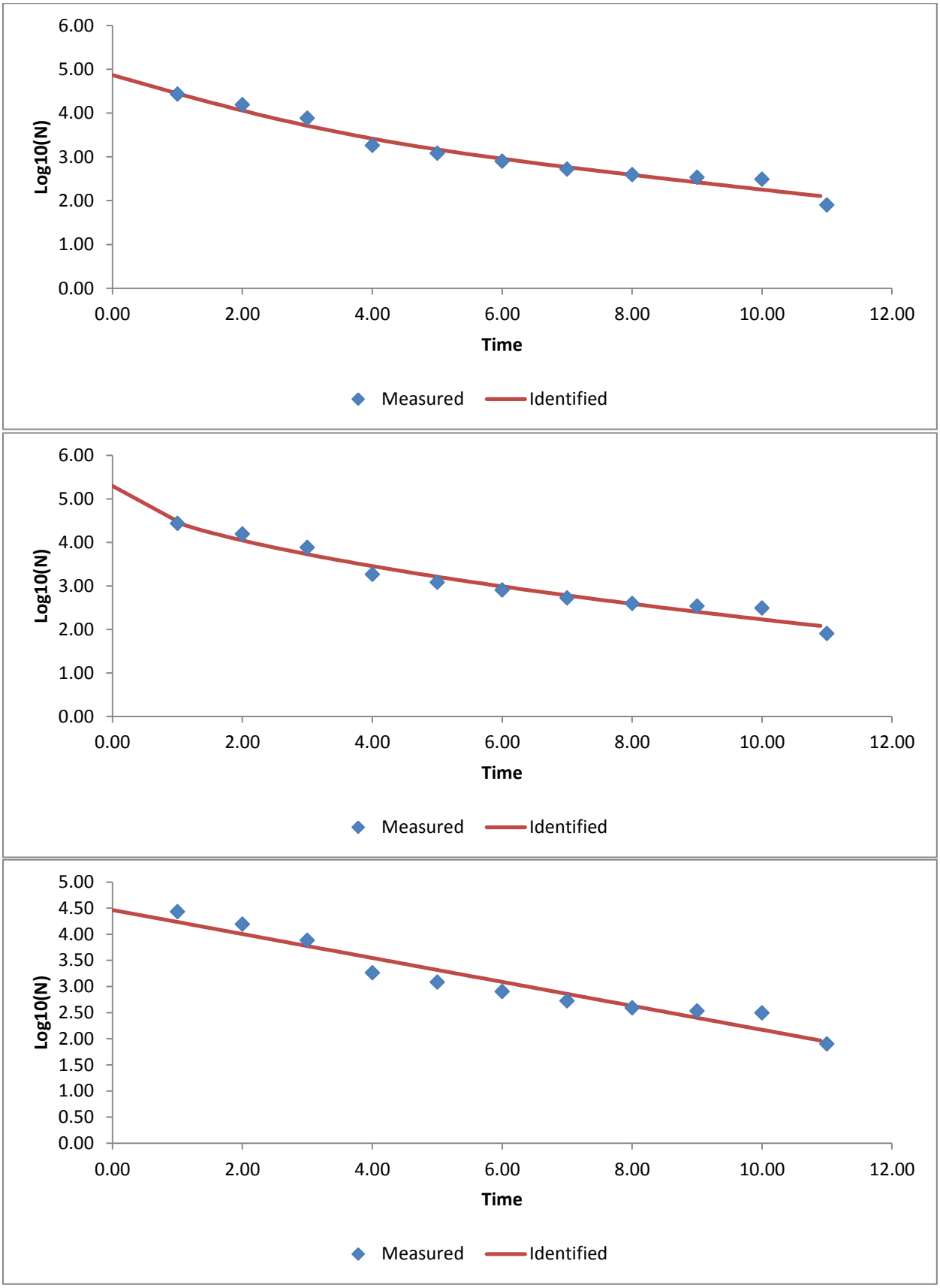
Decay of *E.coli* on spiked lettuce modeled with biphasic (top), log linear+tail, and Weibull+tail (bottom).



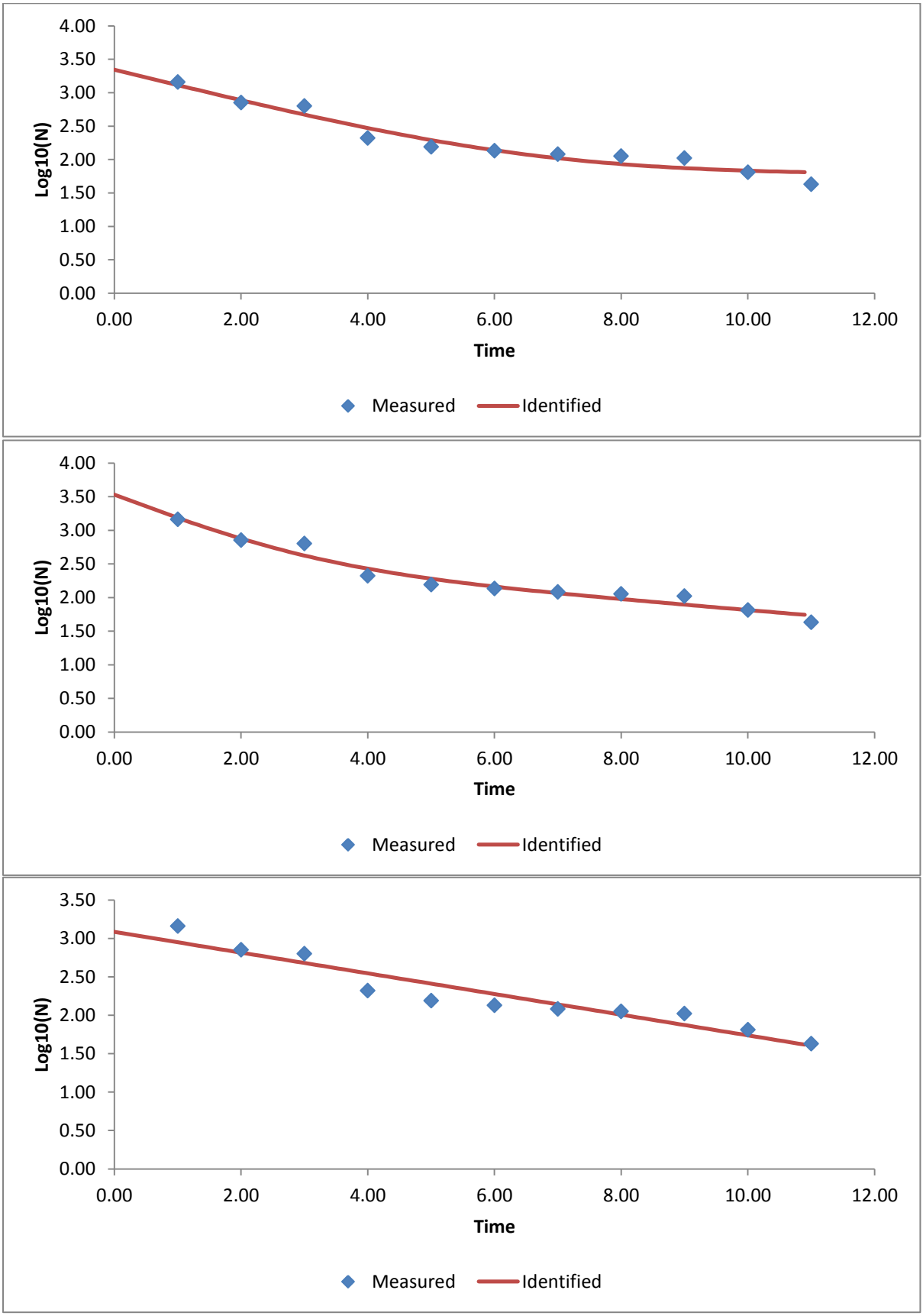
Decay of *E.coli* on lettuce control modeled with biphasic (top), log linear+tail, and Weibull (bottom).



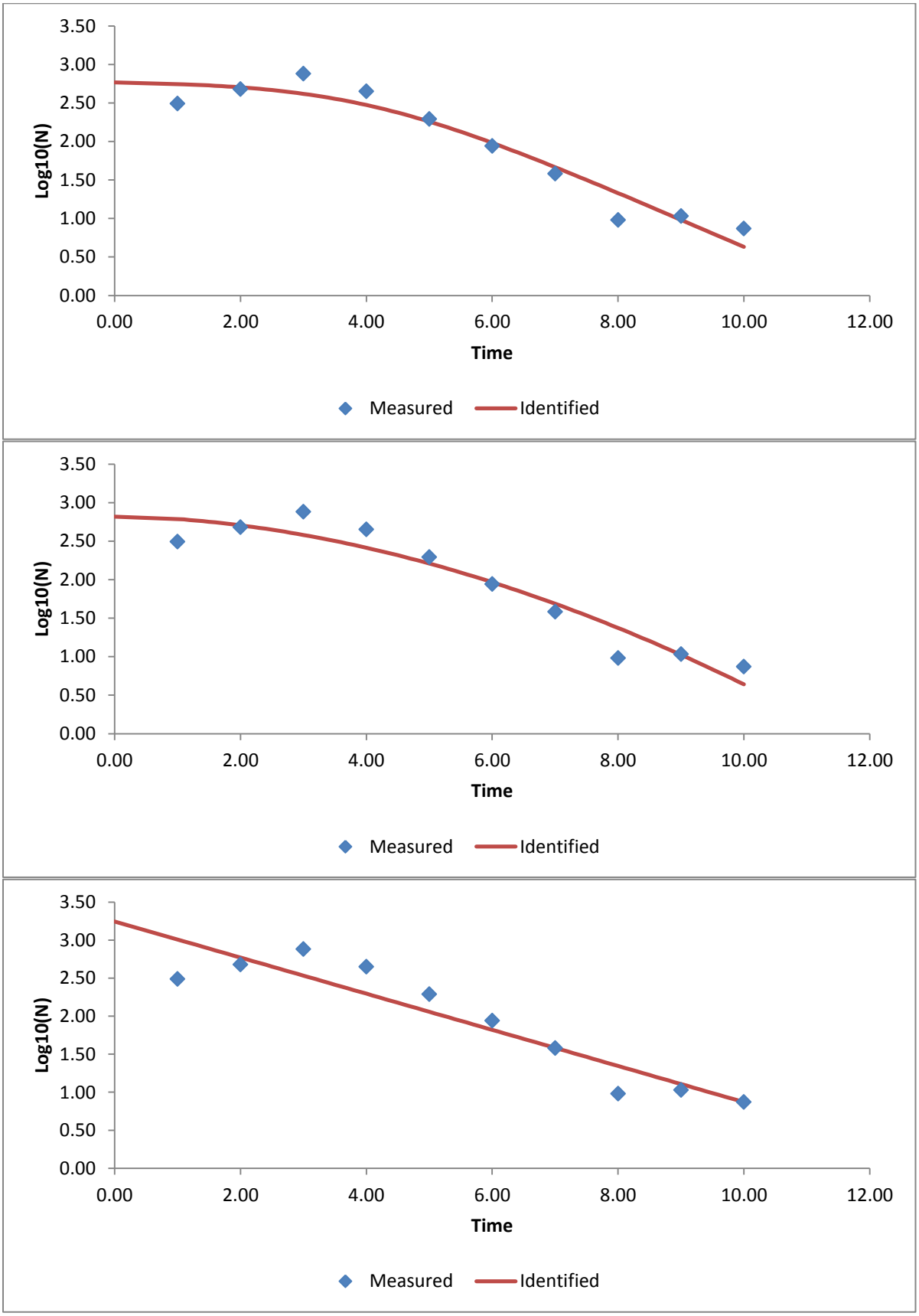
Decay of *E.coli* on outer part of spiked cabbage modeled with biphasic model (top), Weibull, and log linear regression (bottom)



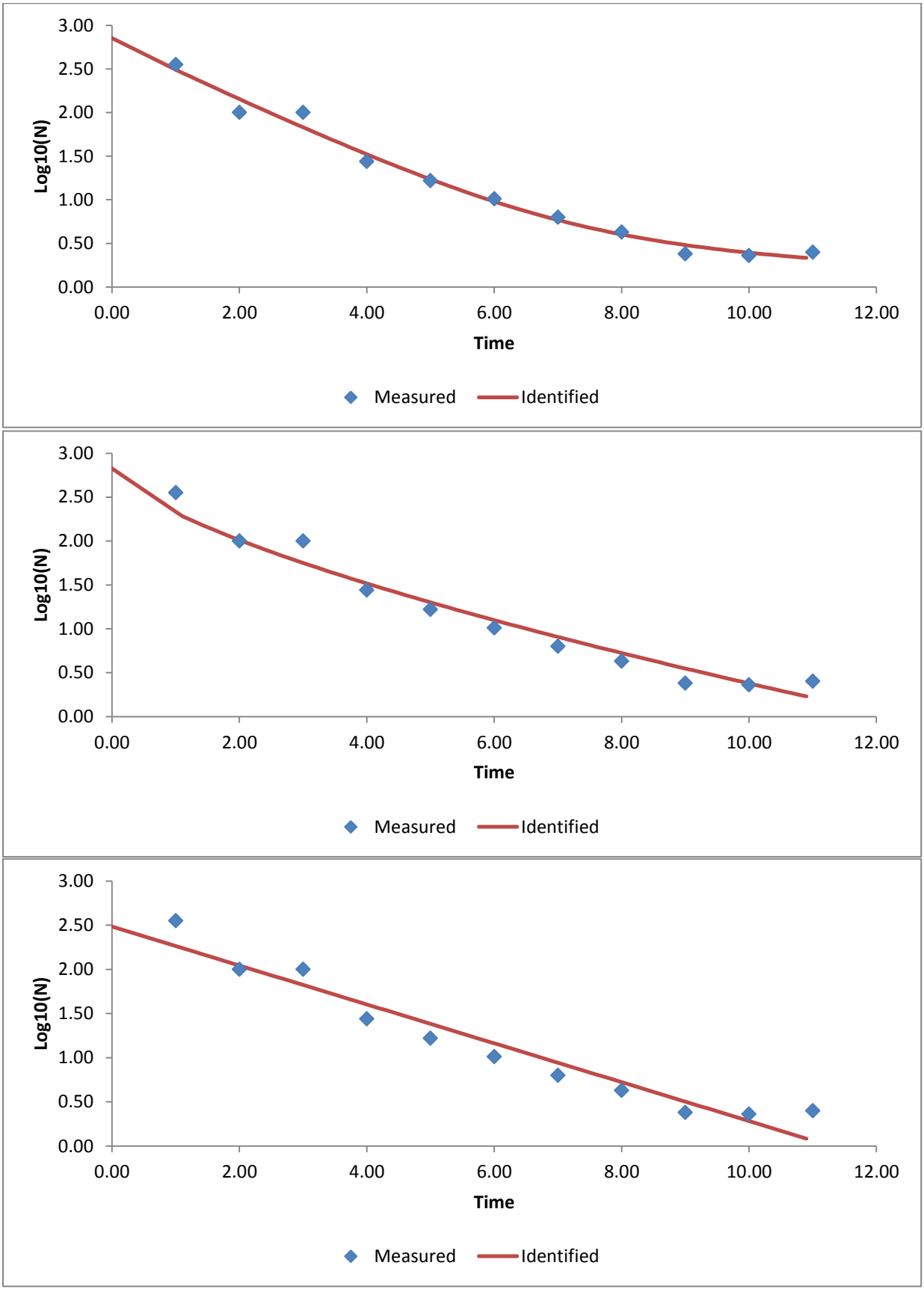
Decay of *E.coli* on outer part of cabbage control modeled with biphasic (top), log linear + tail, and log linear regression (bottom).



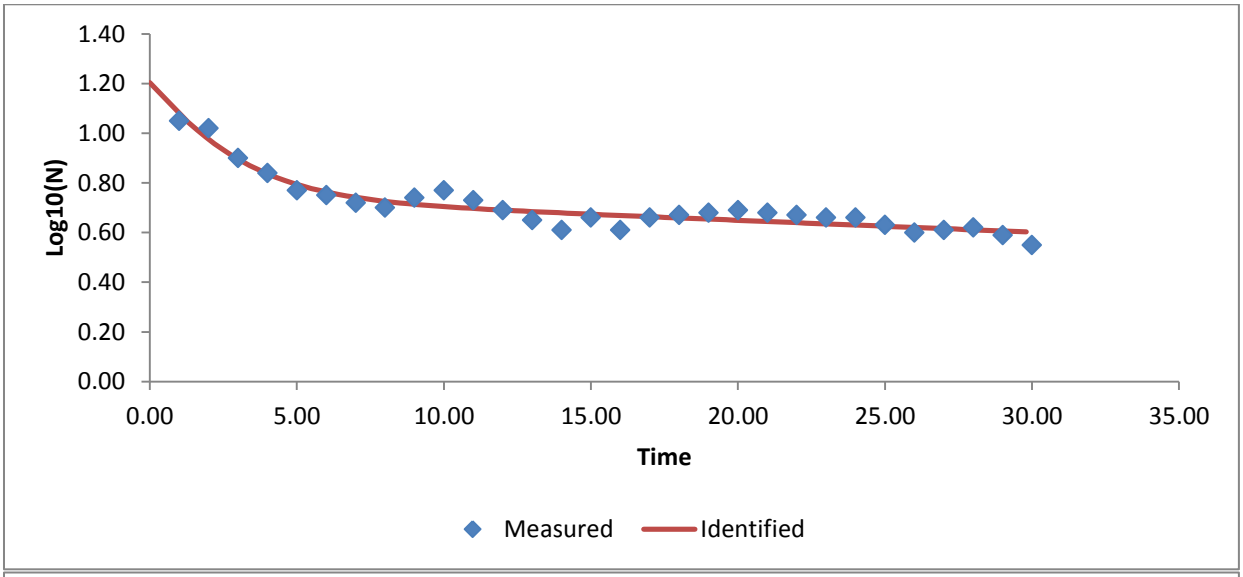
Decay of *E.coli* on the inner part of spiked cabbage modeled with log linear + shoulder (top), weibull, and log linear regression (bottom)

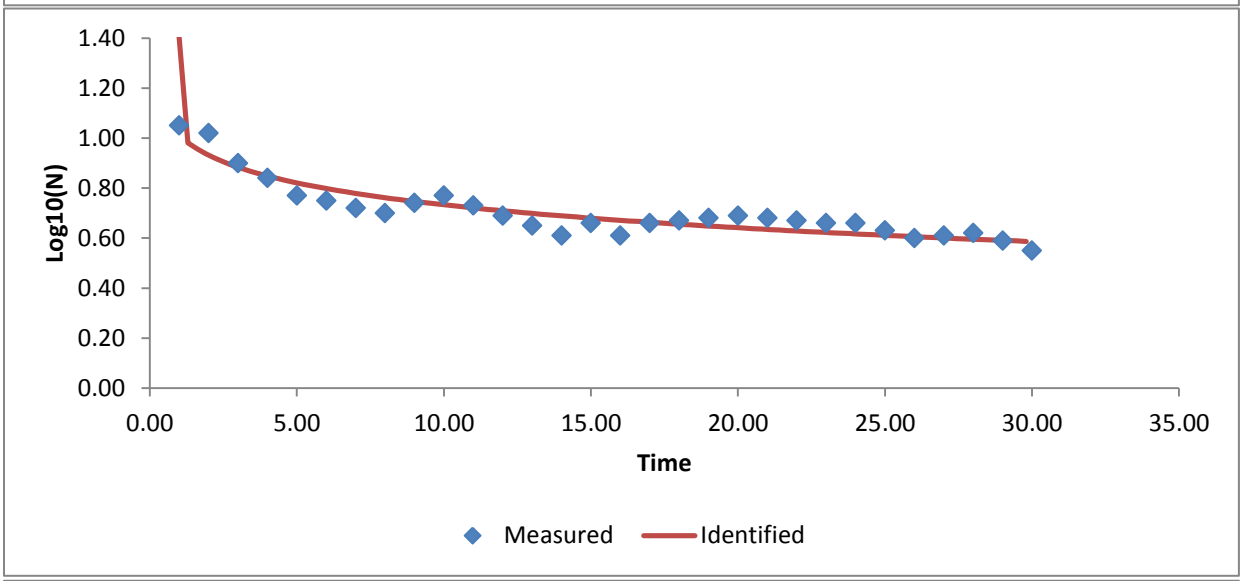


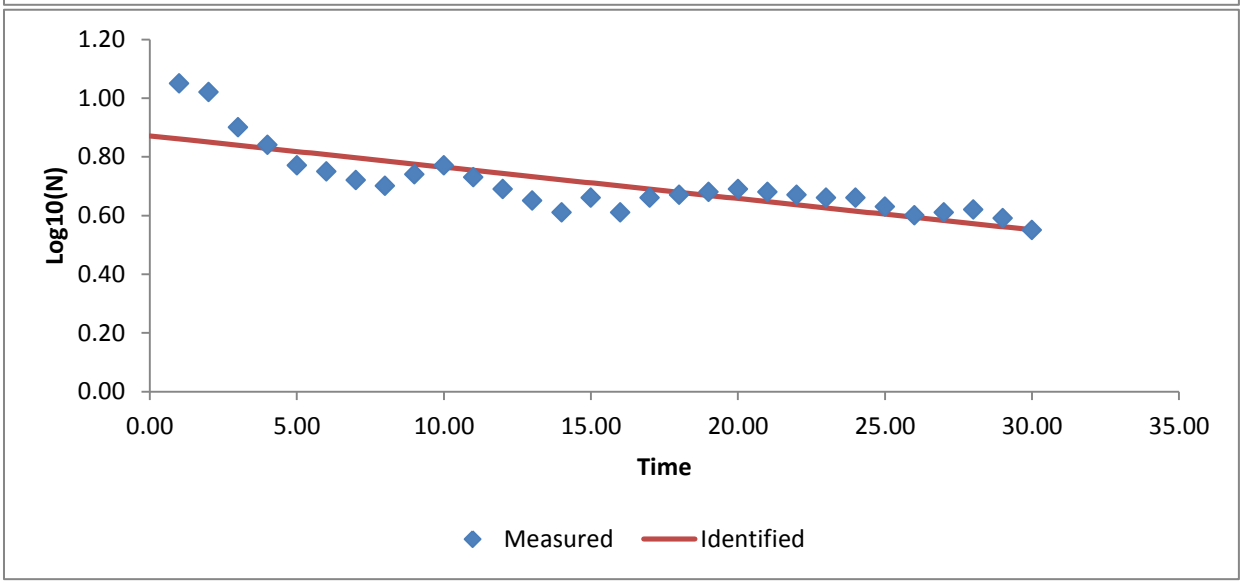
Decay of *E.coli* on inner part of control cabbage modeled with double Weibull (top), Weibull, and log linear regression (bottom).



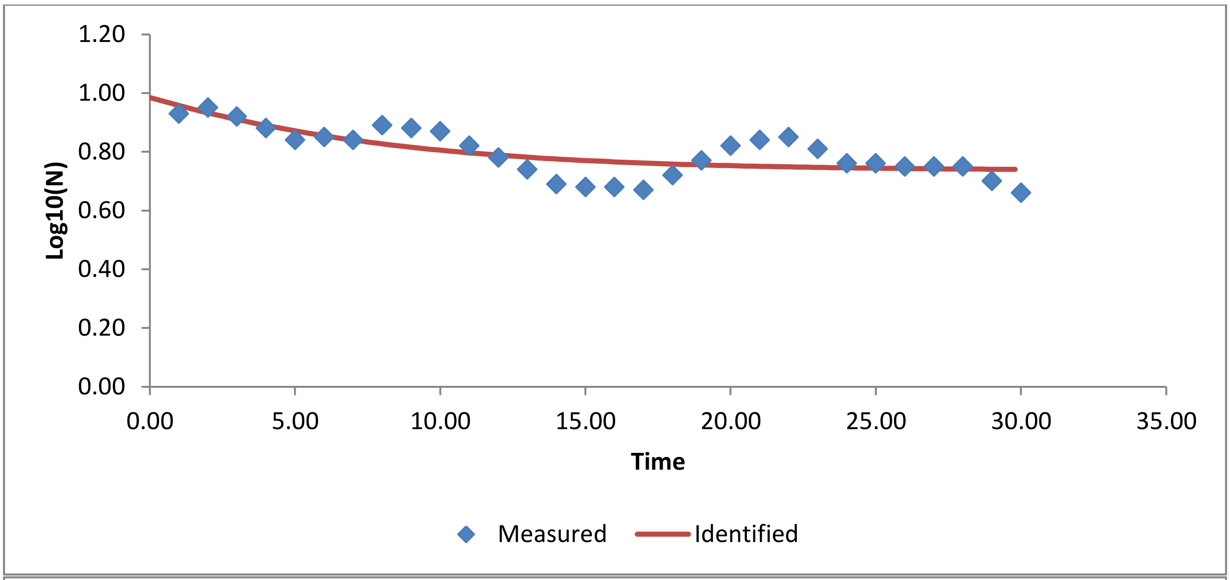
Decay of *Ascaris suum* on spiked lettuce modeled with biphasic model (top), Weibull, and log linear regression (bottom).

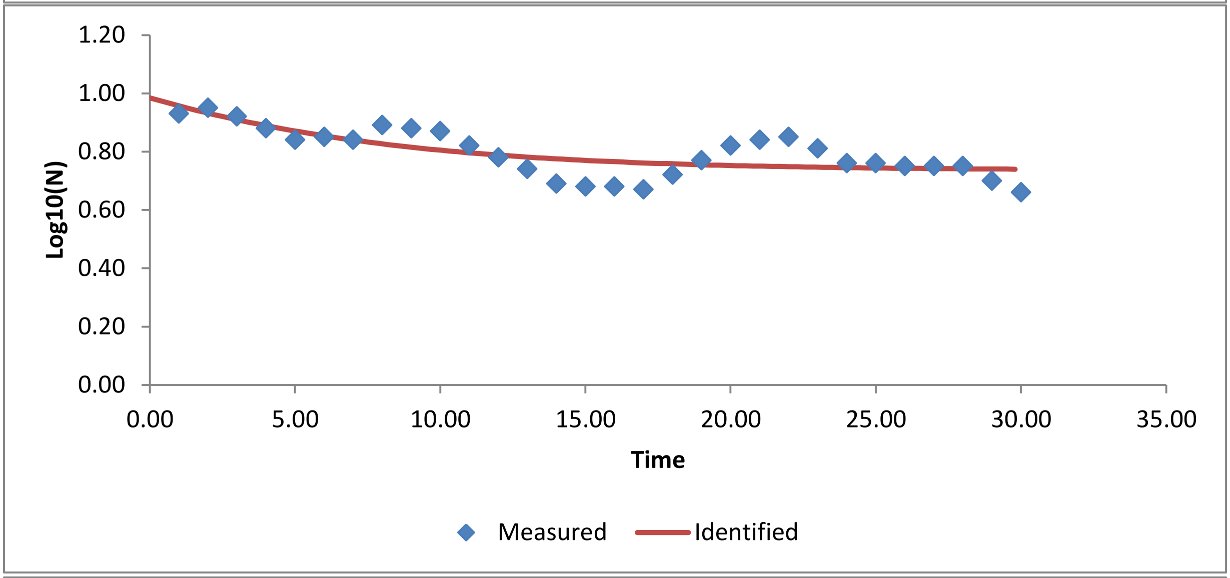


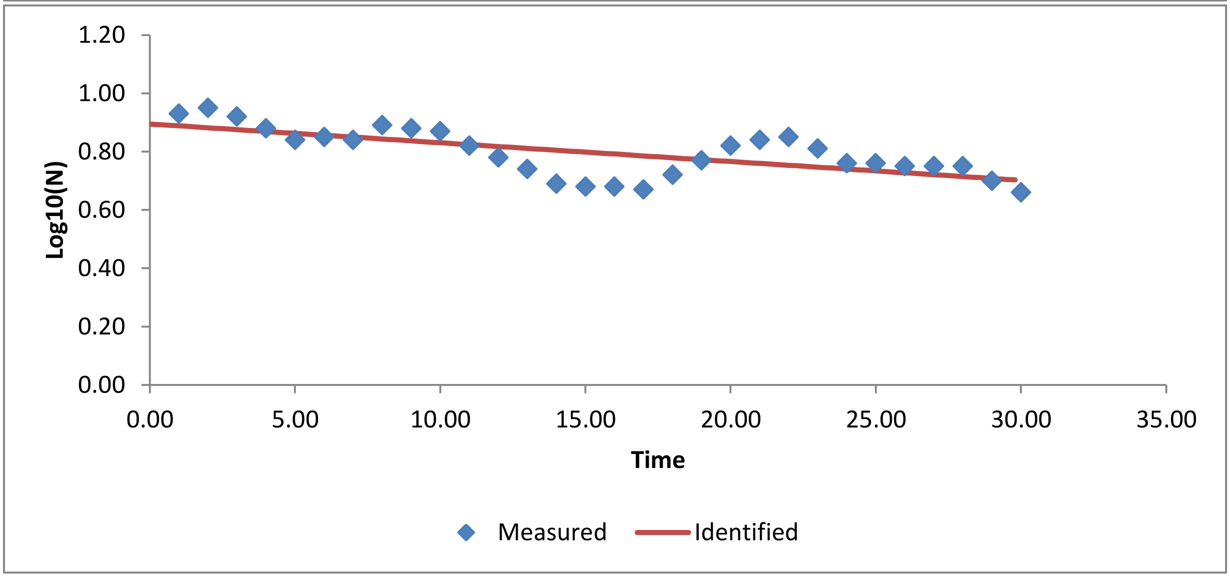




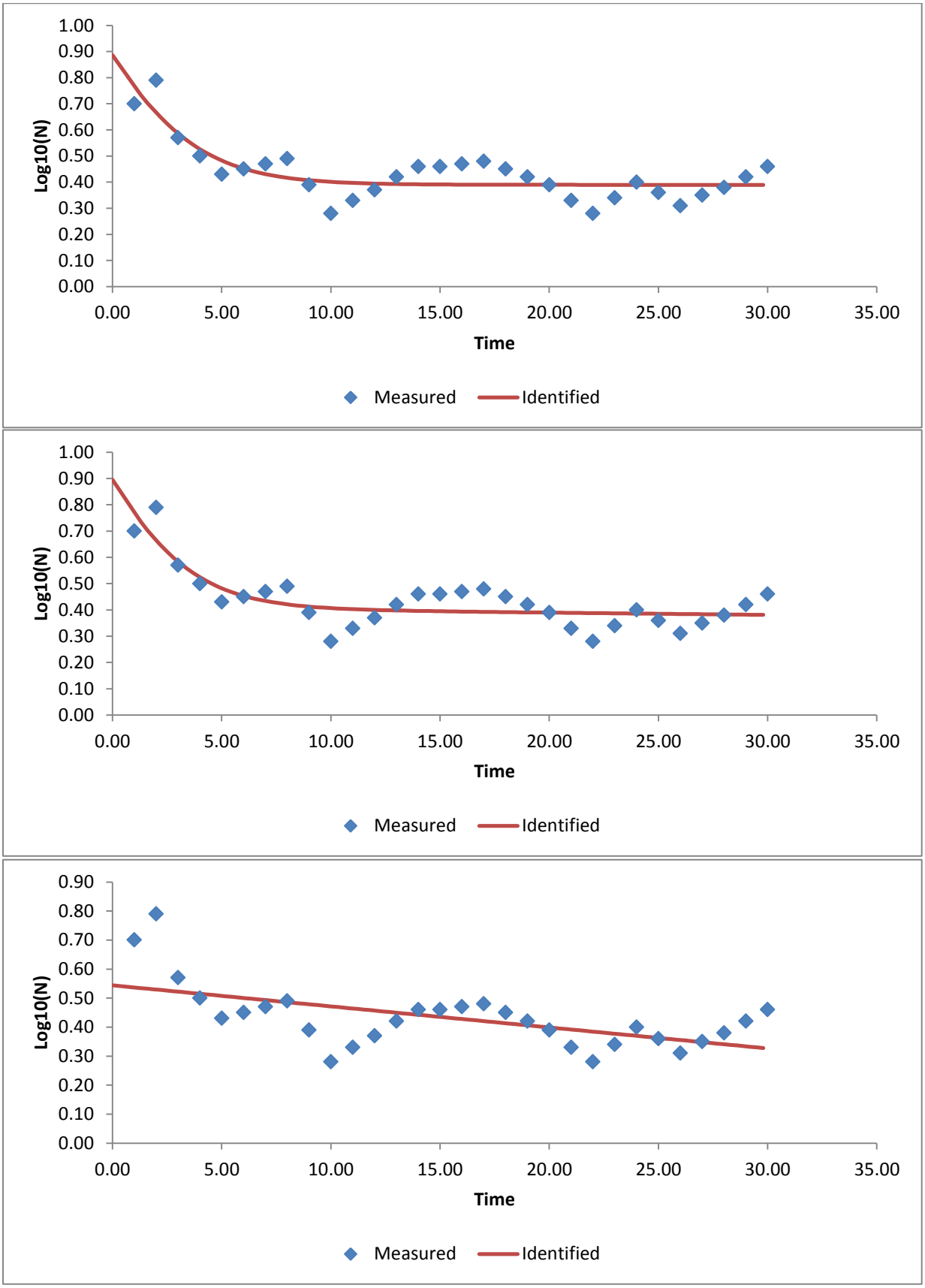
Decay of *Ascaris suum* on control lettuce modeled with log linear + tail, biphasic, and log linear regression (bottom).



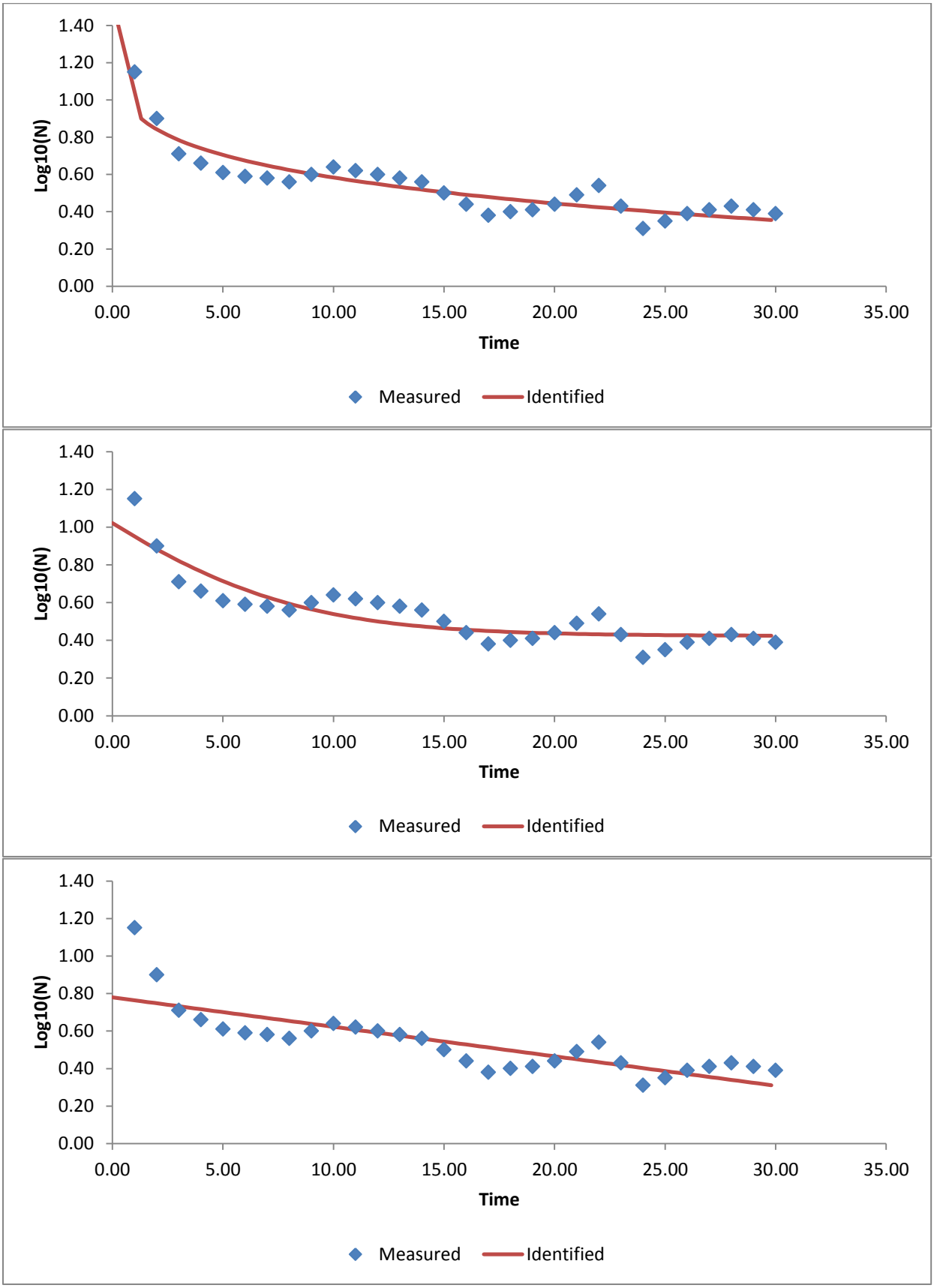




Decay of *Ascaris suum* on spiked cabbage modeled with Log linear + tail (top), biphasic, and log linear regression (bottom).



Decay of *Ascaris suum* on control cabbage modeled with double Weibull (top), log linear + tail, and log linear regression (bottom).



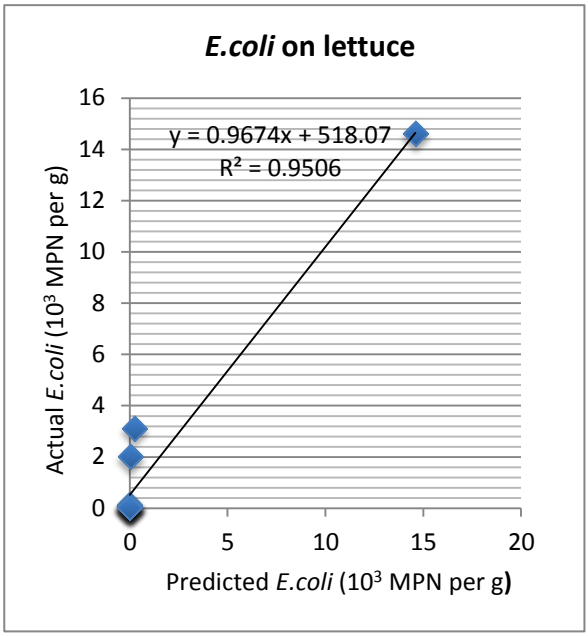
Appendix C – Comparison of results in trial 1 and 2

Because of few data points and linearity, all the curves during the first five days were best fitted with log-linear regression.

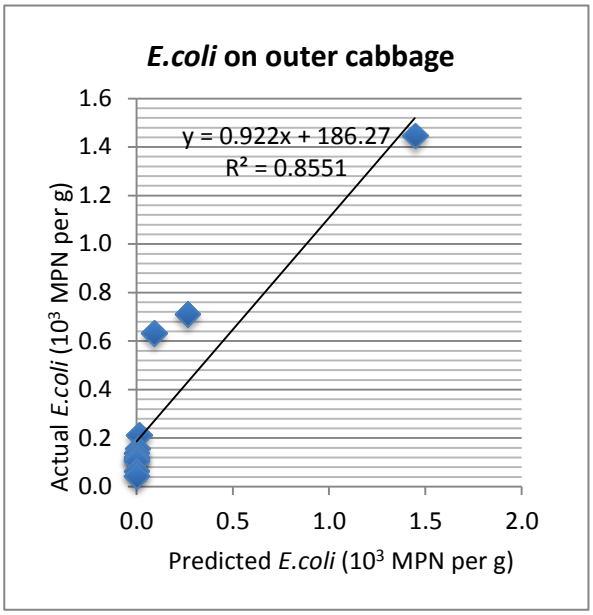
Vegetable	**Kmax in trial 1 (first five days)**	**Kmax in trial 2**
Lettuce	1.68 day^{-1}	1.54 day^{-1}
Inner cabbage	0.10 day^{-1}	0.19 day^{-1}
Outer cabbage	0.84 day^{-1}	0.79 day^{-1}

Appendix D - Validation regression charts and graphs

E.coli- validation of biphasic model on control lettuce		
Day	Predicted (N)	Actual (N0)
0	14600.00	14600
1	248.31	3100
2	23.38	2000
3	0.56	100
4	0.35	83.2
5	0.24	70.8
6	0.16	58.9
7	0.05	21.9
8	0.03	18.6
9	0.02	17.8
10	0.02	15.8

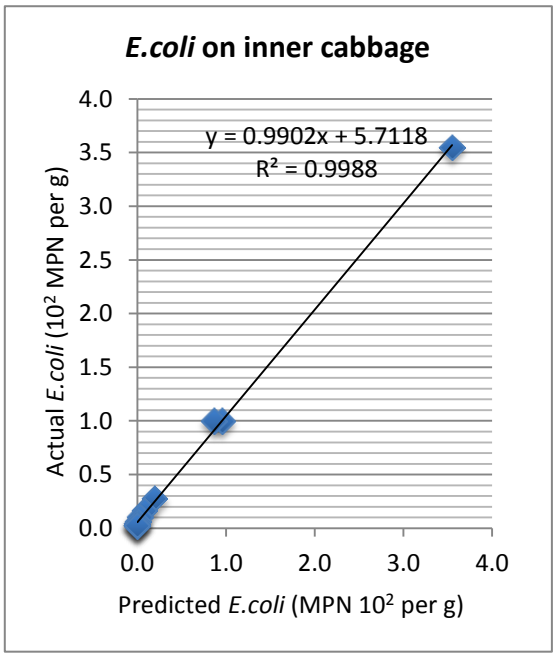


E.coli- validation of biphasic model on control cabbage (outer)		
Day	Predicted (N)	Actual (N0)
0	1445.44	1445.44
1	264.13	707.95
2	91.45	630.96
3	12.42	208.93
4	4.03	154.88
5	1.65	134.90
6	0.74	120.23
7	0.36	112.20
8	0.19	104.71
9	0.06	64.57
10	0.02	42.66



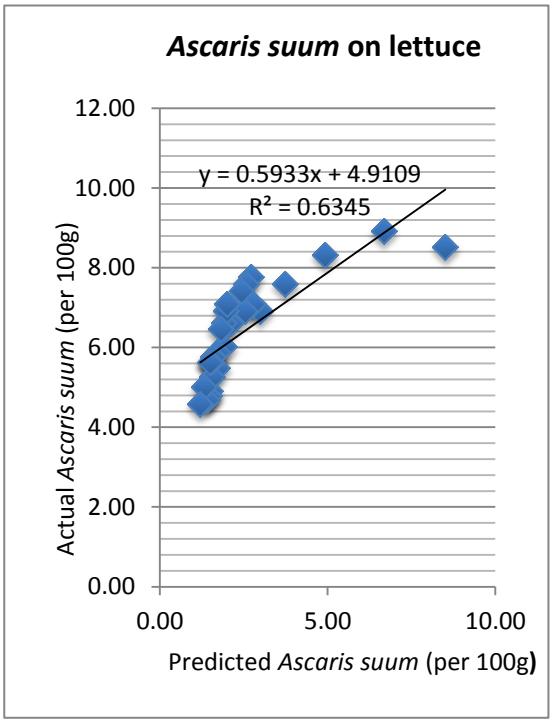
E.coli- validation of log linear + shoulder model on control cabbage (inner)

Day	Predicted (N)	Actual (N0)
0	354.81	354.81
1	95.47	100.00
2	86.57	100.00
3	19.68	27.54
4	8.49	16.60
5	3.18	10.23
6	1.04	6.31
7	0.34	4.27
8	0.09	2.40
9	0.04	2.29
10	0.02	2.51



Ascaris suum - validation of biphasic model on control lettuce

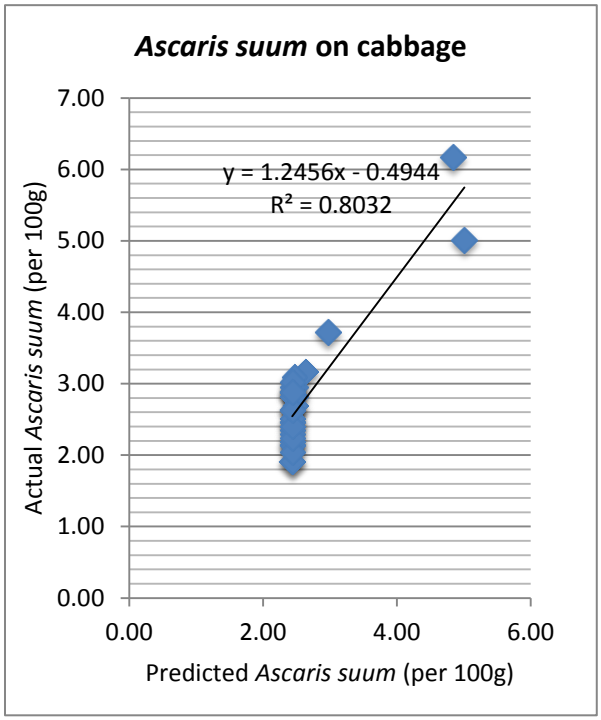
Day	Predicted (N)	Actual (N0)
0	8.51	8.51
1	6.67	8.91
2	4.92	8.32
3	3.74	7.59
4	2.99	6.92
5	2.77	7.08
6	2.53	6.92
7	2.71	7.76
8	2.56	7.59
9	2.44	7.41
10	2.13	6.61
11	1.91	6.03
12	1.72	5.50
13	1.51	4.90
14	1.46	4.79
15	1.44	4.79
16	1.40	4.68
17	1.55	5.25
18	1.72	5.89
19	1.91	6.61
20	1.98	6.92
21	2.01	7.08
22	1.81	6.46



23	1.60	5.75
24	1.58	5.75
25	1.53	5.62
26	1.52	5.62
27	1.50	5.62
28	1.33	5.01
29	1.20	4.57

Ascaris suum - validation of log-linear + tail model on control cabbage

Day	Predicted (N)	Actual (N0)
0	5.01	5.01
1	4.84	6.17
2	2.97	3.72
3	2.64	3.16
4	2.49	2.69
5	2.49	2.82
6	2.49	2.95
7	2.48	3.09
8	2.45	2.45
9	2.44	1.91
10	2.45	2.14
11	2.45	2.34
12	2.45	2.63
13	2.45	2.88
14	2.45	2.88
15	2.45	2.95
16	2.45	3.02
17	2.45	2.82
18	2.45	2.63
19	2.45	2.45
20	2.45	2.14
21	2.45	1.91
22	2.45	2.19
23	2.45	2.51
24	2.45	2.29
25	2.45	2.04
26	2.45	2.24
27	2.45	2.40
28	2.45	2.63
29	2.45	2.88



Appendix E – Measurement of rain catchment and temperature

Rain catchment

Date	February 28th	March 4th	March 6th
Rain catchment	4 mm/m^2	3.5 mm/m^2	44 mm/m^2

Temperature

Date	28.Feb	29.Feb	01.Mar	02.Mar	03.Mar	04.Mar
Temperature	30.6^0C	29.1^0C	31.4^0C	30.3^0C	31.6^0C	30.8^0C

Date	05.Mar	06.Mar	07.Mar	08.Mar	09.Mar	**Average**
Temperature	30^0C	31.5^0C	29.3^0C	31.7^0C	31.1^0C	**30.7^0C**

Appendix F – Daily values *E.coli* and *Ascaris suum* on control vegetables

Contamination of *E.coli* (MPN per g) on control vegetables.

Days of cessation	Lettuce	Outer cabbage	Inner cabbage
0	14600	1445,44	354,81
1	3100	707,95	100,00
2	2000	630,96	100,00
3	100	208,93	27,54
4	83,2	154,88	16,60
5	70,8	134,90	10,23
6	58,9	120,23	6,31
7	21,9	112,20	4,27
8	18,6	104,71	2,40
9	17,8	64,57	2,29
10	15,8	42,66	2,51

Contaminationof *Ascaris suum* (eggs per 100g) on control vegetables.

Days of cessation	Lettuce	Cabbage
0	8,51	5,01
1	8,91	6,17
2	8,32	3,72
3	7,59	3,16
4	6,92	2,69
5	7,08	2,82
6	6,92	2,95
7	7,76	3,09
8	7,59	2,45
9	7,41	1,91
10	6,61	2,14
11	6,03	2,34
12	5,50	2,63
13	4,90	2,88
14	4,79	2,88
15	4,79	2,95
16	4,68	3,02
17	5,25	2,82
18	5,89	2,63
19	6,61	2,45
20	6,92	2,14
21	7,08	1,91
22	6,46	2,19
23	5,75	2,51
24	5,75	2,29
25	5,62	2,04
26	5,62	2,24
27	5,62	2,40
28	5,01	2,63
29	4,57	2,88