University of Alberta

Infectious Prion Inactivation in Water and Wastewater

by

Ning Ding

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DEDICATION

To my husband Dr. Yang Zhan and my parents

for their love and support

ABSTRACT

Misfolded prions (PrP^{Sc}) are well known for their resistance to conventional decontamination processes. The potential risk of contamination of the water environment, as a result of the disposal of specified risk materials (SRM), has raised public concerns. Ozone is commonly utilized in the water industry for the inactivation of microbial contaminants and was tested in this research for its ability to inactivate prions (263K hamster scrapie). With the applied ozone dose of 7.6-25.7 mg/L, the efficacy of ozone inactivation of PrP^{Sc} was both pH and temperature dependent. Treatment variables included applied ozone dose (7.6–25.7 mg/L), contact time (5 s and 5 min), temperature (4°C and 20°C) and pH (pH 4.4, 6.0, and 8.0). The inactivation of PrP^{Sc} was quantified by determining the *in vitro* destruction of PrP^{Sc} templating properties using the protein misfolding cyclic amplification (PMCA) assay and bioassay. Highest levels of prion inactivation (≥4-log) were observed with applied ozone doses of 13.0 and 25.7 mg/L, at pH 4.4 and 20°C. The kinetic modeling of prion inactivation in phosphate buffered saline (PBS) with applied ozone dose of 10.8 mg/L at pHs and temperatures described above was characterized by both Chick-Watson (CW) and efficiency factor Hom (EFH) models. It was found that the EFH model fit the experimental data more appropriately. Based on the EFH model, *CT* values were determined for 2-log, 3-log, and 4-log inactivation. A comparison of ozone *CT* requirements among various pathogens suggests that prions are more susceptible to ozone degradation than some model bacteria and protozoa. Subsequently, the ozone inactivation of infectious prions was assessed

in the raw, gravity separated and dissolved air flotation (DAF) treated rendering wastewater, and in the municipal final effluent. It was found that the organic load highly affected prion inactivation by ozone, while DAF treatment significantly removed the organics and improved the efficacy. At the applied ozone dose of 44.6 mg/L, a >4-log inactivation was achieved after 5 min of exposure in the DAF treated rendering plant wastewater. The results indicated that ozone could serve as a final barrier for prion inactivation in primary and/or secondary treated wastewater.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

°C	degree Celsius
%	percent
µL	micro liter
AcOH	acetic acid
A/S	air to solid ratio
atm	atmosphere
BOD	biochemical oxygen demand
BOD_5	BOD over a 5-day period
BSE	bovine spongiform encephalopathy
C	residual ozone concentration (mg/L) at time t
C_0	residual ozone concentration (mg/L) of the possible measurement closest to 0 s
CDI	conformation dependent immunoassay
CFIA	Canadian Food Inspection Agency
CJD	Creutzfeldt-Jakob disease
COD	chemical oxygen demand
Cl_2	chlorine
CW	Chick-Watson
cm	centimeter
CT	concentration multiplied by time
CWD	chronic wasting diseases
DAF	dissolved air flotation
DI	deionized
EDTA	ethylenediaminetetraacetic acid
EFH	efficiency factor Hom
ELISA	Enzyme-linked immunosorbent assay
ESS	error sum of squares
Fe^{2+}	Fenton
Fe^{3+}	iron

GSS	Gerstmann-Straussler-Scheinker syndrome
h	hour
H_2O_2	hydrogen peroxide
IBH	infectious brain homogenates
ID_{50}	dose that will infect 50% of the experimental group
k	the inactivation rate constant
k'	the first order ozone decomposition rate constant (min^{-1})
L	liter
$\log_{10}(N/N_0)$	$\log_{10}$ of survival of PrP^{Sc}
m	constant for the inactivation rate law which describes deviation from ideal Chick-Watson law
M	mole/liter
MBM	meat and bone meal
n	the constant referred to coefficient of dilution
min	minute
mL	milliliter
mM	millimole per liter
mmol/L	millimole per liter
mol/L	mole per liter
NaClO	sodium hypochlorite
NaOH	sodium hydroxide
NBH	normal brain homogenates
O_3	ozone
ODF	ozone demand-free
•OH	hydroxyl radicals
PBS	phosphate buffered saline
PK	proteinase K
PMCA	Protein misfolding cyclic amplification
PrP^{*}	intermediate state of PrP^{Sc}
PrP^{C}	normal cellular prion protein
PrP^{Sc}	misfolded pathological isoform of PrP^{C}

PTA	phosphotunstic acid precipitation
R^2	correlation coefficient
s	second
SDS	sodium dodecyl sulfate
SRM	specified risk materials
t	time
TDP	thermal depolymerization
TDS	total dissolved solids
THMs	trihalomethanes
TiO_2	titanium dioxide
TME	transmissible mink encephalopathy
TSEs	transmissible spongiform encephalopathies
TSS	total suspended solids
UV	ultraviolet
USEPA	United States Environmental Protection Agency
V	volt
vCJD	variant CJD

Chapter 1 General Introduction

1.1 Background of Prion diseases

PrP^{C} is the normal cellular prion protein present in most tissues in humans and other animals (Krasemann et al. 2010), and is expressed at particularly high level in neuronal tissues (Maddison et al. 2007; Tichopad et al. 2003). PrP^{C} can transform into a protease-resistant, infectious, misfolded pathological isoform (PrP^{Sc}) which causes neurodegenerative diseases called transmissible spongiform encephalopathies (TSEs). TSEs include scrapie in sheep and goats; chronic wasting diseases (CWD) in deer, moose, and elk; bovine spongiform encephalopathy (BSE) in cows; transmissible mink encephalopathy (TME) in minks; and Kuru, Creutzfeldt-Jakob disease (CJD) and Gerstmann-Straussler-Scheinker sundrome (GSS) in humans (Prusiner 1998). Familial CJD and GSS are generic disorders of human, whereas Kuru results from ritualistic cannibalism, a societal practice which has almost vanished in the Fore tribe of Papua New Guinea (Prusiner 1991). At present there is no treatment for these fatal diseases. The most common TSE disease in animals is scrapie. Scrapie and CWD are horizontally transmissible and the infectivity can remain in the environment for a very long time (Brown and Gajdusek 1991). Scrapie has been reported worldwide, except for New Zealand and Australia. Although scrapie has been present for more than 250 years, it is believed not to be harmful to humans (APHIS 2004). CWD has been found in 14 states in the USA, two provinces in Canada, and in Korea (Kim et al. 2005; USGS 2007). A study by Marsh et al. (2005) has demonstrated that CWD is transmissible to squirrel monkeys (Marsh et al. 2005). A risk assessment study of CWD to humans with 81 volunteers who had consumed or been exposed to CWD contaminated venison was performed to assess the risk of CWD to humans (Garruto et al. 2008). It is currently considered unlikely that CWD can cross the species barrier to humans (MaWhinney et al. 2006), however, if it can, Garruto et al. (2008) suggested that people with certain risk factors would be vulnerable. BSE is the most infamous type of prion disease,

although it has just emerged in the past few decades (Ironside et al. 1996). More than 280,000 cattle have been indentified BSE, and 1-3 million are likely been infected (Aguzzi and Heikenwalder 2006; Smith and Bradley 2003). In addition, 176 cases of a new variant of CJD (vCJD) in humans have been reported due to the consumption of BSE contaminated meat in UK up to 2013 (The UK Creutzfeldt Jakob Disease Surveiliance Unit 2013). Owing to the limited information on the incubation time, the possibilities that more people have contacted with or consumed BSE infected meat is still a public health concern (Balter 2001; Soto 2004). Knowing the incidence of BSE, the specific risk materials (SRM) produced by cattle industry containing prion contaminated materials are strictly regulated by the Canadian Food Inspection Agency (CFIA) and disposed separately from other wastes in order to minimize the risks caused by BSE to human and animal health and food safety. SRM includes skull, brain, trigeminal ganglia, eyes, tonsils, spinal cord, and dorsal root ganglia of cattle aged 30 months or older, and the distal ileum of cattle of all ages (Canadian Food Inspection Agency 2011).

1.2 Prion biology

Prions propagate by refolding abnormally into a structure which is able to convert normal molecules of the protein (PrP^{C}) into abnormally structured form (PrP^{Sc}). The conversion of PrP^{C} into PrP^{Sc} induces a conformational change in the protein, in which the α-helical content of the normal protein is reduced and the amount β-sheet increases (Soto 2004). PrP^{C} has approximately 43% α-helical and 3% β-sheet structure, whereas PrP^{Sc} has approximately 30% α-helix and 43% of β-sheet structure (Pan et al. 1993).

Accompanied with these structure changes, some biochemical properties also change. PrP^{Sc} is insoluble whereas PrP^{C} is soluble in non-denaturing detergents (Meyer et al. 1986). PrP^{Sc} is resistant to protease, it can only be partially hydrolyzed by protease to form an amino terminally truncated fragment designated as PrP 27-30 (Oesch et al. 1985). PrP^{Sc} accumulates in brain tissues, whereas PrP^{C} does not (Borchelt et al. 1990). In addition, the distribution of PrP^{C}

and the accumulation of PrP^{Sc} occur in different sites in the brain (Taraboulos et al. 1992).

Although some features of the diseases caused by prions and viruses are similar, nucleic acids, lipids and/or carbohydrates are not the infectious elements of prions; the infectious protein was initially considered the only possibility of the infectivity of prions (Alper et al. 1967; Prusiner 1998). However, later studies suggested that cofactors such as RNA or lipids might also be components of infectious particles (Geoghegan et al. 2007; Piro and Supattapone 2010). It has been demonstrated that removal of PrP gene does not appear to harm mice, thus the prion diseases appear to result from PrP^{Sc} accumulation, rather than deficiency of PrP^{C} (Pan et al. 1993). A prion replication model has been proposed to explain the pathogenesis of prion diseases (Cohen et al. 1994). According to this model, there is an intermediate state of formation of PrP^{Sc}, the unfolded monomer PrP^{*}. PrP^{*} may revert to PrP^{C}, be degraded or generate PrP^{Sc}. Usually, the amount of PrP^{*} is low, thus negligible amount of PrP^{Sc} is formed. However, extraneous PrP^{Sc} would act as templates to promote the conversion from PrP^{*} to PrP^{Sc}. Due to the insolubility of PrP^{Sc}, the conversion is irreversible. PrP mutation would also misfold PrP^{C} and increase the possibility of formation of PrP^{Sc}.

1.3 The potential risks of prions

One of the major concerns is the transmission of prion disease through the surgical instruments and devices among human and animals. Regular disinfectants for viruses and bacteria have little effect on prions. Transmission of scrapie among sheep has occurred due to a prion contaminated vaccine (Gordon 1946). Scrapie has been shown to survive exposure to 0.35% formalin that inactivated louping-ill viruses. The transmission of CJD through ethanol and formaldehyde vapor sterilized brain insertion electrodes in humans further validated that prions are not inactivated by regular disinfectants (Taylor 1999).

There are several potential routes of prions entering the environment. First, prions may shed in urine (Gonzalez-Romero et al. 2008; Seeger et al. 2005), feces (de Motes et al. 2008b; Safar et al. 2008; Williams and Miller 2002), saliva

(Mathiason et al. 2006; Williams and Miller 2002), and blood (Mathiason et al. 2006). Therefore, prions may enter the environment through live or infected animals. Second, prions may enter the environment through infected carcasses (Miller et al. 2004). Also, prions may enter the environment through the contaminated solid or liquid waste from slaughtering houses, rendering plants, agricultural digesters, and some septic systems including private game dressing, and rural meat processors which unknowingly process infected carcasses (Pedersen et al. 2006; Saunders et al. 2008). Environmental reservoirs of scrapie and CWD facilitate the transmission of infectivity (Miller et al. 2004). The infectivity can be transmitted through the contaminated pasture or infected soil (Williams and Miller 2002). It has been reported that scrapie might persist in the pasture for 16 years (Georgsson et al. 2006), and survive in soil for at least three years (Brown and Gajdusek 1991). CWD can remain infectious in pasture for at least two years (Miller et al. 2004).

Prions are believed to be "sticky" and bind to particulates in the environment. In the case of prion entering surface water or wastewater systems, prions appear to aggregate and to be partitioned into sludge and biosolids (Gale et al. 1998). BSE and scrapie were reported to degrade faster in sewage than in PBS (de Motes et al. 2008a), while another study suggested that organic matter in water protects infectious prion from degradation (Miles et al. 2011). PrP^{Sc} survived mesophilic anaerobic digestion of the sludge (Hinckley et al. 2008; Kirchmayr et al. 2006) and was gradually degraded in thermophilic sludge (Kirchmayr et al. 2006).

1.4 Thesis scopes and objectives

Water and wastewater may act as a transporting agent of infectious prions. It has been reported that PrP^{Sc} was detected in the surface water samples from a CWD endemic area (Nichols et al. 2009). In addition, there are concerns that SRM liquid waste may be released into the environment; therefore, there is a possibility that infectious prions may enter wastewater treatment systems. In order to control prion disease transmission through water and wastewater, inactivation

approaches were studied. Ozone as an advanced technology for water and wastewater disinfection was targeted as the potential disinfectant for prion inactivation. In this study, the effectiveness of ozone on prion inactivation in simplex water matrix was assessed, followed by kinetic modeling of the inactivation in both simplex and complex water and wastewater matrices.

The objectives of this research were: (1) to determine the effectiveness of ozone for inactivating infectious prions (263K scrapie), as evaluated by protein misfolding cyclic amplification (PMCA) assay under a variety of experimental conditions; (2) to validate the correlation of PMCA assay with animal bioassay on the detection of infectious prions in ozonated samples; (3) to investigate the reaction kinetics of ozone inactivation of infectious prions under various conditions in an ozone demand-free buffer system; (4) to assess the partition of infectious prions during gravity separation in rendering plant wastewater; (5) and to investigate the reaction kinetics of ozone inactivation of infectious prions in pretreated rendering plant wastewater and municipal final effluent.

1.5 Thesis organization

The thesis consists of seven chapters. Chapter 1 contains background information on infectious prions. Chapter 2 presents a review of literature on inactivation methods of infectious prions from both laboratory and engineering application aspects. The available prion detection methods are also discussed. Chapter 3 contains detailed information with respect to materials and methods used in the whole study. Chapter 4 presents results on the evaluation of ozone's effectiveness in inactivating template-directed misfolding properties of PrP^{Sc}, as determined by PMCA under a variety of experimental conditions (ozone dose, contact time, pH, and temperature) for which *CT* values (disinfectant concentration multiplied by contact time) for ozone could be derived. The fit of previously described water disinfection models for ozone inactivation of infectious prions was compared in Chapter 5. The inactivation effectiveness at various pHs and temperatures were discussed, and the correlation of PMCA assay to animal bioassay on the determination of prion inactivation by ozone was also

verified. Chapter 6 examined the partitioning of infectious prions after gravity separation process in rendering wastewater, and assessed the effectiveness of ozone inactivation of prions in raw plant rendering wastewater. The work presented in this chapter also determined the kinetics of ozone inactivation in rendering plant wastewater after gravity separation and dissolved air flotation (DAF) treatment respectively, and investigated the kinetics of ozone inactivation of infectious prions in municipal final wastewater effluent. Chapter 7 contains the general discussion of the results presented in previous chapters as well as conclusions and recommendations for future work.

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Chapter 2 Infectious Prion Inactivation: A Review

2.1 Introduction

Due to the fatal and unique amplification properties of infectious prions and their resistance to conventional disinfection approaches, studies on prion inactivation have never stopped since 1960s. With the emergence of BSE in cattle, the vCJD in humans due to consumption of BSE infected beef, and the growing problem of CWD in North America, concerns with respect to the spread of prion infectivity are raised. CFIA has enforced “zero tolerance” of the presence of BSE in any of the food product (Canadian Food Inspection Agency 2009b). Prion inactivation has gained a lot of attention among researchers and an increasing number of studies are focused in this field (Giles et al. 2008; Race and Raymond 2004; Taylor 1999). Laboratory scale inactivation studies have been widely carried out on infectious prions in brain suspensions and prions attached to the stainless steel surfaces mimicking surgery tool surfaces (Lehmann et al. 2009; Pritzkow et al. 2011). Rodent passaged prions have been mostly used in these studies because they are easily obtainable, whereas other studies have also suggested different resistance among various strains (Giles et al. 2008). Approaches of inactivation of infectious prions in small volumes of suspensions or attached to medical instruments in laboratories or medical facilities can be classified as chemical inactivation, autoclaving, proteolytic inactivation, and combination of those described above (Taylor 2000). Recently, more research studies have been performed aiming to improve the treatment of SRM in large volumes or infectious prions in the environmental matrices in which prions aggregate and bind to other particles. For engineering application of SRM treatment, incineration is the major approach currently been used (AARI 2005); high temperature and pressure rendering and alkaline hydrolysis have also been approved by the European Commission Scientific Steering Committee for SRM disposal (European Commission 2003).

Various prion strains have been used in prion inactivation studies (Table 2.1). Bioassays have been considered as the "gold standard" for prion inactivation because they reflect the changes in infectivity upon inactivation. However, these assays are extremely expensive and time-consuming. Due to the drawback of bioassays, immunoblotting alone such as western blot has been mostly utilized for the detection of PrP^{Sc}; however, western blot alone may fail to detect infectivity under some circumstances (Barron et al. 2007; McLeod et al. 2004; Triantis et al. 2007). Other methods including cell culture and especially protein misfolding amplification (PMCA) which are closely correlated with bioassays, are gradually applied in prion inactivation studies (Miles et al. 2011; Pritzkow et al. 2011).

Table 2.1 Infectious prion strains discussed in this chapter

Strain	Source
263K	hamster-passaged scrapie
Sc237	hamster-passaged scrapie
HY TME	hamster-passaged TME
22A	mouse-passaged scrapie
139A	mouse-passaged scrapie
ME7	mouse-passaged scrapie
301V	mouse-passaged BSE
BSE	BSE from cattle
S.Co.	guinea pig-passaged CJD
Kitasato-1	mouse-passaged CJD
sCJD	mouse-passaged CJD
CWD	CWD from elk

A greater than 4-log (99.99%) reduction of waterborne pathogens in surface water are regulated by USEPA after disinfection to meet the requirement for drinking water safety (U.S. Environmental Protection Agency 1999a). For prions however, though it was proved that the risk of infection by prions in 1000 time dilution of 1 LD_{50} is considerably low, there is no safe dose of prions (Fryer and McLean 2011). Therefore, complete inactivation is regulated for prion-related materials (i.e. laboratory stream or SRM) (Canadian Food Inspection Agency 2012). In this chapter, recent literature concerning inactivation of infectious

prions in suspensions, prions attached to metal surfaces, as well as SRM disposal and prion inactivation in environmental matrices are reviewed and discussed in terms of the susceptibility of different strains, efficacy and applicability of the inactivation methods, detection methods of prions, and engineering application of some treatments.

2.2 Inactivation of infectious prions in suspensions or attached to metal surfaces

2.2.1 Chemical inactivation

The chemical inactivation methods that has been approved and recommended by CFIA are: a) 2M sodium hydroxide (NaOH) for 1 h for the liquid and surface disinfection; b) sodium hypochlorite (NaClO) with 2% final free chlorine for 1 h for the liquid and instrument disinfection; and c) 5% aqueous acid phenol solution Environ LpH for the surface disinfection for 30 min (Canadian Food Inspection Agency 2009a; Williams 2005). Chemical reagents that have been reported to be able to inactive infectious prions by more than 3-$\log_{10}$ are listed in Table 2.2.

Table 2.2 Effective chemicals for prion inactivation by >3-$\log_{10}$ at room temperature

Disinfectant	Prion concentration	Infectious agent (strain)	Inactivation ($\log_{10}$)	Reference
NaOCl, initial available chlorine 0.5%	$10^{10.14}$ LD_{50}/g	Scrapie (263K)	3.1-$\log_{10}$ at 15 min, 3.5-$\log_{10}$ at 30 min, 4.2-$\log_{10}$ at 1 h	(Brown et al. 1982b)
NaOCl, residual available chlorine 1%	$10^{5.5}$ LD_{50}/g	Scrapie (22A)	≥4.6-$\log_{10}$ at 30 min	(Kimberlin et al. 1983)
NaOCl, residual available chlorine 1%	$10^{7.1}$ LD_{50}/g	Scrapie (139A)	≥5.9-$\log_{10}$ at 30 min	(Kimberlin et al. 1983)
NaOCl, initial available chlorine 0.825%	$10^{3.6}$ LD_{50}/g	BSE	>3.6-$\log_{10}$ at 30 min	(Taylor et al. 1994)
NaOCl, initial available chlorine 2.5%	$10^{5.5}$ LD_{50}/wire	Scrapie (263K)	≥5.5-$\log_{10}$ at 60 min	(Pritzkow et al. 2011)
NaOH, 0.1M	$10^{10.6}$ - $10^{11.3}$ LD_{50}/g	Scrapie (263K)	5.0-$\log_{10}$ at 15 min, 6.0-$\log_{10}$ at 1 h	(Brown et al. 1986)
NaOH, 1M	$10^{10.6}$ - $10^{11.3}$ LD_{50}/g	Scrapie (263K)	6.0-$\log_{10}$ at 15 min, ≥6.8-$\log_{10}$ at 1 h	(Brown et al. 1986)
NaOH, 2M	$10^{9.5}$ LD_{50}/g	Scrapie (263K)	5.1-$\log_{10}$ at 2 h	(Taylor et al. 1994)
NaOH, 0.1M	$10^{6.1}$ - $10^{7.2}$ LD_{50}/g	CJD (S.Co.)	4.8-$\log_{10}$ at 15 min and 1 h	(Brown et al. 1986)
NaOH, 1M	$10^{6.1}$ - $10^{7.2}$ LD_{50}/g	CJD (S.Co.)	4.5-$\log_{10}$ at 15 min, ≥5.0-$\log_{10}$ at 60 min	(Brown et al. 1986)
NaOH, 1M	$10^{3.6}$ LD_{50}/g	BSE	>3.6-$\log_{10}$ at 30 min	(Taylor et al. 1994)
NaOH, 1M	$10^{5.5}$ LD_{50}/wire	Scrapie (263K)	≥5.5-$\log_{10}$ at 60 min	(Pritzkow et al. 2011)
Phenolic, LpH, 0.9%	7.4 LD_{50}	Scrapie (263K)	>5-$\log_{10}$ at 30 min	(Ernst and Race 1993)
Phenolic, LpH, 9%, 81%, 90%	7.4 LD_{50}	Scrapie (263K)	>7.4-$\log_{10}$ at 30 min	(Ernst and Race 1993)
Phenolic, LpH, 0.09%	7.4 LD_{50}	Scrapie (263K)	>7.4-$\log_{10}$ at 30 min	(Ernst and Race 1993)

LD_{50}: 50% lethal dose intracerebral units/g, or units/wire
Reactions were in room temperature if not specified

As shown in Table 2.2, 263K is more resistant to chemical inactivation than other scrapie strains (i.e. 22A, 139A), thus is always used as a model for prion inactivation. BSE appears to be more resistant than scrapie 263K, however, due to the low infectious titer of BSE in the studies, inactivation higher than 3.6-$\log_{10}$ could not be achieved (Taylor et al. 1994). Although the infectious titer of BSE used in the study was low ($10^{3.6}$ LD_{50}), a complete loss of infectivity was observed after inactivation for 30 min by sodium hypochlorite containing 0.825% free chlorine (Taylor et al. 1994). Therefore, it was concluded that the already recommended procedure for achieving inactivation by exposure to sodium hypochlorite solution containing 20 g/L of available chlorine for 1 h was effective with BSE agent (Taylor et al. 1994). The resistance of CJD is between 263K scrapie and BSE. All these chemical disinfection procedures are hazardous either to the surgical instruments or to the operators, thus are not suitable for routine maintenance of equipment.

2.2.2 Autoclaving

Autoclaving is widely used to sterilize laboratory supplies. Autoclaving at intense temperature for required period can also inactivate prions significantly. The effective autoclaving temperatures and duration on prion inactivation are shown in Table 2.3

Table 2.3 Autoclaving conditions effective on prion inactivation

Autoclaving type	Prion concentration	Infectious agent (strain)	Inactivation (log_{10})	Reference
Gravity autoclaving 132°C 1 h	$10^{9.5}$ LD_{50}	Scrapie (263K)	≥8.8 log_{10}	(Brown et al. 1986)
Gravity autoclaving 132°C 1 h	$10^{11.8}$ LD_{50}	Scrapie (263K)	6.4 log_{10}	(Ernst and Race 1993)
Gravity autoclaving 132°C 1.5 h	$10^{11.8}$ LD_{50}	Scrapie (263K)	>7.4 log_{10}	(Ernst and Race 1993)
Gravity autoclaving 126°C 1h	$10^{7.2}$ LD_{50}	Scrapie (139A)	≥6.9 log_{10}	(Kimberlin et al. 1983)
Gravity autoclaving 132 °C 1 h	$10^{6.1\text{-}7.2}$ LD_{50}	CJD (S.Co.)	≥5.0 log_{10}	(Brown et al. 1986)
Gravity autoclaving 121 °C 30 min	$10^{5.8}$ LD_{50}	CJD (Kitasato-1)	3.1 log_{10}	(Taguchi et al. 1991)
Gravity autoclaving 132 °C 1 h	$10^{5.8}$ LD_{50}	CJD (Kitasato-1)	>4.8 log_{10}	(Taguchi et al. 1991)
Autoclaving 134°C 30 min	NA	sCJD	>7.2 log_{10}	(Giles et al. 2008)
Autoclaving 134°C 30 min	NA	BSE (301V)	>8.1 log_{10}	(Giles et al. 2008)
Autoclaving 134°C 2 h	NA	Cattle BSE	>4.0 log_{10}	(Giles et al. 2008)
Autoclaving 134°C 30 min	NA	Scrapie (Sc237)	>7.2 log_{10}	(Giles et al. 2008)
Porous autoclaving 134°C 18 min	$10^{9.5}$ LD_{50}	Scrapie (263K)	≥ 7.3 log_{10}	(Taylor et al. 1994)
Porous autoclaving 136°C 4-32 min	$10^{7.2}$ LD_{50}	Scrapie (139A)	≥6.9 log_{10}	(Kimberlin et al. 1983)
Porous autoclaving 136°C 4-32 min	$10^{6.4}$ LD_{50}	Scrapie (22A)	≥5.6 log_{10}	(Kimberlin et al. 1983)
Porous autoclaving 134 and 136°C	$10^{7.2}$ LD_{50}	Scrapie (22A)	$\geq 7.2_{10}$	(Taylor 1999)

LD_{50}: 50% lethal dose intracerebral log_{10} units/g
NA: not available

Gravity autoclaving at 132°C for 1 h was shown to achieve >4.8-$\log_{10}$ inactivation of scrapie, CJD, and mouse passaged BSE (Brown et al. 1986; Ernst and Race 1993; Kimberlin et al. 1983; Taguchi et al. 1991), however, completely infectivity removal was not guaranteed (Brown et al. 1986; Tamai et al. 1988). The type of autoclaving was not specified in Giles et al. (2008), whereas it has been pointed out that the complete inactivation has been achieved by autoclaving of cattle BSE at 134°C for 2 h, but only 3.2-$\log_{10}$ inactivation could be achieved after autoclaving at 134ºC for 30 min. Comparing with more than 8.1-$\log_{10}$ inactivation of mouse passaged BSE, and >7.2-$\log_{10}$ inactivation of scrapie 237 and sCJD at 134°C for 30 min, it has been demonstrated that the cattle BSE is more resistant to autoclaving than the mouse passaged BSE, CJD and hamster scrapie. The main difference between gravity and porous loading autoclaving is that the steam fills up the chamber rapidly during sterilization stage in the latter. An adequate inactivation level was met with porous autoclaving at shorter time comparing to the gravity autoclaving. For example, porous autoclaving achieved≥7.3 -$\log_{10}$ inactivation of scrapie 263K at 134°C for as short as 18 min, and a 2.5-$\log_{10}$ inactivation was obtained for BSE at the same time. Paradoxically, the thermostability of scrapie 22A was enhanced as temperature increased from 134°C to 138°C; and there was no difference in scrapie 263K residual infectivity among porous autoclaving at 134°C, 136°C and 138°C (Taylor 2000). This suggested that once autoclaved, the dried macerated tissue is likely to be more resistant to inactivation if not fully destroyed.

2.2.3 Multi-barrier treatment of autoclaving and chemical inactivation

It has been demonstrated that the combination of chemical treatment and autoclaving is more efficient and effective than each method alone (Ernst and Race 1993; Giles et al. 2008; Rutala and Weber 2001). The use of sodium hydroxide with autoclaving is very common, as sodium hydroxide is one of the most effective chemical disinfectants of prions. The conditions of effective multi-barrier treatments are listed in Table 2.4.

Table 2.4 Multi-barrier treatment of chemical inactivation and autoclaving

Inactivation methods	Prion concentration	Infectious agent (strain)	Inactivation (log_{10})	Reference
0.09M NaOH 2 h and autoclave 121°C 1 h	$10^{7.4}$ LD_{50}	Scrapie 263K	>7.4 log_{10}	(Ernst and Race 1993)
2M NaOH 0 or 60 min and autoclave 121°C 30 min	~10^{8} LD_{50}	Scrapie 22A	≥8 log_{10}	(Taylor et al. 1997)
1M NaOH 1 h and autoclave 121°C 30 min	$10^{5.8}$ LD_{50}	CJD (Kitasato-1)	≥4 log_{10}	(Taguchi et al. 1991)
4% SDS-1% AcOH 30 min and autoclave 134°C 15 min	~10^{10} LD_{50}	Scrapie 237	100% survival	(Peretz et al. 2006)
4% SDS-1% AcOH 30min and autoclave 134°C 15 min	~10^{10} LD_{50}	sCJD	100% survival	(Peretz et al. 2006)
4% SDS-1% AcOH 2h and autoclave 134°C 15 min	~10^{10} LD_{50}	Mouse BSE (301V)	>8.1 log_{10}	(Giles et al. 2008)
4% SDS-1% AcOH 2h and autoclave 134°C 15 min	~10^{10} LD_{50}	Cattle BSE	>4.0 log_{10}	(Giles et al. 2008)
4% SDS-1% AcOH 2h and autoclave 134°C 15 min	~10^{10} LD_{50}	sCJD	>7.2 log_{10}	(Giles et al. 2008)
4% SDS-1% AcOH 2h and autoclave 134°C 15 min	~10^{10} LD_{50}	Scrapie (263K)	>7.2 log_{10}	(Giles et al. 2008)

$LD_{50:}$ 50% lethal dose intracerebral log_{10} units/g

Treatment of hamster scrapie with 0.09 M sodium hydroxide for 2 hours plus autoclaving at 121°C for 1 hour achieved an inactivation of >7.4-$\log_{10}$ (Ernst and Race 1993), which is much higher than each inactivation method alone (Tables 2.2 and 2.3). Scrapie 22A, which is a relatively thermostable scrapie strain, could be completely inactivated by a combination of 2 M sodium hydroxide with or without incubation, and autoclaving at 121°C for 30 min (Taylor et al. 1997). Treatment of CJD (Kitasato-1) with 1 M sodium hydroxide for 1 hour and autoclaving at 121°C for 30 min could achieve a≥4 -$\log_{10}$ inactivation, which was higher than 3.1-$\log_{10}$ inactivation with autoclaving alone at 121°C for 30 min (Taguchi et al. 1991). The combination of sodium hydroxide and autoclaving has been most widely used in laboratories to disinfect prions. SDS as a detergent had some effect on prion degradation; and the effect was enhanced by heat. Although the effectiveness of combination of 4% sodium dodecyl sulfate (SDS)-1% Acetic acid (AcOH) and autoclaving at 134°C was similar as observed for autoclaving at 134 °C alone for prions inactivation in suspensions (Tables 2.2 and 2.3), the combined treatment was more effective for stainless steel bound prions which are more difficult to inactivate (Giles et al. 2008; Peretz et al. 2006). Therefore, the combination of chemical treatment and autoclaving is more reliable than each treatment alone. However, the harsh reaction conditions (i.e. high concentration of corrosive chemicals, high temperature) prohibit their usage on sensitive medical equipments.

2.2.4 Proteolytic inactivation

Proteases such as trypsin in non-denaturing environment have little effect on prion degradation (Hunter and Millson 1967). After prolonged digestion time, proteinase K (PK) was able to destroy the infectivity of purified scrapie by more than 99.9%; whereas no reduction in infectivity was observed with less purified scrapie (Prusiner et al. 1981). It was reported that six strains of foodborne bacteria exhibited activities to degrade the hamster scrapie *in vitro*, suggesting that the proteases secreted by these bacteria assisting in prion degradation (Muller-Hellwig et al. 2006). Prions and keratins have similar conformations that contain

high content of β-sheet and high levels of aggregation. Subtilisin, or subtilisin-like enzymes, secreted by bacteria of *Bacillus* species, were able to destroy keratins, and most of these enzymes are thermophilic (Tsiroulnikov et al. 2004). These enzymes may also have effect on prions. For example, MSK103 protease, derived from *Bacillus* strain, can cause degradation of hamster scrapie in combination with SDS at 50°C for 20 h (Yoshioka et al. 2007); incubation of BSE infected mouse brain homogenates with the subtilisin enzyme properase at pH 12, 60ºC for 30 min could extend the mouse survival in the bioassays (McLeod et al. 2004); an alkaline protease MC3, represents a genetically engineered variant of the *Bacillus lentus* subtilisin, could reduce the infectivity of BSE by >7-$\log_{10}$ at 60°C (Dickinson et al. 2009). An alkaline serine protease, isolated from the culture medium of *Streptomyces* sp. was able to completely degrade scrapie PrP^{Sc} signal in 3 min at the optimum condition of 60°C and pH 11 (Hui et al. 2004). A serine protease extracted from lichens has also shown ability to degrade prion infectivity (Johnson et al. 2011). Keratinase produced by *Bacillus licheniformis*, Proteinase K and other two subtilisin proteases were also found to be able to accomplish full degradation of PrP^{Sc} in BSE and scrapie infected brain tissue, with >100°C pretreatment in detergent, confirmed by western blot detection of PrP^{Sc} (Langeveld et al. 2003). Microbial consortia taken from the rumen and colon of cattle was found to be able to degrade scrapie within 20 and 40 hours, respectively at 37°C under anaerobic conditions, however, residual infectivity was retained (Scherbel et al. 2006; Scherbel et al. 2007). Clearly, thermophilic enzymes/proteases at high pH and temperatures were more efficient in inactivation of prions, however, complete inactivation of infectivity was not guaranteed at most conditions. For enzymatic treatment, scrapie was suggested to be more resistant than BSE, which is in distinction to chemical treatment and autoclaving to which BSE is more resistant (Baron et al. 2004; de Motes et al. 2008c; Kuczius and Groschup 1999).

2.2.5 Advanced oxidation inactivation

Johnson et al. (2009) demonstrated that UV-ozone treatment of hamster-adapted transmissible mink encephalopathy prions induced inactivation of PrP^{Sc} at

≥5-$\log_{10}$ level. Ozone was generated by UV at a wavelength of 185 nm, and then decomposed by UV at another wavelength of 254 nm to produce hydroxyl radicals. However, the UV-ozone system studied by Johnson et al. (2009) produced limited ozone thus generating limited hydroxyl radicals, requiring exposure times up to several weeks, questioning the practicality of using UV-generated ozone as a decontamination/sterilizing approach to prion inactivation. Prion inactivation has also been studied with other advanced oxidation methods, such as copper and hydrogen peroxide (Lehmann et al. 2009; Solassol et al. 2006), Fenton reaction (Suyama et al. 2007a), photo-Fenton treatment (Paspaltsis et al. 2009), and titanium dioxide photo-catalysis (Paspaltsis et al. 2006). Hydrogen peroxide inactivation (100 mmol/L) in the presence of copper (0.5 mmol/L) was reported to achieve ≥5.2-$\log_{10}$ inactivation of 263K scrapie prion with a contact time of 2 h at a room temperature (Solassol et al. 2006). Increasing the concentration of hydrogen peroxide to 2.2 mol/L reduced the contact time to 30 min for the same level of inactivation (Lehmann et al. 2009). Hydrogen peroxide inactivation (1.5 mol/L) in the presence of Fe^{2+} (15.7 mmol/L), heating at 50 °C for 22 h was able to obtain approximately 6 $\log_{10}$ reduction of prion infectivity (Suyama et al. 2007a). PrP^{Sc} could also be degraded by≥2.4 $\log_{10}$ by photo-Fenton treatment (147 mmol/L H_2O_2, 8.9 mmol/L Fe^{3+}) after 5 h of UV-A exposure (Paspaltsis et al. 2009), and be degraded by≥2 -$\log_{10}$ during titanium dioxide photo-catalysis (25 mmol/L TiO_2 and 118 mmol/L H_2O_2) after 12 h UV-A exposure (Paspaltsis et al. 2006). Due to the low sensitivity of the PrP^{Sc} detection methods used in the last two studies, inactivation higher than 2.4-$\log_{10}$ was not achieved. In these advanced oxidation studies, hydroxyl radicals as a sole component to inactivate PrP^{Sc} demonstrated its capabilities, however, prolonged exposure times (from 30 min to 22 h) were essential to continuously generate potent hydroxyl radicals sufficient for the inactivation.

2.3 Disposal of SRM solid waste

TSE diseases pose serious problems to animal husbandry and food industry. In Canada, the Canadian Food Inspection Agency has implemented an enhanced ban on the use of SRM for all animal feed, pet food and fertilizers

(Canadian Food Inspection Agency 2007b). Large volumes of infected materials are therefore generated with control, requiring safe, effective disposal methods. Stringent decontamination processes of prion infected tissue or carcasses must be applied before their disposal to the environment. As distinct from prions in brain suspensions or attached to medical instruments, prions bound to SRM and infected materials are more resistant and always generated in large volumes. Inactivation methods described earlier may not apply to SRM. The current methods for disposal of a large volume of infected tissue wastes include hyperbaric rendering, incineration, alkaline hydrolysis, and landfilling (burial) (Pedersen et al. 2006), whereas landfilling has been strictly regulated by CFIA (Canadian Food Inspection Agency 2012). The proposed methodologies include thermal hydrolysis, thermal depolymerization, pyrolysis and composting (AARI 2005).

2.3.1 Rendering

Rendering converts waste animal tissues into stable, value-added materials. The inactivation stage of rendering involves the mechanisms of heat treatment at high temperature under hyperbaric conditions, which is similar to steam autocalving. It was demonstrated that no infectivity was detected in meat and bone meal (MBM) after hyperbaric rendering with the steam in either batch or continuous-flow cooker, with end temperature around 134°C; rendering at lower temperature and atmosphere pressure was not able to achieve fully inactivation. BSE is more resistant to rendering than scrapie, especially in the procedure with low temperature and short time (Schreuder et al. 1998; Taylor et al. 1995). Therefore, hyperbaric rendering (133°C, 3 bars for 20 min) which was proved to have 3-$\log_{10}$ reduction of BSE infectivity is endorsed and adopted by the European Commission for rendering TSE materials (Taylor and Woodgate 2003). All the inactivation was verified with rodent (mouse) bioassays, whereas cattle can be 1000 times more susceptible to BSE than transgenic mice (Giles et al. 2008). Hence there is a ban with feeding mammalian-derived proteins to ruminants in Europe and Canada. Since the first case of BSE in Canada, the ban was enhanced and SRMs must be removed from all animal feed, pet food and

fertilizers in Canada (Canadian Food Inspection Agency 2007b). In addition, only waste tissues that are dedicated for products would pass through the rendering processes; SRM other than these however, is required for other decontamination treatment.

2.3.2 Incineration

Although hyperbaric rendering have been shown to inactivate prions, the European Commission recommends the incineration followed by rendering for disposal of large volume of SRM and infected animal carcasses (Taylor and Woodgate 2003). As distinct from autoclaving, incineration involves thermal treatment with dry heat (i.e. without steam). Prions are very resistant to dry heat. It was reported that lyophilized scrapie 263K agent could survive treatment at 360°C for 1 h (Brown et al. 1990), but no infectivity was detected after treatment at 200°C for 1 hour of non-lyophilized scrapie strain ME7 (Taylor et al. 1996). However, it was demonstrated that incineration of scrapie infected hamster brain macerates at 600°C in air for 15 min could not achieve complete inactivation, while treatment at 1000°C for 15 min successfully removed the infectivity (removal of >9-$\log_{10}$) as verified by both of western blot and animal infectivity tests. Air emission from the incineration apparatus at 1000°C was not infectious either (Brown et al. 2000; Brown et al. 2004). These studies have been based on the laboratory scale, the effectiveness of a full-scale incineration facility would also depend on its design (Pedersen et al. 2006) and to the best of our knowledge no data has been reported up to date. European Commission has established the minimum temperatures of 850°C for inactivation of SRM.

Although other incineration studies have been performed, none of those have examined a large quantity of infected wastes. Furthermore, few studies have been focused on BSE, CJD, and CWD incineration. During a large outbreak of BSE, scrapie or CWD, incineration of SRMs to eliminate environmental infectivity would be impractical (Saunders et al. 2008), and there is a big concern on the efficiency of incineration with respect to energy consumption and carbon dioxide emission.

2.3.3 Alkaline hydrolysis

Alkaline hydrolysis processes expose prion positive tissue or carcasses to potassium or sodium hydroxides at a peak operating temperature of 150°C. As described previously, a combination of heat and sodium hydroxide is more effective for prion inactivation than either one alone. Alkaline hydrolysis was proven to degrade all infectious proteins of mouse adapted scrapie infectivity. It has been considered a superior alternative to rendering for disposing prion contaminated biological materials (Murphy et al. 2009). Alkaline hydrolysis in tissue digesters, in which the alkaline solution is circulated, has been used to dispose CWD infected carcasses in some states in the USA (Johnson 2004) and has also been approved by the European Commission to dispose SRM. Compared to other treatment approaches, alkaline hydrolysis does not produce much pollution to the environment, and the small residual sludge and powders can be safely landfilled after pH neutralization (Taylor and Woodgate 2003). However, there are significant drawbacks of the alkaline hydrolysis, which may require up to several days to finish the hydrolysis procedure. This may induce high energy consumption and overall cost (Murphy et al. 2009).

2.3.4 Landfilling

Although incineration and alkaline hydrolysis have been demonstrated to be effective in prion inactivation; landfilling (on-site burial) of carcasses of TSE infected animals has been long used because it is economically attractive. It was demonstrated that soil and soil minerals had a potential to bind purified infectious prions and harbor infectivity which was not easily extracted by water (Johnson et al. 2006). However, based on a modified quantification method reported in another study (Saunders et al. 2009), a slower and reduced adsorption to soil particles was observed with the tissue homogenates. The maximum adsorption requires days to weeks, depending on the type of soils and minerals, thus the transportation of prions in soil environment could not be excluded (Saunders et al. 2009). In another study, the water that washed the soil contaminated by scrapie for 3 years contained about $10^{5.6}$-$10^{6.4}$ IU_{50} prion infectivity (Brown and Gajdusek 1991), suggesting that prion infectivity could migrate from one matrix to another

through runoff water. Scrapie could survive in soil for 3 years, with only ~2-$\log_{10}$ reduction in infectivity and some infectivity leached to the immediate soil beneath (Brown and Gajdusek 1991), with both soil and aqueous extracts from soil retaining the infectivity (Seidel et al. 2007). The immediate soil beneath buried carcasses exhibited higher proteolysis capacity than any other parts of the soil. Decomposition of buried carcasses mainly relies on the microorganisms producing extracellular enzymes. It was suggested that a buried depth of 25-45 cm tended to favor the high proteolytic activity of enzymes thus triggering degradation of ovine prion protein, possibly BSE. However, the proteolytic activity was greatly reduced once carcasses were buried at a larger depth (95–110 cm) (Rapp et al. 2006). Therefore, landfilling cannot significantly reduce the infectivity of prions; in case that infected materials are released to the environment, they would still pose health concerns.

2.3.5 Other disposal technologies

Thermal hydrolysis is a technology which uses high temperature (180°C) and pressure (12 atm) to denature the long chain molecules of SRMs or dead stock into smaller and simpler molecules (Schmidt 2001). It has been reported that the complete inactivation was almost achieved at 138°C (Taylor 2000). As verified by animal infectivity tests (Somerville et al. 2009), the Biosphere thermo hydrolysis technology which combined saturated steam heating to 180°C (10 bar) with stirring was able to achieve 100% infectivity removal ($\geq 10^{5}$ ID_{50}) of BSE. Because thermal hydrolysis breaks proteins into short chain fragments which are biologically suitable for microorganisms, the output may have a higher biogas yield and faster fermentation than conventional digestion processes if used for anaerobic digestion (Schieder et al. 2000).

Thermal depolymerization (TDP) is a process using intense heat and pressure for the reduction of complex organic waste materials into light crude oil. It may convert animal by-product and other wastes into high quality diesel oil, gas, minerals and chemicals. The oil and gas can be used on-site to operate plants (AARI 2005). However, whether this method may completely inactivate prions is still under investigation.

Pyrolysis is a chemical decomposition of condensed substance by heating in the absence of oxygen, generating oil and gas products. Gasification is a process of decomposition of biomass at high temperatures by controlling of oxygen. It has been demonstrated on a laboratory scale that vacuum pyrolysis could generate a combustible gas, high calorific value oil and a solid residue rich in minerals (Chaala and Roy 2003). These methods can contribute to carbon dioxide reduction and may be used to treat animal wastes and are considered as alternatives to incineration for prion contaminated animal tissue disposal.

Although characteristically resistant to enzymatic degradation, there are a few proteases which have the ability to degrade prions at proper conditions, as described in a previous section. In addition to landfilling, composting as another waste disposal approach was expected to be more effective on prion degradation due to its appropriate temperature for thermophilic microbes (Huang et al. 2007). Composting was carried out in one study for 108 and 148 days, with the temperature at above 60°C for 2 weeks then slowly decreasing to ambient temperature (Huang et al. 2007). Although not completely degraded, the western blotting showed that infectious sheep scrapie was partially destroyed by composting (Huang et al. 2007). A biosecure composting system was recently developed in Alberta, Canada, for the treatment of cattle carcasses and manure following infectious disease outbreak; whether it can successfully inactivate TSE infectivity is still under investigation (Xu et al. 2009). SRM degradation has also been studied with a passively aerated laboratory-scale composter (Xu et al. 2013). At the thermophilic temperatures (>55°C), a loss of western blot signal of PrP^{Sc} (scrapie 263K) was observed after 14 days. However, complete degradation of infectious prions was not demonstrated due to the lack of an effective detection method for composting matrices.

2.4 Infectious prion detection methods

The lack of sensitive and efficient detection methods of PrP^{Sc} for both medical and environmental applications has hampered our ability to assess and control prion transmission. At present, the most widely used detection method is a standard western blot assay which is capable of detecting up to a 3 $\log_{10}$ dilution

of the original brain (Lee et al. 2000; Saa et al. 2006). Other available detection methods including filter retention assays (Solassol et al. 2004), ELISA assays (Biffiger et al. 2002; Saa et al. 2006), the phosphotungstic acid precipitation (PTA) incorporating a conformation dependent immunoassay (CDI) assay (Saa et al. 2006; Safar et al. 1998), the tandem mass spectrometry (Nano LC/MS/MS) assay (Onisko et al. 2007) have significantly lower detection limits than the animal infectivity bioassays whose maximum detection was reported to be 9-$\log_{10}$ (Saa et al. 2006). Cell culture assays have similar detection limits and accuracy as bioassays, however, they are only limited to several prion strains (Mahal et al. 2008). A recent study reported a new cell line, epithelial RK13 which is permissive to mouse and bank vole prions as well as natural prion agents from sheep and cervids (Arellano-Anaya et al. 2011). Both flow cytometry and PMCA have been reported to have higher detection limit than bioassays (Castilla et al. 2005; Trieschmann et al. 2005), whereas only PMCA mimics the amplification of PrP^{Sc} *in vivo* since the end products have been shown to be infectious. Comparison of the sensitivity of detection methods is listed in Table 2.5.

Table 2.5 Comparison of sensitivity of methods to detect infectious prions

Assay	Maximum dilution detected of original brain	Reference
Standard western blot	3.0×10^{-3}	(Saa et al. 2006)
ELISA	3.7×10^{-4}	(Saa et al. 2006)
PTA-CDI	$5 \times 10^{-5} – 6 \times 10^{-5}$	(Saa et al. 2006; Safar et al. 1998)
Nano LC/MS/MS	2×10^{-7}	(Onisko et al. 2007)
Cell culture	$\sim 10^{-8}$	(Arellano-Anaya et al. 2011)
Animal bioassay	1×10^{-9}	(Saa et al. 2006)
Flow cytometry	$\sim 10^{-10}$	(Trieschmann et al. 2005)
PMCA	1×10^{-12}	(Saa et al. 2006)

It should be noted that animal infectivity bioassay is a ‘gold standard” for measuring prion infectivity and is well accepted and applied in various disinfection studies, however, this assay is extremely expensive and time-consuming (Brown et al. 1986; Taylor et al. 1994). Therefore, many studies use western blot to detect PrP^{Sc} which is much faster in evaluation of the inactivation

effectiveness (Paspaltsis et al. 2006; Xu et al. 2013). However, the inconsistency of western blot detection to animal infectivity has been reported (Barron et al. 2007; McLeod et al. 2004; Triantis et al. 2007), possibly due to the low detection limit of western blot. On the basis of its high sensitivity and autocatalytic generation of PrP^{Sc} *in vitro* as *in vivo*, PMCA as an assay is ideal for use in PrP^{Sc} decontamination experiments (Murayama et al. 2006; Suyama et al. 2007b). This *in vitro* method of prion detection has several distinct advantages over other *in vitro* algorithms of prion detection and quantification. Firstly, PMCA remains the only published *in vitro* method capable of amplifying infectious prion proteins (Saborio et al. 2001; Soto et al. 2002) (Fig 2.1). Secondly, PMCA relies on template directed misfolding and amplification of normal PrP^{C} (naturally present in normal brain homogenates) as a function of seeding a small quantity of infectious PrP^{Sc} into the *in vitro* reaction, mimicking the pathological process of disease progression *in vivo* (i.e., template directed misfolding) (Castilla et al. 2005). Thirdly, the overall amount of amplification obtained in PMCA is contingent with the amount of infectious seed used to initiate template directed misfolding (Saborio et al. 2001), thus allowing for quantification of PrP^{Sc} found in the original infectious seed. Fourthly, PMCA is extremely sensitive, providing a dynamic range of sensitivity of 12-$\log_{10}$ detection for a 10% infectious brain homogenate after multiple rounds of amplification. By comparison with the methods such as western blot, PMCA enhanced the dynamic range of sensitivity by 9-$\log_{10}$ over traditional western blot (Saa et al. 2006). Fifthly, the PMCA assay considerably reduces the time required for generating results (i.e., several days) compared to animal bioassay models (several months to more than a year). Furthermore, it is amenable to relatively large-scale parallel processing of samples, allowing for more samples to be analyzed under various conditions; outcomes that are vital for intensive data modeling approaches. Studies by Murayama et al. (2006) (Murayama et al. 2006) and Suyama et al. (2007b) (Suyama et al. 2007b) examined heat sterilization of PrP^{Sc} and showed that PMCA results correlated with animal infectivity. Suyama et al. (2007a) reported that advanced oxidation products generated by the Fenton reactivation were

potent inhibitors of prion propagation by PMCA. The PMCA amplification of PrP^{Sc} as a representative of infectivity has been demonstrated to correlate well with the animal bioassays (Pritzkow et al. 2011) and effectively employed as a marker for TSE diagnosis (Saa et al. 2006).

2.5 Prion inactivation in wastewater

2.5.1 Potential contamination of prions in wastewater

It has been reported that the risk of BSE infectivity in aquatic water and wastewater was very low and should not sustain epidemic in cattle herd (Gale and Stanfield 2001; Gale et al. 1998). Low concentrations of PrP^{CWD} has been detected in one surface water sample from a CWD epidemic area, as well as the nearby municipal water treatment facility (Nichols et al. 2009), strongly indicating that water may act as a transport agent for prions. The quantitative assessment proposed that in the event of SRM coming into wastewater systems, it would exclusively be partitioned into the sludge; and the remaining infectivity after non-complete treatment would be retained by soil (Hinckley et al. 2008). This risk assessment was based on the best estimation of BSE contaminated agents entering wastewater systems from slaughtering houses, and no epidemic should happen due to the application of the sewage sludge to agricultural lands. However, provided that the abattoir containment is failed that infected spinal cord and brains are released to the wastewater system, the subsequent wastewater and sludge treatment would not remove much infectivity and epidemic might happen among the herd (Hinckley et al. 2008). Since no prion infectivity has ever been detected in wastewater or sludge due to less sensitive analyzing approaches, whether the assessment model is biased or not is unknown. Besides slaughterhouses, prions may enter wastewater systems through other routes, such as agricultural digesters, particularly those used for disposal of downer cows; some septic systems including private game farms, rural meat processors, and necropsy laboratories doing CWD surveillance; as well as leachate from landfills where prion contaminated materials are buried (Pedersen et al. 2006).

Due to their hydrophobic nature, infectious prions tend to attach to solids in aquatic environments (Gale et al. 1998). However, the extent of attachment

varies depending on many factors, including the solubility of solids, interference with other organic matter, and the subsequent removal of suspended solids, etc (Nichols et al. 2009). The fate of infectious prions in various water matrices has been investigated in several studies (de Motes et al. 2008a; de Motes et al. 2008c; Maluquer de Motes et al. 2012; Miles et al. 2011). Both scrapie and BSE have shown limited degradation (1 to 2-$\log_{10}$) while incubating in phosphate buffered saline (PBS), distilled water and tap water at 20-25°C for 56-265 days (de Motes et al. 2008a; Miles et al. 2011). Higher degradation rates of BSE and scrapie were initially observed when incubating in raw municipal wastewater and slaughterhouses wastewater, evaluated by immunoblotting detection of the presence of PrP^{Sc} (de Motes et al. 2008a; de Motes et al. 2008c). Nevertheless, a recent study has clarified that neither BSE infectivity nor scrapie infectivity could significantly degraded. As a matter of fact, BSE infectivity was not eliminated during incubation in the wastewater for up to 265 days (Maluquer de Motes et al. 2012).

It was reported that slaughterhouse wastewater from abattoirs where one positive BSE case was identified, contained low levels of infectious prions resulting from SRM contamination (de Motes et al. 2008c). After primary treatment of wastewater, the stability of infectious prions was found to be half of that in the untreated wastewater, indicating a higher proteolytic activity from the bacterial load in the concentrated flocculated materials (de Motes et al. 2008c). In addition, flocculation might also assist in aggregation of infectious prions by filtering them out of wastewater systems after primary treatment. However, without conducting bioassays, the absence of infectious agents in the effluent wastewater was not proven (de Motes et al. 2008c). Screening with the filter size less than 6 mm should prevent tissue parts entering wastewater treatment facilities. Provided 1% of the infectivity from slaughterhouses entering wastewater, 0.026% would be ingested by cattle under a qualitative risk assessment (Yoshioka et al. 2007).

In terms of the partition and persistence of prions to conventional wastewater treatment, two studies have examined the fate of prions in the sludge.

During a simulated wastewater treatment process, infectious prions were survived during incubation with activated sludge (Hinckley et al. 2008), though an amyloid-like fibril from lysozyme was reported to be decreased in activated sludge treatment (Morales-Belpaire and Gerin 2008). No infectivity was detected in activated sludge supernatant with animal infectivity tests, but a large fraction of infectious prions survived at simulated activated sludge and mesophilic anaerobic sludge digestion (Hinckley et al. 2008). This again demonstrated that prion infectivity would be mostly sustained in the sludge; and mesophilic proteases were not as powerful as thermophilic proteases to degrade prions. Under thermophilic anaerobic sludge digestion, prion infectivity was significantly reduced as verified by western blot (Kirchmayr et al. 2006). However, bovine BSE showed the highest stability towards thermophilic proteases, followed by mouse BSE and scrapie (Kirchmayr et al. 2006). This was in contrary to the finding that BSE was more easily degraded by proteases than scrapie in sewage (de Motes et al. 2008a; de Motes et al. 2008c). Because of variant conditions in wastewater, more research is required to verify these findings.

2.5.2 Potential inactivation of infectious prions in wastewater

PrP^{Sc} folds into shapes that clumped together into prion plaques in animals' brains; once releasing to environment with SRM, the plaques bind to solids and particles thus become more recalcitrant (Johnson et al. 2007). Stringent disinfectants or inactivation methods are required to inactivation the recalcitrant plaques. As discussed above, alkaline hydrolysis and incineration above 1000°C can completely eliminate prion infectivity. Therefore, prions bound to biosolids and sludge after secondary wastewater treatment can be removed from clarifiers and decontaminated with alkaline hydrolysis and incineration. However, there is a lack of effective approaches for treatment of infectious prions in wastewater.

Chlorine, ozone and UV irradiation are typically used to disinfect bacteria, viruses, and microorganisms in wastewater treatment plants. However, prions are very resistant to UV irradiation (Alper et al. 1967). Chlorine, in the form of sodium hypochlorite has been demonstrated to be effective in prion inactivation. However, the required high concentration (>5000 mg/L) as compared to mg/L as

a regular residual in wastewater (U.S. Environmental Protection Agency 1999a), would result in tremendous formation of trihalomethanes (THMs) which are carcinogenic by-products. As one of the advanced oxidation wastewater treatment disinfectants, ozone has a short half life thus has fewer and less toxic disinfection by-products than chlorine. Ozone has the proven ability to offer an intense oxidation, which is known to be capable of degrading a wide range of organic contaminants in water and wastewater. Advanced oxidation is widely used in wastewater treatment (Agustina et al. 2005) and has also been approved to be effective in prion inactivation as discussed in a previous section. Prion infectivity in wastewater might also be treated by this technology.

2.6 Concluding Remarks

Prion contamination of medical instruments and the environment is a big concern in public health (Gibbs et al. 1994; Saunders et al. 2008). Infectious prions are extremely resistant to conventional decontamination. In summary, chemical treatment, autoclaving, protease enzymatic treatment and advance oxidation have all been reported to be able to inactivate prion infectivity albeit at different extent. Chemical treatment and autoclaving have been intensively studied. The combination of chemical treatment and autoclaving was found to be more effective and efficient than using each method alone, regardless of the inactivation degree. However, both of the commonly used chemicals (sodium hypochlorite and sodium hydroxide) are corrosive at concentrations required for inactivation of prions. The phenolic LpH is expensive compared to the other disinfectants and is toxic if discharged to the environment. In addition, due to the space requirement, autoclaving is not suitable for treatment of large quantities of prion wastes. Enzymatic/protease treatment does not require harsh reaction conditions (i.e. concentration, temperature) as chemical treatments and autoclaving. However, the complete degradation of prion infectivity is also not guaranteed. Advanced oxidation has been used to treat microorganisms and organic materials in water and wastewater, and it might be applied to treat prions in wastewater in the future. Its effectiveness and suitability for disposal of large quantities of infectious materials are still under investigation.

The establishment of effective prion inactivation approaches in different environmental matrices is an urgent issue (Saunders et al. 2008). Most laboratory decontamination approaches cannot be applied to prion inactivation in the environment due to their high cost, toxicity or inability to be applied to a large quantity of infectious tissues. Several inactivation approaches are conducted to dispose SRM on land, whereas only alkaline hydrolysis and incineration have been reported of capable of complete inactivation (Brown et al. 2004; Taylor and Woodgate 2003) and has been approved by European Commission (European Commission 2003). However, these two approaches are highly energy consumed and resulted in emission of large quantities of carbon dioxide. More research on the development of approaches on inactivation of prion infected materials is necessary, and the more reliable and efficient inactivation approaches may be applied as alternates to current methodologies. Compared with the prion inactivation of solid waste, fewer inactivation studies have been performed in wastewater. Although there is lack of clear evidence of prions' emergence in wastewater, the possibility exists and the impact would be significant during an outbreak. Ozone is likely suitable for the treatment of infectious prions in water matrices, as described above; thus this technology should be investigated.

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Chapter 3 Materials and Methods

3.1 Buffers

Phosphate buffered saline (130 mM sodium chloride, 20 mM potassium chloride, 7 mM sodium phosphate, and 3 mM potassium phosphate in Milli-Q water) was prepared and used to make brain homogenates; and for making ozone stock solution in inactivation experiments. An alternate PBS buffer (0.66x) was used as a component of conversion buffer for PMCA assay. The 0.66x PBS was prepared using PBS tablets (BioBasic Inc., Markham, ON, Canada), dissolving 1 tablet per 150 mL Milli Q water.

PMCA conversion buffer was prepared with a final concentration of 0.15 M sodium chloride (Fluka/Sigma-Aldrich, Toronto, Canada), 5 mM EDTA (Gibco Invitrogen Canada Inc., Burlington, ON, Canada), and 1% Triton (MP Biochemicals, Salon, OH, USA) in 0.66x PBS, and adding 1x complete protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada) according to manufacturers' instructions.

3.2 Animals

All animal work procedures were performed under strict accordance with Canadian Council on Animal Care guidelines in such a manner as to minimize suffering. Protocols were approved and monitored by the Animal Care Committee at the Canadian Food Inspection Agency, Ottawa Laboratory – Fallowfield. Three to 6-week old female Syrian golden hamsters (Charles River Laboratories International, Inc., Wilmington, MA, USA) were used to prepare infectious brain homogenates (IBH) and normal brain homogenates (NBH), respectively.

3.3 Preparation of hamster brain homogenates

3.3.1 Infectious brain homogenates (IBH)

Female Syrian Gold hamsters were either exposed orally (100 µL) or by intraperitoneal injection (50 µL) to an inoculum of 263K scrapie positive brain homogenates at the CFIA TSE Laboratory in Nepean, Ontario, Canada. Hamsters displaying clinical signs of scrapie, typically 95–110 days post inoculation, were

euthanized with carbon dioxide and the brains harvested in as short of time as possible. All infected hamsters were confirmed positive by routine diagnosis at the CFIA TSE Laboratory (ELISA and immunohistochemistry). The infectious dose of brain homogenates was subsequently determined to be $10^{9.94}$ ID_{50} per gram of brain tissue as confirmed by hamster infectivity end point titration assays. Ten percent IBH samples (weight/volume in 1x PBS) were manually disrupted (15–20 strokes) on ice using a Potter/glass tissue grinder/homogenizer and allowed to stand on ice for 30 min followed by 1 min centrifugation at 1000x *g*. The clarified 10% IBH supernatant was used as stock solutions for ozone inactivation and PMCA experiments.

3.3.2 Normal brain homogenates (NBH)

Hamsters were sacrificed by exposure to excess carbon dioxide (dry ice in a kill box), upon confirmed death. Cold 1x PBS with 5 mM EDTA (Gibco Invitrogen Canada Inc., Burlington, ON, Canada) was perfused through the hamster circulatory system with the assistance of a peristaltic pump attached to a syringe with the needle puncture to the left ventricle of the heart. A 10% NBH was prepared by adding 1 g of perfused brain suspended in 8 mL of the conversion buffer and 1 mL of 15 USP sodium heparin solution (BD Vacutainer, Franklin Lakes, NJ, USA). The sodium heparin solution was originally prepared by the addition of 10 mL of 0.66x PBS to a pre-coated 150 USP sodium heparin vacutainer (BD Vacutainer), and subsequently aliquoted and froze at –20 °C. Normal brain samples in the conversion buffer/sodium heparin solution were manually disrupted (15–20 strokes) on ice using a Potter/glass tissue grinder/homogenizer and allowed to stand on ice for 30 min followed by 1 min centrifugation at 1000x *g*. The clarified 10% NBH supernatant was used for PMCA/experimentation.

3.4 Ozone inactivation experiments

Ozone stock solutions were generated from ultra pure oxygen using an ozone generator (G30, PCI WEDECO). Concentrated ozone stock solutions in PBS buffer were prepared by bubbling ozone gas through 1 L of PBS buffer

chilled at 4 °C, for around 30 min and ozone dose within the stock solution determined using the Indigo method (APHA 2005).

For ozone inactivation experiments, shell vial reaction tubes (Fisher Scientific, Canada) were mounted on the top of a stir plate with a Teflon-coated magnetic stir bar in each tube to ensure even mixing. The temperature was controlled by submerging the reaction tubes in ice water (4°C) or at room temperature (20°C). Separate reaction tubes were set up to withdraw samples at pre-determined reaction times. The experiment was carried out by adding ozone demand-free (ODF) PBS buffer and diluted IBH into reaction tubes, followed by ozone stock solution with a final volume of 1 mL. During the reaction, reaction tubes were covered with plastic lids. Samples were withdrawn at pre-determined reaction times for residual ozone concentration determination, immediately followed by addition of 20 µL of 1 M sodium thiosulfate to neutralize residual ozone in the reaction tubes. The ozonated samples were then frozen at –80 °C until PMCA assay. The non-ozonated control samples were treated in the same manner except that ozone was completely neutralized prior to addition of diluted IBH. The residual ozone was determined by Indigo method (APHA 2005) with a UV-visible spectrophotometer (Biospec Mini 1240, Shimadzu, Japan) immediately after the experiment. Absorbance measurement was performed at 600 nm in 1 cm quartz cell. The presence of diluted IBH and the applied sodium thiosulfate in the control samples had negligible effect on Indigo method. The absorbance of the control samples was set up as reference for calculation of residual ozone concentration at predetermined reaction time points.

3.5 Protein misfolding cyclic amplification (PMCA) assay

The PMCA assay was used to measure inactivation of the templating properties of ozone treated PrP^{Sc} samples (Soto et al. 2002) (Fig 3.1). It is analogous to PCR, where a minimal seed is needed to trigger a polymerization chain reaction. PMCA results in the template-direct misfolding of a large quantity of PrP^{C}, with a small quantity of PrP^{Sc} acted as the seed, which mimics the process of prion disease progression *in vivo*. The overall amount of amplification

obtained in PMCA is contingent on the amount of infectious seed used to initiate template-directed misfolding.

Control and ozonated samples were serial diluted 10-fold in 10% NBH prior to the PMCA assay. Subsequently, two replicates of 8 µL aliquots samples of each 10-fold dilution series was mixed with 72 µL of 10% NBH in 200 µL flat cap, thin wall PCR tubes (Axygen,Unison City, CA, USA) by inversion. For the experiments performed in Chapter 6, two Teflon beads (2.38 mm in diameter; Cat# 9660K12, McMaster-Carr, Aurora, OH, USA) were filled into 200-µL flat cap thin-wall PCR tubes (Axygen, Union City, CA, USA) before adding samples. As a negative control for PMCA, 80 µL of a 10% NBH was also prepared in the thin wall PCR tubes. PCR tubes were randomly placed in a Sonicator (Qsonica, LLC., Newton, CT, USA) with the sonicator microplate cup horn housed within the acoustic enclosure (provided with the instrument) and the water reservoir temperature set to 37°C. For experiments carried out in Chpater 4 and 5, a sonicator model of 4000 was used. For experiments carried out in Chapter 6, a sonicator of a new model Q700 was used. PMCA was performed for 19 h, with 40 s of sonication followed by 29 min and 20 s incubation periods within each cycle (total number of cycle = 38). Samples were subsequently frozen at –80°C and PrP^{Sc} detected by western blot.

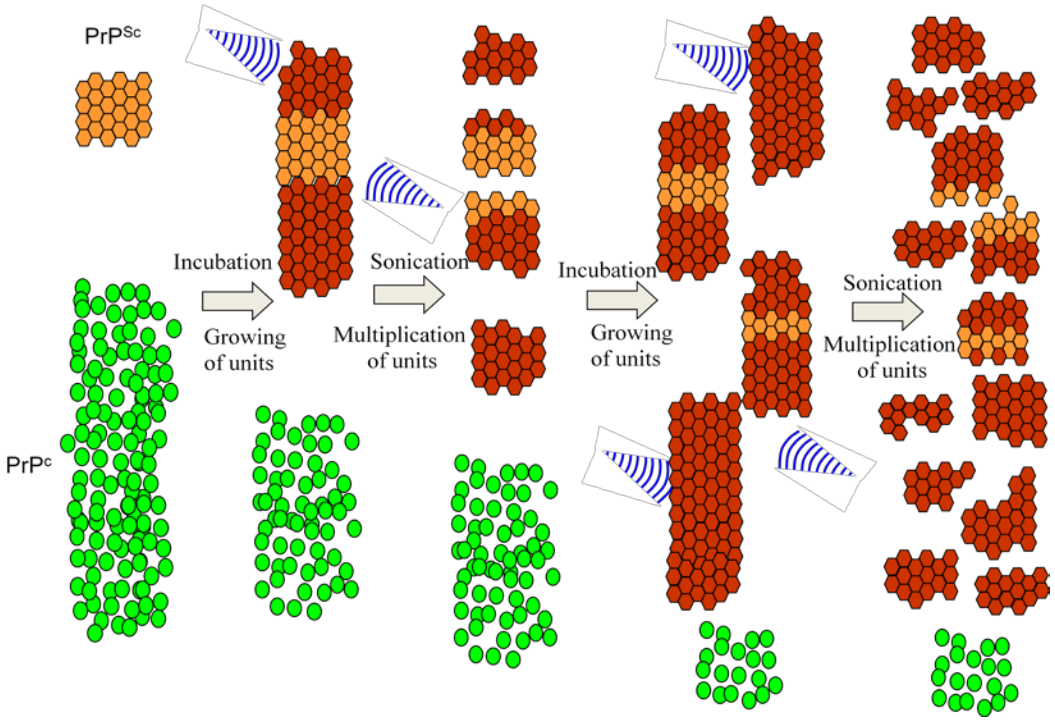

Figure 3.1 Protein misfolding cyclic amplification (adapted from Soto et al. 2002)

3.6 Proteinase K digestion, SDS-PAGE and western blot

Proteinase K (PK) digestion (200 µg PK/mL) was carried out on all PMCA samples and non-PMCA controls (1% IBH in NBH). The digestion was performed at 37 °C for 20 min and stopped with the addition of an equivalent volume of 2x Laemmli buffer [28.5 mL of Laemmli sample buffer (Bio-Rad Laboratories, Mississauga, ON, Canada), 0.6 g SDS (Sigma-Alrich Canada Ltd, Oakville, ON, Canada), and 1.5 mL β-mercapto-ethanol (Sigma-Alrich Canada Ltd, Oakville, ON, Canada)] and incubated at 100 ± 5 °C for 5 min. Twenty five µL of denatured samples were fractionated by SDS-PAGE (Pierce precise 12% precast polyacrylamide gels, Thermo Scientific, Rockford, IL, USA) at 100 V for 1 h, and transferred onto PVDF membrane (Bio-rad laboratories, Mississauga, ON, Canada) at 20 V overnight on ice. The blots were blocked in 5% skim milk in 1x PBS (Bio-rad laboratories, Mississauga, ON, Canada) with 0.1% Tween 20 (Bio-rad laboratories, Mississauga, ON, Canada) for 1 h at room temperature. The blots were then probed with primary anti-prion protein 3F4 antibody (Millipore, Billerica, MA, USA) at 1:20,000 in 1x PBS (10 mM sodium phosphate and 150 mM sodium chloride) containing 0.1% Tween 20 for 1 h at room temperature,

followed by washing three times (10 min each) in 1x PBS-0.1% Tween 20. The conjugated secondary antibody, goat anti-mouse horse-radish peroxidase (HRP, Bio-Rad) (1:10,000 in 1x PBS-0.1% Tween 20), was subsequently added to bind to the primary antibody and incubated for 1 h at room temperature, followed by washing three times (10 min each) in 1x PBS-0.1% Tween 20, and washing twice (5 min each) in 1x PBS without Tween 20. Immunoreactive bands were then visualized using ECL reagent and ImageQuant RT ECL Imager (Amersham, GE Life Sciences, Canada).

3.7 Quantitative analysis of western blot images

In order to quantify the amount of PrP^{Sc} detected by western blot, the blots were analyzed using ImageQuant™ TL software (GE Health Care). The net intensity volume of each active band was normalized by subtracting the intensity of background. Assuming that the normalized intensity volume follows an exponential relationship with dilution before reaching saturation (Saborio et al. 2001), the intensity volume of all dilutions of ozonated samples and lower dilutions (-2 to $-6\log_{10}$) of the positive control samples were standardized against the intensity volume (saturated) of $-1\log_{10}$ of the positive control samples, present in the same blot image. The inactivation of PrP^{Sc} by ozonation was calculated according to Eqn. 1,

$$\log_{10}\frac{N}{N_0} = \log_{10}\left(\frac{\frac{\log_{10}(\text{Intensity of highest dilution of ozonated sample 1})}{\log_{10}(\text{Intensity of } -1\log_{10} \text{ of control})} / (\text{Highest dilution of ozonated sample 1})}{\frac{\log_{10}(\text{Intensity of highest dilution of control})}{\log_{10}(\text{Intensity of } -1\log_{10} \text{ of control})} / (\text{Highest dilution of control})}\right) \quad (1)$$

where $\log_{10}(N/N_0)$ is the $\log_{10}$ of survival of PrP^{Sc} after ozone treatment. The values described as: a) 'Intensity of highest dilution of ozonated sample 1' represents the normalized intensity volume of the signal immediately >0 of one ozonated sample (i.e. $-2\log_{10}$ of 3 s ozonated sample in Fig. 1, first row and left column); b) 'Highest dilution of ozonated sample 1' represents the dilution fold whose signal immediately >0 (in the form of 10^{-n}, where n is an integer) of ozonated sample 1 (i.e. 10^{-2}); c) 'Intensity of $-1\log_{10}$ of control' represents the normalized intensity volume of the signal of $-1\log_{10}$ dilution of positive control sample (i.e. $-1\log_{10}$ of control in Fig. 1, first row and left column); d) 'Intensity of

highest dilution of control' represents the normalized intensity volume of the signal immediately >0 of positive control sample (i.e. -4$\log_{10}$ of control in Fig. 1, first row and left column); and e) 'Highest dilution of control' represents the dilution fold whose signal immediately >0 (in the form of 10^{-n}, where n is an integer) of positive control sample (i.e. 10^{-4}).

3.8 Kinetic modeling and estimation of CT values

The ozone decomposition rate constants, *k'* for each condition, were calculated using the Solver function in Microsoft Excel 2007 to regress the first-order kinetic equation

$$C = C_0 \exp(-k't) \tag{2}$$

where C and C_0 are residual ozone concentration (mg/L) at contact time t (min) and 3 s or 5 s (the possible measurement closest to 0 s), respectively; *k'* is the first order ozone decomposition rate constant (min^{-1}). The inactivation of PrP^{Sc} under each condition grouped by pH and temperature, were fit into both of the Chick-Watson (CW) model and the efficiency factor Hom (EFH) model, as shown in Eqn. 3 and 4, respectively.

$$\log_{10}\frac{N}{N_0} = -\frac{k}{k'n}C_0^n[1-\exp(-nk't)] \tag{3}$$

$$\log_{10}\frac{N}{N_0} = -kC_0^n t^m\left[\frac{1-\exp(-\frac{nk't}{m})}{(\frac{nk't}{m})}\right]^m \tag{4}$$

where k is the inactivation rate constant, n is the constant referred to coefficient of dilution, and m is the constant for the inactivation rate law which describes deviation from ideal Chick-Watson law. The value $\log_{10}(N/N_0)$ is as presented in Eqn. 1, C_0, t and *k'* are as presented in Eqn. 2. To determine the unknown parameters in Eqn. 3 and 4, the Solver function in Microsoft Excel 2007 was used to minimize the sum of squares of the differences (Error sum of squares, ESS) between observed (Eqn. 1) and predicted (Eqn. 3 and 4) survival under each condition.

The *CT* values were estimated for the purpose of assessing the ozone inactivation of PrP^{Sc}. Assuming that ozone decomposition follows pseudo first-order kinetics after initial ozone demand, the *CT* values ($mg \cdot L^{-1}$ min) were estimated by the area under the ozone decay curve at the specific time, using Eqn. 5.

$$CT = \int C(t)dt = \frac{C_0}{k'}[1 - \exp(-k't)] \quad (5)$$

where C_0, k' and t are as presented in Eqn. 2.

3.9 Statistical Analysis

The correlation coefficient (R^2) for each kinetic model was calculated by regression (Microsoft Excel 2007) to determine the fit of predicted CW or EFH model of inactivation curve to the observed inactivation data. The differences of inactivation at various conditions were analyzed by *t*-test with 95% confidence level using Graph Pad Prism 4.

3.10 References

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Chapter 4 Inactivation of Template-Directed Misfolding of Infectious Prion Protein by Ozone[1]

4.1 Introduction

Tissues associated with the nervous and immune systems are considered specified risk materials (SRM) in the food animal production industry. Due to the resistant nature of PrP^{Sc} and the potentially high levels of PrP^{Sc} present in SRM tissues, the liquid and solid wastes generated from the disposal of SRM pose serious concerns due to the possible release and bioaccumulation of misfolded infectious prion proteins in the environment. PrP^{Sc} has been found to be extremely recalcitrant in the environment. PrP^{Sc} appears to be resistant to conventional municipal water and wastewater treatment regimens, such as chlorination (usually ~1 mg/L available Cl_2 in water) (Taylor et al. 1994), UV irradiation (Hartley 1967), and mesophilic anaerobic sludge digestion (Hinckley et al. 2008). The high resistivity of PrP^{Sc} to conventional inactivation in water and wastewater intensifies concerns about prion contamination of the environment, thus effective approaches for prion decontamination in aqueous environment are desirable.

Ozone as an advanced oxidation technology is widely used in water and wastewater treatment processes for inactivation of bacteria (Zuma et al. 2009a), viruses (Lim et al. 2010; Thurston-Enriquez et al. 2005), and protozoa (Li et al. 2001). Although certain advanced oxidation processes have been shown to inactivate infectious prions (Johnson et al. 2009; Paspaltsis et al. 2009; Paspaltsis et al. 2006; Solassol et al. 2006; Suyama et al. 2007a), several major knowledge gaps exist, in particular how interactions between oxidant reaction conditions (e.g., temperature, pH, duration of exposure, ozone dose and organic load) affect inactivation levels of prions. Consequently, the derivation of a *CT* disinfection value for prions has never been reported. The *CT* value is defined as the product of the residual disinfectant concentration (e.g., mg/L) and the contact time (e.g., minutes) required to achieve a certain level of inactivation of a particular target

[1] A version of this chapter has been published. Ding et al. (2012) *Applied and Environmental Microbiology*. 78(3): 613-620.

organism (i.e., CT_{99} is the *CT* product required for 99 % inactivation [or 2-$\log_{10}$]). The United States Environmental Protection Agency (USEPA) routinely uses a *CT* concept for characterizing disinfection requirements for microbial pathogens under a given set of reaction conditions (pH and temperature), and for which a comparative assessment of the susceptibility of the pathogens can be made (USEPA 1991; USEPA 2006). Consequently, the *CT* value is commonly used as an engineering target for inactivation of pathogens in water matrices, as regulatory standards in water treatment, and for modeling the inactivation kinetics of physicochemical disinfectants (Lim et al. 2010; Thurston-Enriquez et al. 2005).

The objective of this study was to determine the effectiveness of ozone for inactivating the template directed misfolding properties of PrP^{Sc} (263K scrapie) under a variety of experimental conditions (ozone dose, contact time, pH, and temperature) and for which *CT* values for ozone could be derived.

4.2 Experimental design

Ozone inactivation experiments were performed under three pHs (4.4, 6.0 and 8.0), and two temperatures (4 and 20°C). Most natural waters and municipal wastewater have pH range of 6-8 (Spellman 2009), and the pH of wastewater from slaughter houses can be as low as 3 due to pH adjustment for blood removal (Masse and Masse 2000). Therefore, we selected three pHs (4.4, 6.0 and 8.0) within the range for evaluation. Two temperatures of 4 and 20°C were chosen to represent ambient and cold water temperatures. Two applied ozone doses were selected (~10 mg/L and ~20 mg/L). Samples were collected at two contact time points (5 s and 5 min), to evaluate the instantaneous and extended inactivation. PMCA assay was applied for PrP^{Sc} detection. Detailed materials and methods were described in Chapter 3.

4.3 Results

4.3.1 Evaluation of ozone demand

Prior to investigating the effect of ozone inactivation on PrP^{Sc}, preliminary experiments were carried out to determine the ozone demand associated with inactivation of PrP^{Sc} in experimental brain homogenates. At an ozone dose of ~12

mg/L, ozone was completely consumed by a 0.1% IBH suspension within 5 s, while a residual ozone concentration was maintained for up to 5 min when a 0.01% IBH was used (Table 4.1). For this reason a 0.01% IBH was used for all subsequent ozonation experiments for determining the inactivation efficiency of PrP^{Sc} by ozone and for calculation of an ozone *CT* product for PrP^{Sc}.

Table 4.1 Ozone demand of PrPSc at pH 6.0, 4°C

Ozone dose (mg/L)	Concentration of IBH	Contact time	Residual ozone (mg/L)
12.9	0.1%	5 s	0
		30 s	0
		2 min	0
		5 min	0
12.5	0.01%	5 s	8.3
		30 s	8.2
		2 min	6.0
		5 min	5.0

4.3.2 Detection of PrP^{Sc} by PMCA assay

A 0.1% suspension of IBH represented the lowest concentration of IBH for which PrP^{Res} could be detected by western blot alone (Fig. 4.1A), indicating that western blot was insufficient as detection tool for characterizing ozone inactivation of PrP^{Sc}. Incorporation of PMCA upstream of western blot increased the sensitivity of detection of PrP^{Sc} upwards of 6-7 $\log_{10}$ compared to western blot alone (Fig. 4.1). A 10% IBH sample could be diluted 100 million-fold (10^8) and still be detected after only a single round of PMCA (38 cycles) (Fig. 4.1B). This expanded range of sensitivity allowed for detection and quantification of ozone inactivation of PrP^{Sc} under the conditions necessary to maintain an ozone residual (i.e., 0.01% IBH suspension), and consequently for derivation of a range of *CT* values based on approximately 4 orders of magnitude of inactivation (i.e., up to $CT_{99.99}$ [detection of prion signal between 0.01% IBH and 0.000001% IBH using PMCA]).

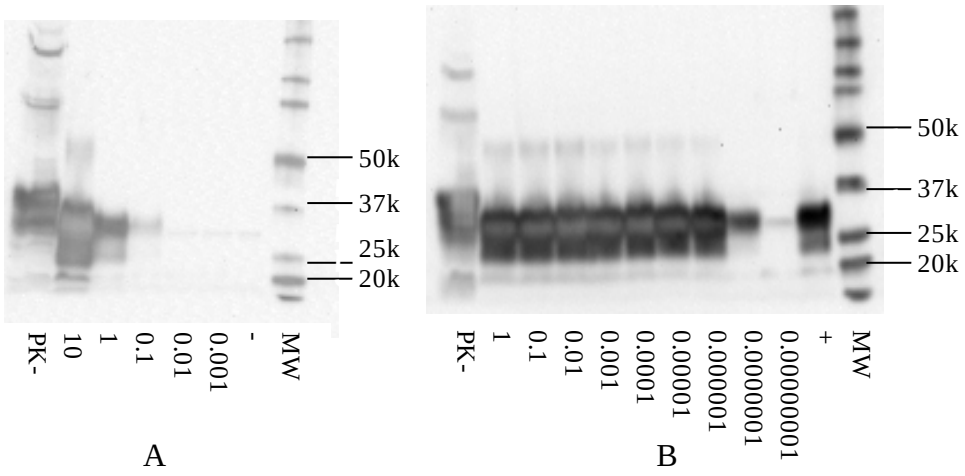

Figure 4.1 A comparison between traditional western blot methodologies and PMCA for detection of 263K scrapie.

Panel (A): Western blot of a 10-fold serially diluted IBH (10%) without PMCA. Lanes labeled 10 to 0.001 represent IBH in percentage (%), respectively. Panel (B): Western blot of a 10-fold serially diluted IBH (10%) with PMCA. Lanes labeled 1 to 0.00000001 represent IBH in percentage (%), respectively. Lanes labeled as 'PK-' represent 1% IBH not treated with PK. Lane labeled as '-' represents 10% NBH treated with PK. Lane labeled as '+' represents 1% IBH treated with PK. Molecular weight markers (lane labeled MW) at 50, 37, 25 and 20 are indicated.

4.3.3 Ozone inactivation of PrP^{Sc}

Ozone inactivation experiments were performed under varying initial ozone doses (7.6 mg/L to 25.7 mg/L), contact time (5 s and 5 min), pH (4.4, 6.0, and 8.0), and temperatures (4 and 20 °C), to assess optimal conditions for inactivating the templating properties of PrP^{Sc}. In preliminary low dose ozone reactions (7.6 mg/L) carried out at pH 8.0 and 20 °C, a PrP^{Sc} reactive band was observed in control blots (i.e., samples in which ozone was neutralized by sodium thiosulfate) as low as 5 $\log_{10}$ dilution of a 0.01% IBH sample using PMCA (Fig. 4.2, Panel C). However, after only 5 s of exposure to this ozone dose, pH and temperature, a loss of 1 $\log_{10}$ in PrP^{Sc} western blot signal intensity was observed (Fig. 4.2, Panel C), and exposure to ozone at this dose for 5 min resulted in 3 $\log_{10}$ loss in observable PrP^{Sc} signal intensity (Fig. 4.2, Panel C). In fact, exposure of an IBH (0.01%) sample to ozone at any condition tested always resulted in a measureable loss of signal intensity by PMCA compared to controls (Fig. 4.2). At pH 4.4 and 20 °C, both low dose (13.0 mg/L) and high dose ozone (23.5 mg/L)

readily inactivated PrP^{Sc}, as assessed by the inability of ozone-treated IBH to act as a seeding template for conformational misfolding of PrP^{C} by PMCA (Fig. 4.2, Panel A). Exposure to ozone for as little as 5 s at low pH (4.4) and 20ºC appeared to completely inactivate the templating properties of the PrP^{Sc} present in a 0.01% IBH (Fig. 4.2, Panel A). In this context, PMCA represented a valuable tool for examining ozone inactivation of the templating properties of PrP^{Sc}. The western blot image after PMCA of samples ozonated at 4°C is shown in Fig. B1.

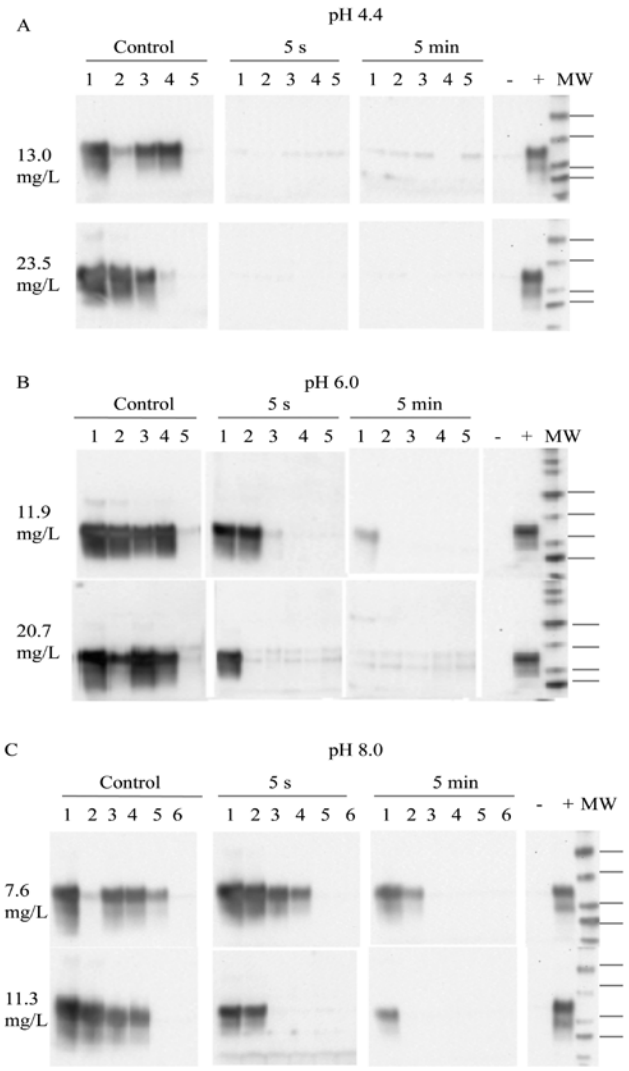

Figure 4.2 Western blots from 0.01% IBH samples treated with ozone at 20°C and amplified by PMCA.

The images in each panel, from left to right, represent non-ozone treated control samples, samples treated with ozone for 5 s, and samples treated with ozone for 5 min. The applied ozone doses are provided to the left of each panel. Panel A = pH 4.4; Panel B = pH 6.0; and Panel C = pH 8.0. The numbers at the top of each image represent dilutions of 0.01% IBH in $\log_{10}$. Lane labeled as '-' represents 10% NBH treated with PK. Lane labeled as '+' represents 1% IBH treated with PK. Molecular weight markers (lane labeled MW) at 50, 37, 25 and 20 kD are indicated.

To generate a more accurate quanititative estimate of ozone inactivation, western blot images obtained from PMCA reactions were analyzed by densitometry (Fig. 4.3). Densitometric analysis of western blots were normalized against saturated signal intensity of PMCA non-ozonated control samples and the normalized intensity was used to estimate $\log_{10}$ reduction of signal intensity of ozone treated samples, using Eq. 1. (Fig. 4.3).

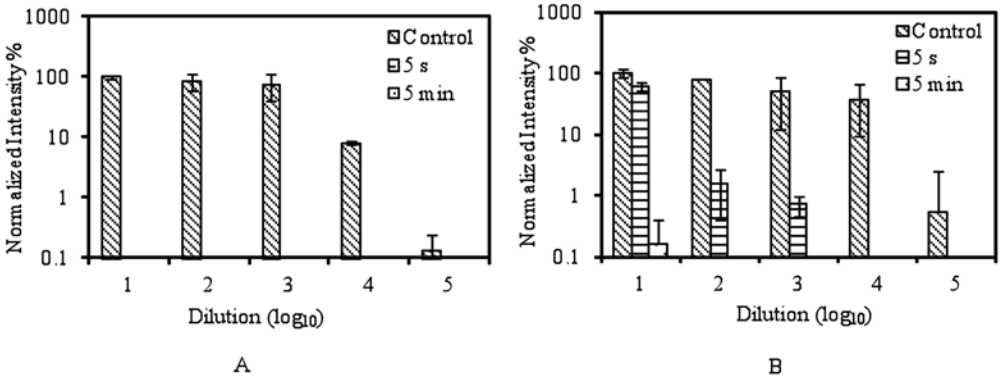

Figure 4.3 Densitometry analysis of Western blot images in Panel A and B of Fig. 4.2.
(A) Normalized intensity of images in Panel A, Row 2 (pH 4.4, ozone dose of 23.5 mg/L). (B) Normalized intensity of images in Panel B, Row 2 (pH 6.0, ozone dose of 20.7 mg/L).

A summary of the log estimates of the levels of ozone inactivation of PrP^{Sc} based on PMCA densitometric analysis of western blots under the various experimental conditions is provided in Table 4.2. Ozone dose, contact time, pH, and temperature were all shown to affect ozone inactivation of PrP^{Sc}. Higher ozone doses and longer contact times resulted in greater PrP^{Sc} inactivation at any given pH and temperature. For example, at pH 6.0 and 4 °C, low dose ozone exposure (12.5 mg/L), resulted in 1.9 $\log_{10}$ inactivation of the templating properties of PrP^{Sc} after 5 s, while a higher inactivation of 3.6 $\log_{10}$ was achieved at 5 min exposure (Table 4.2). At this same pH and temperature, but with a higher ozone dose (20.7 mg/L), greater inactivations were achieved (2.2 $\log_{10}$ and $\geq$4 $\log_{10}$ after exposure of PrP^{Sc} to ozone for 5 s and 5 min, repectively [Table 4.2]).

Table 4.2 Summary of ozone inactivation of PrP^{Sc} under various conditions

pH	Temperature (°C)	Ozone dose (mg/L)	Contact time	*CT* (mg·L^{-1} min)	Inactivation $\log_{10}$ (N_0/N)
4.4	4	13.7	5 s	0.59	2.8
			5 min	31.6	≥4
		25.7	5 s	1.52	≥4
			5 min	67.8	≥4
	20	13.0	5 s	0.59	≥4
			5 min	28.6	≥4
		23.5	5 s	1.17	≥4
			5 min	56.9	≥4
6.0	4	12.5	5 s	0.69	1.9
			5 min	32.2	3.6
		20.7	5 s	1.33	2.2
			5 min	56.5	≥4
	20	11.9	5 s	0.66	2.4
			5 min	26.0	4.4
		20.7	5 s	1.15	2.9
			5 min	41.5	≥4
8.0	4	9.4	5 s	0.40	0.2
			5 min	14.7	2.4
		14.1	5 s	0.72	1.1
			5 min	25.5	2.9
	20	7.6	5 s	0.02	0.9
			5 min	NA	2.9[a]
		11.3	5 s	0.36	1.9
			5 min	6.51	3.0

[a] No residual ozone maintained

In addition to ozone dose and contact time dependency, ozone inactivation of PrP^{Sc} was shown to be pH and temperature dependent. A reaction pH of 4.4 provided greater levels of inactivation of PrP^{Sc} compared to a higher pH (6.0 or 8.0) at the same temperature and ozone dose. For example, when the pH of the reaction was increased from 4.4 to 6.0 at 20 °C, low dose ozone (11.9 mg/L) did not completely inactivate the templating properties of the infectious IBH seed after 5 s or 5 min contact time (Fig. 4.2, Panel B). Increasing the ozone dose to 20.7 mg/L at this same pH and temperature inactivated the templating properties of the IBH seed after a 5 min exposure but not after a 5 s exposure (Fig. 4.2, Panel B). This is in contrast to experiments carried out at pH 4.4 where a dose of 13.0 mg/L of ozone completely inactivated the templating properties of the PrP^{Sc} in the

IBH after only 5 s exposure (Fig. 4.2, Panel A). When the pH of the reaction was further increased to pH 8.0, ozone doses of 11.3 mg/L did not completely inactivate templating properties of PrP^{Sc} in the IBH after 5 min (Fig. 4.2, Panel C). Greater inactivation of PrP^{Sc} was observed at higher temperatures. For example, at pH 4.4, and ozone dose of 13.0 mg/L and contact time of 5 s, PrP^{Sc} inactivation increased from 2.8 to ≥ 4 $\log_{10}$ as temperature was increased from 4 to 20 °C (Table 4.2). Overall, the ideal reaction conditions for PrP^{Sc} inactivation were at pH 4.4 and 20°C (Table 4.2).

Ozone inactivation *CT* values for PrP^{Sc} under various treatment conditions were generated using Eq. 2 and are presented in Table 4.2. With a range of initial ozone doses between 11.3 and 14.1 mg/L, at 4°C, a *CT* between 0.59 and 0.72 $mg \cdot L^{-1}$ min resulted in 2.8 $\log_{10}$ inactivation of PrP^{Sc} at pH 4.4, followed by 1.9 $\log_{10}$ at pH 6.0, and 1.1 $\log_{10}$ at pH 8.0. At 20°C, a *CT* between 0.36 and 0.66 $mg \cdot L^{-1}$ min resulted in ≥ 4 $\log_{10}$ of inactivation at pH 4.4, followed by 2.4 $\log_{10}$ at pH 6.0, and 1.9 $\log_{10}$ at pH 8.0. At *CT* between 25.5 and 32.2 $mg \cdot L^{-1}$ min, PrP^{Sc} inactivation was ≥ 4 $\log_{10}$ at pH 4.4 and both temperatures, and at pH 6.0 and 20°C, while the inactivation was 3.6 and 2.9 $\log_{10}$ at 4°C, pH 6.0 and 8.0, respectively (Table 4.2).

CT values of PrP^{Sc} were compared to other well studied waterborne pathogens (Table 4.3). In general, PrP^{Sc} was found to be less resistant to ozone than some encysted waterborne protozoa (i.e., *Cryptosporidium*) or spore-forming bacteria (*Bacillus subtilis*) under similar experimental conditions. For example, inactivation of *Cryptosporidium* oocysts at pH 6-7 at 5 °C required an ozone *CT* value of 32 $mg \cdot L^{-1}$ min for 2 $\log_{10}$ inactivation, whereas PrP^{Sc} at pH 6 and 4°C required 1.33 $mg \cdot L^{-1}$ min for 2.4 $\log_{10}$ inactivation (i.e., *Cryptosporidium* was >24 times more resistant to ozone than PrP^{Sc}).

Table 4.3 Comparative summary of the efficacies of ozone inactivation of assorted microorganisms and PrP^{Sc} in water

Microorganisms / PrP^{Sc}	pH	Temperature (°C)	*CT* ($mg \cdot L^{-1}$ min)	Inactivation (log_{10})	Reference
E. coli	6-7	5	0.02	2	(Hoff 1986)
Rotavirus	6-7	5	0.006 – 0.06	2	(Hoff 1986)
Adenovirus 40	7	5	0.01 – 0.02	2	(Thurston-Enriquez et al. 2005)
	7	5	0.07 – 0.60	4	
Poliovirus	7.2	5	0.60	2	(USEPA 1991)
			1.20[a]	4[a]	
		20	0.25	2	
			0.50	4	
Giardia lamblia cysts	7	5	1.30	2	(USEPA 1991)
			1.90	3	
		20	0.48	2	
			0.72	3	
Cryptosporidium parvum oocysts	6-7	5	32	2	(USEPA 2006)
			47	3	
		20	7.8	2	
			12	3	
PrP^{Sc} (263K scrapie)	4.4	4	0.59	2.8	This chapter
		20	0.59	≥4	
	6.0	4	0.69	1.9	
		20	0.66	2.4	
	8.0	4	0.72	1.1	
		20	0.36	1.9	

[a] extrapolated by applying first order kinetics with a safety factor of 3

4.4 Discussion

This study investigated the effectiveness of ozone inactivation for infectious prion protein (263K hamster scrapie, PrP^{Sc}) in aqueous solution, and represents the first report to generate ozone inactivation *CT* values for infectious prion protein (263K hamster scrapie; PrP^{Sc}). The current paper sets a base for understanding the conditions affecting ozone inactivation of prion proteins and consequently lays the foundation for modeling the kinetics of ozone inactivation of PrP^{Sc} (and other chemical disinfectants). A detailed understanding of the

kinetics of ozone inactivation of PrP^{Sc} is instrumental for assessing the applicability and efficacy of new or existing technologies for mitigating prion contamination risks in water (i.e., SRM generated wastewater).

Our data suggests that PrP^{Sc} is highly susceptible to inactivation by ozone. The ozone *CT* values derived for PrP^{Sc} in this study were considerably lower than those described for certain waterborne pathogens (i.e., *Cryptosporidium*) and spore forming bacteria (i.e., *B. subtilis*) at comparative temperatures and pHs (Table 4.3). Although the applied ozone dose in this study was higher than is normally done for ozone applications in drinking water disinfection (due to the high ozone demand of the IBH), *CT* values provide a normalized approach to characterizing susceptibility of a particular microbial contaminant to ozone in an aqueous matrix. Since ozone is widely used in the drinking water and wastewater treatment industries for inactivation of encysted protozoan parasites and spore-forming bacteria, ozone treatment may hold significant promise for application in large-scale treatment systems for prion inactivation in water and wastewater streams (i.e., rendering plants' effluents) as well as decontamination of instruments in the medical field. Ozone treatment has been well established for industrial wastewater detoxification (Rice 1981) and minicipal wastewater treatment application (Oneby et al. 2010). Ozone application has also been studied for olive mill wastwater (Bettazzi et al. 2007) and industrial wastewater (Gunukula and Tittlebaum 2001) which contain high concentration of grease and oil. Similarly, SRM wastewater also containes higher level grease and oil, as well as higher concentration of IBH which contribute to more ozone demand. For industrial applications, continuous flow reactors are in place for ozone supply. Different from batch reactors used in this study, continuous flow reactors provide constant and considerably higher ozone dose. It has been reported that in a continuous flow reactor, using an impinging bubble column ozone contactor led to significant increase in the mass transfer rate, and the cumulative ozone dose for a pulp mill effluent reached more than 300 mg/L with 20 min ozonation (El-Din and Smith 2001). Therefore, in practical situation, SRM wastewater treatment can be performed by optimizing the design of reactors and ozone contactor, with

consideration of the characteristics of orgaincs present in the wastewater. Experiments carried out in batch reactors in this study is essential for understanding the efficacy of ozone on PrP^{Sc} inactivation in simplex matrix, providing fundamentals for carrying out prion inactivation studies under complex matrices.

Like viruses, prions lack certain biological characteristics directly associated with self-maintenance and replication, and therefore require infection of a living tissue. Prions were once considered to be 'protein only' (Prusiner 1998) whereas studies have shown that cofactors such as RNA and lipids might also be components of the infectious particles (Piro and Supattapone 2010; Wang et al. 2010). The conformational misfolding of PrP^{C} to PrP^{Sc} results in an increase in β-sheet content (Pan et al. 1993), which makes the protein aggregate and resistant to regular disinfectants. Ozone has been reported to target susceptible amino acids, such as cysteine, methionine, tryptophan, tyrosine, histidine, cystine and phenylalanine, affecting the primary structure of the infectious prion protein (Mudd et al. 1969). The oxidation and cleavage of selected amino acids or monomeric units can induce modification or significant changes in the secondary, tertiary and quaternary structure, or hindering protein folding abilities (Cataldo 2006).

The effect of pH on PrP^{Sc} inactivation by ozone was significant ($p < 0.05$) at both 4 and 20°C, and PrP^{Sc} was inactivated more rapidly at pH 4.4 than at pH 6.0 and 8.0. The effect of pH on ozone inactivation of microorganisms in water has been investigated for a variety of water pathogens. In some studies, the effect of pH has been reported to be insignificant for ozone inactivation of *Cryptosporidium parvum* oocysts at pH 6-10 (Gyurek et al. 1999; Li et al. 2001; Ran et al. 2010; Rennecker et al. 2001) and poliovirus at pH 3-10 (Katzenelson et al. 1979). However, ozone was also reported to be more effective for *Giardia muris* cysts (Wickramanayake et al. 1984), and *Bacillus subtilis* spores (Cho et al. 2003; Dow et al. 2006; Larson and Mariñas 2003) with inactivation greater at higher pH (pH 5-10). While our data is distinct from other studies which demonstrate greater inactivation at higher pH (Cho et al. 2003; Dow et al. 2006;

Larson and Mariñas 2003), ozone inactivation of *E. coli* and norovirus has also been shown to be more effective at a lower pH (Lim et al. 2010; Zuma et al. 2009a). Thus, the pH effect for ozone inactivation appears to be microorganism specific, and is likely to be dependent on the structural components of the microorganisms (Wickramanayake et al. 1984).

In addition to the pH specificity of ozone against various microorganisms, one of the most important aspects affecting pH during ozonation is the application and interpretation of ozone doses. The variance of applied ozone doses may directly lead to the difference of inactivation (Ding et al. 2012; Uhm et al. 2009; Wickramanayake et al. 1984). Farooq et al. (1977) reported that ozone inactivation of *Mycobacterium fortuitum* was higher at pH 5.7 than at pH 10.1 with similar applied ozone doses; while the difference was insignificant when residual ozone was maintained at close levels (Farooq et al. 1977). It was suggested that the high inactivation rate was due to the lower ozone decay rate at lower pH (Langlais et al. 1991). In our study, the applied ozone doses at various pHs were at the same level, which indicated that low pH favored molecular ozone and acted as the major oxidant for PrP^{Sc} inactivation. At a pH above 4, ozone gradually decomposes into hydroxyl radicals (·OH) with the initiator of OH^- in a chain reaction (Langlais et al. 1991), as outlined in Eqn. 6 and 7.

$$O_3 + OH^- \rightarrow HO_2^- + O_2 \quad (6)$$

$$O_3 + HO_2^- \rightarrow \cdot OH + O_2\cdot^- + O_2 \quad (7)$$

It was shown that ·OH was generated by O_3 in reaction with OH- in water, and the reactions were expedited as pH increased (Langlais et al. 1991). Our study indicates that ·OH formed by O_3 decomposition is less effective than the molecular O_3 on inactivating PrP^{Sc}. With the demonstration that the conformational stability of infectious prion agent is not affected over pH range 2 to 10 (Mould et al. 1965), our data suggests that oxidation of infectious prion agents by molecular ozone was more effective than indirect oxidation associated with ozone decomposition to hydroxyl ions (·OH).

Temperature has effects on ozone solubility in water, ozone decomposition rate and inactivation rate. Under similar conditions (i.e. water

characteristics, applied ozone doses), ozone decays faster at higher temperatures (Dow et al. 2006). However, ozone inactivation of microorganisms in water has been reported to be even higher at higher temperatures. Ozone inactivation of *Giardia lamblia* at pH 7 (Wickramanayake et al. 1984), *Naegleria gruberi* at pH 7 (Wickramanayake et al. 1984), *Cryptosporidium* at pH 6-8 (Li et al. 2001; Ran et al. 2010), and *Bacillus subtilis* spores at pH 7 (Larson and Mariñas 2003) was higher as temperature increased towards 25°C, which is consistent with ozone inactivation of PrP^{Sc} observed in this study.

PMCA as an *in vitro* method of prion detection has several distinct advantages over other *in vitro* algorithms of prion detection and quantification. Firstly, PMCA remains the only published *in vitro* method capable of amplifying infectious prion proteins (Saborio et al. 2001). Secondly, PMCA relies on template directed misfolding and amplification of normal PrP^{C} (naturally present in normal brain homogenates) as a function of seeding a small quantity of infectious PrP^{Sc} into the *in vitro* reaction, mimicking the pathological process of disease progression *in vivo* (i.e., template directed misfolding) (Castilla et al. 2005). Thirdly, the overall amount of amplification obtained in PMCA is contingent on the amount of infectious seed used to initiate template directed misfolding (Saborio et al. 2001), thus allowing for quantification of PrP^{Sc} found in the original infectious seed. Fourthly, PMCA is extremely sensitive, providing a dynamic range of sensitivity of 8 $\log_{10}$ detection for a 10% infectious brain homogenate after a single round of amplification in this study. By comparison to methods such as Western blot, PMCA enhanced the dynamic range of sensitivity by 6 $\log_{10}$ over traditional Western blot in our experiments, similar to what other researchers have found (Saa et al. 2006). PMCA has also been shown to have a lower detection limit than animal infectivity bioassays. The dynamic range of sensitivity of PMCA allowed for the optimal conditions of inactivation of PrP^{Sc} to be determined, and consequently the derivation of *CT* products for ozone. Fifthly, the PMCA assay considerably reduces the time required for generating results (i.e., 3 days) compared to animal bioassay models (several months to more than a year). Furthermore, it is amenable to relatively large-scale parallel processing of

samples, allowing for more samples to be analyzed under various conditions; outcomes that are vital for the intensive data modeling approaches which we are currently pursuing. Studies by Murayama et al. (2006) (Murayama et al. 2006) and Suyama et al. (2007b) (Suyama et al. 2007b) examined heat sterilization of PrP^{Sc} and showed that PMCA result correlated with animal infectivity. Furthermore, Suyama et al. (2007a) (Suyama et al. 2007a) reported that advanced oxidation products generated by the Fenton reactivation were potent inhibitors of prion propagation by PMCA. Therefore, it is suggested that PMCA serves as the most appropriate approach for determination of prion inactivation by ozone and advanced oxidation. We are currently in the process of validating the levels of ozone inactivation of PrP^{Sc} using animal infectivity studies.

A recent study by Johnson et al. (2009) demonstrated that UV-ozone treatment of hamster-adapted transmissible mink encephalopathy prions induced inactivation of PrP^{Sc} ≥ 5 $\log_{10}$. Ozone was generated by UV at a wavelength of 185 nm, and then decomposed by UV at another wavelength of 254 nm to produce hydroxyl radicals. However, the UV-ozone system studied by Johnson et al. (2009) produced limited ozone thus generating limited hydroxyl radicals, requiring exposure times up to several weeks, questioning the practicality of using UV-generated ozone as a decontamination/sterilizing approach to prion inactivation. Prion inactivation has also been studied with other advanced oxidation methods, such as copper and hydrogen peroxide (Lehmann et al. 2009; Solassol et al. 2006), iron and hydrogen peroxide (Suyama et al. 2007a), photo-Fenton treatment (Paspaltsis et al. 2009), and titanium dioxide photo-catalysis (Paspaltsis et al. 2006). Hydrogen peroxide inactivation (100 mmol/L) in the presence of copper (0.5 mmol/L) was reported to achieve ≥ 5. 2-$\log_{10}$ inactivation of 263K scrapie prion with a contact time of 2 h at room temperature (Solassol et al. 2006). Increasing the concentration of hydrogen peroxide to 2.2 mol/L reduced the contact time to 30 min for the same level of inactivation (Lehmann et al. 2009). Hydrogen peroxide inactivation (1.5 mol/L) in the presence of Fe^{2+} (15.7 mmol/L), heating at 50°C for 22 h was able to obtain approximately 6-$\log_{10}$ reduction of prion infectivity (Suyama et al. 2007a). PrP^{Sc} could also be degraded

by ≥2.4 $\log_{10}$ by photo-Fenton treatment (147 mmol/L H_2O_2, 8.9 mmol/L Fe^{3+}) after 5 h UV-A exposure (Paspaltsis et al. 2009), and be degraded by≥2 $\log_{10}$ by titanium dioxide photo-catalysis (25 mmol/L titanium dioxide and 118 mmol/L H_2O_2) after 12 h UV-A exposure (Paspaltsis et al. 2006). Due to the low sensitivity of the PrP^{Sc} detection methods used in the last two studies, inactivation higher than 2.4-$\log_{10}$ was not derived. In these advanced oxidation studies, hydroxyl radicals as a sole component to inactivate PrP^{Sc} demonstrated its capabilities, however, prolonged exposure times (from 30 min to 22 h) were essential to continuously generate potent hydroxyl radicals sufficient for the inactivation. In contrast, ozone at a pH of 4.4 and 20°C in this study caused a very rapid ≥4 $\log_{10}$ inactivation of PrP^{Sc} after 5 s of exposure, suggesting that ozone treatment might be more efficient for PrP^{Sc} inactivation than other advanced oxidants.

4.5 Conclusion

PMCA is an extremely sensitive tool for detection and quantification of PrP^{Sc} and for measuring ozone inactivation of the template directed misfolding properties of PrP^{Sc}. Ozone inactivation of scrapie 263K was shown to be dose, contact time, temperature, and pH dependent. In addition, ozone was found to be extremely effective on inactivation of 263K scrapie, with more than 4-$\log_{10}$ inactivation observed at *CT* of 0.59 $mg \cdot L^{-1}$ min at pH 4.4 and 20°C. The derived ozone *CT* product for PrP^{Sc} was similar to that of poliovirus, and considerably less than encysted protozoan parasites such as *Cryptosporidium* spp. and *Giardia* spp.

4.6 References

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Chapter 5 Kinetics of Ozone Inactivation of Infectious Prions[2]

5.1 Introduction

The identification of a new variant CJD (vCJD), due to consumption of BSE contaminated meat, has raised great public concerns (Will et al. 1999). Since then, bovine tissues containing high levels of PrP^{Sc} (including skull, brain, trigeminal ganglia, eyes, tonsils, spinal cord and dorsal root ganglia of cattle aged 30 months or older, and the distal ileum of cattle of all ages) were designated as specified risk materials (SRM), and cannot be used for animal feed, pet food and fertilizer by the industry (Canadian Food Inspection Agency 2011). Effective inactivation and disposal of SRM is important for limiting risks posed to public health through contamination of the environment, as PrP^{Sc} is notable for its recalcitrance in the environment (Pedersen et al. 2006).

Water and wastewater may act as a transporting agent of infectious prions. It has been reported that PrP^{Sc} was detected in surface water samples from a CWD endemic area (Nichols et al. 2009). With the concerns that SRM liquid waste may be released into the environment, there is a possibility that infectious prions may enter wastewater treatment systems. In the case of PrP^{Sc} present in wastewater, BSE infectivity has been demonstrated to persist in raw sewage for longer than 150 days (Maluquer de Motes et al. 2012), and anaerobic digestion had very limited impact on prion degradation (Hinckley et al. 2008), which indicated that infectious prions would survive conventional wastewater treatment.

We recently examined the effectiveness of PrP^{Sc} inactivation by ozone (Ding et al. 2012). PrP^{Sc} (scrapie 263K) was inactivated by ozone under a variety of conditions, with inactivation levels dependent upon the applied ozone dose (7.6–25.7 mg/L), contact time (5 s and 5 min), pH (pH 4.4, 6.0 and 8.0), and temperature (4 and 20°C). The previous chapter of this thesis reported that higher

[2] A version of this chapter has been accepted for publication. Ding et al. (2012) *Applied and Environmental Microbiology*. Published ahead of print 15 February 2013.

ozone doses, greater contact time (5 min), low pH (4.4) and elevated temperature (20°C) resulted in the highest level of PrP^{Sc} inactivation, as evaluated by protein misfolding cyclic amplification (PMCA). With the demonstration of the effectiveness of ozone for inactivation of PrP^{Sc} and the foundation of preliminary derivation of *CT* under various conditions in our previous study (Ding et al. 2012), an understanding of the kinetics of ozone inactivation of PrP^{Sc} is required for the design of competent water treatment technologies capable of inactivating infectious prions. The objectives of this study were to: (1) compare the fit of previously described water disinfection models for ozone inactivation of PrP^{Sc}, (2) document the ozone inactivation of PrP^{Sc} at various pHs and temperatures based on the best fit models, and (3) compare PMCA assay and animal bioassay on determination of prion inactivation by ozone.

5.2 Experimental design

Ozone inactivation experiments were performed under three pHs (4.4, 6.0 and 8.0), and two temperatures (4 and 20°C), as described in Chapter 4. Applied ozone doses of 11 mg/L were carried out in the ozonation experiments. Samples were collected at five contact time points (3 s, 15 s, 2 min, 3 min, and 5 min), to build the kinetic models. The PMCA assay was primarily used in this chapter to evaluate the inactivation of infectious prions by ozone. Results obtained from Chapter 4 were validated by animal bioassays in this chapter. Detailed materials and methods were described in Chapter 3.

5.3 Results

Ozone inactivation experiments were conducted under various conditions including pH (4.4, 6.0, and 8.0) and temperatures (4 and 20°C). With exposure to ozone, PrP^{Sc} templating properties were gradually inactivated over the course of 5 min of exposure (Fig. 5.1 and 5.2). At 4°C, a loss of 2-3 $\log_{10}$ in PrP^{Sc} signal intensity was observed after 3 s at pH 4.4, and no signal was detected after contact time of 5 min (Fig. 5.1). The loss of ability of PrP^{Sc} as a seeding template for misfolding of PrP^{C} by PMCA was also observed at other pHs (pH 6.0 and 8.0) after ozone treatment, however, at pH 6.0 and 8.0 the complete loss of the seeding

effect was not achieved after 5 min (Fig. 5.1). This is in accordance with the findings in our previous study (Ding et al. 2012). The PMCA western blot images of samples treated at 20°C are shown in Fig. 5.2. To generate a quantative estimate of ozone inactivation, triplicate western blot images were analyzed by desitometric analysis and the values '$\log_{10}(N/N_0)$' were estimated as described in Materials and Methods. Observed survivial of PrP^{Sc} exposures to ozone for each testing condition are illustrated in Fig. 5.3.

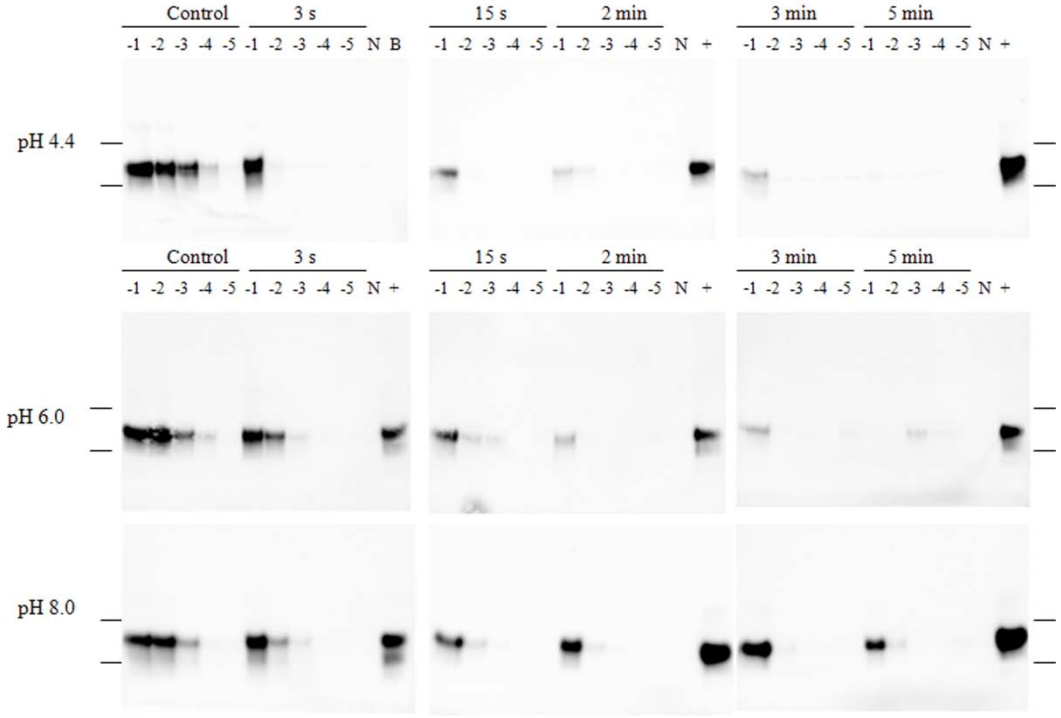

Figure 5.1 Western blot images of 0.01% IBH samples ozonated at 4°C and amplified by PMCA.

The images in each row, from left to right, represent positive control samples, samples treated with ozone for 3 s, 15 s, 2 min, 3 min and 5 min. The pHs of the reactions are shown to the left of each row. The numbers on top of each blot image (-1 to -5) represent $\log_{10}$ dilutions of samples. Lanes labeled 'N' represent 10% NBH treated PK. The lane labeled 'B' represents a blank lane. Lanes labeled with '+' represent $-1\log_{10}$ of positive control samples (same samples as $-1\log_{10}$ of control in the first lane in left column). Molecular weights of 37 and 25 were indicated to the left and right of images in the left and right columns, respectively.

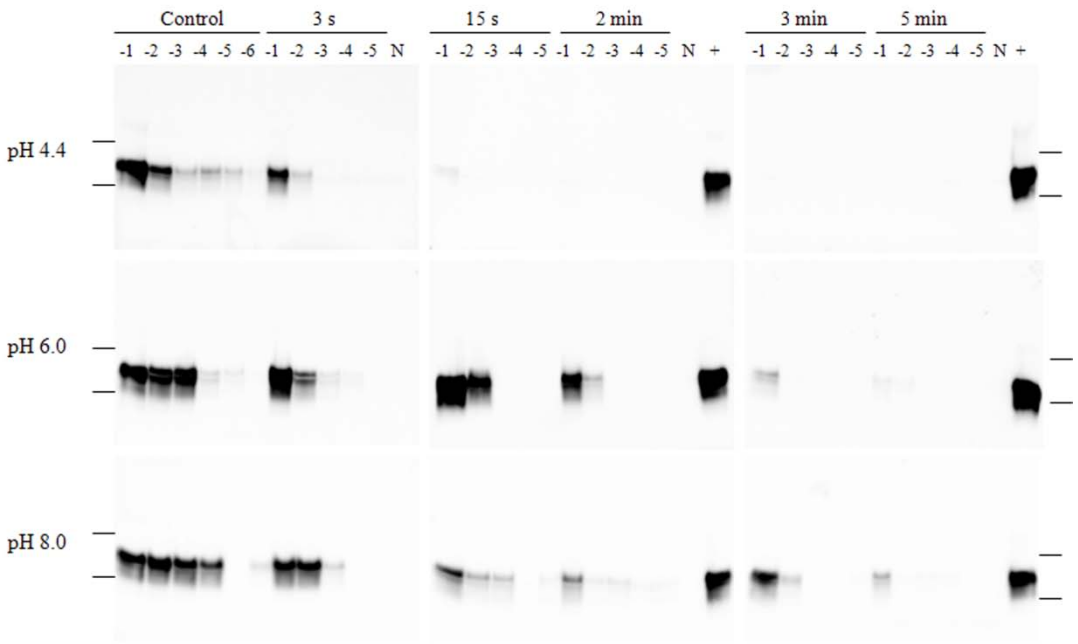

Figure 5.2 Western blot images of 0.01% IBH samples ozonated at 20°C and amplified by PMCA.

The images in each row, from left to right, represent positive control samples, samples treated with ozone for 3 s, 15 s, 2 min, 3 min and 5 min. The pHs of reactions are shown to the left of each row. The numbers on top of each blot image (-1 to -6) represent $\log_{10}$ dilutions of samples. Lanes labeled 'N' represent 10% NBH treated by PK, lanes labeled with '+' represent -1$\log_{10}$ of positive control samples (same samples as -1$\log_{10}$ of control in the left column). Molecular weights of 37 and 25 were indicated to the left and right of images in the left and right columns, respectively.

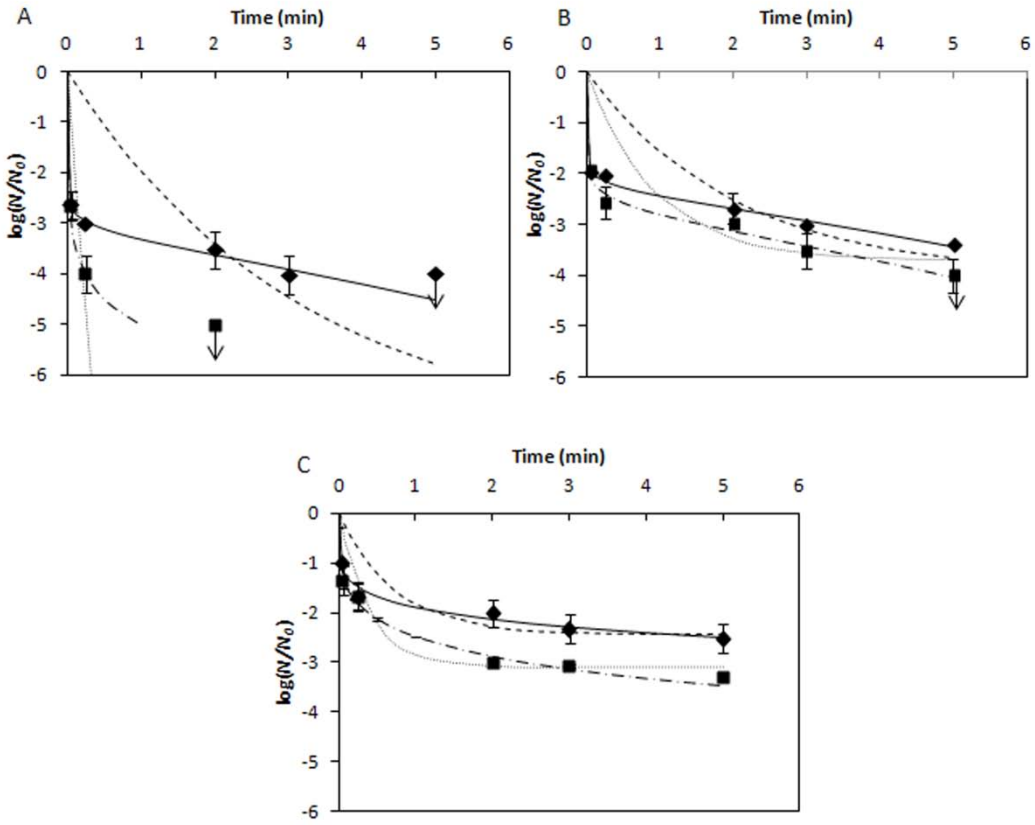

Figure 5.3 Inactivation of PrP^{Sc} by ozone at 4 and 20°C and at (A) pH 4.4, (B) pH 6.0, (C) pH 8.0.

'♦' represents observed data at 4°C, '■' represents observed data at 20°C; '——' represents EFH predicted model at 4°C, '– · –' represents EFH predicted model at 20°C, '- - - -' represents CW predicted model at 4°C, '——' represents CW predicted model at 20°C. ⇓ indicates the values were smaller than shown.

As shown in Fig. 5.3, the observed survival data were characterized by an apparent rapid inactivation, followed by a tailing effect. This non-linear appearance of survival suggested that a non-linear model was essential for adequate description of ozone inactivation kinetics of PrP^{Sc}. Both Chick-Watson (CW) and efficiency factor Hom (EFH) models were used to predict the inactivation kinetics. The applied ozone doses, ozone concentration C_0 (t = 3 s), and parameters for CW and EFH models of inactivation at each condition are provided in Table 5.1. The initial demand of ozone at pH 8.0 was much higher than at other pHs; as C_0 decreased from 10.49 to 7.58 mg/L in at 4°C, and C_0 decreased from 12.54 to 7.14 mg/L in at 20°C (Table 5.1). It was also observed

that, ozone decay was fastest at pH 8.0 and 20°C (k' = 0.36 min^{-1}), followed by pH 8.0 and 4°C, pH 6.0 and 20°C, and pH 4.4 and 20°C. Minimum ozone decay was observed at pH 4.4 (k' = 0.05 min^{-1}) and pH 6.0 (k' = 0.07 min^{-1}) at 4°C (Table 5.1). Compared with CW model, the EFH model was better fit for the observed values. For the prediction of the inactivation kinetics for all testing conditions, the ESS values of EFH model were more than 20 times smaller than ESS values of CW model, and the correlation coefficients (R^2) of EFH predicted values were closer to 1 than CW predicted values (Table 5.1), indicating that the EFH model was more appropriate for the prediction of the inactivation of PrP^{Sc} by ozone.

Table 5.1 Summary of estimated parameters for fitted CW and EFH model

Condition	Applied ozone dose (mg/L)	C_0 (at 3s) (mg/L)	k'[a] (min^{-1})	Model	k	n	m	ESS[b]	R^{2}[c]
pH 4.4, 4°C	11.79	10.07	0.05	CW	5.92×10^{-7}	6.57		12.58	0.95
				EFH	97.32	-1.48	0.06	0.03	0.98
pH 4.4, 20°C	12.81	10.95	0.13	CW	1.49×10^{-5}	5.92		8.30	1.00
				EFH	0.80	0.79	0.19	0.00	1.00
pH 6.0, 4°C	8.82	7.67	0.07	CW	1.14×10^{-6}	7.06		6.11	0.97
				EFH	22.88	-1.12	0.07	0.02	0.98
pH 6.0, 20°C	12.19	10.66	0.17	CW	1.14×10^{-6}	6.37		6.09	0.84
				EFH	8.11	-0.46	0.09	0.07	0.97
pH 8.0, 4°C	10.49	7.58	0.20	CW	3.42×10^{-6}	6.81		1.71	0.88
				EFH	1.86	0.01	0.17	0.07	0.95
pH 8.0, 20°C	12.54	7.14	0.36	CW	6.79×10^{-6}	7.11		1.01	0.95
				EFH	2.27	0.05	0.23	0.05	0.98

[a]Average pseudo first-order ozone decomposition rate constant of three replicates
[b]Error sum of squares.
[c] Coefficients of determination between predicted and observed values

At all testing conditions in our study, the inactivation curve predicted by EFH model steeply declined (in the first 15 s), followed by a slower tailing effect. As observed in Chapter 4, ozone inactivation of PrP^{Sc} was pH and temperature dependent. As shown in Fig. 5.3, the inactivation was higher at lower pH. At 4°C,

the inactivation reached 2.6-$\log_{10}$ in 3 s at pH 4.4, followed by a gradual increase to >4-$\log_{10}$ in 5 min (Fig. 5.3A). In contrast, at pH 6.0, the inactivation was 2.0-$\log_{10}$ in 3 s, and gradually increased to 3.4-$\log_{10}$ in 5 min (Fig. 5.3B). The inactivation at pH 8.0 was even lower, with 0.8-$\log_{10}$ inactivation in 3 s and 2.5-$\log_{10}$ in 5 min (Fig. 5.3C). Similarly, the inactivation of PrP^{Sc} was the highest at pH 4.4, followed by pH 6.0 and pH 8.0, at 20°C. The inactivation of infectious prions at various pHs were significantly different ($p < 0.05$). The ozone inactivation of PrP^{Sc} was also found to be more efficient at a high temperature (20°C) than at a low temeprature (4°C). At pH 4.4, the inactivation was raised from 3.0 to 4.0-$\log_{10}$ at 15 s, and raised from 3.5 to >5-$\log_{10}$ at 2 min, as temperature was increased from 4 to 20°C (Fig. 5.3A). Greater inactivation at higher temperature was also observed at pH 6.0 and 8.0. The effect of temperature was also found to be significant for all pHs tested ($p < 0.05$) (Fig. 5.3B and 5.3C).

The estimated *CT* values from EFH models for ozone inactivation of PrP^{Sc} under various treatment conditions are presented in Table 5.2. In general, the required *CT* values were lower for higher temperature and lower pH. For example, to achieve 4-$\log_{10}$ inactivation at pH 4.4, the required *CT* value for 20°C was much lower than for 4°C. As modeled by EFH, the *CT* value was as low as 2.62 mg·L^{-1} min at pH 4.4 and 20°C, for 4-$\log_{10}$ PrP^{Sc} inactivation. A ≥3 -$\log_{10}$ inactivation could not be achieved in 5 min at pH 8.0 and 4°C, and a ≥4 -$\log_{10}$ inactivation was not obtained in 5 min at pH 6.0 and 4°C, and at pH 8.0 and 20°C. Overall, ozone inactivation of PrP^{Sc} was most effective at pH 4.4 and 20°C and least effective at pH 8.0 and 4°C.

Table 5.2 Predicted *CT* values of PrP^{Sc} inactivation by ozone

Condition	Estimated *CT* (mg·L^{-1} min)		
	2-$\log_{10}$ (99%) inactivation	3-$\log_{10}$ (99.9%) inactivation	4-$\log_{10}$ (99.99%) inactivation
pH 4.4, 4°C	0.01	3.02	30.68
pH 4.4, 20°C	0.06	0.55	2.62
pH 6.0, 4°C	0.73	22.69	-
pH 6.0, 20°C	0.41	14.31	35.16
pH 8.0, 4°C	9.07	-	-
pH 8.0, 20°C	2.59	11.68	-

To investigate whether PMCA results were associated with infectivity, ozone treated samples were also analyzed by animal bioassay. Samples treated by ozone at 4°C and at three pHs (4.4, 6.0, and 8.0) processed in our previous study (Ding et al. 2012) were examined. A linear standard curve of IBH dilution up to 0.00001% (2.94 ID_{50}/g brain, incubation time = 118 days) was generated to determine the ID_{50} of ozone treated samples with incubation time less than 118 days (Figure S1). A four-parameter standard curve of IBH dilution up to 0.0000001% (0.94 ID_{50}/g brain, incubation time >300 days) was also plotted to determine the ID_{50} of ozone treated samples with a latent period more than 118 days in bioassay (Fig. B.2). By using the four-parameter standard curve, both of the delay in incubation and the variation of sick percentage were considered (Lehmann et al. 2009). The log_{10} inactivation at each treatment condition was calculated by taking the difference from readings of the ID_{50} of positive control and ozone treated samples from the standard curve. As shown in Table 5.3, the reduction of infectivity of ozone treated samples for both 5 s and 5 min at pH 4.4 and 4°C were >4.1-log_{10}. The reduction of ozone treated samples for 5 s and 5 min at pH 6.0 and 4°C were 3.1-log_{10} and 3.5-log_{10}, respectively. The reduction of ozone treated samples for both 5 s and 5 min at pH 8.0 and 4°C were 1.0-log_{10} and 3.1-log_{10}, respectively. The PMCA assay data correlated with the bioassay findings to a large extent, most notably on reflecting the trends of inactivation as functions of reaction time and pHs (Table 5.3).

Table 5.3 Comparison of infectivity reduction evaluated by bioassay and PMCA assay

Sample	Attack Rate (number of clinical animals / number animals infected)	Incubation time (d) (SD)	Infectivity reduction (log_{10})	
			Bioassay	PMCA (Ding et al. 2012)
Scrapie 263K IBH, dilution				
10^{-1}	100% (8/8)	63 (4)	-	-
10^{-2}	100% (9/9)	77 (6)	-	-
10^{-3}	100% (10/10)	85 (6)	-	-
10^{-4}	100% (10/10)	86 (5)	-	-
10^{-5}	100% (10/10)	96 (2)	-	-
10^{-6}	100% (10/10)	103 (2)	-	-
10^{-7}	100% (9/9)	118 (11)	-	-
10^{-8}	22% (2/9)	219 (243, 194[a])	-	-
10^{-9}	0% (0/10)	>300	-	-
Bioassay negative control (10% NBH)	0/2[b]	>300	-	-
Bioassay positive control (10% IBH)	5/5	68 (0)	-	-
Positive control 0.01% IBH (ozone quenched with sodium thiosulfate)	6/6	89 (4)	-	-
Ozone (13.7 mg/L) treated for 5 s at pH 4.4 and 4°C	0/4[b]	>300	>4.1	2.8
Ozone (13.7 mg/L) treated for 5 min at pH 4.4 and 4°C	0/2[b]	>300	>4.1	>4
Ozone (12.5 mg/L) treated for 5 s at pH 6.0 and 4°C	5/5	124 (30)	3.1	1.9
Ozone (12.5 mg/L) treated for 5 min at pH 6.0 and 4°C	5/6	145 (24)	3.5	3.6
Ozone (14.1 mg/L) treated for 5 s at pH 8.0 and 4°C	6/6	97 (4)	1.0	1.1
Ozone (14.1 mg/L) treated for 5 min at pH 8.0 and 4°C	6/6	123 (5)	3.1	2.9

[a] Incubation time of two clinical animals.

[b] Groups each contained animals euthanized prior to 300 days (range 202 to 238 days) due to illness unrelated to scrapie, tested negative for scrapie and were not included in the calculations. The remaining hamsters had no indication of scrapie at >300 days and were PrP^{Sc} negative as verified by immunohistochemistry after euthanasia.

5.4 Discussion

As reported in our previous study, the PMCA assay has a dynamic range of sensitivity of detection for 263K scrapie between 7-8 $\log_{10}$ after one round of amplification (19 hours) (Ding et al. 2012), making it an amenable assay for determining inactivation kinetics of chemical disinfectants on prions. In this study, we found that the infectivity reduction evaluated by bioassay was similar or about 1-$\log_{10}$ higher than PMCA assay, which corresponds with another study which also demonstrated consistency within 1-$\log_{10}$ between the two assays (Pritzkow et al. 2011). It has recently been reported that the PMCA assay may be 10^1 to 10^3 times more sensitive than bioassay, depending on the strain of PrP^{Sc} (Makarava et al. 2012; Wilham et al. 2010), however, the authors emphasized that the applicability of PMCA as a quantitative assay are its advantages on quantification of low titer PrP^{Sc}, and significant reduction of time, labour and ethical animal usage (Makarava et al. 2012). Furthermore, the correlation between the prion seeding activity (i.e., PMCA) and prion infectivity (i.e., animal bioassay) based on inactivation parameters such as pH, reaction time, and ozone dose, as demonstrated in our study, provide further support for the use of the PMCA assay as an effective approach for generation of prion inactivation kinetics. In this respect, modeling kinetics based on the PMCA assay appear valid but may conservatively underestimate the reduction of infectivity. For water and wastewater treatment engineering principles, conservative approaches or estimation is usually applied in order to ensure an extra measure of safety, especially in the situation that real conditions are difficult or impossible to monitor/simulate (U.S. Environmental Protection Agency 2006). The models reported in our study best represent these situations.

To our knowledge, this is the first study describing the kinetics of ozone inactivation of infectious prion protein. We applied the CW and EFH models that are typically used in water disinfection under disinfectant demand conditions. Compared to the CW model, the EFH model more accurately described the initial steep inactivation curve and the tailing-off behavior for PrP^{Sc}. It has been reported that the CW model adequately described chlorine-induced inactivation, but not

ozone-induced inactivation of microbes (Finch et al. 1988; Katzenelson et al. 1974). The Hom model accounts for the deviation from the CW model in practice (Hom 1972). In the presence of obvious ozone decay during reactions, the modified Hom models (i.e. EFH) provided the best-fit for viral, protozoa and bacterial inactivation by ozone in water (Gyurek and Finch 1998; Haas and Kaymak 2003; Lim et al. 2010). The tailing effect observed in these studies was attributed to clumping of organisms, presence of pathogen subpopulations with varying resistance to the disinfectant, or distributed resistance within a single population (Cerf 1977; Hiatt 1964).

Ozone inactivation was shown to be dose, contact time, pH, and temperature dependent. The pH conditions of 4.4, 6.0 and 8.0 were chosen to be representative of moderate acidic, slightly acidic and slightly alkaline conditions for disinfection; higher pH was not selected due to the high ozone decomposition rate at alkaline conditions (Ku et al. 1996). Temperatures of 4°C and 20°C were chosen to represent typical disinfection temperatures associated with temperate climate conditions. Inactivation of PrP^{Sc} was greatest at a pH of 4.4 and lowest at the highest pH (8.0). The pH effect alone does not appear to act directly on the conformational stability of the infectious property of the prion agent, as pH by itself has been shown to have little effect on scrapie infectivity over the range of pH 2–10 (Mould et al. 1965). Since ozone decomposition has been shown to be more rapid at a higher pH (Ku et al. 1996), acidic conditions may favor the sustained and direct attack of molecular ozone on the prion protein itself or on biomolecular targets affecting the stability of the misfolded conformer (i.e., lipids) (Appel et al. 2001). Direct oxidation of protein targets by molecular ozone predominate reactions kinetics at lower pH as indirect oxidative byproducts (i.e., hydroxyl radical generation) comprise a minor component of the oxidative potential at low pH (Chu and Ma 2000). High inactivation rates of ozone at more acidic pHs have also been observed for *E. coli* (Suyama et al. 2007a), norovirus (Lim et al. 2010) and helminth eggs (Velasquez et al. 2004). We are currently addressing what contributions molecular ozone and its oxidative derivatives ($OH^{\cdot}$, H_2O_2, etc) have on inactivation of PrP^{Sc} as a means of characterizing the

molecular mechanisms responsible for inactivation. Furthermore, optimization of the experimental methods and approaches to prion inactivation, as described in this manuscript, lays the foundation for a more thorough examination of the kinetics of ozone inactivation of prions.

The effect of temperature on the inactivation of microorganisms and the degradation of organic pollutants by ozone has also been investigated by us and others (El-Din et al. 2006; Li et al. 2001). In general, elevated temperatures result in more rapid inactivation of microbial pollutants by ozone, an outcome also observed in our study. Interestingly, temperature has opposing effects on ozone solubility and disinfection rates (Bablon et al. 1991).

Inactivation of prions has also been studied with other oxidants in various studies (Johnson et al. 2009; Kimberlin et al. 1983; Lehmann et al. 2009; Paspaltsis et al. 2009; Paspaltsis et al. 2006; Rutala and Weber 2001; Solassol et al. 2006; Suyama et al. 2007a; Taylor et al. 1994). Chlorine and hydroxyl radials are the primary oxidants in these studies. It has been reported that 4.4-$\log_{10}$ inactivation of scrapie was obtained with 30-min exposure with residual chlorine of 1000 mg/L (Kimberlin et al. 1983), which is extremely higher than the concentration used in water disinfection, and the concentration of ozone applied in this study. A UV-ozone treatment system, which primarily generated hydroxyl radicals as oxidants was used to treat infectious prions (Johnson et al. 2009). Although more than 5-$\log_{10}$ inactivation was achieved, the exposure time was up to a couple of weeks. Other studies also demonstrated that hydroxyl radicals inactivated infectious prions, more or less, with exposure time from 30-min to several hours (Lehmann et al. 2009; Paspaltsis et al. 2009; Paspaltsis et al. 2006; Solassol et al. 2004; Solassol et al. 2006; Suyama et al. 2007a). However, none of these studies investigated the reaction kinetics. In these studies, infectious brain homogenates were mixed with oxidants at predetermined concentrations, followed by incubation for a period. The effectiveness of oxidants was assessed at the end of reaction, with or without neutralizing the oxidants. This is a biological rationale of analyzing the effectiveness of prion inactivation, without considering consumption of oxidants as a function of time. In this study, we measured residual

ozone at each reaction time point, and took account of ozone decomposition in the generation of inactivation curves, which is imperative in water disinfection engineering studies.

Due to the lack of fast and sensitive detection methods, prions had not been reported in water and wastewater until the development of PMCA. CWD has been detected at very low concentrations in the samples from one stream water and the nearby water treatment plant of a CWD endemic area (Nichols et al. 2009). In our study, the PrP^{Sc} was spiked at a concentration, although lower than that in some prion decontamination studies (Rutala and Weber 2001), was higher than what might be expected in real situations during water and wastewater treatment. Our results indicate that ozone can be used for the inactivation of infectious prion protein in surface water or aqueous environment with limited ozone demand, and lays a foundation for understanding the inactivation of infectious prions using ozone in more complex water matrices. Rendering plants generate large volume of wastewater that may contain infectious prion protein. While blood collection is highly regulated to ensure no contamination with SRM (Canadian Food Inspection Agency 2007a) and all SRM are required to be destroyed (i.e. through controlled incineration), or permanently contained (i.e. landfill) (Canadian Food Inspection Agency 2007b), the disposal of SRM generated wastewater is not regulated. Ozone technology, as discussed above, may serve as a barrier for prion present in wastewater from slaughter houses or SRM rendering facilities.

Different from clean water or ozone-demand free buffered systems, wastewater coming from rendering plants contains grease, oil and other organics which lead to high level of ozone demand. Discharges from rendering plants without SRM are usually pretreated with screening, gravity separation, flow equalization, chemical pretreatment and dissolved air flotation, followed by biological treatment (Sindt 2006). However, with the concern of infectious prion contamination, and the sequential cost of disposal of sediments and floats, the ozone treatment should be added as an additional barrier to the overall process of SRM generated wastewater treatment. Due to the higher biological oxygen

demand (BOD) and/or chemical oxygen demand (COD) (i.e. carbonaceous BOD between 4,000 and 10,000 mg/L) in the rendering wastewater (Sindt 2006), high applied ozone doses would be needed for an appropriate level of inactivation of PrPSc, depending on the characteristics of the organics and competitive reactions in the wastewater. Ozone technology has been tested for wastewater treatment having high organic content, such as olive mill wastewater (COD $\approx$ 300,000 mg/L) in a semi-batch reactor (Bettazzi et al. 2007). Ozone has also been applied to treat diluted landfill leachate (COD = 1010 mg/L, BOD = 186 mg/L). The initial feed gas ozone concentration of 8.3 mg/L (33.3 mg/min) resulted in effective inactivation after 2-hour contact time (Zouboulis et al. 2007). From this information it is predicted that when ozone is used for inactivation of PrP^{Sc} in rendering plant wastewater, a long reaction time will be required for effective inactivation. On an industrial scale, the rate capacity of a typical oxygen-fed ozone generator in a wastewater treatment plant is around 3000 kg/d (U.S. Environmental Protection Agency 1999b), and ozone production can be further enhanced by increasing ozone mass transfer rate, using multi-jet ozone contactors in continuous flow reactors (Baawain et al. 2011). Therefore, full-scale ozonation systems used for industrial wastewater treatment may hold promise for controlling prion contaminated wastewater from SRM rendering facilities.

Ozone may also be a potential treatment for sterilization of medical instruments contaminated with prions in hospitals. Transmission of CJD due to neurosurgical instruments and intracerebral electrodes contamination has been identified (Brown et al. 1994), consequently, regulatory preventative methods have been proposed, including using disposable instruments or decontamination by stringent methods (WHO 1999). The decontamination methods usually involve high temperature and/or high concentration of sodium hydroxide or bleach, which may not be compatible with sensitive medical instruments. Alternative methods have been proposed including Fenton reaction (Lehmann et al. 2009), gaseous hydrogen peroxide (Fichet et al. 2007), enzymatic treatment (Jackson et al. 2005) and other organic chemical treatment (Peretz et al. 2006). These methods either require long reaction time (i.e. >30 min to several hours), or pretreatment and/or

sequential treatment, to obtain optimum inactivation level (i.e. >5-$\log_{10}$). Due to the capability of ozone on prion inactivation in short period of time, the application of ozone on medical instruments may also be feasible with further research.

5.5 Conclusion

In conclusion, Ozone inactivation of PrP^{Sc} (scrapie 263K) in ozone-demand free phosphate buffered saline was more accurately modeled by efficiency factor Hom model than Chick-Watson model. The survival curves showed a rapid inactivation followed by a tailing-effect. The effect of both pH and temperature were significant ($p < 0.05$) on ozone inactivation efficacy, and ozone was found to be more effective at low pH (4.4) and high temperature (20°C). Further studies on PrP^{Sc} inactivation in complex water matrices (SRM wastewater) and PrP^{Sc} bound medical instruments are needed to test ozone application in various areas.

5.6 References

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Chapter 6 Ozone Inactivation of Infectious Prions in Rendering Plant and Municipal Wastewaters[3]

6.1 Introduction

Infectious prions are very recalcitrant in the environment (Brown and Gajdusek 1991; Georgsson et al. 2006; Miller et al. 2004). Scrapie and CWD are horizontally transmissible in the environment (Hadlow et al. 1982; Miller et al. 2004). It has been reported that scrapie persisted in the pasture for 16 years (Georgsson et al. 2006), and survived in soil for at least 3 years (Brown and Gajdusek 1991). CWD could remain infectious in pasture for at least 2 years (Miller et al. 2004). Low concentrations of CWD has been detected in one surface water sample from a CWD epidemic area, as well as the nearby municipal water treatment facility (Nichols et al. 2009), strongly indicating that water may act as a transport agent for prions. In addition, the fate of infectious prions in various water matrices have been investigated in several studies, which suggested that infectious prions could also survive in water and wastewater for fairly long time (de Motes et al. 2008a; de Motes et al. 2008c; Maluquer de Motes et al. 2012; Miles et al. 2011). Both scrapie and BSE have shown limited degradation (1-2 $\log_{10}$) while incubating in phosphate buffered saline (PBS), distilled water, and tap water at 20-25°C for 56-265 days (de Motes et al. 2008a; Miles et al. 2011). Higher degradation rates of BSE and scrapie were initially observed when incubating in a raw municipal wastewater and a slaughterhouse wastewater, evaluated by western blot detection of the presence of PrP^{Sc} (de Motes et al. 2008a; de Motes et al. 2008c). Nevertheless, further studies using the "gold standard" animal infectivity test and an alternative cell culture assay have demonstrated minute degradation of BSE and scrapie in the municipal wastewater (Maluquer de Motes et al. 2012; Miles et al. 2011), verifying that western blot

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detection of PrP^{Sc} does not correlate with infectivity (Maluquer de Motes et al. 2012; McLeod et al. 2004). Prions can also survive in water and wastewater which has been treated with UV irradiation (Hartley 1967), chlorine disinfection (Miles et al. 2011; Taylor et al. 1994), and mesophilic anaerobic digestion (Hinckley et al. 2008; Kirchmayr et al. 2006).

We have demonstrated that ozone is effective in the inactivation of prions in PBS in our previous studies in Chapter 4 and 5. In the wastewater matrices, however, organic matter may shield organisms from the attack of disinfectants (Geldreich 1996), and ozone demand is significantly higher in the presence of the large amount of organics (Hart et al. 1983). The goal of this study was to provide further investigation on the effectiveness of ozone inactivation of prions in the rendering plant wastewater and municipal final effluent. Gravity separation and dissolved air flotation (DAF) as primary treatments were applied on raw rendering wastewater to evaluate their effects of organic removal on prion inactivation by ozone. The specific objectives of this study were to: a) examine the partition of prions during the gravity separation process in raw rendering wastewater; b) assess the effectiveness of ozone inactivation of prions in raw rendering wastewater; c) determine the kinetics of ozone inactivation of prions in rendering wastewater after organics removal by gravity separation and DAF treatment, respectively; and d) investigate the kinetics of ozone inactivation of infectious prions in municipal final effluent.

6.2 Experimental design

6.2.1 Partition of prions during gravity separation in raw rendering plant wastewater

Raw rendering plant wastewater (after screening and settling of coarser solids) was collected from an undisclosed rendering plant in Canada in March 2012. A 100 µL aliquot of 10% IBH was added into 100 mL of the rendering plant wastewater in a 120-mL glass container. Samples were mixed by inverting and gently shaking the container. An aliquot (1 mL) of the sample was transferred into a 1.5-mL plastic tube immediately after mixing and kept static at a room

temperature (20 ± 1°C). Samples in the container were gravity separated after 1.5 h, of natural gravity separation, into three layers, with the scum at the top, supernatant wastewater in the middle, and a very thin layer of scattered precipitated solids at the bottom. After gravity separation, a 1 mL aliquot was withdrawn from the scum and from the supernatant wastewater, respectively. All three samples were subjected to protein misfolding cyclic amplification (PMCA) assay to assess any possible partitioning of prions. This prion partitioning experiment was performed in triplicate. All prion-related work was performed in the biocontainment facilities at the Center for Prion and Protein Folding Diseases (CPPFD) at the University of Alberta, Edmonton, Canada. Optimization experiments for natural gravity separation of raw rendering plant wastewater not containing prions were performed first in the wastewater treatment laboratories in the Department of Civil and Environmental Engineering at the University of Alberta to decide on the best conditions for performing the natural gravity separation of the raw rendering plant wastewater in the CPPFD.

6.2.2 Dissolved air flotation (DAF) treatment of raw rendering plant wastewater

A bench-scale DAF jar test unit (Aztec Environmental Control, UK) having a jar volume of 1400 mL was used to simulate the dissolved air flotation (DAF) process for treating raw rendering plant wastewater. The DAF experiment was started with the pressure of the DAF unit maintained at 70 psi and a temperature of 20 ± 1°C. The jar was filled with 1000 mL of the raw rendering plant wastewater. DAF treatment was initiated by rapidly mixing (200 rpm) the wastewater for 30 s, followed by slow mixing at 20 rpm for 10 min. Air flow of micro bubbles was introduced to the bottom of the jar for 40 s, with an air to solids ratio of 0.006, followed by a resting period for 1.5 h to allow for flocculation followed by solids-liquid separation to occur. Samples were separated into three layers including the scum, the supernatant wastewater, and the scattered solids precipitated at the bottom. Samples from the supernatant wastewater were taken from a sampling port and set aside for ozone inactivation experiments. The DAF treatment experiment was carried out in duplicate in the

wastewater treatment laboratories in the Department of Civil and Environmental Engineering at the University of Alberta.

6.2.3 Analysis of wastewaters

Municipal final effluent was collected from the municipal Gold Bar wastewater treatment plant (GBWWTP) in Edmonton, AB, Canada in April 2012. The wastewater treatment unit operations and processes at GBWWTP consist of pretreatment, primary treatment, two stages of biological secondary treatment and final UV inactivation. GBWWTP final effluent, together with rendering plant wastewater (raw, gravity separated and DAF treated) were characterized in terms of pH, chemical oxygen demand (COD), 5-day biochemical oxygen demand (BOD_5), total suspended solids (TSS), and total dissolved solids (TDS). These quality wastewater parameters were analyzed in triplicate according to the Standard Methods (American Public Health Association 2005) and are presented in Table 6.1. The standard curve of COD is shown in Fig. B.3.

Table 6.1 Characteristics of original wastewaters

Parameter[a]	**Rendering wastewater**			**Municipal final effluent**
	Raw	**Gravity separation**[b]	**DAF**[b]	
pH[c]	5.7 ± 0.1	5.9 ± 0.1	5.9 ± 0.1	6.1 ± 0.1
COD (mg/L)[c]	96975 ± 6525	13400 ± 500	7900 ± 1000	16 ± 2
BOD_5 (mg/L)[c]	56175 ± 2625	3367 ± 133	2184 ± 329	1 ± 0.2
TSS (mg/L)[c]	20610 ± 1686	2845 ± 315	80 ± 30	0
TDS (mg/L)[c]	6230 ± 258	6540 ± 200	6570 ± 216	540 ± 13

[a] Parameters of wastewater characteristics without dilution
[b] Supernatant wastewater
[c] Mean ± standard deviation

6.2.4 Prion inactivation by ozone in wastewaters

Ozone inactivation experiments were performed in raw, gravity separated, and DAF treated rendering plant wastewater, and municipal final wastewater effluent. The contents of reaction vials were assembled as follows: a 10% IBH

stock was diluted in the raw, gravity separated, DAF treated wastewater, or municipal final effluent into a 0.1% working stock. For raw and pre-treated rendering plant wastewater, 100 µL of the 0.1% IBH were added into each reaction vial, followed by adding 900 µL of ozone stock solution, to yield an applied ozone dose of around 44 mg/L. Samples were collected at five contact time points (5 s, 30 s, 2min, 3min, and 5 min). For municipal final wastewater effluent, two ozone doses were applied in independent experiments. For the experiment with the higher ozone dose, 200 µL of final effluents were added into each reaction vial, followed by adding 700 µL of the stock solution, to yield an applied ozone dose of 23 mg/L. For the experiment with the low ozone dose, 200 µL of the final effluents were added into each reaction vial, followed by adding 350 µL of PBS at pH 6, and 350 µL of ozone stock solution, to yield an applied ozone dose of 13 mg/L and the same dilution of final effluent as that treated with the higher ozone dose. Samples were collected at five contact time points (5 s, 30 s, 2min, 5min, and 10 min), to build kinetic models. PMCA assay was applied for PrP^{Sc} detection. Detailed materials and methods were described in Chapter 3.

6.3 Results

6.3.1 Partition of infectious prions by natural gravity separation in raw rendering plant wastewater

To investigate the partitioning of infectious prions by natural gravity separation process, we spiked IBH into the raw rendering plant wastewater to yield a final concentration of 0.01%, and examined the partitioning of prions between the scum layer at the top and wastewater underneath the scum layer. After 1.5 h of natural gravity separation, the wastewater was separated into three layers: the top layer consisted of the dark-brown concentrated scum, the middle layer was light-brown wastewater, and the bottom layer consisted of scattered precipitated solids. As shown in Fig. 6.1, PrP^{Sc} in the raw rendering plant wastewater was amplified up to 4-$\log_{10}$ after 19 h of PMCA. After natural gravity separation, PrP^{Sc} was shown to partition primarily to the scum layer at the top as determined by the ability of PMCA to detect approximately 1.0-$\log_{10}$ more PrP^{Sc}

in the scum layer (approximately 4.5-$\log_{10}$) compared to wastewater (middle layer after gravity separation) (approximately 3.5-$\log_{10}$). Although PrP^{Sc} preferentially partitioned into the scum layer, a large proportion of PrP^{Sc} still remained in the wastewater. Replicate western blot images are shown in Fig. B.4.

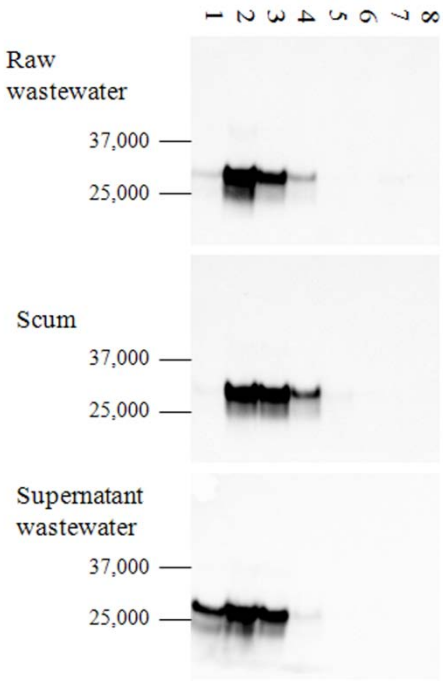

Figure 6.1 Western blot images amplified by PMCA of infectious prions after gravity separation for 1.5 h of raw rendering plant wastewater.

Lanes 1-8 in each panel represent 1 to 8-$\log_{10}$ dilution of 0.01% IBH in raw rendering plant wastewater, scum layer, and gravity-separated (i.e., supernatant) wastewater, respectively, after 1.5 h gravity separation. Molecular weights of 37,000 and 25,000 were indicated to the left of panels.

6.3.2 Ozone inactivation of infectious prions in the rendering plant raw, gravity separated and DAF-treated wastewater

Ozone inactivation experiments using raw and DAF-pretreated rendering plant wastewaters were performed with applied ozone doses of 43.4 - 44.6 mg/L, IBH concentration of 0.01%, and a wastewater diluted by 1:10 at 20°C. As shown in Fig. 6.2, a PrP^{Sc} reactive signal was observed in the positive control blots at as low as 5-$\log_{10}$ dilutions in all three wastewaters. After 5 min of exposure to ozone, a loss of greater than 4-$\log_{10}$ in PrP^{Sc} signal intensity was observed in DAF-treated wastewater, and the approximate losses of 3- and 2-$\log_{10}$ in PrP^{Sc} signal

intensity were observed in the gravity-separated and raw rendering plant wastewaters, respectively. To generate quantitative estimates of the levels of ozone inactivation and prion inactivation kinetic parameters, western blot images obtained from PMCA assays were further measured by the densitometric analyses as described previously in Chapter 3. Replicate western blot images are shown in Fig. B.5-B.7.

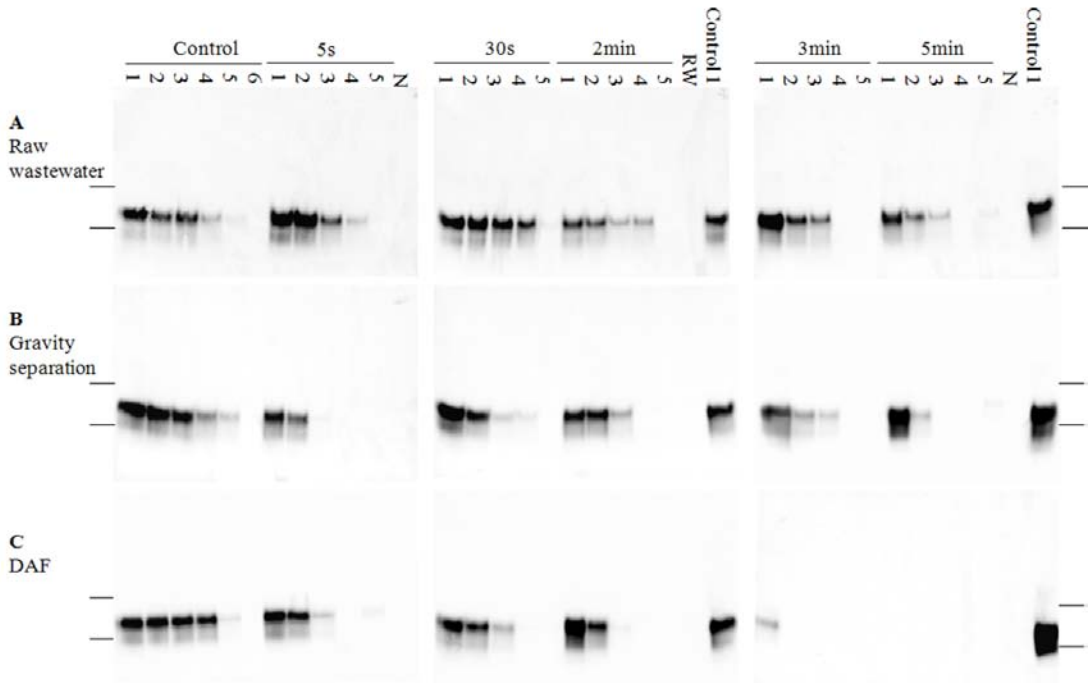

Figure 6.2 Western blot images amplified by PMCA of ozonated samples in (A) raw rendering plant wastewater; (B) gravity-separated rendering plant wastewater; (C) DAF-treated rendering plant wastewater.

The images in each row, from left to right represent positive control, samples ozonated for 5 s, 30 s, 2 min, 3 min and 5 min. The numbers at the top of each panel represent $\log_{10}$ dilution of 0.01% IBH. Lanes labeled "N" represent 10% NBH, the lane labeled "RW" represents negative control raw rendering wastewater in the same wastewater dilution as ozonated samples, lanes labeled "Control 1" represents 1-$\log_{10}$ dilution of the positive control sample (the first lane in the left panel). Molecular weights of 37,000 and 25,000 were indicated to the far left and right of panels, respectively. Samples were all treated with PK.

An interesting observation related to an apparent inhibition of the PMCA assay when prions were spiked directly into raw rendering plant wastewater and diluted by 10^{-1} during PMCA (Fig. 6.1), suggesting that organic matter within the PMCA

assay may inhibit *in vitro* replication of prions by PMCA. However, further dilutions of the wastewater matrix in conversion buffer (10^{-2}) resulted in amplification by the PMCA assay. In addition, no inhibition of the PMCA assay was observed for the control sample in the ozonation experiment using the raw rendering plant wastewater diluted by 1:10 (Fig. 6.2). This data suggest that PMCA may be inhibited by organic matter and this inhibition can be overcome by subsequent dilutions of the wastewater matrix in a conversion buffer during PMCA (i.e., analogous to polymerase chain reaction [PCR] where inhibitors of the PCR assay can often be diluted out) (Drosten et al. 2002).

Ozone demand was significantly higher in the raw rendering plant wastewater compared to pretreated rendering plant wastewater matrices (Table 6.2), indicating that organic matter and particulates in the wastewater competitively reacted with ozone. During the prion inactivation in the raw rendering plant wastewater, the residual ozone was instantaneously reduced from 43.4 mg/L to 0.6 mg/L after only 5 s of exposure (Table 6.2), resulting in an estimated 1.4-log$_{10}$ inactivation achieved after 5 min exposure (Fig. 6.3). For the raw rendering plant wastewater, no kinetic model of prion inactivation was generated due to the low concentration of residual ozone (0.6 mg/L) remaining in the solution after the instantaneous ozone demand was satisfied. In the experiment with gravity-separated rendering plant wastewater, residual ozone concentration, after satisfying the instantaneous ozone demand, was 12.0 mg/L, and followed a first-order decomposition kinetics up to exposure times of 3 min (Table 6.2). An inactivation of 2.8-log$_{10}$ was obtained in this matrix after 5 min of exposure (Fig. 6.3). Due to the enhanced removal of organic matter in DAF-treated rendering plant wastewater, the concentration of residual ozone, after satisfying the instantaneous ozone demand, was 16.8 mg/L, and followed first-order decomposition kinetic during the entire reaction (Table 6.2); thus leading to greater than 4-log$_{10}$ inactivation after 5 min of exposure (Fig. 6.3). Both CW and EFH models were used to model the ozone-induced prion inactivation kinetics in PBS in Chapter 4 and 5, as well as in each pretreated rendering plant wastewater in the current chapter. In all cases, the EFH model produced better fit to the

observed inactivation data and was subsequently used for kinetic modeling. The parameters of EFH model for prion inactivation in the gravity-separated and DAF-treated rendering plant wastewaters are shown in Table 6.2. The parameters of CW models are not shown. It was shown that EFH models provided a better fit to the gravity-separated and DAF-treated rendering plant wastewaters observed ozone-induced prion inactivation data, resulting in smaller error sum of squares (ESS) and higher coefficients of determination (R^2) between the modeled and observed values (Table 6.2). Because the residual ozone concentration was only detected up to the reaction (i.e., exposure) time of 3 min in the gravity-separated rendering plant wastewater, the EFH kinetic model was used to model the prion experimental inactivation data up to a reaction time of 3 min. All experimental inactivation data (i.e., up to 5 min of exposure time) were used for EFH kinetic modeling for prion inactivation in the DAF-treated rendering plant wastewater. As shown in Fig. 6.3, the inactivation efficiency in the DAF-treated rendering plant wastewater was significantly ($p < 0.0001$) higher than that in the gravity-separated rendering plant wastewater.

Table 6.2 Summary of ozone decomposition rate constants and estimated parameters for the EFH kinetic model for gravity-separated and DAF-treated rendering plant wastewater

Wastewater		Applied ozone dose (mg/L)[a]	C_0 (at 5 s) (mg/L)[a]	k' (min^{-1})[a]	k	n	m	ESS[b]	R^{2c}
Rendering	Raw	43.4 ± 0.8	0.6 ± 0.2	-	-	-	-	-	-
	Gravity-separated	44.6 ± 0.3	12.0 ± 0.2	1.09 ± 0.14	3.02	-0.37	0.07	0.001	0.998
	DAF-treated	44.6 ± 1.5	16.8 ± 1.1	0.64 ± 0.04	3.88	-0.20	0.07	0.119	0.958
Municipal final effluent		22.5 ± 1.1	17.1 ± 0.5	0.19 ± 0.06	4.01	-0.12	0.19	0.121	0.982
		13.4 ± 0.4	9.0 ± 1.9	0.21 ± 0.06	2.08	-0.11	0.29	0.062	0.988

[a] Mean ± standard deviation
[b] Error sum of squares
[c] Coefficients of determination between modeled and observed values

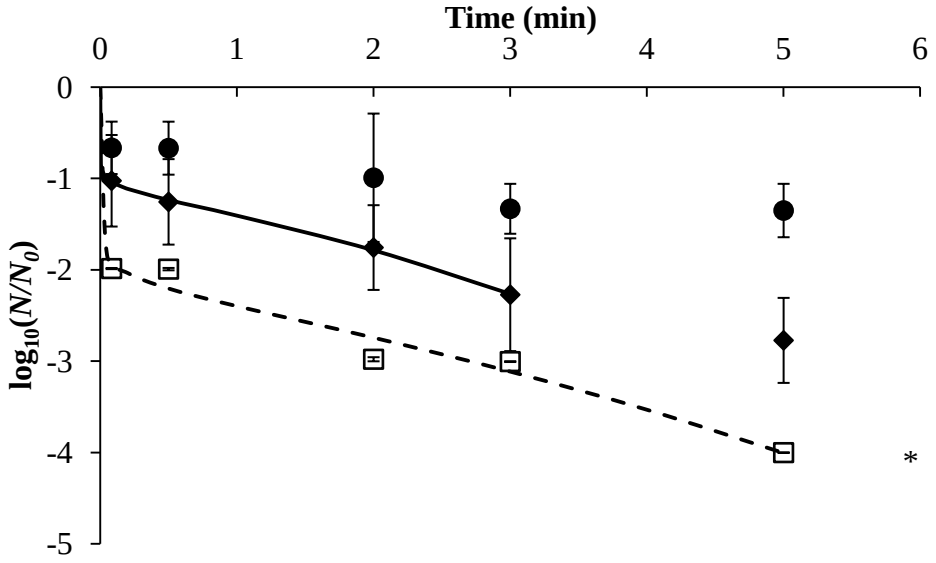

Figure 6.3 Ozone inactivation of prions in raw, gravity separated and DAF treated rendering wastewater.

"●" represents observed values in raw rendering plant wastewater, "♦" represents observed values in gravity- separated rendering plant wastewater, "□" represen ts observed values in DAF-treated wastewater, " — " represents predicted EFH model in gravity-separated wastewater, " - - - " represents predicted EFH model in DAF-treated wastewater. No model was predicted for inactivation in raw wastewater as no residual ozone was maintained during the reaction. Error bars show the standard deviations. * This point was calculated as an average of two values because the third replicate was below the detection limit.

6.3.3 Ozone inactivation of infectious prions in the municipal final effluent

Ozone inactivation of prions in the municipal GBWWTP final effluent was carried out with two levels of applied ozone doses of 13.4 and 22.5 mg/L, an IBH concentration of 0.01%, and with a wastewater dilution 3:10 at room temperature (20 ± 1°C). Similar to the control samples in the diluted rendering plant wastewaters, a PrP^{Sc} reactive signal was observed at as low as 5-$\log_{10}$ dilution of positive control samples, while the disappearance of a PrP^{Sc} signal over time was dependent on the applied ozone doses (Fig. 6.4). The quantitative estimates of prion inactivation using densitometric analysis are shown in Fig 6.5. Ozone-induced prion inactivation kinetics was modeled using both CW and EFH models with EFH model producing a better fit to observed experimental inactivation data (CW model data not shown) (Table 6.2). Prion inactivation using the applied ozone doses of 13.4 and 22.5 mg/L resulted in significantly different ($p < 0.01$) inactivation levels (Fig. 6.5). The *CT* values for 2- and 3-$\log_{10}$ inactivation in the municipal final effluent treated with the lower ozone dose of 13.4 mg/L were calculated from EFH models, and are presented in Table 6.3. A 4-$\log_{10}$ inactivation could not be achieved with the applied ozone dose of 13.4 mg/L. The obtained *CT* values were comparable with *CT* values required for prion inactivation in PBS (Chapter 5) (Table 6.3). The replicate western blot images are shown in Fig. B.8 and B.9.

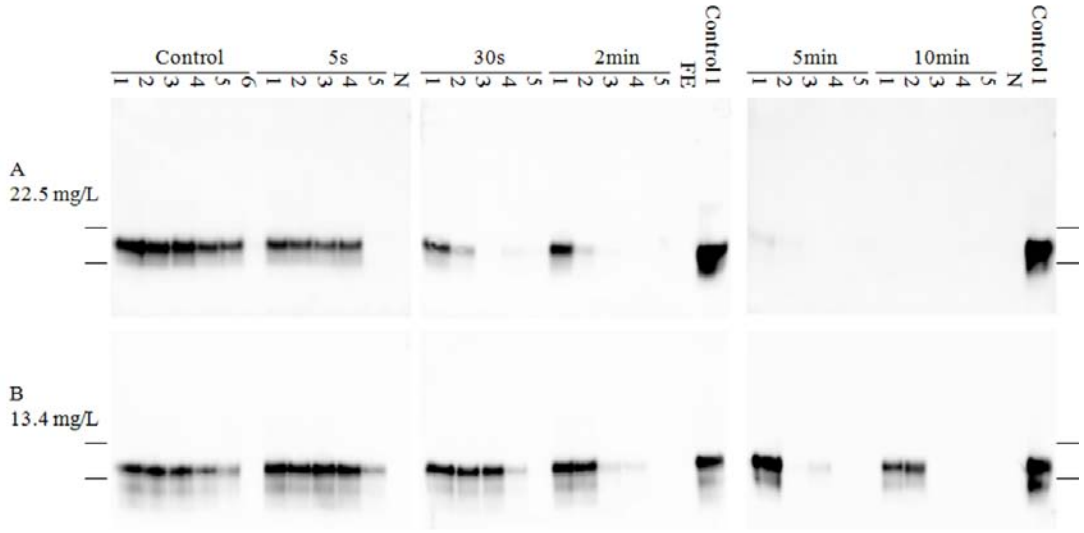

Figure 6.4 Western blot images amplified by PMCA of ozonated samples in municipal final effluent with ozone doses of (A) 22.5 mg/L and (B) 13.4 mg/L.

The images in each row, from left to right represent positive control, samples ozonated for 5 s, 30 s, 2 min, 5 min and 10 min. The numbers at the top of each panel represent $\log_{10}$ dilution of 0.01% IBH. Lanes labeled "N" represent 10% NBH, the lane labeled "FE" represents negative control final wastewater effluent in the same wastewater dilution as ozonated samples, lanes labeled "Control 1" represents 1-$\log_{10}$ dilution of the positive control sample (the first lane in the left panel). Molecular weights of 37000 and 25000 were indicated to the far left and right of panels. Samples were all treated with PK.

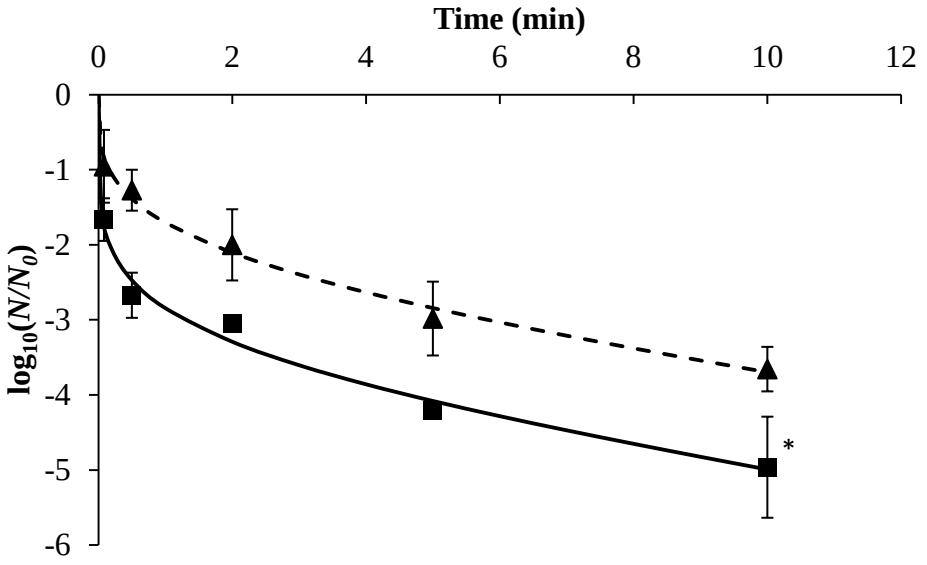

Figure 6.5 Ozone inactivation of prions in municipal final effluent.

"▲" represents observed experimental data with applied ozone dose of 13.4 mg/L, "- - -" represents predicted EFH model with applied ozone dose of 13.4 mg/L, "■" represents observed experimental data with applied ozone dose of 22.5 mg/L, "—" represents predicted EFH model with applied ozone dose of 22.5 mg/L. Error bars show the standard deviations. * This point was calculated as an average of two values because the third replicate was below the detection limit.

Table 6.3 Comparison of *CT* values of prion inactivation in PBS and municipal final effluent

Wastewater	Applied ozone dose (mg/L)	C_0 (mg/L)	k'(min^{-1})	Estimated *CT* ($mg \cdot L^{-1}$ min)		
				2-log_{10} inactivation	3-log_{10} inactivation	4-log_{10} inactivation
Municipal final effluent	13.4	9.0	0.21	10.47	24.49	-
PBS (pH 6.0)*	12.2	10.7	0.17	0.41	14.31	35.61

*Chapter 5

6.4 Discussion

Due to their hydrophobic nature, infectious prions tend to adhere to solids and particles in aquatic environments (Adkin et al. 2012). However, the extent of this adhesion varies depending on many factors, including the type and

concentration of solids, the interference with other organic matter, and subsequent removal of suspended solids (Nichols et al. 2009). It has been reported that in the case of infectious prions present in wastewater, the majority of prions would likely partition into the biosolids during biological treatment (Hinckley et al. 2008). However, less sensitive prion detection approaches used by researchers (i.e., western blot and animal infectivity) reaching the lower limits of detection for these assays precluded accurate assessment of the presence of prions in the treated wastewater. Despite the small risk, the possibility of prions present in wastewater cannot be discounted, especially under circumstances where compliance with environmental regulatory requirements may be difficult for some small SRM processing facilities (Adkin et al. 2012; Sindt 2006). Gravity separation is one of the most widely used unit operations to remove suspended and colloidal materials from wastewater (Metcalf & Eddy Inc. 2003). In this study, we found that after gravity separation of raw rendering plant wastewater, a large proportion (approximately 1-$\log_{10}$) of the infectious prions partitioned into the scum layer and possibly the settled solids, but a significant proportion of infectious prions still remained in the gravity-separated rendering plant wastewater due to the incomplete removal of suspended solids. Because of the limitation of instruments in the biocontainment facility, we were not able to conduct an experiment on prion partitioning during DAF treatment. However, it has been reported that infectious prions are found to adhere to solids and/or particles while in aquatic environments (Gale et al. 1998). Therefore, since DAF treatment removed 99.6% of TSS, it could be assumed that approximately 2.4-$\log_{10}$ (99.6%) removal of prions could be achieved after DAF treatment of the raw rendering plant wastewater. As it has been reported that the infectivity of BSE remained for up to 265 days in raw municipal wastewater (Maluquer de Motes et al. 2012), in this context, the residual infectious prions would still be present in the primary treated wastewater.

Ozone has been widely used in the wastewater treatment industry for disinfection and micropollutants removal purposes (Chelme-Ayala et al. 2010; Chelme-Ayala et al. 2011; Martin et al. 2010; Perez-Estrada et al. 2011; U.S.

Environmental Protection Agency 1999). The application of ozone for pig slurry treatment was proposed because of its availability and strong virucidal effect (Turner and Burton 1997). We previously demonstrated that an applied ozone dose in the range of 12.2 - 12.8 mg/L led to a 4-$\log_{10}$ inactivation of prions after as little as 5 to 15 s of reaction time at pH 4.4 and 20°C, and after 5 min of reaction time at pH 6.0 and 20°C in an ozone demand-free buffered systems (Chapter 4 and 5). However, in the present study a much higher ozone dose of 43.4 - 44.6 mg/L was almost depleted instantaneously in the raw rendering plant wastewater due to its high ozone demand due to its high organic matter content. As a result, a much lower prion inactivation (1.5-$\log_{10}$) was obtained as compared to that achieved in PBS after 5 min of exposure (Ding et al. 2013). To investigate the effect of organics and solids removal on prion inactivation, we pretreated the rendering plant raw wastewater using gravity separation and DAF treatment prior to ozonation, and found that the effect of ozone on prion inactivation was significantly improved by both, with the DAF treatment being more effective.

In this chapter, we used batch reactors and jar test units for gravity separation and DAF treatment, respectively. Higher organic removal with these processes was obtained in our experiments compared to industrial-scale rendering wastewater treatment plants (Johns 1995; Mittal 2006). Typically, BOD_5 removal during gravity separation in industrial full-scale raw rendering plant wastewater treatment operation is in the range of 25% - 40% (Mittal 2006), whereas a 94% BOD_5 removal was obtained in this study. Typical COD and BOD_5 reductions after DAF treatment are in the range of 32% - 92%, and TSS reduction is in the range of 70% - 97% in rendering wastewater treatment plants (Johns 1995), while we observed 91.9%, 96.1% and 99.6% removal of COD, BOD_5 and TSS, respectively. In full-scale industrial wastewater treatment facilities, an enhanced organic (COD and BOD_5) removal is usually achieved by using primary treatment followed by biological treatment (Sindt 2006). After primary and biological treatments, BOD_5 can be reduced by 97.5% to 99.8% (Sindt 2006). Under treatment conditions that led to enhanced organic matter reduction, the results of

this study showed that the effectiveness of ozone for prion inactivation in rendering plant wastewater could be significantly increased ($p < 0.0001$).

The efficacy of ozone disinfection against a variety of microbes in different wastewater and sludge matrices has been investigated in several studies (Finch and Smith 1989; Greene and Stenstrom 1994; Millamena 1992; Watkins et al. 1997). Ozonation has been applied to swine manure sludge to control odors and reduce concentration of pathogens in a continuously stirred batch reactor (Watkins et al. 1997). Ozone was continuously bubbled into 1 L of the swine manure sludge (COD = 46,900 - 76,500 mg/L) with ozone feed ozone gas of 1-3 g/L (Watkins et al. 1997). More than half of malodors were successfully removed, and a 3-$\log_{10}$ of *E.coli* was inactivated. However, a greater than 6-$\log_{10}$ inactivation of *E.coli* was achieved with a much lower applied ozone dose of 22 mg/L in a secondary wastewater effluent (COD = 18 - 29 mg/L) (Finch and Smith 1989), which has much lower level of organic matter than the manure sludge. Ozone has also been used for the disinfection of urban storm drainage water (Greene and Stenstrom 1994). It was found that the residual ozone and levels of inactivation of the total coliforms were negatively correlated with the suspended solids, turbidity and total organic carbon concentrations. In addition, a low ozone dose stream (0.11 $g \cdot h^{-1}$) with 1 h retention time did not effectively remove organic matter in a slaughterhouse wastewater sample (Millamena 1992). Therefore, in agreement with our study, higher applied ozone doses were required, in the current study, for the inactivation of prions in the wastewaters with higher levels of organic materials as was the case for microorganisms inactivation. The application of ozone to wastewaters with high ozone demand requires sufficient ozone concentration and maximum ozone dissolution efficiency in order to treat large volumes of wastewater. This study was a laboratory-scale prion-inactivation-kinetics study based on a batch ozonation system. As such, ozone was not continuously bubbled into the treatment reactor. Instead, it was supplied as an ozone stock solution and it was consumed quickly due to the ozone demand of the wastewater. This is different than an industrial treatment system where flow-through reactors (both for the liquid and gas phases) with much higher ozone

generation capacity for disinfection are employed. A typical oxygen-fed ozone generator in a wastewater treatment plant can generate ozone at a 3,000 kg per day (U.S. Environmental Protection Agency 1999), and the mass transfer efficiency of ozone gas to the liquid phase as a key factor affecting the disinfection can be improved by using multijet ozone contactors (Baawain et al. 2011). As discussed, although the ozone disinfection performance is negatively affected by the ozone-consuming materials (i.e., to satisfy the ozone demand) in the wastewater, by effective removal of organics in the wastewater through pretreatment, ozone treatment provides a strong promise for prion inactivation in wastewaters.

USEPA adopts a *CT* concept as an indicator for the effectiveness of inactivation of waterborne pathogens in public surface waters (USEPA 2003). The comparison of *CT* values for the disinfection of microorganisms under different treatment conditions needs to be conducted with caution. The disparities in the applied ozone doses, instantaneous ozone demand, ozone decomposition rate, and/or ozone residuals could influence the inactivation efficiency (Ding et al. 2012; Farooq et al. 1977; Thurston-Enriquez et al. 2005; Uhm et al. 2009; Wickramanayake et al. 1984). The efficacy of prion inactivation by ozone with the applied dose of 13.4 mg/L in the municipal final effluent was compared to the efficacy of prion inactivation in ozone demand-free buffered system (PBS at pH 6.0), with the applied ozone dose, C_0, and ozone decay (i.e., decomposition) rate being at similar levels (Table 3) (Chapter 5). It was shown that the required *CT* values for prion inactivation in the municipal final effluent were higher than those in the ozone demand-free PBS. The municipal final effluent sample was collected after secondary wastewater treatment (i.e., after total suspended solids and organic matter were mostly removed [TSS and BOD_5 in the final effluent of 0 and 1 mg/L, respectively] and microorganisms were removed and/or inactivated). In agreement with our results, Miles et al. (Miles et al. 2011) showed that infectious prions degraded slower in a 0.22 μm filtered wastewater than in PBS, DI water, and tap water. Since most of the microorganisms and particulate matter were removed from the wastewater after filtration, only dissolved organics remained in the

wastewater and affected prion stability. The authors suggested that some organic matter may protect the target organism from disinfectants (Geldreich 1996; Miles et al. 2011). Moreover, it has been found that viruses were more resistant to chlorine disinfection in groundwater and secondary municipal wastewater effluents than in PBS, suggesting that the constituents in these waters increased the resistance of viruses (Li et al. 2011; Thurston-Enriquez et al. 2003). Therefore, it was suggested that the dissolved materials may affect the susceptibility of prions to ozone in secondary-treated municipal wastewater (i.e., municipal final effluent).

In summary, we have investigated the partitioning of infectious prions in the gravity-separated (after 1.5 h of gravity separation) rendering plant wastewater, and evaluated the effects of pretreatment such as gravity separation and dissolved air floatation on raw rendering plant wastewater and the resulting impacts on ozone inactivation of infectious prions before and after pretreatment. After gravity separation and by using the PMCA assay, we observed that a large proportion of infectious prions partitioned into the scum layer whereas some prions still remained in the gravity-separated rendering plant wastewater. The effectiveness of ozone inactivation of infectious prions was evaluated in the diluted rendering plant wastewater and the municipal final effluent. It was shown that the efficacy of ozone inactivation was substantially increased after the removal of most of the organic matter and solids from the raw rendering plant wastewater. The efficacy of ozone inactivation of infectious prions was lower in the municipal final effluent than that in the ozone demand-free PBS. Until now, the presence of infectious prions in wastewater after secondary treatment has not been analyzed using any reliable and sensitive method. Under the situation where secondary wastewater treatment is not in place or is not effectively partitioning infectious prions into biosolids, ozone as a final barrier would effectively inactivate infectious prions in such wastewaters.

6.5 References

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Chapter 7 Thesis Overview, General Discussion and Conclusions

7.1 Thesis overview

The emergence of vCJD in humans, due to the consumption of BSE infected beef, has raised great public concerns on the treatment of large volumes of BSE infected tissue. Due to the extremely recalcitrant nature of infectious prions, studies on searching effective and efficient approaches for their treatment have never been stopped. Chemical treatment including sodium hydroxide, sodium hypochlorite, Environ LpH at certain concentrations and reaction times, porous autoclaving, and the combination of the previous methodologies have been proved to be able to successfully decontaminate infectious prions, while the harsh reaction condition inhibits their usage on treatment of large volumes of liquid waste, as well as sensitive medical devices. Protease treatment has also shown its ability on degrading prion templates, whereas the complete elimination of infectivity was not guaranteed. In addition, these approaches are not amenable to large amount of prion contaminated wastewater. Advanced oxidation has shown its effectiveness in prion inactivation, with different incubation time depending on the detailed methods and less harsh reaction conditions than chemical treatment. Ozone as one of the advanced oxidation technology has been widely used for the disinfection of waterborne pathogens such as bacteria, viruses, and protozoan. As an advanced technology, ozone has the higher oxidizing potential than other chemicals used in water disinfection, i.e. chlorine and chlorine dioxide. This research focused on assessing the feasibility and efficacy of ozone application on prion inactivation at various conditions (including pH, applied ozone doses, contact time, temperature and wastewater matrices) in water and wastewater.

In order to achieve the objectives, a phenomenological and methodological study was first carried out to address the potential of ozone as a potent inactivator of prion replication (i.e., template-directed misfolding), a process believed to underlie the pathology of all TSEs. The effects of the applied

ozone doses (13 mg/L and 23 mg/L), pHs (4.4, 6.0, and 8.0), temperatures (4 and 20°C) and contact time (5 s and 5 min) on the effectiveness of ozone inactivation of prion template direct misfolding abilities were preliminary evaluated. Ozone was found to be more effective at higher applied ozone dose, lower pH, higher temperature and higher contact time. This provided the foundation for the following ozone inactivation kinetic modeling studies. Determining ozone inactivation kinetics requires the mathematical modeling of large, replicate datasets. Animal bioassays represent the "gold standard" for prion infectivity, whereas the assays are extremely expensive and are not amenable to the large-scale experimentation needed for derivation of *CT* values or for kinetic modeling studies. Although the PMCA assay has been demonstrated qualitatively for its good correlation with animal infectivity in several studies, and the correlation has been semi-quantified in one prion disinfection study, however, the correlation has never been quantitatively verified in any inactivation study. The second part of this research was to quantitatively verify this correlation and generate the ozone inactivation kinetics by PMCA assay. With the development of an efficient and ultrasensitive prion detection approach of PMCA assay, very small amount of infectious prions could be detected in biological samples as well as in environmental samples such as water and soil. Therefore, the inactivation of prion in water and wastewater at lower concentrations than in biological samples could be evaluated. Consequently, the kinetics of prion inactivation in wastewater matrices was further developed. Rendering plant wastewater, as one of the potential sources containing infectious prions, was chosen as one wastewater to be tested. The municipal final wastewater effluent was also chosen as a type of wastewater having less organics than rendering plant wastewater. It was found that ozone demand was significantly high in the raw rendering plant wastewater, thus pretreatments of gravity separation and dissolved air flotation (DAF) were applied, to evaluate the effects of organic removal on prion inactivation by ozone. In summary, ozone is extremely effective on prion inactivation in ozone-demand free water, and could serve as a final barrier for prion inactivation in wastewater after primary or secondary treatment.

7.2 General Discussion

The main purposes of this research were to assess the effectiveness of ozone on the inactivation of infectious prions in different water matrices. This was done by investigating the effect of applied ozone doses, pHs, contact time, temperatures in PBS, and the organic matter in different wastewater matrices. The log_{10} inactivation levels of infectious prions were assessed by the protein misfolding cyclic amplification (PMCA) assay. The results shown that in PBS, ozone inactivation of infectious prion depended on the applied ozone dose, contact time, temperature and pH. As indicated in Chapter 4, the inactivation was $\geq$4-log_{10} with applied ozone dose of 13 mg/L, contact time of 5 s and at pH 4.4 and 20°C.

The inactivation levels were 2.4-log_{10} and 1.9-log_{10} at pH 6.0 and 8.0, respectively, which suggested that infectious prions are more susceptible to ozone at lower pHs. It has been reported that pH itself has shown little effect on the scrapie infectivity over the range of pH 2 to 12 (Mould et al. 1965), hence it was indicated that the molecular ozone reaction is more effective on attacking the prion protein or the biomolecular targets affecting the stability of the misfolded conformer. Ozone reaction in water has two pathways: a) direct molecular ozone reaction which predominates under acidic pH, and b) indirect reaction in which ozone decomposes into hydroxyl radicals to react and predominates under basic conditions (Chu and Ma 2000). The high inactivation rates at more acidic pHs were also observed for *E. coli.* (Zuma et al. 2009) and norovirus (Lim et al. 2010). However, the effect of pH on ozone disinfection has shown to be insignificant on *Cryptosporidium parvum* oocysts (Gyurek et al. 1999; Li et al. 2001; Ran et al. 2010; Rennecker et al. 2001) and poliovirus (Katzenelson et al. 1979), whereas the inactivation by ozone favored high pH for *Giardia muris* cysts (Wickramanayake et al. 1984) and *Bacillus subtilis* spores (Cho et al. 2003; Dow et al. 2006; Larson and Mariñas 2003). This is an indication that the pH effect for ozone inactivation appears to be microorganism specific, and is likely to be dependent on the structural components of the microorganisms (Wickramanayake et al. 1984). Another important point that needs to be mentioned is the pH effect

related to interpretation of ozone dose. Farooq et al. (1977) reported that ozone inactivation of *Mycobacterium fortuitum* was higher at pH 5.7 than at pH 10.1 with similar applied ozone doses; while the difference was insignificant when residual ozone was maintained at similar levels. Some authors also suggested that the high inactivation rate was due to the lower ozone decay rate at lower pH (Langlais et al. 1991). Therefore, it is critical to elaborate the experimental conditions and the criteria for pH effect descriptions. In the present study, the applied ozone doses at various pHs were at similar levels, while the residual ozone were at different levels according to different ozone decomposition rates. Due to the high ozone decomposition rate at high pH, this is an indication that the oxidation of infectious prions by molecular ozone was more effective than indirect oxidation associated with the ozone decomposition to hydroxyl radicals. Prion inactivation using hydroxyl radicals have been reported in several studies (Johnson et al. 2009; Lehmann et al. 2009; Paspaltsis et al. 2009; Paspaltsis et al. 2006; Solassol et al. 2006; Suyama et al. 2007). Incubation time of 30 min to several weeks was required to obtain >5-$\log_{10}$ inactivation. As a comparison, the contact time of ozone inactivation was only 5 s to achieve a >4-$\log_{10}$ inactivation.

It is interesting to note that prion has 8 copper binding sites (Brown et al. 1997). Copper concentration as low as 1 μM in the brain may cause the conversion of PrP^{C} into PrP^{Sc}, thus inducing prion diseases (Requena et al. 2001). On the other hand, a higher copper concentration (i.e., 500 μM) in the presence of hydrogen peroxide can reversely inactivate PrP^{Sc} (Lehmann et al. 2009; Solassol et al. 2006). Both PrP^{C} and PrP^{Sc} undergo specific cleavage on exposure to copper (Cu^{2+}) and hydrogen peroxide (H_2O_2) (McMahon et al. 2001; Solassol et al. 2006). This process is described as Fenton reaction, from which Cu^{2+} was oxidized by H_2O_2 to Cu^{+}, and H_2O_2 is further oxidized by Cu^{+} to generate hydroxyl radicals (·OH). More research is needed to explore the effect of concentration of copper and hydrogen peroxide on prion inactivation. In our study, we have demonstrated that molecular ozone is extremely effective in prion inactivation macroscopically; however, reactions at the molecular level near copper binding sites (i.e. molecular ozone reaction or Fenton reaction) was not differentiated. To further investigate

reactions in the core of prion aggregates near copper binding sites, Fenton reaction may be ceased with EDTA, by which molecular ozone reaction can be differentiated from hydroxyl radical reactions. In this way, whether the reactions near these sites are hydroxyl radical reaction, molecular ozone reaction or combination of both can be determined.

Temperature has also shown to affect prion inactivation by ozone. Temperature affects ozone solubility in water, ozone decomposition rate and disinfection rate. Under similar conditions, ozone decomposes faster at higher temperature (Dow et al. 2006). Moreover, the solubility of ozone is lower at higher temperatures (Bablon et al. 1991). In the present study, the prion inactivation was found to be even higher at higher temperatures, which was in agreement with the ozone inactivation of *Giardia lamblia* (Wickramanayake et al. 1984), *Naegleria gruberi* (Wickramanayake et al. 1984), *Cryptosporidium* (Li et al. 2001; Ran et al. 2010), and *Bacillus subtilis* spores (Larson and Mariñas 2003).

By demonstrating that ozone is effective on prion inactivation, the inactivation kinetics at various conditions was further developed and described in detail in Chapter 5. Experimental data were fitted on both Chick-Watson and efficiency factor Hom models that are usually used for modeling under disinfectant demand conditions (Gyurek and Finch 1998). Compared to the CW model, the EFH model described more accurately the initial steep inactivation curve and the tailing-off behavior for PrP^{Sc}. It has been reported that the CW model adequately described chlorine-induced inactivation, but not ozone-induced inactivation of microbes (Finch et al. 1988; Katzenelson et al. 1974), whereas the Hom model accounts for the deviation from the CW model in practice (Hom 1972). Based on EFH models, the required *CT* values for $\log_{10}$ inactivation were calculated.

One of the most important features of this research was the derivation of the *CT* values for prions which has never been reported. Although many studies have reported prion inactivation by chemical reagents (Brown et al. 1982; Kimberlin et al. 1983; Pritzkow et al. 2011; Taylor et al. 1994), none of these studies investigated the reaction kinetics. In these studies, infectious brain

homogenates were mixed with chemical reagents at predetermined concentrations, followed by incubation. The effectiveness of the oxidants was evaluated at the end of reaction, with or without neutralizing the oxidants. This is a biological rationale of analyzing the effectiveness of prion inactivation, without considering the consumption of oxidants as a function of time. In this research, the residual ozone was measured at each reaction time point, and of the ozone decomposition was taken into account for the generation of inactivation curves, which is imperative in water disinfection engineering studies. The *CT* values were thereafter derived based on the kinetic modeling curves, and provided a normalized approach for evaluating the susceptibility of a particular microbial contaminant in water matrices. The USEPA routinely uses the *CT* concept for characterizing disinfection requirements for water-borne pathogens under a given set of conditions (U.S. Environmental Protection Agency 1991; U.S. Environmental Protection Agency 2006). Consequently, the *CT* value is commonly used as an engineering target for the inactivation of pathogens in water matrices, as a regulatory standard in water treatment, and modeling the inactivation kinetics for physiochemical disinfectants (Lim et al. 2010; Thurston-Enriquez et al. 2005). Based on the *CT* values, the present research demonstrated that prions are highly susceptible to inactivation by ozone. The *CT* values for prion inactivation were considerably lower than those described for certain waterborne pathogens (i.e. *Cryptosporidium*) and spore-forming bacteria (i.e. *B. subtilis*). Although the applied ozone doses in the present research were slightly higher than those applied in other disinfection studies, due to the high ozone demand by non-target macromolecules in the brain suspensions, compared to other disinfectants, ozone was found to be very effective on prion inactivation in water.

PMCA as a power *in vitro* tool for the detection and quantification of PrP^{Sc} has been reported. However, its correlation with animal infectivity in prion decontamination process has only been semi-quantified in one study (Pritzkow et al. 2011). More than 300 days were spent on the bioassay in this research and it was found that the infectivity reduction was similar or ~1 $\log_{10}$ higher than PMCA assay, which agreed with the results reported by Pritzkow et al. (2011) that the

two assays were within 1-$\log_{10}$ range of difference. In this aspect, modeling kinetics based on PMCA assay may conservatively underestimate the reduction of infectivity. In water and wastewater treatment engineering principles, conservative approaches or estimation is usually applied in order to ensure an extra measure of safety, especially under the situation that real conditions are difficult to monitor or simulate. Compare with bioassays, PMCA significantly reduces the time for analysis, labour and ethical animal usage, thus this technique was an ideal alternate of bioassays in prion decontamination studies.

Infectious prions were reported to attach to particles and solids in the aquatic environments (Gale et al. 1998). It was shown that after gravity separation for 1.5 h, large amounts of infectious prion still remained in the liquid phase, due to the incomplete removal of suspended particles. In addition, the infectivity would remain in wastewater for more than 265 days (Maluquer de Motes et al. 2012). The results of the present research described in Chapter 6 shown that the effectiveness of ozone inactivation of infectious prions was highly affected by the presence of ozone consuming organics. A much higher ozone dose of 44 mg/L was instantaneously depleted in the rendering wastewater (initial COD = 96975 mg/L). Due to the higher organic load, pretreatments by using gravity separation and DAF treatment were applied on the rendering wastewater. With gravity separation, the COD of the rendering wastewater was reduced to 13400 mg/L, and further reduced to 7900 mg/L by DAF treatment. With the same level of applied ozone dose, a >4-$\log_{10}$ inactivation was obtained in DAF treated rendering wastewater at contact time of 5 min. Other studies also reported that the presence of high organic matter affected ozone disinfection in wastewaters or sludge (Finch and Smith 1989; Greene and Stenstrom 1994; Millamena 1992; Watkins et al. 1997). A 3-$\log_{10}$ of *E.coli* was inactivated with 2 g/L of the applied ozone and 1-$\log_{10}$ of total coliforms were removed with 1 g/L of the applied ozone in a swine manure sludge (COD = 46,900-76,500 mg/L) (Watkins et al. 1997), while a >6-$\log_{10}$ inactivation of *E.coli* was achieved with a much lower applied ozone dose of 22 mg/L in a secondary effluent (COD = 18-29 mg/L) (Finch and Smith 1989).

The *CT* values for prion inactivation in the municipal final effluent (3:10 in dilution) was compared to the *CT* values in PBS. It was found that *CT* values in the former wastewater were higher than the latter, indicating that the presence of dissolved organics in the final effluent affects the susceptibility of prions to ozone, under the condition of similar ozone decomposition rate, similar applied ozone dose and concentration after instantaneous ozone demand. In another study it was shown that the prions degraded more slowly in 0.22 μm filtered wastewater than in PBS, deionized water, and tap water (Miles et al. 2011). In addition, viruses have also shown to be more resistant to chlorine disinfection in groundwater than in PBS (Thurston-Enriquez et al. 2003). Therefore, it was suggested that the dissolved materials may affect the susceptibility of prions to ozone in final treated wastewater.

In this research, hamster scrapie 263K was used as the model for the inactivation study. The reasons for its use are as follows: a) hamster scrapie is the most readily available prion and has been commonly used in prion research (Ernst and Race 1993; Kimberlin et al. 1983; Lehmann et al. 2009). Inactivation studies have been widely carried out on hamster scrapie and 263K is the most resistant strain to heat and chemicals among other scrapie strains (Taylor 1998); and b) the PMCA of hamster scrapie 263K has been well established and has been shown to correlate well with animal infectivity (Chen et al. 2010; Pritzkow et al. 2011), while detailed PMCA procedures are still under research for BSE and just published lately for CWD (Johnson et al. 2012); validation of PMCA to animal infectivity of BSE/CWD inactivation has not been reported to date. For the generation of kinetic modeling, animal bioassays could not be utilized because more than 500 days are required to complete this bioassay. However, rodent scrapie might be less resistant to some chemicals (Giles et al. 2008) and this issue should be explored in ozone inactivation.

7.3 Conclusions

- The efficacy of ozone inactivation of prions in PBS was dependent on the applied ozone dose, contact time, pH and temperature.

- Ozone was effective for infectious prions inactivation in PBS, especially at acidic pH (4.4) and high temperature (20°C).
- By comparison of *CT* values, prion was more susceptible to ozone than encysted protozoan parasites such as *Crypstosporidium spp.*
- PMCA is an extremely sensitive tool for the detection and quantification of PrP^{Sc} and for measuring ozone inactivation of the template-directed misfolding properties of PrP^{Sc}.
- PMCA assay was found to be slightly conservative on evaluating the inactivation of infectious prions by ozone, whereas this is applicable in water treatment engineering application to ensure extra measure of safety.
- The efficiency factor Hom model was more appropriately fit to the inactivation data than the Chick-Watson model.
- By gravity separation for 1.5 h, infectious prions could not be predominately partitioned into the floc phase; a large of amount of them still remained in the liquid phase.
- The organic load in the rendering wastewater competitively reacted with ozone, significantly reducing the efficacy of ozone on prion inactivation.
- By pretreating the wastewater by gravity separation and DAF treatment, a significant amount of organic could be removed from the wastewater, and the efficacy of ozone inactivation of prions was increased.
- After DAF treatment, a >4-$\log_{10}$ inactivation of prions was obtained with applied ozone dose of 44 mg/L and contact time of 5 min, at 20°C, in 1:10 dilution of rendering wastewater.
- A >4-$\log_{10}$ inactivation was obtained with applied ozone dose 23 mg/L in the municipal final effluent. However, a lower ozone dose (13 mg/L) shown a lower inactivation efficacy in this effluent than that observed in PBS.

7.4 Recommendations

Based on the results and discussion from the current research, the following recommendations are made for future research:

- The molecular level reactions near copper binding sites in prion aggregates during ozone inactivation should be further explored.
- Prion inactivation by ozone in wastewater could be carried out in a semi-batch reactor in which ozone concentration could be maintained at a much higher level than that in the batch reactors.
- The partition of infectious prions after DAF treatment in rendering wastewater should be addressed.
- The fate of infectious prions during the process of wastewater treatment should be investigated, by using the fast and sensitive PMCA assay for prion detection, in order to better understand the distribution of prions in different treatment stages.
- Different prion strains which exist in the environment (i.e. BSE, CWD, scrapie) should be used for future research instead of rodent-passaged srapie.

7.5 References

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Appendix A Other experimental procedures

A1. Preparation of ozone demand-free buffer, tips, stir bars, tubes and reaction vials

Ozone demand-free (ODF) PBS and water were made by purging ozone gas through 1 L glass container containing PBS or deionized (DI) water for 20 min at room temperature, with the power of ozone generator set at 20%. The ODF PBS or water was kept in the containers for 1 week with the lid loose, to remove residual ozone. Pipett tips, stir bars, tubes and reaction vials were submerged in deionized in a big Teflon container. Ozone gas was purged into the container for 20 min at room temperature. The pipette tips, stir bars, tubes and reaction vials were kept in the ozonated water overnight, dried at room temperature until use.

A2. Determination of ozone concentration

Ozone concentration was measured by a spectrophotometer directly and by the indigo method.

A2.1 Direct UV method

The direct measurement is based on ozone adsorption in the UV wavelength of 260 nm. Ozone concentration was measured immediately using quartz cell with 10 mm light path. The ozone convention was estimated by Eq. A1:

$$[O_3] = \frac{AMU}{k_{260}} \times l \times ABS \qquad \text{(A1)}$$

Where AMU is the atomic mass unit for ozone (48000 mg/mol), k_{260} is the molar absorptivity for ozone (3300 M^{-1} cm^{-1}), l is the length of the light passage through the quartz cell (10 mm), ABS is the absorbance at 260 nm.

A2.2 Indigo method

The indigo method involves the reaction of ozone with potassium indigo trisulfonate in acid solution. An indigo stock solution was prepared by adding 770 mg potassium indigo trisulfonate into 1L ODF water, followed by adding 1 mL phosphoric acid. This stock solution is stable when stored in the dark for period up to four months. It should be discarded when the absorbance of a hundredfold dilution falls below 0.16 absorbance units/cm. The Indigo reagent II was prepared

by adding 100 mL indigo stock solution, 10 g NaH_2PO_4, 7 mL concentrated phosphoric acid into 1 L ODF water.

The spectrophometric procedure for samples containing between 0.05 -0.5 mg/L ozone is to add 10 mL indigo reagent II in 100 mL volumetric flask. For blank to the 10 mL indigo reagent, add 90 mL ODF water. For samples to be measured to the 10 mL indigo reagent add 1-90 mL of sample in accordance to the ozone residue concentration and then use ODF water to the volume of 100 mL. Read the absorbance of the blank at 600 nm. The concentration of ozone was calculated by Eq. A2.

$$[O_3] = \frac{100 \times \Delta A}{f \times b \times V} \qquad \text{(A2)}$$

where 100 is the flask volume (mL), ΔA is the difference of absorbance between the blank and sample, V is the sample volume (mL), f = 0.42.

A3. Determination of wastewater characteristics

A3.1 COD

Measurement of COD was using the Novaspec II spectrophotometer. COD digestion solution was prepared by mixing 35 mM potassium dichromate and 3.0 M sulfuric acid. Sulfuric acid regent was 10 g/L dissolved in concentrated sulfuric acid. The entire digestion (90 min at 140°C on the micro digestion block), and subsequent COD determination (absorbance at 600 nm on the Novaspec II) took place in a sealed 10 mL test tube containing 3.5 mL sulfuric acid reagent, 2.0 mL of micro-COD digestion solution and a 2.0 mL aliquot of sample, standard or blank to be analyzed. Ensure that the test tubes are tightly closed before digestion.

A3.2 BOD_5

The following reagents wee prepared before BOD test. A. phosphate buffer solution: dissolve 8.5 g KH_2PO_4, 21.75 g K_2PO_4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g NH_4Cl in about 500 mL distilled water and dilute to 1 L. The pH should be 7.2 without further adjustment. B. magnesium sulfate solution: Dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ in distilled water and dilute to 1 L. C. Calcium chloride solution: dissolve 27.5 g $CaCl_2$ in distilled water and dilute to 1 L. D. Ferric chlorite solution: dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ in distilled water and dilute to 1 L. E.

Glucose-glutamic acid solution: dry reagent-grade glucose and reagent grade glutamic acid at 103°C for 1 h. Add 150 mg glucose and 150 mg glutamic acid to distilled water and dilute to 1 L. Prepare fresh immediately before use. The dissolved oxygen meter was used for measurement. Dilution water check, seed check, standard check were prepared along with the samples. The DO uptake attribute to the seed added to each bottle generally should be between 0.6 and 1.0 mg/L, the amount of the seed should be adjusted from this range to the required to provide glucose-glutamic acid check results of 198±30.5 mg/L after 5-day. BOD_5 was calculated as Eq. A3

$$BOD_5, mg/L = \frac{(D_1 - D_2) - (S)V_s}{P} \qquad \text{Eq. A3}$$

where D_1 = DO of diluted sample immediately after preparation, mg/L, D_2 = DO of diluted sample after 5 day incubation at 20°C, S=oxygen uptake of seed, V_s = volume of seed in the respective bottle, mL, and P=decimal volumetric fraction of sample used; 1/P = dilution factor.

A3.3 TSS and TDS

To measure TSS and TDS, 25 mL of municipal final effluent or diluted rendering plant wastewater was filtered through 2.0 μm filter in the crucibles. The effluent was transferred into evaporation dishes. The procedure was completed by placing the dish in a steam table for 90 min to reduce water volume, and Drying was completed in the oven (180°C) overnight. TSS = (W_f - W_i)/ sample size ×1000mL, where W_f is the final weight of crucibles, and W_i is the initial weight of the crucibles. TDS = (W_f' - W_i')/sample size ×1000mL, where W_f' is the final weight of evaporation dishes, and W_i is the initial weight of evaporation dishes.

Appendix B Supporting Figures

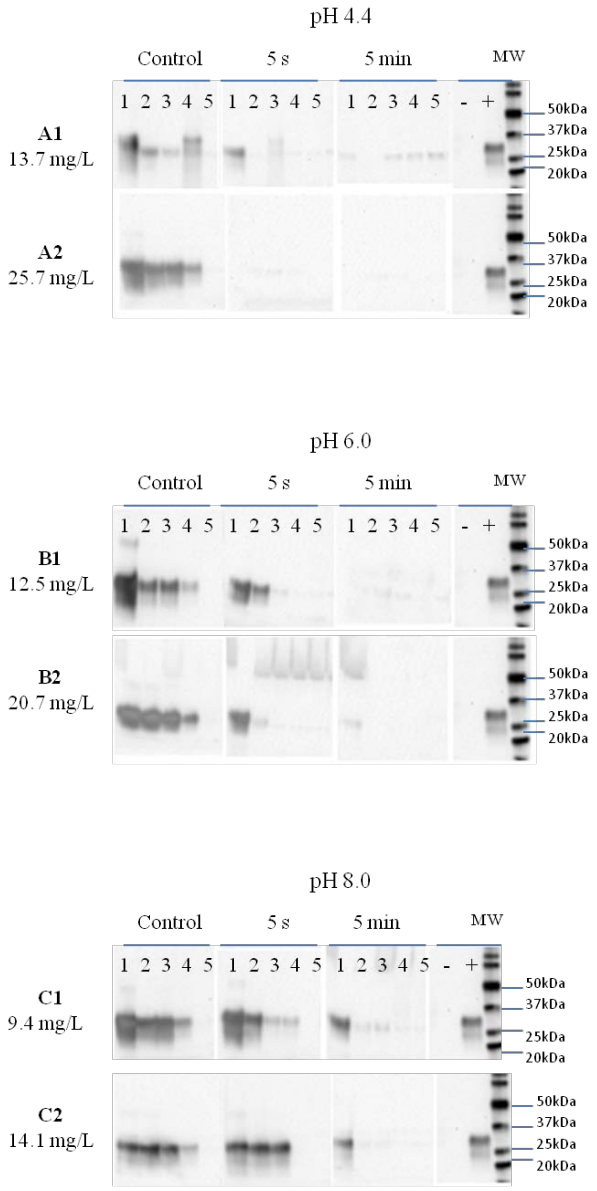

Figure B.1 Western blots from 0.01% IBH samples treated with ozone at 4°C and amplified by PMCA.

The images in each panel, from left to right, represent non-ozone treated control samples, samples treated with ozone for 5 s, and samples treated with ozone for 5 min. The applied ozone doses are provided to the left of each panel. Panel A = pH 4.4; Panel B = pH 6.0; and Panel C = pH 8.0. The numbers at the top of each image represent dilutions of 0.01% IBH in $\log_{10}$. Lane labeled as '-' represents 10% NBH treated with PK. Lane labeled as '+' represents 1% IBH treated with PK. Molecular weight markers (lane labeled MW) at 50, 37, 25 and 20 are indicated.

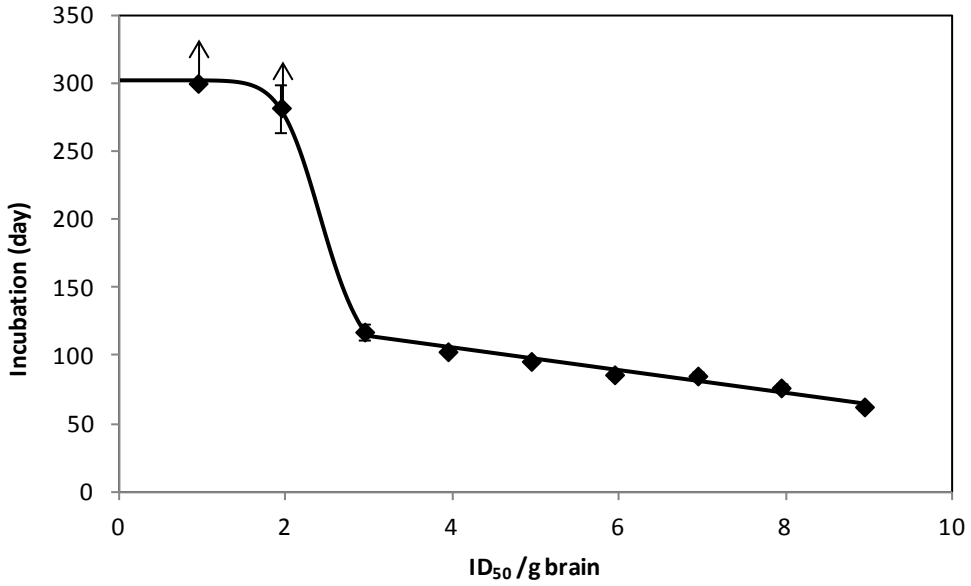

Figure B.2 Standard curve of the incubation time on serial dilution of scrapie 263K infected hamster brains.

Values represent the means of each dilution with error bars indicating the standard deviation or range. Arrows indicate that real incubation times were larger than values shown.

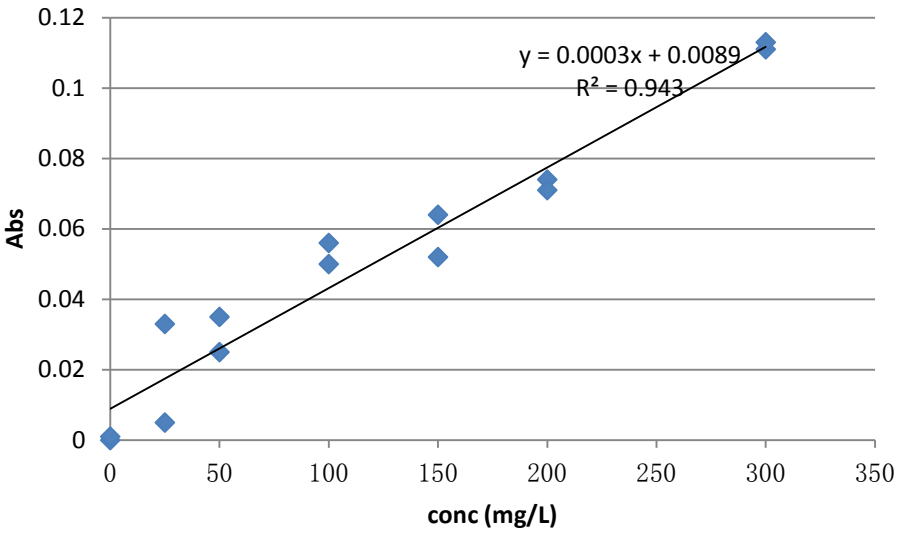

Figure B.3 COD standard curve of wastewater.

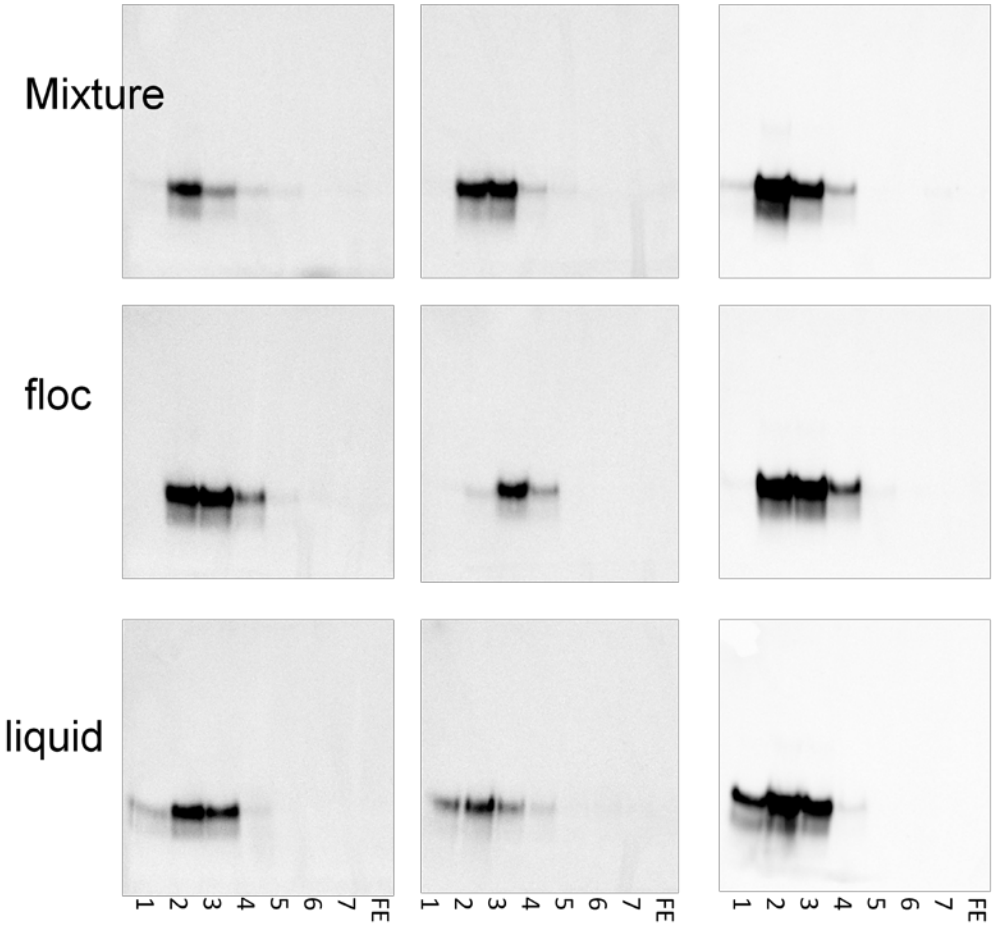

Figure B.4 Partition of infectious prions after gravity separation for 1.5 h in replicate.

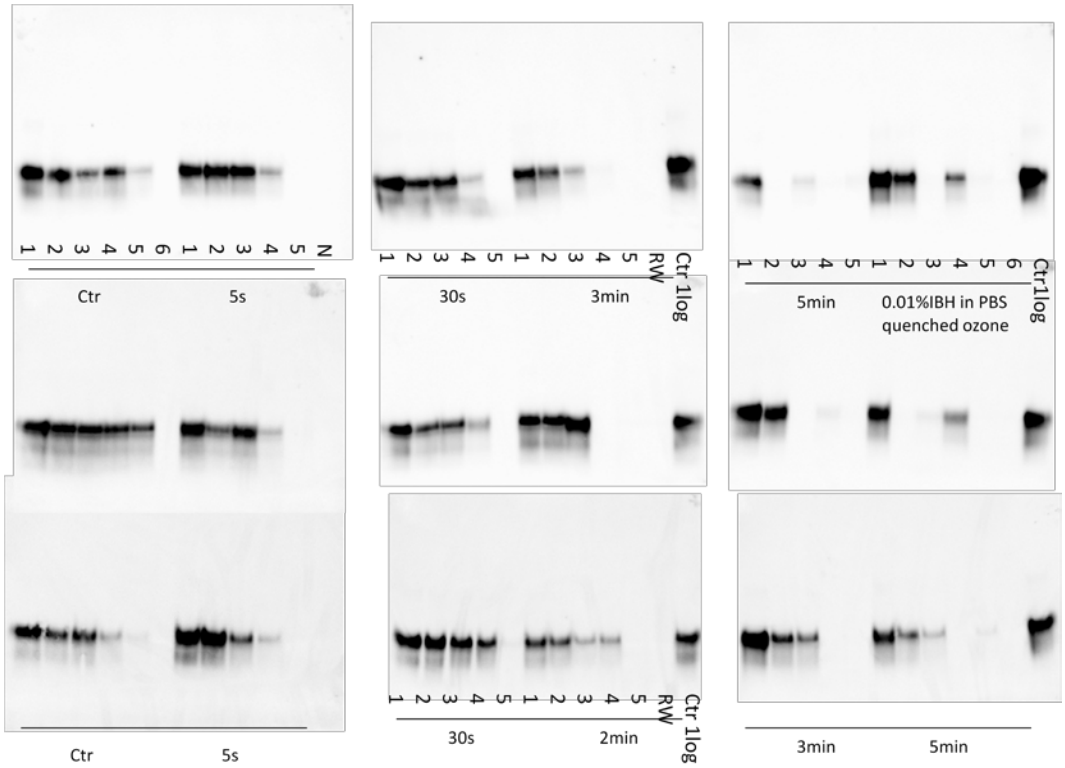

Figure B.5 Replicate western blot images of prion inactivation in raw rendering plant wastewater after PMCA.

Applied ozone dose = 44 mg/L, wastewater dilution = 1:10. The left panel in the top row represents a PMCA control of IBH serial dilution in PBS. The images in each row, from left to right, represent positive control samples, samples treated with ozone for 3 s, 15 s, 2 min, 3 min and 5 min. The pHs of the reactions are shown to the left of each row. The numbers on top of each blot image (1 to 6) represent $\log_{10}$ dilutions of samples. Lanes labeled 'N' represent 10% NBH treated PK. The lane labeled 'RW' represents a rendering wastewater control sample. Lanes labeled with 'ctr1log' represent -1$\log_{10}$ of positive control samples (same samples as -1$\log_{10}$ of control in the first lane in left column).

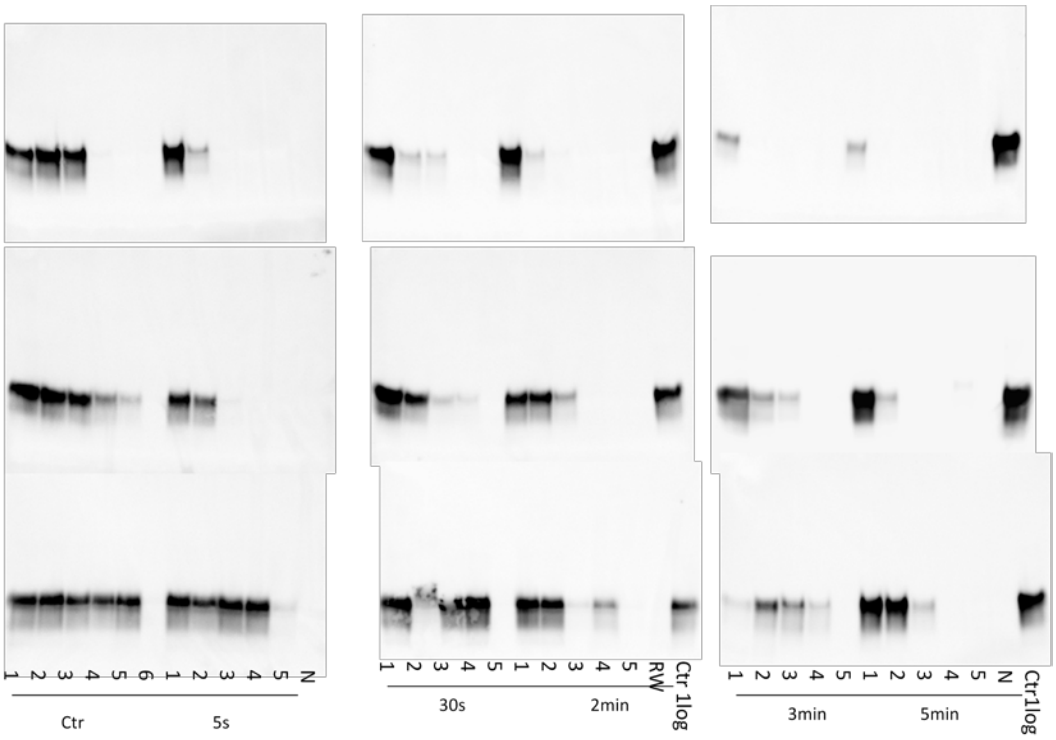

Figure B.6 Replicate western blot images of prion inactivation in gravity separated wastewater after PMCA.

Applied ozone dose = 44 mg/L, wastewater dilution = 1:10. The images in each row, from left to right, represent positive control samples, samples treated with ozone for 3 s, 15 s, 2 min, 3 min and 5 min. The pHs of the reactions are shown to the left of each row. The numbers on top of each blot image (1 to 6) represent $\log_{10}$ dilutions of samples. Lanes labeled 'N' represent 10% NBH treated PK. The lane labeled 'RW' represents a rendering wastewater control sample. Lanes labeled with 'ctr1log' represent -1$\log_{10}$ of positive control samples (same samples as -1$\log_{10}$ of control in the first lane in left column).

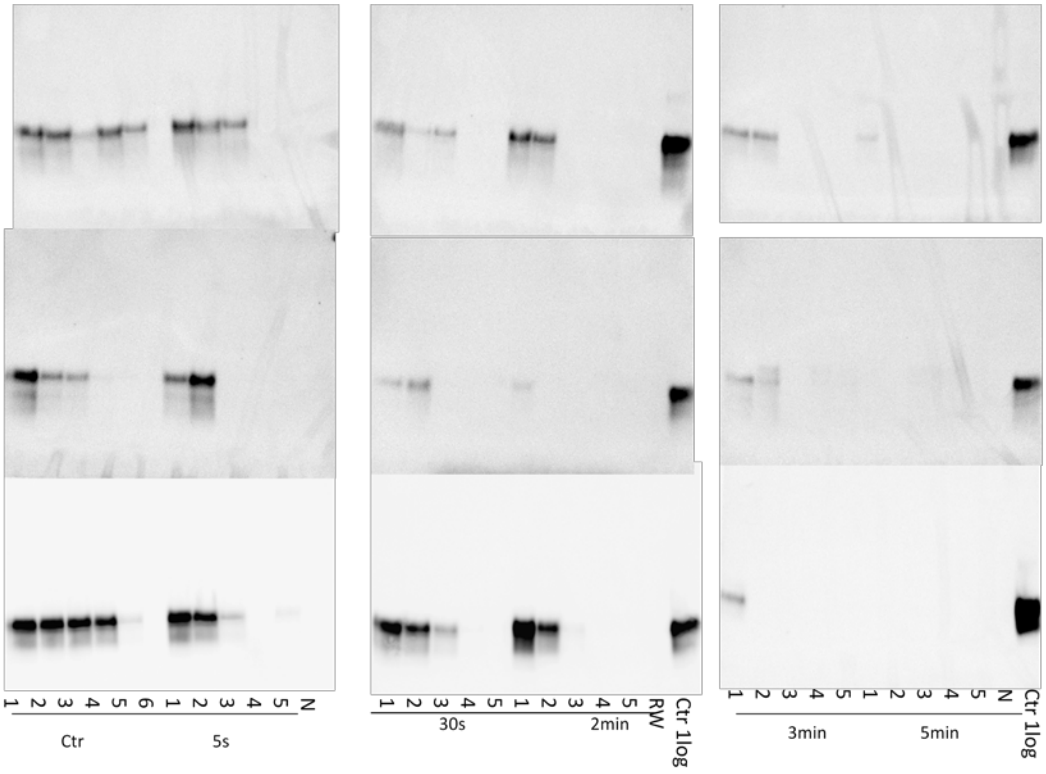

Figure B.7 Replicate western blot images of prion inactivation in DAF treated rendering wastewater after PMCA.

Applied ozone dose = 44 mg/L, wastewater dilution = 1:10. The images in each row, from left to right, represent positive control samples, samples treated with ozone for 3 s, 15 s, 2 min, 3 min and 5 min. The pHs of the reactions are shown to the left of each row. The numbers on top of each blot image (1 to 6) represent $\log_{10}$ dilutions of samples. Lanes labeled 'N' represent 10% NBH treated PK. The lane labeled 'RW' represents a rendering wastewater control sample. Lanes labeled with 'ctr1log' represent -1$\log_{10}$ of positive control samples (same samples as -1$\log_{10}$ of control in the first lane in left column).

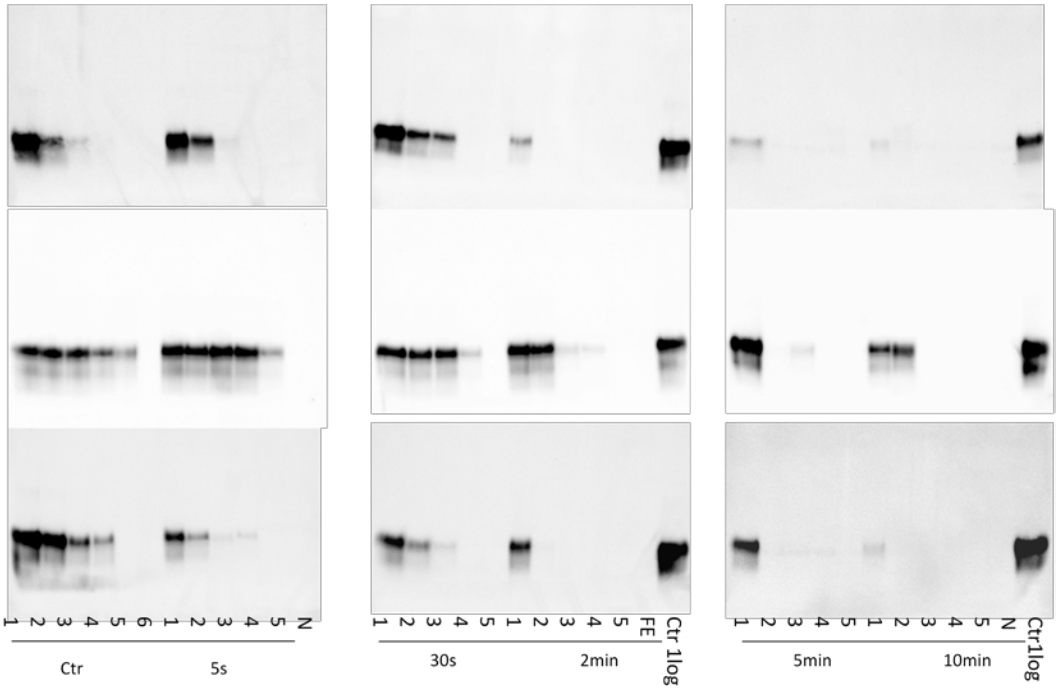

Figure B.8 Replicate western blot images of ozone inactivation of prions in municipal final effluent (3:10 in dilution) with applied ozone dose of 13 mg/L.

The images in each row, from left to right represent positive control (0 s), samples ozonated for 5 s, 30 s, 2 min, 5 min and 10 min. The numbers at the top of each panel represent $\log_{10}$ dilution of 0.01% IBH. Lanes labeled "N" represent 10% NBH, the lane labeled "FE" represents negative control final effluent in the same dilution as ozonated samples, lanes labeled "Ctr 1log" represents 1-$\log_{10}$ dilution of the positive control sample. Samples were all treated with PK.

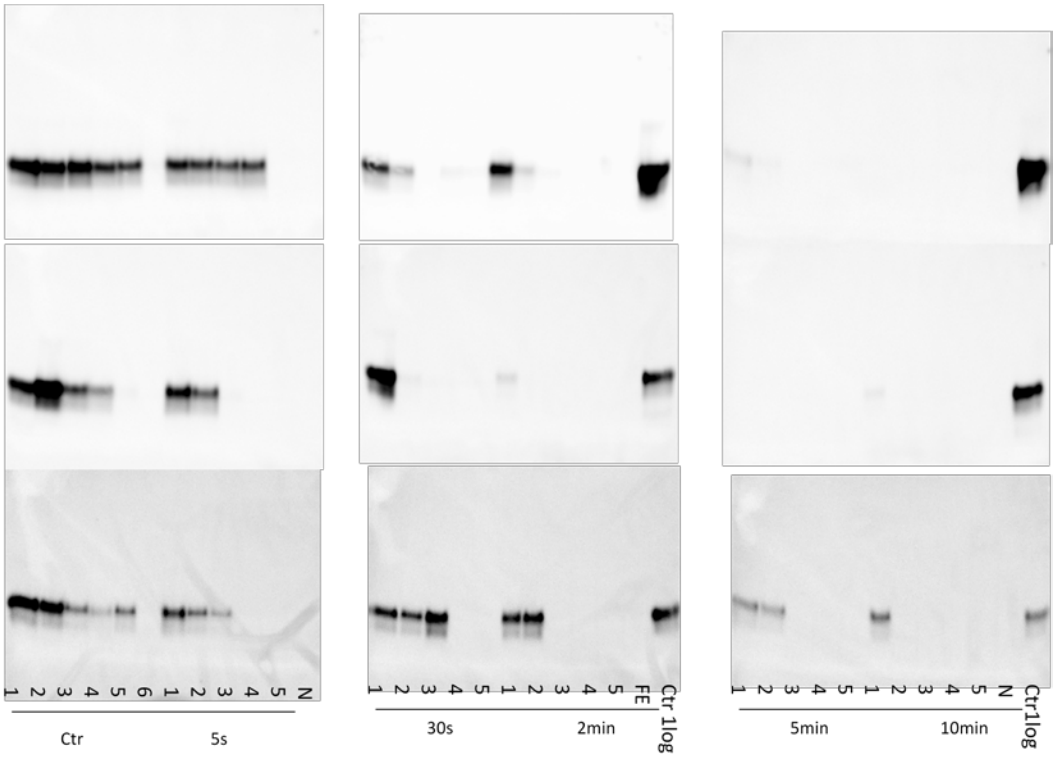

Figure B.9 Replicate western blot images of ozone inactivation of prions in municipal final effluent (3:10 in dilution) with applied ozone dose of 23 mg/L.

The images in each row, from left to right represent positive control (0 s), samples ozonated for 5 s, 30 s, 2 min, 5 min and 10 min. The numbers at the top of each panel represent $\log_{10}$ dilution of 0.01% IBH. Lanes labeled "N" represent 10% NBH, the lane labeled "FE" represents negative control final effluent in the same dilution as ozonated samples, lanes labeled "Ctr 1log" represents 1-$\log_{10}$ dilution of the positive control sample. Samples were all treated with PK.