Université de Montréal

Impact of pesticides on indicator and pathogenic microorganisms persistence under laboratory and field conditions

par

Tran Thi Phuong Hoa

Département de pathologie et microbiologie

Faculté de médecine vétérinaire

Mémoire présenté à la Faculté de médecine vétérinaire

en vue de l'obtention du grade de

maître ès sciences (M.Sc.)

en sciences vétérinaires

option hygiène vétérinaire et innocuité des aliments

Août, 2012

© Tran Thi Phuong Hoa, 2012

RÉSUMÉ

On s'intéresse aux impacts des pesticides sur la microflore des plantes surtout dans le contexte des légumes contaminés par des agents pathogènes. Le but de cette étude est d'évaluer l'impact de certains pesticides sur la persistance de micro-organismes indicateurs et pathogènes. En laboratoire, la persistance d'*E. coli* et de *Salmonella* en présence de quatre pesticides (Ripcord 400EC, Copper 53W, Bioprotec CAF, Serenade MAX) a été étudiée. Les plaques de Pétrifilm et le milieu sélectif XLD sont utilisés pour énumérer les populations d'*E. coli* et de *Salmonella*. Il a été démontré que le Serenade MAX favorisait la croissance microbienne, le Bioprotec CAF et le Ripcord 400EC soutenaient la survie microbienne et le Copper 53W inhibait la croissance, à la fois d'*E. coli* et de *Salmonella*. En conditions terrain, Ripcord 400EC, Copper 53W, Bioprotec CAF ont été étudiés sur une culture de brocoli irriguée avec de l'eau expérimentalement contaminée par *E. coli*. Dans tous les traitements, un impact de l'irrigation a été observé sur les populations de levures et de moisissures (diminution) et les bactéries aérobies totales (augmentation). Une prévalence supérieure d'*E. coli* a été observée dans les parcelles traitées avec le Bioprotec CAF comparativement aux traitements au Copper 53W, ce qui est en accord avec les résultats observés lors de l'essai en laboratoire. Cependant, l'analyse statistique n'a montré aucune différence significative entre les traitements appliqués. Les effets directs des pesticides sur les micro-organismes sont confirmés dans des conditions de laboratoire mais demeurent méconnus dans les conditions expérimentales au champ.

Mots-clés : Pesticides, persistance, *E. coli*, *Salmonella*, microflore des plantes, brocoli.

SUMMARY

There is a concern about the impact of pesticide on plant microflora, especially in the context of vegetables contaminated with pathogens. The aim of this study was to evaluate the impact of various pesticides on indicator and pathogenic microorganisms' persistence. In laboratory, survival of *E. coli* and *Salmonella* in four pesticides (Ripcord 400EC, Copper 53W, Bioprotec CAF, Serenade MAX) was evaluated. Petrifilm count plates and XLD agar were used to enumerate *E. coli* and *Salmonella* counts. Results showed a direct effect of various pesticides on microorganisms: Serenade MAX promoted microbial growth; Bioprotec CAF and Ripcord 400EC supported microbial survival; and Copper 53W inhibited both *E. coli* and *Salmonella* growth. In field conditions, three pesticides (Ripcord 400EC, Copper 53W, Bioprotec CAF) were studied on broccoli irrigated with *E. coli* - contaminated water. Broccoli samples were analyzed to determine *E. coli,* mold and yeast, and total aerobic counts. Irrigation resulted in mold and yeast counts decline but aerobic bacteria populations increased slightly in all treatments. Higher *E. coli* prevalence in Bioprotec CAF treatments compared to Copper 53W treatments was consistent with results observed during the laboratory assay. However, statistical analysis showed no significant difference between treatments. The direct effect of pesticides on microorganisms under laboratory conditions was demonstrated but it is still unclear under experimental field conditions.

Keywords: Pesticides, persistence, *E. coli*, *Salmonella*, plant microflora, broccoli

TABLE OF CONTENTS

Université de Montréal .... **i**

RÉSUMÉ .... **ii**

SUMMARY .... **iii**

TABLE OF CONTENTS .... **iv**

LIST OF TABLES .... **vii**

LIST OF FIGURES .... **viii**

LIST OF ABBREVIATIONS .... **ix**

ACKNOWLEDGMENTS .... **xi**

Chapter 1. INTRODUCTION **1**

Chapter 2. LITERATURE REVIEW **5**

2.1 Human pathogens associated with vegetable 6

2.1.1 *Escherichia coli* 6

2.1.1.1 Features, history 6

2.1.1.2 Effects on human health 8

2.1.2 *Salmonella* 11

2.1.2.1 Features, history 11

2.1.2.2 Effects on human health 12

2.2 Plant indigenous microflora 13

2.3 Interaction between human pathogens and plant indigenous microflora 14

2.4 Factors affecting persistence of human pathogens on vegetables 17

2.5 Outbreaks of human pathogens associated with vegetables 20

2.6 Microbial contamination sources for vegetables 22

2.7 Human pathogens and contaminated water 24

2.7.1 Presence of human pathogens in water 24

2.7.2 Factors influencing persistence of human pathogens in water 26

2.7.3 Survival and growth of human pathogens in pesticide solutions 27

2.8 Pesticides 28

2.8.1. Pesticide types 30

2.8.1.1 Chemical pesticides 30

2.8.1.1.1 Organic pesticides 30

2.8.1.1.2 Inorganic pesticides 34

2.8.1.2 Bio-pesticides 36

2.8.2 Water used for pesticide preparation 38

2.8.3 Pesticide degradation 39

2.8.4 Effects of pesticide on microorganisms 42

2.9 Strategies to reduce vegetable contamination by human pathogens 45

HYPOTHESIS 48

Chapter 3. Impact of Pesticides on Indicator and Pathogenic Microorganism Persistence under Laboratory and Field Conditions.....49

ARTICLE IN PREPARATION.....49

Chapter 4. GENERAL DISCUSSION.....77

Chapter 5. CONCLUSION.....91

BIBLIOGRAPHICAL REFERENCES.....94

LIST OF TABLES

Literature review

Table I. Potential sources of pathogenic microorganisms and conditions influencing their survival and growth on fresh produce 23

Article

Table I. Description of pesticides used for the laboratory assay and field trial 65

Table II. Source of bacterial strains used for the laboratory assay 66

Table III. Treatments in field experiments 67

Table IV. Spraying, irrigation and broccoli sampling schedule 68

LIST OF FIGURES

Literature review

Figure 1. Chemical structure of DDT, organophosphorus, carbamates and pyrethrins.......31

Figure 2. Chemical structure of cypermethrin 32

Figure 3. Environmental degradation of pesticides..40

Article

Figure 1. *E. coli* and *Salmonella* in pesticide solutions at 4°C and 21°C.............................70

Figure 2. *E. coli* in pesticide solutions at 4°C 71

Figure 3. Overall *E. coli* prevalence during the sampling period 72

Figure 4. *E. coli* positive rates in each treatment according to sampling time. 73

Figure 5. Mold and yeast counts in broccoli samples according to pesticide 74

Figure 6. Total aerobic microflora counts in broccoli samples according to pesticide........75

LIST OF ABBREVIATIONS

B.	*Bacillus*
BCPC	British Crop Production Council
CCME	Canadian Council of Ministers of the Environment
CDC	Centre for Disease Prevention and Control
CFU	Colony-Forming Unit
CPA	2,2-dimethyl-3(2,2-dichlorovinyl) cyclopropane-carboxylic acid
DAEC	Diffusely adherent *E. coli*
DDT	Dichlorodiphenyltrichloromethane
DCVA	2,2-dimethylcyclopropane carboxylic acid
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EC	Emulsifiable Concentrate
ECDC	European Centre for Disease Prevention and Control
EHC	Environmental Health Criteria
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FDR	Food and Drug Regulations
IPCS	International Programme on Chemical Safety

IRDA	Research and Development Institute for the Agri-environment
IU	International Unit
IWMI	International Water Management Institute
JMPS	FAO/WHO Joint Meeting on Pesticide Specifications
LC_{50}	Lethal concentration, 50%
LD_{50}	Lethal dose, 50%
log	Logarithm
MARD	Ministry of Agriculture and Rural Development of Vietnam
MRL	Maximum Residue Level
PBA	3-phenoxybenzoic acid
S.	*Salmonella*
SC	Aqueous Suspension Concentrate
SL	Soluble Concentrate
SP	Water Soluble Powder
STEC	Shiga toxin producing *E. coli*
US	United States of America
USEPA	United States Environmental Protection Agency
WG	Water dispersible granules
WHO	World Health Organization
WP	Wettable Powder

ACKNOWLEDGMENTS

I would like to thank my director, Dr. Ann Letellier for her guidance during this entire research project and her assistance in the completion of this thesis. Thanks also to my co-directors Dr. Francis Beaudry for his help and participation in my research and Dr. Caroline Côté for her patience, for always being available to answer questions, and for her on-going interest in my progress throughout my graduate studies.

Appreciation is extended to the Faculty of Veterinary Medicine, University of Montreal and Food Agricultural Products Quality Development Control Project (FAPQDC), who provided me with financial support.

There are many people in the Research and Development Institute for the Agri-environment who I would like to thank: Katie Roseberry and Mylène Généreux for their help in lab navigation and technical support and Nicole, Robert, Sylvain, Louis David, Marie-Josée, Mariline, Catherine and Alexandre for their help in the lab and the field and for their friendship.

Special thanks go to my family for their love and encouragement from the very beginning of my studies, and especially to my parents who have been taking good care of my son while I have been pursuing this path.

Thank you to Hélène Bergeron, Serge Charron, Martin Michaud and others in the FAPQDC project who have been so supportive and caring of me and my cohorts from Vietnam. Thanks to my Vietnamese friends who have helped me out during difficult or discouraging moments. Thank you all for being there for me.

Chapter 1. INTRODUCTION

The presence of human pathogens on edible plants can be a source of human illnesses. Many human pathogen outbreaks associated with the consumption of fruits and vegetables have been documented in which the causative agents could be *E. coli* and *Salmonella*. In the US, 21% of *E. coli* O157:H7 outbreaks were linked to vegetable products from 1991 to 2002 (Rangel *et al.*, 2005), and from 1996 to 2007, 33 outbreaks were associated with *Salmonella*-contaminated fruits and vegetables (Callaway, 2008). Multistate outbreaks of *Salmonella* infections linked to tomatoes occurred in 2004 in the US and Canada (CDC, 2005a).

Vegetables can be contaminated by human pathogens during any stage of production. Potential pre-harvest sources of contamination include soil, feces, irrigation water, water used for pesticide applications, dust, insects, inadequately composted manure, wild and domestic animals, and human handling (Beuchat, 1996). Human pathogens can be recovered on harvested products and many among them are consumed raw. In Mexico, 5.8% of vegetable samples were found contaminated with *Salmonella* (Quiroz-Santiago *et al.*, 2009). This percentage is higher in countries where adequate sanitation practices are not applied. For example, up to 76% of vegetable samples were found to be *E. coli*-positive in Vietnam (Ha *et al*, 2008).

In the field, pesticides are dissolved and diluted in water and sprayed to ensure crop coverage. Study results show that pesticide application does not only control pests, but also

influences the population of non-targeted microorganisms. Pesticides can have a direct effect on human pathogens by promoting or inhibiting their growth and survival (Peter *et al*., 2005; Guan *et al*., 2001, 2005). The ability of some pesticides to promote microbial growth reinforces concerns that pesticide application can be associated with human pathogens after using contaminated water to prepare pesticide solutions.

On the other hand, under field conditions, pesticides can also have an indirect effect on human pathogen persistence by affecting plant microflora, which includes many different genera of bacteria, filamentous fungi and yeasts (Lindow and Brandl, 2003). Pesticide application can alter the microbial biomass and microbial community structure on plants by providing carbon and nitrogen - energy sources for microorganisms - or by reducing plant microflora population or diversity (Zhang *et al.*, 2008, 2009a, 2009b). These changes could have an effect on the competitions between human pathogens and other microorganisms in the plant microflora (Brandl, 2006; Aruscavage, 2006), result in the changes of the human pathogen persistence on crops. Carlin et *al.*, (1996) determined that the increase or decrease of the microflora population on minimally processed fresh broad-leaved endive by chemical disinfection can affect the persistence of human pathogens such as *Listeria monocytogenes*.

Very few studies focused on the effect of pesticide application on human pathogen persistence and in addition, in the field where continuous interactions between pathogens

and plant indigenous microflora are present, the effect of pesticides on pathogen survival is still unclear. Therefore, the objectives of this study were (1) to evaluate the survival of *E. coli* and *Salmonella* in four pesticides solutions (Ripcord 400EC, Copper 53W, Serenade MAX, and Bioprotec CAF) under laboratory conditions; and (2) to study the persistence of *E. coli* (used as a fecal contamination indicator) on broccoli crops exposed to three pesticides (Ripcord 400EC, Copper 53W, and Bioprotec CAF). Expected results were to be able to observe the effects of pesticides on the survival and growth of *E. coli* and *Salmonella* under laboratory conditions and also to clarify the effects of pesticides on *E. coli* under in field conditions.

Chapter 2. LITERATURE REVIEW

2.1 Human pathogens associated with vegetable

Human pathogens associated with vegetable can be divided into several groups such as: bacteria including *Campylobacter jejuni*, *Clostridium botulinum*, *Escherichia coli* O157 and other Shiga-toxin producing *Escherichia coli* (STEC), *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *Vibrio cholerae*, *Yersinia enterocolitica*; protozoa including *Cyclospora cayetanensis*, *Cryptosporidium parvum*, *Giardia lamblia*; and viruses including hepatitis A and Norwalklike viruses (Steel and Odumeru, 2004). These pathogens can be transmitted to humans through consume vegetables contaminated. According to the report of FAO/WHO (2008), *E. coli* O157 and *Salmonella* are among the pathogens most commonly associated with fresh fruit and vegetable.

2.1.1 *Escherichia coli*

2.1.1.1 Features, history

Escherichia coli (*E. coli*) is a member of the *Enterobacteriaceae* family. It is a Gram-negative, non-sporulating, facultative anaerobic, rod-shaped bacterium, about 1.1-1.5 x 2.0-6.0 µm in size. *E. coli* can live on a wide variety of substrates and obtains carbon and energy through oxidizing organic compounds (Schulze *et al.*, 2006). The suitable

temperature for the development of *E. coli* is around 37 °C, but some strains can grow at temperatures of up to 49 °C (Fotadar *et al.*, 2005). In optimal growing conditions, the rate of cell division of the *E. coli* bacteria is very fast; the number of bacterial cells can double every 20 minutes (Schulze *et al.*, 2006).

E. coli can be isolated from the intestines of humans and animals, but represent less than 1% of the total intestinal bacterial biomass of humans and warm blooded animals (Todar, 2007). The bacterium is ingested in foods, water or obtained directly from other individuals. Because it is ubiquitous in human and animal feces and is also able to survive outside the body, the presence of *E. coli* is considered to be an indicator of fecal contamination (Bopp *et al.*, 2005). Its detection indicates the presence of fecal contamination, and therefore the possible presence of other pathogenic microorganisms.

E. coli strains are essentially avirulent and are part of the intestinal flora (Todar, 2007), but some strains such as serotype O157: H7 are virulent and can cause serious food poisoning in humans (Vogt and Dippold, 2005). In 1945, Enteropathogenic *E. coli* was first isolated after a massive diarrhea outbreak in children in Great Britain (Kaper *et al*., 2004). Since then, a series of *E. coli* strains have been isolated and classified into categories of virulence. Enterotoxigenic *E. coli* was recognized as a cause of human diarrheal illness in the 1960s. Enteroinvasive *E. coli* strains were first shown to be capable of causing diarrhea in volunteer studies conducted in 1971 (Nataro and Kaper, 1998). Enterohaemorrhagic *E.*

coli or Shiga toxin producing *E. coli* was recognized as a distinct category of intestinal pathogenic *E. coli* in 1982 (Kaper *et al*., 2004). Enteroaggregative *E. coli* and diffusely adherent *E. coli* have been more recently isolated.

2.1.1.2 Effects on human health

E. coli was first detected as the cause of a diarrheal outbreak in children in 1935 and it was many years later considered to be a commensal organism of the intestinal microflora (Todar, 2007). Human diseases caused by pathogenic *E. coli* strains are divided into three types based on the infection they can result in: intestinal diseases, urinary tract infections, and neonatal meningitis. Although *E. coli* is best known for its ability to cause intestinal diseases, uropathogenic *E. coli* causes 90% of the urinary tract infections that affect millions of people each year (Todar, 2007) and meningitis-causing *E. coli* produces meningitis in neonates that cause many newborns die and who survive sustain permanent brain damage (Tiskumara *et al.*, 2009).

Enterotoxigenic *E. coli* (ETEC) is considered as a major bacterial pathogenic cause of diarrhea in travelers and children in developing countries. Approximately 200 million diarrhea cases and several of thousands of deaths each year are linked to ETEC (Qadri *et al*., 2005). ETEC usually enters the body through the consumption of food or contaminated

drinking water. It causes watery diarrhea that lasts for 3 to 7 days on average and sometimes longer resulting in dehydration and malnutrition in infants (WHO, 2009).

Enteropathogenic *E. coli* (EPEC) strains remain the most prevalent enteropathogen isolated from children under 2 years old in low income countries (Nguyen *et al*., 2006). It can cause persistent diarrhea that lasts for two weeks or more, leading to weight loss, malnutrition and even death (CIDRAP, 2006). These bacteria spread to humans through contact with contaminated water or infected animals.

E. coli O157:H7 and other serotypes belong to Shiga toxin producing *E. coli* (STEC also known as enterohemorrhagic *E. coli*). *E. coli* O157 causes at least 80% of the cases of hemolytic uremic syndrome in North America and they are the main cause of bloody diarrhea in developed countries (Lynn *et al*., 2005). Food containing raw milk, sausage, roast beef, untreated water, apple cider, raw vegetable, salads and mayonnaise are common sources of infection. Illness caused by STEC often results in bloody diarrhea, from mild to severe (Bopp *et al.*, 2005).

Enteroinvasive *E. coli* (EIEC) strains invade colon cells and produce a generally watery but occasionally bloody diarrhea. EIEC is very rare in North America and is much less common than ETEC or EPEC in developing countries (Kaper *et al.*, 2004). Human has

been known as a reservoir for this pathotype and contaminated food has been considered as one among the vectors.

Enteroaggregative *E. coli* (EAEC) was isolated from children with diarrhea during an outbreak in Japan (Itoh *et al.*, 1997). It was also involved in a massive diarrheal outbreak in children in Chile, Mexico, Kenya and India and associated with chronic diarrhea among human immunodeficiency virus infected patients (Cennimo *et al.*, 2007). Exposure to contaminated food and person-to-person contact are the causes of EAEC diarrheal illness.

Diffusely adherent *E. coli* (DAEC) also causes diarrhea in the developing world, particularly among children through consumption of contaminated food, drinking water or contact with people who are carrying the pathogens (Kaper *et al*., 2004).

Neonatal meningitis *E. coli* causes meningitis in neonates. They are transferred from the mother to the neonate during birth, and can trigger meningitis in the newborn child. The majority of cases of *E. coli* meningitis are associated with a serotype of *E. coli* that contains a capsular antigen called K1 (Tiskumara *et al.*, 2009).

2.1.2 *Salmonella*

2.1.2.1 Features, history

Salmonella is a member of the *Enterobacteriaceae* family, a Gram-negative, non-sporulating, rod-shaped, facultative anaerobic, with a diameter of about 0.7-1.5 µm, and a length of about 2.0-5.0 µm. The optimal growth temperature of *Salmonella* is between 35-37°C. They are chemoorganotrophs which oxidize organic substances to obtain carbon and energy (Bopp *et al.*, 2005). *Salmonella* is found in the intestines of humans and animals, and also in water, soil and plants. Sometimes it can be found in food, particularly meat and eggs (CIDRAP, 2006). *Salmonella* organisms can survive in water or in soil from several weeks to years if conditions of temperature, humidity, and pH are favorable *(*Todar, 2007).

Currently, there are three species recognized in the genus *Salmonella*: *Salmonella enteric*, *Salmonella bongori,* and *Salmonella subterranea. Salmonella enterica* has six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, *S. enterica* subsp. *indica* (Euzéby, 2005). These subspecies are differentiated on the basis of biochemical traits and genomic similarities and contain multiple serotypes (Bopp *et al.*, 2005). The DNA of the *Salmonella* genus is closely related to another genus of the *Enterobacteriaceae* family: *Escherichia (*Todar, 2007).

There are over 2579 *Salmonella* serotypes based on the presence of three major types of antigens: somatic (O) or cell wall antigens; flagellar (H) antigens; and surface antigens (Todar, 2007). More than 99.5% of the *Salmonella* strains isolated from humans and other warm blooded animals belong to *S. enterica* subsp. *enterica* strains. *S. enterica* subspecies and *S. bongori* are isolated from cold blooded animals and the environment (Josefsen *et al.*, 2011).

2.1.2.2 Effects on human health

In humans, *Salmonella* is the cause of diseases called salmonellosis: typhoid resulting from bacterial invasion of the bloodstream, and acute gastroenteritis resulting from a foodborne infection (Josefsen *et al.*, 2011).

Typhoid fever is a serious bloodstream infection associated with *S*. Typhi. The symptoms often present as prolonged high fever, fatigue, weakness, and headache, without diarrhea. It is transmitted through contact with an infected person or fecal contaminated food or water (Pegues *et al.*, 2005). Typhoid fever is endemic in poor and developing countries. In fact, 80% of reported cases come from Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, and Vietnam (Chau *et al.*, 2007). In North America, most cases of typhoid fever are linked to international traveling (Lynch *et al.*, 2009).

Non-typhoidal *Salmonella* usually causes gastroenteritis similar to that caused by other bacterial enteric pathogens with symptoms like abdominal cramps, diarrhea, and fever occurring within 8 to 72 hours after contaminated food is eaten (Klotchko and Wallace, 2009). Symptoms are usually mild, however for those at risk such as infants, the elderly and those with weakened immune systems, *Salmonella* infections can become very serious and lead to death. Some common isolates are *S*. Enteritidis, *S*. Typhimurium, *S*. Newport, *S*. Javiana, *S*. Heidelberg, *S* Montevideo. The main reservoirs for non-typhoidal *Salmonella* are animals such as poultry, livestock, pests and reptiles. *Salmonella* infection is usually related to the consumption of food or water contaminated by animal feces. An estimated 96% of all *Salmonella* cases are caused by foods (Mead *et al.*, 1999).

2.2 Plant indigenous microflora

Plants provide habitats for many species of microorganisms such as bacteria, yeasts and filamentous fungi (Lindow and Brandl, 2003). Bacteria density is often found in the range of 10^6 to 10^7 cells/cm^2 of leaf (Andrews and Harris, 2000). Yeasts that also often colonize in this habitat have density lower than bacteria. Filamentous fungi are not regularly present. Gram-positive bacteria are more numerous than Gram-negative bacteria (Zhang *et al*., 2010). Most of these bacteria are non-pathogenic organisms and often play important roles in influencing the environment and the health of the host plant, even contributing to carbon and nitrogen cycles. The distribution of bacterial populations on

plants is different depending on crop and bacterial species. For example, pigmented bacteria colonize leaf surfaces to access the sunlight necessary for their activity. For this reason, they are rarely found in the rhizosphere (Lindow and Brandl, 2003). According to Zhang *et al* (2010), bacterial populations on spinach and rape phyllosphere were larger compared to celery, broccoli, and cauliflower.

2.3 Interaction between human pathogens and plant indigenous microflora

Human pathogens, principally, *E. coli* O157:H7 and *Salmonella* are transient residents of plants. To survive and grow in the plant environment, human pathogens have to compete with indigenous members of plant microbial communities (Brandl, 2006) for nutrition, energy and colonization on the host. In this competition, the advantage belongs to the microorganisms that have the ability to absorb nutrients more quickly, to cope with more extreme conditions or be able to restrict the activities of other antagonistic microorganisms (Beattie and Lindow, 1994).

Study results in the laboratory found that the interation between pathogens and other members of plant indigenous microflora can result in an inhibition or an enhancement of their growth. In many cases, interaction of human pathogens with the members of plant pathogens is beneficial to their growth on plants. *Cladosporium* spp and *Alternaria alternata* enhances the growth of *S. enterica* in tomatoes (Wade and Beuchat, 2003). Well

and Butterfield (1997) observed an increase of *S.* Typhimurium numbers in samples of rotten potatoes, carrots, and peppers caused by *Pseudomonas viridiflava* and *Erwinia carotovora*. After an incubation time of 72 hours at room temperature, *S.*Typhimurium numbers in these samples were higher than in healthy samples by approximately 3 and 10 times, respectively.

Conversely, the growth of human pathogens can be inhibited by the interaction with plant microflora. Janisiewski *et al* (1999) observed that high concentrations of *Pseudomonas syringae* limited the proliferation of *E. coli* O157 in plant lesions due to competition for carbon and energy sources. When *Pseudomonas syringae* was not present, there was at least a 2-log increase in the numbers of *E. coli* O157. *Pseudomonas* strains also acted as growths inhibitors for one or more of the following pathogens: *Staphylococcus aureus, E. coli* O157*, S.* Montevideo*,* and *Listeria monocytogenes* on shredded lettuce (Schuenzel and Harrison, 2002). Liao and Fett (2001) tested 120 strains of indigenous microflora from green bell peppers, romaine lettuce, pre-peeled baby carrots and sprouting seeds (alfalfa and clover) for their ability to inhibit the growth of *Salmonella* Chester, *Listeria monocytogenes*, *Escherichia coli*, or *Erwinia carotovora*. They found that six isolates had the ability to inhibit the growth of one or more pathogens: *Bacillus* spp. (3 strains), *Pseudomonas aeruginosa* (1 strain), *Pseudomonas fluorescens*, and yeast. *Pseudomonas fluorescens* and yeast reduced the growth of *S.* Chester and *Listeria monocytogenes* by 1 and 2 logs respectively over a period of 3 days.

In the same conditions, the interactions between members of plant microflora can cause diverse effects. Riordan *et al* (2000) found that the presence of *Penicillium expansum* in apple wounds inoculated with *E. coli* O157:H7 made the levels of this strain decline from 3 to less than 1 log at 4°C or room temperature. However, in the presence of *Glomerella cingulata* at room temperature, *E. coli* O157:H7 numbers increased from 3.18 to 6.81 log (Riordan *et al.*, 2000). *Listeria monocytogenes* was co-inoculated with one of four plant pathogens (*Pseudomonas fluorescens, Pseudomonas viridiflava, Erwinia carotovora and Xanthomonas campestris*) on potato tuber slices. The growth of *Listeria monocytogenes* was strongly inhibited by *Pseudomonas fluorescens* and *Pseudomonas viridiflava* but was not affected by *Erwinia carotovora* or *Xanthomonas campestris.* Competition for iron via production of siderophores was suggested as a possible mechanism for the antagonism of *Pseudomonas* (Liao and Sapers, 1999).

The interaction between the microorganisms of plant microflora can be affected by the application of chemicals. For example, the application of hydrogen peroxide reduced the commensal microflora of broad leaved endive (Carlin *et al.*, 1996), leading to an increase in *Listeria monocytogenes* populations. Abamectin, an insecticide, significantly affected the bacterial community on broccoli leaves. It decreased bacterial biomass and altered the proportion of Gram-negative and Gram-positive bacteria, favouring the Gram-negative ones (Zhang *et al.*, 2009b).

2.4 Factors affecting persistence of human pathogens on vegetables

The survival and growth of human pathogens on vegetables depends on many factors such as temperature, pH, moisture, sunlight, vegetable type, chemicals disinfections, and competition with other microorganisms on vegetable.

In a study on the survival and growth of *E. coli* O157:H7 on vegetables, the influence of storage temperature and vegetable type on survival and growth of *E. coli* O157:H7 was determined. Populations of *E. coli* O157:H7 declined 1 to 4 logs on vegetables stored at 5°C, but increased 1 to 3.3 logs on vegetables stored at 12 and 21°C. The most rapid increases in O157:H7 populations occurred on lettuce and cucumbers stored at 21°C. However, no growth was observed on shredded carrots under the same conditions (Abdul-Raouf *et al.*, 1993).

In another study investigating the effects of storage temperature on growth and survival of *E. coli* O157:H7 on vegetables (lettuce, Swedish turnip, dry coleslaw mix, soybean sprouts), populations of *E. coli* O157:H7 were found to have increased 1.5 to 2.5 logs during a 12 days storage period on shredded lettuce (8°C). *E. coli* O157:H7 populations on packaged coleslaw and soybean sprouts increased by 1.5 to 2.5 logs up to day 5, but declined during subsequent storage at 8°C. Reducing the storage temperature from 8 to 4°C reduced the growth of *E. coli* O157:H7 on ready to use packaged vegetables.

However, viable populations remained at the end of the storage period at 4°C (Francis and O'Beirne, 2001).

Cooler temperature promotes the survival of pathogenic microorganisms. In one study, *S.* Enteritidis survived in all restaurant-made salsa samples stored at room temperature (20°C) and survived longer in refrigerated samples (4°C) than in samples stored at room temperature after 24 hours (Franco *et al.*, 2010). *E. coli* O157:H7 and *Salmonella* can survive on frozen cut pineapple at - 20°C for at least 180 days but did not grow at each of the studied temperatures (4°C, 12°C, and 23°C) (Strawn and Danyluk, 2010).

Other studies also suggested that bacteria could survive but could grow only slowly or be inactive at low temperature. *S.* Montevideo survived on the surfaces of whole tomatoes stored at 10°C for 18 days, but increased within 7 days and within 1 day when stored at 20 and 30°C, respectively (Zhuang *et al.*, 1995). This pathogen had remained unchanged in chopped tomatoes stored at 5°C and at a 4.1 pH for 9 days but increased significantly after storage for 96 or 22 hours at 20 or 30°C, respectively (Zhuang *et al.*, 1995).

In a study evaluating the survival and growth of five *Salmonella* strains on whole and chopped vegetables at 4, 12, and 21°C for 7 days (Ma *et al.*, 2010), it was found that

Salmonella did not grow at 4°C, but survived on whole tomatoes and jalapeno peppers. Significant growth at 12 and 21°C was observed on whole cilantro and on all chopped vegetables, with chopped jalapeno peppers being the most supportive for *Salmonella* growth. Regardless of differences in salsa formulation, no growth of *Salmonella* was observed in salsa stored at 4°C.

In a recent study, the persistence of *Salmonella* spp. and *E. coli* on whole and sliced zucchini squash at 25°C and 3 to 5°C was tested. Both *Salmonella* and *E. coli* grew when inoculated onto sliced squash after 24 hours at 25°C. At 3 to 5°C, the bacterial growth was inhibited but they still survived (Castro *et al.*, 2010).

Low pH in vegetables was found to correlate with decreases in *E. coil* O157:H7 populations and naturally occurring microflora. *E. coli* O157:H7 survived well at pH values of 3.4 to 6.8 at 4°C, but the number of damage cells increased as pH decreased and incubation time increased. At 37°C, *E. coli* O157:H7 was inactivated at pH less than or equal to 3.6, but could grow at pH above 4.7 (Han and Linton, 2004).

The shorter survival times of pathogenic bacteria on crops in comparison to survival time in water and soil, reflect the increased exposure to sunlight and subsequent desiccation of pathogens on crop surfaces (Steel and Odumeru, 2004). Survival of *S.* Montevideo on leaves of tomato plants was greatly affected by relative humidity. Reductions of 3-4 log

CFU/leaf occurred when leaves were dried after inoculation. When leaves growth was supported by a hydroponic nutrient medium and incubated at 100 percent relative humidity, there was no significant reduction (Rathinasabapathi, 2004).

Chemicals disinfections also affect the persistence of human pathogens. The mixture of copper and sodium hypochlorite can reduce by 5 logs *Listeria monocytogenes* and *E. coli* O157:H7 counts (Rodgers and Ryser, 2004).

2.5 Outbreaks of human pathogens associated with vegetables

Although fresh fruits and vegetables are implicated less frequently than meat, meat products, eggs, dairy products and seafood in outbreaks, *E. coli* and *Salmonella* infection from consumption of fruits and vegetables have been often documented.

Most recently, a large outbreak occurred in Europe where German scientists found the *E. coli* strains that caused 46 deaths and more than 3900 illnesses in bean sprouts (ECDC, 2011). In 2005, a large outbreak involving *E. coli* O157:H7 was associated with lettuce. One hundred and thirty-five cases were confirmed, including 11 cases of hemolytic uremic syndrome. All these cases were infected by the *E. coli* strain after consumption of lettuce in contaminated water (Soderstrom *et al.*, 2008). In 2006, two outbreaks of foodborne illness caused by *E. coli* occurred back to back in the US. One outbreak caused

199 cases of infection, 3 deaths and 31 cases of hemolytic uremic syndrome after spinach contaminated with *E. coli* O157:H7 was consumed (CDC, 2006b). Another outbreak linked to *E. coli* O157:H7 involved in contaminated lettuce in Taco Bell restaurants that caused 71 infections, with 52 cases due to the same *E. coli* strain, 8 cases of hemolytic uremic syndrome (CDC, 2006a). One of the largest outbreaks occurred in 1996, resulted in about 9451 cases of *E. coli* O157:H7 infection in Japan and caused 12 deaths. Epidemiological investigation suggested that hydroponically grown radish sprouts were the suspect food. This raised concerns about transmission of *E. coli* O157:H7 through sprouts (Hara-Kudo *et al.*, 1997).

Salmonella outbreaks resulting from consumption of vegetables have also been recorded. In 2008, outbreak of *S.* Saint-Paul involving jalapeno and serrano peppers was the largest foodborne outbreak in more than a decade in the US. At least 1442 infected cases in 43 US states, 286 hospitalized cases and two deaths were recorded. The investigation showed that Mexican-grown raw jalapeno and serrano peppers were the major vehicles by which the pathogens were transmitted. Irrigation water on the farm was pinpointed as the contamination source (CDC, 2008). In 2007, an outbreak of *S.* Wandsworth was linked to contaminated seasoning mix used in Veggie Booty, causing 65 cases of infection. Six patients were hospitalized, no deaths were reported (Clark, 2009). In 2006, *S.* Typhimurium associated with tomatoes caused a multistate outbreak in the US, with 190 confirmed cases of infection, 24 cases of hospitalization, no deaths (Behravesh *et al.*, 2012). *S.* Javiana and

S. Braenderup contaminated tomatoes also caused many food poisoning cases in Canada and the US in 2004 (CDC, 2005a).

2.6 Microbial contamination sources for vegetables

Vegetables can be contaminated by pathogenic bacteria throughout the entire process, from planting to processing to consumption. Potential pre-harvest and post-harvest sources of contamination are shown in Table I.

Table I. Potential sources of pathogenic microorganisms and conditions influencing their survival and growth on fresh produce.

Period	Source
Pre-harvest	Soil Irrigation water Green or inadequately composted manure Air (dust) Wild and domestic animals Human handling Water for other uses (for example, pesticides, foliar treatments)
Post-harvest	Human handling (workers, consumers) Harvesting equipment Transport containers (field to packing shed) Wild and domestic animals Air (dust) Wash and rinse water Sorting, packing, cutting and further-processing equipment Ice Transport vehicles Improper storage (temperature, physical environment) Improper packaging (including new packaging technologies) Cross contamination (other foods in storage, preparation areas) Improper display temperature Improper handling after wholesale or retail purchase Cooling water (for example, hydrocooling)

Source: Beuchat, 1996

2.7 Human pathogens and contaminated water

Contaminated water is a source of pathogens found on vegetables. Solomon *et al* (2002) found that contaminated soil and irrigation water are could be a source of contamination for lettuce plants by showing how the *E. coli* O157:H7 migrate throughout the plant. *E. coli* could associate with cabbage crops irrigated with contaminated creek water (Wachtel *et al.*, 2002). Six different *E. coli* serotypes on cabbage roots were identified. They were correlated well with the serotypes in the irrigated water. *Salmonella* associated with stems and leaves of tomato plants grown hydroponically in inoculated nutrient solution has also been reported (Guo *et al.*, 2002). Pathogens transferred from contaminated water to vegetables were the cause of many foodborne illness outbreaks such as the case of *Salmonella* Newport associated with tomatoes (Greene *et al.*, 2008) and *E. coli* O157:H7 contaminated spinach (CDC, 2006b), both of which were irrigated by contaminated water.

2.7.1 Presence of human pathogens in water

The presence of human pathogens in water has been shown in many studies. For example, 9.4% of water samples contained *Salmonella* in a study on the risk factors involved in pathogenic bacteria contamination in Texas (Duffy *et al.*, 2005). Some bacterial pathogens commonly found in water such as *Campylobacter* spp., *E. coli* 157: H7,

Salmonella spp., *Shigella* spp. are also known as fecal-associated pathogenic bacteria. They can be transmitted from water to vegetables through irrigation or spraying pesticide.

The microbial quality water varies with water source. Groundwater is less contaminated by microbial pathogens because of its location deep in the ground where there are relatively impermeable rock aquifers protected from pollution (Borchardt *et al*., 2007). However, it can be also contaminated by the introduction of surface water. A recent study was conducted to evaluate possible contamination of untreated and treated groundwater by fecal coliform and *E. coli* in 100 randomly selected boreholes from different parts of Ibadan metropolis (Nigeria). Data showed that 73% of the borehole water samples had coliform and 18% had detectable *E. coli* (Olusegun, 2010).

Surface water can be contaminated by waste water from cities, industry, agriculture and wildlife, so it is very susceptible to becoming contaminated by pathogenic microorganisms. For example, to investigate a cause of an outbreak in Connecticut (USA), samples from sediment, drinking water, lake water, and ice were obtained and cultured for *E. coli* and tested for Shiga- toxin. Shiga-toxin produced by *E. coli* serotype O121 was detected from lake water (McCarthy *et al.*, 2001). A survey of surface waters in southern Alberta (Canada) looking for the presence of *E. coli* O157:H7 and *Salmonella* showed that prevalence of *E. coli* O157:H7 and *Salmonella* in water samples was 0.9% and 6.2%, respectively (Johnson *et al.*, 2003).

Wastewater and sewage sludge is highly contaminated. In large population and low income countries, incidence can be high. In Vietnam, the presence of *Salmonella* was detected in 70% of untreated septage and 60% of sewage sludge samples (Yen-Phi *et al*, 2010), that should be discharged into the sewage system.

2.7.2 Factors influencing persistence of human pathogens in water

Factors influencing the persistence of pathogenic microorganisms in water and sewage include temperature, pH, organic content, and exposure to sunlight (Feachem *et al.*, 1983).

E. coli O157 could survive longer in natural mineral water than in distilled deionized water and sterilized natural mineral water because it contains nutrients and minerals. With an inoculum of 10^3 CFU/mL, the persistence of the pathogen at 15°C was 70 days for natural mineral water, 49 days for the sterilized natural mineral water and 21 days for distilled deionized water (Kerr *et al.*, 1999).

Temperature also influences bacteria survival time in water. In groundwater, bacteria survived longer than in surface water because groundwater tends to be cooler and the bacteria in groundwater experienced less microbiological and biological activity (Feachem *et al.*, 1983) and they are protected from sunlight. From 20 to 30°C, bacteria such

as *E. coli* and *Salmonella* usually live for 30 days in water and sewage sludge (Feachem *et al.*, 1983). However, *E. coli* O157:H7 in 27 organic wastes originating from slaughterhouses, waste water treatment plants, creameries and farms was observed to be still viable after 64 days, at an incubating temperature of 10°C (Avery *et al.*, 2005).

2.7.3 Survival and growth of human pathogens in pesticide solutions

Although human pathogens can survive in some pesticide solutions, laboratory experiments showed that the effect of pesticides on human pathogens is quite variable. Some pesticides can promote the growth of microorganisms. Populations of *Salmonell*a, *Shigella*, *Listeria* and *E. coli* O157: H7 in solutions of Afolan, Bravo 500, Lorsban 4E and Ambush 500EC for 96 hours at 22°C were increased 100-1060 times. The growth of *E. coli* in pesticide solutions was affected by changes in the inoculum level of *E. coli* O157:H7 from 10^2 to 10^4 CFU/mL, by temperature (from 20 - 22 to 31°C) and by concentration (0.5 to 1.5 times) (Guan *et al.*, 2001). Ten types of pesticides were examined for their capacity of supporting microbial contaminant at 30°C for 48 h. The results showed that two pesticide solutions, Kumulus and Pirimor, dissolved in sterile water, supported the survival and growth of *Pseudomonas*, *Salmonella* and *E. coli*, whereas nine pesticide solutions diluted or dissolved from different sources of agricultural water supported the growth of these bacteria (Peter *et al.*, 2005). At 21°C for 96 h, a solution of the pesticide Bravo 500 could support all inoculated bacteria survival or growth. Among tested bacteria, *Salmonella*

spp. was able to survive in the pesticide solutions, but its survival varied depending on the type of formulation, incubation temperature and pesticide concentration (Guan *et al.*, 2005).

The effect of pesticides on human pathogens highly depends on the pesticide concentration. Abamectin 1.8% decreased bacterial biomass and altered the proportion of Gram-negative and Gram-positive bacteria at a dose recommended by the manufacturer. However, reducing the dose of abamectin by half did not cause any significant effects on bacterial community (Zhang *et al.*, 2009b).

2.8 Pesticides

Pesticide refers to any substance or mixture of substances intended for preventing, destroying or controlling any pest. Three types of pesticides are commonly used in agriculture production including fungicides to kill fungi, herbicides to kill weeds and insecticides to kill insects. According to British Crop Production Council (BCPC), there are 908 pesticide active ingredients worldwide (BCPC, 2009). From these active ingredients, ten of thousand of pesticides are formulated. In the US, approximately 16,000 pesticide products are registered with the Environmental Protection Agency (USEPA) stemming from about 600 active ingredients (CDC, 2005b).

Pesticides play a significant role in agricultural production, preventing crop damage from pests and reducing yield losses. Production yield can be reduced by up to 10% when pesticides are not used (Kuniuki, 2001). However, pesticides have also had an adverse impact on humans and the environment. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, soil and water (Miller, 2004). Pesticides applied to crops can evaporate and cause air and water pollution. According to Gilliom *et al* (2007) pesticides were found to be present in most stream water samples and over 90% of wells sampled in the US. Many pesticides are persistent soil contaminants which decrease soil bio-diversity (Pal *et al.*, 2005). Pesticides may also cause massive death tolls in bees, birds and fish (Boone *et al.*, 2007; Whitehorn *et al.*, 2012; Mohammad *et al.*, 2008). Exposure to pesticides causes acute or chronic toxicity for humans (Mishra *et al.*, 2009). These effects can range from simple skin and eye irritation to serious effects such as damaging the nervous system and causing mutations, teratogenic effects, or cancer (EHC 83, 1989; Mishra *et al.*, 2009).

Besides the pest-control effect of pesticides, their adverse effects on non-target organisms have been recognized by scientists. Some studies demonstrated the effect of pesticide application on microbial diversity (Wang *et al.*, 2012; Krauss *et al.*, 2011). Some pesticides promote microbial activity and biomass by adding nutrients such as carbon or nitrogen to microorganisms (Cycon and Piotrowska, 2009; Perucci *et al.*, 2000; Wardle and Parkinson, 1990; Zelles *et al.*, 1985). For example, under field conditions, microbial

biomass in soil increased following an application of fosthiazate (containing nitrogen and phosphorous) (Eisenhauer *et al*., 2009). Other pesticides decreased microbial activity, biomass or populations (Vig *et al*., 2008) due to the direct toxicity of some pesticides for microorganisms, among other reasons. According to Zhang *et al* (2009), when applied alone, copper stressed microbial communities, but the mixture of copper and cypermethrin (a pyrethroid insecticide containing nitrogen) increased microbial biomass carbon.

2.8.1. Pesticide types

2.8.1.1 Chemical pesticides

2.8.1.1.1 Organic pesticides

Chemical pesticides include inorganic and organic pesticides (which are largely synthetic) for crop protection (Unsworth, 2010). The main elements of organic pesticides are carbon and hydrogen in combination with other elements such as oxygen, nitrogen, phosphorus, sulfur or chlorine. The first synthetic organic pesticides developed were organochlorine compounds (Daly *et al.*, 1998). Other classes of synthetic pesticides include organophosphorus, carbamates, and pyrethroids (Figure 1). Pyrethroid includes synthetic pesticides that were modeled on pyrethrins of chrysanthemums. Pyrethroids are also toxic

to the nervous system but are safer for humans than organochlorines, organophosphorus, and carbamates (BCPC, 2009).

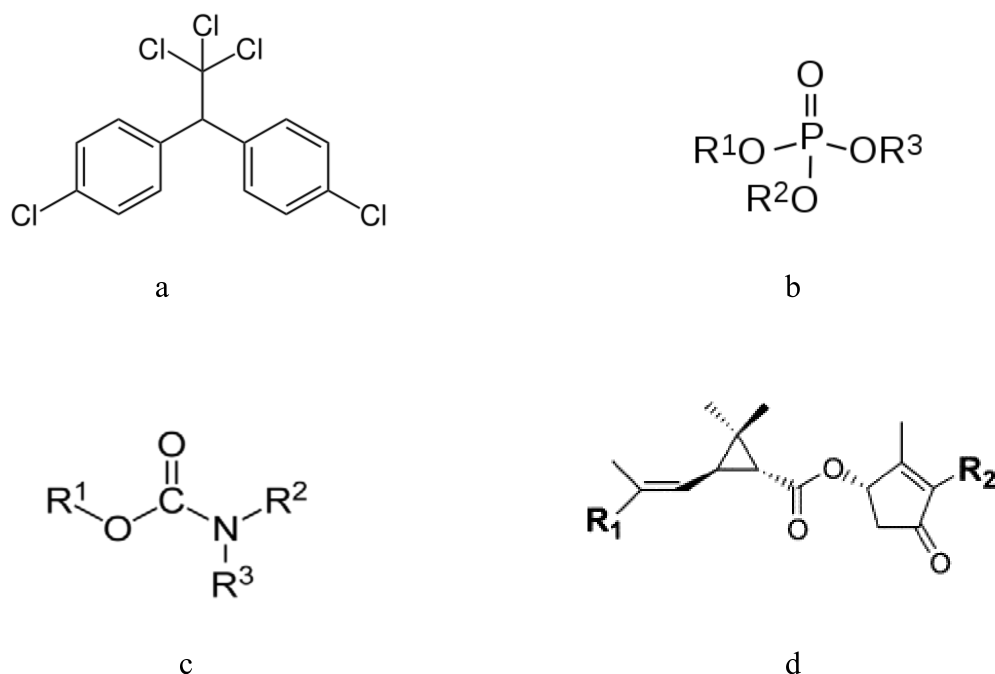


Figure 1. Chemical structure of a) DDT (an organochlorine insecticide) b) organophosphorus c) carbamates d) pyrethrins (R is methyl, ethyl or another functional group). (Source: EHC 63, 1986; EHC 64, 1986; EHC 83, 1989; Kazuhiko *et al.*, 2005)

Cypermethrin is a pyrethroid insecticide (Figure 2). Its chemical formula is $C_{22}H_{19}O_3NCl_2$ and its molecular weight is 416.3 g/mol. Its colour is yellow, physical state varies from a viscous liquid to a semi-solid that has a viscosity and rigidity intermediate between that of a solid and a liquid. It melts at 80°C and decomposes at 220°C. Its density at 22°C is 1.12 g/mL. It is slightly soluble in water (0.009 mg/L) and soluble in organic

solvents (hexane 103 g/L, xylene > 450 g/L). Its vapour pressure at 20°C is 1.4 x 10^{-9} mmHg (EHC 82, 1989).

Cypermethrin is moderately toxic to mammals. In rats, LD_{50} varies from 250 to 4000 mg/kg depending on the solvents used in the test and on the proportions of cypermethrin's isomers (Cox, 1996). It is irritating to the skin and eyes of rats and causes allergic skin reactions for guinea pigs. Cypermethrin does not cause teratogenic effects or mutations and no adverse effects on reproduction were observed in tests with rats, mice and dogs (EHC 82, 1989). EPA has classified cypermethrin as a possible human carcinogen because it causes lung tumors in female mice. In animals, cypermethrin is rapidly metabolized by the action of cytochrome P450 enzymes producing hydroxylated metabolites (Kasai, 2004), with over 99% being eliminated within hours. The remaining 1%becomes accumulated in fat tissues. This portion is eliminated slowly, with a half-life of 3.4-18 days for the trans-isomer and the cis-isomer (USEPA, 1989).

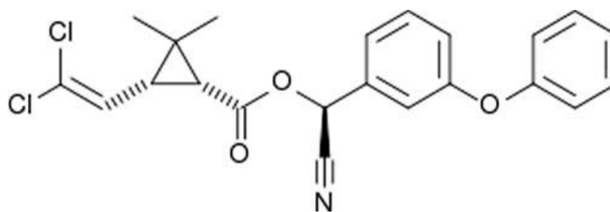


Figure 2. Chemical structure of cypermethrin

Cypermethrin has low avian toxicity. Its acute oral LD_{50} in mallard ducks is greater than 10,000 mg/kg and dietary LC_{50} in mallards and bobwhite quail is greater than 20,000 ppm. Cypermethrin is highly toxic to fish and aquatic organisms. LC_{50} in rainbow trout in 96 hours is 0.82 ppb and LC_{50} in *Daphnia magna* is 0.26 ppb (USEPA, 1989).

Cypermethrin is used to control many pests, including moth pests of cotton, fruit, and vegetable crops, by acting on the central nervous systems of insects. Cypermethrin prolongs the transient increase in sodium permeability of the nerve membrane by causing sodium channels gating to stay open longer than normal, resulting in repetitive activity in the nervous system. In turn, nerve continuously releases the neurotransmitter acetylcholine lead to acetylcholine depletion, fasciculations, and muscular weakness (Cox, 1996).

In soil under aerobic conditions, the half-life of cypermethrin is from 4 days to 8 weeks. Cypermethrin is more persistent under anaerobic conditions. It breaks down rapidly with a half-life of 8 to 16 days when exposed to sunlight. Cypermethrin is slightly soluble in water and has the tendency to adsorb in soil particles. In neutral or acid solutions, cypermethrin is slowly hydrolyzed (EHC 82, 1989). This process is more rapid in alkaline solutions (EHC 82, 1989). On wheat, residue of cypermethrin was 4 ppm immediately after spraying and 0.2 ppm after 27 days (USEPA, 1989).

2.8.1.1.2 Inorganic pesticides

Inorganic pesticides often do not contain carbon atoms in their composition although some inorganic pesticides contain carbon in the form of cyanide or carbonate. They occupy a very small percentage of chemical pesticides. Some inorganic substances are used as pesticides such as copper compounds and sulfur; the antibacterial activity of copper compounds has been known for a long time (Dollwet and Sorenson, 2001).

In crop protection, copper sulfate is used as an inorganic fungicide. Its chemical formula is $CuSO_4$, and its molecular weight is 159.6. It consists of grayish-white to greenish-white rhombic crystals that melt and slowly decompose at 200°C and completely breaks down into copper oxide and sulfur trioxide at 650°C. It is soluble in water (143 g/L, at 0°C) and insoluble in ethanol (EHC 200, 1998).

Copper sulfate is moderately toxic upon acute oral exposure. The acute oral LD_{50} for copper sulfate in rats is 300 mg/kg (EHC 200, 1998). It can be corrosive to the skin and eyes. It is considered as a skin sensitizer and can cause allergic reactions. The acute dermal LD_{50} is greater than 1124 mg /kg in rats. The inhalation LC_{50} is greater than 1303 mg /kg in rabbits (EHC 200, 1998). Studies on its neurotoxicity have not shown any effects on behavior with doses of 20 to 40 mg/kg per day (EHC 200, 1998). Copper sulfate does not

cause reproductive problems, developmental toxicity, mutagenic effects or carcinogenic effects.

In humans and warm blooded animals, copper sulfate is absorbed into the blood under the acidic conditions of the stomach. After ingestion, more than 99% of copper is eliminated through excretion. It is distributed primarily in the liver, brain, heart, kidneys and muscles (Extonet, 1994).

Copper sulfate is very toxic to aquatic life. LC_{50} (96 h) ranges from 3-7340 μg/L for freshwater fish and from 60-1400 μg /L for marine fish. It is nontoxic to birds. Copper sulfate treatment causes a decrease in the population of earthworms (EHC 200, 1998).

In soil, copper sulfate is mobile due to the high solubility in water. It has low leaching potential because it can bind to soil particles. When applied with irrigation water, copper sulfate does not accumulate in the surrounding soil. It binds to organic and mineral particles. In water, it binds to suspended particles and sediment (Extonet, 1994).

Copper sulfate is used against a number of diseases caused by fungi such as mildew, black spot and white mold. The fungicidal mechanism of copper compounds is the disruption of the metabolism of cells by replacing essential ions. Excess copper causes the inactivation of enzymes responsible for the destruction of lignocellulosic materials; it

damages cell membranes, leading to the interruption of nutrient transport into and out of the cell, causes the misregulation of protein functions, and release hydrogen peroxide free radicals that attack amino acids (Nies, 1999), leading to cell death.

2.8.1.2 Bio-pesticides

Bio-pesticides are substances from natural sources such as plants, fungi and bacteria. Microorganisms including bacteria, fungi, viruses, and protozoa that affect pest populations are also considered as bio-pesticides. Bio-pesticides fall into three major classes: microbial pesticides, biochemical pesticides and plant-incorporated protectants. As of 2010, there were 32 microbial pesticides registered in Canada. Twelve of which are bacterial species (Kabaluk *et al.*, 2010). According to the Ministry of Agriculture and Rural Development of Vietnam (MARD), in 2009, bio-pesticides accounted for more than 20% of all pesticides (MARD, 2009).

Microbial pesticide products contain living microorganisms (e.g., a bacterium, fungus, virus or protozoan) or the toxins they produce as active ingredients. Bacteria are the microorganisms most frequently associated with the control of insects. One hundred species are specifically entomopathogenic but only a few types have been considered for the production of bio-pesticides (Miller *et al.*, 1983).

The most widely used microbial pesticide is *Bacillus thuringiensis* (*B. thuringiensis*) which account for about 90% of the current bio-pesticide market (EHC 217, 1999). *B. thuringiensis*, a facultative anaerobic, Gram-positive bacterium, can be isolated from soil, insects, and plant surfaces. Each subspecie of *B. thuringiensis* specifically kills one or a few related species of insect larvae by producing a different mix of protein. For example, *B. thuringiensis* kurstaki is against moth and butterfly caterpillar; *B. thuringiensis* israelensis is against mosquito and blackfly larvae; and *B. thuringiensis* tenebrionis is against beetle larvae (Kabaluk *et al.*, 2010).

B. thuringiensis kurstaki is commonly found in soil, is completely biodegradable and does not persist in the digestive systems of birds or mammals (EHC 217, 1999). It produces a range of insecticidal toxins, of which the most important delta-endotoxins are known as Cry proteins in reference to their crystalline nature. Toxic crystals formed during bacterial sporulation are dissolved when the spores germinate in the insect gut and are activated by insect digestive enzymes. They bind to receptors in the membrane of midgut cells and affect formation of pores for uncontrolled ion movement (Yamamoto, 2001). The insect usually dies of starvation and septicemia rather than as a direct result of *B. thuringiensis* toxicity (Whalon and Wingerd, 2003).

Another strain also used as a microbial pesticide is *Bacillus subtilis* (*B. subtilis*). It is also a Gram-positive, aerobic, rod-shaped, motile bacterium able to produce endospores

and is commonly recovered from soil, water and air. Strain QST 713 can be used as a microbial fungicide to control *Sclerotinia sclerotiorum* on crucifer crops. Modes of action of *B. subtilis* QST 713 on fungi involve its competing for nutrients on the leaf surface and the antimicrobial activity of iturins, a pore-forming lipopeptide produced by *B. subtilis* causing changes of the cytoplasmic membrane and subsequent disruption of the pathogen cells (Edgecomb and Manker, 2006).

2.8.2 Water used for pesticide preparation

Pesticides are sold in several formulations such as Emulsifiable Concentrate (EC), Wettable Powder (WP), Soluble Concentrate (SL), Water Soluble Powder (SP), Aqueous Suspension Concentrate (SC), Water dispersible granules (WG), etc (JMPS, 2006). All these formulations need to be dissolved or diluted in water for application in the field by foliar spraying. The water volume used to apply these pesticides can range from hundred to thousand of liters per hectare according to manufacturer recommendations (Furness and Thompson, 2008).

Water may contain pathogens so it must be clean to prevent the spread of infectious diseases and environmental pollution and to ensure pesticide effectiveness. However, in many poor areas such as Vietnam, people still use water from rivers, lakes and canals to prepare pesticides, to irrigate crops or to wash vegetable products before being sold (Ha *et*

al., 2008). Report of International Water Management Institute (IWMI, 2006) showed that across 50 cities in Asia, Africa and Latin America, the use of untreated water in crop irrigation was a common reality in three-fourths of these cities.

2.8.3 Pesticide degradation

Pesticides may be absorbed by soil particles, vegetation, or other surfaces. The environmental degradation of pesticides occurs by either microbial degradation, chemical degradation or photodegradation (Figure 3). Pesticides may be broken down by the chemical reactions which hydrolysis is the most common reaction. Sunlight can break down pesticides that are on the surface of soil and above-ground parts of plants.

Microbial degradation of chemicals is one of the most important mechanisms for the breakdown of pesticides (Singh and Walker, 2006). It is the breakdown of chemicals by microorganisms into biomass and less complex compounds, and finally to water, carbon dioxide, the oxides or mineral salts. The substances degraded or transformed by microorganism are used as a source of energy, carbon, nitrogen. The rate of microbial degradation depends on numerous factors, including pesticide properties, activity of microorganisms, density of microbial community, moisture, sunlight, temperature and pH.

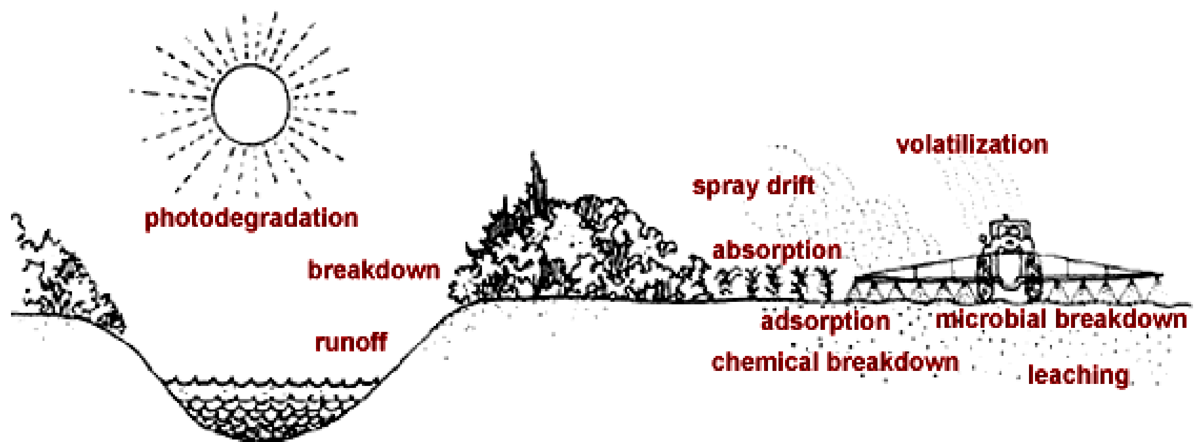


Figure 3. Environmental degradation of pesticides (Source: Ministry of Agriculture, British Columbia, Canada).

Temperature has a great influence on pesticide biodegradation since microbial degradation is mediated by enzymes and enzyme activity increase with higher temperatures. In fact, in the temperature range from 10 to 45°C, most reactions catalyzed by enzymes tend to double in rate for each 10 degree increase (Kerle *et al.*, 2007). This is also the favorable temperature range for growth of microorganisms.

Some bacteria participate in the decomposition process of cypermethrin in soil, such as *Pseudomonas flourescens, Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus cereus*, *Aspergillus niger, Klebsiella* sp., *Achromobacter* sp., *Ochrobactrum lupine, Streptomyces aureus.*In aerobic conditions, 84% of cypermethrin was metabolized in natural soil that contained the microorganisms, while only 8% was in sterilized soil (EHC 82, 1989).

Pseudomonas plays an active role in the biodegradation of cypermethrin. Cypermethrin's half-life time was estimated at 20 days when in presence of *Pseudomonas* under laboratory conditions, whereas it lasted from 4 days to 8 weeks under field conditions (Grant *et al.*, 2002). Cleavage of ester linkage to give 2,2-dimethyl-3(2,2-dichlorovinyl) cyclopropane-carboxylic acid (CPA), 3-phenoxybenzoic acid (PBA), and carbon dioxide is the main step in the biodegradation process of cypermethrin (Roberts and Standen, 1977).

Microflora composition can be changed by increasing the microbial decomposition process. Microbial populations of *Pseudomonas aeruginosa*, *E. coli* and *Klebsiella* sp. increased from 1.1 x 10^5 CFU/g to 118 x 10^5 CFU/g in 24 hours in 1% cypermethrin, whereas *Bacillus* sp. and *Corynebacterium* only grew in 0.1% cypermethrin (Murugesan *et al.*, 2010). In the presence of pyrethroids, the growth of *Pseudomonas* and *Serratia* increased in the first 7 days and then decreased rapidly (Grant *et al.*, 2002) because cypermethrin levels had exceeded the microbial decomposition capacity.

As an inorganic pesticide, copper is not readily bioavailable. In the environment, it can be transformed into other forms. According to the assessment of the International Programme on Chemical Safety (EHC 200, 1998), copper is usually adsorbed by organic matter, carbonate minerals, clay minerals, hydrous iron and manganese oxides in soil. The Cu tolerance levels of bacterial communities in soil have been correlated with enhanced copper concentrations (Kunito *et al.*, 1999). High copper residue in a field reduced the

growth of plant roots and caused lack of water and nutrients for plants (Remans *et al.*, 2012).

Microbial pesticides originate from many different *Bacillus* species which are isolated from soil, plant and other habitats. After application of these bio-pesticides to a field, the microbes persist as a component of the natural microflora at gradually decreasing concentrations for weeks, months or years (EHC 217, 1999). Sunlight and temperature appear to be the factors reducing the bioactivity of *Bacillus thuringiensis* (Pusztai *et al.*, 1991). Most studies demonstrated that *Bacillus thuringiensis* products are unlikely to pose any hazard to humans and the environment (EHC 217, 1999).

2.8.4 Effects of pesticide on microorganisms

Cypermethrin does not have bactericidal properties but can affect microbial communities. This matter relates to the biodegradation of cypermethrin by some microorganisms that can produce the enzymes. Two major enzymes play the important roles in the metabolism of cypermethrin are cytochrome P450 and carboxylesterase. Cypermethrin can be metabolized by oxidation of cytochrome P450 enzymes and by ester hydrolysis of carboxylesterases (Kasai, 2004). Enzyme-producing microorganisms use the metabolites from cypermethrin as a source of energy, carbon, nitrogen, result in they were enhanced. In a series of studies on the effect of cypermethrin application on the microbial

community of pepper and cucumber plant phyllosphere, results showed that cypermethrin application increased both total and bacterial biomass and made the growth of Gram-negative bacteria greater than Gram-positive bacteria (Zhang *et al.*, 2008, 2009a). Permethrin, an active ingredient in the pyrethroid group, together with cypermethrin may support the existence of *Salmonella*, *E. coli* O157:H7, *Listeria monocytogenes* and *Shigella.* It was also demonstrated that *S.* Typhimurium can grow in a permethrin solution incubated at 21°C within 96 hours (Guan *et al.*, 2005, 2001).

Copper compounds are toxic to many kinds of bacteria and fungi. The bactericidal mechanism of copper compounds is the disruption of the metabolism of cells by replacing essential ions. Enzymes responsible for the destruction of lignocellulosic materials of cell are inactivated. Cell membranes of bacteria are destroyed. Elevated copper concentrations have been shown to reduce beneficial mycorrhizal associations and their functions (Liao *et al.*, 2003) and also reduce microbial activity and microbial biomass of other microorganisms in soil (Zwieten *et al.*, 2004). Results of recent research indicated that lactic acid in combination with copper sulfate could be used to inhibit the growth of *Salmonella* spp. and *E. coli* O157:H7 (Salam *et al.*, 2008). Copper plated surfaces used in food processing operations have been shown to have significant antibacterial activity against *Salmonella enterica* and *Campylobacter jejuni* (Faúndez *et al.*, 2004).

Bacillus thuringiensis can produce antimicrobial peptides called bacteriocins that are toxic to bacterial strains closely related to the producer strain (Woo *et al.*, 2008; Gray *et al.*, 2006; Kamoun *et al.*, 2005). *B. thuringiensis* strain B439 produces thuricin 439, which acts as a bactericidal peptide. Thuricin 439 was shown to affect growth of *B. cereus* and *B. thuringiensis* strains as well as *L. innocua* 4202 but it had no effect on the growth of some other Gram-positive bacteria (several *B. subtilis* strains, *Bacillus coagulans*, *Bacillus firmus*, several *Clostridium* species, *Lactobacillus sakei*, *Lactococcus lactis*, *Micrococcus luteus*, *Staphylococcus aureus*). Neither Gram-negative bacteria (*Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, several *Pseudomonas* species, *Salmonella* Typhimurium) nor mold (*Aspergillus niger*, *Penicillium roqueforti*) were affected by Thuricin 439 (Ahern *et al.*, 2003). However, Cyt1Aa, a delta-endotoxin protein produced by *B. thuringiensis* subsp. israelensis was found to be bactericidal for *E. coli* (Cahan *et al.*, 2008).

Some strains of *B. subtilis* are known to be antagonistic toward many fungal plant pathogens. This antagonism may be achieved in several ways including nutrient competition, colonization and attachment of the bacteria to the fungal pathogen (Demoz and Korsten, 2006). The modes of action of *B. subtilis* QST 713 are colonizing leaf surfaces, competing with pathogens for nutrients and space, and physically preventing attachment and penetration of the pathogen. In addition, *B. subtilis* has been shown to produce a wide variety of antibacterial and antifungal compounds. *B. subtilis* QST 713

produces Iturin A that can stop spores of *Botrytis cinerea* and *Rhizoctonia solani* from germinating, disrupt germ tube growth, and inhibit plant pathogen attachment to leaves (Kloepper *et al.*, 2004). *B. subtilis* 6051 forms a biofilm and produces surfactin to prevent the attachment of *Pseudomonas syringae* on plant roots (Bais *et al.*, 2004). The antimicrobial produced by *B. subtilis* MIR 15 appears to be mainly active against Gram-negative bacteria including *E. coli* and *Pseudomonas aeruginosa* (Perez *et al.*, 1992).

2.9 Strategies to reduce vegetable contamination by human pathogens

Determining the exact origin of a contamination cannot be ignored when building strategies and interventions to minimize the presence of pathogenic microorganisms on vegetables. Even if these pathogenic bacteria can contaminate vegetables at any point throughout the production system (Buck *et al.*, 2003), this can be solved by set up the critical control points. Depending on contamination sources, numerous strategies should be applied to reduce pathogenic bacteria on vegetables during pre and post-harvest operations.

Some studies have shown that pesticide solutions may create suitable environments for the survival and growth of pathogenic bacteria such as *Salmonella, E. coli, Listeria monocytogenes* and *Shigella* (Guan *et al.*, 2005, 2001; Peter *et al.*, 2005). Therefore, pre-harvest applications of pesticide solutions mixed with contaminated water onto vegetable produce could be an additional source of microbial contamination. Some microbiological

issues are related to the application of pesticides on vegetables as a source of microbial contaminants should thus be considered. Pesticide compositions could be either stimulatory or inhibitory to microbial growth and this could be a significant aspect to consider either before or after their application on the produce. The microbial quality of the water used for dilution of the pesticide could be a factor if the pesticide supports the growth of microorganisms.

Water used to irrigate and to dilute pesticides can be obtained from dams, rivers and lakes. These sources may contain coliforms, fecal coliforms, *E. coli* and various food associated pathogens such us *Salmonella, Campylobacter jejuni* and *Listeria monocytogenes*, which can grow even with the addition of pesticide. A critical aspect in any strategy for food safety at this stage is to improve water quality to ensure water used for preparation of pesticides has been adequately treated, especially when using highly contaminated water such as surface water or wastewater. Wastewater containing human pathogens must be treated and strictly monitored before being used on vegetables. There are many methods to reduce the presence of microorganisms in water such as filtration, disinfection with chlorine, ozone, ultraviolet light exposure, electronic beam processing and heat treatment. However, scientists continue to search for new antibacterial measures because the measures mentioned above have certain limitations: high cost; potential of destroying beneficial flora; and reactions of higher concentrations of chlorine with organic

materials like carbohydrates which are found on the surface of fresh produce, leading to reduction of disinfection efficacy (Hyun-Gyun *et al.*, 2005).

Many pesticides can inhibit or kill bacteria, but many types of pesticides can support the growth of bacteria (Peter *et al.*, 2005). The modes of action of chemical pesticides to bacteria are mainly related to the enzyme activities of bacteria. For example, copper ions inactivate enzymes responsible for the destruction of lignocellulosic materials (Nies, 1999) and cypermethrin can stimulate the enzyme production of bacteria that use cypermethrin as carbon and energy sources (Kasai, 2004). Whereas, the mode of action of microbial pesticides do not involved in bacterial enzymes. Some *Bacillus* strains can produce the bateriocins which have a selective effect on a few strains close to it or can affect other bacteria by antagonistic mechanism (Ahern *et al.*, 2003; Demoz and Korsten, 2006). To reduce the risk of contaminated vegetables by pathogenic bacteria due to the application of pesticides in pre-harvest periods, there is a need to identify which types of pesticides can inhibit or promote the growth and survival of pathogenic bacteria. The identification can base on mode of action of the pesticides. Most previous studies focused on evaluating the growth and survival of pathogenic microorganisms in solutions of chemical pesticides. Given that chemical pesticides can pose a risk to consumer health due to toxic residues left behind on vegetables, identification of bio-pesticides that are able to inhibit the growth of pathogenic microorganism without leaving harmful residues is one of the strategies proposed to reduce the risk of microorganism contamination on vegetables.

HYPOTHESIS

Under laboratory conditions, the pathogenic microorganism persistence in pesticide solutions is significantly affected. Under field conditions, where many environmental factors are present, especially the presence of plant indigenous microflora, pesticides directly or indirectly influence the persistence of pathogenic microorganisms.

OBJECTIVES

Two objectives of this study are (1) Evaluate the survival of *E. coli* and *Salmonella* in four pesticides solutions - Ripcord 400EC, Copper 53W, Serenade MAX, and Bioprotec CAF - under laboratory conditions; (2) Study the persistence of *E. coli* (used as a fecal contamination indicator) on broccoli crops exposed to three pesticides - Ripcord 400EC, Copper 53W, and Bioprotec CAF. These objectives will contribute to assess the risk of microbial contamination due to pesticide application.

Chapter 3. Impact of Pesticides on Indicator and Pathogenic Microorganism Persistence under Laboratory and Field Conditions

ARTICLE IN PREPARATION

Journal of Food Protection

Running head:

Full title: Impact of Pesticides on Indicator and Pathogenic Microorganism Persistence under Laboratory and Field Conditions

Hoa Tran Phuong[1], Caroline Côté [2], Mylène Généreux[2], Vicky Toussaint[3], Francis Beaudry[1], Ann Letellier[1 *]

[1] Faculty of Veterinary Medicine, Université de Montréal, 3200, rue Sicotte, Saint-Hyacinthe, QC J2S 2M2, Canada.

[2] Research Centre, Research and Development Institute for the Agri-environment, 3300, rue Sicotte, Saint-Hyacinthe, QC J2S 7B8, Canada.

[3] Horticulture Research and Development Centre, Agriculture and Agri-Food Canada, 430 Gouin Blvd., St-Jean-sur-Richelieu, QC J3B 3E6, Canada

Key words: *E. coli*, *Salmonella*, microflora, pesticide, broccoli, persistence.

---

[*] Author for correspondence: telephone: (450)773-8521, fax: (450)778-8128

Abstract

The impact of pesticides on indicator and pathogenic microorganism persistence was examined under laboratory and field conditions. Under laboratory conditions, four pesticide solutions (Ripcord 400EC, Copper 53W, Bioprotec CAF, Serenade MAX) dissolved or diluted in sterile water as a control were tested to determine the persistence of *E. coli* and *Salmonella*. Petrifilm count plates and XLD agar were used to enumerate *E. coli* and *Salmonella* counts. Under field conditions, three pesticides (Ripcord 400EC, Copper 53W, Bioprotec CAF) were tested on broccoli irrigated with *E. coli*-contaminated water to determine their effects on persistence of *E. coli* and other microorganisms. Broccoli samples were analyzed to determine bacteria, total aerobics and, mold and yeast counts. For the laboratory experiments, results showed a direct effect of various pesticides on microorganisms: Copper 53W inhibited both *E. coli* and *Salmonella* growth; Bioprotec CAF and Ripcord 400EC supported microbial survival; especially, Serenade MAX promoted microbial growth (over 57 days at 21°C). In the field, irrigation made mold and yeast counts decline on broccoli but slightly increased total aerobic bacteria populations. Higher *E. coli* prevalence on broccoli in Bioprotec CAF treatments compared to Copper 53W treatments is consistent with results observed during the laboratory assay. However, no significant statistical difference was observed between treatments for *E.coli* prevalence. The direct effect of pesticides on microorganisms under laboratory conditions was demonstrated but it is still unclear under experimental field conditions.

INTRODUCTION

The presence of human pathogens on crops represents a significant potential source of human illness. According to Beuchat *(3)*, vegetables may become contaminated by pathogenic bacteria at any point throughout the entire process, from planting to consumption. Potential pre-harvest sources of contamination include soil, feces, irrigation water, water used to apply pesticides, dust, insects, inadequately composted manure, wild and domestic animals, and human handling.

Pesticides play a significant role in agricultural production by protecting the crops from insects, fungi, weeds and others pests. They are usually diluted or dissolved in water to ensure plant coverage. According to manufacturer recommendations, clean water should be used to prepare pesticides. However, farmers can use surface water sources from lakes, ponds, rivers or canals that could harbor human pathogens. Concerns have been raised about the ability of pesticide solutions to become vectors of human pathogens given recent outbreaks linked to contaminated vegetables *(6, 7)*.

Pesticide application does not only control pests but can also have an impact on non-target microorganisms. The direct effect of pesticides on human pathogens by promoting or inhibiting microbial growth can be found in earlier studies *(9, 10, 17)*. These studies suggested that water used for the preparation of pesticide solutions can be a source of microbial contamination. Pesticides can also have an indirect effect on plant microbial

communities, which can include many different genera of bacteria, filamentous fungi, yeasts, algae, and less frequently, protozoa and nematodes *(14)*. The interactions between this microflora and enteric pathogens *(1,4)* can be influenced by pesticide; Zhang *et al. (20)* found that pesticide application changed microbial biomass and microbial community structure. The effect of a pesticide on the plant microflora depends on its properties and concentration, type of microorganisms and their persistence in the environment *(12)*. Carlin *et al. (5)* determined that increase or decrease of microbial flora populations by chemical disinfection can affect the persistence of human pathogens on plants.

The aims of this study were 1) to evaluate the survival of *E. coli* and *Salmonella* in four pesticide solutions incubated at two temperatures under laboratory conditions, and 2) to study the impact of three pesticide applications on the persistence of generic *E. coli* (used as a fecal indicator microorganism) and on the general microflora in broccoli under field conditions before or following irrigation with contaminated water.

MATERIALS AND METHODS

Laboratory assay

Pesticides evaluated in this assay are described in Table I. All four pesticides are registered by the Pest Management Regulatory Agency of Canada for use in broccoli crops (Agri-Reseau, 2010). Pesticides were diluted or dissolved in 44 mL of sterile water in

sterile 50 mL tubes to reach manufacturer recommended concentrations for cole crops (Table I). After reconstitution, pesticide solutions were homogenized for 5 minutes to ensure homogeneous solutions. Dilutions were performed immediately prior to inoculation with microorganisms. Absence of *E. coli* and *Salmonella* in pesticide solutions was verified before microbial inoculation. As a control, tubes containing sterile water were also inoculated.

Description of *E. coli* and *Salmonella* strains used for inoculation is shown in Table II. Bacteria were maintained by subculture on Columbia blood agar 5% (OXOID, MP0351). The *Salmonella* inoculum solution was obtained after inoculating 1 liter of nutrient broth (OXOID, CM0001) with three full loops of fresh bacteria culture (18-24 h) plated on Columbia agar. The broth was vigorously homogenized with a vortex mixer for 10 seconds. After incubating for 24 hours at 35°C, the final bacterial concentration was approximately $2x10^{8}$ CFU/mL which was verified by serial dilution and plate counting. This broth was diluted in 0.1% peptone water (Difco, REF211677) in order to get a bacterial concentration of $45x10^{3}$ CFU/mL. A 1 mL portion of this solution was transferred into a tube that contained 44 mL of pesitices solution to obtain a bacterial concentration of 10^{3} CFU/mL in each tube. The same steps were performed for the *E. coli* inoculum solution, but the final 10^{3} CFU/mL concentration was made from the 5 strain mixtures.

In order to avoid potential interactions between bacteria, *E. coli* and *Salmonella* were inoculated in different pesticide tubes. After inoculation, tubes were vigorously shaken by hand and incubated at 4°C and 21°C. For each pesticide, a total of 3 tubes for each microorganism and for each incubation temperature were analyzed. *E. coli* and *Salmonella* counts were determined immediately after inoculation and on day 1, 2, 3, 4, 7, 10, 14, 21, 29, 49, 57 following inoculation. Samples were serial-diluted in 0.1% peptone water to determine bacterial counts. For *E. coli* samples, 1 mL of diluted samples was inoculated on Petrifilm™ *E. coli*/Coliform plates (3M Canada Microbiology, 6414) and incubated at 35°C for 48 h. For *Salmonella* samples, 0.1 mL of diluted samples was inoculated on XLD agar (Difco, REF 278850) and incubated at 35°C for 24 h. Typical colonies were counted after incubation and positive and negative controls were performed for each procedure.

Field experiment

The field trial was carried out at the Research and Development Institute for the Agri-environment (IRDA) experimental farm located in Saint-Hyacinthe, Québec, Canada. Broccoli (*Brassica oleracea* 'Everest') plots were set using a completely randomized design including 7 treatments repeated 3 times, for a total of 21 plots, which were 4 m x 4 m and 8 m apart from each other. There were 6 broccoli rows in each plot with 17 plants per row for a total of 102 broccoli plants per plot. Treatment description is shown in Table

III. The irrigation, spraying and sampling schedule is presented in Table IV. Pesticide concentrations applied on broccoli followed manufacturer recommendations for this crop (Table I). Pesticides were diluted or dissolved in drinking water prior to field application. Irrigation water was taken from an old quarry. It was not analyzed for the chemical properties, but it did not contain detectable levels of *E. coli.* It was artificially contaminated with swine and bovine slurries to reach a concentration of *E. coli* at 1047 CFU/100 mL which was verified.

Each broccoli sample consisted of three broccoli subsamples per plot. They were aseptically taken from the randomly inside positions of plot. Subsamples (n=567) were cut into approximately one gram pieces and thoroughly mixed to form a composite sample. All samples were kept at 4°C before analyses which was performed within 48 hours following sampling. Broccoli samples were analyzed to determine *E. coli,* mold and yeast, as well as total aerobic counts. *E. coli* populations were determined using the Health Canada (2001) MFHPB-34 procedure. In brief, 25 g of broccoli were aseptically weighed in a sterile bag and 225 mL of 0.1% peptone water was added. One milliliter of the sample was inoculated on 3M Petrifilm^{TM} *E. coli*/Coliform count plates (3M Canada Microbiology, 6414). After incubation at 35°C for 48 h, typical colonies of *E. coli* were counted. An enrichment procedure using the Colilert medium (IDEXX Laboratories, W200I) was also performed on broccoli samples to evaluate the presence of *E. coli* in case of bacterial counts below the method detection limit Previously weighed 25 g in 225 mL of peptone water were

incubated at 35°C for 24 h. Following this pre-enrichment step, 1 mL was transferred into a transparent, non-fluorescent tube containing 10 mL of Colilert reagent. The tube was aseptically capped, shaken and incubated for 24 hours at 44.5°C. Results were read by comparing samples to the positive and negative controls under ultraviolet light (366 nm). Mold and yeast counts were determined using the 3M Petrifilm™ Yeast and Mold count plate (3M Canada Microbiology, 6417). The inoculated plates were incubated at 21°C and checked for growth at both 3 and 5 days. Total aerobic bacteria counts were determined using the 3M Petrifilm™ Aerobic count plate (3M Canada Microbiology, 6400). Incubation was performed at 35°C for 48 h.

Statistical analysis

Correlation between *E. coli* and *Salmonella* in laboratory assays was analyzed using the Spearman correlation. Differences between treatments in the field experiment were analyzed by the SAS GLIMMIX procedure.

RESULTS AND DISCUSSION

Impact of pesticides on microorganism survival under laboratory conditions

Survival of *E. coli* and *Salmonella* in pesticide solutions in sterile water at 4°C and 21°C is presented in Figure 1. *E. coli* survived longer in sterile water than *Salmonella* and was recovered 2 days after inoculation at 4°C. *E. coli* and *Salmonella* were not detected in Copper 53W at either 4°C or 21°C, after 24 hours of incubation. Copper sulfate in this solution could have inhibited the survival of both microorganisms, which is consistent with many reported results *(8, 17, 18, 19)*. Indeed, small quantities of free copper ions are toxic to many groups of bacteria *(16)*. In Ripcord 400EC, *Salmonella* was not found after 24 hours of incubation at 4°C and 21°C but *E. coli* still survived at 4°C after 24 hours of incubation. According to the studies of Guan *et al. (10)*, *E. coli* populations increased in a permethrin solution, which is a pyrethroid insecticide like cypermethrin in Ripcord 400EC. In Bioprotec CAF, following inoculation at 4°C, *E. coli* and *Salmonella* were detected on day 29 and 14, respectively. Survival time was shorter at 21°C while *E. coli* and *Salmonella* were no longer found on day 7 and 4, respectively. In this study, the Serenade MAX solution promoted microbial growth, especially at 21°C, where microorganism populations increased strongly (from 3.18 to 8.29 log for *E. coli* and from 3.47 to 7.51 log for *Salmonella*) after 24 hours of incubation. Counts were maintained at high levels until day

57 when the experiment ended. Serenade MAX supported *E. coli* and *Salmonella* survival at 4°C for at least 29 days and 57 days, respectively.

Statistical analysis of fluctuations in the microbial population showed a high correlation (0.89) between *E. coli* and *Salmonella* in the pesticide solutions. This suggested that behavior was similar for both bacteria.

Temperature strongly affected microbial survival, with different responses depending on pesticide. In Serenade MAX, microorganism counts increased and populations were maintained at 21°C, while a general reduction was observed at 4°C. In Ripcord 400EC and Bioprotec CAF, higher temperature shortened the survival time of microorganisms. This was consistent with studies of Guan *et al. (9,10)*, where increasing the temperature from 21°C to 25°C or 30°C reduced microorganism survival depending on the pesticide.

Pesticide solutions influenced microorganism persistence as shown in Figure 2 for *E. coli* at 4°C. Bioprotec CAF and Serenade MAX were found to have the ability to support the growth of *E. coli* and *Salmonella.* However, most pesticides cannot be used as pure active ingredients; they must be mixed with other active ingredients and additives or adjuvants (often referred to as inerts) that promote efficacy, stability and to facilitate application or improve safety and shelf-life. The exact composition of inerts and adjuvants

in pesticide formulations are not mentioned by manufacturers due to proprietary protection. Therefore, the persistence of microorganisms in pesticide solutions may be due to the effects of both the active ingredients and inerts mixed in the finished formulations.

Persistence of *E. coli* on broccoli under field conditions

Result of *E. coli* population determined by 3M^{TM} Petrifilm^{TM} *E. coli* count plates showed that most of samples were below the method detection limit. *E. coli* could be quantified in 5 broccoli samples taken from experimental plots, varying between 10 and 20 CFU/g. These *E. coli* samples were taken in treatments 5 (Bioprotec CAF sprayed before irrigation), 6 (Bioprotec CAF sprayed before irrigation) and 7 (no spraying). The *E. coli* numbers under dection limit can be resulted from adverse conditions like ultraviolet sunlight exposure and desiccation that if *E. coli* could not attach to plant surfaces, then they would have fallen down with the water and diffused into the soil. The competition between *E. coli* and indigenous microflora could also make it difficult for *E. coli* to colonize the phyllosphere *(4)*.

E. coli was detected in 77 out of 189 broccoli samples using the enrichment procedure. Results for the overall *E. coli* prevalence during the sampling period are shown in Figure 3. Higher *E. coli* prevalence in Bioprotec CAF treatments compared to Copper 53W treatments is consistent with results observed during the laboratory assay, while

microbial growth was inhibited in Copper 53W and supported in Bioprotec CAF. However, statistical analysis showed no significant difference between treatments. The observation of the changes of *E. coli* positive rates (i.e. *E. coli*-positive samples/ 3 samples) in each treatment according to sampling time (Figure 4) showed that *E. coli* mostly appears after irrigation. This could be explaned by the supplying of *E.coli* from irrigation water.

Mold and yeast populations in broccoli are presented in Figure 5 for each treatment. Their counts were reduced 20 hours after irrigation. This could be due to many factors such as the washout effect of irrigation, stress when environmental conditions change suddenly, and competition between microorganisms on broccoli crops *(1, 4, 14)*. However, the washout effect is less likely because the samples that had mold and yeast population increases were taken 20 hours after irrigation. In addition, the aerobic bacteria would have also been influenced by the washout but they tended to increase their populations. The increase of moisture after irrigation could possibly be associated with the growth rate of bacteria. These results suggested that there was a competition between molds and yeasts on broccoli and microorganism from irrigation water. The effect of pesticides on mold and yeast populations after pesticide applications could not be assessed because it were very different according to treatment.

Aerobic bacterial counts in broccoli according to treatment are presented in Figure 6. They tended to increase after irrigation due to the supply from artificially contaminated

irrigation water. A competition between aerobic bacteria and microorganisms from irrigation water could also occur. They had to compete with each other for attachement to the plant *(15)*, for iron via production of siderophores *(13)*, and also for the same carbon and energy sources *(11)*. In this competition the organisms that have the capacity to absorb nutrients more efficiently, that adapt quickly to harsh environmental conditions will gain the advantage *(2).*Aerobic microorganism population behavior after pesticide application was very different between treatments. In Copper 53W treatments, they decreased after spraying. Whereas, in Ripcord 400EC and Bioprotec CAF spray treatments, the increase and decrease of aerobic bacterial counts do not follow the spraying. So, the direct effect of pesticides on aerobic microflora was difficult to establish.

Overall, the effect of irrigation water on *E. coli*-positive rates, molds and yeasts and total aerobic bacteria counts could be observed. However, the impact of pesticide on *E. coli* molds and yeasts, and aerobic bacteria populations were less clear.

CONCLUSION

This study highlighted how pesticide can inhibit, support or increase enteric bacteria survival under laboratory conditions. These impacts could not be clearly established on broccoli crops under the field conditions of this study. Future work should focus on further

determining if pesticides play a role in food safety in terms of supporting the proliferation of human pathogens on different crops.

REFERENCES

1. Aruscavage, D., Lee, K., Miller, S., LeJeune, J.T. 2006. Interactions affecting the proliferation and control of human pathogens on edible plants. *J. Food Sci*. 71(8): 89-99.
2. Beattie, G.A. and Lindow, S.E. 1994. Epiphytic fitness of phytopathogenic bacteria: physiological adaptations for growth and survival. *In* Dang1J.L (ed.), Bacterial pathogenesis of plants and animals, molecular and cellular mechanisms. Springer-Verlag, Berlin. 1-28.
3. Beuchat, L.R. 1996. Pathogenic microorganisms associated with fresh produce. *J. Food Prot*. 59:204-16.
4. Brandl, M. T. 2006. Fitness of human enteric pathogens on plants and implications for food safety. *Annual Review of Phytopathology*. 44: 367-92.
5. Carlin, F., Nguyen, T.C., Morris, C.E. 1996. Influence of background flora on *Listeria Monocytogenes* on minimally processed fresh broad-leaved endive (*Cichorium endivia* var. latifolia). *J. Food Prot*. 59:698-703.

6. Centers for Disease Control and Prevention (CDC). 2008. Outbreaks of *Salmonella* serotype Saintpaul infection associated with multiple raw produce items - United States, 2008. Morb Mortal WKly 2008. 57(34):929-34.

7. European Centre for Disease Prevention and Control (ECDC). 2011. Shiga toxin-producing *E. coli* (STEC): Update on outbreak in the EU (27 July 2011, 11:00). Retrieved May 18, 2012. Available at: http://ecdc.europa.eu/en/activities/sciadvice/Lists/

8. Faúndez, G., Troncoso, M., Navarrete, P., Figueroa, G. 2004. Antimicrobial activity of copper surfaces against suspensions of *Salmonella enterica* and *Campylobacter jejuni*. *BMC Microbiology*. 4: 19.

9. Guan, T., Blank, G., Holley, R.A. 2005. Survival of pathogenic bacteria in pesticide solutions and on treated tomato plants. *J. Food Prot*. 68:296-304.

10. Guan, T., Blank, G., Ismond, A., Acker, R.V. 2001. Fate of foodborne bacterial pathogens in pesticide products. *J. Sci. Food Agri*. 81:503-12.

11. Janisiewski, W.J., Conway, W.S., Leverentz, B. 1999. Biological control of postharvest decays of apple can prevent growth of *Escherichia coli* O157:H7 in apple wounds. *J. Food Prot*. 62(12):1372-5.

12. Johnsen, K., Jacobsen, C. S., Torsvik, V., Sørensen, J. 2001. Pesticide effects on bacterial diversity in agricultural soils – a review. *Biol Fertil Soils*. 33:443-53.

13. Liao, C.H. and Sapers, G.M. 1999. Influence of soft rot bacteria on growth of *Listeria monocytogenes* on potatoes tuber slices. *J. Food Prot*. 62: 343-48.

14. Lindow, S.E. and Brandl, M.T. 2003. Minireview: Microbiology of the Phyllosphere. *Appl. Environ. Microbiol*. 69(4): 1875-83.

15. Mandrell, R., Gorski, L., Brandl, M. 2006. Attachment of microorganisms to fresh produce. In: Sapers, G. M., Gorny, J.R., Yousef, A.E. eds., *Microbiology of Fruits and Vegetables*. Boca Raton, FL: CRC Press. 33-73

16. Menkissoglu, O. and Lindow, S.E. 1991. Relationship of free ionic copper and toxicity to bacteria in solutions of organic compounds. *Phytopathology*. 81(10): 1258-63.

17. Peter, J. Ng., Fleet, G.H., Gillian, T., Heard, G.M. 2005. Pesticides as a source of microbial contamination of salad vegetables. *Int. J. Food Microbiol*. 101: 237- 50.

18. Rodgers, S.L. and Ryser, E. 2004. Reduction of microbial pathogens during apple cider production using sodium hypochlorite, copper ion, and sonication. *J. Food Prot*. 67:766-71.

19. Salam, A.I., Yang, H., Chung, W.S. 2008. Antimicrobial activity of lactic acid and copper on growth of *Salmonella* and *Escherichia coli* O157:H7 in laboratory medium and carrot juice. *Food Chemistry* 109:137-43.

20. Zhang, B., Zhang, H., Jin, B., Tang, L., Yang, J., Li, B., Zhuang, G., Bai, Z. 2008. Effect of cypermethrin insecticide on the microbial community in cucumber phyllosphere. *J. Environ Sci* 20(11): 1356-62.

.

Table I. Description of pesticides used for the laboratory assay and field trial.

Trade name	Manufacturer	Active ingredient	Formulation	Active ingredient content	Active ingredient content per tube
Ripcord 400EC[1]	BASF Canada Inc.	Cypermethrin	Emulsifiable concentrate	407 g/L	0.01 mL
Copper 53W[1]	United Agri Products Canada Inc.	Copper sulfate	Wettable powder	53%	0.06 g
Bioprotec CAF[1]	AEF Global Inc.	*Bacillus thuringiensis* kurstaki HD-1	Aqueous	1.14×10^7 IU/g	0.08 mL
Serenade MAX	AgraQuest Inc.	*Bacillus subtilis* QST 713	Wettable powder	14.6 % 7.3×10^9 CFU/g	0.6 g

[1]Pesticides used in the field trial.

Table II. Source of bacterial strains used for the laboratory assay.

Bacteria	Source
E. coli CC2001-119	Pig slurry
E. coli CC2001-127	Poultry manure
E. coli CC2001-151	Dairy cow manure
E. coli CC2004-245	Paper mill biosolid-amended soil
E. coli ATCC 25922	Reference strain
Salmonella Typhimurium ATCC 14028	Reference strain

Table III. Treatments in field experiments.

Treatment	
1	Copper 53W sprayed 3 days before irrigation
2	Copper 53W sprayed 3 days after irrigation
3	Ripcord 400EC sprayed 3 days before irrigation
4	Ripcord 400EC sprayed 3 days after irrigation
5	Bioprotec CAF sprayed 3 days before irrigation
6	Bioprotec CAF sprayed 3 days after irrigation
7	Irrigation only (no pesticide spraying)

Table IV. Spraying, irrigation and broccoli sampling schedule.

Date	Sampling time (hours)	Treatment application
12-07-2011	0[1]	Pesticide spraying (treatments 1, 3, 5)
14-07-2011	36	
15-07-2011	60[2]	Irrigation (all treatments)
	65[3]	
16-07-2011	85	
17-07-2011	109	
18-07-2011	133[1]	Pesticide spraying (treatments 2, 4, 6)
20-07-2011	181	
21-07-2011	205	

[1] Sampling was done 1 hour before spraying

[2] Sampling was done 1 hour before irrigation

[3] Sampling was done 1 hour after irrigation

FIGURE LEGEND

Figure 1. *E. coli* and *Salmonella* in pesticide solutions at $4^{o}C$ and $21^{o}C$.

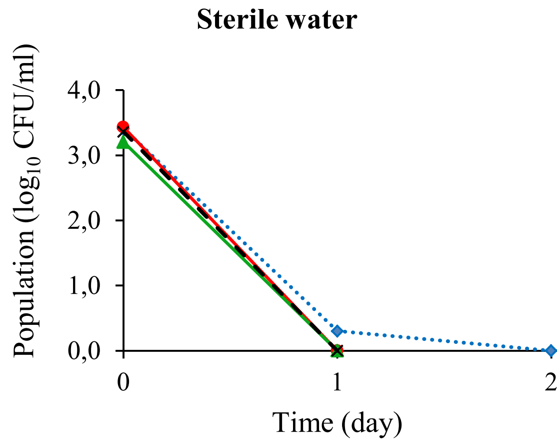
Figure 2. *E. coli* in pesticide solutions at $4^{o}C$.

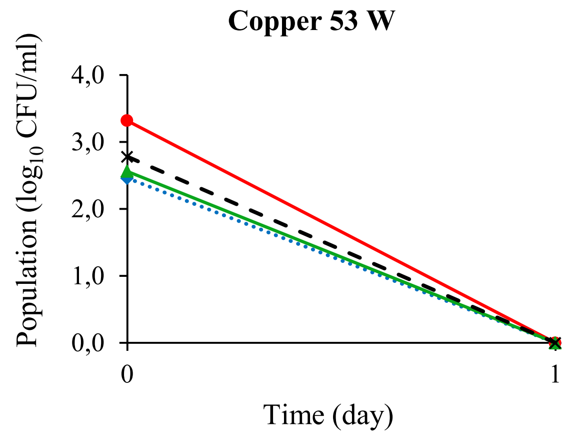
Figure 3. Overall *E. coli* prevalence during the sampling period

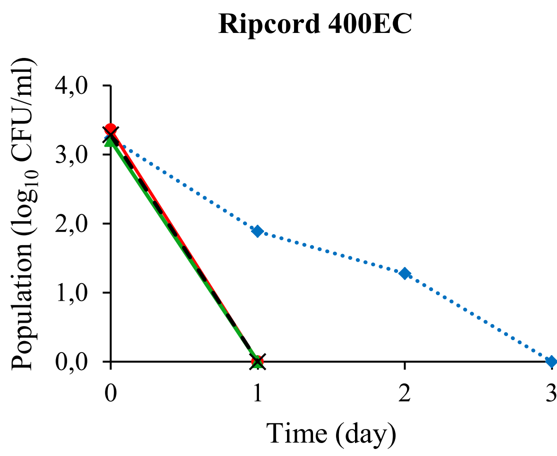
Figure 4. *E. coli* positive rates in each treatment according to sampling time.

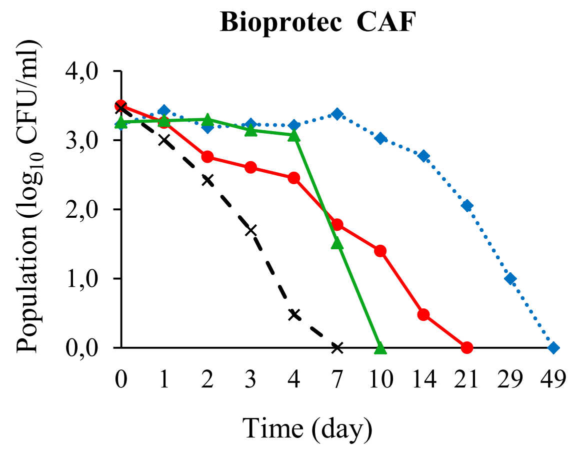
Figure 5. Mold and yeast counts in broccoli samples according to pesticide.

Figure 6. Total aerobic microflora counts in broccoli samples according to pesticide.


Sterile water
Population ($\log_{10}$ CFU/ml)
4,0
3,0
2,0
1,0
0,0
0
1
2
Time (day)


Copper 53 W
Population ($\log_{10}$ CFU/ml)
4,0
3,0
2,0
1,0
0,0
0
1
Time (day)


Ripcord 400EC
Population ($\log_{10}$ CFU/ml)
4,0
3,0
2,0
1,0
0,0
0
1
2
3
Time (day)


Bioprotec CAF
Population ($\log_{10}$ CFU/ml)
4,0
3,0
2,0
1,0
0,0
0 1 2 3 4 7 10 14 21 29 49
Time (day)

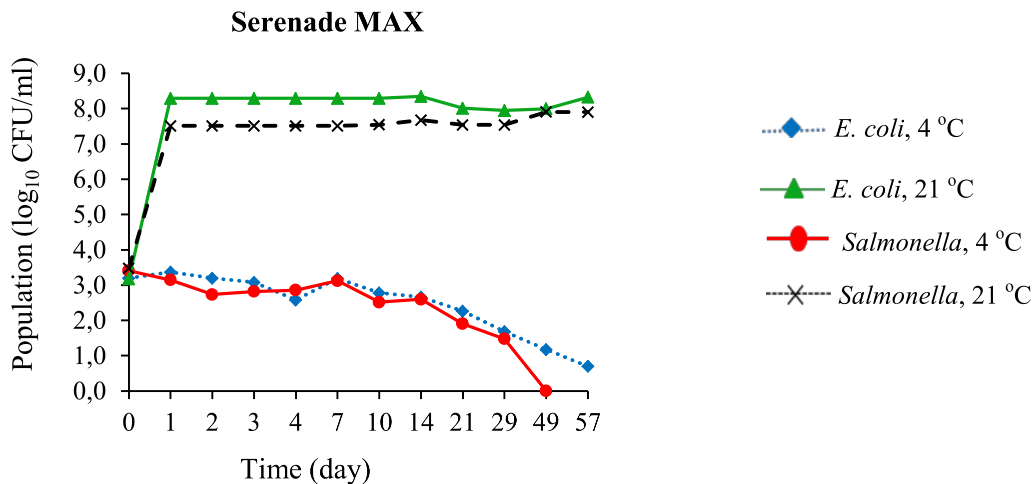


Figure 1. *E. coli* and *Salmonella* in pesticide solutions at 4°C and 21°C.

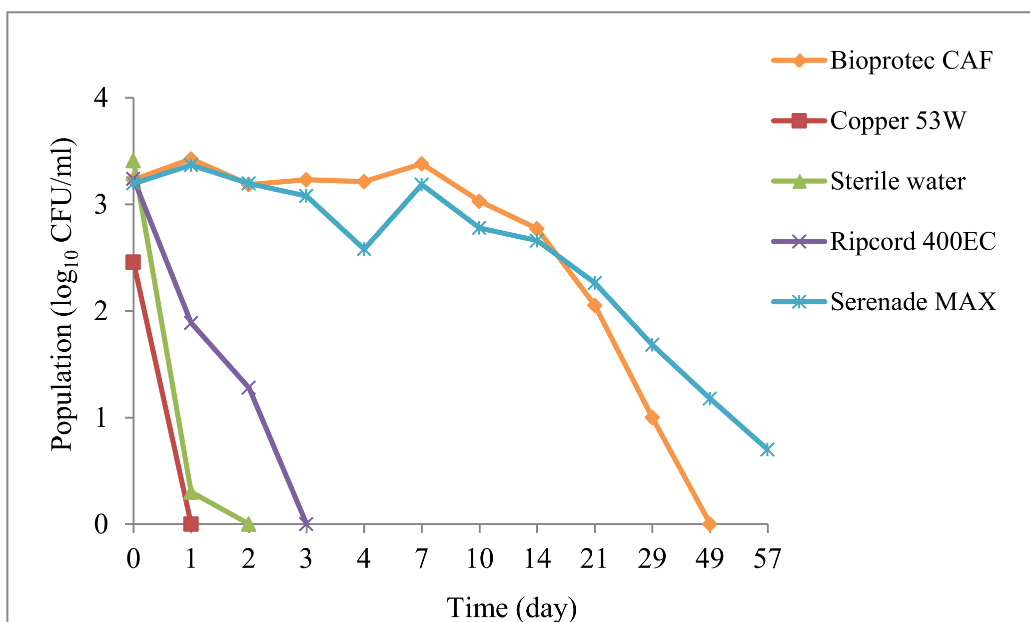


Figure 2. *E. coli* in pesticide solutions at 4°C.

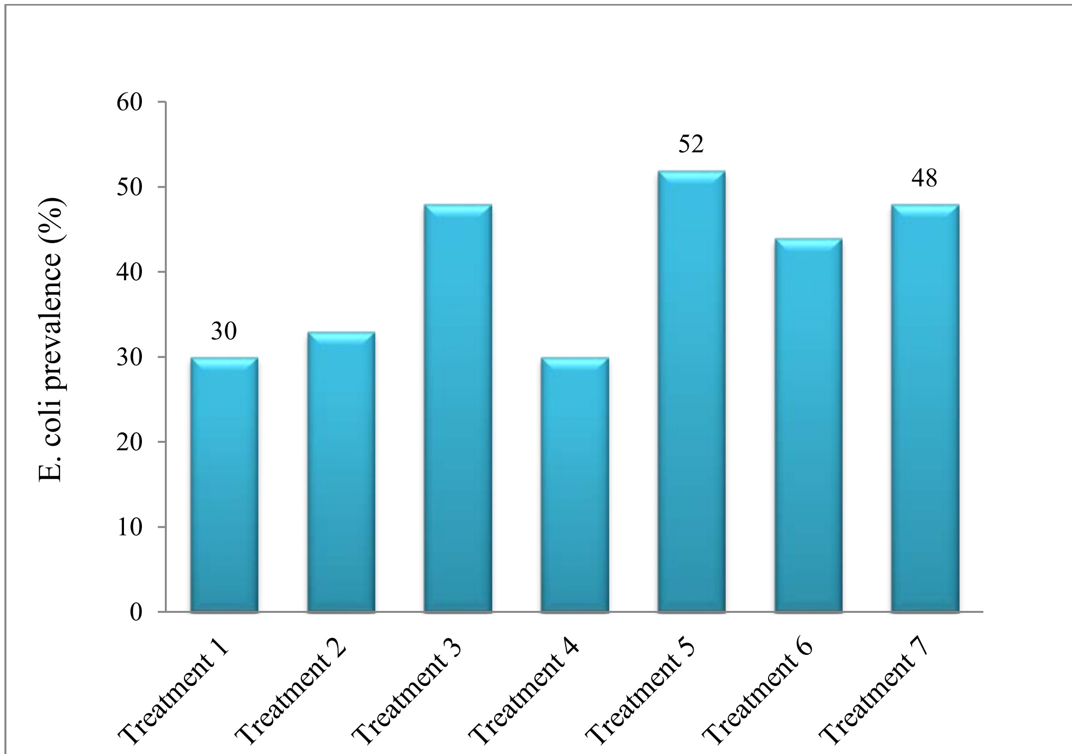


Figure 3. Overall *E. coli* prevalence during the sampling period

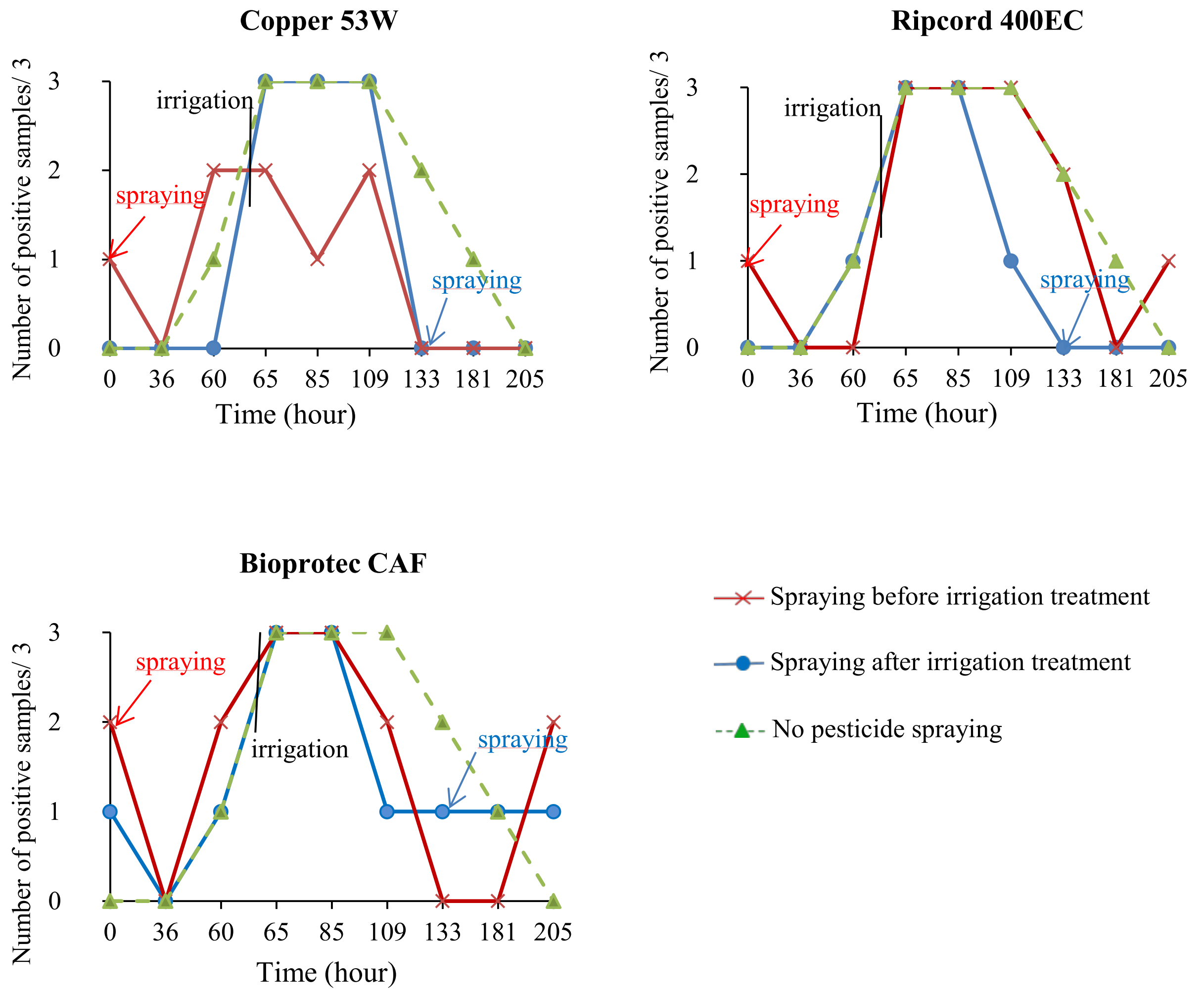


Figure 4. *E. coli* positive rates in each treatment according to sampling time.

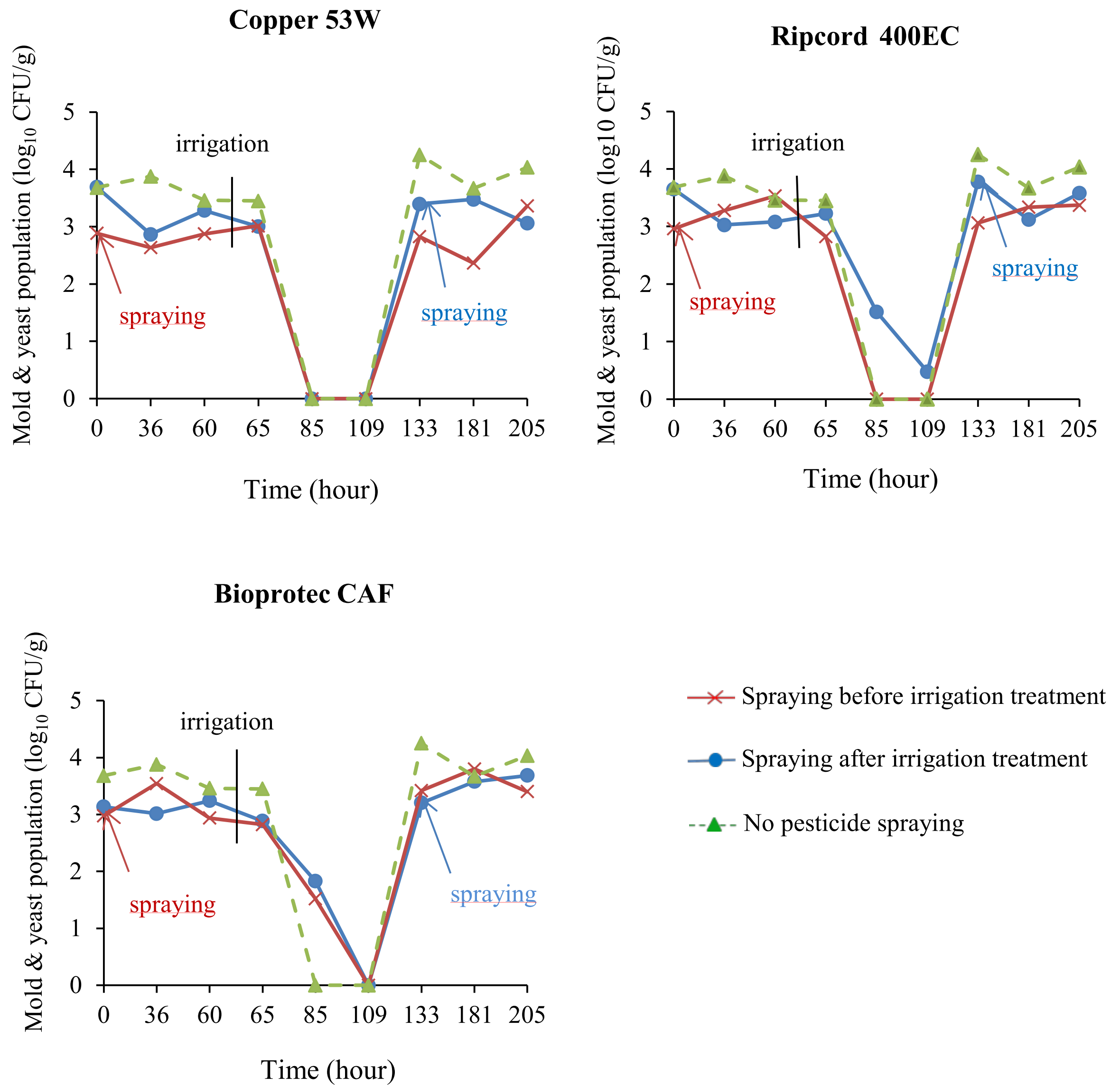


Figure 5. Mold and yeast counts in broccoli samples according to pesticide.

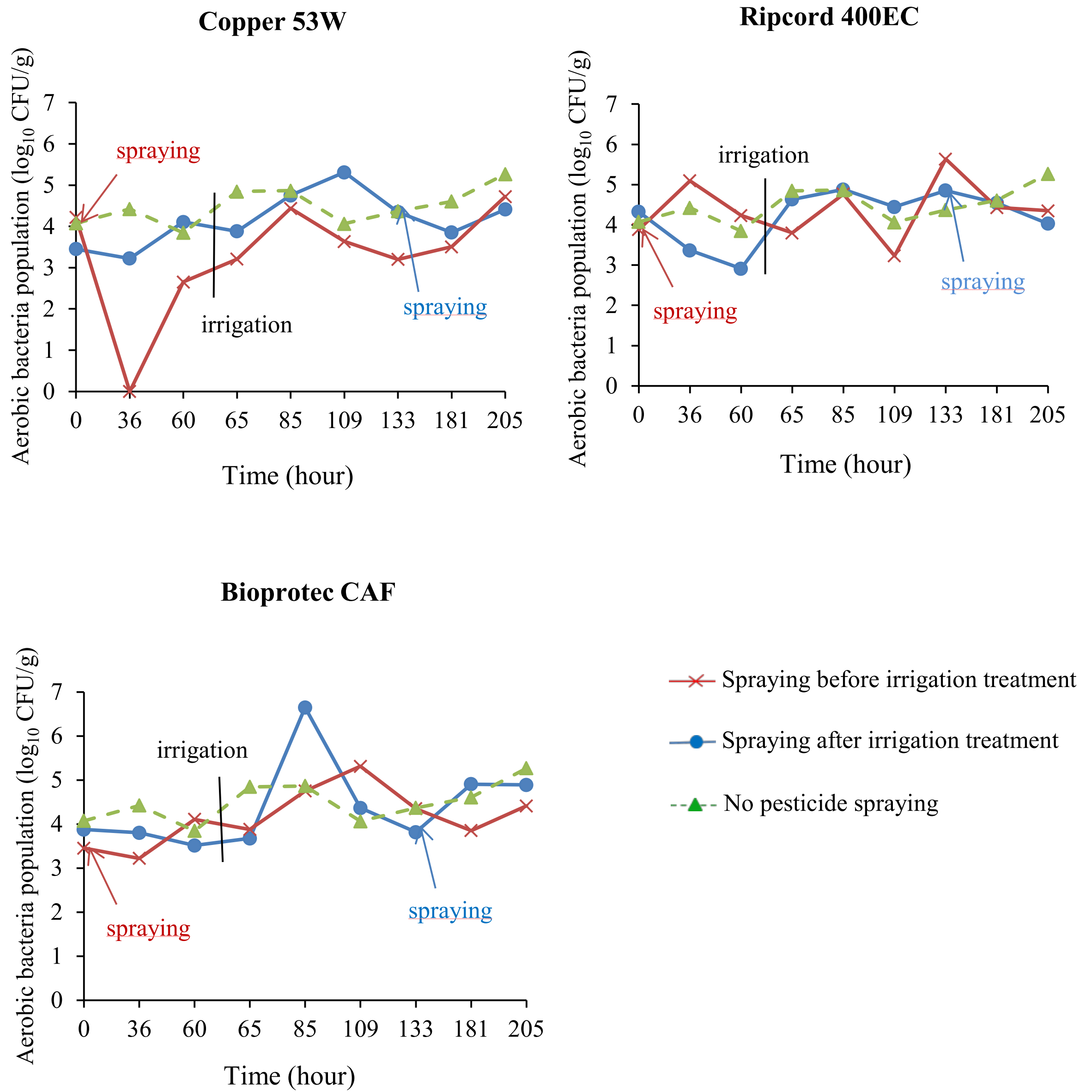


Figure 6. Total aerobic microflora counts in broccoli samples according to pesticide

Chapter 4. GENERAL DISCUSSION

Pesticides are common used in the protection of agricultural production. Some studies have shown that some pesticide types can support the pathogen microorganism persistence. So, an assessment on the potential spread of pathogenic microorganisms due to the pesticide application in the field is essential.

Pesticides are usually dissolved or diluted in water and sprayed with large amounts of water to ensure plant coverage. Recommendations of the manufacturer include the requirement to use clean water to prepare pesticide solutions. However, there are still many parts in the world using water from ponds, lakes, rivers or canals. These sources of water are associated with a high risk of contamination by pathogenic agents in agricultural production (Scott *et al.*, 2004), especially in densely populated areas of poor countries, where there is no capacity to effectively treat wastewater. For example in Vietnam, about 80% of suburban canal waters were positive for *E. coli* and only 20% met the Vietnamese standard for coliform in irrigation water, *i.e.* 10,000 CFU/100 mL (Ha *et al*., 2008). Contaminated water alone could contribute to the spread of pathogenic agents like *E. coli* and *Salmonella* to crops, and if contaminated water is used for diluting pesticides that have the ability to support microbial survival and/or growth, the risk of contamination of vegetables could increase considerably.

Four different pesticides were used to determine their impacts and/or effect on *E. coli* and *Salmonella*. Selected pesticides in this study were: Ripcord 400EC, whose active ingredient is cypermethrin, a synthetic organic chemical insecticide of the pyrethroid group; Copper 53W, whose active ingredient is copper sulfate, an inorganic fungicide; Bioprotec CAF, which contains *Bacillus thuringiensis* kurstaki HD-1 toxins; and Serenade MAX, which contains *Bacillus subtilis* QST 173. Cypermethrin and copper are commonly used in crop protection and there have been a few studies on the effects of these chemical pesticides on pathogenic agents (Peter *et al*., 2005; Guan *et al*., 2005, 2001). Both Bioprotec CAF and Serenade MAX are bio-pesticides. These two bio-pesticides are commonly used in organic vegetable production where many types of vegetables are eaten raw or are minimally processed. They are also considered to be safe for human health and environmentally sound. Most studies (Guan *et al.*, 2001; 2005; Peter *et al.*, 2005) have focused on evaluating the impact of chemical pesticides and have ignored similar types of testing with bio-pesticides.

Sterile water was used for dissolving pesticides in laboratory assays to ensure that under laboratory conditions, the human pathogens would only affected by pesticides and did not interact with other organisms. Using of drinking water for diluting pesticides in field trials helped us narrow the objects of observation, focusing on the impact of pesticides to the human pathogens in present of other microorganisms on plant. Manufacturer recommendations on dilution rates were followed. In other studies, different sources of

agricultural water (wells, dams and rivers) were used for diluting pesticides to examine the persistence of microorganisms naturally present in these waters (Peter *et al*., 2005). Saline water and sterile water were also used in the studies of Guan *et al.* (2001; 2005) as control conditions.

For laboratory assays, we used an indicator microorganism (*E. coli*) and a pathogenic microorganism (*Salmonella*) instead of testing with a variety of bacterial pathogens. The results of statistical analysis indicated that the fluctuations of *E.coli* and *Salmonella* numbers in the pesticide solutions had a high correlation (0.89), suggesting that the response of *E. coli* and *Salmonella* to the pesticide solutions is quite similar. The populations of these bacteria had the same decrease and increase rates in solutions but were different in terms of time and concentration. In field experiments, we used *E. coli* as an indicator microorganism. It is easier and cheaper to test for indicator *E. coli* than for the other possible pathogens that might be present. On the other hand, we did not use pathogenic microorganisms in order to avoid a cross contamination to neighboring areas.

In the laboratory, each pesticide solution was separately spiked with a definite amount of *E. coli* and *Salmonella.* The initial microbial populations were 1000 CFU/mL. This is 1,000 times higher than the guidelines set out by the Canadian Council of Ministers of the Environment (CCME), which is set at a maximum concentration of 100 fecal coliforms (*E. coli*)/100 mL and 1000 total coliform bacteria/100 mL in irrigation water

from surface water (CCME, 2002). The high initial population of bacteria used in this study was set to help track the change of bacterial populations under the influence of pesticides.

In the field, pesticides are usually sprayed immediately after dilution or within 24 hours to avoid any decrease in active ingredient concentration or its stability. However, laboratory experiments in this study did not stop at 24 hours but extended until microorganisms could no longer be found in the solution in order to examine the impact of the pesticides on microbial growth and survival.

Most pesticides are used in formulated product, which the composition contains active and other ingredients. Active ingredients have the pesticidal efficacy but other ingredients do not have. These other ingredients can be solvents, carriers, adjuvants or others. Solvent intended to reduce the concentration of active ingredient, carriers are liquids or solid chemicals that support the delivery of the active ingredient, and adjuvants often help make the pesticide stick to or spread out on the application surface. Other adjuvants aid in the mixing of some formulations. For example, technical grade cypermethrin is semi-solid and insoluble in water so the formulation of the active ingredient as an emulsifiable concentrate is required (EHC 82, 1989). This formulation needs some non-pesticidal ingredients such as a solvent to dissolve cypermethrin, an emulsifier to make the emulsifiable form, and a dispersant to facilitate dispersion in water, etc. Pure copper sulfate is a solid and is insoluble in water. It is commonly formulated in wettable powder form

which the carrier, the adhesive, the suspending agent are indispensable. The exact composition of the non-pesticidal ingredient is closely maintained secret and considered confidential business information falling under proprietary protection. Farmers directly apply or dilute the pesticide before application as recommended by the manufacturer. In this study, the other ingredients of four pesticides used in this study are not mentioned by the manufacturers. The degradation of the active ingredient by pathogens or the mode of action of the active ingredient on pathogens for each pesticide was not studied. This was also the case for the other ingredient. Therefore, the persistence of microorganisms in pesticide solutions might be due to both the active ingredients and the other ingredients contained in the formulated product that could cause the toxic or nutritional effects on microorganisms.

The results of the initial investigation, indicated that microorganisms did not only survive (in Ripcord 400EC, Bioprotec CAF, Serenade MAX solutions), but also grew (in the Serenade MAX solution). The effect of each pesticide on microorganisms was demonstrated by the survival time and the change in the number or titer of microorganisms.

In the Copper 53W solution (Copper sulfate), there was no bacterial colony detected after incubation of the medium for 24 h. It is probable that microorganisms were killed by copper sulfate. Other studies on the effects of pesticides on human pathogens (Guan *et al.*, 2005; Peter *et al.*, 2005) also gave similar results in the testing of original copper

pesticides. According to Nies (1999), copper can kill bacteria by mechanism of action related to the displacement of essential ions, thereby disrupting protein function, inactivating enzymes, producing hydrogen peroxide free radicals, or disrupting membranes. Indeed, the bactericidal capacity of copper compounds has been shown in several studies (Salam *et al.*, 2008; Rodgers and Ryser, 2004; Faúndez *et al.*, 2004; Zwieten *et al.*, 2004; Liao *et al.*, 2003). The International Program on Chemical Safety (EHC 200, 1998) warned that misuse of agricultural chemicals containing copper on fields can cause a decrease of microbial activity in soil.

In the Ripcord 400EC solution (Cypermethrin), at 4^oC *E. coli* survived after incubation for 48 h. Guan *et al* (2005; 2001) also found that many species of human pathogens, including *E. coli* 0157: H7 and *Salmonella*, survived and grew in Ambush 500EC for 96 hours at room temperature, whose active ingredient is permethrin, the same pyrethroid group as cypermethrin. Cypermethrin is subjected to microbial degradation. It stimulates some bacterial groups that can produce the enzymes contribute to the metabolism of cyperethrin (Kasai, 2004). Enzyme-producing microorganisms use the metabolites from cypermethrin as a source of energy, carbon. It can be concluded that cypermethrin was not bactericidal, but could support microbial survival. Another reasons explained for the survival of *E. coli* at 4°C is that its optimal growth temperature is 37°C. Its growth rate decreases at lower temperatures and growth completely stops below 7°C. At 4°C, *E. coli* does not take much energy to replicate compared to at 21^oC, so it may survive longer.

In this study, we observed that *E. coli* indicators survived longer than *Salmonella* in the control solutions, Ripcord 400EC, Bioprotec CAF and Serenade MAX. However, in the study of Guan et *al.* (2004), *Salmonella* was the bacteria with the better growth in comparison with *E. coli* O157:H7, *Listeria monocytogene* and *Shigella* during 21°C incubation for 96 hours in the solution of pesticide Bravo 500 (chlorothalonil as an active ingredient). This pesticide was diluted by saline water. This could be related to bacterial strain differences, which suggests that different microorganisms can diversely adapt to the environment and that these survival capacities are complex.

In a Bioprotec CAF solution (*Bacillus thuringiensis* kurstaki HD-1 toxins), *E. coli* and *Salmonella* survived but did not grow at both 4°C and 21°C. Gram-positive bacteria can produce bacteriocins that are antimicrobial peptides toxic to bacterial strains closely related to the producer strain (Ahern *et al.*, 2003). However, at this time, no studies demonstrated that the presence of bacteriocins of *B. thuringiensis* kurstaki can affect the survival of *E. coli.* We believe that in the composition of Bioprotec CAF there are some nutrients helping *E. coli* and *Salmonella* to survive. In other words, this microbial pesticide could support or contribute to the survival of *E. coli* and *Salmonella*, not only related to *B. thuringiensis* but also to the nutritional support included within this commercial product.

In the Serenade MAX solution (*Bacillus subtilis* QST 713), *E. coli* and *Salmonella* survived at 4°C, grew and maintained high population at 21°C. *B. subtilis* has been shown

to produce a wide variety of antibacterial and antifungal compounds. It produces antibiotics such as difficidin and oxydifficidin that have activity against a wide spectrum of aerobic and anaerobic bacteria (Zimmerman *et al.*, 1987). The antimicrobial metabolites produced by *B. subtilis* MIR 15 appear to be mainly active against Gram-negative bacteria including *E. coli* and *P. aeruginosa* (Perez *et al.*, 1992). But in this study, *Bacillus subtilis* QST 713 could not produce antibacterial compounds that inhibit *E. coli* and *Salmonella*; in fact, it had the opposite effect of promoting the proliferation of these Gram-negative bacteria. In addition, a high increase of both *E. coli* and *Salmonella* populations can be explained by the fact that at 21°C, some of the compounds present in the finished pesticide formulation may have created a very favorable environment for microbial proliferation.

Temperature was a factor influencing the persistence of *E. coli* and *Salmonella* in pesticide solutions. However, in each type of pesticide solution, this influence was different. In the Serenade MAX solution, a temperature of 21°C was favorable for microbial growth and produced a high increase over more than 57 days, whereas a temperature of 4°C was inappropriate and resulted in a prolonged reduction of microorganism numbers. In contrast, increasing the temperature from 4°C to 21°C shortened the survival time of *E. coli* in the Ripcord 400EC and Bioprotec CAF formulations, probably due to an increase of bacterial metabolism and acceleration of the nutrients degradation rate. This was consistent with the study of Guan (2005, 2001), where increasing the temperature from 21 to 25 or 30°C had variable effects on the survival of

microorganisms depending on the pesticide. It is recognized that under stressful conditions, such as exposure to pesticide solutions at high temperatures, there is a greater energy cost for bacteria in order to maintain cytoplasmic homeostasis.

Serenade MAX and Bioprotec CAF, which presented the ability to support the growth of *E. coli* and *Salmonella*, are bio-pesticides commonly used in organic vegetable production. We usually pay attention to the risks of pesticides in term of food residues but for most microbial pesticides, maximum residue limits may be ignored because they pose a minimal risk to human health. Update to 2010, USEPA (2010) established exemption from the requirement of a tolerance for about 53 active substances. Provision B.15.002 (2) of the Food and Drug Regulations (FDR, 2008) in Canada, provides a list of 7 agricultural chemicals which are exempt from the requirement of setting MRLs.

Compared to chemical pesticides, bio-pesticides are safer and sounder. However, since these bio-pesticides have been shown to support the survival of *E. coli* and *Salmonella*, adequate sanitation practices should be established.

For field experiments, three pesticides including Ripcord 400EC, Copper 53W and Bioprotec CAF were further tested. These are widely used compared to Serenade MAX because they have a broad spectrum of pest eradication and a faster killing effect. Due to the fact that the experimental area was quite large, in order to avoid the spread of

microorganisms under uncontrolled conditions, as well as for safety purposes, we did not use human pathogens in the field study. Instead of testing with pathogenic *E. coli* we used a generic *E. coli* indicator. Irrigation water was artificially contaminated with a concentration of *E. coli* at 1047 CFU/100 mL from swine and bovine slurry, 10 times higher than the guidelines of CCME which allows for 100 CFU/100 mL in irrigation water from surface water (CCME, 2002).

In this part of the study, we expected to observe the impact of pesticides on the microbial community of broccoli and *E. coli* indicator bacteria. However, we did not observe any impact of pesticides on the persistence of *E. coli*, with 97.35% (184/189) of samples under the detection limit of the counting method (< 10 CFU/g). There was no significant difference in *E. coli* positive samples between the plots. Possible explanations for the low *E. coli* numbers may be adverse conditions like ultraviolet sunlight exposure and desiccation that if *E. coli* could not attach to plant surfaces, then they would have fallen down with the water and diffused into the soil or a competition between *E. coli* and indigenous microflora could have occurred, making it impossible for *E. coli* to colonize the phyllosphere (Brandl, 2006). That was the reason why Colilert medium was continuously used to evaluate the presence of *E. coli.*

There was also no significant difference of *E. coli* prevalence between the treatments during the entire experiment period. The effects of pesticide application on *E.*

coli prevalence in each treatment were not detectable. However, the *E. coli* prevalence in broccoli sprayed with Bioprotec CAF before irrigation was high at 52% (14/27 of samples were positive with *E. coli*) while the prevalence was lower at 30% (8/27 of samples were positive with *E. coli*) on broccoli sprayed with Copper 53W before irrigation. These results were consistent with laboratory assay results, where Copper 53W could inhibit and Bioprotec CAF could support microbial growth. Whereas, the *E. coli* prevalence in the broccoli sprayed with Ripcord 400EC before irrigation was same as the broccoli without pesticide spraying. It may be due to the effect of Ripcord 400EC *E. coli* prevalence was weak. Overall, pesticide application did not pose a risk elevation of *E. coli* prevalence in the field.

The observation of the changes of *E. coli* positive rates in each treatment according to sampling time showed that *E. coli* mostly appears after irrigation (based on results of determination by Colilert medium). This was expected because irrigation water was artificially contaminated by *E. coli*. Some samples taken before irrigation were positive for *E. coli* because the bacterial had probably already been brought in to the environment by carriers such as air, wind, insects, birds and human handling. We did not conduct an analysis to identify them.

The impact of pesticides on plant microflora in this study was very diverse before and after irrigation. For example, the mold and yeast numbers in broccoli treated by Copper

53W were reduced when spraying before irrigation but increased when spraying after irrigation; for Ripcord 400EC, they were increased when spraying before irrigation but reduced when spraying after irrigation; for Bioprotec CAF, they were increased after spraying. The aerobic bacteria numbers on broccoli also changed as diversely as the one of mold and yeast. But there were no significant differences in the population change of molds, yeasts and bacteria in treatments with pesticide. It reflects the fact that microorganisms have the ability to adapt to environment changes.

All treatments had the common characteristic of an increase of *E. coli*-positive rates after irrigation. For molds and yeasts, the populations were reduced 20 hours after irrigation. Aerobic bacteria populations tended to increase with *E. coli* prevalence due to the supply from artificially contaminated irrigation water. Mold and yeast population decrease could be due to many factors such as the washout effect of irrigation, stress when environmental conditions change suddenly, and competition between microorganisms on broccoli crops (Warriner and Namvar, 2010; Brandl, 2006; Aruscavage, 2006; Lindow and Brandl, 2003; Liao and Fett, 2001). However, the washout effect is less likely because the samples that had mold and yeast population increases were taken 20 hours after irrigation. In addition, the aerobic bacteria would have also been influenced by the washout but they tended to increase their populations. The increase of moisture after irrigation could possibly be associated with the growth rate of bacteria. These results indicated that there was an interaction between plant microflora on broccoli and *E. coli* from irrigation water

The results of laboratory studies showed direct effects of pesticides on *E. coli* and *Salmonella*. However, the field study results did not show any indirect effects of pesticides on indicator microorganisms.

The quality of water used for diluting pesticide plays an important role in preventing the application from becoming source of pathogens. The risk of spreading pathogens after pesticide application may occur particularly when using contaminated water to prepare the pesticide. The problem is real, especially in countries where surface water is still widely used for agricultural production and high rates of pathogenic contamination of surface water are found.

Chapter 5. CONCLUSION

Usually, pesticides pose a risk to consumer health because of residues. Their use in agricultural production is generally not believed to pose any microbial risks to public health, but this point has not been assessed by researchers in the province of Québec. Based on the results of this study, several conclusions can be made:

Seranade MAX, Bioprotec CAF and Ripcord 400EC directly impact the persistence of *E. coli* and *Salmonella* by supporting microbial survival and/or growth. They may contribute to a microbial contamination risk of vegetables and fruits. Conversely, Copper 53W inhibited microbial growth.

Temperature plays an important role in the potential for *E. coli* and *Salmonella* to survive and grow in pesticide solutions, but whether that role is negative or positive depends on the pesticide solution. In the Serenade MAX solution, a temperature of 21°C was favorable for microbial growth whereas in the Ripcord 400EC and Bioprotec CAF, this temperature was 4°C.

The effects of each pesticide on persistence of *E. coli* and *Salmonella* were observed at different levels and the behavior was similar for both bacteria (correlation between *E. coli* and *Salmonella* equals 0.89).

Irrigation water influenced on *E. coli*-positive rates, molds and yeasts and total aerobic bacteria counts, whereas the impact of pesticide on *E. coli*, molds and yeasts, and aerobic bacteria populations could not assessed.

This study indicates that pesticide applications in these experimental conditions did not result in the increase of *E. coli* in the field. However, if pesticides are prepared with contaminated water, they can potentially spread pathogens. To reduce the risk of vegetables being contaminated by human pathogens due to pesticide application during a pre-harvest period, the quality of water used to dilute pesticides needs to be controlled, especially given the fact that some pesticides can support the survival and growth of some pathogenic

BIBLIOGRAPHICAL REFERENCES

Abdul-Raouf, U.M., Beuchat, L.R., Ammar, M.S. 1993. Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. Appl Environ Microbiol. 59:1999-2006.

Agri-Reseau. 2010. Coût des insecticides et des fongicides homologués dans la culture des crucifères en 2010. Agriculture, pêcheries et alimentation Québec. Centre de référence en agriculture et agroalimentaire du Québec. Retrieved Sep, 5, 2010. Available at http://www.agrireseau.qc.ca/Rap/documents/b01cru12.pdf

Ahern, M., Verschueren, S., Sinderen, D.V. 2003. Isolation and characterisation of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. FEMS Microbiol Letters 220. 127-31.

Andrews, J. H. and Harris, R. F. 2000. The ecology and biogeography of microorganisms on plant surfaces. Annu. Rev. Phytopathol. 38:145-80.

Aruscavage, D., Lee, K., Miller, S., LeJeune, J.T. 2006. Interactions Affecting the Proliferation and Control of Human Pathogens on Edible Plants. J. Food Sci. 71(8): 89-99.

Avery, L.M., Killham, K., Jones, D.L. 2005. Survival of *E. coli* O157:H7 in organic wastes destined for land application. J. Appl Microbiol. 98:814-22.

Bais, H.P, Fall, R., Vivanco, J.M. 2004. Biocontrol of *Bacillus subtilis* against infection of arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiology. 134:307-19.

Beattie, G.A. and Lindow, S.E. 1994. Epiphytic fitness of phytopathogenic bacteria: physiological adaptations for growth and survival. In: Dang JL (ed). Bacterial

pathogenesis of plants and animals, molecular and cellular mechanisms. Springer-Verlag, Berlin.1-28.

Behravesh, C.B., Blaney, D., Medus, C., Bido, S.A., Phan, Q., Soliva, S., Daly, E.R., Smith, K., Miller, B., Taylor, T., Nguyen, T., Perry, C., Hill, T.A., Fogg, N., Kleiza, A., Moorhead, D., Al-Khaldi S., Braden, C., Lynch, M.F. 2012. Multistate outbreak of *Salmonella* serotype Typhimurium infections associated with consumption of restaurant tomatoes, USA, 2006: hypothesis generation through case exposures in multiple restaurant clusters. Epidemiol Infect, 1-9.

Beuchat, L.R. 1996. Pathogenic microorganisms associated with fresh produce. J. Food Prot. 59:204-16.

Boone, M.D., Semlitsch, R.D., Little, E.E., Doyle, M.C. 2007. Multiple stressors in Amphibian communities: Effects of chemical contamination, Bullfrog and Fish. Ecological Applications 17(1):291-301.

Bopp, C.A., Brenner, F.W., Wells, J.G., Strokbine, N.A. *Escherichia, Shigella* and *Salmonella.* 2005. Eds. Murray, P. R., Baron, E.J., P. Faller, M. A., Tenover, F.C., Yolken, R.H. Manual of clinical microbiology, 7^{e} edition. American society for microbiology, USA. 3: 23-30, 28:459-74.

Borchardt, M.A., Bradbury, K.R., Gotkowitz, M.B., Cherry J.A., Parker, B.L. 2007. Human enteric viruses in groundwater from a confined bedrock aquifer. Environ Sci Technol. 41(18):6606-12.

Brandl, M. T. 2006. Fitness of Human Enteric Pathogens on Plants and Implications for Food Safety. Annu. Rev. Phytopathol. 44: 367-92.

British Crop Protection Council (BCPC). 2009. The Pesticide Manual, 15th edition. Tomlin C.D.S editor. p vii.

Buck, J.W., Walcott, R.R., Beuchat, L.R. 2003. Recent trends in microbiological safety of fruits and vegetables. Plant Health Progress.10.1094.

Cahan, R., Friman, H., Nitzan, Y. 2008. Antibacterial activity of Cyt1Aa from *Bacillus thuringiensis* subsp. israelensis. Microbiology. 154:3529-36.

Callaway, E. 2008. US *Salmonella* Outbreak Explained. Retrieved Nov 14, 2010. Available at:http://www.newscientist.com/article/dn14110-us-salmonella-outbreak-explained-.htm.

Canadian Council of Ministers of the Environment (CCME) 2002. Canadian water quality guidelines for the protection of agricultural water use. Retrieved Nov 4, 2012. Available at: http://ceqg-rcqe.ccme.ca.

Carlin, F., Nguyen, T.C., Morris, C.E. 1996. Influence of background flora on *Listeria Monocytogenes* on minimally processed fresh broad-leaved endive (*Cichorium endivia* var. latifolia). J. Food Prot. 59:698-703.

Cox, C. 1996. Insecticide factsheet - Cypermethrin. Journal of pesticide reform. 16(2) 15-20.

Castro, J.R., Santos, L.E.M., Gomez, C.A.A., Gonzalez, C.A.R., Villagomez, J.R.I., Gordillo, A.J.M., Lopez, A.V., del Refugio, M.T.V. 2010. Incidence and behavior of

Salmonella and *Escherichia coli* on whole and sliced zucchini squash (Cucurbitapepo) fruit. J. Food Prot. 73(8):1423-9.

Cennimo, D.J., Koo, H., Mohamed, J.A., Huang, D.B., Chiang, T. 2007. Enteroaggregative *Escherichia coli*: A review of trends, diagnosis, and treatment. Infect Med. 24:100-10.

Centers for Disease Control and Prevention (CDC). 2005a. Outbreaks of *Salmonella* infections associated with eating Roma tomatoes-United States and Canada, 2004. Morb Mortal WKly Rep. 54(13):325-8.

Centers for Disease Control and Prevention (CDC). 2005b. Pesticide-Related Illness and Injury Surveillance: A How-To Guide For State-Based Programs. NIOSH Publication No. 2006–102, p 217.

Centers for Disease Control and Prevention (CDC). 2006a. Multistate outbreak of *E. coli* O157 infections, November - December 2006. Retrieved Nov 14, 2010. Available at http://www.cdc.gov/ecoli/2006/december/121406.html.

Centers for Disease Control and Prevention (CDC). 2006b. Update on Multi-State Outbreak of *E. coli* O157:H7 Infections From Fresh Spinach. Retrieved Nov 14, 2010. Available at http://www.cdc.gov/foodborne/ecolispinach/100606.htm

Centers for Disease Control and Prevention (CDC). 2008. Outbreaks of *Salmonella* serotype Saintpaul infection associated with multiple raw produce items - United States, 2008. Morb Mortal WKly 2008. 57(34):929-34.

Chau, T.T., Campbell, J.I., Galindo, C.M., Hoang, N.V.M, Diep, T.S., Nga, T.T. 2007. Antimicrobial drug resistance of *Salmonella enterica* serovar Typhi in Asia and molecular mechanism of reduced susceptibility to the fluoroquinolones. Antimicrob Agents Chemother. 51(12):4315-23.

CIDRAP. Update 2006. Common causes of foodborne disease. Center for Infectious Disease Research & Policy, University of Minnesota. Retrieved Nov 7, 2012. Available at: http://www.cidrap.umm.edu/cidrap/content/fs/food-desease/causes/ index.html.

Clark, M. 2009. Veggie Booty *Salmonella* Outbreak-Nationwide. Retrieved Feb 14, 2011. Available at:http://www.marlerclark.com/case_news/view/veggie-booty-salmonella-outbreak-nationwide.

Cycon, M. and Piotrowska-Seget, Z. 2009. Changes in bacterial diversity and community structure following pesticides addition to soil estimated by cultivation technique. Ecotoxicology. 18(5):632-42.

Daly, H., Doyen, J.T., Purcell, A.H. 1998. Introduction to insect biology and diversity, 2nd edition. Oxford University Press. New York. 14:279-300.

Demoz, B. T. and Korsten, L. 2006. *Bacillus subtilis* attachment, colonization, and survival on avocado flowers and its mode of action on stem-end rot pathogens. Retrieved Jan 25, 2011. Available at: http://www.up.ac.za/dspace/bitstream/2263/1263/1/Demoz_ Bacillus%282006%29.pdf.

Dollwet, H.H.A. and Sorenson, J.R.J. 2001. Historic uses of copper compounds in medicine. Trace Elements Med. 2: 80-7.

Duffy, E.A., Lucia, L.M., Kells, J.M., Castillo, A., Pillai, S.D., Acuff, G.R. 2005. Concentration of *Escherichia coli* and genetic diversity and antibiotic resistance profiling of *Salmonella* isolated from irrigation water, packing shed equipment, and fresh produce in Texas. J. Food Prot. 68:70-9.

Edgecomb, D.W. and Manker, D. 2006. *Bacillus subtilis* strain QST 713, bacterial disease control in fruit, vegetable and ornamental production. In: Mtteilungen aus der Biologishen Bundesanstalt fur Land-und Forstwirtschaft. Zeller, W., Ullrich, C. (Ed). No. 408. 167-9.

Eisenhauer, N., Klier, M., Partsch, S., Sabais, A.C.W., Scherber, C., Weisser, W.W., Scheu, S. 2009. No interactive effects of pesticides and plant diversity on soil microbial biomass and respiration. Applied Soil Ecology. 42: 31–6.

Environmental Health Criteria (EHC) Monographs No. 217. 1999. *Bacillus thuringiensis*. International Programme on Chemical Safety. Retrieved Nov 17, 2010. Available at: http://www.inchem.org/documents/ehc/ehc/ehc217.htm

Environmental Health Criteria (EHC) Monographs No. 200. 1998. Copper. International Programme on Chemical Safety. Retrieved March 17, 2012. Available at: http://www.inchem.org/documents/ehc/ehc/ehc200.htm#SectionNumber:1.4

Environmental Health Criteria (EHC) Monographs No. 82. 1989. Cypermethrin. International Programme on Chemical Safety. Retrieved May 28, 2011. Available at: http://www.inchem.org/documents/ehc/ehc/ehc82.htm.

Environmental Health Criteria (EHC) Monographs No. 83. 1989. DDT and its derivatives – environmental aspects. International Programme on Chemical Safety. Retrieved Nov, 6, 2012. Available at: http://www.inchem.org/documents/ehc/ehc/ehc83.htm

Environmental Health Criteria (EHC) Monographs No. 64. 1986. Carbamate pesticides: a general information. International Programme on Chemical Safety. Retrieved Nov, 6, 2012. Available at: http://www.inchem.org/documents/ehc/ehc/ehc64.html.

Environmental Health Criteria (EHC) Monographs No. 63. 1986. Organophosphorus insecticides: a general introduction. International Programme on Chemical Safety. Retrieved Nov, 6, 2012. Available at: http://www.inchem.org/documents/ehc/ehc/ehc63.html

European Centre for Disease Prevention and Control (ECDC). 2011. Shiga toxin-producing *E. coli* (STEC): Update on outbreak in the EU (27 July 2011, 11:00). Retrieved May 18, 2012. Available at http://ecdc.europa.eu/en/activities/sciadvice/Lists/.

Euzéby, J.P. 2005. Nomenclature des salmonelles. Dictionnaire de Bactériologie Vétérinaire. Retrieved Feb 14, 2011. Available at: http://www.bacterio.cict.fr/bacdico/systematique/nomensalmonelles.html

Extonet. 1994. Pesticide information profile: Copper sulfate. Retrieved Aug 20, 2012. Available at: http://pmep.cce.cornell.edu/profiles/extoxnet/carbaryl-dicrotophos/copper-sulfate-ext.html

FAO/WHO. 2008. Microbiological hazards in fresh leafy vegetables and herb. Microbiological risk assessment series 14. Retrieved Nov 6, 2012. Available at ftp://ftp.fao.org/docrep/fao/011/i0452e/i0452e00.pdf.

FAO/WHO Joint Meeting on Pesticide Specifications (JMPS). 2006. Manual on development and use of FAO and WHO specifications for pesticides. Retrieved Aug 18, 2012. Available at: http://whqlibdoc.who.int/publications/2006/9251048576_eng_update2.pdf.

Faúndez, G., Troncoso, M., Navarrete, P., Figueroa, G. 2004. Antimicrobial activity of copper surfaces against suspensions of *Salmonella enterica* and *Campylobacter jejuni*. BMC Microbiology. 4: 19.

Feachem, R.G., Bradley, D.J., Garelick, H., Mara, D.D. 1983. Sanitation and Disease: Health Aspects of Excreta and Wastewater Management. In: World Bank Studies in Water Supply and Sanitation 3. New York, NY: John Wiley & Sons.

Food and Drug Regulations (FDR), 2008. Provision B.15.002 (2). Retrieved Mar, 2011. Available at: http://laws-lois.justice.gc.ca/eng/regulations/C.R.C.,_c._870/section-B.15.002-20080616.html

Fotadar, U., Zaveloff, P., Terracio, L. 2005. Growth of *Escherichia coli* at elevated temperatures. J Basic Microbiol. 45(5): 403-04.

Francis, G.A. and O'Beirne, D. 2001. Effects of vegetable type, package atmosphere and storage temperature on growth and survival of *Escherichia coli* O157:H7 and *Listeria monocytogenes,* J Ind Microbiol & Biotec. 27: 111-6.

Franco, W., Hsu, W.Y., Simonne, A.H. 2010. Survival of *Salmonella* and *Staphylococcus aureus* in Mexican red salsa in a food service setting. J Food Prot. 73(6):1116-20.

Furness, G. and Thompson, A. 2008. Using point of first run-off and spray volume in litres per 100 metres per metre of canopy height for setting pesticide dose. Agricultural engineering international: the GIGR Ejournal. Retrieved December 10, 2011. Available at: http://www.cigrjournal.org/index.php/ Ejounral/article/viewFile/1247/ 1105.

Gilliom, R.J., Barbash, J.E., Crawford, G.G., Hamilton, P.A., Martin, J.D., Nakagaki, N., Nowell, L.H., Scott, J.C., Stackelberg, P.E., Thelin, G.P., Wolock, D.M. 2007. The Quality of our nation's waters: Pesticides in the nation's streams and ground water, 1992–2001. US Geological Survey. Fact Sheet 2006–3028. 1: 4.

Grant, R.J., Daniell, T.J., Betts, W.B. 2002. Isolation and identification of synthetic pyrethroid-degrading bacteria. J. Appl Microbiol. 92:534-40.

Gray, E.J., Lee, K.D., Souleimanov, A., Di Falco, M.R., Zhou, X., Ly, A., Charles, T.C., Driscoll, B.T., Smith, D.L. 2006. A novel bacteriocin, thuricin 17, produced by PGPR strain *Bacillus thuringiensis* NEB17: Isolation and classification. J. Appl. Microbiol. 100: 545-54.

Greene, S.K., Daly, E.R., Talbo, E.A., Demma, L.J., Holzbauer, S., Patel, N.J., Hill, T.A., Walderhaug, M.O., Hoekstra, R.M., Lynch, M.F., Painter, T.A. 2008. Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields. Epidemiol Infect. 136(2): 157–65.

Guan, T., Blank, G., Holley, R.A. 2005. Survival of pathogenic bacteria in pesticide solutions and on treated tomato plants. J. Food Prot. 68:296-304.

Guan, T., Blank, G., Ismond, A., Acker, R.V. 2001. Fate of foodborne bacterial pathogens in pesticide products. J. Sci. Food Agri. 81:503-12

Guo, X., van Iersel, M.W., Chen, J., Brackett, R.E., Beuchat, L.R. 2002. Evidence of association of *Salmonella* with tomato plants grown hydroponically in inoculated nutrient solution. Appl Environ Microbiol. 68:3639-43.

Ha, N.T., Kitajima, M., Hang, N.V., Matsubara, K., Takizawa, S., Katayama, H., Oguma, K., Ohgaki, S. 2008. Bacterial contamination of raw vegetables, vegetable-related water and river water in Ho Chi Minh City, Vietnam. Water Sci Technol. 58(12):2403-11.

Han, Y. and Linton, R.H. 2004. Fate of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in strawberry juice and acidified media at different pH values and temperature. J. Food Prot. 67, 2443-49.

Hara-Kudo, Y., Konuma, H., Iwaki, M., Kasuga, F., Sugita-Konishi, Y., Ito, Y.Y., Kumagai, S. 1997. Potential hazard of radish prout as a vehicle of *E. coli* O157:H7. J. Food Prot. 60: 1125-7.

Health Canada. 2001. Enumeration of *E. coli* and coliforms in food products and food ingredients using 3M™ Petrifilm™ *E. coli* count plates. Retrieved Mar 20, 2011. Available at: http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume 2/mfhpb34-01-eng.php

Hyun-Gyun, Y., Bartz, J.A., Schneider, K.R. 2005. Effectiveness of individual or combined sanitizer in treatments for inactivating *Salmonella* spp. on smooth surface, stem scar, and wounds of tomatoes. J Food Sci. 70(9): 409-14.

Itoh, Y., Nagano, I., Kunishima, M., Ezaki, T. 1997. Laboratory investigation of enteroaggregative *Escherichia coli* O untypable: H10 associated with a massive outbreak of gastrointestinal illness. J. Clin Microbiol. 35: 2546-50.

International Water Management Institute (IWMI). 2006. Recycling Realities: Managing risks to make wastewater an asset. Water Policy Briefing.Retrieved Nov 10, 2012. Available at : http://www.iwmi.cgiar.org/Publlications/Water_Policy_Briefs/ PDF/Wpb17.pdf

Janisiewski, W.J., Conway, W.S., Leverentz, B. 1999. Biological control of postharvest decays of apple can prevent growth of *Escherichia coli* O157:H7 in apple wounds. J. Food Prot. 62(12):1372-5.

Johnson, J.Y.M., Thomas, J.E., Graham, T.A., Townshend, I., Byrne, J., Selinger, L.B., Gannon, V.P.J. 2003. Prevalence of *Escherichia coli* O157:H7 and *Salmonella* spp. in surface waters of southern Alberta and its relation to manure sources. Canadian J. Microbiol. 49: 326-35.

Josefsen, M.H., Lofstrom, C., Olsen, K.E.P., Molbak, K., Hoorfar, J. 2011. Molecular detection of human bacterial pathogens. Ed. Dongyou Liu. *Salmonella*. CRC Press. 87:1023-15.

Kabaluk, J.T., Brookes, V.R., Svircev, A.M. 2010. Canada. In: The use and regulation of microbial pesticides in representative jurisdictions worldwide. Kabaluk, J.T., Svircev, A.M., Goettel, M.S., Woo, S.G. (ed.). IOBC Global. 59-73.

Kamoun, F., Mejdoub, H., Aouissaoui, H., Reinbolt, J., Hammami, A., Jaoua, S. 2005. Purification, amino acid sequence and characterization of bacthuricin F4, a new bacteriocin produced by *Bacillus thuringiensis*. J. Appl. Microbiol. 98: 881-8.

Kaper, J.B., Nataro, J.P., Mobley, H. 2004. Pathogenic *Escherichia coli*. Nat rev. Microbiol. 2: 123-40.

Kasai, S. 2004. Role of cytochrome P450 in mechanism of pyrethroid resistance. J. Pestic. Sci. 29**:**220-221.

Kazuhiko, M., Yukio, K., Atsushi, H., Koji, N., Yoshio, Katsuda., Aikikazu, H., Koichiro, Komai. 2005. Biosynthesis of pyrethrin I in seedlings of *Chrysanthemum cinerariaefolium.* Phytochemistry. 66(13) :1529-1535.

Kerle, E.A., Jenkins, J.J., Vogue, P.A. 2007. Understanding pesticide persistence and mobility for groundwater and surface water protection. Oregon State University Extension Service. EM 8561-E. Retrieved Nov 4, 2012. Available at http://ir.library.oregonstate.edu/xmlui/bitstream/handle/1957/20565/em8561-e.pdf?sequence=1.

Kerr, M., Fitzgerald, M., Sheridan, J.J., Mcdowell, D.A., Blair, I.S. 1999. Survival of *Escherichia coli* O157:H7 in bottled natural mineral water. J. Appl. Microbiol. 87: 833-41.

Kloepper, J.W, Ryu, C.M., Zhang, S. 2004. Induce systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94:1259-66.

Klotchko, A. and Wallace, M.R. 2009. Salmonellosis. Retrieved Feb 19, 2011. Available at: http://emedicine.medscape. com/article/228174-overview.

Krauss, J., Gallenberger, I., Steffan-Dewenter, I. 2011. Decreased functional diversity and biological pest control in conventional compared to organic crop fields. PLoS One. 6(5):e19502.

Kunito, T., Saeki, K., Oyaizu, H., Matsumoto, S. 1999. Influence of copper forms on the toxicity to microorganisms in soils. Encotoxicol Environ Saf. 44(2): 174-81.

Kuniuki, S. 2001. Effects of organic fertilization and pesticide application on growth and yield of field-grown rice for 10 years. Japanese J. Crop Sci. 70(4): 530-40.

Liao, C.H. and Fett, W.F. 2001. Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. J. Food Prot. 64(8): 1110-15

Liao, C.H. and Sapers, G.M. 1999. Influence of soft rot bacteria on growth of *Listeria monocytogenes* on potatoes tuber slices. J. Food Prot 62: 343-48

Liao, J.P., Lin, X.G., Cao, Z.H., Shi, Y.Q., Wong, M.H. 2003. Interactions between arbuscular mycorrhizae and heavy metals under sand culture experiment. Chemosphere 50:847-53.

Lindow, S.E. and Brandl, M.T. 2003. Minireview: Microbiology of the Phyllosphere. Appl environ microbiol 69(4): 1875-83.

Lynch, M.F., Blanton, E., Bulens, S., Polyak, C., Vojdani, J., Stevenson, J. 2009. Typhoid fever in the United States, 1999-2006. JAMA. 302(8):859-65.

Lynn, R.M., O'Brien, S.J., Tayor, C.M. 2005. Childhood hemolytic uremic syndrome, United Kingdom and Ireland. Emerg Infect Dis. (4):590-6.

Ma, L., Zhang, G., Gerner-Smidt, P., Tauxe, R.V., Doyle, M.P. 2010. Survival and growth of *Salmonella* in salsa and related ingredients. J. Food Prot. 73(3):434-44.

McCarthy, T.A., Barrett, N.L., Hadler, J.L. 2001. Hemolytic-uremic syndrome and *Escherichia coli* O121 at a lake in Connecticut, 1999. Pediatrics.108:59.

Mead, P.S., Slutsker, L., Dietz, V., McCraig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V. 1999. Food-related illness and death in the United States. Emerging Infectious Diseases, 5: 607-25.

Miller, G.T. 2004. Sustaining the Earth, 6th edition. Thompson Learning, Inc. Pacific Grove, California. 9: 211-16.

Miller, L.K., Lingg, A.J., Bulla, L.A.jr. 1983. Bacterial, viral and fungal insecticides. Sci. 219:715-21.

Ministry of Agriculture, British Columbia, Canada. Environmental Protection. Retrieved May 20, 2011. Available at http://www.agf.gov.bc.ca/pesticides/c_2.htm#1b

Ministry of Agriculture and Rural Development, Vietnam (MARD). 2009. Circular No. 09/2009/TT-BNN.

Mishra, P.K., Samarth, R.M., Pathak, N., Jain, S.K., Banerjee, S., Maudar, K.K. 2009. Bhopal gas tragedy: review of clinical and experimental finding after 25 years. Int J Occup Med Environ Health. 22(3): 193-202.

Mohammad, F.K., Al-Badrany, Y.M., Al-Jobory, M.M. 2008. Acute toxicity and cholinesterase inhibiton in chick dosed orally with organophosphate insecticides. Arh Hig Rada Toksikol. 59(3):145-51.

Murugesan, A. G., Jeyasanthi, T., Maheswar, S. 2010. Isolation and characterization of cypermethrin utilizing bacteria from Brinjal cultivated soil. African J. Microbiol. Research. 4 (1) 10-13.

Nataro, J.P. and Kaper, J.B. 1998. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. 1998.11:142-201 (erratum in: Clin Microbiol Rev. 1998.11:403).

Nguyen, R.N., Taylor, L.S., Tauschek, M. 2006. A typical enteropathogenic *Escherichia coli* infection and prolonged diarrhea in children. Emerg Infect Dis. 12(4):597-603

Nies, D. H. 1999. Microbial heavy metal resistance. Appl Microbiol Biotech 51:730-50

Olusegun, P.A. 2010. Lead and coliform contaminants in potable groundwater sources in Ibadan, South-West Nigeria. J Environ Chem Ecotox 2(5): 79-83.

Pal, R., Chakrabarti, K., Chakraborty, A., Chowdhury, A. 2005. Pencycuron application to soils: Degradation and effect on microbiological parameters. Chemosphere 60(11):1513-22.

Pegues, D.A., Ohl, M.E., Miller, S.I. 2005. *Salmonella* species, including *Salmonella* Typhi. Eds. Mandell, G.L., Bennett, J.E., Dolin, R., Mandell, Douglas, and Bennett's Principles and practice of infectious diseases. Philadelphia: Elsevier Churchill Livingstone, 2:2636-54

Perez, C., Suarez, C., Castro, G.R. 1992. Production of antimicrobials by *Bacillus subtilis* MIR 15. Biotechnol. 26: 331-36.

Perucci, P., Dumontet, S., Bufo, S.A., Mazzatura, A., Casucci, C. 2000. Effects of organic amendment and herbicide treatment on soil microbial biomass. Biol. Fertil. Soils, 32: 17-23.

Peter, J. Ng., Fleet, G.H., Gillian, T., Heard, G.M. 2005. Pesticides as a source of microbial contamination of salad vegetables. Int J. Food Microbiol. 101: 237-50.

Pusztai, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., Carey, P.R. 1991. The mechanism of sunlight-mediated inactivation of *Bacillus thuringiensis* crystals. Biochem J. 273: 43-47.

Qadri, F., Svennerholm, A.M., Faruque, A.S. 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev.18(3): 465-83.

Quiroz-Santiago, C., Rodas-Suarez, O., Vazquez, Q.C.R., Fernadez, F.J., Quinones-Ramirez, E.I., Vazquez-Salinas, C. 2009. Research note: Prevalence of *Salmonella* in vegetables from Mexico. J. Food Prot. 72(6): 1279-82.

Rangel, J.M., Sparling, P.H., Crowe, C., Griffin, P.M., Swerdlow, D.L. 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. Emerg Infect Dis 11(4): 603-9.

Rathinasasabapathi, B. 2004. Survival of *Salmonella* Montevideo on tomato leaves and mature green tomatoes, J. Food Prot. 67:2217-9.

Remans, T., Thijs, S., Truyens, S., Weyens, N., Schellingen, K., Keunen, E., Gielen, H., Cuypers, A., Vangronsveld, J. 2012. Understanding the development of roots exposed to contaminants and the potential of plant associated bacteria for optimization of growth. Ann Bot. 110(2):239-52.

Riordan, D.C.R., Sapers, G.M., Annous, B. A. 2000. The survival of *Escherichia coli* O157:H7 in the presence of *Penicillium expansum* and *Glomerella cingulata* in wounds on apple surfaces. J. Food Prot. 63:1637-42.

Roberts, T.R. and Standen, M.E. 1977. Degradation of the pyrethroid cypermethrin NRDC 149(±)-alpha-cyano-3-phenoxy-benzyl(±)-*cis,trans*-3-(2,2-dichlorovinyl-2,2-dimethylcyclo-propanecarboxylate and the respective *cis*-(NRDC 160) and the *trans*-(NRDC 159) isomers in soils. Pesticide Sci. 8: 305-19.

Rodgers, S.L. and Ryser, E. 2004. Reduction of microbial pathogens during apple cider production using sodium hypochlorite, copper ion, and sonication. J. Food Prot 67:766-71.

Salam, A.I., Yang, H., Chung, W.S. 2008. Antimicrobial activity of lactic acid and copper on growth of *Salmonella* and *Escherichia coli* O157:H7 in laboratory medium and carrot juice. Food Chemistry 109:137-43.

Schuenzel, K.M., and Harrison, M.A. 2002. Microbial antagonists of foodborne pathogens on fresh, minimally processed vegetables. J Food Prot 65(12):1909-15.

Schulze, J., Schiemann, M., Sonnenborn, U. 2006. 120 years of *E. coli*. Its importance in research and medicine. Alfred-Nissle-Gesellschaft e.V. 58089 Hagen, Germany. 7-54.

Scott, C.A., Faruqui, N.I., Raschid-Sally, L. 2004. Wastewater Use in Irrigated Agriculture: Confronting the Livelihood and Environmental Realities. CABI Publishing, Oxfordshire, UK. 1-10.

Singh, B.K. and Walker, A. 2006. Microbial degradation of organophosphorous compounds. FEMS Microbiol. Rev. 30, 428-71.

Soderstrom, A., Osterberg, P., Lindqvist, A., Jonsson, B., Lindberg, A., Blide Ulander, S., Welinder-Olsson, C., Lofdahl, S., Kaijser, B., De Jong, B., Kuhlmann-Berenzon, S., Boqvist, S., Eriksson, E., Szanto, E., Andersson, S., Allestam, G., Hedenstrom, I., Ledet Muller, L., Andersson, Y. 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. Foodborne Pathogens and Disease. 5(3): 339-49.

Solomon, E. B., Yaron, S., Matthews, K.R. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl. Environ. Microbiol. 68: 397-400.

Steel, M. and Odumeru, J. 2004. Irrigation water as source of foodborne pathogens on fruit and vegetables. J.Food Prot. 12: 2839-49.

Strawn, L.K and Danyluk, M.D. 2010. Fate of *Escherichia coli* O157:H7 and *Salmonella* on fresh and frozen cut pineapples. J. Food Prot. 73(3):418-24.

Tiskumara, R., Fakharee, S.H., Liu, C.Q., Nuntnarumit, P., Lui, K.M., Hammoud, M. 2009. Neonatal infections in Asia. Arch Dis Child Fetal Neonatal. 94:144-8.

Todar, K. 2007. Bacterial Pathogens and Diseases of Humans. Available Textbook of Bacteriology. University of Wisconsin–Madison Department of Bacteriology. Retrieved Oct 20, 2010. Available at: http:///www.textbookofbacteriology.net/ kt_toc.html.

United States Environmental Protection Agency (USEPA). Update to 2010. Biopesticide Federal Register Notices – 2010. Retrieved Mar, 2011. Available at: http://www.epa.gov/pesticides/biopesticides/regtools/frnotices2010.html

United States Environmental Protection Agency (USEPA). 1989. Pesticide Fact Sheet Number 199: Cypermethrin. Office of Pesticides and Toxic Substances. 2-9

Unsworth, J. 2010. History of Pesticide Use. Retrieved Nov 09, 2011. Available at: http://agrochemicals.iupac.org/index.php?option=com_sobi2&sobi2Task=sobi2Details&catid=3&sobi2Id=31

Vig, K., Singh, D.K., Agarwal, H.C., Dhawan, A.K., Dureja, P. 2008. Soil microorganisms in cotton fields sequentially treated with insecticides. Ecotoxicol. Environ. Saf. 69: 263-76.

Vogt, R.L., Dippold, L. 2005. *Escherichia coli* O157: H7 Outbreak Associated with Consumption of ground beef, June-July 2002. Public Health Rep 120(2): 174-78.

Wachtel, M. R., Whitehand, L. C., Mandrell, R. E. 2002. Prevalence of *Escherichia coli* associated with a cabbage crop inadvertently irrigated with partially treated sewage wastewater. J. Food Prot. 65:471-75.

Wade, W.N and Beuchat, L.R. 2003. Metabiosis of proteolytic moulds and *Salmonella* in raw, ripe tomatoes. J. Appl Microbiol. 95(3):437-50.

Wang, X., Song, M., Wang, Y., Gao, C., Zhang, Q., Chu, X., Fang, H., Yu, Y. 2012. Response of soil bacterial community to repeated applications of carbendazim. Ecotoxicol Environ Saf. 75(1):33-9.

Wardle, D.A. and Parkinson, D. 1990. Effects of three herbicides on soil microbial biomass and activity. Plant Soil, 122: 21-28.

Warriner, K. and Namvar, A. 2010. The tricks learn by human enteric pathogens from phytopathogens to persist within the plant environment. Current Opinion Biotechnology. 21(2): 131-36.

Wells, J. M. and Butterfield, J. E. 1997. *Salmonella* contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. The American Phytopathological Society. 81(8) 867-72.

Whalon, M.E. and Wingerd, B.A. 2003. Bt: Mode of action and use Archives of Insect Biochemistry and Physiology, 54: 200-11.

Whitehorn, P.R., O'Connor, S., Wackers, F.L., Goulson, D. 2012. Neonicotinoid pesticide reduces bumble bee colony growth and queen production. Science. 336(6079):351-2.

Woo, J.J., Mabood, F., Souleimanov, A., Zhou, X., Jaoua, S., Kamoun, F., Smith, D.L. 2008. Stability and Antibacterial Activity of Bacteriocins Produced by *Bacillus thuringiensis* and *Bacillus thuringiensis* ssp. Kurstaki. J. Microbiol. Biotechnol. 18(11): 1836-40.

World Health Organization (WHO). Updated 2009. Enterotoxigenic *Escherichia coli* (ETEC) Diarrhoeal Diseases. Retrieved Oct 22, 2010 Available at: http://www.who.int/vaccine_research/diseases/diarrhoeal/en/index4.html.

Yamamoto, T. 2001. One hundred years of *Bacillus thuringiensis* research and development: Discovery to transgenic crops. J. Insect Biotechnol. Sericol. 70:1–23.

Yen - Phi, V.T., Rechenburg, A., Vinneras, B., Clemens, J., Kistemann, T. 2010. Pathogens in septage in Vietnam. Sci Total Environ. 408(9): 2050-3.

Zelles, L., Scheunert, I., Korte, F. 1985. Side effects of some pesticides on non-target soil microorganisms. J. Environ. Sci. Health, 20: 457-88.

Zhang, Y., Zhu, W., Sun, C., Xiao, L., Yang, L.Y. 2009. Effect of combined application of copper and cypermethrin on structural diversity of soil microbial community. J. Agro-Environ Sci. 28(4): 673-79.

Zhang, B., Bai, Z., Hoefel, D., Wang, X., Zhang, L., Li, Z. 2010. Microbial diversity within the phyllosphere of different vegetable species. A. Méndez-Vilas (ed). In Current research, technology and education topics in applied microbiology and microbial biotechnology. Formatex 2010. 1067-77.

Zhang, B., Bai, Z., Hoefel, D., Tang, L., Wang, X., Li, B., Li, Z., Zhuang, G. 2009a. The impacts of cypermethrin pesticide application on the non-target microbial community of the pepper plant phyllosphere. Sci Total Environ. 407(6):1915-22.

Zhang, B., Tang, L., Li, Z.M., Wang, H.L., Xu, W.T., Zhang, H.X., Zhuang, G.Q., Bai, Z.H. 2009b. Effect of abamectin insecticide on the microbial community in broccoli phyllosphere. Huan Jing Ke Xue. 30(5):1292-7.

Zhang, B., Zhang, H., Jin, B., Tang, L., Yang, J., Li, B., Zhuang, G., Bai, Z. 2008. Effect of cypermethrin insecticide on the microbial community in cucumber phyllosphere. J.Environ. Sci. 20(11): 1356-62.

Zhuang, R.Y., Beuchat, L.R., Angulo, F.J. 1995. Fate of *Salmonella* Montevideo on and in raw tomatoes as affected by temperature and treatment with chlorine. Appl Environ Microbiol. 61: 2127-31.

Zimmerman, S.B., Schwartz, C.D., Monaghan, R.L., Pleak, B.A., Weissberger, B., Gilfillan, E.C., Mochales, S. Hernandez, S., Currie, S.A., Tejera, E., Stapley, E.O. 1987. Difficidin and oxydifficidin: Novel broad spectrum antibacterial antibiotics produced by *Bacillus subtilis*. J. Antibiotics 40(12):1677-81.

Zwieten, L.V., Merrington, G., Zwieten, M.V. 2004. Review of impacts on soil biota caused by copper residues from fungicide application. SuperSoil. 3rd Australian New Zealand Soils Conference, December 2004, University of Sydney, Australia. Retrieved Mard 19, 2011. Available at : http:/www.regional.org.au/au/asssi/supersoil2004/s3/oral/1573_vanzwieten.htm.