Microbial Status of Irrigation Water for Vegetables as Affected by Cultural Practices

Agronomic Aspects

Mehboob Alam

Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Biosystems and Technology
Alnarp

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Cover: Irrigation water pond (upper left) used during field study, photocatalytic unit (upper right) used for water decontamination at field, rocket (lower left) grown for greenhouse experiments and SEM image (lower right) showing bacterial cells in leaf crevices.
(Photos by: Mehboob Alam and Kerstin Brismar)

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Abstract

Human pathogens present in irrigation water can be transmitted to plants. Consumption of fruits and vegetables irrigated with pathogen-contaminated water can cause illness in humans. Leafy vegetables that are consumed fresh are particularly prone to cause such illnesses. Understanding the microbiota of irrigation water and its decontamination and introducing some preventative pre-harvest cultural practices can help procure hygienically safe horticultural produce.

Variations were found in water indicator organisms, including heterotrophic plate counts, total coliforms, thermotolerant coliforms, *Escherichia coli* and faecal enterococci, at five different sampling sites in an irrigation water distribution system (IWDS) on a commercial vegetable-growing farm. 454-pyrosequencing data showed that the IWDS bacterial community was dominated by *Bacteriodetes* and *Proteobacteria*, with classes within these phyla, including *Flavobacteriia*, *Sphingobacteriia, α-, β- and γ-Proteobacteria*, being found at all five sampling sites. The genera *Arcicella*, *Flavobacterium*, *Limnohabitans*, *Sejongia*, *Fluviicola*, *Escherichia*, *Clostridium* and *Legionella* were present at various sites. Indicator organisms and the pathogen *Salmonella* in the IWDS were significantly reduced by photocatalytic treatment in most cases.

Pre-harvest cultural practices to reduce pathogen load, including cessation of irrigation with contaminated water three days before harvest and decreasing the water regime of the growing medium for leafy vegetables, were assessed. The results showed that an attenuated *gfp*-tagged *E. coli* O157:H7 decreased with increasing time to harvest after cessation of irrigation, but were present in the plant phyllosphere three days after cessation, irrespective of dose applied. Similarly, both attenuated *gfp*-tagged *E. coli* O157:H7 and an attenuated strain of *L. monocytogenes* persisted in vegetables grown at a reduced water regime in the growing medium. Total microbiota and *Enterobacteriaceae* remained unchanged on plants after cessation of irrigation with contaminated water and on plants grown on different water regimes. Use of contaminated irrigation water for leafy vegetable production should thus be avoided. Photocatalytic treatment can be used to decontaminate irrigation water.

Keywords: decontamination, food safety, human pathogens, irrigation water hygiene, pre-harvest cultural practices, rocket, spinach, Swiss chard

Author's address: Mehboob Alam, SLU, Department of Biosystems and Technology, Microbial Horticulture Unit, P.O. Box 103, SE-230 53 Alnarp, Sweden
E-mail: Mehboob.Alam@slu.se

Dedication

To my family, teachers and friends

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Alsanius, B.W., **Alam, M.**, Ahlström, C., Sylla, J., Rosberg, A-K., Olsson, C., Mogren, L., Ahrné, S., Molin, G. & Jensén, P. Microbial community structure of the free water phase in a field irrigation system. (provisionally accepted for publication in *Science of the Total Environment*).

II **Alam, M.**, Ahlström, C., Rosberg, A-K., Mogren, L., Ahrné, S., Molin, G., Jensén, P. & Alsanius, B.W. Impact of photocatalysis on microbial decontamination of irrigation water. (manuscript).

III **Alam, M.**, Ahlström, C., Burleigh, S., Olsson, C., Ahrné, S., El-Mogy, M., Molin, G., Jensén, P., Hultberg, M. & Alsanius, B.W. (2014). Prevalence of *Escherichia coli* O157:H7 on spinach and rocket as affected by inoculum and time to harvest. *Scientia Horticulturae* 165, 235-241.

IV **Alam, M.**, Mogren, L., Ahrné, S., Molin, G., Jensén, P., Boqvist, S., Vågsholm, I. & Alsanius, B.W. Does growing medium water regime affect *Escherichia coli* O157:H7 and *Listeria monocytogenes* occurrence on leafy vegetables? (manuscript).

The contribution of Mehboob Alam to the papers included in this thesis was as follows:

I Participated in the field and laboratory part of the experiments together with co-authors and participated in writing and commenting on the manuscript.

II Participated in planning and performing the field and laboratory experiments together with co-authors and participated in data analysis and writing the manuscript with other co-authors.

III Planned and performed the greenhouse and laboratory experiments together with co-authors and evaluated the data and wrote the manuscript together with co-authors.

IV Planned and performed most of the experimental work in the greenhouse and laboratory together with co-authors and evaluated the data and participated in the writing of the manuscript together with co-authors.

Abbreviations

$^{\bullet}OH$	Hydroxyl radical
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAB	Blood agar base
bp	Base pair
CO_2	Carbon dioxide
ddNTP	dideoxynucleotides
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotides
FAO	Food and Agriculture Organisation
FE	Faecal enterococci
gs	Stomata gaseous conductance
HPC	Heterotrophic plate count
IWDS	Irrigation water distribution system
LB	Luria-Bertani broth
NaCl	Sodium chloride
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PAST	Palaeontological statistics software package
PCR	Polymerase chain reaction
PPi	Pyrophosphate
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
TC	Total coliform bacteria
TiO_2	Titanium dioxide
TSA	Tryptic soy agar
TTC	Thermotolerant coliform bacteria
UV	Ultraviolet

VRBD	Violet red bile dextrose agar
vwc	Volumetric water content
WHO	World Health Organisation

1 Background

Irrigation is an essential step in crop production in areas with deficient or sporadic rainfall and therefore millions of hectares are irrigated world-wide for food production. As high quality irrigation water is becoming scarce, the risk of outbreaks of foodborne illnesses due to consumption of crops irrigated with contaminated water is increasing (Miraglia *et al.*, 2009; Klonsky, 2006). Verotoxin-producing *Escherichia coli*, *Salmonella* spp. and *Listeria* spp. are some of the prominent foodborne pathogens that can be transferred via irrigation water to plant surfaces (Liu *et al.*, 2013; Beuchat, 1996b). Most of the foodborne pathogens linked with fresh produce consumption are associated with gastrointestinal diseases. Decontamination of irrigation water is highly recommended for hygienically safe agricultural production. It has been found that the pathogens can survive for varying periods on the plant surface depending on environmental conditions such as temperature, nutrient availability, humidity and UV radiation (Brandl, 2006).

The main objectives of this thesis were to investigate (i) the bacterial community structure in the free water phase of an irrigation water distribution system (IWDS) on a commercial vegetable-growing farm and (ii) to assess the impact of cultural management on irrigation water quality, by photocatalytic treatment and on the plant phyllosphere colonisation of selected human pathogens by cessation of irrigation with contaminated water before harvest and growing plants using different water regimes in the growing medium.

2 Introduction

2.1 Irrigation water

Water used to replace or supplement precipitation in the production of crops is called irrigation water (Hargreaves & Merkley, 1998). Irrigation of crops is an important and long-used practice to increase agricultural and horticultural production. According to the Food and Agriculture Organisation (FAO), the drinking water requirement for one person is 2-4 L d^{-1}, but to produce one person's daily food takes 2000-5000 L of water. It requires almost 1000-3000 L of water to produce 1 kg of rice and 13 000-15 000 L to produce 1 kg of grain-fed beef (FAO, 2010; Pimentel *et al.*, 1997). Demand for irrigation water is increasing (Shiklomanov, 1998) and in 2000 almost 274 million hectares of agricultural land were irrigated world-wide, which is about 16% of the total cultivated area on Earth (Sieberta *et al.*, 2006). In Sweden, during 2003 an area of almost 53,000 ha was under irrigation (Wriedt *et al.*, 2008).

2.1.1 Sources of irrigation water

In many cases irrigation water is stored in a source, natural or artificial, prior to use. Fresh water that can be used for irrigation and which is accessible to humans comprises less than 1% of the Earth's total water resources (Zia *et al.*, 2013). There are different sources of irrigation water, including rainwater, groundwater, surface water and untreated or treated wastewater.

Rainwater

Rainwater use is considered the easiest method of crop production (Li *et al.*, 2000). 'Rainwater harvesting' is a term used for collecting and storing rainwater in man-made reservoirs (Makoto, 1999; Prinz, 1999) for subsequent use for irrigation of crops.

Groundwater

Groundwater can be accessed through wells and springs. Groundwater is comparatively hygienically safer than surface water for crop production (Ayers & Westcot, 1985).

Surface water

In general, various surface water sources can be utilised for crop irrigation (Winter *et al.*, 1999). Surface water is ultimately hydraulically connected to groundwater, but it can become contaminated with the addition of wastewater, stormwater and agricultural run-off, which in many cases contain loads of pathogens (Winter *et al.*, 1999).

Wastewater

Lack of freshwater for irrigation has forced growers to utilise any type of available water, including wastewater, and around 20 million hectares (7% of all irrigated land) are irrigated with different types of wastewater (Scott *et al.*, 2004). Wastewater use in the developing countries has increased because it contains ample amounts of nutrients and is a reliable source of water supply (Hussain *et al.*, 2001).

2.1.2 Irrigation methods

Application of water to plants can be through different means or irrigation methods. There are two main types of irrigation method, surface irrigation and localised irrigation (Cuenca, 1989). An easy way of crop irrigation is through surface irrigation, in which water flows under gravity without pumping. Surface irrigation can be performed as furrow, flood or border strip irrigation and the water is not applied directly to the plant canopy, so the plant phyllosphere cannot be directly contaminated if unhygienic water is used (Solomon *et al.*, 2002).

When water is applied to each plant with the help of connected pipes, this is called localised irrigation (Vermeiren & Jobling, 1983). With this irrigation method, water can be supplied through drip irrigation (water is applied to the root zone of each plant), spray or micro-sprinkler irrigation (water is supplied directly to the plant canopy) or bubbler irrigation (water is applied in low quantities to the soil adjacent to plants) (Frenken, 2005). Micro-irrigation of crops can apply the required water either directly to the plant canopy or to the root zone, improving the quality and quantity of the produce. Vegetables are mostly irrigated with localised irrigation systems, and therefore in this thesis a sprinkler irrigation system was used in experiments with leafy vegetables (Papers III and IV).

In areas with a high groundwater level, a sub-irrigation method can be utilised in which water is raised by pumps and pipes to open ditches or underground conduits and is made available through capillary force to the plant root zone (Smajstrla *et al.*, 1991).

2.1.3 Water circuit

Open irrigation systems result in larger water losses due to insufficient control (mainly evaporation and technical faults in the distribution system) (Rivas *et al.*, 2007). For example, in Zimbabwe 50% of water is lost through evaporation during surface irrigation of the traditional irrigated gardens (Batchelor *et al.*, 1996). Therefore, installation of a water circuit is important for improving the efficacy in IWDS and 10-50% water can be saved (Postel, 1992).

The hygiene quality of IWDS is affected by the microbiological status of the water source, the complex environment, nutrient availability, microbial interactions and accumulation of sediment, which can contain vibrant microbial communities important for food safety (Pachepsky *et al.*, 2012). The use of partly treated or untreated wastewater for irrigation increases the risk of microbes occurring in the water delivery system. Research indicates that these microorganisms can then persist in the water circuit within biofilms (Yan *et al.*, 2009; LeChevallier *et al.*, 1987). Pathogen survival and growth in the water system is affected by various environmental factors as well as nutrient availability, microbial interactions, pipe material, system hydraulics, use of disinfectants and residuals, and sediment accumulation, with carbon accumulation in particular acting as a limiting factor (Pachepsky *et al.*, 2011; USEPA, 2002).

Pathogenic microorganisms have been found in the water remaining in pipes between irrigation events (Pachepsky *et al.*, 2012; Juhna *et al.*, 2007). In a study in the USA on membrane bioreactor treatment plants, regrowth of pathogens, including *Legionella* and *Aeromonas*, has been reported (Jjemba *et al.*, 2010). These microbes mix with irrigation water passing through irrigation systems and may reach the plant surface. Flushing of the irrigation system is one way to decrease the risk of microbial contamination in pipes (Pachepsky *et al.*, 2012). With advances in technology, water circuit irrigation systems have been adopted for many crops, including vegetables. This thesis focused on an IWDS used for irrigating vegetables at commercial level and evaluated the microbial community in this system. For decontamination of the irrigation water, a prototype photocatalytic treatment unit installed in the IWDS was evaluated (Papers I and II).

2.2 Pathogens in irrigation water

A wide range of microbial pathogens have been found in water and can be transferred to crops during irrigation. Okafo (2003) recovered *E. coli*, *Salmonella* spp. and *Vibrio* spp. from irrigation stream water in Nigeria and found that these microbes were also present on the irrigated plants. Some possible routes for contamination of irrigation water are shown in Figure 1. Aquatic plants and sediments can help pathogen survival in the open irrigation system, whereas in case of pipe-based irrigation systems pathogens can survive through biofilms. Survival of pathogens in the water and surrounding environment is mainly dependent on factors such as nutrient availability, temperature, organic matter content, competition with other microorganisms, pH and radiation (Pachepsky *et al.*, 2011). It has been shown that *E. coli* can survive for up to 300 days in autoclaved, filtered river water at 4 °C (Flint, 1987). Use of contaminated water for irrigation of crops is considered to be responsible for several outbreaks of disease following consumption of such crops (Beuchat & Ryu, 1997).

Bottom sediment could be one of the major reservoirs of pathogenic microorganisms as it provides nutrient availability and protection from UV sunlight (Burton *et al.*, 1987; Lewis *et al.*, 1986). For example, Pachepsky *et al.* (2011) showed that faecal coliforms are multiple-fold higher in sediments than in the water column. Therefore, to obtain maximum decontamination in water treatment, it is recommended that the total suspended solids (TSS) content be reduced before treatment (Rose *et al.*, 1996).

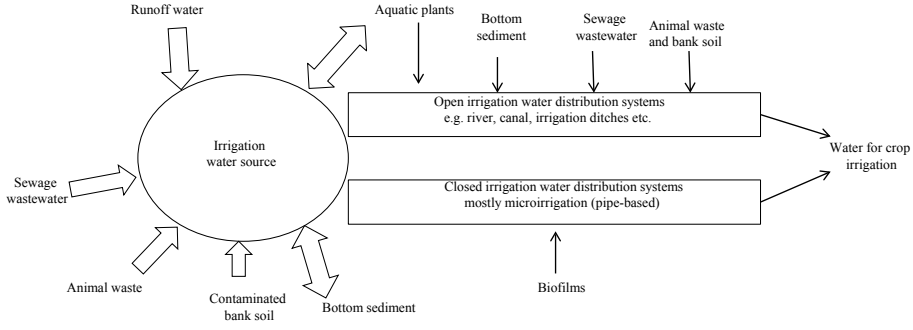

Figure 1. Potential factors affecting the microbiological quality of irrigation water sources and irrigation water distribution systems. Modified from Pachepsky *et al.*(2011), reprinted with kind permission from Elsevier.

In a water circuit, complex environment, good nutrient availability, microbial interactions, sediment accumulation and protection from UV light are some of

the favourable conditions for pathogen survival (Pachepsky *et al.*, 2012). Biofilms can be formed in the water circuit by certain pathogens in order to persist and survive (LeChevallier *et al.*, 1987). Important pathogens including *E. coli* O157:H7, *Salmonella, Listeria monocytogenes, Cryptosporidium oocysts, Vibrio* spp. and *Yersinia* spp. have been found in different irrigation systems (Pachepsky *et al.*, 2012; Wilkes *et al.*, 2009; Doyle, 1990). These can subsequently mix with the irrigation water and may reach the plant surface. Therefore, in this thesis the pathogens *E. coli* O157:H7 and *Listeria monocytogenes* were analysed in order to determine their prevalence in the phyllosphere of leafy vegetables, as affected by pre-harvest cultural practices (Papers III and IV).

2.2.1 Water indicator organisms

For water quality assessment, heterotrophic plate counts (HPC) at 22 ºC, total coliforms (TC), faecal (thermotolerant) coliform bacteria (TTC), *Escherichia coli* (*E. coli*) and faecal enterococci (FE) are normally used (DIN-19650, 1999). These indicator organisms are not necessarily pathogenic, but indicate possible contamination of the water by different pathogens. Heterotrophic plate counts indicate the general pollution state, consisting of all aerobic microorganisms, including yeasts and moulds. For water hygiene standards, measurements of TC and 'faecal coliform' organisms are often used in combination (Blumenthal *et al.*, 2000). The group TC includes Gram-negative, non-spore forming, rod-shaped bacteria, comprising the genera *Escherichia*, *Citrobacter*, *Enterobacter* and *Klebsiella*. These indicate the general sanitary level of water. The TTC include the genera *Escherichia* and *Klebsiella* and indicate the level of faecal contamination (Paruch & Mæhlum, 2012). Faecal coliforms are broadly equivalent to 'thermotolerant coliforms'. These and *E. coli* indicate short-term faecal contamination, with *E. coli* being the faecal indicator bacterium and a comparatively more reliable and consistent predictor of illness (Paruch & Mæhlum, 2012; Edberg *et al.*, 2000). The FE, or faecal streptococci, are not a taxonomic-systematic class of microorganisms, but mainly comprise species belonging to *Enterococcus* (*E. avium*, *E. durans*, *E. faecalis*, *E. faecium*) and some streptococci (*S. bovis*, *S. equinus*) (Leclerc *et al.*, 1996). They are Gram-positive and survive for a long time in water, and are therefore used as indicators for long-term faecal contamination (Pourcher *et al.*, 1991). In this thesis, all these water indicators were used to assess the microbial quality of irrigation in an IWDS and water decontamination by photocatalysis (Papers I and II).

2.2.2 Guidelines for using irrigation water

For hygienically safe agricultural production, the World Health Organisation (WHO) and various countries have established guidelines for using irrigation water. The experiments described in this thesis were performed with leafy vegetables (Papers III and IV), which are mostly consumed raw, and therefore the guidelines presented below are for raw crops. Recommendations on sampling frequency of irrigation water vary between different guidelines from daily sampling to five times a month (Jamieson *et al.*, 2002), once a month (Strang, 2010), or once a year (Pachepsky *et al.*, 2011). The United States Food and Drug Administration (USFDA) guidelines recommend site-specific analysis for the specific crop, pathogen, irrigation system, water source and management practice/s (Pachepsky *et al.*, 2011). General recommendations for water sampling frequency include using the geometric mean from five weekly measurements or five sampling events per month (Pachepsky *et al.*, 2011; British Columbia Ministry of Environment, 2001).

According to the guidelines in British Columbia, Canada, the geometric mean of five sampling events per month for various indicator organisms in irrigation water should be: faecal coliforms <200 CFU 100 mL^{-1}, *E. coli* <77 CFU 100 mL^{-1} and faecal *Enterococci* <20 CFU 100 mL^{-1} (British Columbia Ministry of Environment, 2001).

In the guidelines set by Alberta, Canada, the geometric mean of five sampling events per month for indicator organisms should be: total coliforms <1000 CFU 100 mL^{-1}, *E. coli* <200 CFU 100 mL^{-1} and *Enterococci* <35 CFU 100 mL^{-1} (Alberta Environment, 1999).

According to DIN 19650 (1999) there are four classes of irrigation water. For crops consumed raw, specifications for the classes EK-1 and EK-2 should be observed (DIN-19650, 1999). In EK-1, the water quality is the same as drinking water quality and no *E. coli* or faecal streptococci should be present in the water. In class EK-2, the guidelines recommend that *E. coli* should be $\leq$200 CFU 100 mL^{-1} and faecal streptococci should be $\leq$100 CFU 100 mL^{-1}. DIN 19650 (1999) also recommends no *Salmonella* presence in 1000-mL samples of irrigation water and no potentially pathogenic stages of human parasites.

2.3 Human pathogens and vegetables

Routes of transmission of human pathogens through vegetables are summarised in Figure 2. One major cause of vegetable contamination could be the unavailability of hygienic irrigation water. Pathogens can be transmitted to vegetables and cause outbreaks of illnesses when these are consumed (EFSA, 2013). A wide range of pathogens can be transferred to plants via irrigation

water, surviving several days both on external and internal parts of the plant (Islam *et al.*, 2004). In many cases pathogens have the ability to enter into the plant tissues through wound surfaces and stomatal cavities (Barker-Reid *et al.*, 2009; Gomes *et al.*, 2009; Aruscavage *et al.*, 2008). In the plant tissues, pathogens are protected from various disinfection treatments and UV-light and have ample amounts of nutrients available (Heaton & Jones, 2008). Experiments on human pathogen population dynamics and survival, applied with irrigation water either in the plant canopy or by the root system, have given differing results, so it is difficult to make general statements on pathogen survival and their populations on plants (Berger *et al.*, 2010).

Leafy vegetables are normally irrigated near harvest to increase their market value, so they can be responsible for a large proportion of foodborne illnesses if contaminated irrigation water is used (EFSA, 2013; Harris *et al.*, 2003). Irrigation can also lead to a humid microenvironment in the plant canopy and result in better survival of pathogens (Dreux *et al.*, 2007). Studies have reported high numbers of pathogenic infections, especially diarrhoea, due to consuming uncooked vegetables irrigated with contaminated water (Harris *et al.*, 2003).

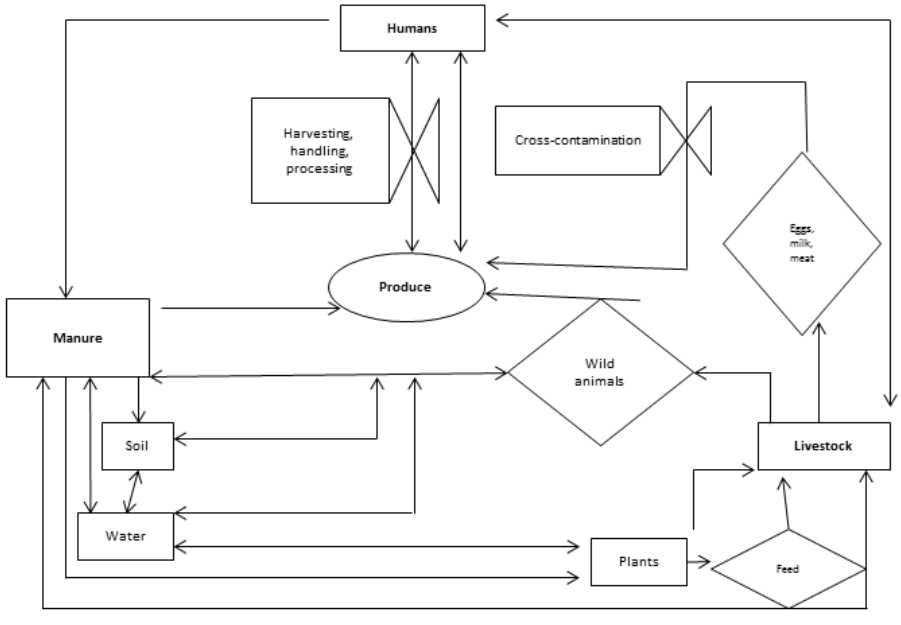

Figure 2. Field crop contamination by human pathogens via different sources. Adapted from (Köpke *et al.*, 2007; Beuchat, 1996b), reprinted with the kind permission of Woodhead Publishing Limited.

Escherichia coli O157:H7 and *L. monocytogenes* are among the dominant causal agents of certain food illnesses transmitted through fresh fruits and vegetables (EFSA, 2013; Brackett, 2001). The infective dose of both bacteria is low (Ramaswamy *et al.*, 2007; Ackers *et al.*, 1998). The two pathogens are compared in Table 1. Both have been found in surface water (Wilkes *et al.*, 2009) and can be carried to the plant phyllosphere via irrigation water (Steele & Odumeru, 2004). Both *E. coli* O157:H7 and *L. monocytogenes* have been documented in numerous disease outbreaks linked to fruits and vegetables world-wide (EFSA, 2013). An *E. coli* O157 outbreak in Sweden was attributed to consumption of fresh lettuce irrigated with contaminated water (Söderström *et al.*, 2008). In many cases the initial concentration of pathogens in irrigation water is critical for produce contamination, but Pachepsky *et al.* (2011) concluded that the concentration in irrigation water may not necessarily be the dominant factor if the microorganism is able to internalise in produce or colonise it.

The plant phyllosphere can be considered a hostile environment for enteric pathogens. This environment is typically characterised by fluctuating temperatures, inconsistent nutrient availability, competition with resident microbiota, UV-light and water activity (Heaton & Jones, 2008; Cooley *et al.*, 2006). Therefore, human pathogens (outside their host) are considered not to be part of the phyllosphere. However, as evidenced from the outbreaks of foodborne illness, these pathogens can be considered capable of adapting to phyllosphere conditions (Berger *et al.*, 2010). Studies suggest that bacterial processes, including gene expression, motility and extracellular compound production, can be important in colonisation and survival in the phyllosphere (Aruscavage *et al.*, 2006; Solomon & Matthews, 2006). It has been shown that plants have defence mechanisms against undesired bacterial proliferation, *e.g.* the plant hormone ethylene can inhibit certain plant pathogens, but there are no such reports about plant defence against human pathogens. One possible reason could be that since plants do not recognise human pathogens as potentially harmful, they do not prevent their colonisation. Therefore, human pathogens can exist as a part of the plant phyllosphere (Berger *et al.*, 2010).

Table 1. *Comparison of the human pathogens* Escherichia coli *O157:H7 and* Listeria monocytogenes

	E. coli O157:H7	*L. monocytogenes*
Category	Human pathogen	Human pathogen
Family	*Enterobacteriaceae*	*Listeriaceae*
Gram test	Gram-negative	Gram-positive
Transmission to human	Contaminated food	Contaminated food
Habitat	Intestine of warm-blooded animals	Plants, soil, animal faeces
Impact on humans	Bloody diarrhoea, homolytic uraemic syndrome, kidney failure	Listeriosis, gastroenteritis, miscarriage (newborn mortality)

Both *E. coli* O157:H7 and *L. monocytogenes* have been found to colonise plant surfaces and, depending on environmental factors and nutrient availability, both can survive for long periods on plants (Islam *et al.*, 2004; Beuchat, 1996a). The abiotic factors required for the growth of both pathogens are shown in Table 2. Takeuchi *et al.* (2000) observed that *E. coli* O157:H7 can attach better to surfaces and the cut edge of lettuce leaves than *L. monocytogenes*. Pathogen colonisation in the plant phyllosphere is mainly dependent on moisture and nutrients and bacteria need active motility or simple diffusion for colonisation (Cooley *et al.*, 2003). The colonisation may advance to microbial aggregate formation, especially near stomatal depressions and intercellular junctions, and thus pathogens can protect themselves from adverse environmental conditions, as well as post-harvest sanitisation treatments (Heaton & Jones, 2008). Internalisation within the plant and aggregate formation in the plant phyllosphere are considered to be potential factors for long-term survival of human pathogens on plants (Heaton & Jones, 2008).

It has been indicated that environmental factors affect the contamination of leafy vegetables mainly during pre-harvest (Liu *et al.*, 2013). Studies have shown that environmental conditions, particularly temperature increases and precipitation pattern changes, can affect the survival of human pathogens on leafy vegetables (Liu *et al.*, 2013). Plant characteristics, *e.g.* leaf water content, nutrient content, antioxidants and leaf morphology, may affect the phyllosphere microbiota. Leaf physiology and morphology may also affect the development of microbial populations in the phyllosphere and it is possible that certain spots on the leaf surfaces that are suitable for microbial growth can develop. Pre-harvest cultural practices that can alter leaf morphology and physiology, and consequently the prevalence of human pathogens on the leaf surfaces, could be exploited in order to prevent proliferation of human pathogens.

As water of good hygienic quality is a scarce commodity, pre-harvest cultural management strategies are important to consider. The main focus of this thesis was on adopting irrigation water-related cultural practices, namely (i) decontamination of irrigation water before application to vegetables; (ii) cessation of irrigation to reduce moisture on the plant surface (Keraita *et al.*, 2007) and (iii) reducing the moisture content of the growing medium to develop dry conditions on the leaf surface. These practices may be helpful in reducing the load of pathogenic microorganisms in the irrigation water, as well as in the plant phyllosphere.

Table 2. *Abiotic factors required for growth of* Escherichia coli *O157:H7 and* Listeria monocytogenes.

		E. coli O157:H7	*L. monocytogenes*
pH	Minimum	4.4	4.4
	Optimum	6-7	7
	Maximum	9.0	9.4
Temperature (°C)	Minimum	7-8	1.5
	Optimum	37	37
	Maximum	46	45
Water activity (a_w)	Minimum	0.950	0.920
	Optimum	0.995	-
	Maximum	-	-

2.4 Irrigation water hygiene

The hygiene quality of irrigation water and crops can be assured either by supplying pathogen-free water or by disinfecting water before it reaches the plants. Possible water disinfection treatments include heat treatment or pasteurisation, filtration, UV irradiation, chlorination, ozonation (Newman, 2004), waste stabilisation, use of sedimentation ponds, waste storage or filtration through sand and soil (Keraita *et al.*, 2010; Mara & Silva, 1986). All these have been shown to decrease the levels of microorganisms in irrigation water.

Water decontamination can be achieved through physical, chemical or biological methods. Every treatment system has its own advantages and disadvantages. Use of chlorine for water disinfection is an old and relatively inexpensive technique with a high oxidising potential and the chlorine used can be in different forms, *e.g.* chlorine gas, hypochlorite and chlorine dioxide (Newman, 2004). Chlorine dioxide is very effective in killing bacteria and viruses, but it is very unstable and needs to be produced at the site of

application. Chlorine exists as hypochlorous acid and hypochlorite in water and can react with organic matter in water to create mutagenic and carcinogenic products (Nieuwenhuijsen *et al.*, 2000). Furthermore, it has been observed that in many cases chlorine treatment fails to inactivate oocysts of *Cryptosporidium parvum* (Korich *et al*., 1990; Peeters *et al*., 1989). Ozonation of water is also an effective disinfectant treatment. Ozone can lyse microbial cell membranes due to its highly oxidative properties. However, ozone releases some byproducts that may be deleterious to humans (Glaze & Weinberg, 1993; Haag & Hoigne, 1983). Hydrogen peroxide is an unstable, strong oxidiser that can inactivate the cell membrane of microorganisms. It has been found to be useful against fungi, bacteria and algae, and can therefore be used for disinfection of irrigation water (Glaze *et al.*, 1987).

Water filtration is a very useful method to remove microbes, especially protozoan oocysts and helminth eggs (Landa *et al.*, 1997). As the water passes through a porous granular medium, microbes are removed. Filtration is a simple and relatively safe method, as there is no danger of chemicals forming. However, water filtration normally requires large land areas and environmental factors can sometimes affect the efficacy of the system (Huisman & Wood, 1974). Wetlands are an appropriate low-cost technology for inactivation of water microbes (Greenway, 2005). Constructed wetlands are suitable for pathogen removal through physical, chemical and biological processes (Greenway, 2005; Zdragas *et al.*, 2002; Davies & Bavor, 2000). For example, wetlands are able to remove faecal coliforms, *Enterococci* and the total bacterial load from water (Greenway, 2005; Bolton & Greenway, 1999).

Ultraviolet light in the form of UV-C (λ=254 nm) can be used against microorganisms, resulting in DNA and RNA damage and inactivation. A UV dose of 330 mJ cm^{-2} can completely inactivate faecal coliforms, faecal streptococci and *E. coli* (Caretti & Lubello, 2003). However, some microorganisms are resistant to UV radiation, *e.g*. *Enterobacter cloacae* (Ibáñez *et al.*, 2003). In recent years, semiconductor photocatalytic processes that work on the basis of active oxidative treatment have been developed for water disinfection. Hydroxyl (•OH) radicals generated during this process can be used to inactivate bacteria and viruses. UV-radiation (λ<385 nm) is normally used to activate the semiconductor, which in many cases is titanium dioxide (TiO_2), to form free hydroxyl •OH radicals (Hoffmann *et al.*, 1995). The radicals can degrade microbial cell walls, which leads to the release of essential cell components, resulting in microbial death (Kiwi & Nadtochenko, 2005). In this process, TiO_2 can be used either in powder form or fixed in lines with a UV-lamp (Malato *et al.*, 2007). Gram-positive and Gram-negative bacteria, yeast and algae can be inactivated using metal halide lamp irradiation

and TiO_2 (Matsunaga *et al.*, 1985). The photocatalytic unit can be installed online in the IWDS and hence no water storage is required. The water decontamination experiment described in this thesis (Paper II) used a prototype photocatalytic unit that was installed directly in the IWDS.

2.5 Microbial analysis

Culture-dependent methods represent only 0.1-3% of the total microbiota within the community (Whipps *et al.*, 2008). Thus although most standardised laboratory procedures for describing water quality are based on culture-dependent techniques using semi-selective media and enrichment, the majority of the microorganisms inhabiting water and the phyllosphere cannot be cultured on standard laboratory media. Therefore, culture-independent techniques can make it possible to identify the unexploited constituents of the microbial community. Water-related microbial communities can be assessed through DNA-based techniques (Bernhard & Field, 2000; Toze, 1999). Important DNA-based techniques include gene cloning and sequencing (Díez *et al.*, 2001), denaturing gradient gel electrophoresis (Emtiazi *et al.*, 2004; Araya *et al.*, 2003), terminal restriction fragment length polymorphism (Bernhard & Field, 2000), and a recently developed next generation sequencing (NGS) technique, 454 pyrosequencing (Douterelo *et al.*, 2013; Telias *et al.*, 2011). Microbial DNA is very stable in the environment and therefore can persist for extended periods after cell death (Josephson *et al.*, 1993; Novitsky, 1986). Thus the DNA-based techniques sequence both viable and dead members of the community and the results obtained do not discriminate between living and dead organisms.

In bacteria, 16S rRNA genes can be used as phylogenetic markers to assess the microbial community and phylogenetic information on the dominant members of the community can be generated by sequencing the 16S rRNA genes obtained through PCR (Osborn *et al.*, 2000). Basically, RNA-based microbial community analysis provides information on active members of the community (Griffiths *et al.*, 2000). Recent advances in sequencing techniques have made it possible to assess microbial communities in detail.

The Sanger sequencing method works by utilising 16S rRNA gene amplification, followed by cloning and the chain termination method with dye-labelled dideoxynucleotides (ddNTPs) (Siqueira Jr *et al.*, 2012). This method can generate sequence reads with a length of around 1000 bp (Luo *et al.*, 2012), and has been used for investigation of bacterial communities for several years (Siqueira Jr *et al.*, 2012). However, the high cost of cloning and subsequent sequencing with the Sanger sequencing method make it difficult to

examine a large number of clones from a large number of samples (Siqueira Jr *et al.*, 2012). Therefore, a recently developed next generation sequencing (NGS) technique that can generate high throughput sequences has been adopted for large-scale bacterial community analyses. The five most widely used NGS technologies at present are 454-pyrosequencing, Illumina, SOLiD, the HeliScope Single Molecule Sequencer and Single Molecule Real Time technology. All can perform massive parallel sequencing (Siqueira Jr *et al.*, 2012). The NGS techniques are usually employed for metagenomic studies of complex microbial communities (Luo *et al.*, 2012).

Of the NGS techniques, the most commonly used are the pyrosequencing methods (Metzker, 2009). Pyrosequencing provides a large number of sequence reads in a single run and thus allows microbial communities to be studied in depth (Edwards *et al.*, 2006). This technology is a sequencing-by-synthesis method (Siqueira Jr *et al.*, 2012), in which the isolated DNA is bound to small beads. An oil-water emulsion polymerase chain reaction (PCR) is performed and DNA is amplified on beads containing oligonucleotide primers. A million copies of a specific DNA template are generated on each bead. These beads, which contain the enzymes that are subsequently used in the pyrosequencing reaction steps, are deposited in picotitre wells (Mardis, 2008). A mixture of the single-stranded DNA template, sequencing primer, DNA polymerase, ATP sulfurylase, luciferase and apyrase helps in the pyrosequencing reaction. The four deoxynucleotides (dNTPs) are added to the pyrosequencing reaction. If a nucleotide is incorporated into a sequence, a phosphodiester bond between the dNTPs is formed, releasing pyrophosphate (PPi) in a quantity equivalent to the amount of nucleotide incorporated. This is followed by conversion of PPi to adenosine triphosphate (ATP). The ATP helps conversion of luciferin to oxyluciferin, which emits light in an amount proportional to the amount of ATP used. The emitted light is detected and the sequence can be determined by repeated incorporation of the complementary nucleotide and light emission (Siqueira Jr *et al.*, 2012).

The Illumina/Solexa sequencing technology is also based on the sequencing-by-synthesis method. It works on the principle of dye terminator nucleotides incorporated into the sequence by a DNA polymerase similar to that in the Sanger sequencing method (Siqueira Jr *et al.*, 2012). In Illumina, a flow cell surface is used for the immobilisation of DNA fragments, followed by bridge PCR for amplification (Shendure & Ji, 2008).

The sequence read length generated by most of the NGS technologies is shorter than that needed for identification of bacterial gene length (Luo *et al.*, 2012). Therefore bacterial identification using these methods has focused primarily on hypervariable regions of the 16S rRNA gene (Huse *et al.*, 2008).

Hypervariable regions (V1-V6) have been commonly used for microbial identification (Siqueira Jr *et al.*, 2012). The read length of Illumina is about 100 bp (Siqueira Jr *et al.*, 2012) and is not suitable for bacterial identification. The 454-pyrosequencing technology has progressed over time and the recently developed GS FLX+ can generate read lengths up to 1000 bp (454 LifeSciences, 2014; Luo *et al.*, 2012), which can be utilised for identification of microorganisms in an environment. This technique has been used for exploring microbial communities in different environments (Douterelo *et al.*, 2013; Petrosino *et al.*, 2009; Edwards *et al.*, 2006).

In this thesis, both culture-dependent and culture-independent (454-pyrosequencing) techniques were used, to assess indicator organisms and general bacterial microbiota, respectively, in the free water phase of a field irrigation system (Papers I and II).

3 Objectives

The main aims of this thesis were to find ways to increase the food safety of irrigated leafy vegetables and to identify cultural practices to minimise the prevalence of human pathogens on fresh produce.

Specific objectives were to:

- Identify the dominant bacterial microbiota in a commercial irrigation water distribution system (IWDS) (Paper I).
- Explore the efficacy of photocatalysis in decontaminating irrigation water (Paper II).
- Investigate the population of introduced human pathogens as affected by cessation of irrigation before harvest of leafy vegetables (Paper III).
- Investigate the effect of water regime of the growing medium on introduced human pathogens on leafy vegetables (Paper IV).

The starting hypotheses in Papers I-IV were as follows:

(i) The microbial community structure within the IWDS changes during irrigation events (Paper I).

(ii) The microbial community varies at different sampling sites within the IWDS (Paper I).

(iii) A photocatalytic unit installed in the IWDS can improve the water microbial hygiene quality (Paper II).

(iv) The prevalence of *E. coli* O157:H7 decreases with increasing time interval between irrigation and harvest (Paper III).

(v) The decline in *E. coli* O157:H7 inoculated into the phyllosphere is a function of its concentration applied through irrigation water (Paper III).

(vi) Different water regimes applied to the growing medium can affect the occurrence of human pathogenic bacteria on leafy vegetables (Paper IV).

4 Materials and Methods

Issues related to the microbial quality of irrigation water were investigated from two perspectives: (i) microbial community structure and water quality indicators in the free water phase in the IWDS, including the effects of decontamination (Papers I and II) and (ii) the impact of contaminated irrigation water on pathogen occurrence on leafy vegetables, as affected by cultural practices (Papers III and IV). Water samples were collected from the IWDS on a commercial vegetable-growing farm in southern Sweden (Papers I and II). Experiments on the persistence of *gfp*-tagged *E. coli* O157:H7 (Papers III and IV) and *L. monocytogenes* (Paper IV) on leafy vegetables were carried out in the greenhouse, to exclude background contamination and to comply with Swedish legislation. Table 3 shows the irrigation water parameters used in the two approaches.

Table 3. *Irrigation water parameters used in field and greenhouse experiments*

	Field (Papers I, II)	**Greenhouse (Papers III, IV)**
Water source	Pond	Potable water
Water pH	7.7	8.3
Water electrical conductivity (mS cm^{-1})	0.7	0.18
Water temperature (°C)	10-20	15-18
Decontamination method	Photocatalysis (Paper II)	Filtration &mild chlorination

4.1 Field experiments

4.1.1 Water sample collection (Papers I, II)

Irrigation water samples at the commercial vegetable-growing farm were collected from the IWDS. Water collected in a pond and originating from a nearby stream, surface run-off and rainfall was pumped through the IWDS

(Figure 3). The water was pre-filtered (50 µm polyester cartridge filter, Harmsco) before entering the field pipeline.

A prototype photocatalytic unit (Wallenius Water M900BE 160 W) was mounted on the irrigation ramp. In this photocatalytic unit, TiO_2 was fixed with the UV-radiation lamp and installed online in the IWDS. Triplicate water samples were collected at five sampling sites on three and five occasions during 2009 and 2011, respectively. Water samples were collected from (i) the pond, (ii) after coarse filtration, (iii) at the start of the field water pipe and (iv) at the end of the field water pipe for analyses of the microbial community structure of the free water phase in the IWDS during 2011 (see Paper I). Decontamination was studied at the end of the IWDS and included water samples from (i) the pond, (iv) the end of the field water pipe (before photocatalytic unit) and (v) after treatment with the photocatalytic unit during 2009 and 2011 (Paper II). All water samples were immediately cooled and brought to the laboratory within 1.5 h of sampling for further analysis.

4.2 Greenhouse experiment

The experimental procedures used in the two greenhouse experiments are summarised in Figure 4.

4.2.1 Plant material (Papers III, IV)

For the experiments described in Paper III, seeds of spinach (*Spinacia oleracea* L. cv. Island) and rocket (*Diplotaxis tenuifolia* L. cv. Grazia) treated with metalaxyl-M/thiram/thiophanate-methyl (Seminis, Oxnard CA, USA) were sown in trays (0.52 m x 0.42 m x 0.09 m) at a rate of about 400 seeds per tray. The trays were filled with three layers of growing medium: a 1-cm bottom layer of sand (particle size 0.2-1 mm), a 4.5-cm middle layer of fertilised peat-based growing medium (K-soil) and a 2.5-cm top layer of peat-based growing medium (S-soil), both from Hasselfors Garden AB, Örebro, Sweden. For the experiments described in Paper IV, spinach and rocket seeds were also used as described above and, in addition, Swiss chard (*Beta vulgaris* L. cv. Bull's blood) seeds were used. The trays in this case were filled with two layers of growing medium: a 4.5-cm bottom layer of fertilised peat-based growing medium (K-soil) and a 1.5-cm top layer of peat-based growing medium (S-soil). The seeds in this experiment were sown at a density of 0.10 g, 4.76 g and 4.50 g per tray for rocket, spinach and Swiss chard, respectively.

The trays were placed in the experimental greenhouse (21$\pm$2 °C, relative humidity 60-80% and at least 12 h light d^{-1}) (Figure 5). For inoculation with attenuated strains of human pathogens, the plant trays were transferred to a

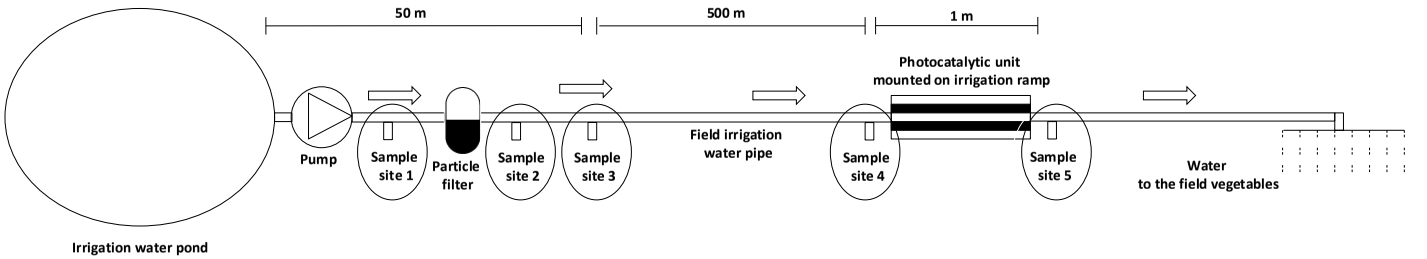

Figure 3. Water samples collected from an irrigation water distribution system on a commercial farm growing vegetables. Samples were collected from (i) the pond, (ii) after a pre-filter, (iii) at the start of the field pipe and (iv) after the field pipeline, on five occasions during 2011 (Paper I). Water samples were collected from (i) the pond, (iv) after the field pipe (before the photocatalytic unit) and (v) after the photocatalytic unit on three and five occasions during 2009 and 2011, respectively (Paper II).

greenhouse section approved for experiments with genetically modified organisms (REK 2011/1072; ID202100-2817v28) and kept under the same environmental conditions as described above.

The experiments in Paper III were repeated two times each, with three replicates for four harvest times and three inoculum densities and controls. In the experiments in Paper IV, five replicates were performed in trial one and three replicates in trial two, each divided for two water regimes, with parallel controls.

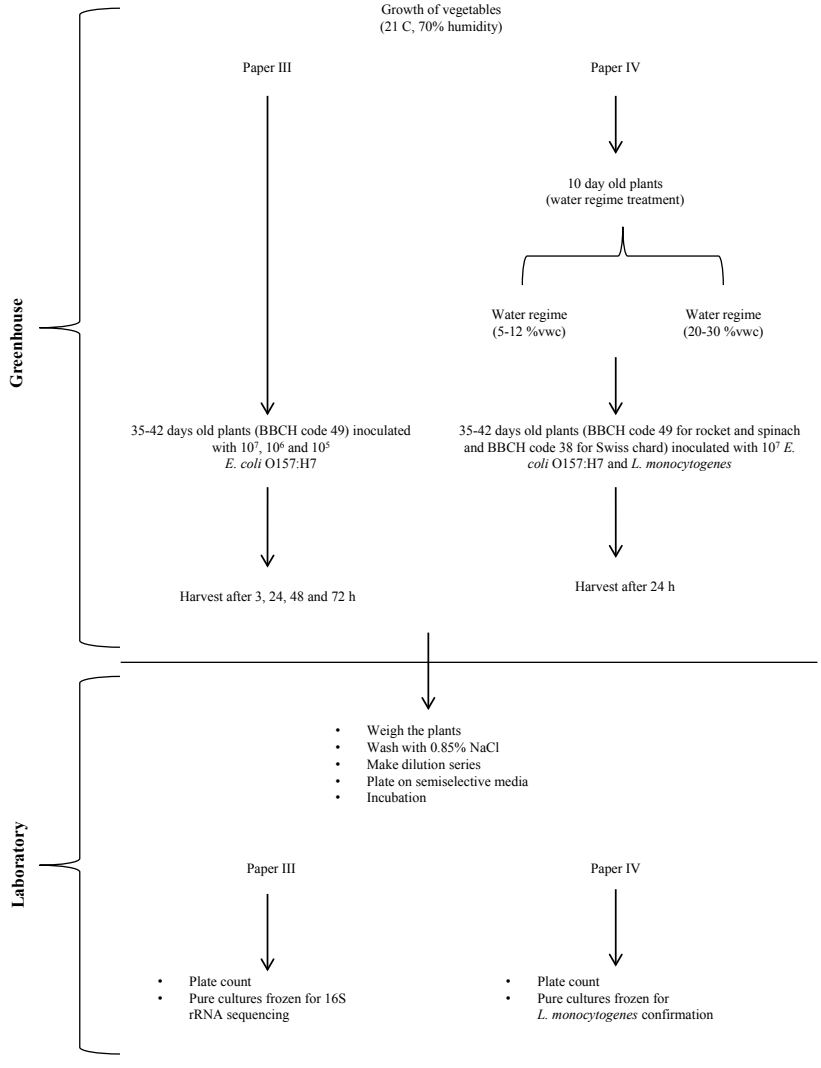

Figure 4. Greenhouse and laboratory procedures used in experiments on leafy vegetables (Papers III, IV).

4.2.2 Bacterial inoculum preparation and inoculation (Papers III, IV)

Bacterial strains of *E. coli* serotype O157:H7 (registered, E81186, verotoxin-1 and -2 absent and *eae*-gene present) for use in Papers III and IV were procured from the Swedish Institute for Communicable Disease Control, Solna, Sweden, and non-pathogenic *Listeria monocytogenes* for use in Paper IV from the National Veterinary Institute, Uppsala, Sweden.

The *E. coli* O157:H7 (*gfp*-tagged) cells were prepared as explained in Papers III and IV for the experiments using Luria-Bertani broth (LB, L3022-1kg, Sigma, Stockholm, Sweden), supplemented with 100 µg mL^{-1} ampicillin and 0.1% L-arabinose, solidified with 1.5% Bacto Agar (DIFCO 214010, DeMoines, USA) and incubated for 18 h at 37 °C. The cell density (OD_{620}) for *E. coli* O157:H7 was adjusted to 1.0 (Expert 96™ spectrophotometer, AsysHiTech, Eugendorf, Austria), corresponding to 10^9 CFU mL^{-1}. Preparation of *L. monocytogenes* cells for the experiment is described in detail in Paper IV. In brief, the cells were prepared using Blood Agar Base (BAB, OXOID, CM0055, Hampshire, England) supplemented with 200 µg mL^{-1} rifampicin and incubated (48 h, 26 °C). For *L. monocytogenes* the cell density was adjusted to (OD_{620}) 0.8, which corresponds to 10^9 CFU mL^{-1}.

A final density of *gfp*-tagged *E. coli* O157:H7 of 10^7, 10^6 or 10^5 CFU mL^{-1} was sprayed on leafy vegetables at a rate of 25 mL $tray^{-1}$ (see Paper III). Similarly, *gfp*-tagged *E. coli* O157:H7 and *L. monocytogenes* (10^7 CFU mL^{-1}) were sprayed at a rate of 25 mL $tray^{-1}$ (Paper IV).

Figure 5. Leafy vegetables (left: Swiss chard, right: spinach) grown in the greenhouse during the experiments on leafy vegetables inoculation with human pathogens. (Photo: Mehboob Alam).

After 35-42 days, (BBCH code 49 for rocket and spinach and BBCH code 38 for Swiss chard), the plants were transferred to the greenhouse section approved for experiments with genetically modified organisms and kept under the same environmental conditions as explained above. In the experiments in Paper III, 12 trays (rocket or spinach) per treatment were inoculated with *E. coli* O157:H7 suspension. In the experiments in Paper IV, plants of the same age (rocket, spinach and Swiss chard) were transferred to the greenhouse section as explained above and 10 plant trays during trial one and 6 trays during trial two were inoculated with *E. coli* O157:H7 or *L. monocytogenes* suspension. In the control treatments, the same numbers of trays containing plants were sprayed with an equivalent volume of sterile 0.085% NaCl solution. The plant trays then remained in the greenhouse until harvest. All plants from each tray were considered an individual replicate and were harvested 1.5 cm above the growing medium using sterile scissors and the material kept separately in plastic bags. In Paper III, the plants were harvested at 3, 24, 48 and 72 h after inoculation, each with three replicates per treatment. In Paper IV, the plants were harvested after 24 h of inoculation, with five and three individual replicates in trials one and two, respectively. The plastic bags of plant material were then brought to the Risk Class II laboratory for analysis.

4.3 Analyses

4.3.1 Growing medium water regime analyses (Paper IV)

Two water regimes were used in Paper IV to examine the effect of water regime in the growing medium on human pathogens on leafy vegetables. A Fieldscout TDR 300 device (Spectrum Technologies, Plainfield, Illinois, USA) was used for measuring the water regime of the growing medium. It was determined that 62% volumetric water content (vwc) was equivalent to 100% field capacity of the growing medium. Ten days after sowing the seeds, the trays were divided into two groups (five replicates in trial one and three replicates in trial two), one with a growing medium moisture content of 20-30% vwc (32% of field capacity) and the other with a growing medium moisture content of 5-12% vwc (16% of field capacity). This difference between the two treatments was maintained during the remainder of the experiment.

4.3.2 Plant analyses (Papers III,IV)

Plant fresh weight was determined immediately after harvest. For control treatments, leaf area (cm^2) was also determined (LI-3100 Area meter, LI-COR Inc., Lincoln, USA) (Papers III and IV). In Paper III, plant dry weight was

measured after five days of desiccation at 70 °C, while in Paper IV, plant dry weight was measured after freeze-drying. Leaf stomata gaseous conductance (gs) of CO_2 was measured with the help of gas exchange photosynthesis meter (LCpro, ACD Bioscientific, Hoddesdon, UK) to evaluate the effect of water regime (20-30% and 5-12% vwc) on this parameter (Paper IV).

4.3.3 Microbial analyses

Culture-dependent analyses

Assessment of the microbiological quality of irrigation water in Papers I and II was based on determination of indicator organisms in the water (see section 2.2.1), namely heterotrophic plate counts (HPC), total coliform bacteria (TC), thermotolerant coliform bacteria (TTC), *Escherichia coli* (*E. coli*), faecal enterococci (FE) and *Salmonella*. A detailed description of the procedure for enumeration of the indicator organisms can be found in Papers I and II. Table 4 summarises the semi-selective media used during the experiments.

In Papers III and IV, after harvest and fresh weight determination, aliquots of 100 mL sterile NaCl (0.85%) were added to the plant bags to wash off phyllosphere-associated microorganisms. The bags were then shaken by hand (208 rpm) for one minute and 50 mL aliquots of the suspension were poured into sterile tubes. A 10-fold dilution series was made using 0.85% NaCl and from a determined dilution series, 50 µL of the suspension were spread on semi-selective media, using a spiral plater (WASP2, Don Whitley Scientific Limited, Shipley, UK) to enumerate the strains introduced (*E. coli* and *L. monocytogenes*), *Enterobacteriaceae* and total aerobic counts from the phyllosphere, as explained in Papers III and IV. The specific incubation conditions used are listed in Table 4. Only plates with 30 to 300 colonies were considered for analysis.

Table 4. *Semi-selective media used for determination of irrigation water indicator organisms, including heterotrophic plate counts (HPC), total coliforms (TC), thermotolerant coliforms (TTC),* Escherichia coli (E. coli)*, faecal enterococci (FE) and* Salmonella *(Papers I, II); the bacterial strains (gfp-tagged* Escherichia coli *O157:H7 and* Listeria monocytogenes*) introduced into the plant phyllosphere;* Enterobacteriaceae*; and total aerobic counts in the phyllosphere (Papers III, IV).*

	Filter (0.45 µm)	Medium	Incubation time (h)	Temperature (°C)	Comments
Water indicator organisms					
HPC	-	Yeast peptone agar	68±4	22±2	(Papers I,II)
TC	+	Les endo agar	24±4	35±0.5	(Papers I,II)[a]
TTC	+	mFC agar supplemented with rosolic acid	24±4	44±0.5	(Papers I,II)[a]
E. coli	+	mFC agar supplemented with rosolic acid	24±4	44±0.5	(Papers I,II)[a]
FE	+	Slanetz-Bartley agar	44±4	35±1	(Papers I,II)[a]
Salmonella	+	Rappaport-Vassiliadis broth	16-20	37±1	(Papers I,II)[a]
Bacterial strains introduced to the vegetable phyllosphere					
E. coli O157:H7	-	Luria-Bertani (supp. with ampicillin and L-arabinose)	18	37	(Papers III,IV)[b]
L. monocytogenes	-	Blood agar base (supp. with rifampicin)	42	26	(Paper IV)[c]
Bacteria from the vegetable phyllosphere					
Enterobacteriaceae	-	Violet red bile dextrose agar	24	37	(Papers III,IV)
Total aerobic counts	-	Tryptic soy agar	72	25	(Papers III,IV)

[a]Confirmation procedure for irrigation water indicator organisms explained in Paper I.
[b]Green fluorescing colony counting under UV-light.
[c]*Listeria monocytogenes* confirmation procedure explained in Paper IV.
+ = filtered through 0.45 µm membrane; - = no filtration performed.

Culture-independent analyses

In Papers I and II, three independent water samples (1 L) from five collection events during 2011 at each sampling site within the IWDS were filtered separately through a 0.45 µm filter (VWR 514-0605) to assess microbial community in the IWDS. Repeated centrifugation (30 min, 4 ^{o}C, 3000 xg) and resuspension with 0.85% NaCl were performed on the filter residues. First the filter residues were centrifuged (3000 xg, 30 min) in 5 mL 0.85% NaCl, then the suspension was discarded and the pellets were subjected to a second centrifugation (10,000 rpm, 3 min) in 1 mL 0.85% NaCl. The suspension was again discarded and the pellets were stored at -80 ^{o}C. For 454-pyrosequencing, the pellets were processed as explained in Papers I and II.

In Paper III, dominant *Enterobacteriaceae* from rocket and spinach canopies were characterised by randomly selecting solitary colonies from VRBD plates after incubation (24 h, 37 °C), pure-cultured on full-strength TSA and incubated (72 h at 25 °C). Pure cultures were transferred to sterile cryovials with freezing medium (4.28 mM K_2HPO_4, 1.31 mM KH_2PO_4, 1.82 mM Na-citrate, 0.87 mM $MgSO_4$ x $7H_2O$, 1.48 mM glycerol 98%) (Fåk *et al.*, 2012) and stored at -80 °C. Cryopreserved cultures were grown on 0.1 TSA plates and incubated (72 h at 25 °C) and single colonies were randomly selected and transferred to freezing medium and preserved at -80 °C. For sequencing, the cryotubes with pure culture were treated as described in Paper III.

As explained in Papers I-III, DNA was extracted using BioRobot® EZ1 with EZ1 DNA tissue card and EZ1 DNA tissue kit (QIAGEN®, Hilden, Germany). In Papers I and II, the DNA amplification was performed using multiple displacement amplification (Illustra Genomiphi V2 DNA amplification kit, GE Healthcare, UK). The quantity and purity of the amplified DNA were assessed using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

In Papers I-III, the amplification of the 16S rRNA genes was performed using universal forward primer ENV1 and reverse primer ENV2. The correct size of amplified fragments was determined by running the amplified fragments against DNA Molecular Weight Marker VI (Roche Diagnostics) on 1.5% agarose gel (type III: High EEO, Sigma, St Louis, MO, USA).

For 454-pyrosequencing in Papers I and II, the purified DNA was sent for pyrosequencing to LGC Genomics GmbH (Berlin, Germany). For sequencing in Paper III, the amplicons were sent to MWG (Ebersberg, Germany).

4.3.4 Statistical analyses

For experiments with plate counts, the data were log-transformed and analysed for statistically significant differences using Minitab Version 16. General linear model followed by Tukey's test was applied to identify the differences. In Papers I and II, non-parametric Kruskal-Wallis-ANOVA, regressions and principal component analysis were performed to compare the results from weather data and viable counts.

Diversity indices (Papers I, II) were calculated using paleontological statistics software package (PAST) Version 2.17b (Hammer *et al.*, 2001). Statistical analyses performed in the different experiments are described in detail in the individual papers.

5 Results and Discussion

5.1 Analyses of field water samples (Papers I, II)

5.1.1 Microbiota of the irrigation water distribution system

Irrigation water systems have been shown to harbour many different microorganisms, including human pathogenic microorganisms, as explained in section 2.2. Some of these microorganisms are capable of forming biofilms on the surfaces of the IWDS and can persist for a long time, contaminating the water (Pachepsky *et al.*, 2012).

Indicator organisms evaluated in Papers I and II, varied markedly. Heterotrophic plate count (HPC) was found to be significantly lower after the water passed through the field pipe (before photocatalysis) and after photocatalysis. No differences were found in total coliform bacteria (TC), thermotolerant coliform bacteria (TTC), *E. coli* and faecal enterococci (FE) between the four sampling sites before photocatalysis (Paper I). However, the levels of all these organisms were significantly lower in most cases in samples after photocatalysis (Paper II). *Salmonella umbilo* was also found in water samples collected from the pond during 2009. Apart from HPC, indicator organisms were significantly affected by abiotic factors in samples collected from the first four sampling sites (Paper I).

In this thesis, plate count methods were used for the culturable water indicator organisms. For more detailed assessment of the bacterial microbiota in the irrigation water community, samples collected during 2011 were subjected to 454-pyrosequencing. The 454-pyrosequencing data presented in this thesis are based on relative abundance. In total, 42,586 16S rRNA gene sequences were obtained from all water samples and these were clustered (at >97% similarity) into bacterial operational taxonomic units (OTUs) and taxonomically classified from phylum to genus level. The *Bacteroidetes* and *Proteobacteria* were the most dominant phyla at all sampling sites (Figure 6).

Bacteroidetes comprised more than half of all bacterial phyla in samples from the pond, after coarse filtration, at the start of the field water pipe and before photocatalysis and <50% of all bacterial phyla in samples collected after photocatalysis. *Proteobacteria* comprised >10% of the community at each of the five sampling sites. In contrast, a higher number of *Proteobacteria* was found after photocatalysis compared with in the pond, after coarse filtration, at the start of the field water pipe and before photocatalysis. These two phyla have previously been found to dominate in different water sources and systems (Douterelo *et al.*, 2013; Kwon *et al.*, 2011; O'Sullivan *et al.*, 2006). *Actinobacteria* was also found at all sampling sites. *Firmicutes* and *Tenericutes* were found at the four sites before photocatalysis, but not after photocatalysis. *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Fusobacteria*, *Nitrospirae*, *Planctomycetes* and TM7 were occasionally found at different sampling sites in the IWDS.

Sequences similar to classes belonging to the phylum *Bacteroidetes* were abundant, and *Flavobacteriia* and *Sphingobacteriia* dominated the classes and were present at all five sites. *Flavobacteriia* were reduced in number after photocatalysis. The most abundant *Proteobacteria* were the *α-Proteobacteria*, *β-Proteobacteria* and *γ-Proteobacteria,* and all three classes were present at all sampling sites. Interestingly, *γ-Proteobacteria* occurrence was lower in the pond water than in samples from before and after photocatalysis, which shows that this group of bacteria can persist in IWDS. Therefore, it is highly recommended that water samples be analysed for microbial quality at the irrigation ramp rather as well as at the water source or at the start of the IWDS (Alsanius *et al.*, submitted). To get maximum disinfection effect, the photocatalytic treatment unit should be installed at the end of the IWDS.

In the work described in this thesis, it was found that more than 50% of all OTUs could not be assigned to specific genera. However, these results need to be confirmed using high-throughput analysis tools. This may lead to the construction of new bacterial groups, as has been seen previously (Kalmbach *et al.*, 1997). Members of the phyla *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Proteobacteria* were highly represented at genus level. The genera *Arcicella* and *Flavobacterium*, which belong to the phylum *Bacteroidetes,* were the most abundant genera and were found at all five sampling sites. The genus *Arcicella* has three known species, namely *Arcicella aquatica* (Nikitin *et al.*, 2004), *Arcicella rosea* (Kämpfer *et al.*, 2009) and *Arcicella aurantiaca* (Sheu *et al.*, 2010), which have been isolated from different aquatic environments (Chen *et al.*, 2013). Members of the genus *Flavobacterium* can be found in soil and freshwater and some are pathogens of fish (Bernardet *et al.*, 1996).

Two important genera that may contain human pathogens (McGuigan *et al.*, 2002; Friedman *et al.*, 1998), *Clostridium* and *Legionella*, were found at the first four sampling sites of the IWDS, *i.e*. before photocatalysis, and were absent (*Clostridium*) or comprised <1% (*Legionella*) of the total bacterial community after photocatalysis. *Clostridium* and *Legionella* have previously been found in different water systems (Kwon *et al.*, 2011; Al-Saif & Brazier, 1996). The genus *Escherichia* was found only after photocatalysis. Various strains of this genus have been found in many foodborne illnesses and have been studied in various experiments involving application of contaminated irrigation water to different fruits and vegetables. Members of the *Escherichia*, including pathogenic strains, have been isolated from many different water sources (Söderström *et al.*, 2008; Tsen *et al.*, 1998). In this thesis a model strain from this genus, *E. coli* O157:H7, was used to study its prevalence on leafy vegetables (Papers III and IV). It was shown that dominant strains in the phyllosphere of leafy vegetables were *Stenotrophomonas*, *Raoultella, Pseudomonas* and *Enterobacter* (see Paper III). It would be interesting to compare the phyllosphere microbiota on leafy vegetables with the microbiota in irrigation water in future studies and to determine the effect they have on interactions with microorganisms carrying food illnesses.

Pachepsky *et al*. (2012) has shown that certain microorganisms have the ability to continue to affect water quality through their persistent presence in the IWDS. Therefore, certain groups of microorganisms may be retained in the system by biofilm formation and survive for a longer time. The pipeline used in these studies (Papers I and II) was new and there were possibilities of more biofilm-forming bacteria being released to the irrigation water passing through the pipe, as explained by Shelton *et al*. (2013).

The important finding from the analysis of IWDS microbiota was that there were variations in the microbiota at the five sampling sites. In field situations, the decontamination unit should be installed at the end of the IWDS. It appears that the genus *Clostridium* occurs at various sampling sites in the IWDS, leading Payment and Franco (1993) to suggest that a member of this genus (*Clostridium perfringens*) could be used as a water indicator organism.

5.1.2 Decontamination of irrigation water (Paper II)

Low hygiene quality irrigation water can be one of the important factors in fruit and vegetable contamination (Steele & Odumeru, 2004). Therefore decontamination of irrigation water is an important step in producing hygienically safe agriculture produce. Irrigation water decontamination was performed in Paper II using a photocatalytic unit. The indicator organisms assessed were in most cases significantly ($p<0.05$) reduced after photocatalysis.

The highest reduction was found for HPC and TC (around log 1 CFU mL^{-1} and log 1 CFU 100 mL^{-1}, respectively). Ireland *et al*. (1993) were also able to reduce HPC and TC by log 1 in pond water using photocatalysis. A reduction of log 0.5-1 CFU 100 mL^{-1} was seen in TTC and *E. coli*, while FE was reduced by log 0.5 CFU 100 mL^{-1}. In many cases, it has been observed that water treatment is dependent on the concentration of microorganisms prior to treatment (Rincón & Pulgarin, 2004). Therefore, the data were divided into high and low loads of microorganisms before treatment and evaluated. A high percentage reduction (88-97%) of the indicator organisms was observed for high loads compared with low loads (41-87%).

The highest reduction through the action of photocatalysis was seen in TC and the lowest in FE. Similar observations have been reported previously (Rincón & Pulgarin, 2004). The difference in decontamination efficacy can be explained by the action of $^{\bullet}OH$ radicals on the microorganism cell walls (Saito *et al.*, 1992). The TC normally consists of Gram-negative, non-spore forming bacteria which are sensitive to physical stress and can easily be eliminated by photocatalysis. The FE comprise cells of Gram-positive bacteria with thicker and denser cell walls and are more difficult to remove by photocatalysis (Kühn *et al.*, 2003). As the decontamination was also dependent on bacterial load before treatment, a possible reason for the low reduction in the FE could be that this group of microorganisms was low before the treatment and hence the efficacy of photocatalysis in reducing this group was low. For improvement of the efficacy, lowering the flow rate through the photocatalytic unit, increasing the number of reactors installed online or mounting phototcatalytic units close to each nozzle on the irrigation ramp should be considered.

Previous studies have indicated that DNA from organisms can persist for several days to weeks after cell death. For example, *Salmonella* DNA can persisted in a seawater microcosm for 10-55 days even if the cells were heat-killed (Dupray *et al.*, 1997). As explained in section 2.5 of this thesis, the ability of 454-pyrosequencing to sequence both viable and dead bacteria does not give an indication about the decontamination efficacy of the photocatalytic unit. In future studies, techniques including the use of propidium monoazide and flow cytometry may help discriminate between viable and dead cells in the community (Nocker *et al.*, 2010).

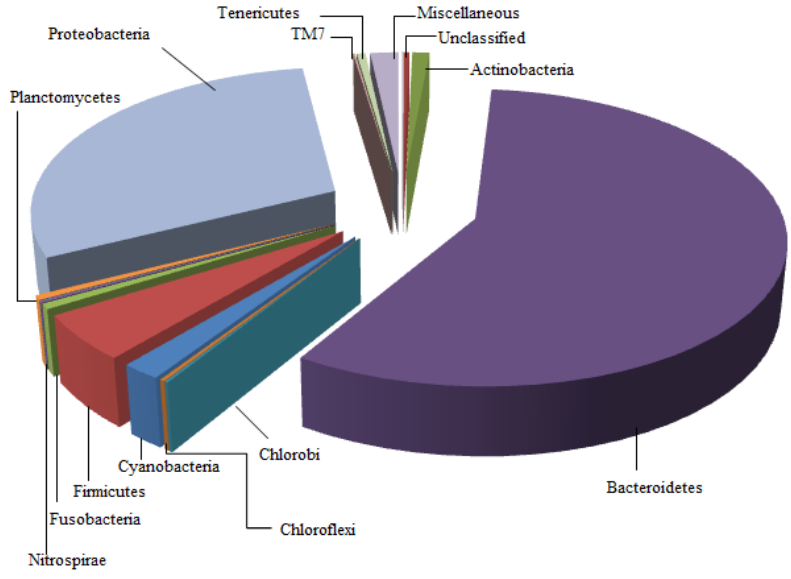

Figure 6. Relative abundance of bacterial phyla in the free water phase of the irrigation water distribution system (IWDS) studied. The data represent the means of all sampling sites and events (n=25).

5.2 Human pathogen interactions in leafy vegetables

5.2.1 General phyllosphere biota (Papers III, IV)

The ambient phyllosphere microbiota of the plant plays an important role in the plant environment. The native microbiota of the plants can affect the prevalence of enteric pathogens introduced from outside the plant environment. Cooley *et al.* (2006) showed that *Wausteria paucula* enhanced the survival of *E. coli* O157:H7 on lettuce foliage and *Enterobacter asburiae* decreased *E. coli* O157:H7 survival. A possible reason could be that *E. asburiae* and *E. coli* O157:H7 utilise almost the same secondary metabolites produced by plants, so competition may develop between the two bacterial strains. On the other hand, commensalism may exist between *E. coli* O157:H7 and *W. paucula* on foliage. Therefore, cultural practices that encourage the growth of competing bacteria, *e.g. E. asburiae*, may reduce the incidence of produce contamination (Cooley *et al.*, 2006). In another study, Wilson *et al.* (1999) exposed plant pathogenic (*Pseudomonas syringae*) and non-pathogenic microorganisms (*Stenotrophomonas maltophilia*, *Pantoea agglomerans*, *Methylobacterium organophylum*) to stress and found that on dry leaves, the population size of the non-pathogenic phyllosphere strains was lower than that of the plant pathogenic strains. The data presented in this thesis did not demonstrate any

interaction between the plant local microbiota and the enteric pathogens introduced, as affected by cultural practices. However, background ambient phyllosphere biota and *Enterobacteriaceae* in the leafy vegetable phyllosphere were assessed and explained in Papers III and IV. In most cases there were no changes in the total microbiota or *Enterobacteriaceae* under pre-harvest cultural practices, as assessed by culture-dependent methods.

The phylogenetic analysis of the sequenced isolates on spinach and rocket (Paper III) revealed that the genera *Stenotrophomonas*, *Raoultella*, *Pseudomonas* and *Enterobacter* were the dominant culturable microbiota, as assessed by VRBD. Members of these genera have been shown to colonise different plant parts (Berg *et al.*, 2005) and they may include human opportunistic pathogens (Alves *et al.*, 2007; Denton & Kerr, 1998; John *et al.*, 1982). It has been shown that members of these genera can be present in different water systems and can be transferred to vegetables through irrigation (Papers I and II; see also section 2.2 of this thesis).

5.2.2 Prevalence of *E. coli* O157:H7 and *L. monocytogenes* on leafy vegetables as affected by pre-harvest cultural practices (Papers III, IV)

Irrigation of leafy vegetables close to the time of harvest is a common practice to increase the market value of the crop, but this practice can promote survival of human pathogens on plant surfaces if contaminated water is used (Solomon *et al.*, 2003). Pathogens may colonise both internal and external plant parts and can survive for long periods depending on environmental factors and nutrients (Olaimat & Holley, 2012; Brandl, 2006). Pathogens can also form aggregates on plant surfaces and can proliferate over longer periods (see section 2.3). The experiments in Papers III and IV were performed under greenhouse conditions, and thus there is a risk that certain important environmental factors, *e.g.* UV-radiation that can directly affect the prevalence of enteric pathogens in the phyllosphere were excluded. A high inoculation density was used in the experiments, due to the fact that a low density may result in low probabilities at average natural concentrations and result in an erroneous conclusion on absence of pathogens in the phyllosphere. Furthermore, as mentioned earlier (Chapter 4), in order to comply with legislation in Sweden and also in order to eliminate background contamination of the crops, the experiments with attenuated human pathogens on leafy vegetables had to be conducted in the greenhouse. Therefore, a significant proportion of the pathogens introduced in these experiments may have attached to the growing medium instead of the plant canopy.

Poor hygiene conditions in the pre-harvest phase cannot necessarily be counteracted in later stages of the production chain. Therefore, cultural

practices could be an option to reduce the contamination of field vegetables (see section 2.3). It has been shown that early cessation of irrigation can change the moisture conditions, as well as causing mild water stress in the phyllosphere. Lower moisture conditions in the phyllosphere may affect the survival of pathogenic bacteria (Cooley *et al.*, 2003). Cessation of crop irrigation using contaminated water may reduce the survival of pathogens on plants (Keraita *et al.*, 2007). To assess the survival of *E. coli* O157:H7 on leafy vegetables after irrigation with contaminated water, an experiment on cessation of irrigation with contaminated water was performed. No *E. coli* O157:H7 was found in the control treatments. However, *E. coli* O157:H7 colonies were more abundant when water with high inoculum densities was used and were significantly higher for all treatments and harvest events in most cases, on both spinach and rocket. There was a reduction in colonies with delayed harvest for both crops, as reported previously by Wood *et al* (2010). Various trends have been found in the decline/survival of *E. coli* O157:H7 on various vegetables (Moyne *et al.*, 2011; Wood *et al.*, 2010; Hutchison *et al.*, 2008; Islam *et al.*, 2004; Abdul-Raouf *et al.*, 1993), which is mainly dependent on initial inoculum, moisture, temperature, nutrients and irradiation (Webb *et al.*, 2008; Solomon *et al.*, 2003). Reductions in the population could be due to dry conditions developing on the leaf surface and affecting nutrient availability to the epiphytic microorganisms (Ibekwe & Grieve, 2004). In Paper III, it was observed that *E. coli* O157:H7 persistence was dependent on the initial inoculum density, with a high density being able to persist for longer periods. This supports previous findings (Webb *et al.*, 2008; Solomon *et al.*, 2003).

In recent years, research on deficit irrigation (irrigation to below the crop water requirement) has been conducted for various horticultural crops, mainly for reasons of sustainability and product quality improvements (Stefanelli *et al.*, 2010). This practice may have a mild effect on human pathogen survival on crops. In this thesis, the effect of water regime in the growing medium on human pathogens on vegetables was assessed (Paper IV). The moisture content of the growing medium was significantly ($p<0.05$) higher for water regimes based on 20-30% vwc than for water regimes based on 5-12% vwc. A difference of more than 50% vwc was found between the two water regimes.

Both *E. coli* O157:H7 and *L. monocytogenes* were absent from the un-inoculated control treatments. Vegetables grown at a water regime of 20-30% vwc had significantly higher ($p<0.05$) numbers of *E. coli* O157:H7 and *L. monocytogenes* colonies than those grown at a water regime of 5-12% vwc (Figure 7). Significant differences were observed between the two water regimes, but the difference in the overall means for the treatments was low and

is only of technical interest. These results do not suggest any improvements in cultural practices with respect to the persistence of human pathogens on plants. Lower number of colonies on plants grown with a water regime of 5-12% vwc in the growing medium could be due to the development of antioxidants (Esteban *et al.*, 2001), which can inhibit the growth of human pathogens (Alberto *et al.*, 2006; Wen *et al.*, 2003). Experiments on apple antioxidants (phenols) have shown that growth of *E. coli* and *L. monocytogenes* can be inhibited by the high amount of phenols in extract from apple skin (Alberto *et al.*, 2006). Similarly, Delaquis *et al.* (2006) observed an antilisterial action of phenols from wounded lettuce in storage. Other studies have shown that phenolic compounds are present in different vegetables, including rocket (Bennett *et al.*, 2006), spinach (Fry, 1982) and Swiss chard (Pyo *et al.*, 2004). These compounds may exert an antibacterial action against human pathogens in the plant phyllosphere.

A difference of more than 50% in stomata gaseous conductance of CO_2 was found between the two water regimes, with higher stomata conductance for plants grown at a water regime of 20-30% vwc than 5-12% vwc. Stomata conductance may affect the water activity on the leaf surfaces. Thus low stomata conductance may result in dryness on the leaf surfaces of plants grown at 5-12% vwc, which may subsequently affect the prevalence of human pathogens (Dreux *et al.*, 2007; Aruscavage *et al.*, 2006; Ibekwe & Grieve, 2004; Chen *et al.*, 1999). As shown by Hirano and Upper (2000), the absence of water on leaf surfaces (dryness) may lead to unavailability of nutrients to microorganisms.

The most important finding regarding pathogen persistence on leafy vegetables was that *E. coli* O157:H7 was still found in the phyllosphere of leafy vegetables at all densities, even after 72 h of desiccation treatment (Paper III). Similarly, different water regimes in the growing medium could not completely reduce the prevalence of *E. coli* O157:H7 and *L. monocytogenes* on leafy vegetables (Paper IV). Previous studies have shown that *E. coli* O157:H7 can persist on fruits and vegetable, *e.g*. on parsley for 177 days (Islam *et al.*, 2004), on lettuce for 25-77 days (Islam *et al.*, 2004) and about 21 days on salad vegetables, watermelons and iceberg lettuce (Diaz & Hotchkiss, 1996; Del Rosario & Beuchat, 1995; Abdul-Raouf *et al.*, 1993). *Listeria monocytogenes* can survive comparatively longer in different plant materials (Beuchat, 1996a). In conclusion, as both *E. coli* and *L. monocytogenes* cause disease at very low doses (Ramaswamy *et al.*, 2007; Ackers *et al.*, 1998), cessation of irrigation at three days before harvest or changing the water regime of the growing medium is not an adequate sanitisation treatment to exclude the probability of viable *E. coli* O157:H7 or *L. monocytogenes* cells on leafy vegetables.

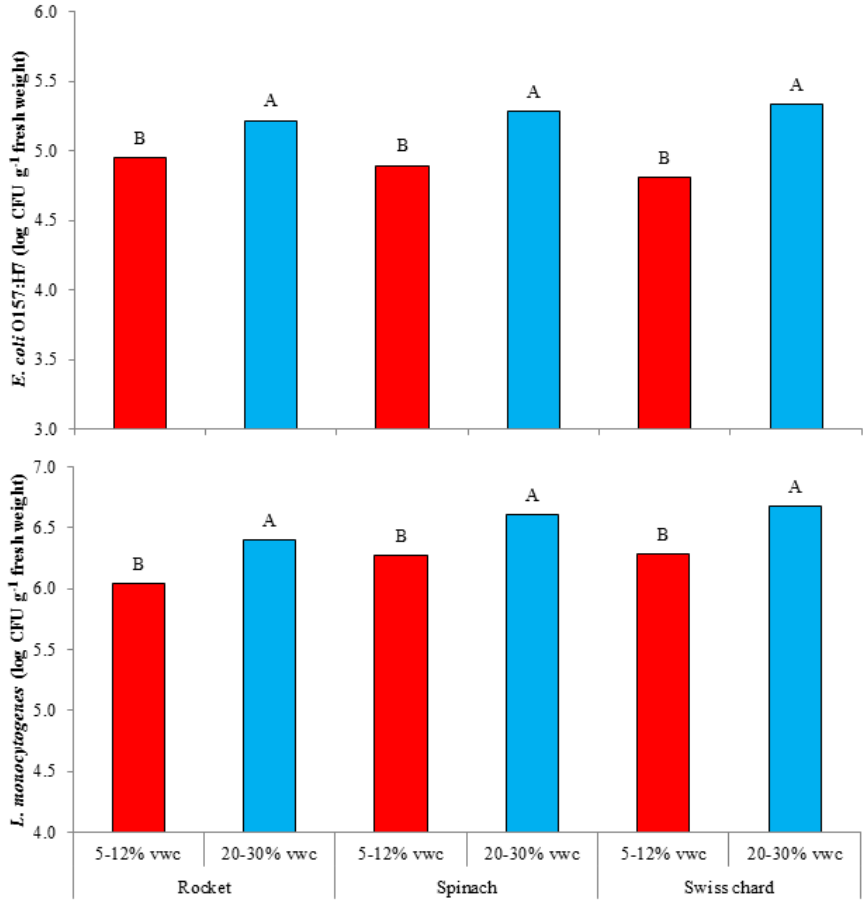

Figure 7. Effect of water regime (5-12% vwc and 20-30% vwc) in the growing medium on the prevalence of *E. coli* O157:H7 and *L. monocytogenes* inoculated onto leafy vegetables and harvested 24 h after inoculation. Data shown as log CFU g^{-1} fresh weight of plants. No *gfp* tagged *E. coli* O157:H7 or *L. monocytogenes* were detected on non-inoculated plants (control groups). Bars with different letters shows significant differences ($p<0.05$) between the two water regimes for each crop, based on Turkey's test (n=8).

6 Main Conclusions and Future Perspectives

The following main conclusions can be drawn from the results presented in this thesis:

- Bacterial community structure varies along the irrigation pipeline. More than half the bacterial microbiota found in irrigation water belonged to unknown genera.

- For maximum decontamination, the water treatment unit should be installed at the end of the irrigation water distribution system.

- Irrigation water can be decontaminated using photocatalysis if there is a high load of microbes in the irrigation water. The prototype photocatalytic unit tested here needs to be optimised.

- Cessation of irrigation with contaminated water three days before harvest did not eliminate *E. coli* O157:H7 from the phyllosphere.

- Low water content of the growing medium did not eliminate human pathogens from the phyllosphere.

In future experiments, more water samples from the free water phase of the IWDS and biofilm samples should be taken to make it possible to draw general conclusions on the microbiota of the irrigation water distribution system. It would be interesting to relate irrigation water microbiota to dynamics in the phyllosphere exposed to the same water source over time. It would also be interesting to evaluate the same photocatalytic unit at different water flow rates, thereby varying the time of exposure of the microbes to photocatalysis. More than one photocatalytic unit may be needed in the irrigation water

distribution system. It would also be interesting to install a photocatalytic unit close to the nozzles, so as to evaluate the efficacy close to the outlet of the irrigation system.

Cultural practices that may enhance development of antioxidants, *e.g*. phenolic compounds, and practices that encourage the growth of competing bacteria such as *Enterobacter asburiae* should be adopted to reduce the numbers of enteric pathogens in the plant phyllosphere.

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