JAWAD NAZIR

PERSISTENCE OF H4N6, H5N1, AND H6N8 AVIAN INFLUENZA VIRUSES, H1N1 HUMAN INFLUENZA VIRUS, AND TWO MODEL VIRUSES (NDV AND ECBO) IN VARIOUS TYPES OF WATER, LAKE SEDIMENT, DUCK FECES, AND MEAT

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Persistence of H4N6, H5N1, and H6N8 avian influenza viruses, H1N1 human influenza virus, and two model viruses (NDV and ECBO) in various types of water, lake sediment, duck feces, and meat

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eingereicht von

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Veterinarian from Lahore, Pakistan

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To my parents for their endless efforts for their children and my life partner for her absolute faith in me and finally to my daughter Zoya, wishing her a bright future

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- Haumacher, R., **Nazir, J.,** Ike, A., Böhm, R., Marschang, R.E. Untersuchungen zur Tenazität von aviären Influenzaviren in Wasser, Sediment und Vogelkot, dargestellt am Beispiel Bodensee. Gemeinsame wissenschaftliche Schlusstagung der Forschungsprojekte „Constanze“ und „Wildvögel und Vogelgrippe“. June 8-9, 2009. Arbon, Switzerland.

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- **Nazir, J.,** Haumacher, R., Ike, A., Stumpf, P., Böhm, R., Marschang, R.E. Tenacity of avian influenza viruses in water. Current developments in food and environmental virology. October 9-11, 2008. Pisa, Italy.

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Abbreviations

°C	Degree centigrade
°dH	Degree of hardness
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
µS/cm	Micro Siemens per centimeter
AAF	Amniotic allantoic fluid
AG	Aktiengesellschaft
AIV	Avian influenza viruses
ATCC	American type culture collection
BD	Becton Dickenson
BE	Beef extract
bp	Base pair
BSL	Bio-safety level
Ca	Calcium
CDC	Centers for Disease Control and Prevention
CEK	Chicken embryo kidney cells
CFU	Colony forming units
cm	Centimeter
CO_2	Carbon dioxide
CPE	Cytopathic effects
Ct	Cycle threshold
CVUA	Chemisches- und Veterinäruntersuchungsamt
DAAD	Deutscher Akademischer Austausch Dienst
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco`s modified Eagle Medium
Dr.	Doctor
DVG	Deutsche Veterinärmedizinische Gesellschaft
DW	Distilled water
ECBO	Enteric cytopathogenic bovine orphan virus

ECE	Embryonating chicken eggs
EDTA	Ethylenediamine-tetraaceticacid
EID_{50}	50 % Embryo infectious dose
et al	et alteri or et alii
FCS	Fetal calf serum
fg	Femtogram
FLI	Friedrich-Loeffler Institute
For	Forward
g	Gram
GC	Germ carrier
GI	Gastrointestinal
GmbH	Gesellschaft mit beschränkter Haftung
H	Hemagglutinin
HA	Hemagglutination
HCl	Hydrochloric acid
HEC	Higher Education Commission
HPAI	High pathogenicity avian influenza
IVPI	Intravenous pathogenicity index
KGaA	Kommanditgesellschaft auf Aktien
l	Liter
LB	Lysis buffer
LGA	Landesgesundheitsamt
LPAI	Low pathogenicity avian influenza
LRM	Linear regression model
M	Molar
M1	Matrix protein
M2	Membrane ion channel
MDBK	Madin Darby bovine kidney cells
MDCK	Madin Darby canine kidney cells
Mg	Magnesium
mg	Milligram
M-gene	Matrix-gene
min	Minutes

ml	Millilitre
mm	Millimetre
mM	Millimolar
MRC-5	Human lung embryonated cells
N	Neuraminidase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDV	Newcastle disease virus
NEA	Non essential amino acids
ng	Nanogram
nm	Nanometre
NP	Nucleoprotein
NS	Normal saline
NVD	No virus detectable
OIE	World Organisation for Animal Health
PA	Polymerase acidic protein
PB	Polymerase basic protein
PBS	Phosphate buffered saline
PCM	Polycarbonate membrane
PCR	Polymerase chain reaction
PCS	Proteolytic cleavage site
pg	Picograms
pH	Potentia Hydrogenii (potential of hydrogen)
p*I*	Isoelectric point
PLB	Phosphate loading buffer
pmol	Picomole
ppm	Parts per million
ppt	Parts per thousand
qRRT-PCR	Quantitative RRT-PCR
R^2	Coefficient of determination
rcf	Relative centrifugal force
Rev	Reverse
RNA	Ribonucleic acid

RRT-PCR	Real time reverse transcriptase-PCR
RT-PCR	Reverse transcriptase-PCR
SiO_2	Silicon dioxide
SPF	Specific pathogen free
SW	Surface water
T-90	Time required for one log reduction in the virus titer
TBC	Total bacterial counts
$TCID_{50}$	50 % Tissue culture infectious dose
TM	Trade mark
TV	Trypsin-versen
U	Units
USA	United States of America
UV	Ultraviolet
UVAS	University of Veterinary and Animal Sciences
VALO	Vakzine Lohmann
WB	Washing buffer
WHO	World Health Organization

1 Introduction

Avian influenza is an infectious viral disease of birds caused by type A influenza viruses which commonly infect poultry and wild birds. Wild aquatic birds serve as the primary reservoir of avian and mammalian influenza viruses and it has been hypothesized that all pandemic human strains have evolved from avian strains (Webster et al., 1992). Avian Influenza viruses (AIV) are transmitted and maintained in wild bird populations by the fecal oral route and water plays an important role in this indirect transmission (Stallknecht and Brown, 2009). Infected birds excrete a large number of virus particles via feces and respiratory secretion into the environment (Swayne, 2008). After shedding, AIV are mostly associated with organic matter, poultry waste, and certain inanimate objects in the environment (Stallknecht and Brown, 2009; Swayne and Halvorson, 2003). Likewise, high amounts of the viruses can be detected in the meat and internal organs of birds infected with high pathogenicity avian influenza (HPAI) viruses (Swayne and Beck, 2005; Thomas and Swayne, 2007) and AIV can survive for several days in poultry carcasses at ambient temperatures (Animal Health Australia, 2008). In order to understand the behavior of these viruses in the environment, it is therefore important to know how long contaminated environments remain infectious.

It is difficult to measure the tenacity of influenza viruses in the environment as a number of physicochemical factors affect their persistence. Water-borne transmission of AIV is well established (Hinshaw et al., 1979; Markwell and Shortridge, 1982) but information on survival of these viruses in aquatic habitats is not sufficient to completely understand the epidemiology of these viruses. Lake sediments and environmental ice have also been proposed as long term reservoirs of influenza viruses (Lang et al., 2008, Zhang et al., 2006) but no information is available on the persistence of AIV in these substrates. Moreover, there is scarcity of data on the survival of AIV in avian feces and duck meat. The available information on the tenacity of AIV in various substrates is mostly based on experiments that were conducted for short periods of time and the temperature range selected was also unable to completely examine the survival of AIV under diverse environmental

conditions. The present study was therefore designed to assess the survival of three low pathogenic AIV (H4N6, H5N1, and H6N8), one human influenza virus (H1N1), and two model viruses [Newcastle disease virus (NDV) and enteric cytopathogenic bovine orphan virus (ECBO)] in sterile distilled water (DW), normal saline (NS), and unsterile surface water (SW) by suspending the virus in the water while a germ carrier technique was adapted to measure the tenacity of these viruses in SW, lake sediment, duck feces, and duck meat at a wide range of temperatures for extended periods of time.

2 Review of literature

2.1 Influenza viruses

2.1.1 Taxonomy

The influenza viruses belong to the family *Orthomyxoviridae* which is divided into five genera: Type A, B, and C influenza viruses, *Isavirus* and *Thogotovirus*. Type A influenza viruses have a wide host range including birds and mammals. Type B viruses infect humans while type C influenza viruses are human pathogens but may also infect pigs. Isaviruses infect fish while thogotoviruses are tick-borne arboviruses infecting livestock and humans (Kawaoka et al., 2005). The viruses are classified into genera on the basis of antigenicity of viral nucleoproteins and matrix proteins (Kobasa and Kawaoka, 2005, Swayne et al., 1998). The influenza A viruses are of great zoonotic importance and are further categorized into subtypes based upon two surface glycoproteins, the hemagglutinin (H) and neuraminidase (N). At present 16 H subtypes (H1-H16) and 9 N subtypes (N1-N9) are recognized (Olsen et al., 2006).

2.1.2 Morphology and composition

Influenza viruses are pleomorphic or spherical in shape ranging from 80-120 nm in diameter but filamentous forms may also occur which are several micrometers in length. They are enveloped viruses and their surface is covered with two types of projections, H and N. The viral envelop is derived from the host cells during the replication cycle. The viral nucleocapsid is helical in shape harboring a negative-sense, segmented, single-stranded RNA genome. There are 8 segments of viral RNA in the case of influenza A viruses (Kawaoka et al., 2005; Swayne and Halvorson, 2003). The genome of influenza A viruses encode for ten proteins, of which eight are structural and two nonstructural proteins. The structural proteins can be divided into three surface proteins that include H, N, and the membrane ion channel (M2) protein and the internal proteins including the nucleoprotein (NP), the matrix protein (M1), and the polymerase complex composed of the polymerase basic protein1 (PB1), PB2, and the polymerase acidic protein (PA). Two additional proteins produced by

influenza A viruses are non structural protein 1 and non structural protein 2 and are prevalent only in the host cells (Suarez, 2008). The H and N are major antigenic proteins. Additionally, H is responsible for the attachment of virus to the target cells and N serves as a cell receptor destroying enzyme. M1 is the most abundant viral protein and plays a role in virus budding while M2 is a small protein that functions as an ion channel and is important for triggering viral uncoating. PB1, PB2, and PA collectively form the polymerase complex which in association with NP is responsible for the transcription, translation, and replication of viral RNA (Swayne and Halvorson, 2003).

2.1.3 Influenza A viruses and their host range

Influenza A viruses infect a wide range of species including humans, pigs, horses, sea mammals (whales and seals), and birds. Wild aquatic birds are the primary reservoir of these viruses as all of the known virus subtypes can be isolated from these birds (Webster et al., 1992). In aquatic birds, influenza A virus infection remains usually asymptomatic, and the viruses are in evolutionary stasis (Webby and Webster, 2001). Generally, influenza A viruses exhibit host species adaptation with easy transmission to individuals of the same species but occasionally there is also interspecies transmission to related species while viral transmission to humans is a rare phenomenon (Swayne and Halvorson, 2003). However, there is clear evidence that influenza A viruses have crossed the species barrier several times in the past (Webster et al., 1992).

The host range of influenza A viruses is mainly determined by the ability of the viral H protein to attach to the host cell sialic acid receptors. The sialic acid receptors can be classified into α-2,3 and α-2,6 based on their molecular configuration. Different animals have different tissue patterns and levels of expression of α-2,3 or α-2,6 sialic acid receptors (Suarez, 2008). The poultry respiratory epithelium is rich in α-2,3 receptors while human respiratory epithelium contains more α-2,6 receptors (Thompson et al., 2006). In contrast, pigs express high levels of both α-2,3 and α-2,6 receptors (Webster et al., 1997). The viral H, based on amino acid sequence, has a

strong specificity for either the α-2,3 or α-2,6 linkage and this can be one factor in host specificity. Avian viruses preferably bind to α-2,3 receptors while human viruses rather bind to α-2,6 receptors (Suarez, 2008). Pigs have been suggested as a mixing vessel for avian and human influenza viruses since they express high levels of both α-2,3 or α-2,6 receptors in their respiratory epithelium. It has been hypothesized that pigs can be simultaneously infected with avian and human strains and reassortment could occur between these two viruses, resulting in new viruses that could become pandemic strains (Webster et al., 1997).

2.2 Avian influenza

Avian influenza is a contagious viral disease of birds mainly affecting the respiratory tract. In domestic poultry, infection with AIV may range from asymptomatic illness to severe systemic disease with 100 percent mortality. The disease severity depends on virus strain, host species and certain environmental factors (Swayne and Halvorson, 2003). Wild birds serve as a reservoir of influenza viruses and usually become infected without showing clinical signs of disease. Overall, AIV have been reported from more than 100 species of free-living birds belonging to 12 avian orders (Stallknecht and Brown, 2008).

2.2.1 Low and high pathogenicity AIV

AIV can be classified into two pathotypes, low pathogenicity avian influenza (LPAI) and HPAI viruses, based on their ability to cause disease in poultry. Most AIV are of low pathogenicity and cause only mild disease while HPAI viruses can cause severe illness with high mortality in poultry populations (Swayne and Halvorson, 2003). The pathotyping can be done by calculating the intravenous pathogenicity index (IVPI) of a given virus through injecting it into 4-8 week old specific pathogen free (SPF) chicks and/or by sequencing the viral genome at the proteolytic cleavage site (PCS) of the H gene (OIE, 2008; Swayne and Suarez, 2000). H is a major surface glycoprotein of AIV that plays a vital role in the initiation of infection process and to attain its full function the H must be proteolytically cleaved into H1 and H2 (Perdue, 2008). Normally, trypsin or trypsin-like proteases cleave H by recognizing a single

arginine at the PCS. The presence of these enzymes in the respiratory and intestinal epithelium explains the distribution of LPAI viruses in infected birds (Kido et al., 1992; Klenk et al., 1975). However, the presence of multiple basic amino acids at the PCS makes it accessible to furin or other ubiquitous proteases that are present in most cells of the body (Stieneke-Grober et al., 1992). Mutations in the virus that result in substitutions of non-basic with basic amino acids or insertions of multiple basic amino acids at the PCS provide a different template for cleavage by proteases and make the viruses capable of replication in a wide range of tissues resulting in more severe systemic illness (OIE, 2008; Perdue, 2008). For all H5 and H7 viruses, if the amino acid sequence of the connecting peptide of the H glycoprotein is similar to that observed for other HPAI isolates, the isolate being tested will be considered to be highly pathogenic (OIE, 2008). All HPAI viruses described so far belong to H5 and H7 subtypes but most H5 and H7 viruses are LPAI viruses and it is believed that HPAI subtypes arise by mutation of LPAI viruses following continuing circulation in domestic poultry (Suarez, 2008).

Previously, HPAI viruses were believed to replicate poorly in wild birds and ducks (Swayne and Halvorson, 2003) but since 2002, following an influenza outbreak caused by HPAI H5N1 viruses in waterfowl and captive birds in Hong Kong (Ellis et al., 2004) this HPAI virus has been detected in more than 50 wild bird species (Stallknecht and Brown, 2008). Also, several studies showed that experimental infection of wild birds with HPAI viruses may lead to clinical illness and in some cases even death of the infected birds (Brown et al., 2006; Perkins and Swayne, 2002).

2.2.2 Zoonotic potential of H5N1 HPAI viruses

In humans, three major influenza A virus pandemics (Spanish flu caused by H1N1 in 1918, Asian Flu caused by H2N2 in 1957, and Hong Kong Flu by H3N2 in 1968) were all related to AIV. A recent human pandemic caused by a “swine flu” H1N1 virus was started in 2009, disseminated to more than 214 countries and resulted into over 18,449 recorded deaths worldwide till August 1, 2010 (WHO, 2010). The newly emerged H1N1 virus is a mixture of avian, porcine, and human influenza viruses

(Fitzgerald, 2009) which resulted from the reassortment of recent North American H3N2 and H1N2 swine viruses (i.e., avian/human/swine 'triple' reassortant viruses) with Eurasian avian-like swine viruses (Neumann et al., 2009). Sporadic cases of human infection with swine influenza have been reported over the past 10 years in the USA. Keeping in view the comparative slower evolution rate of swine influenza viruses than the human influenza viruses the appearance of a new pandemic strain which resulted from the mixture of the genetic material of human and swine viruses was not expected (Fitzgerald, 2009).

Although a number of low and high pathogenicity AIV have been reported to transmit directly from avian to human hosts, the transfer of H5 and H7 HPAI viruses to humans has become more frequent in the past few years (Kalthoff et al., 2010). The first fatal case of H5N1 HPAI virus infection in a human was recorded in 1997 in Hong Kong where 18 people were hospitalized and 6 died of infection with bilateral pneumonia. All of the affected individuals had direct contact with poultry (Mounts et al., 1999; Yuen et al., 1998). In subsequent years, H5N1 HPAI viruses have been documented to have infected at least 516 people, and caused severe illness and death in 306 humans till January 2011 (WHO, 2011). The Asian HPAI H5N1 virus is exceptionally virulent in humans with an estimated case fatality ratio of about 60 %. In addition to being efficiently replicated in humans, the prerequisite for a new pandemic strain to evolve is its easy and sustainable spread among humans. The Asian HPAI H5N1 virus does not seem to fulfill this criterion as person-to-person transmission is reported as a very rare event (Wang et al., 2008).

Besides human infections, some HPAI viruses can infect other mammals. The transmission of HPAI H5N1 virus from infected poultry or wild birds to large felids and domestic cats has been reported from eight countries in Asia and Europe (Harder and Vahlenkamp, 2010). Recently, an HPAI H5N1 virus was isolated from donkeys living in contact with diseased birds in Egypt (Abdel-Moneim et al., 2010).

2.2.3 Laboratory host system for the propagation of AIV

Embryonating chicken eggs (ECE) have been used as a preferred method for the isolation and propagation of influenza viruses because they are supposed to be the most sensitive system for AIV propagation and because viruses grow to a high titer in eggs (Swayne et al., 1998). Avian, human, swine, and equine influenza viruses were all originally propagated in ECE and this method is still commonly used for diagnostic purposes and vaccine production. Recently, there is more emphasis, particularly for mammalian viruses, to grow influenza viruses in cell culture (Suarez, 2008). Moreover, duck, and turkey embryos have also been found to be equally sensitive compared to ECE for the propagation of H1-H16 LPAI viruses (Moresco et al., 2010). In addition to avian embryos, a number of primary cells as well as several cell lines have also been used for the growth of AIV (Swayne and Halvorson, 2003). In one study, three primary cell cultures, and ten established cell lines were evaluated for the growth of twelve AIV strains. Chicken embryo kidney cells (CEK) were found to be more sensitive followed by embryonic swine kidney and Madin-Darby canine kidney cells (MDCK) for virus growth while trypsin supplementation increased the susceptibility of MDCK (Sugimura, et. al., 2000). Similarly, Moresco et al. (2010) compared two primary cell cultures of avian origin and six permanent cell lines for the propagation of AIV. Among all tested cell types the two primary cell cultures, chicken embryo fibroblasts and CEK as well as MDCK are equally sensitive to the tested AIV and overall replication efficiency of the viruses was lower in each of the cell lines tested compared to ECE. Although ECE are the most efficient system for growth of influenza viruses, they are also costly and require much forethought concerning scheduling because embryos must be incubated 9-11 days prior to use. The MDCK has been the most consistently used for culturing and propagating AIV. In a comparative study, MDCK were found to be more sensitive than Vero and human lung embryonated cells (MRC-5) cell lines for the isolation of influenza A viruses from clinical specimens (Reina et al., 1997). Recently, an MDCK based 6:2 reassortant H5N1 vaccine was proposed to be a good candidate as an alternative to egg-based vaccines (Murakami et al., 2008).

2.2.4 Molecular methods for the detection of AIV

Molecular methods including reverse transcriptase-polymerase chain reaction (RT-PCR) and real time reverse transcriptase-PCR (RRT-PCR) are largely used in the diagnosis of AIV from clinical samples (Spackman et al., 2008). These methods have a high sensitivity and specificity comparable to virus isolation in chicken embryos or cell culture (Atmar et al., 1996; Cattoli et al., 2004; Spackman et al., 2002). Although conventional RT-PCR methods have been used for the detection of influenza A viruses from many different species (Fouchier et al., 2000), the RRT-PCR is considered the preferred method as it is faster, more specific, and has a lower risk of cross contamination than the conventional RT-PCR (Spackman et al, 2008). The RRT-PCR for influenza A viruses was developed by Spackman et al. (2002). The method is based on AIV M-gene amplification with a detection limit of 10 femtogram (fg) or approximately 1,000 copies of target RNA and can detect 0.1 50 % egg infective dose of virus. Additionally, based on H gene amplification, RRT-PCRs for H5 and H7 subtypes have also been developed and have a detection limit of 100 fg of target RNA or approximately 10^3 to 10^4 gene copies (Spackman et al., 2002).

2.3 Modes of transmission

2.3.1 Virus shedding and transmission

AIV are excreted from the nares, mouth, conjunctiva, and cloaca of infected birds into the environment (CDC, 2005; Swayne and Halvorson, 2003). In preliminary work done by Webster et al. (1978) it was reported that experimentally infected Muscovy ducks shed 6.4 g of fecal material per hour with an infectivity titer of 7.8 $\log_{10}$ embryo infectious $dose_{50}$ (EID_{50}) per gram. In this way one infected duck can shed about 10^{10} EID_{50} virus in a 24 hour period and virus shedding was recorded for a period of 6-7 days through respiratory secretions and feces. Also, ducks infected with H5N1 virus subtype were found to shed high titers of the virus through trachea and cloaca, with peak levels of virus shedding after three days (Sturm-Ramirez et al., 2005). Similarly, following an experimental infection with an H7N2 virus subtype, virus excretion was recorded as early as two days post inoculation from cloacal swabs and

no virus was detectable from the infected birds by the third week post inoculation while the first week following exposure to the virus was the most active period of virus shedding, which occurred through the respiratory and intestinal tracts (Lu et al., 2003). However, virus shedding through the cloaca of infected ducks can be prolonged for a period of 28 days (Hinshaw et al., 1980).

2.3.2 Water borne transmission

Susceptible birds become infected by direct contact with infected birds or indirectly through contact with surfaces or materials (such as water) that have been contaminated with the virus (CDC, 2005). The fact that influenza viruses are transmitted and maintained in the wild bird population by the fecal oral route indirectly through contaminated water is well established (Stallknecht and Brown, 2009). Isolation of influenza A viruses from unconcentrated lake water and from fecal samples near the shores of these lakes was first reported by Hinshaw et al. (1979). Subsequent to that work, influenza viruses have been isolated several times from SW (Halvorson et al., 1985; Hinshaw et al., 1980; Sivanandan et al., 1991; Ito et al., 1995). In one study, two duck farms were examined monthly for a period of one year for the occurrence and persistence of influenza viruses within the duck communities. An influenza virus was isolated monthly throughout the year from feces or pond water or both, indicating a cycle of waterborne transmission (Markwell and Shortridge, 1982). Another report confirmed the isolation of several AIV subtypes from water samples obtained from different lakes in Alaska in consecutive years. The viruses were still isolated from the water even after the migration of birds suggesting that influenza viruses can be maintained in waterfowl populations by waterborne transmission (Ito et al., 1995).

2.4 Persistence of AIV in aquatic habitats

2.4.1 Persistence in water

The persistence of AIV in water was first studied by Webster et al., (1978) who mixed infected feces obtained from ducks naturally infected with A/duck/Memphis/546/74

(H3N6) influenza virus in non-chlorinated river water and stored the suspension at 4 and 22 °C. With a starting viral concentration of $10^{8.10}$ EID_{50}/ml, virus infectivity did not drop after 7 days at 4 °C and gradually decreased thereafter, but residual infectivity was still detectable after 32 days. At 22 °C the infectivity of virus in the water decreased more rapidly. Significant levels of virus were detected after 4 days but no virus was detectable at 7 days and afterwards. Later, for better understanding of virus persistence in water, Stallknecht et al. (1990a) used five AIV isolates derived from four waterfowl species in Louisiana. The experiment was conducted to study the effects of water temperature on the persistence of AIV under sterile laboratory conditions at 17 °C and 28 °C for 60 days while one virus was tested over 91 days at 4 °C. Linear regression models for these viruses predicted that an initial concentration of $1 \times 10^{6.00}$ tissue culture infectious $dose_{50}$ ($TCID_{50}$)/ml of water could remain infective for up to 207 days at 17 °C and up to 102 days at 28 °C. It was estimated that at 4 °C the contaminated water with a concentration of $1 \times 10^{6.00}$ $TCID_{50}$/ml could remain infective for 1,333 days. Significant differences in slopes for AIV persistence models were detected between treatment temperatures and among viruses.

2.4.2 Effect of pH and salinity

The infectivity of influenza viruses is highly influenced by any change in the physical and chemical conditions of the surrounding environment (Swayne and Halvorson, 2003). As influenza viruses are transmitted in wild aquatic birds through the fecal oral route via contaminated water, water chemistry could play an important role in the persistence of AIV in this medium (Stallknecht and Brown, 2009). Preliminary work to study the effect of water temperature, salinity, and pH on the persistence of AIV was performed by Stallknecht et al. (1990). They designed a lab-based model distilled-water system adjusted to salinity and pH that are normally associated with surface water. The individual as well as interactive effects of the variables were tested using three influenza viruses isolated from ducks in Cameron Parish, Louisiana. Differences were detected between temperature (17 °C and 28 °C), pH (6.2, 7.2, and 8.2), and salinity [0 and 20 parts per thousand (ppt)], with a strong interactive effect

observed between pH and salinity. Estimated persistence of infectivity for 1 x $10^{6.00}$ $TCID_{50}$ of A/mottled duck/LA/38M/87 (H6N2) was longest at 17 °C/0 ppt/pH 8.2 (100 days) and shortest at 28 °C/20 ppt/pH 8.2 (9 days). Differences in the response to these variables were apparent between the viruses. Based on this varied response of different viral strains Brown et al. (2007) included eight wild bird origin LPAI H5 and H7 viruses and two HPAI H5N1 viruses in their study to determine the effects of temperature and water salinity on the persistence of AIV. Viruses were tested at two temperatures (17 °C and 28 °C) and three salinity levels (0, 15, and 30 ppt) of DW. The pH value of the water samples was kept constant at 7.40. Experimental data showed that H5 and H7 AIV can persist for extended periods of time in water, with duration of infectivity comparable to AIV of other subtypes. The persistence of AIV was inversely proportional to temperature and salinity of water and there was a significant interaction between the effects of temperature and salinity on the persistence of AIV, with the effect of salinity more prominent at lower temperatures. Results from the two HPAI H5N1 viruses indicate that these viruses did not persist as long as the LPAI viruses. Later on, for more elaborative work on the persistence of AIV in water, Brown et al. (2009) studied twelve wild bird origin influenza viruses using the same laboratory based model with an extended range of variables (salinity and pH). The AIV varied in their response to each of the examined variables but generally were most stable at a slightly basic pH (7.4-8.2), at low temperatures (< 17 °C), and at fresh to brackish salinities (0-20 ppt). Alternatively, the viruses had a much shorter persistence in acidic conditions (pH < 6.6), at warmer temperatures (> 32 °C), and at high salinity (25 ppt).

Influenza A viruses are sensitive to acidic pH conditions, although their retention of infectivity is dependent upon the degree of acidity and the virus strain involved (Puri et al., 1990). Lower pH values generate a conformational change in the H, which allows fusion with the membrane of the host cell. This conformational change is reversible at pH between 6.00 and 6.40 but irreversible below pH 5.0 (Sato et al., 1983). To check the strain variation of influenza A virus stability at lower pH values Webster et al. (1978) exposed two human and one duck influenza viruses to pH 3.00, 4.00, 5.00, and 7.00. None of the viruses was detectable after 10 minutes (min) at pH

3.00 but duck influenza viruses were more stable at pH 4.00 than human strains. The pH stability of infectious influenza A viruses was also investigated by Scholtissek (1985) who calculated the threshold pH of several influenza virus subtypes where infectivity of the viruses was lost. The pH stability of the virus subtypes tested ranged from 5.1-6.0. The H3 strains were relatively stable against low pH values (threshold between 5.1 and 5.4), while H7 and H5 strains were relatively labile (pH threshold 5.6-6.0). Infectivity of strains with non-cleavable H was much more stable to treatment at low pH than that of strains with cleavable H. In a recent study the viability of an H9N2 isolate at pH 5.00 and 7.00 and at 4 and 20 °C was investigated. Incubation at pH 5.00 had a much greater affect on virus viability than at pH 7.00, as at 4 °C the virus did not survive beyond the second week. Moreover, even immediately after adjusting the pH of the virus suspension to 5.00, at the initiation of the experiment, the original virus titer decreased from $10^{8.00}$ EID_{50}/ml to $10^{4.70}$ EID_{50}/ml (Davidson et al., 2010).

The pH stability of four H7N2 AIV isolates was investigated by adjusting the pH of infective amniotic allantoic fluid (AAF) to 2.00, 5.00, 10.00, and 12.00. All of the isolates with an initial virus concentration of $10^{7.00}$ EID_{50}/ml or more lost 100 % of their infectivity after 5 min following exposure to pH 2.00 and after 15 min following exposure to pH 5.00, 10.00, and 12.00 (Lu et al., 2003). Three HPAI H5N1 virus strains from Thailand were also checked for their sensitivity to pH inactivation. The viruses as infective AAF were exposed to pH 3.00, 5.00, 7.00, 9.00, and 12.00 but none of the pH levels were able to inactivate the tested H5N1 viruses within the exposure times of 5 and 10 min (Wanaratana et al., 2010).

2.4.3 Biotic components

The biotic components of an ecosystem can influence the persistence of viruses in that environment. It has been documented that microorganisms present in a medium are associated with in situ inactivation of the viruses (Fujioka et al., 1980) and viral persistence is generally higher under sterile conditions as compared to non sterile ones (John and Rose, 2005). Alternatively, biofilms or filter-feeding bivalve shellfish

may accumulate viruses and act as efficient vehicles for their transmission (Lees, 2000; Skraber et al., 2005). Limited information is available on the tenacity of AIV in intact biological systems (Stallknecht et al., 2010). In one study, Zarkov (2006) compared the persistence of AIV in sterile and non-sterile natural water samples. The viruses survived for shorter times in unsterile water samples and the loss of viral infectivity was directly related to the increasing concentrations of naturally occurring microorganisms. A recent study on the persistence of HPAI H5N1 virus in SW also described that the virus quickly disappeared in an unfiltered fraction of seawater as compared to other water types (Domanska-Blicharz et al., 2010).

The influence of filter-feeding bivalves on the infectivity of AIV in water has recently been studied by Faust et al. (2009). They put clams in contaminated water samples and afterwards checked the water for residual viral infectivity. The clams were also tested for virus accumulation by feeding them to susceptible wood ducks. None of the wood ducks inoculated intranasally with HPAI virus contaminated water that was filtered by clams or fed with tissues from these clams exhibited morbidity or mortality while all of the ducks exposed to either HPAI virus contaminated water without clams or the original viral inoculums died. This shows that filter-feeding bivalves can remove and reduce the infectivity of AI viruses in water. The role of the biotic community of natural water on the ecology of influenza viruses was further investigated by Stumpf et al. (2010) and Abbas (2009). They checked the ability of zebra mussels (*Dreissena polymorpha)* and water fleas (*Daphnia magna)* to serve as vectors of influenza A virus in lake water. The mussels and water fleas were exposed to H5N1 LPAI virus contaminated lake water and checked afterwards for the presence and accumulation of the virus. The results of both studies, as revealed by RRT-PCR detection and titration on ECE or cell culture, suggest that the mussels and water fleas were capable of accumulating the influenza viruses from the surrounding water.

2.4.4 Abiotic reservoirs

Viable microbes present in the atmosphere may be incorporated into fog, rain, sleet, hail or snow. Environmental ice appears to be an important abiotic reservoir for

pathogenic microbes (Rogers et al., 2004; Smith et al., 2004). Influenza A virus genomes have been detected in the ice of frozen lakes (Smith et al., 2004) and based on the detection of viral RNA, Siberian lake ice associated with waterfowl habitats has been suggested as a long-term environmental reservoir of influenza A viruses (Zhang et al., 2006). Although these findings have not been confirmed by virus isolation, high amounts of virus excreted through feces and nasal secretions may lead to heavy contamination of water (CDC, 2005) and subsequent encapsulation and preservation of virus in the ice. It has been hypothesized that after being incorporated into environmental ice, viruses that survive freezing and thawing may persist for years, centuries or longer (Rogers et al., 2004). The effect of freeze-thawing on the titer of influenza virus subtype H5N7 indicated that each freeze-thaw resulted in a loss of 0.196 $TCID_{50}$/ml or a total loss of 2.15 $TCID_{50}$/ml during 11 freeze-thaw cycles (Stallknecht et al., 2010). Thawing of contaminated ice may release entrapped viruses over ages and water may become contaminated with concurrent strains. Before the refreezing of water, viruses of the present and past may be contracted by the waterfowl, whereas the remaining viruses would again be encapsulated by the formation of ice (Zhang et al., 2006).

2.5 Tenacity of AIV in sewage, excreta, waste, and poultry products

2.5.1 Inactivation in sewage

Considerable differences have been observed in the stability and inactivation rates of viruses in sewage, biosolids, animal manure, natural waters, and other environmental media. These differences exist not only among viruses of different families and genera, but also among viruses of the same family, genus, and even among similar types or strains of virus (Sobsey and Meschke, 2003). Much work has been done on the survival, transport, and fate of enteric and respiratory viruses (Sobsey and Meschke, 2003) but little is known about the persistence of AIV in sewage, waste treatment and management systems (WHO, 2007). Generally, enveloped viruses are less persistent in sewage treatment processes than non-enveloped virus as in one study the time required for one log reduction in virus titer was less than 1 week for a

herpesvirus (enveloped) while more than 6 months were required for a rotavirus (non-enveloped) in liquid animal waste (Pesaro et al., 1995). Inactivation of AIV by ultraviolet irradiation, chlorination, and aerobic digestion in typical domestic wastewater and drinking water systems was studied by Lucio-Forster et al. (2006). The infectious virus was not detectable in wastewater effluent. In bench scale anaerobic digesters, the virus was undetectable after 72 hours with an initial concentration of $10^{3.60}$ EID_{50}/ml.

To evaluate the inactivation of AIV in media representative of a land-disposal scenario, the survival of an H6N2 virus subtype was measured in a methanogenic landfill leachate. Virus persistence was evaluated in the sterile leachate and reverse osmosis water as a function of temperature, pH, and conductivity. Elevated temperature and non-neutral pH resulted in the quickest inactivation of the virus in both media, whereas conductivity did not have a significant influence on virus survival. Media effects were significant and virus inactivation in leachate was consistently the same as or faster than in water (Graiver et al., 2009).

2.5.2 Persistence in bird feces and manure

Keeping in view the high amount of AIV excreted through the feces of infected birds, viral persistence in this medium is of great concern for the spread of the disease (CDC, 2005; WHO, 2006). The stability of influenza viruses in fecal material has been investigated to some extent. The survival of AIV in feces is influenced by: the strain of virus, type of feces (species from which the feces were obtained), physical properties of the feces, and the temperature at which feces was kept (De Benedictis et al., 2007). Fecal material from ducks experimentally inoculated with H3N6 virus subtype showed no detectable decrease in the infectivity titer of the virus over a period of 2 weeks at 0 °C but over the following 2-week period there was an appreciable loss of viral infectivity (3.0 log EID_{50}). Significant residual viral infectivity was detectable after 32 days of storage at 4 °C while an identical sample stored at 22 °C showed a more rapid drop in infectivity. Infectious virus was present for at least 8 days at this temperature but was not detectable after 13 days (Webster et al., 1978).

H5N1 virus subtypes with a concentration of 2.25-3.75 $\log_{10}$ EID_{50} per g of fresh duck feces became undetectable after the feces were dried overnight at room temperature (20 °C). In wet feces, the virus remained viable for 4-6 days at 37 °C. The titers dropped when kept at 25 °C but remained detectable for 7 days, while at 4 °C the viruses were detectable for more than 20 days (WHO, 2007). The persistence of an HPAI H5N1 virus isolated from Thailand was studied by Songersam et al. (2006) under different environmental conditions after mixing the virus (with a titer of $10^{6.30}$ EID_{50}/ml) in fresh feces. The virus was inactivated after 30 min under sunlight at 32-35 °C but was still detectable after 4 days in the shade at 25-32 °C. In another study, a similar virus strain (with a virus titer of $2.38 \times 10^{5.25}$ EID_{50}/ml) mixed with normal chicken manure was inactivated within 24 hours at 25 °C and within 15 min at 40 °C (Chumpolbanchorn et al., 2006).

To check the effect of various types of manures on virus tenacity, chicken manure from three different sources were used to assess the persistence of an H7N2 influenza virus: 1) SPF chickens housed in a bio-safety level-2 (BSL-2) facility, 2) experimental commercial layers removed from a field farm and also housed separately in the BSL-2 facility, and 3) commercial layers in field chicken houses,. The field chicken manure had a significant effect on virus inactivation at ambient temperature (15–20 °C) and up to 56 °C. At such temperatures, virus mixed with field chicken manure lost its infectivity about 5 to 10 times faster than unmixed virus control. In manure types 1, 2, and 3, viral infectivity was lost after 30, 20, and 15 min following incubation at 56 °C, after 16 days, 36 hours, and 24 hours at 30-37 °C, and after 23, 6, and 2 days at 15-20 °C. Of all the types of manures, the field chicken manure had the most inactivating effect and SPF chicken manure had the least inactivating effect on virus persistence.

2.5.3 Persistence in poultry waste and byproducts

Following an outbreak of HPAI virus, all poultry that have potentially been exposed to the virus should be killed and disposed of in an efficient manner. On-site composting is one of the most efficient and environmentally acceptable methods for the disposal

of poultry waste and carcasses (Brglez and Hahn, 2008). Experiments related to the survival of an H6N2 influenza virus strain during the composting process based on virus isolation and RRT-PCR method showed that composting is an efficient method for the removal of AIV. Various poultry waste and parts were mixed with virus suspension and exposed to the composting process. On day 0 the specimens buried in compost contained at least 5.0 $\log_{10}$ of virus and 7.7 $\log_{10}$ of viral RNA. By day 7, temperatures in the compost ranged from 50 to 65 °C and the viruses were killed in all of the specimens and no viral RNA was detectable (Guan et al., 2009).

Animal by-products derived from poultry can be used in poultry and pet foods. Such poultry by-products or derived products should be treated at 150 °C and a pressure of 4 bar for one hour without interruption according to the directives of the European Commission (Anonymous, 2010) which is sufficient to inactivate AIV. However, if the procedure is not carried out properly or the cooked product is subsequently contaminated by unprocessed product, AI viruses could persist in the byproduct for several weeks (Animal health Australia, 2008). The safe and humane disposal of spent laying hens by converting them to acidified pulp is practiced in some countries. Inactivation of a low pathogenic H5N2 during this process was studied to investigate whether acidification would inactivate AIV in biological material. After mixing the virus with acidified and non-acidified pulp the samples were incubated at room temperature and attempts were made to re-isolate the virus after 10 min, 2 hours, 4 hours, 12 hours, and 24 hours, respectively. The virus was not re-isolated from any tube containing acidified pulp, whereas it was cultured from all of the non-acidified samples (Kabell et al., 2009).

2.5.4 Tenacity in bird carcasses and meat

The AIV vary in their virulence and the distribution of lesions in infected birds depending upon the virus strain and pathotype involved (Swayne and Halvorson, 2003). Experimental studies show that following intranasal inoculation, LPAI viruses cause localized infections in the respiratory and gastrointestinal (GI) tracts while HPAI viruses cause respiratory and GI tract infections with systemic spread, and

virus may be detectable in blood, bone marrow, and breast and thigh meat (Swayne and Beck, 2005). Although LPAI strains are typically not present in chicken meat, virus particles in respiratory secretions or feces are possible sources of carcass contamination. In addition, birds processed during the viraemic stage can contaminate other carcasses through virus containing blood or fecal material (Animal health Australia, 2008, Thomas et al., 2008). The virus titer can be as high as $10^{8.00}$ EID_{50}/g of thigh meat and $10^{7.50}$ EID_{50}/g of breast meat (Thomas and Swayne, 2007). Feeding breast or thigh meat from HPAI H5N1 virus-infected chickens to other chickens resulted in virus infection and death (Swayne and Beck, 2005). Birds can therefore be exposed to virus through predation or cannibalization of contaminated carcasses of dead birds (Swayne, 2008). Similarly, other mammals including cats and dogs have been infected after eating raw poultry products (Kuiken et al., 2004; Songserm et al., 2006a).

Several AIV subtypes including H5N1 and H10N7 have been isolated from frozen duck and poultry meat following export to other countries (Mase et al., 2005; Serena Beato et al., 2006; Tumpey et al., 2002) and virus contaminated frozen carcasses have been linked to disease outbreaks in backyard poultry (Harder et al., 2009), which confirms that the influenza viruses can survive in carcasses: for several days at ambient temperatures and for weeks at refrigeration temperatures (Animal health Australia, 2008).

Poultry carcasses infected with HPAI H5N2 virus subtype were composted to assess virus inactivation during the composting process. No infectious virus was detectable form the carcasses after 10 days of composting (Senne et al., 1994). In a recent study, the persistence of an HPAI virus (H7N1) was evaluated in infected poultry carcasses at 22-23 °C and 30-31 °C (Busquets et al., 2010). Samples of skin, pectoral muscle, brain swabs, feathers, oropharyngeal and cloacal swabs were taken from the contaminated carcasses every 24 hours for a period of 6 days and checked for virus infectivity. At 30-31 °C the virus could not be recovered from muscle and cloacal swabs after 24 hours, in brain and oropharyngeal swabs the virus was detectable after one day while feather pulp and skin retained infectious virus for 2 and 3 days, respectively. At 22-23 °C the virus was detectable in cloacal swabs,

muscles, and oropharyngeal swabs for 1, 3, and 4 days, respectively while feather pulp and brain swabs maintained infectious viruses for 5 days or longer.

2.5.5 AIV in poultry eggs

Outbreak descriptions and studies in experimentally infected birds suggest that some HPAI viruses are likely to be found in the eggs before an infected flock is recognized (Spickler et al., 2008). In laying hens, AIV infections produce lesions in the ovaries and oviducts of infected birds. Therefore, influenza viruses could potentially be transmitted via eggs either through virus within the egg contents or on the surface from virus-infected feces (Swayne and Beck, 2004). LPAI virus was detected in the albumin of the eggs laid by experimentally infected breeder turkeys by RRT-PCR and chicken embryo inoculation (Pillai, 2010). Influenza virus has also been recovered from the yolk, albumen, and shell surface of eggs obtained from naturally infected chicken flocks (Cappucci et al., 1985). AIV inactivation in egg and egg products requires heat treatment of 64 °C for 4.5 min, 60 °C for 5 min or over 55 °C for more than 15 min (Animal health Australia, 2008). In one study, LPAI viruses were inactivated in all of the four tested egg products when treated by industry-standard pasteurization protocols. In contrast, an HPAI virus was inactivated in liquid egg products but not in dried egg whites when using a low-temperature industry pasteurization protocol (Swayne and Beck, 2004).

2.6 AIV in the environment

2.6.1 Role of the environment in virus transmission

The environment plays a vital role in the spread of AIV to susceptible avian species (Swayne, 2008). An epidemic may lead to heavy contamination of the environment as revealed by influenza A virus (H5N1) outbreaks among backyard poultry in 3 villages of Cambodia (Vong et al., 2008). Viral RNA was detected in 27 (35 %) of 77 specimens of mud, pond water, water plants, and soil swabs collected from the area. The contaminated environment not only transmits virus between members of one species but also provides a bridge for virus transmission between many diverse

hosts, including wild and domestic animals and man (Stallknecht and Brown, 2009). Recently, it has been proposed that an environmental virus reservoir gives rise to indirect transmission and neglecting environmentally generated transmission chains could underestimate the explosiveness and duration of AIV epidemics (Rohani et al., 2009).

2.6.2 Virus persistence on fomites

Fomites consist of both porous and non-porous surfaces or objects that can become contaminated with pathogenic microbes and serve as vehicles in transmission (Boone and Gerba, 2007). Fomites play an important role in the indirect transmission of AIV to susceptible birds (CDC, 2005; Swayne, 2008). Survival of viruses on fomites is influenced by intrinsic factors such as fomite's properties or virus characteristics and extrinsic factors like environmental temperature, humidity, and suspending medium (Boone and Gerba, 2007).

2.6.2.1 Survival of AIV on environmental surfaces

Early work on the survival of influenza A and B viruses on environmental surfaces showed that both of the viruses studied survived for 24-48 hours on hard, nonporous surfaces (stainless steel and plastic) and 8-12 hours on cloth, paper, and tissues. Measurable amounts of influenza A virus were transferred from stainless steel to hands for 24 hours and from tissues to hands for up to 15 min. Virus survived on hands for up to 5 min after transfer from the environmental surfaces (Bean et al., 1982). Survival of an avian influenza virus subtype H13N7 was evaluated on 12 different porous and non-porous surfaces related to poultry husbandry. The virus was detectable on some of the surfaces for up to 6 days post contamination but not after 9 days. Survival was longer on non-porous surfaces (steel, latex, ceramic tiles, gum boot, tire, and plastic) than on porous surfaces (cotton, polyester fabrics, wood, and egg tray). Poor survival on porous surfaces could be due to inefficient elution of virus from these surfaces (Tiwari et al., 2006). To check the effect of metal type on the survival of influenza A virus on environmental surfaces Noyce et al. (2007) inoculated the influenza virus particles ($2 \times 10^{6.00}$) onto copper and stainless steel

surfaces and incubated at 22 °C at 50-60 % relative humidity. On stainless steel $5 \times 10^{5.00}$ virus particles were still infectious after 24 hours while on copper only $5 \times 10^{2.00}$ virus particles were viable after a period of 6 hours indicating a lower survival rate on copper than on stainless steel surfaces.

2.6.2.2 Survival of AIV on feathers and dust particles

The mode of virus transmission from one farm to another was investigated during an outbreak caused by high pathogenicity H7N3 virus in Canada. The experimental data and epidemiological investigations suggested that wind dispersion of the viruses through contaminated aerosols and dust particles played a vital role in virus transmission (Power, 2005). It has been reported that H5N1 influenza virus can replicate in feather epidermal cells of domestic ducks. The feathers can easily drop off, blow away or be reduced to dust, suggesting that affected feathers of waterfowl infected with influenza virus can be potential sources of infection (Yamamoto et al., 2008). The survival of influenza A virus on the feathers of various bird species was investigated by Yilmaz and Kaleta (2004). After addition of 0.1 ml of the virus suspension with a titer of $10^{6.7}$ $TCID_{50}$/ml, the feathers were placed at room temperature and checked for virus recovery at regular intervals. The important findings were: 1) within one hour of coating with virus, a drop of one log of virus titer was recorded through the drying process; 2) infectious virus was detectable for up to 24 hours after application on the feathers of all bird species; 3) there was no difference in the survival of AIV on the feathers of chickens, doves, and hawks while the longest survival time of 96 hours was recorded on the feathers of Pekin ducks; 4) infectious virus was still detectable after 48 hours on chicken feathers.

2.6.3 Prevalence/persistence of AIV in sediment

The presence of viruses in estuarine sediments has been extensively investigated and it has been observed that viruses may be present in polluted estuarine sediment at higher concentrations than in the overlying water (Gerba et al., 1977; LaBelle et al., 1980). Enteric viruses have mostly been studied in this regard as they are major contaminants of water and also pose a danger for transmission to the susceptible

population via contaminated water. Enteric viruses can readily adsorb to the sediment (LaBelle and Gerba, 1979) and can survive longer in the estuarine sediment than in seawater alone under experimental as well as field conditions (LaBelle, and Gerba, 1980; Smith et al., 1978). Up till now only one report is available on the detection of AIV from lake sediments. This study demonstrates that AIV RNA can be detected for long periods of time in sediments of habitats utilized by waterfowl. The detection rate of influenza virus from the sediment samples was very high (> 50 %). Although this study did not attempt to detect infectious virus, this culture independent approach provides a suitable tool for the determination of influenza virus prevalence and diversity in environmental reservoirs (Lang et al., 2008). It is likely that influenza viruses present in the environment associated with feces or other organic substrates within or at the sediment surface may present the best opportunity for birds (dabbling ducks) that feed at the sediment interface to become infected (Stallknecht and Brown, 2009).

2.7 Germ carrier technique to study the tenacity of viruses

2.7.1 Germ carrier tests

Germ carrier tests have been employed to calculate inactivation rates of viruses under natural inactivating factors in the environment and by disinfectants (Maillard, 2004; ASTM, 2002). It is difficult to detect and quantify virus as a separate entity in nature (Gerba, 1984) as viruses are mostly adsorbed to surfaces and/or embedded in organic or cellular debris, so carriers are more relevant for predicting the activity of biocides under field situations (Sattar et al., 2003). The protocols used include application of test organisms on the carrier followed by drying and subsequent exposure to certain physical or chemical insults. Commonly used materials for germ carriers include stainless steel, plastic, glass, and wood (Maillard, 2004). It is generally recommended that the test surfaces should contain at least $10^{4.00}$ $TCID_{50}$/ml of the recoverable virus and a 3 $\log_{10}$ reduction in the virus titer without cytotoxicity should be measured (Sattar et al., 1989). Non-porous hard surfaces are most commonly used in carrier tests (Maillard, 2004) but under veterinary field conditions

like in poultry houses the viruses may also come into contact with rough and porous surfaces so the assessment of virus persistence on such surfaces is also essential (Tiwari et al., 2006). While discussing a suitable carrier with a rough surface Yilmaz and Kaleta (2003) described the properties of a suitable carrier as: 1) capacity to absorb a virus suspension, 2) no effect on the infectivity titer of the absorbed virus, 3) no easy elution from the carrier in a liquid phase of product test solution, but high virus recovery using a shaker, ultrasound or any other method, 4) international availability, 5) specified and consistent quality, and 6) easy to sterilize.

2.7.2 Carrier tests used for inactivation studies of influenza viruses

The standard protocols for testing the virucidal activity of various biocides in suspension as well as carrier tests have been described in detail by ASTM (2002) CEN (2005), OECD (2009) and several other official bodies all over the world. In Germany, the virucidal testing of the chemical disinfectants used in the veterinary field is performed according to the guidelines of the DVG (2007), where the basic protocols involve the use of NDV and ECBO viruses (representative enveloped and non-enveloped viruses) as test organisms. However, no specific method is available for investigating the efficacy of the chemical disinfectants against influenza viruses.

To investigate the virucidal activity of 6 commercial disinfectants against LPAI viruses, carriers made up of materials (metal, plastic, and wood) typically present in a poultry house were used. All disinfectants were effective at the maximum concentrations tested, although not all of the tests on porous surfaces were conclusive. A lower neutralization index for wood was believed to be due to better recovery from the media rather than poor inactivation on the surface (Lombardi et al., 2008). In another study, the efficacy of two commercial disinfectants was tested against AIV. The experiments were performed in suspension tests and poplar wood carrier tests loaded with serum to simulate the field conditions considering organic soiling and surface porosity. The tests were carried out at 20 °C for reaction times of 15-120 min and additionally at 10 and 4 °C for reaction times of 5 and 10 min. Both disinfectants were initially effective but showed losses of efficacy when organic load increased and

temperature decreased. Both of the disinfectants were suitable at 20 °C but for safe inactivation at 4 °C the contact time had to be extended up to 120 min (Yilmaz et al., 2004).

2.7.3 Filter-based carrier tests

Filter or membrane-based carrier tests have been successfully used to study the survival kinetics of animal viruses in liquid or semi-liquid environmental conditions (Moce-Llivina et al., 2006; Pesaro et al., 1995; Spillmann et al., 1987; Traub et al., 1986). Positively or negatively charged filters have long been used for the concentration of viruses from water (Karim et al., 2009), but the use of such filters to study the inactivation of viruses during sludge treatment was first described by Traub et al. (1986) who used bacteriophage f2 as a test virus. The virus was adsorbed onto an electropositive membrane filter which was then sandwiched between two polycarbonate membranes (PCM) with pores smaller than the virus diameter. The resulting sandwich was fixed in an open filter holder and exposed to the sludge in the digesting tanks. The device described prevented uncontrolled virus escape, but allowed direct contact of the various inactivating or stabilizing substances present in the environment tested with the virus adsorbed to the carrier. After exposure to an environment, the surviving fraction of virus was eluted from the filter and determined by plaque counting. By using PCM without pores for sandwiching, the influence of temperature alone on virus inactivation could be measured. The technique proved useful and was successfully employed to calculate the inactivation of the test virus during sludge digestion processes under anaerobic conditions. Subsequent to this work the same technique was used by Spillman et al. (1987) with minor modifications to further study the inactivation of animal viruses (rotavirus, coxsackievirus B5, and a bovine parvovirus) during a sewage sludge treatment process. A similar technique was used by Pesaro et al. (1995) to study the persistence of five animal viruses, representing picorna-, rota-, parvo-, adeno-, and herpesviruses in liquid and semi-liquid animal waste.

The use of germ carrier techniques to calculate the persistence of AIV in the environment was described by Haumacher et al. (2009) who checked various types of germ carriers (metal, wood, volume, and filter carriers) to determine an appropriate carrier for influenza viruses. Of the germ carriers tested, the metal and wood carriers were inappropriate since a poor viral recovery was achieved after inoculation with the virus suspension, and the volume germ carriers were unsuitable as they resulted in leakage of virus particles into the surrounding environment. However, the filter germ carriers proved good in virus adsorption properties.

3 Materials and methods

3.1 Viruses and cells

3.1.1 Viruses

The description of viruses used to study viral persistence in water, lake sediment, duck feces, and duck meat is given in Table 3.1.

Table 3.1: Description of viruses used in the tenacity trials

Virus type	Strain designation	Source
LPAI H4N6	A/Mallard/Wv1732-34/03 (H4N6)	Friedrich- Loeffler Institute (FLI), Insel Riems, Germany
LPAI H5N1	A/Teal/Wv632/Germany/05 (H5N1)	FLI, Insel Riems, Germany
LPAI H6N8	A/Muteswan/Germany/R2927/07 (H6N8)	FLI, Insel Riems, Germany
Human influenza virus H1N1	A/Puerto Rico/8/1934 (H1N1)	Institute of Virology, Justus Liebig University, Giessen, Germany
NDV	Lasota	Institut für Umwelt-und Tierhygiene, Universität Hohenheim, Stuttgart, Germany
ECBO	LCR-4	Institut für Umwelt-und Tierhygiene, Universität Hohenheim, Stuttgart, Germany

3.1.2 Cells and chicken embryos

The details of cells and chicken embryos used for the propagation and titration of the viruses are given in Table 3.2.

Table 3.2: Description of cell lines and chicken embryos used in the experimental trials

Cell type	Reference No.	Source
Madin Darby canine kidney cells (MDCK)	CCLV-RIE 671	FLI, Insel Riems, Germany
Madin Darby bovine kidney cells (MDBK)	ATCC-CCL-22	FLI, Insel Riems, Germany
Vero cells	ATCC-CCL-81	American type culture collection, USA
Specific pathogen free-embryonating chicken eggs (SPF-ECE)	-	Lohman Tierzucht, GmbH, Cuxhaven, Germany

3.2 Chemicals, reagents and solutions

3.2.1 Cell culture

Water, deionised and filtered

Milli-Q® water (Millipore GmbH, Eschborn)

Penicillin-G solution

60 mg/ml Penicillin-G (1,664 U/mg) (Biochrom AG, Berlin, Germany)

Dissolved in Milli-Q® water. Stock solution has a concentration of approx. 100,000 U/ml.

Streptomycinsulfate solution

256 mg/ml Streptomycinsulfate (758 U/mg) (Biochrom AG, Berlin, Germany)

Dissolved in Milli-Q® water. Stock solution has a concentration of approx. 190,000 U/ml.

Gentamicinsulfate solution

5 mg/ml Gentamicinsulfate (640 U/mg) (Biochrom AG, Berlin, Germany)

Dissolved in Milli-Q® water. Stock solution has a concentration of approx. 3,200 U/ml.

Amphotericin B

Amphotericin B (Biochrom AG, Berlin, Germany)

The lyophilized material was suspended in 5 ml Milli-Q® water to achieve a concentration of 250 µg/ml in the stock solution.

Fetal calf serum (FCS)

(Biochrom AG, Berlin, Germany)

Non essential amino acids (NEA)

(Biochrom AG, Berlin, Germany)

Trypsin-versen (TV) solution 0.05 %

8.00	g/l	136 mM	NaCl (Merck KGaA, Darmstadt, Germany)
0.20	g/l	3 mM	KCl (Merck KgaA, Darmstadt, Germany)
0.20	g/l	1 mM	KH_2PO_4 (Merck KgaA, Darmstadt, Germany)
2.31	g/l	6 mM	Na_2HPO_4 x 12 H_2O (Merck KgaA, Darmstadt, Germany)
0.132	g/l	0.9 mM	$CaCl_2$ x 2 H_2O (Merck KgaA, Darmstadt, Germany)
0.5	g/l		Trypsin-dry substance (Biochrom AG, Berlin, Germany)
1.25	g/l	3 mM	Versen (Titriplex III) (Merck KgaA, Darmstadt, Germany)
0.05	g/l	37,900 U/l	Streptomycin sulfat (Biochrom AG, Berlin, Germany)
0.06	g/l	100,000 U/l	Penicillin-G (Biochrom AG, Berlin, Germany)

The mixture was dissolved in Milli-Q®, adjusted to pH 7.00 with 1 M NaOH, sterile filtered, stored at -20 °C and melted at 4 °C before use.

0.01 M, 0.1 M, and 1 M hydrochloric acid (HCl) solution

HCl 6 mol/l (Carl Roth GmbH & Co, Karlsruhe, Germany)

For making 0.01, 0.1, or 1 M HCl solutions, an amount of 0.167, 1.67, or 16.7 ml of the HCl was pipetted into a bottle and Milli-Q® water was added to a final volume of 100 ml.

0.01 M, 0.1 M, and 1 M sodium hydroxide (NaOH) solution

NaOH 99 %, p.a. (Carl Roth GmbH & Co, Karlsruhe, Germany)

An amount of 0.04, .4, or 4 g of the NaOH was mixed in 100 ml of Milli-Q® water to make 0.01, 0.1, or 1 M NaOH solutions.

Cell culture medium

Dulbecco`s modified Eagle Medium (DMEM) as a powder with 4.5 g/l D-Glucose, L-Glutamin, without $NaHCO_3$ and without Sodium-Pyruvat (Biochrom AG, Berlin, Germany) was dissolved in Milli-Q® water at a concentration of 13.4 g/l, 2.2 g/l of $NaHCO_3$ was added, the medium was sterile filtered, and prepared for further use as outlined in Tables 3.3 and 3.4.

Table 3.3: Composition of growth and maintenance medium for MDCK and MDBK

Ingredients	Growth medium	Maintenance medium
FCS	5 %	2 %
NEA	1 %	1 %
Gentamicinsulfate solution	-	6.4 U/ml
Penicillin-G solution	-	200 U/ml
Streptomycinsulfat solution	-	380 U/ml
Amphotericin B solution	-	0.5 µg/ml

Note: The quantity of ingredients is listed as a final concentration

Table 3.2: Composition of growth and maintenance medium for Vero cells

Ingredients	Growth medium	Maintenance medium
FCS	5 %	2 %
Gentamicinsulfate solution	-	6.4 U/ml
Penicillin-G solution	-	200 U/ml
Streptomycinsulfate solution	-	380 U/ml
Amphotericin B solution	-	0.5 µg/ml

Note: The quantity of ingredients is listed as a final concentration

Standard 1 nutrient agar

Standard 1 nutrient agar (Merck KgaA, Darmstadt, Germany)

37 g of the agar was dissolved in one liter of Milli-Q® water, pH was adjusted to 7.50 ± 0.2, autoclaved at 121 °C for 15 min and poured into 92 x 16 mm sterile plastic petri dishes.

3.2.2 Embryo inoculation and HA test

Normal saline (NS) solution

9 g/l (136 mM) NaCl (Merck KgaA, Darmstadt, Germany) in Milli-Q® water, sterilized, and stored at room temperature.

Phosphate buffered saline (PBS)

8.00	g/l	137	mM	NaCl (Merck KgaA, Darmstadt, Germany)
0.20	g/l	26	mM	KCl (Merck KgaA, Darmstadt, Germany)
0.12	g/l	1.23	mM	KH_2PO_4 (Merck KgaA, Darmstadt, Germany)
0.91	g/l	5.1	mM	$Na_2HPO_4 \times 2H_2O$ (Merck KgaA, Darmstadt, Germany)

Dissolved in Milli-Q® water and pH was adjusted to 7.50 with the help of 1 M NaOH solution.

Iodine solution

Betaisodona® Povidon-Iod (Mundipharm GmbH, Limburg, Germany)

Erythrocytes

The chicken erythrocytes were purchased from Lohman Tierzucht, GmbH, (Cuxhaven, Germany) as a 1 % (V/V) suspension and stored at 4 °C. Before use, the erythrocytes were washed three times with NS solution by centrifugation at 1,000 rcf for 5 min and resuspending the sedimented erythrocytes to get a final concentration of 1 percent.

3.2.3 RRT-PCR

Nuclease-free water

(QIAGEN Sciences, Maryland, USA)

Diethylpyrocarbonate (DEPC) water

DEPC (Fluka, Buchs SG, Switzerland)

DEPC-water was used as nuclease free water. First, a 10 % DEPC stock solution was prepared: 10 ml DEPC was dissolved in 90 ml of absolute ethanol. The stock solution was stored in brown bottles in the dark. When required, a 1 % working solution (DEPC water) was prepared by mixing the stock solution in sterile Milli-Q® water, autoclaved at 120 °C for 30 min and stored at 4 °C.

Silica-matrix

Silicon dioxide (SiO_2), approx. 99 % (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)

An amount of 60 g SiO_2 particles were added to a measuring cylinder and filled up to 500 ml with DEPC water. After thorough mixing, the cylinder was allowed to stand at room temperature for 24 hours. Then, 430 ml of the supernatant was discarded. To re-suspend the silica, the cylinder was again filled up to 500 ml with DEPC water. After a further sedimentation of 5 hours, 440 ml of the supernatant was discarded. Afterwards, 600 µl of 25 % HCl (6.85 M) solution was added to adjust the pH of the solution to 2.00. The solution was slightly shaken to re-suspend the silica particles, aliquoted in 1 ml portions into 1.5 ml safe-lock nuclease free centrifuge tubes, autoclaved at 121 °C for 15 min and stored at room temperature in the dark.

0.1 M Tris-HCl

Tris-HCl (Carl Roth GmbH & Co, Karlsruhe, Germany)

An amount of 12.1 g Tris-HCl was dissolved in 800 ml Milli-Q® water. The pH of the solution was adjusted to 6.40 with 0.01 M HCl solution. Finally, the bottle was filled up to 1,000 ml with Milli-Q® water, and the pH was adjusted once again to 6.40. The solution was stored at 4 °C.

0.2 M Ethylenediamine-tetraaceticacid (EDTA)

EDTA (Carl Roth GmbH & Co, Karlsruhe, Germany)

An amount of 7.44 g EDTA was dissolved in 100 ml DEPC water. The pH of the solution was adjusted to 8.00 with 5 N NaOH, autoclaved at 120 °C for 30 min and stored at 4 °C.

Lysis buffer (LB)

Guanidinethiocyanate (Carl Roth GmbH & Co, Karlsruhe, Germany)

After putting 48 g Guanidinethiocyanate in a 250 ml-glass beaker, 1 ml Triton x-100 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added followed by 8.8 ml of 0.2 M EDTA (pH 8.00) and 40 ml 0.1 M Tris HCl (pH 6.40). The beaker was covered with aluminium foil and placed in a water bath at 56 °C for 15 min to dissolve the ingredients. The solution was distributed into sterile 50 ml plastic tubes, covered with aluminium foil and stored in the dark at room temperature.

Washing buffer (WB)

After putting 48 g Guanidinethiocyanate in a 250 ml-glass beaker, 40 ml 0.1 M Tris HCl (pH 6.40) was added. The beaker was covered with aluminium foil and placed in a water bath at 56 °C for 15 min to dissolve the ingredients. The solution was distributed into sterile 50 ml plastic tubes, covered with aluminium foil and stored in the dark at room temperature.

70 % Ethanol

Ethanol Rotipuran® 99.8 % (Carl Roth GmbH & Co, Karlsruhe, Germany)

After putting 35 ml of absolute ethanol into a sterile, nuclease free 50 ml tube, 15 ml Milli-Q® water was added. The tube was stored at room temperature.

Acetone

Acetone Rotipuran® 99.8 % (Carl Roth GmbH & Co, Karlsruhe, Germany)

RNase inhibitor

RiboLock™ RNase Inhibitor 40 U/µl (Fermentas GmbH, St. Leon-Rot, Germany)

One-step real time PCR reagents

TaqMan® One-Step RT-PCR Master Mix Reagents Kit, consists of:

- Taq Man® 2x Universal PCR Master Mix No AmpErase® UNG (= Reaction mix)
- 40x Multi Scribe™ and RNase Inhibitor Mix (=Enzyme mix)
  (Applied Biosystems Deutschland GmbH, Darmstadt, Germany)

3.2.4 Tenacity trials

Phosphate loading buffer (PLB)

88.9 parts of: Potassium di-hydrogen phosphate (KH_2PO_4) 98 % (Carl Roth GmbH & Co, Karlsruhe, Germany), 9.073 g/l dissolved in Milli-Q® water

11.1 parts of: di-Sodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_20) (Merck KgaA, Darmstadt, Germany), 11.87 g/l dissolved in Milli-Q® water

The pH of the loading buffer was adjusted to 6.00, 6.50, and 7.40 using 1 M NaOH and 1 M HCl.

Elution medium

Beef extract (BE) dry, granulated (Merck, KgaA, Darmstadt, Germany)

NaCl (Merck, KgaA, Darmstadt, Germany)

For AIV and NDV 4 % BE and 1 M NaCl with a pH 7.00 were mixed at a ratio of 1:1, while for ECBO 4 g BE and 2.9 g NaCl were dissolved in 100 ml Milli-Q® water and the pH was adjusted to 8.50 using 1 M NaOH and 1 M HCl.

3.3 Other materials and instruments

3.3.1 Cell culture and virology

96-well plates

Nunclon™ 96-well cell culture plates flat bottom	(Nunc GmbH, Wiesbaden, Germany)
BD Falcon™ Microtest cell culture plates flat bottom	(BD Biosciences Discovery Labware, Heidelberg, Germany)
Nunclon™ 96-well HA test plates, round bottom	(Nunc GmbH, Wiesbaden, Germany)

Cell culture flasks

Nunclon™ cell culture bottles with gas exchange cap (24 cm^2, 80 cm^2, and 175 cm^2)	(Nunc GmbH, Wiesbaden, Germany)
BD Falcon™ cell culture bottles with gas exchange cap 25 cm^2, 75 cm^2, and 175 cm^2	(BD Biosciences Discovery Labware, Heidelberg, Germany)

Egg incubator

With full automatic turning system	(Dipl. Ing. W. Ehret GmbH, Emmendingen, Germany)

Incubators for cell culture

Innova® CO-170 CO_2 incubator	(New Brunswick Scientific, Edison, USA)
Cytoperm Heraeus CO_2 incubator	(Kendro Laboratory Products, Hanau, Germany)

Electronic weighing balance

Precision balance Mettler PC 440 delta range®	(Mettler-Toledo GmbH, Giessen, Germany)

Microscopes

Inverted light microscope, Wilovert	(Will, Wetzlar, Germany)
Inverted light microscope, Epivert	(Leitz, Wetzler, Germany)

3.3.2 RRT-PCR

Step-one real-time PCR system	(Applied Biosystems Deutschland GmbH, Darmstadt, Germany)
Bench centrifuge 5415 D	(Eppendorf AG, Hamburg, Germany)
Ultrospec 2100 Pro UV/visible spectrophotometer	(Amersham Biosciences, Uppsala, Sweden)

48-well plates

(Applied Biosystems Deutschland GmbH, Darmstadt, Germany)

MicroAmp™ Fast Optical 48-well reaction plate (0.1 ml)

MicroAmp ™ 48-well optical adhesive film

3.3.3 Tenacity trials

Centrifuge

Varifuge 3.2 RS rotor number 5315	(Heraeus Sepatech GmbH, Osterode, Germany)

Incubators and environmental chambers

APT. line™ KB 115	(Binder GmbH, Tuttlingen, Germany)
Heraeus B 6420	(Kendro Laboratory Products, Hanau, Germany)
Refrigerator with ventilated air UG 1300	(Brown Boveri and Cie-Aktiengesellschaft Mannheim, Germany)
Dry sterilizer	(Willi Memmert, Schwabach, Germany)
Vortex shaker , EU Plug	(VWR International GmbH, Darmstadt, Germany)
Microprocessor pH meter pH 539	(Wissenschaftlich-Technische Werkstätten, Weilheim, Germany)
Ultrasonic bath (40 KHz)	(Bandelin Electronics, Berlin, Germany)

Syringe filter holder 13 mm	(Sartorius Stedim Biotech GmbH, Göttingen, Germany)
1.5 and 2 ml polypropylene tubes	(Eppendorf AG, Hamburg, Germany)
BD Falcon™ 15 ml and 50 ml polypropylene conical tubes	(BD Biosciences Discovery Labware, Heidelberg, Germany)
0.2 µm and 0.45 µm syringe filters	(Th. Geyer, Renningen, Germany)
Zeta Plus Virosorb 1MDS Filter 90 mm disc number 6408501	(Cuno Inc., Meriden, USA)
Glass fibre filter-GF 50	(Schleicher and Schuell Microscience GmbH, Dassel, Germany)
Glass fibre filter with organic binder MN 85/70	(Macherey-Nagel GmbH Düren, Germany)
PCM pore size 10 nm	(Pieper Filter GmbH, Bad Zwischenahn, Germany)

Surface water (SW)

Water was collected from Lake Constance at Langenargen, Germany, about 2 meters away from the shore, transported to the laboratory at low temperature, and stored at 4 °C until use.

Lake sediment

The freshly collected lake sediment from Lake Constance was procured from the Institut für Seenforschung, Langenargen, Germany, shortly before the beginning of each trial and stored in the laboratory at 4 °C until use. The sediment was collected from the “Gnadensee” part of Lake Constance partly beside the shore and partly about 1 kilometer away from the coast. A sediment sampler (“Sedimentstecher”) was used to collect the sediment from the upper 10 cm of the lake floor. The sediment was dense and pasty in consistency.

Duck feces

Initially, the duck feces samples were collected from a free-range duck farm situated in Schwäbisch Hall, Germany, by Dr. Werner Philipp and used for tenacity trials with H5N1 virus. Due to snow fall in the winter season the collection of feces from that farm was not possible. Later, the duck feces were collected from a duck farm situated at Sachsenheim, Germany, by Dr. Renate Haumacher. These ducks are kept indoors in the winter so the feces was slightly mixed with straw and feed residues. These feces were used for the tenacity studies with all other viruses.

Duck meat

Duck meat was purchased as frozen whole ducks from a supermarket. The carcasses were incised to separate the breast meat which was used for the tenacity studies.

3.4 Virus propagation

3.4.1 Propagation in SPF chicken embryos

The influenza viruses and NDV were propagated in the allantoic sac of VALO-SPF chicken embryos. The VALO-SPF-ECE were purchased from Lohmann Tierzucht GmbH, Cuxhaven, Germany, and incubated for 9-11 days in the egg incubator at 37 °C and 50-60 % humidity. The eggs were candled and only live embryos were used for virus propagation. To protect against cross-contamination, each virus was propagated separately. The virus inocula were filtered through 0.2 µm syringe filters and the embryos were inoculated by the method described by Senne (1998). After disinfection of the surface, a small hole was pricked into the egg shell and 0.1 ml of the virus suspension was injected into the allantoic sac. The hole was sealed with glue and eggs were incubated once again at 37 °C for another 72 hours. The embryos were checked after each 24 hours and any dead embryo was kept in the refrigerator. After 72 hours all of the embryos were chilled overnight and processed for the harvesting of AAF using sterile instruments. The harvested AAF was centrifuged at 5,000 rcf for 15 min, aliquoted in 1 ml, 10 ml, and 50 ml sterile plastic tubes and stored at -80 °C.

3.4.2 Propagation in cell culture

The ECBO was propagated in MDBK. The cells were sub cultured in 175 cm^2 cell culture flasks. After 24 hours of incubation, when the cell monolayer was 90 % confluent, the growth medium was discarded and 5 ml of virus suspension was pipetted onto the cell monolayer and incubated for one hour at 37 °C. Afterwards, 50 ml of maintenance medium was added to the flask and incubated at 37 °C and 5 % CO_2. The flasks were checked after 24 hours for cytopathic effects (CPE). Complete destruction of the cell monolayer occurred within 2-3 days, after which the flasks were placed in the freezer at -80 °C and subjected to 3 freeze-thaw cycles. The virus suspension was then removed from the flasks, centrifuged at 5,000 rcf for 15 min, aliquoted in 10 and 50 ml sterile plastic tubes and stored at -80 °C.

3.5 Cell culture procedure and preparation of 96-well plates

3.5.1 MDCK

After the removal of growth medium from a 75 cm^2 flask, the cell monolayer was washed with 5 ml TV solution. Five ml of TV solution was then added to the flask and incubated at room temperature for 5 min. Four ml of TV solution was removed, leaving 1 ml in the bottle followed by incubation at 37 °C for 20 min. When all of the cells were detached, 4 ml of growth medium was added and mixed by pipette in order to separate the cells. For sub-culturing in the flasks, a split ratio of 1:10 was used while for preparing 96-well plates the cells were diluted to a final concentration of 10^6 cells /ml in the growth medium and dispensed in a volume of 0.1 ml to each well of the 96-well plate. The plates and flasks were incubated at 37 °C with 5 % CO_2. The plates reached 90 % confluency after 24 hours of incubation and were then used for virus titration. The cells in the flasks formed a complete monolayer within 2-3 days.

3.5.2 MDBK and Vero cells

After the removal of growth medium from a 75 cm^2 flask, the cell monolayer was washed with 5 ml TV solution. Five ml of TV solution was then added to the flask, 4 ml was removed after washing and 1 ml left behind. The flasks were incubated at

room temperature for 5 min. When all of the cells were detached, 4 ml of growth medium was added and mixed by pipette in order to separate the cells. For sub-culturing in the flasks, a split ratio of 1:10 was used while for preparing 96-well plates the cells were diluted to a final concentration of 10^6 cells /ml in the growth medium and dispensed in a volume of 0.1 ml to each well of the 96-well plate. The plates and flasks were incubated at 37 °C with 5 % CO_2. The plates reached 90 % confluency after 24 hours of incubation and were then used for virus titration. A complete monolayer was formed in the flasks within 4-5 days.

3.6 Virus titration

The virus titrations were carried out by end point serial dilution method in 96-well microtitration plates. 0.9 ml of maintenance medium was added to each of several sterile plastic tubes. 0.1 ml of the virus suspension was then added to the first tube, mixed by vortexing and 0.1 ml of the suspension was then transferred to the next tube. Serial tenfold dilutions were thus made from 10^0 to 10^8. The cell culture plates (96-well) with 90 % confluency were used for the titrations. Growth medium was discarded from the plates and 100 µl of the virus-containing maintenance medium was added to the respective wells of the cultured cells (4 wells per dilution step). Growth and maintenance medium for Vero cells were used without NEA. In the wells inoculated with undiluted virus, 100 µl virus suspension was added, incubated at 37 °C for 1 hour, virus suspension was then removed and replaced with 100 µl maintenance medium, while cell control wells received 100 µl of the maintenance medium. Plates were incubated for 7 days at 37 °C and 5 % CO_2 and checked afterwards for CPE by light microscopy. A complete destruction of the cell monolayer was regarded as positive for virus growth. Tissue culture infective $dose_{50}$ ($TCID_{50}$) values were calculated by the Spearman-Kärber method (Villgas, 1998). Minimum detectable limit of the assay was $10^{1.75}$ $TCID_{50}$/ml. The infectivity titers below the detection limit were regarded as zero.

3.7 Bacterial Counts

The total bacterial counts (TBC) in the SW samples were determined using the method described by Drca (2007). Briefly, the TBC was determined by spread plate method on Standard 1 nutrient agar. The water samples were serially tenfold diluted in sterile NS. Then, 0.1 ml of the diluted sample was poured on the agar plates and uniformly spread on the surface of the agar using a sterile glass rod. Duplicate plates were inoculated for each dilution. Plates were incubated at 37 °C under aerobic conditions for a period of 24 hours. Afterwards, colonies were counted from those plates having between 20-200 colonies. The average of two plates was taken and multiplied by the dilution factor to calculate the colony forming units (CFU)/ml of the sample.

3.8 HA test procedure

For the influenza viruses, virus replication were confirmed by HA test which was carried out in accordance with the directives of the OIE (2008). The U bottom 96-well plates were used for the test and 25 µl of NS was pipetted into each well of the plates. An amount of 25 µl of cell culture supernatant from the titration plate was then transferred independently into the respective well of the U bottom plate. Afterwards, 25 µl of 1 % washed chicken erythrocytes were added to each well. Negative control wells contained only NS while positive control wells received the known virus suspension. The plates were incubated at room temperature for 20-30 min. The agglutinated erythrocytes made a carpet on the bottom of the plates showing positive results while free erythrocytes accumulated in the centre as a small bead showing negative results.

3.9 Extraction of viral RNA

Extraction of viral RNA was performed as described by Boom et al. (1990) using the silica matrix method. An amount of 0.3 ml of the sample was pipetted into a 1.5 ml nuclease free plastic tube. Then 0.9 ml of LB and 0.04 ml silica matrix were added to the sample, incubated at room temperature for 10 min and vortexed every 3 min. The

sample was centrifuged at 14,000 rcf for 30 seconds at 4 °C. The supernatant was discarded. Afterwards, 1 ml WB was added to each tube, vortexed, centrifuged at 14,000 rcf for 30 seconds at 4 °C and supernatant was discarded. This washing step was repeated once, followed by two washing steps with 70 % ethanol. Subsequently, 1 ml acetone was added to the sediment, the tubes were vortexed and centrifuged at 14,000 rcf for 3 min at 4 °C. The supernatant was discarded and the tubes were turned over onto paper to remove the rest of the acetone. The pellet was dried at 60 °C for 10 min. After drying, 0.075 ml nuclease-free water and 0.7 µl RNase-inhibitor were added, vortexed and incubated at 60 °C for 15 min, during which the tubes were vortexed at 5 min intervals. The tubes were then centrifuged again at 14,000 rcf for 3 min at 4 °C. An amount of 60 µl supernatant from each tube was transferred to a new 0.5 ml RNase free tube without silica matrix. These tubes were centrifuged again, and 50 µl of supernatant from each tube was collected, transferred to a new RNase free tube, labelled, and stored at -80 °C.

3.10 PCR analysis

3.10.1 RRT-PCR

The RRT-PCR was performed according to the method described by Spackman et al. (2002), based on the detection of a portion of the M-gene (fragment of ~ 100 bp in segment 7). The PCR protocol was adopted from the CVUA, Stuttgart (Hoferer, 2006). The internally modified primer sequences used in the PCR protocol were obtained from the Cantonal Laboratory, Basel, Switzerland (Vogel and Alt, 2005). Sequences of the primer pair and probe are shown in Table 3.4. The master mix was collectively prepared for all of the samples processed at once in a 1.5 ml reaction tube. For each reaction, 5 µl nuclease free water, 12.5 µl reaction mix, 0.5 µl enzyme mix, and 2 µl primer-probe mix (Table 3.5) were added to a final volume of 20 µl. The master mix was calculated with a safety margin of two samples per plate. After adding the reaction mixture to the wells of a 48-well plate, 5 µl of the sample was added to the respective wells. The plate was sealed with MicroAmp™ 48-well optical adhesive film and immediately placed in the step one real-time PCR system.

Table 3.4: Sequences of the primers and probe used in RRT-PCR for influenza A virus M-gene detection as described by Spackman et al. (2002) and modified by Vogel & Alt, (2005)

Designation	Sequence
InfA_2_For (Forward)	5'-AGATGAGYCTTCTAACMGAGGTC-3'
InfA_2_Rev (Reverse)	5'-GCAAAIACATCYTCAAGTYTCTG-3'
InfA_2_FAM (Probe)	6FAM-TCAGGCCCCCTCAAAGCCGA-BHQ2

Table 3.5: Primer probe mix as described by Hoffmann (2006)

Reagent	Volume (concentration of stock solution)	Final concentration
Primer 1 (InfA_2_For)	20 µl (100 pmol/µl)	10 µM
Primer 2 (InfA_2_Rev)	30 µl (100 pmol/µl)	15 µM
Probe (InfA_2_FAM)	2,5 µl (100 pmol/µl)	1.25 µM
Milli-Q®	147.5 µl	-

The PCR was performed according to following temperature profile

1. Reverse Transcription	30 min	50 °C	
2. Inactivation / Activation	15 min	95 °C	
3. Denaturation	30 sec	95 °C	**42 Cycles**
4. Annealing	30 sec	57 °C	
5. Elongation	30 sec	72 °C	

3.10.2 Quantitative RRT-PCR (qRRT-PCT)

For a comparative quantitation of viral RNA in the samples a qRRT-PCR was performed. A serial dilution of a defined amount of viral RNA was used to make a standard curve for the quantitation of RNA in the experimental samples. The H6N8 AIV RNA extracted by RNeasy Kit (QIAGEN Sciences, Maryland, USA) from the infective AAF was diluted in nuclease free water to a final concentration of 1 nanogram (ng)/ml as measured by spectrophotometry at 260 nm wavelength. One ng of the viral RNA was serial tenfold diluted from 10^0-10^4. For each PCR run the diluted defined viral RNA was added to separate wells in the 48-well plates along with the negative and positive controls and the samples to be tested. The extracted viral RNA samples from the tenacity trials were diluted tenfold before they were added to the 48-well plates. For each sample (known viral RNA, positive controls, and the samples to be tested), the reaction was performed in duplicate wells while for negative controls nuclease free water was added in 8 wells. The qRRT-PCR run was performed as described in section 3.10.1 and the results were analysed by the software: StepOne^{TM} Software Version 2.0 (Applied Biosystems, Deutschland GmbH, Darmstadt).

3.11 Germ carrier technique

Before starting the tenacity studies in various substrates, virus recovery from a virus loaded substrate was assessed. These spiking trials were performed using lake sediment as a substrate. During several repeats of the experiments it was noticed that the virus titer dropped by three $logs_{10}$ immediately after addition of the virus to the sediment. Ten ml of AAF with the AIV H5N1 (with a virus titer of $10^{6.25}$ $TCID_{50}$/ml) was mixed with 10 g of lake sediment and allowed to stand for 15 min. The supernatant collected afterwards had a virus titer of $10^{3.0}$ $TCID_{50}$/ml, indicating a loss of more than 99.9 % of the virus titer. This phenomenon necessitated the use of an appropriate germ carrier for the tenacity studies.

Initially, 3 each Zeta Plus Virosorb 1MDS filters, glass fibre filter-GF 50 and glass fibre filter with organic binder MN 85/70 were analyzed to determine the appropriate

filter to be used as a germ carrier. Of these, the Zeta Plus Virosorb proved better in virus adsorption properties while the other two filters were thin and became flimsy after the filtration was carried out, and were therefore inappropriate for use in germ carrier studies. Virosorb filters were purchased as 90 mm discs (disc number 6408501) and then cut into small circular pieces of 15 mm diameter, sterilized by autoclaving, and kept in a drying oven overnight before use. The term germ carrier is hereafter used for these 15 mm Zeta Plus Virosorb discs. Optimization experiments were performed using the H5N1 virus.

3.11.1 Effect of loading medium on virus adsorption to the carriers

To facilitate the adsorption of virus particles to the germ carrier, phosphate loading buffers (PLB) with pH 6.00, 6.50, and 7.40 were tested. After mixing virus containing AAF with PLB at a ratio of 1:10, the required pH was adjusted using 1 M NaOH and 1 M HCl. An amount of 5 ml of the mixture was filtered through each disc with the help of a sterile syringe filter holder device. After filtration each disc was placed in 2 ml of elution medium (4 % BE and 1 M NaCl, pH 7.00 mixed in a ratio of 1:1) adopted from Traub et al. (1986). Germ carriers containing elution medium were subjected to sonication in an ice bath for 5 min and then centrifuged at 2,000 rcf for 15 min as described by Haumacher et al. (2009). The eluted virus was then titrated on MDCK as described in section 3.6. Duplicate germ carriers were tested each time and the whole experiment was repeated three times.

3.11.2 Effect of drying on the titer of filter bound virus

A total of twenty germ carriers were prepared as described in section 3.11.1 by mixing infective AAF in PLB pH 7.40 at a ratio of 1:10. Six germ carriers were kept covered in a Petri dish containing tissue paper soaked with sterile DW and twelve were placed in an open Petri dish without moisture. Both dishes were kept at ambient temperature in the safety cabinet. Virus quantitation was carried out at the beginning and after each hour for dry and every 2 hours for wet germ carriers for a period of 6 hours. Elution followed by titration for each germ carrier was performed as

described in sections 3.11.1 and 3.6. Duplicate germ carriers were checked each time and three repetitions of the whole experiment were performed.

3.12 Tenacity of the influenza and model viruses in various types of water

Three different water types were used for the tenacity studies: DW with a pH 7.80; NS, pH 7.20, and SW obtained from Lake Constance.

3.12.1 Persistence of the viruses in DW and NS

DW and NS were autoclaved after adjustment of the pH to the respective values using sterile 0.1 M HCl and 0.1 M NaOH. For the influenza viruses as well as NDV, the infective AAF and for ECBO infective cell culture supernatant was mixed into the water at a ratio of 1:10 for DW and NS, and aliquoted into sterile 1.5 ml polypropylene tubes at a volume of 1 ml per tube. The tubes were equally divided into five groups and placed in thermostatically controlled incubators or environmental chambers whose temperatures were previously adjusted to -10, 0, 10, 20, and 30 °C. Titrations of the samples were carried out on day 0 and then afterwards at regular intervals: after each 2 days at 30 °C, 4 days at 20 °C, 14 days at 10 °C, and 28 days at 0 and -10 °C.

Duplicate samples were tested at each time point for all of the treatment groups. The samples were tested for a maximum of 36 weeks. However, the titration of respective samples was terminated if no virus was detectable for two successive attempts. In virus inoculated water samples, pH was tested at the termination of the experiments and found to be around the adjusted values with a difference of ± 0.1.

3.12.2 Persistence of the viruses in SW

3.12.2.1 Persistence of viruses after suspending in the SW

SW used for the trials was checked for the presence of any endogenous influenza viruses by cell culture inoculation as well as cytotoxicity to MDCK and found to be negative. SW was used for tenacity trials without any treatment and also subjected to chemical analysis. Bacterial counts in the SW were performed at the beginning of the

experiment and when no virus was detectable in the H5N1 virus inoculated water samples. The virus suspensions were mixed in SW at a ratio of 1:100, as in spiking trials adding a high concentration of AAF to SW promoted intense microbial growth while adding too little AAF provided insufficient virus titers for tenacity studies. Samples were distributed into small tubes and placed at five different temperatures as described in section 3.12.1. Similarly, titration of all of the samples was carried out at the start of the experiments and then afterwards at regular intervals i.e. daily at 30 °C, every 2 days at 20 °C, every 7 days at 10 °C, and every 14 days at 0 and -10 °C. Before titration, each of the SW samples was centrifuged at 5,000 rcf for 10 min and filtered through 0.45 µm pore size disposable syringe filters. Duplicate samples were tested at each time point for all of the treatment groups. The samples were tested for a maximum of 36 weeks. However, the titration of respective samples was terminated if no virus was detectable in two successive attempts.

The samples containing H4N6, H5N1, and H6N8 AIV were also processed for the quantitation of viral RNA by qRRT-PCR. RNA extraction was performed as described in section 3.9 from each of the duplicate samples at the beginning of each trial and afterwards when no virus was detectable by titration on cell culture.

3.12.2.2 Persistence of the viruses in SW using germ carrier technique

To check the persistence of the six viruses in SW filter germ carriers were prepared by mixing H1N1, H4N6, H5N1, H6N8, or NDV virus stock solutions in PLB, pH 7.40 and ECBO stock solution in PLB, pH 6.00 at a ratio of 1:10 as modified from Traub et al. (1986). An amount of 5 ml of the mixture was filtered through each disc with the help of a sterile syringe filter holder device. The filter discs were then sandwiched between sterile PCM with pore size 10 nm as shown in Figure 3.1. Several such sandwich germ carriers were prepared for each virus and always kept moist before use. About 10 ml SW was filled in sterile 15 ml plastic tubes and two germ carriers were placed in each tube. The tubes were then transferred to incubators previously adjusted to temperatures of 30, 20, 10, 0, and -10 °C. Titration of virus samples was performed at regular intervals: after each 4 days at 30 °C for 16 days, 8 days at 20 °C for 32 days, 2 weeks at 10 °C for 2 months, and 4 weeks at 0 and -10 °C for

4 months. Each time duplicate samples were tested by removing sandwich germ carriers from the water. The outer surface of the PCM was wiped with tissue paper and filter discs were removed with sterile forceps and placed immediately in 2 ml of the elution medium (3.2.4) for the respective viruses. The elution process was performed as described in section 3.11.1 and titration of eluted viruses was performed on respective cells as described in section 3.6. The bacterial count and viral titrations were also performed from the water samples containing H5N1 germ carriers in the beginning and at the termination of each trial at five temperatures

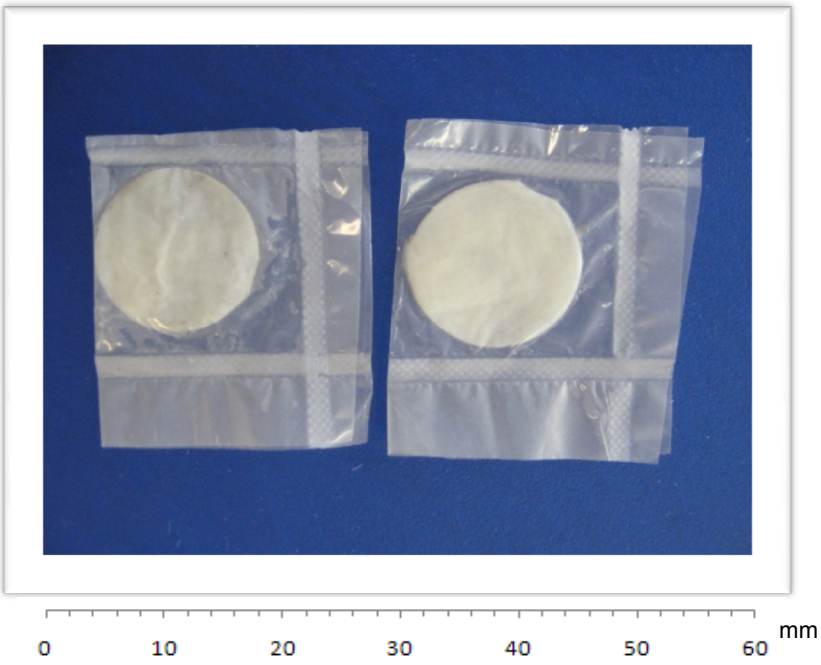


Figure 3.1: Filter germ carriers sandwiched between PCM

3.13 Tenacity in lake sediment

Lake sediment collected from Lake Constance was used to study the tenacity of the six viruses. Sandwich germ carriers were prepared for all of the viruses as described in section 3.12.2.2. Several such carriers were prepared for each virus and always kept moist before use under the sterile cabinet and were used within 2-3 hours of

their preparation. The trials with all of the viruses were started at different times: after starting with one virus the trial with another virus was started a week later. About 30-40 ml of sediment was filled in sterile 50 ml plastic tubes and three germ carriers were placed in each tube in such a way that they were surrounded by sediment (Figure 3.2). The tubes were then transferred to incubators previously adjusted to temperatures of 30, 20, 10, 0, and -10 °C. Titration of virus samples was performed at regular intervals: after each 2 days at 30 °C for 30 days, 4 days at 20 °C for 60 days, weekly at 10 °C for 14 weeks, and every 2 weeks at 0 and -10 °C for 6 months. Each time triplicate samples were tested by removing sandwich germ carriers from the sediment. The germ carriers were placed in a Petri dish and the outer surface of the PCM was washed with sterile Milli-Q® water to remove the sediment. Then after tearing the PCM open, the filter discs were removed with sterile forceps and placed immediately in 2 ml of the elution medium and further processed for the elution and titration of residual viral infectivity in the samples as described in sections 3.11.1 and 3.6.

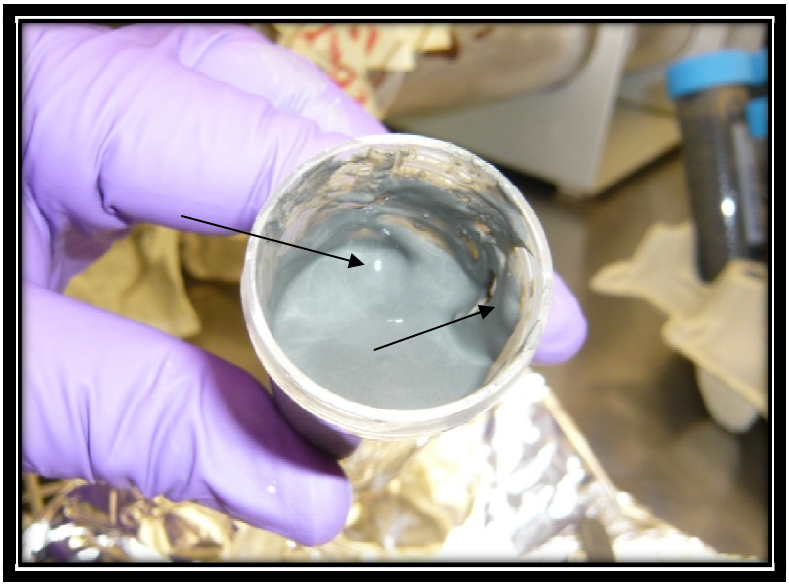


Figure 3.2: Sandwich germ carrier embedded in the lake sediment

3.14 Tenacity in duck feces

Sandwich germ carriers were prepared for all of the viruses as described in section 3.12.2.2. Several such carriers were prepared for each virus and always kept moist before use under the sterile cabinet and were used within 2-3 hours of their preparation. The trials with all of the viruses were started independently with an interval of one week between each trial. About 30-40 g of duck feces was filled into each of several sterile 50 ml plastic tubes and three germ carriers were placed in each tube. For close contact between feces and germ carrier adsorbed virus, the germ carriers were placed separately and deeply embedded in the feces. The tubes were then transferred to incubators previously adjusted to temperatures of 30, 20, 10, 0, and -10 °C. Virus titrations from the samples were performed at regular intervals: daily for influenza viruses and every 2 days for model viruses at 30 °C for 14 days, after each 2 days for influenza viruses and 4 days for model viruses at 20 °C for 28 days, weekly for influenza viruses, and every two weeks for model viruses at 10 °C for 12 weeks, every 2 weeks for influenza viruses, and monthly for model viruses at 0 and -10 °C for 6 months. Each time triplicate samples were tested by removing sandwich germ carriers from the duck feces. The germ carriers were placed in a petri dish and the outer surface of the PCM was washed with sterile Milli- Q® water to remove the faecal debris. Then after tearing the PCM open, the filter discs were removed with sterile forceps and placed immediately in 2 ml of elution medium. Elution and virus titration for the respective viruses was performed as described in sections 3.11.1 and 3.6.

3.15 Tenacity in duck meat

Sandwich germ carriers were prepared for all of the viruses as described in section 3.12.2.2. Several such carriers were prepared for each virus and always kept moist before use under the sterile cabinet and were utilized within 2-3 hours of their preparation. The trials with all of the viruses were started independently with an interval of one week between each trial. About 20-30 g of duck breast meat was placed in each of several sterile 50 ml plastic tubes and three germ carriers were

placed in each tube. The meat was sliced to make small pockets for putting in the germ carriers and each carrier was placed in a separate pocket for close contact of the filter adsorbed virus with the meat contents (Figure 3.3). The tubes were then transferred to incubators previously adjusted to temperatures of 30, 20, 10, 0, and -10 °C. Titration of virus samples was performed at regular intervals: every 2 days at 30 °C for 14 days, every 4 days at 20 °C for 28 days, weekly at 10 °C for 12 weeks, and every 2 weeks at 0 and -10 °C for 6 months. Each time triplicate samples were tested by removing sandwich germ carriers from the meat. The germ carriers were placed in a petri dish and the outer surface of the PCM was washed with sterile Milli-Q® water and wiped with tissue paper. Then, after tearing the PCM open, the filter discs were removed with sterile forceps and placed immediately in 2 ml of elution medium. Elution and virus titration for the respective viruses was performed as described in sections 3.11.1 and 3.6.

Figure 3.3: Sandwich germ carriers placed in the meat

3.16 Analysis of experimental results

For the assessment of tenacity trials of the six viruses in various types of water in all treatment groups, infectivity titers of duplicate samples were recorded for each titration as $TCID_{50}$/ml in the form of log base 10. For the tenacity studies of all virus types in other substrates (lake sediment, duck feces, and duck meat) the virus titrations form three germ carriers were recorded each time as $TCID_{50}$/ml in the form

of log base 10. For calculating the arithmetic mean, the 2 or 3 logarithmic figures were changed to arithmetic numbers and their mean was again converted to logarithmic values. The serial data thus obtained was analyzed by a linear regression model with the help of Microsoft excel (Microsoft office excel 2007; Microsoft corporation, Redmond, Washington, USA). The T-90 values (time required for one log reduction in the virus titer) were calculated using this model. The estimated persistence of viral infectivity with a starting viral concentration of $10^{6.00}$ $TCID_{50}$/ml was also calculated using this model.

3.16.1 Linear regression analysis for the samples stored at -10 °C

The germ carrier technique was used to estimate the persistence of the viruses in SW, sediment, duck feces, and meat. The virus recovery data showed that the single freeze-thaw cycle at -10 °C resulted in an abrupt decline in the titer of the filter bound viruses. Due to the effect of freeze-thawing on viral infectivity, the zero day time point looks like an outlier in the regression analysis. Hence the regression analyses on -10 °C samples were also performed excluding the 0 day data point.

3.16.2 Calculation of viral RNA per ml of the sample

For the calculation of the amount of viral RNA per ml of the original sample, the amount of RNA as measured by qRRT-PCR was multiplied by the dilution factor (50) which is calculated according to following formula:

Total volume of RRT-PCR mixture (25 µl)/ Volume of RNA sample (5 µl) = 5.

The extracted RNA was diluted tenfold before addition in the RRT-PCR mixture so

5 X10 = 50 (dilution factor).

The end value of viral RNA was calculated as picograms (pg)/ml of the sample.

4 Results

4.1 Microbiological and physicochemical characteristics of the SW used in the tenacity trials

The TBC in SW was 1.15 x 10^2 CFU/ml. The count increased in the virus inoculated samples to 1.8 x 10^7 CFU/ml at 30 °C after 7 days, 3.3 x 10^7 CFU/ml at 20 °C after 14 days, and 7.9 x 10^6 CFU/ml at 10 °C after 21 days. No significant increase in the bacterial count was observed in the samples stored at 0 and -10 °C. Furthermore, fungal growth in the form of small balls was evident in some of the tubes stored at 10 °C and associated with comparatively low virus titers. The other parameters checked in the beginning of the trials were pH: 7.84, hardness: 7.4 °dH, electrical conductivity: 280 µS/cm, salinity: 150 parts per million (ppm), Ca concentration: 42 mg/l and Mg concentration: 7.9 mg/l.

In case of SW used to study the persistence of the viruses by germ carrier technique, the TBC was 1.7 x 10^2 CFU/ml at the beginning of the studies. This increased to 9 x 10^4, 3.6 x 10^4, and 11 x 10^3 CFU/ml at 30, 20, and 10 °C while no increase in the microbial count was observed in the samples stored at 0 or -10 °C. No virus was detected by titration on MDCK in those water samples tested in which LPAI H5N1 virus containing germ carriers were immersed and stored at all of the five temperatures.

4.2 Tenacity of influenza and model viruses in various types of water

The linear regression models along with the estimated persistence of influenza and model viruses with a starting viral concentration of $10^{6.00}$ $TCID_{50}$/ml in various types of water at different temperatures are presented in the appendix as Tables 9.1-9.5. Linear regression models show that persistence of all of the viruses was highest at -10 °C followed by 0, 10, 20, and 30 °C. In general, influenza viruses persisted for shorter times than the model viruses while ECBO had the highest survival time in lake water as apparent from Figures 4.1 to 4.30. There were clear differences between the individual influenza virus strains in their tenacity at the test temperatures.

4.2.1 Tenacity of LPAI H4N6 virus in DW, NS, and SW

The linear regression models for the persistence of LPAI H4N6 virus in DW, NS, and SW are presented in Figures 4.1 to 4.5. Virus persistence was inversely proportional to the storage temperature. In DW with a starting virus titer of $10^{5.14}$ $TCID_{50}$/ml the virus was detectable for 30, 60, ≥ 252, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively. In NS with a starting virus titer of $10^{5.14}$ $TCID_{50}$/ml the virus was detectable for 22, 44, 224, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively, while in SW with a starting virus titer of $10^{4.14}$ $TCID_{50}$/ml the virus was detectable for 4, 8, 21, 56, and 182 days at 30, 20, 10, 0, and -10 °C, respectively.

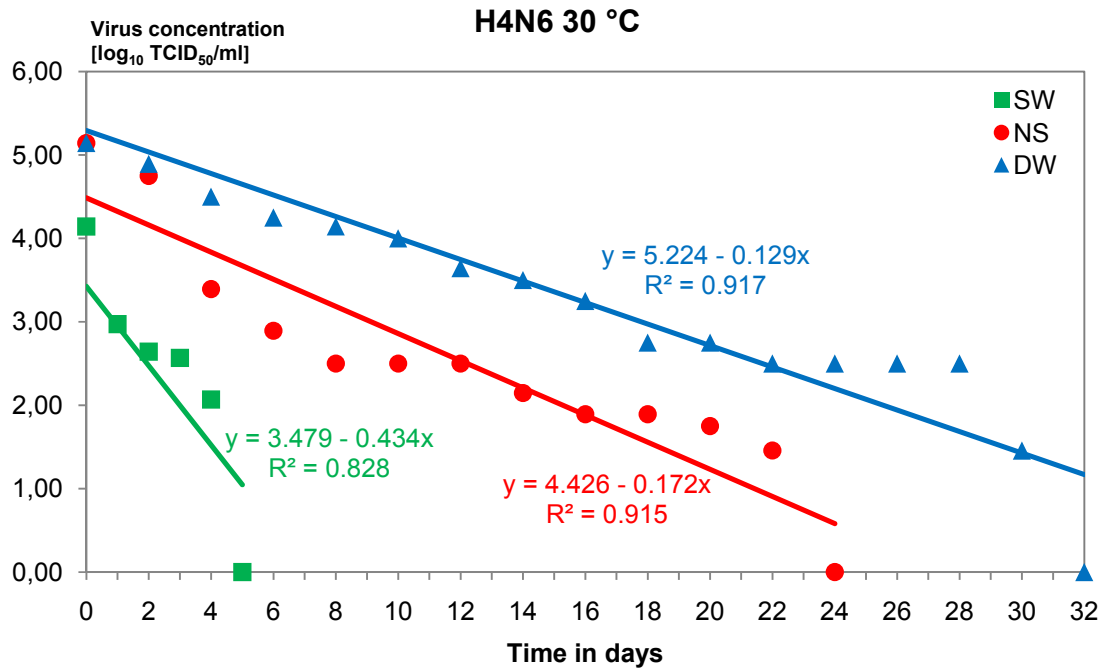


Figure 4.1: Persistence of LPAI H4N6 virus in DW, NS, and SW at 30 °C

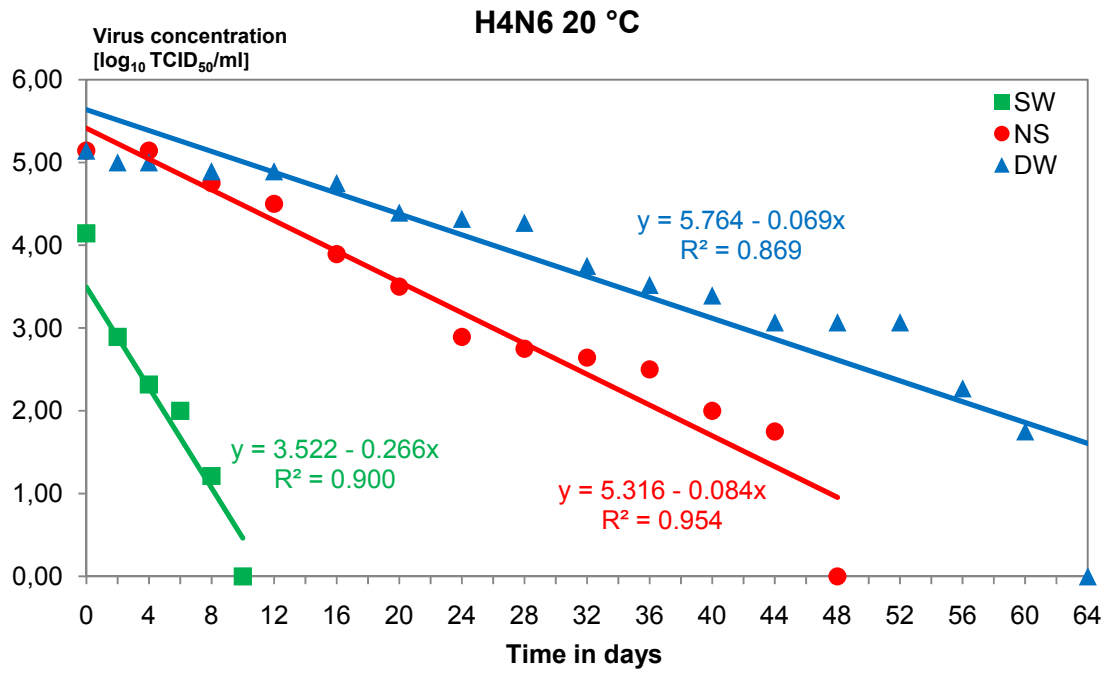


Figure 4.2: Persistence of LPAI H4N6 virus in DW, NS, and SW at 20 °C

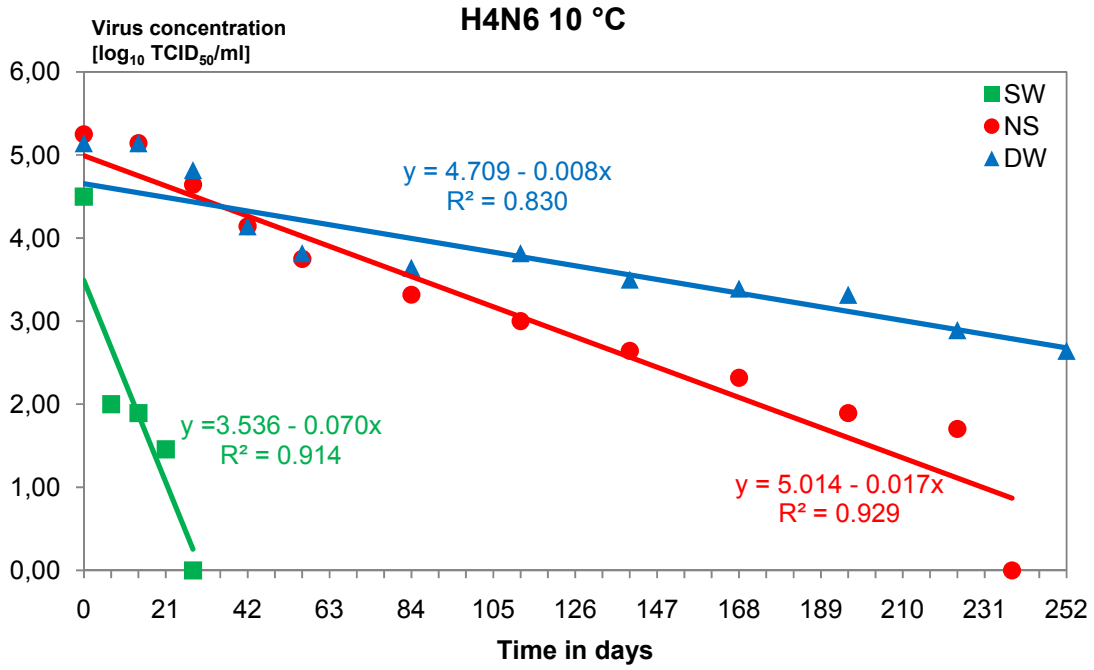


Figure 4.3: Persistence of LPAI H4N6 virus in DW, NS, and SW at 10 °C

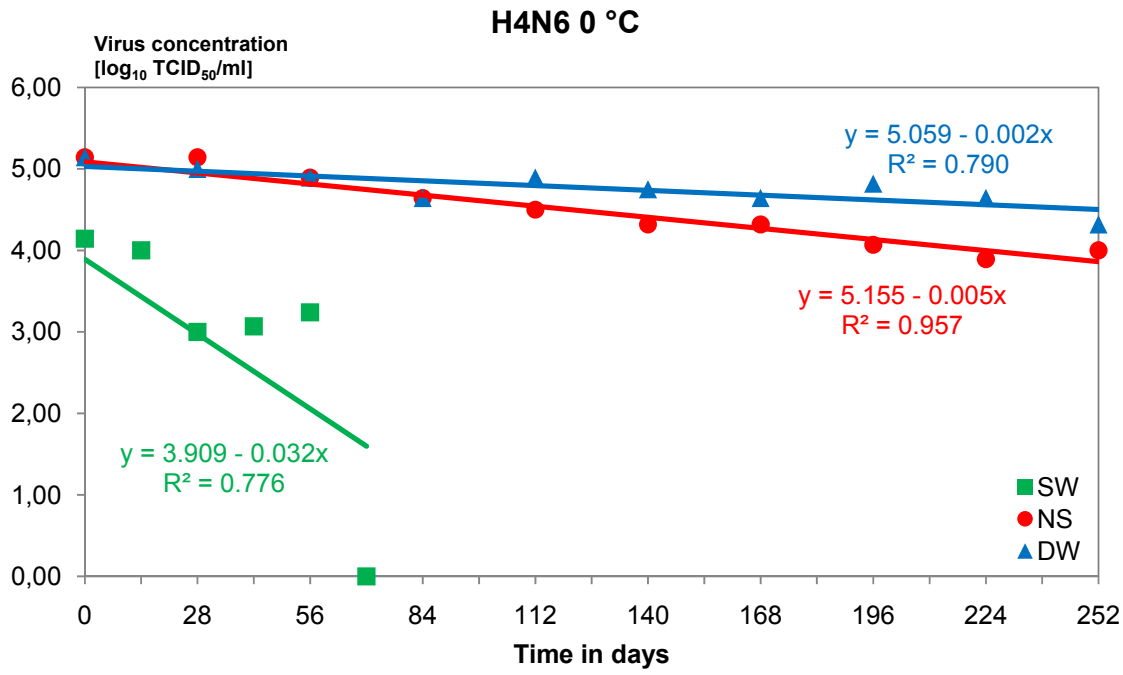


Figure 4.4: Persistence of LPAI H4N6 virus in DW, NS, and SW at 0 °C

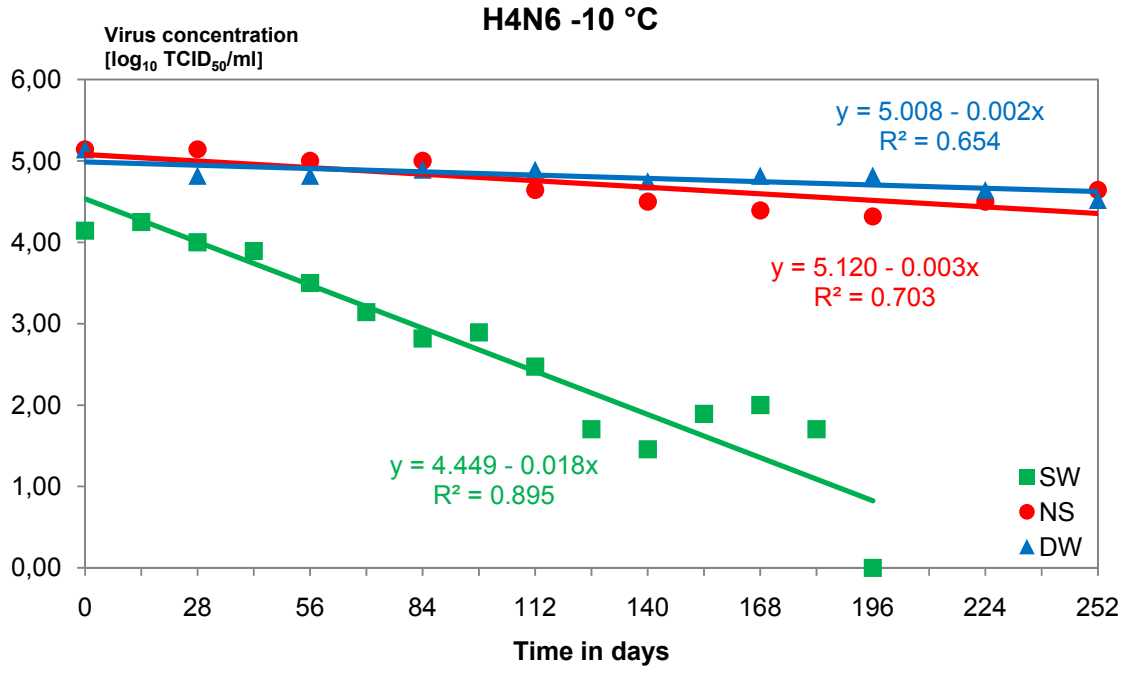


Figure 4.5: Persistence of LPAI H4N6 virus in DW, NS, and SW at -10 °C

4.2.2 Tenacity of LPAI H5N1 virus in DW, NS, and SW

The linear regression models showing the persistence of LPAI H5N1 virus in DW, NS, and SW are presented in Figures 4.6 to 4.10. Virus persistence was inversely proportional to the storage temperature. In DW with a starting virus titer of $10^{5.00}$ $TCID_{50}$/ml the virus was detectable for 20, 52, ≥ 252, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively. In NS with a starting virus titer of $10^{5.25}$ $TCID_{50}$/ml the virus was detectable for 20, 52, 224, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively, while in SW with a starting virus titer of $10^{4.50}$ $TCID_{50}$/ml the virus was detectable for 3, 6, 21, 42, and 182 days at 30, 20, 10, 0, and -10 °C, respectively.

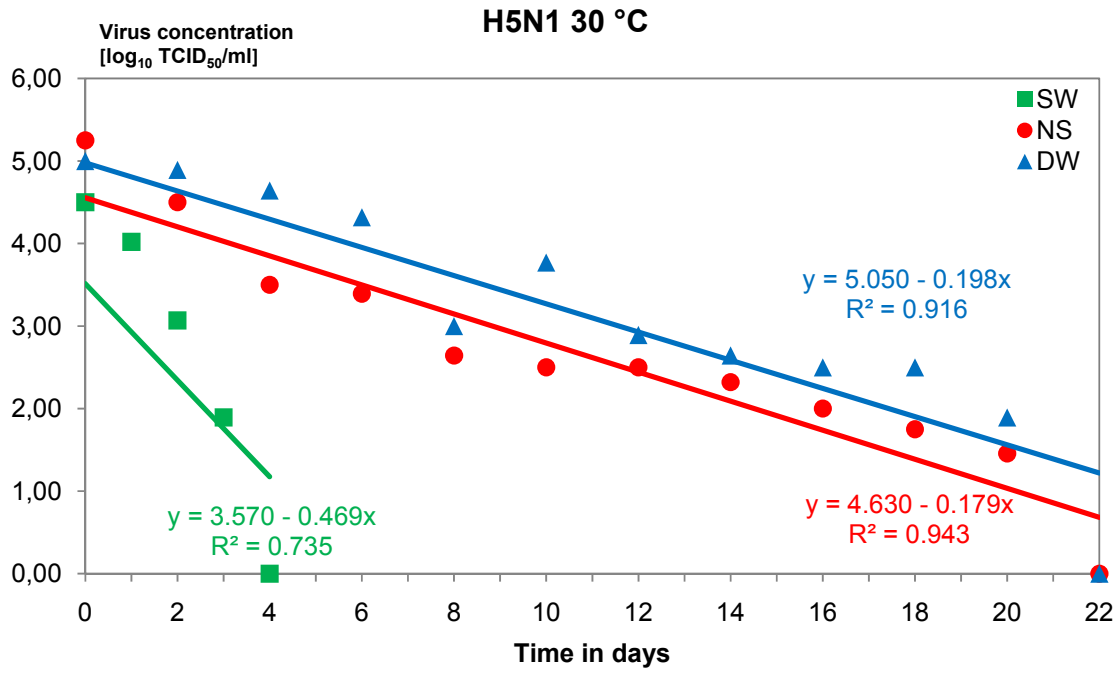


Figure 4.6: Persistence of LPAI H5N1 virus in DW, NS, and SW at 30 °C

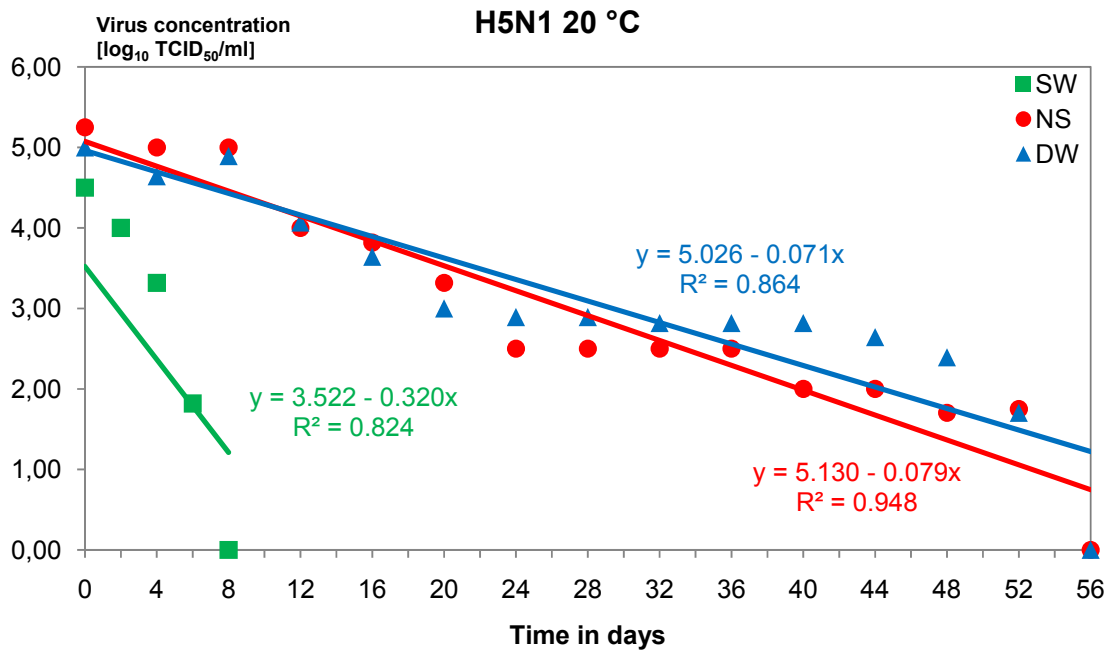


Figure 4.7: Persistence of LPAI H5N1 virus in DW, NS, and SW at 20 °C

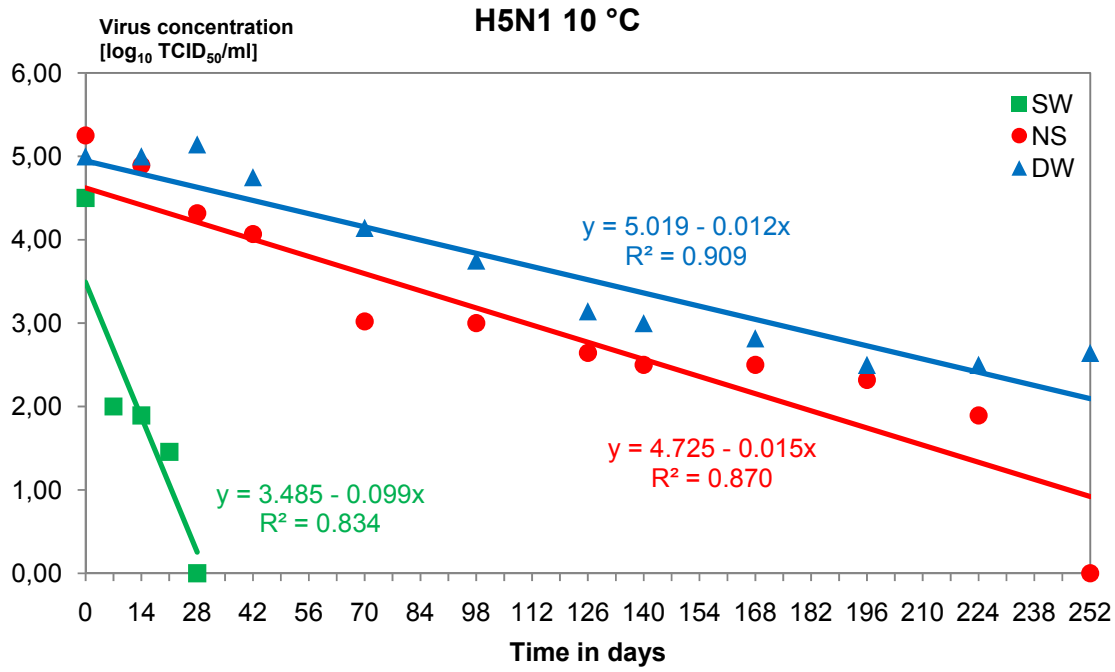


Figure 4.8: Persistence of LPAI H5N1 virus in DW, NS, and SW at 10 °C

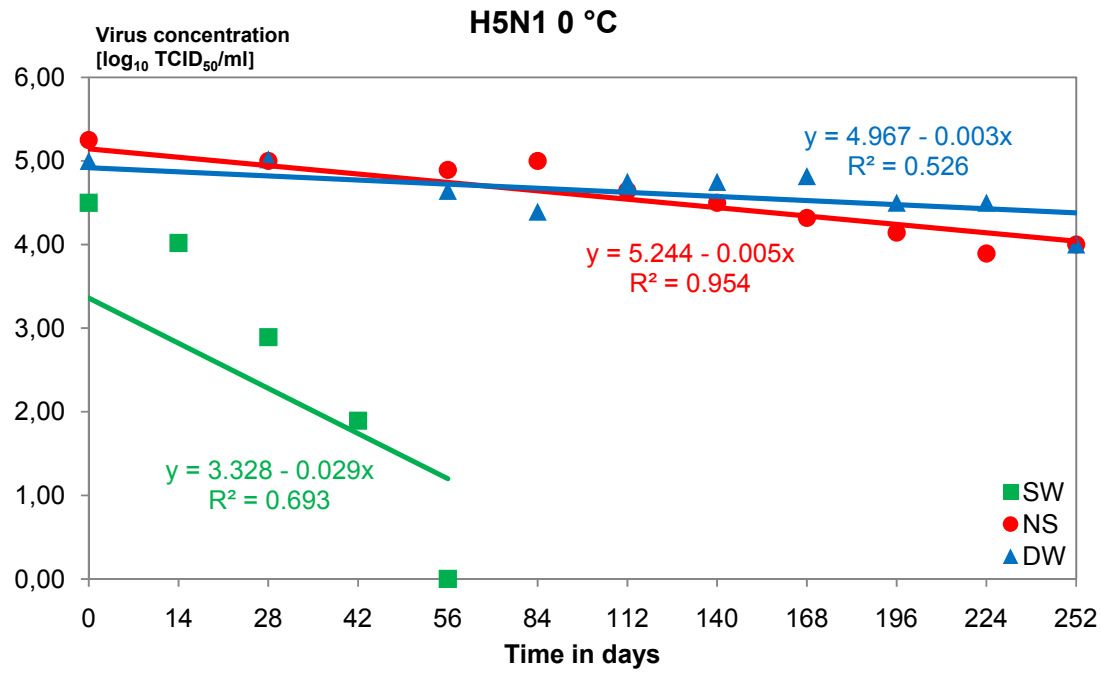


Figure 4.9: Persistence of LPAI H5N1 virus in DW, NS, and SW at 0 °C

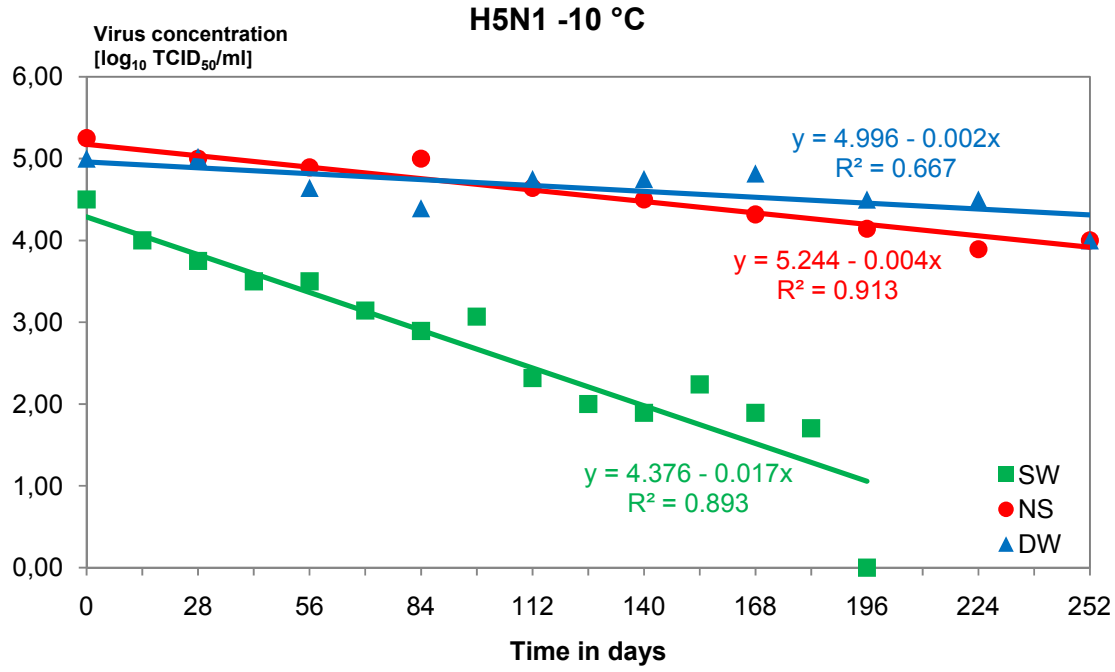


Figure 4.10: Persistence of LPAI H5N1 virus in DW, NS, and SW at -10 °C

4.2.3 Tenacity of LPAI H6N8 virus in DW, NS, and SW

The linear regression models showing the persistence of LPAI H6N8 virus in DW, NS, and SW are presented in Figures 4.11 to 4.15. The persistence was inversely proportional to the storage temperature. In DW with a starting virus titer of $10^{5.39}$ $TCID_{50}$/ml the virus was detectable for 42, 144, ≥ 224, ≥ 224, and ≥ 224 days at 30, 20, 10, 0, and -10 °C, respectively. In NS with a starting virus titer of $10^{5.50}$ $TCID_{50}$/ml the virus was detectable for 10, 28, ≥ 224, ≥ 224, and ≥ 224 days at 30, 20, 10, 0, and -10 °C, respectively, while in SW with a starting virus titer of $10^{5.14}$ $TCID_{50}$/ml the virus was detectable for 7, 12, 42, 112, and ≥ 224 days at 30, 20, 10, 0, and -10 °C, respectively.

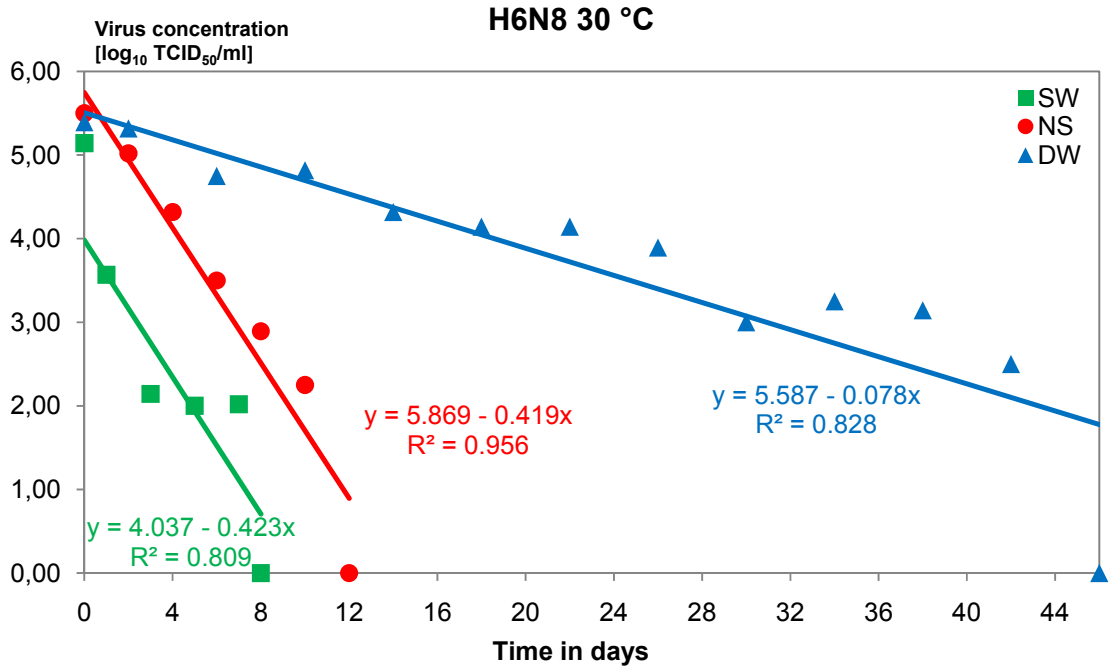


Figure 4.11: Persistence of LPAI H6N8 virus in DW, NS, and SW at 30 °C

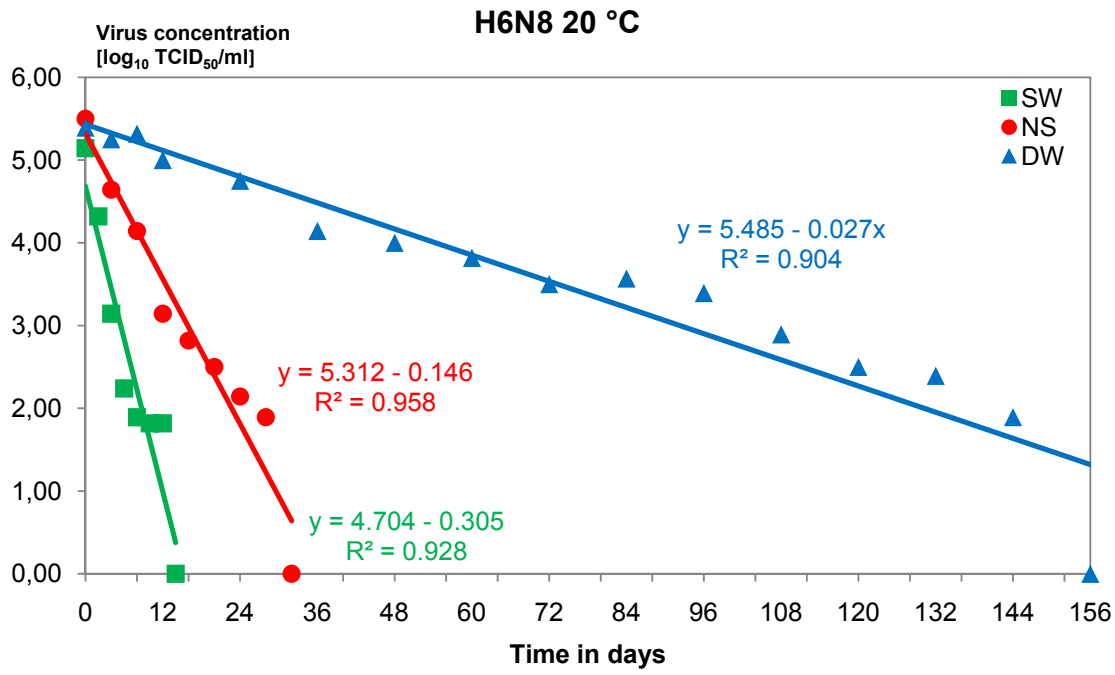


Figure 4.12: Persistence of LPAI H6N8 virus in DW, NS, and SW at 20 °C

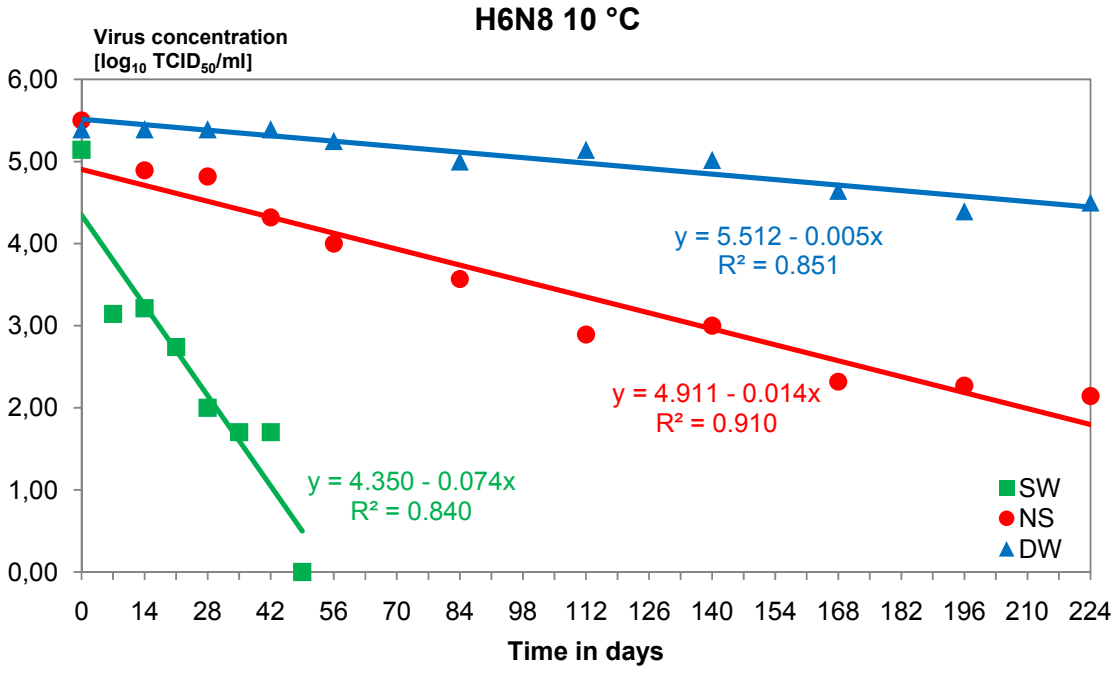


Figure 4.13: Persistence of LPAI H6N8 virus in DW, NS, and SW at 10 °C

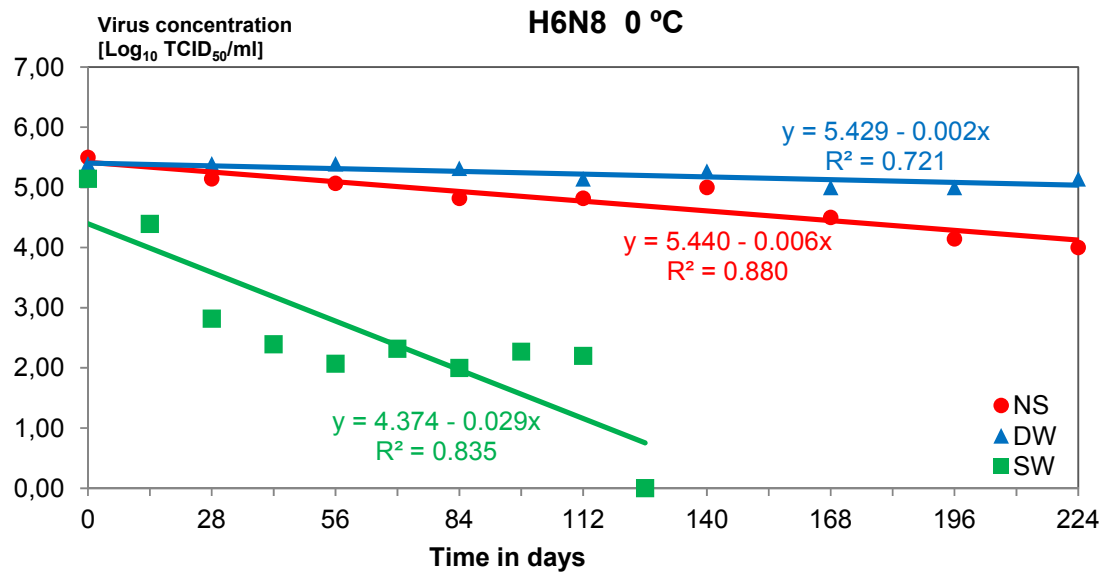


Figure 4.14: Persistence of LPAI H6N8 virus in DW, NS, and SW at 0 °C

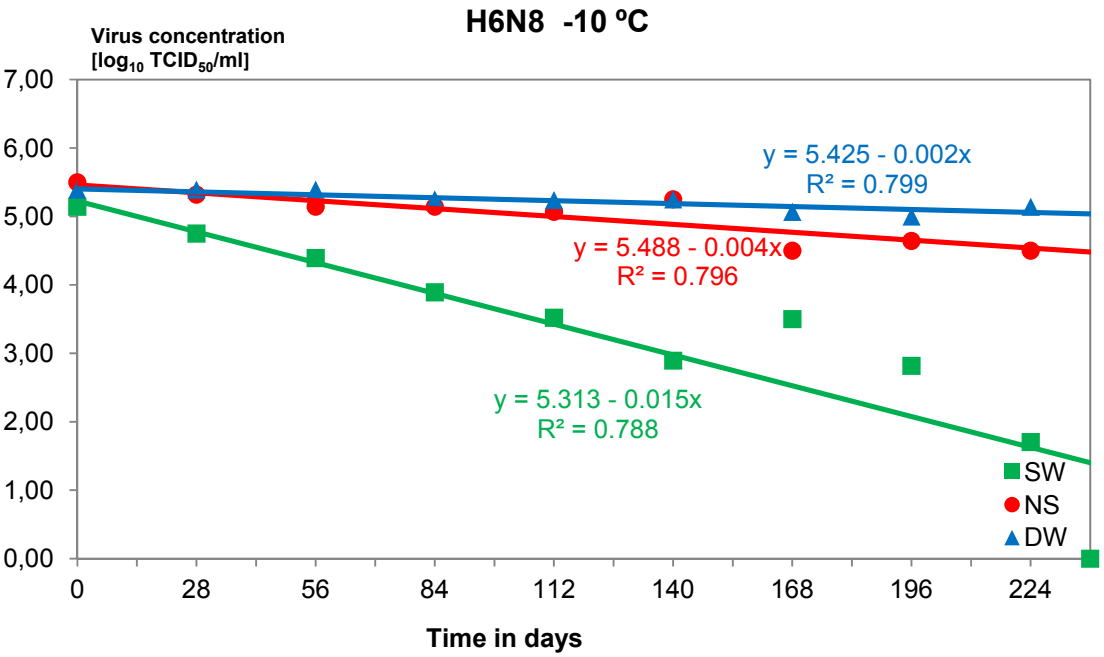


Figure 4.15: Persistence of LPAI H6N8 virus in DW, NS, and SW at -10 °C

4.2.4 Tenacity of human influenza virus H1N1 in DW, NS, and SW

The linear regression models showing the persistence of human influenza virus H1N1 in DW, NS, and SW are presented in Figures 4.16 to 4.20. The persistence was inversely proportional to the storage temperature. In DW with a starting virus titer of $10^{6.32}$ $TCID_{50}$/ml the virus was detectable for 40, 104, ≥ 168, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively. In NS with a starting virus titer of $10^{6.32}$ $TCID_{50}$/ml the virus was detectable for 8, 64, ≥ 168, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively, while in SW with a starting virus titer of $10^{4.39}$ $TCID_{50}$/ml the virus was detectable for 1, 6, 14, 28, and 140 days at 30, 20, 10, 0, and -10 °C, respectively.

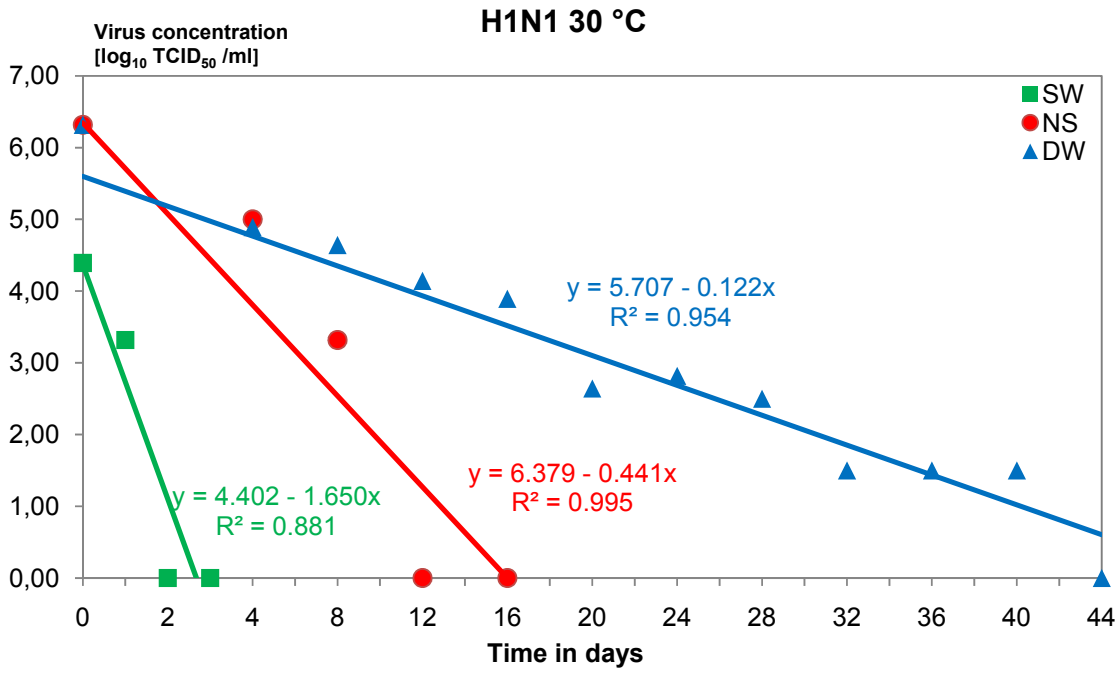


Figure 4.16: Persistence of human influenza virus H1N1 in DW, NS, and SW at 30 °C

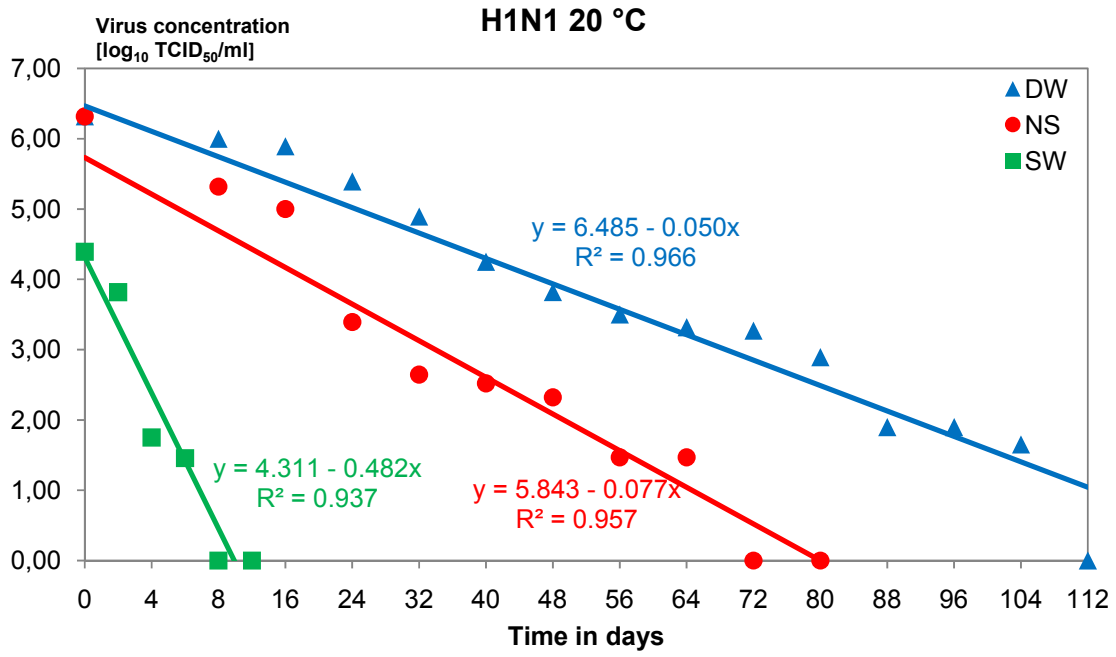


Figure 4.17: Persistence of human influenza virus H1N1 in DW, NS, and SW at 20 °C

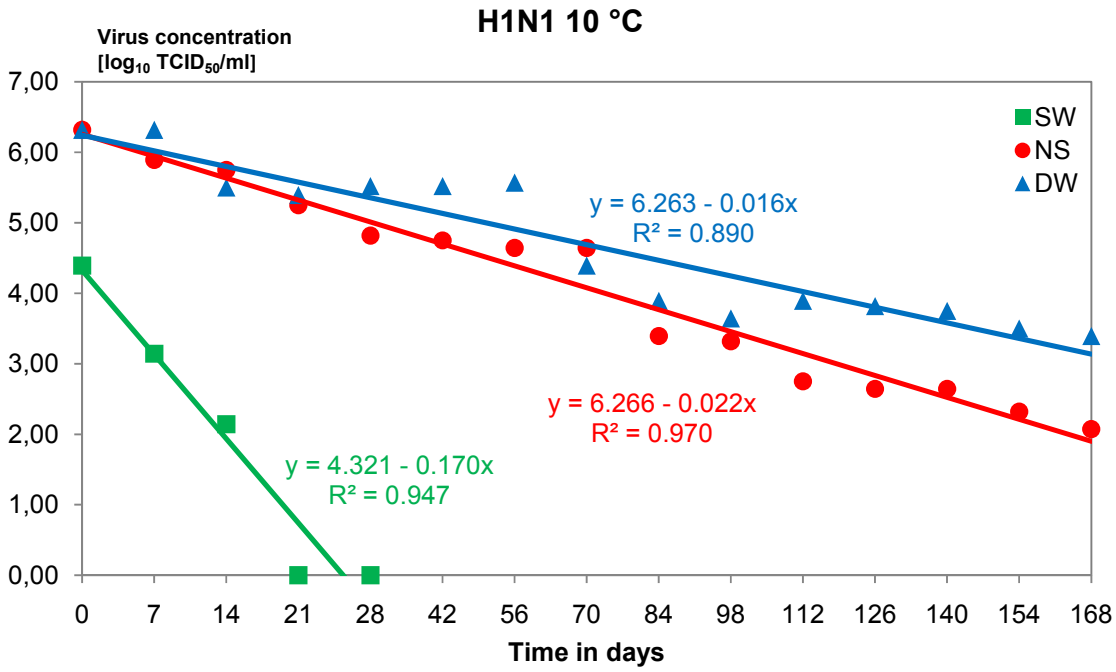


Figure 4.18: Persistence of human influenza virus H1N1 in DW, NS, and SW at 10 °C

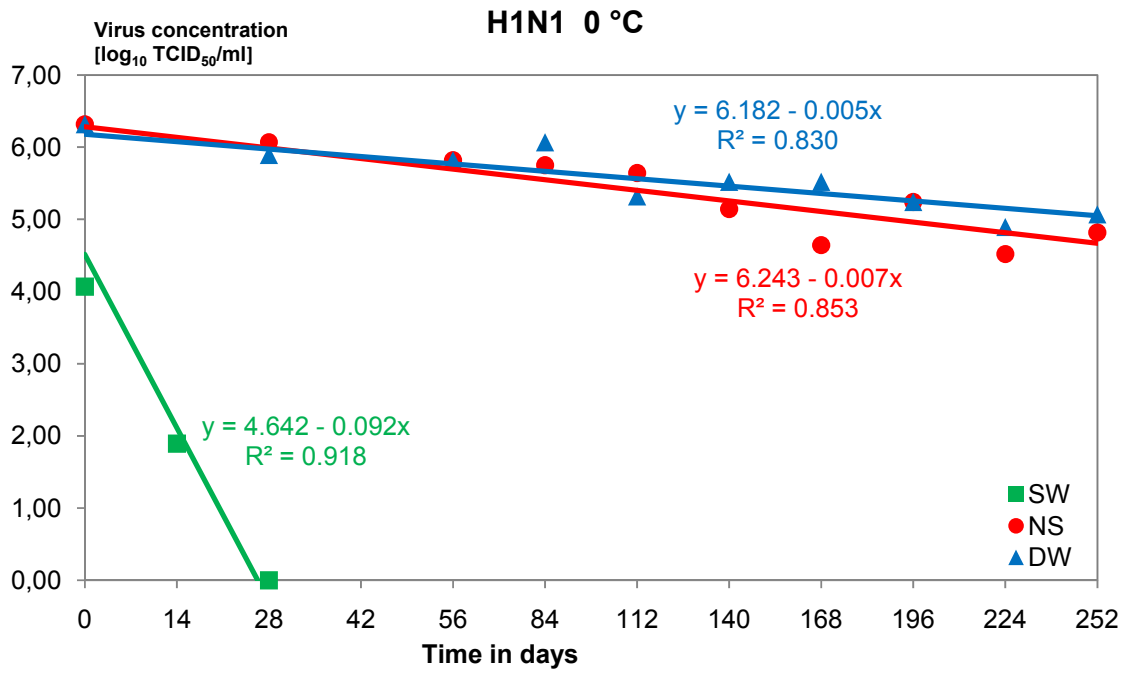


Figure 4.19: Persistence of human influenza virus H1N1 in DW, NS, and SW at 0 °C

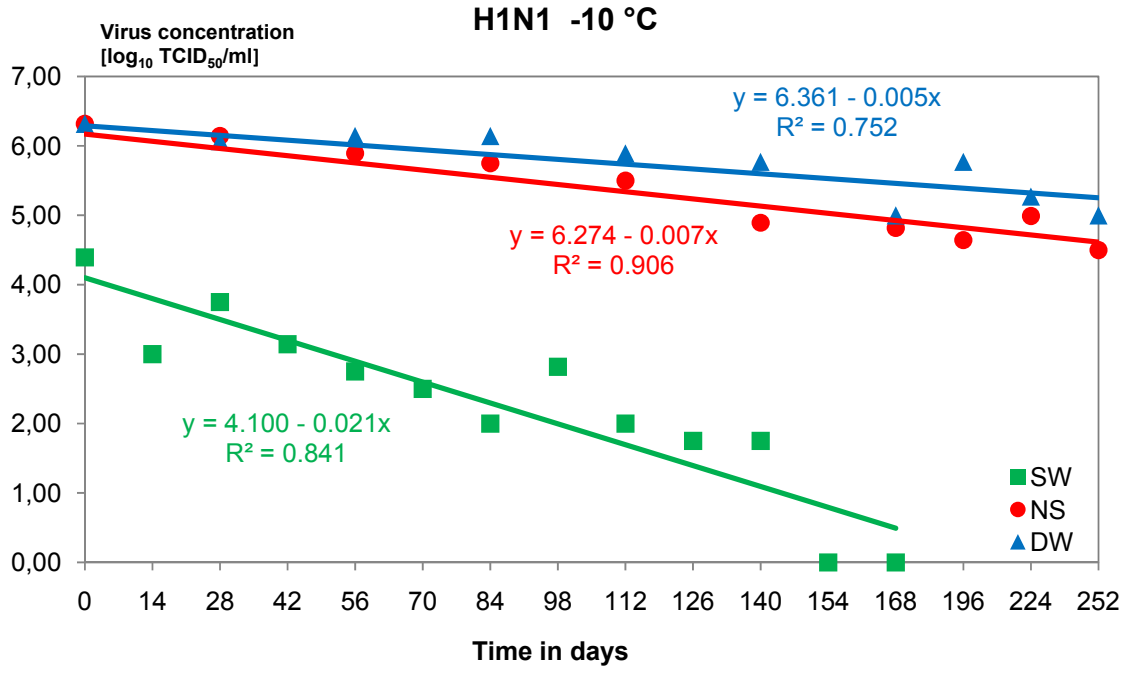


Figure 4.20: Persistence of human influenza virus H1N1 in DW, NS, and SW at -10 °C

4.2.5 Tenacity of NDV in DW, NS, and SW

The linear regression models showing the persistence of NDV in DW, NS, and SW are presented in Figures 4.21 to 4.25. The persistence was inversely proportional to the storage temperature. In DW with a starting virus titer of $10^{6.89}$ $TCID_{50}$/ml the virus was detectable for ≥ 60, ≥ 168, ≥ 238, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively. In NS with a starting virus titer of $10^{6.89}$ $TCID_{50}$/ml the virus was detectable for 48, 128, ≥ 238, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively, while in SW with a starting virus titer of $10^{4.81}$ $TCID_{50}$/ml the virus was detectable for 3, 6, 28, 168, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively.

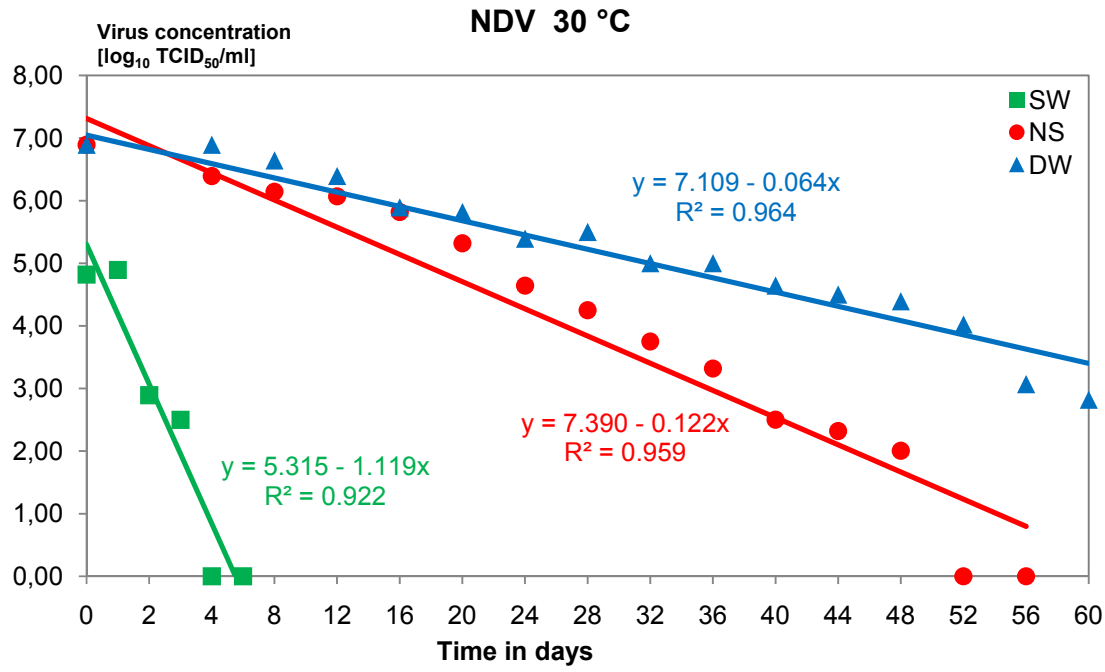


Figure 4.21: Persistence of NDV in DW, NS, and SW at 30 °C

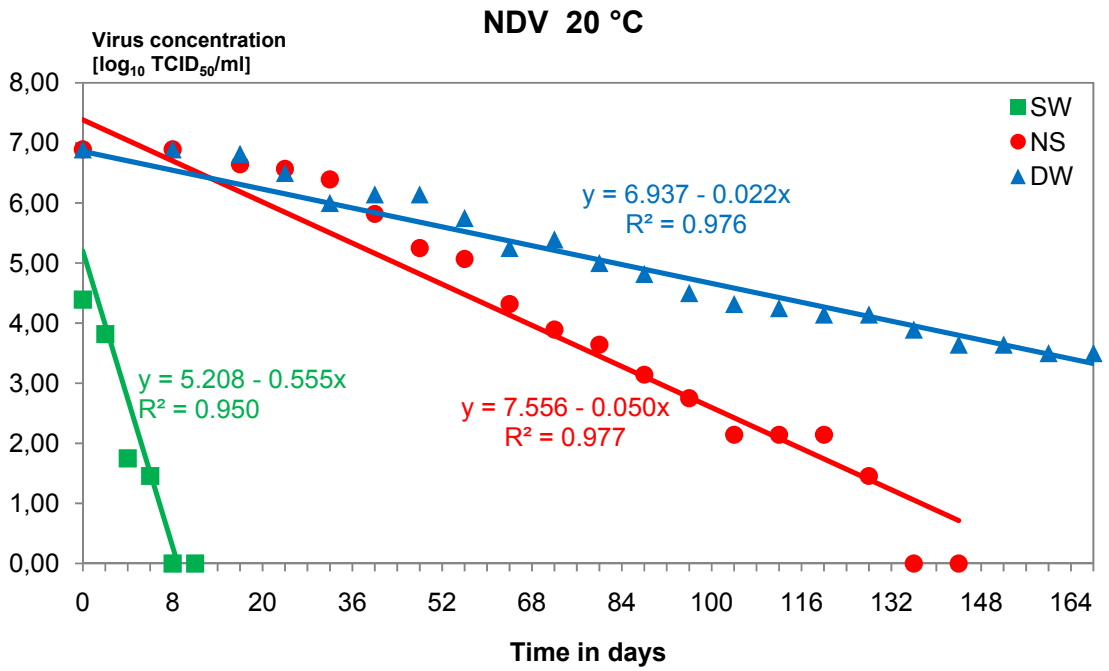


Figure 4.22: Persistence of NDV in DW, NS, and SW at 20 °C

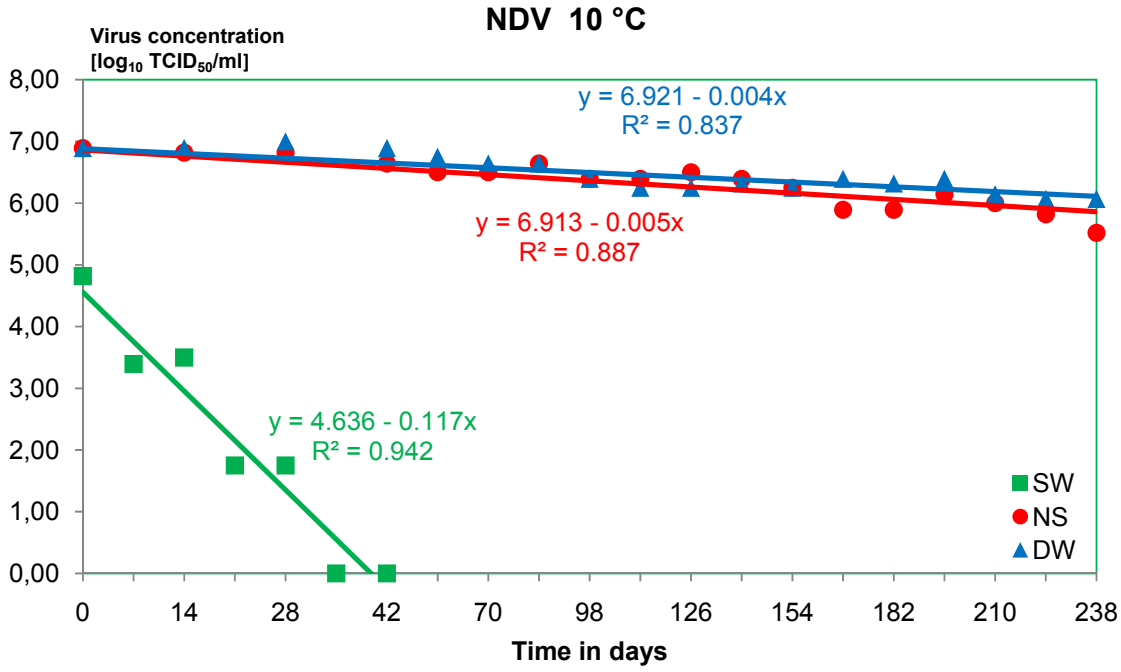


Figure 4.23: Persistence of NDV in DW, NS, and SW at 10 °C

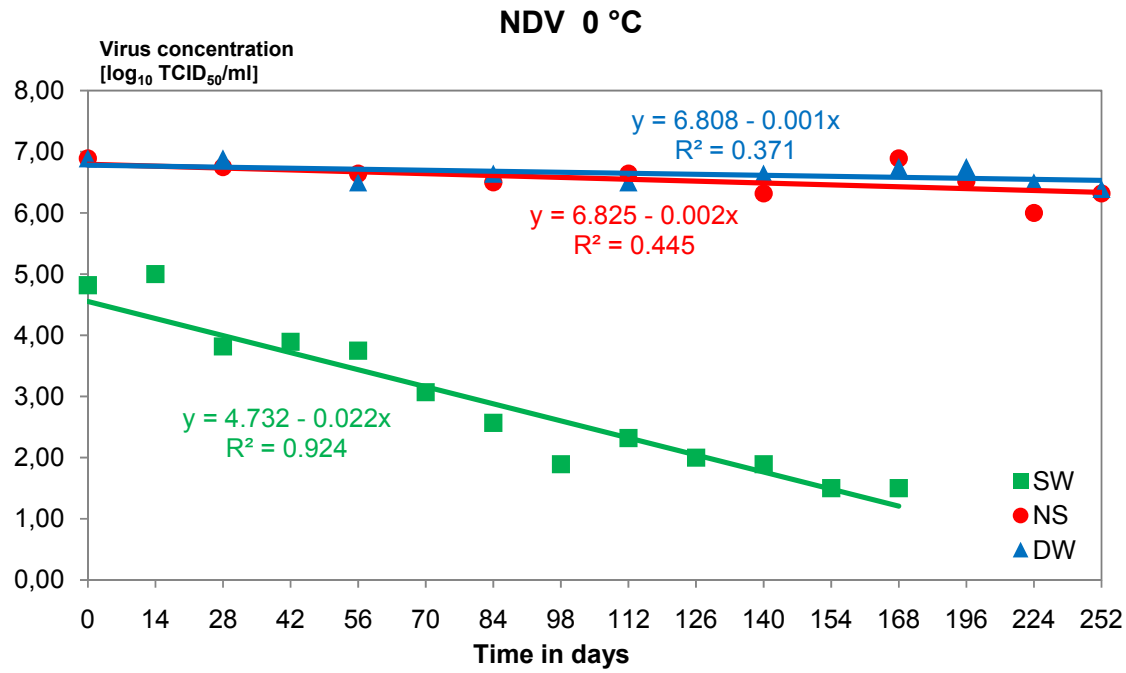


Figure 4.24: Persistence of NDV in DW, NS, and SW at 0 °C

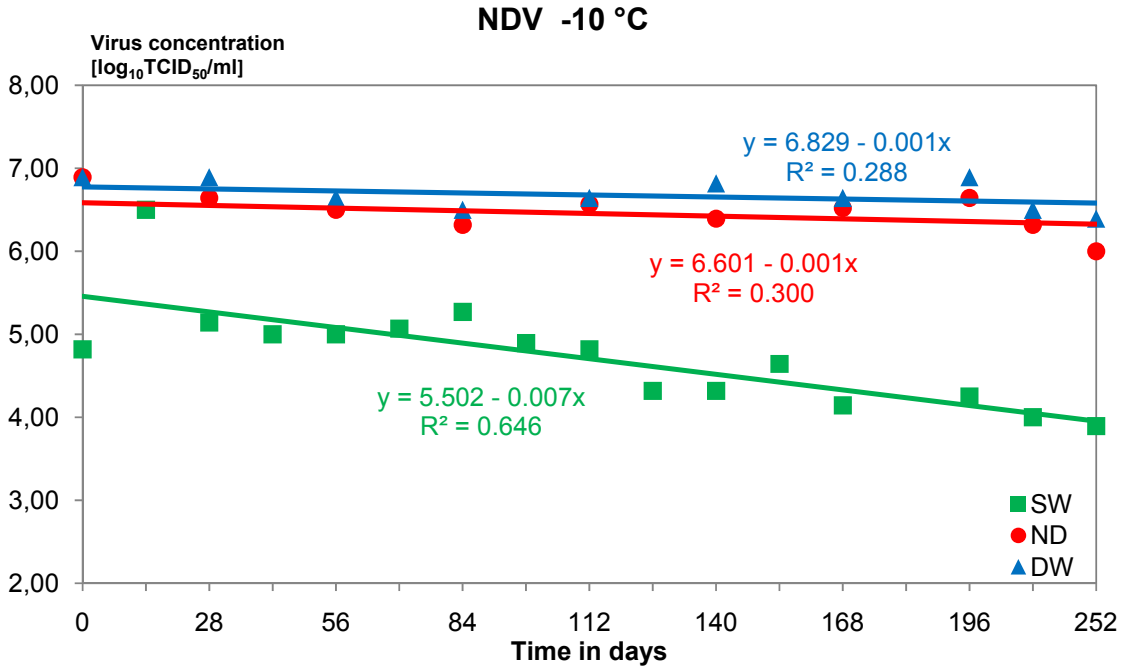


Figure 4.25: Persistence of NDV in DW, NS, and SW at -10 °C

4.2.6 Tenacity of ECBO in DW, NS, and SW

The linear regression models showing the persistence of ECBO in DW, NS, and SW are presented in Figures 4.26 to 4.30. The persistence was inversely proportional to the storage temperature. In DW with a starting virus titer of $10^{6.82}$ $TCID_{50}$/ml the virus was detectable for ≥ 60, ≥ 168, ≥ 196, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively. In NS with a starting virus titer of $10^{6.52}$ $TCID_{50}$/ml the virus was detectable for 32, 80, ≥ 196, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively, while in SW with a starting virus titer of $10^{5.07}$ $TCID_{50}$/ml the virus was detectable for 28, ≥ 56, ≥ 154, ≥ 252, and ≥ 252 days at 30, 20, 10, 0, and -10 °C, respectively.

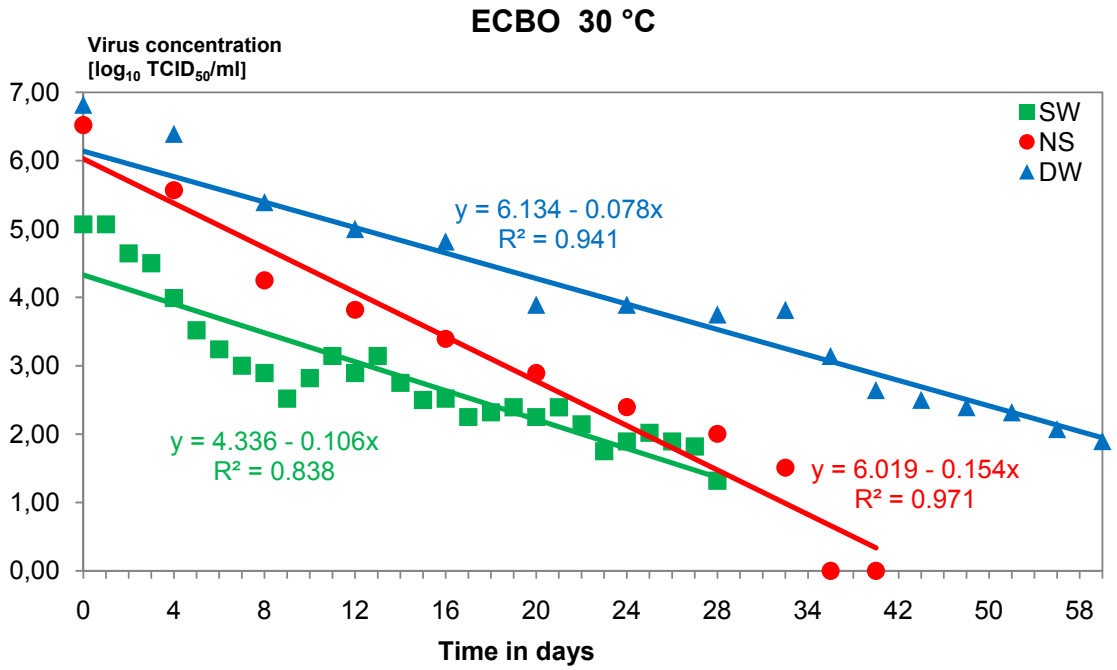


Figure 4.26: Persistence of ECBO in DW, NS, and SW at 30 °C

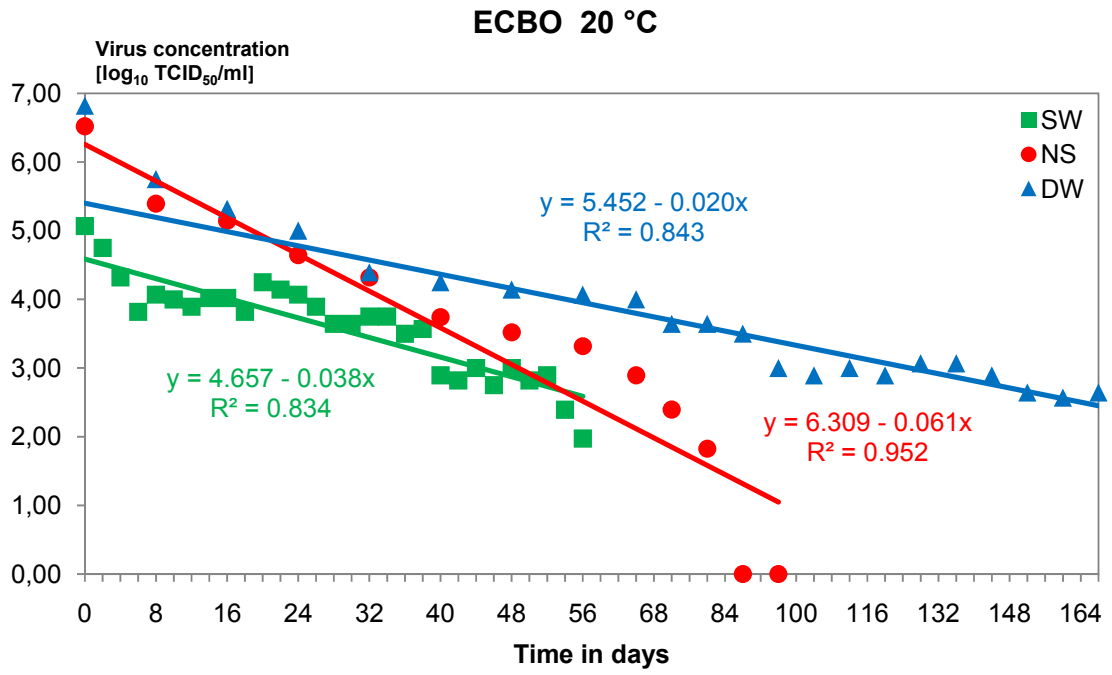


Figure 4.27: Persistence of ECBO in DW, NS, and SW at 20 °C

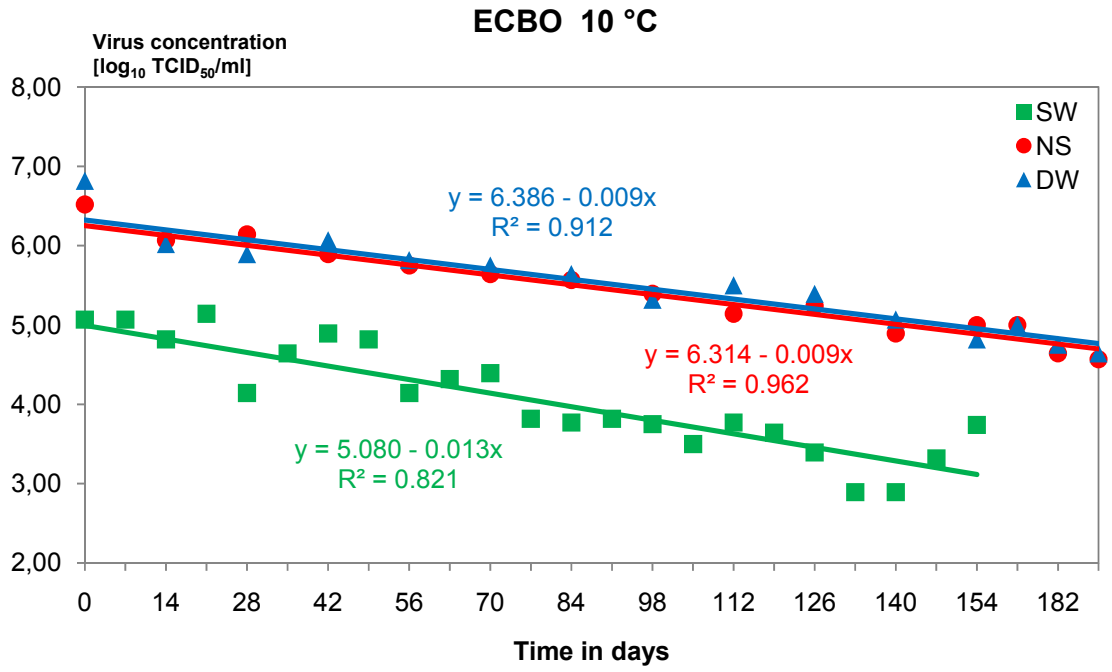


Figure 4.28: Persistence of ECBO in DW, NS, and SW at 10 °C

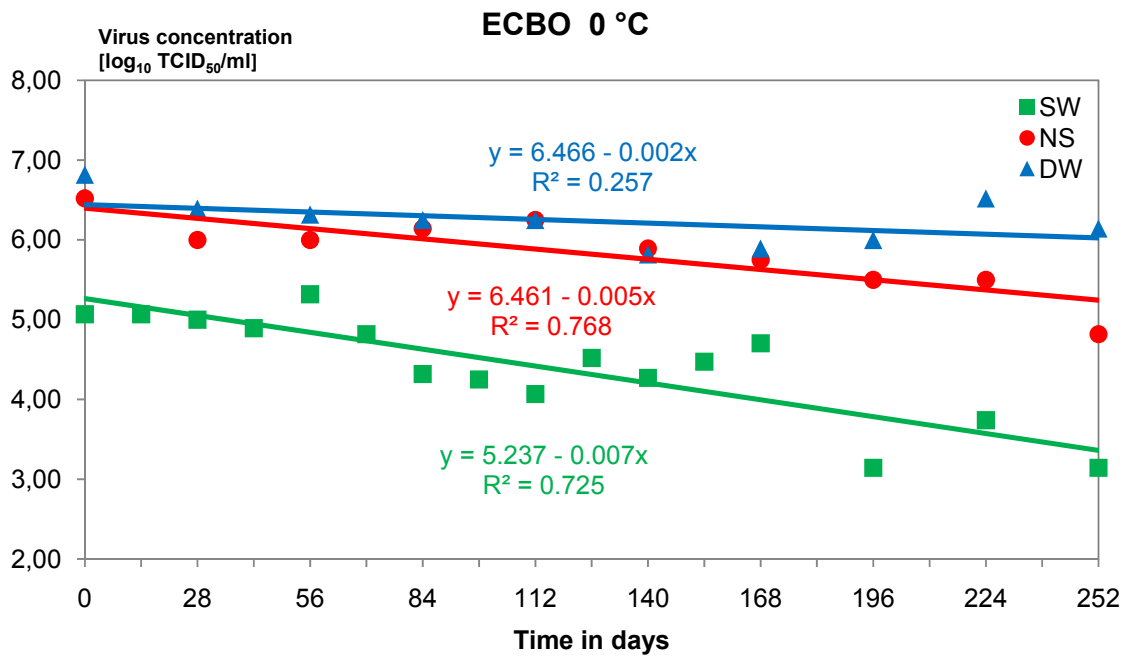


Figure 4.29: Persistence of ECBO in DW, NS, and SW at 0 °C

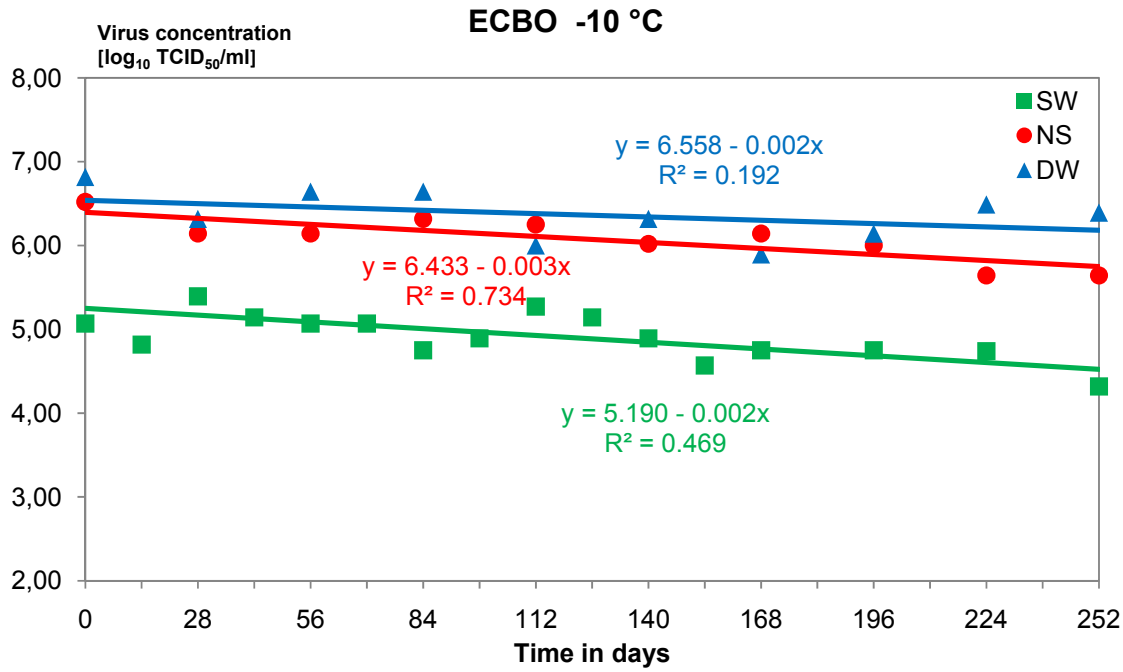
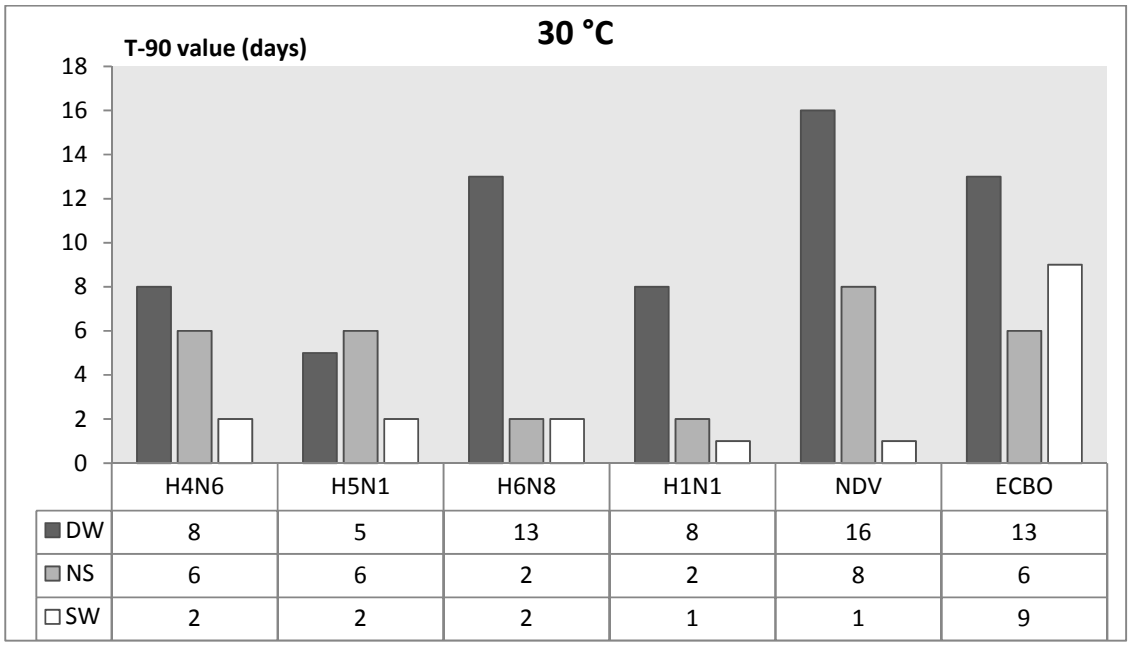


Figure 4.30: Persistence of ECBO in DW, NS, and SW at -10 °C

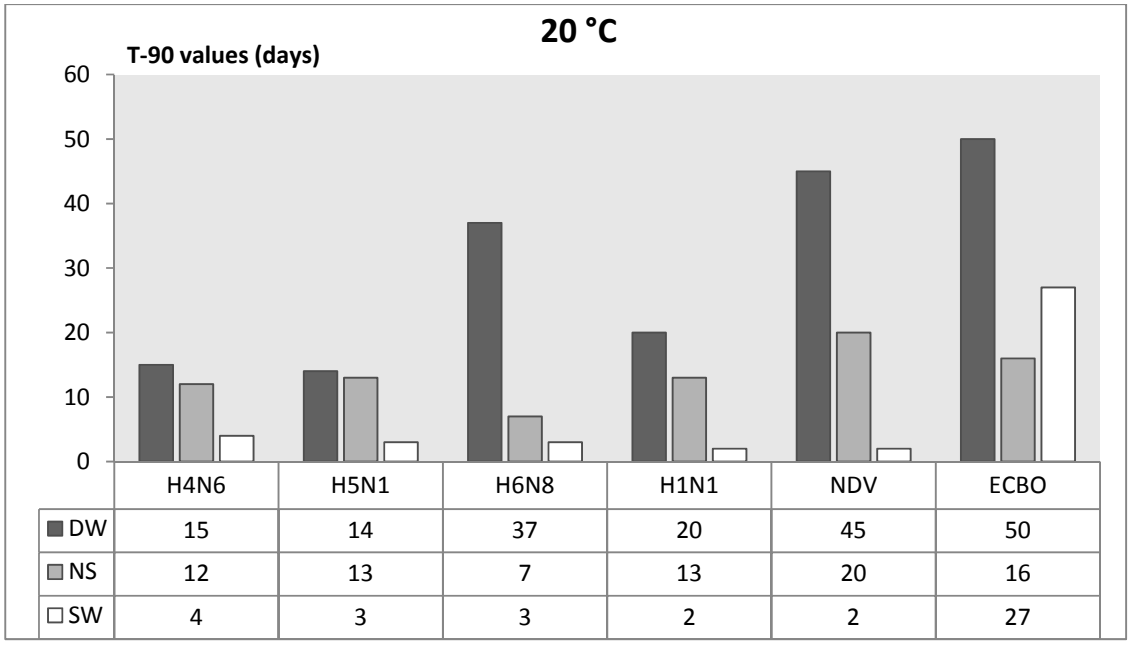
4.3 Comparison of T-90 values of the viruses in water

For a better comparison of the persistence of influenza and model viruses in three different water types the T-90 values calculated from linear regression models are displayed in Figures 4.31 to 4.35. It is clear from T-90 values that individual influenza viruses have equivalent survival time in various types of water at all of the temperatures while the model viruses have comparatively higher T-90 values than that of influenza viruses. Within different types of water the viruses have higher T-90 values in DW followed by in NS and the lowest in SW.



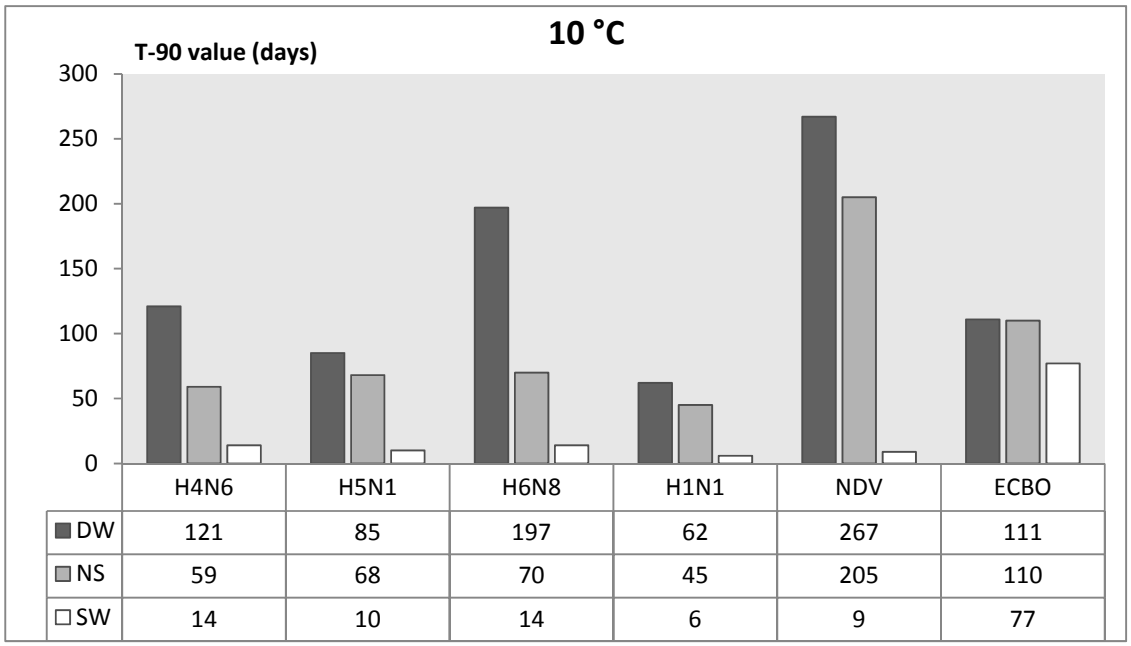
	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
DW	8	5	13	8	16	13
NS	6	6	2	2	8	6
SW	2	2	2	1	1	9

Figure 4.31: Comparison of T-90 values of the influenza and model viruses in various water types at 30 °C



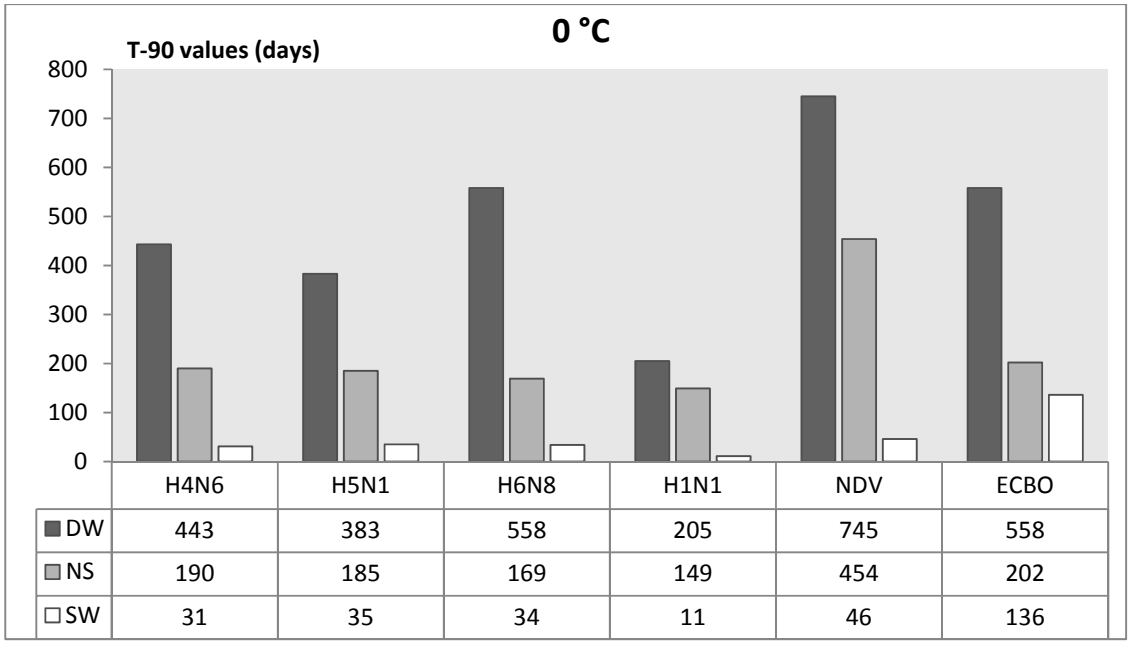
	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
DW	15	14	37	20	45	50
NS	12	13	7	13	20	16
SW	4	3	3	2	2	27

Figure 4.32: Comparison of T-90 values of the influenza and model viruses in various water types at 20 °C



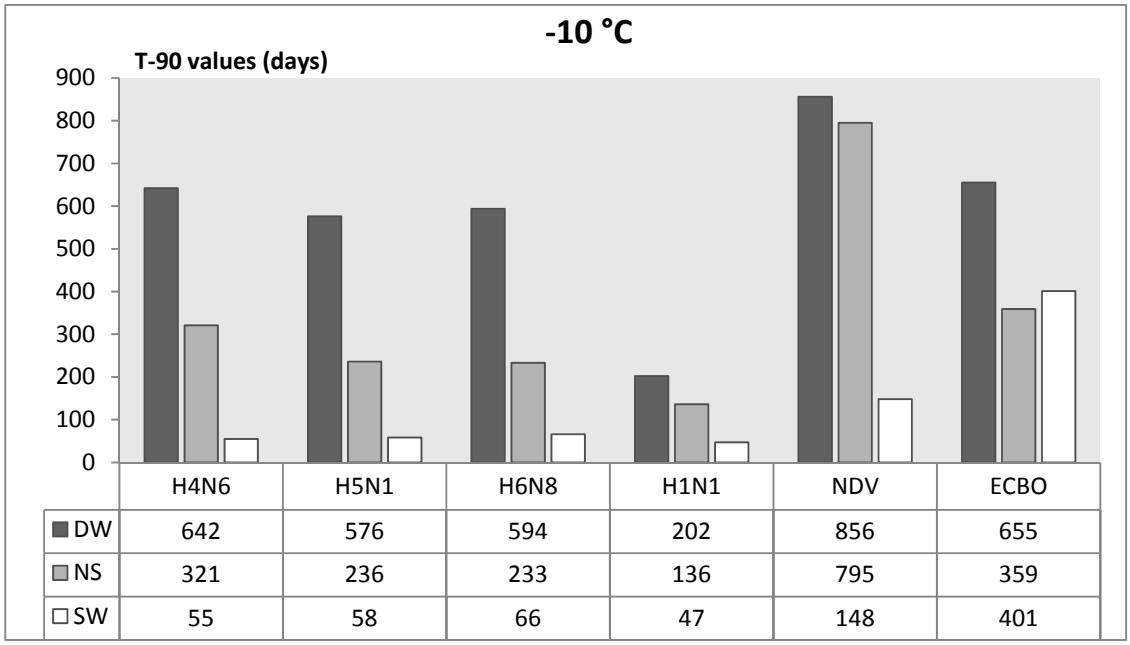
	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
DW	121	85	197	62	267	111
NS	59	68	70	45	205	110
SW	14	10	14	6	9	77

Figure 4.33: Comparison of T-90 values of the influenza and model viruses in various water types at 10 °C



	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
DW	443	383	558	205	745	558
NS	190	185	169	149	454	202
SW	31	35	34	11	46	136

Figure 4.34: Comparison of T-90 values of the influenza and model viruses in various water types at 0 °C



	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
DW	642	576	594	202	856	655
NS	321	236	233	136	795	359
SW	55	58	66	47	148	401

Figure 4.35: Comparison of T-90 values of the influenza and model viruses in various water types at -10 °C

4.4 Persistence of viral RNA in the inoculated water samples

For the quantitation of viral RNA a standard curve was determined using 1 ng of viral RNA and serial diluting it tenfold from 10^1-10^4 to have 1, 10, 100, and 1,000 pg of viral RNA as shown in Figures 4.36 and 4.37. The amount of viral RNA detected in the H4N6, H5N1 and H6N8 inoculated SW samples at the start of the experiments and after storage at various temperatures are shown in Tables 4.1 to 4.3. A significant amount of viral RNA was still detectable in the contaminated water samples stored at 30, 20, 10, 0, and -10 °C after the virus was no longer detectable by cell culture titration. The rate of viral RNA degradation was faster at high temperatures than at lower ones.

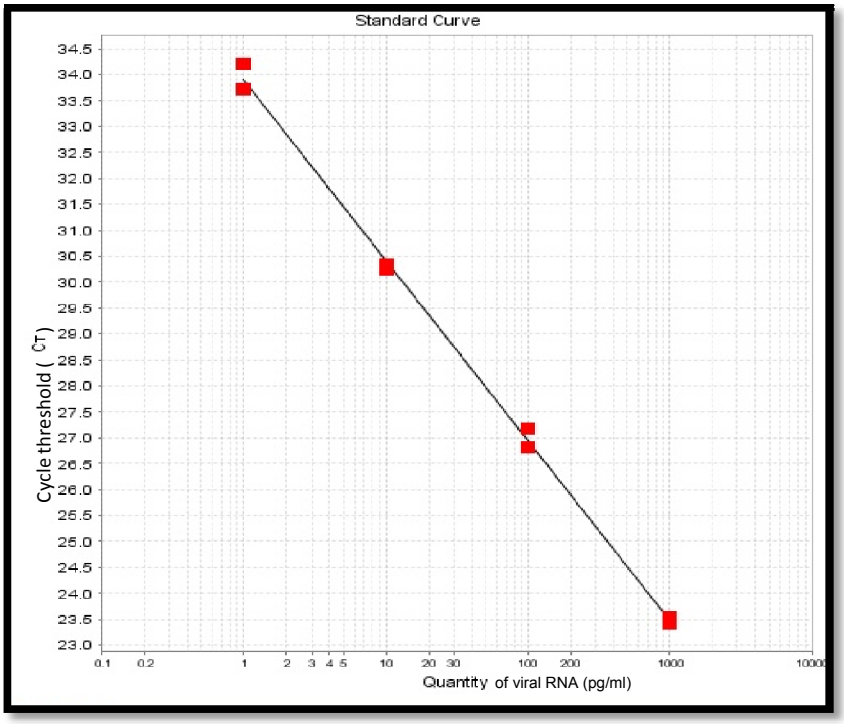


Figure 4.36: Standard curve for the quantitation of viral RNA

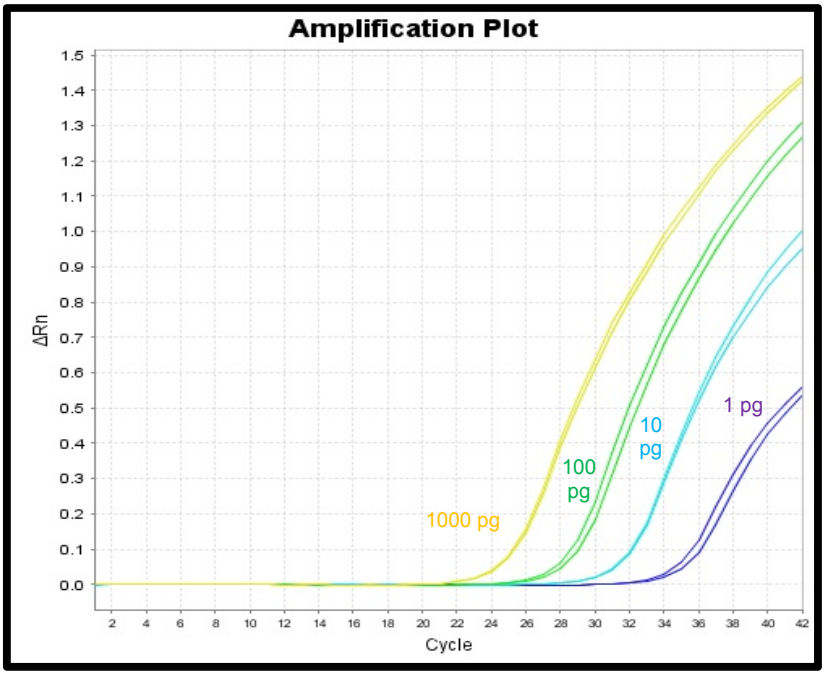


Figure 4.37: Amplification plot showing cycle thresholds for 1, 10, 100, and 1,000 pg of viral RNA diluted in nuclease free water

Table 4.1: Quantitation of viral RNA in H4N6 AIV inoculated SW at the beginning and after incubation at various temperatures

Temperature, incubation time	Amount of viral RNA in pg/ml			
	*Value 1	*Value 2	*Average	RNA in the original sample
Day 0	117	81	99	4,950
30 °C, 10 days, NVD	44	23	33.5	1,675
20 °C, 14 days, NVD	33	46	39.5	1,975
10 °C, 42 days, NVD	40	7	23.5	1,175
0 °C, 84 days, NVD	51	45	48	2,400
-10 °C, 196 days, NVD	73	71	72	3,600

NVD: No virus detectable by titration on cell culture

*Amount of RNA in the PCR reaction mix, diluted 1:50 from original sample (see section 3.16.2)

Table 4.2: Quantitation of viral RNA in H5N1 AIV inoculated SW at the beginning and after incubation at various temperatures

Temperature, incubation time	Amount of viral RNA in pg/ml			
	*Value 1	*Value 2	*Average	RNA in the original sample
Day 0	118	120	119	5,950
30 °C, 10 days, NVD	19	14	16.5	825
20 °C, 14 days, NVD	17	11	14	700
10 °C 35 days, NVD	24	14	19	950
0 °C, 84 days, NVD	47	48	47.5	2,375
-10 °C, 196 days, NVD	62	82	72	3,600

NVD: No virus detectable by titration on cell culture

*Amount of RNA in the PCR reaction mix, diluted 1:50 from original sample (see section 3.16.2)

Table 4.3: Quantitation of viral RNA in H6N8 AIV inoculated SW at the beginning and after incubation at various temperatures

Temperature, incubation time	Amount of viral RNA in pg/ml			
	*Value 1	*Value 2	*Average	RNA in the original sample
Day 0	23	20	21.5	1,075
30 °C, 12 days, NVD	8	6	7	350
20 °C, 18 days, NVD	13	14	13.5	675
10 °C, 49 days, NVD	11	15	13	650
0 °C, 140 days, NVD	9	7	8	400
-10 °C, 238 days, NVD	15	12	13.5	675

NVD: No virus detectable by titration on cell culture

*Amount of RNA in the PCR reaction mix, diluted 1:50 from original sample (see section 3.16.2)

4.5 Suitability of the germ carrier technique

For efficient adsorption of influenza viruses to the filter discs, phosphate buffer adjusted to three different pH values (6.00, 6.50, and 7.40) was evaluated for use as a loading medium. The H5N1 virus as infective AAF was mixed in a ratio of 1:10 in the PLB. An amount of 5 ml of the mixture was filtered through each filter disc. The virus adsorbed to the filter disc was recovered by the elution process as described in section 3.11.1. The virus concentration in the influent and effluent virus suspension as well as in the elution medium was determined. The results of three independent trials showed that loading medium with pH 7.40 produced germ carriers with high virus titers as shown in Table 4.4. The use of loading buffer resulted in adsorption of 44-82 % of the virus particles onto filter discs (Table 4.4). Drying the filter carriers resulted in a decline of at least 3 logs of eluable virus titer after a period of 6 hours while a negligible loss of virus titer was observed after the same time in germ carriers kept under moist conditions (Table 4.5). No virus was detected in water samples in which germ carriers were incubated while successful recovery of virus from filter discs stored at low temperatures was possible for influenza and model viruses during the whole study period. These findings confirm that Virosorb filter discs wrapped in polycarbonate membrane are suitable germ carriers for studies on the persistence of influenza viruses under wet environmental conditions.

Table 4.4: Effect of loading medium on adsorption of H5N1 avian influenza virus to zeta plus virosorb filters[a]

Virus mixed in loading buffer[b]	Trial 1			Trial 2			Trial 3		
	Influent	*Effluent (% adsorption)*	*Eluent*	*Influent*	*Effluent (% adsorption)*	*Eluent*	*Influent*	*Effluent (% adsorption)*	*Eluent*
PLB pH 6.00	5.00	4.50 *(68)*	4.64	5.00	4.75 *(44)*	4.82	5.00	4.50 *(68)*	4.64
PLB pH 6.50	5.50	4.75 *(82)*	4.89	5.50	5.00 *(68)*	4.64	5.50	5.25 *(44)*	4.75
PLB pH 7.40	6.00	5.50 *(68)*	5.32	5.75	5.25 *(68)*	5.00	5.75	5.25 *(68)*	5.14

Note: All values are virus titer in $\log_{10}TCID_{50}$/ml, PLB: Phosphate loading buffer

[a] Five ml of virus mixed in loading buffer was filtered through each germ carrier which was then eluted in 2 ml of the elution medium (2 % BE and 0.5 M NaCl, pH 7.00)

[b] H5N1 AIV infective AAF mixed in loading buffer at a ratio of 1:10

Table 4.5: Effect of drying on infectivity of filter bound LPAI H5N1 virus[a]

Time Period (Hrs)	*Dry Germ carriers*			*Wet Germ Carriers*		
	Trail 1	**Trail 2**	**Trail 3**	**Trail 1**	**Trail 2**	**Trail 3**
0	5.07	5.27	5.00	5.07	5.27	5.00
1	4.75	4.82	4.00			
2	3.64	3.74	3.14	5.02	5.32	4.89
3	2.89	3.00	2.82			
4	3.02	1.71	2.27	5.07	5.14	5.14
5	2.50	ND	2.00			
6	1.90	1.47	ND	5.00	5.00	5.07

Note: All values are virus titer in $\log_{10}$ $TCID_{50}$/ml, ND: Not detectable

[a] Five ml of the virus mixed in phosphate loading buffer (pH 7.40) was filtered through each germ carrier which was then incubated at room temperature (24-26 °C) for the time indicated. Thereafter, the virus was eluted from the filters in 2 ml of the elution medium (2 % BE and 0.5 M NaCl, pH 7.00)

4.6 Tenacity of the influenza and model viruses in various substrates using the germ carrier technique

The linear regression models along with the estimated persistence of influenza and model viruses with a starting viral titer of $10^{6.00}$ $TCID_{50}$/ml in various substrates at different temperatures are presented in the Appendix as Tables 9.6-9.10. The virus recovery data and T-90 values as calculated by the linear regression models demonstrate that the persistence of the influenza as well as model viruses was highest in the sediment followed by in the SW and the shortest in both the duck feces and duck meat.

4.6.1 Tenacity of LPAI H4N6 virus in SW, sediment, duck feces, and meat at various temperatures

The linear regression models showing the persistence of H4N6 AIV in SW, sediment, duck feces, and meat at various temperatures are presented in Figures 4.38 to 4.42. In SW with a starting virus titer of $10^{4.39}$ $TCID_{50}$/ml the virus was detectable for 8, 16, 42, ≥ 112, and ≥ 112 days at 30, 20, 10, 0, and -10 °C, respectively. In sediment with a starting virus titer of $10^{4.02}$ $TCID_{50}$/ml the virus was detectable for 28, 52, 91, 140, and 126 days at 30, 20, 10, 0, and -10 °C, respectively. In duck feces with a starting virus titer of $10^{4.14}$ $TCID_{50}$/ml the virus was detectable for 4, 12, 70, 154, and 126 days at 30, 20, 10, 0, and -10 °C, respectively, while in duck meat with a starting virus titer of $10^{5.14}$ $TCID_{50}$/ml the virus was detectable for 7, 12, 35, 154, and 154 days at 30, 20, 10, 0, and -10 °C, respectively.

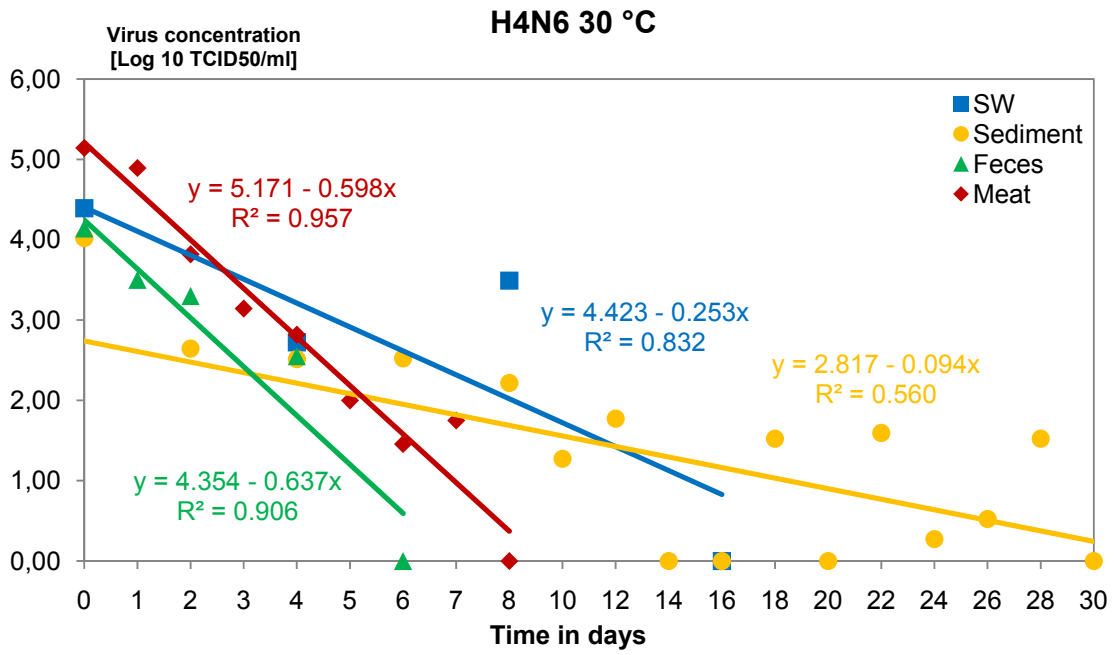


Figure 4.38: Persistence of LPAI H4N6 virus in SW, sediment, duck feces, and meat at 30 °C

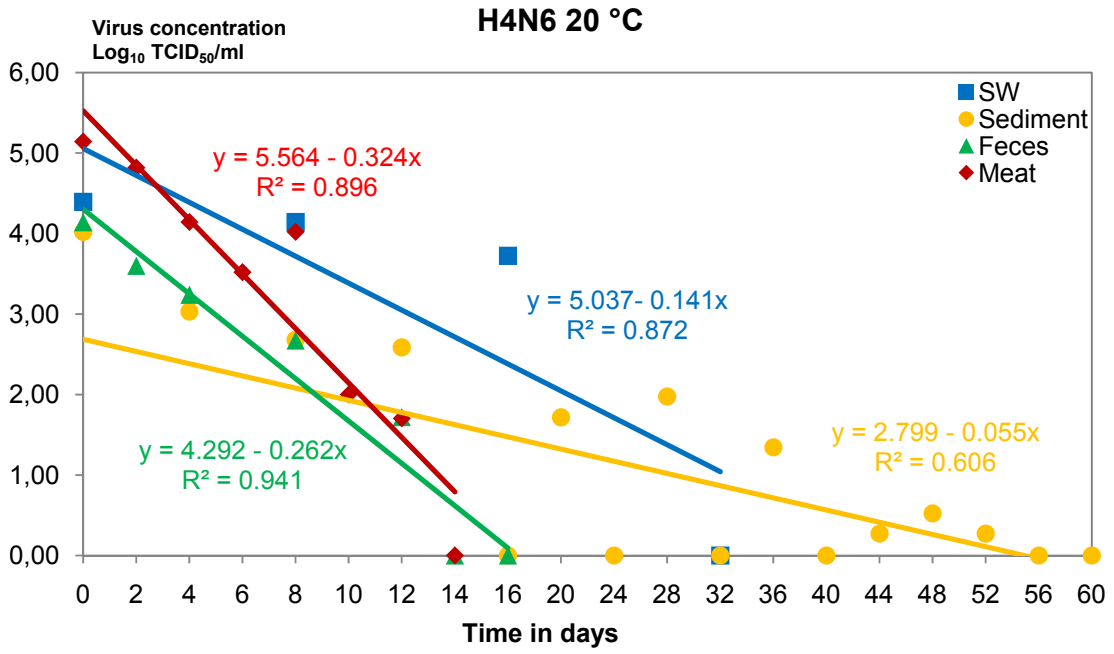


Figure 4.39: Persistence of LPAI H4N6 virus in SW, sediment, duck feces, and meat at 20 °C

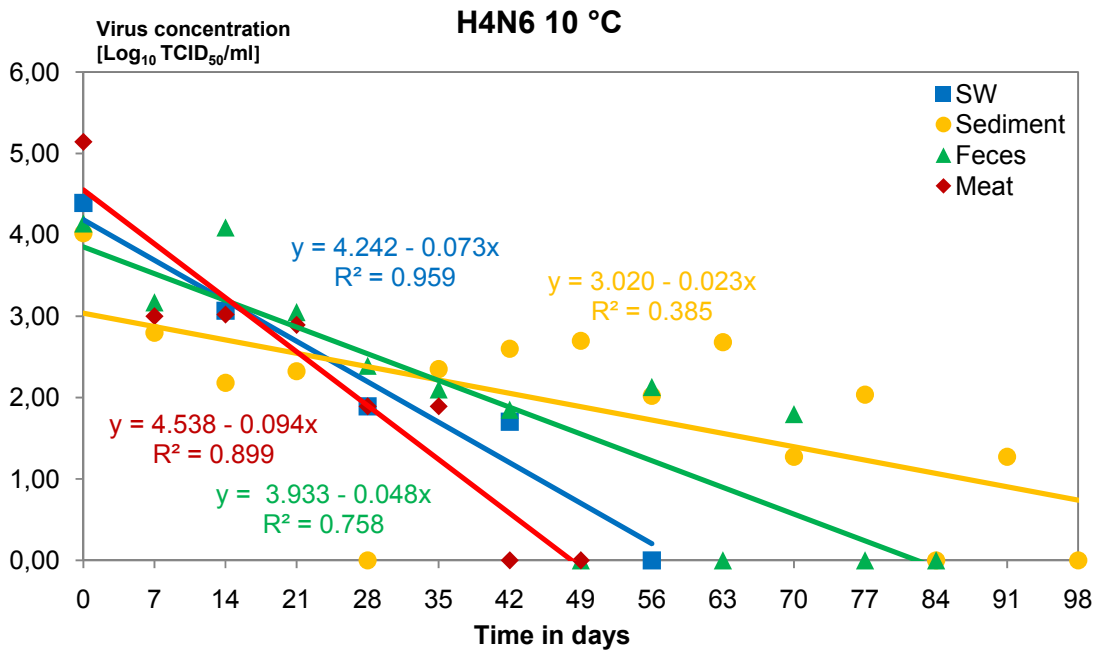


Figure 4.40: Persistence of LPAI H4N6 virus in SW, sediment, duck feces, and meat at 10 °C

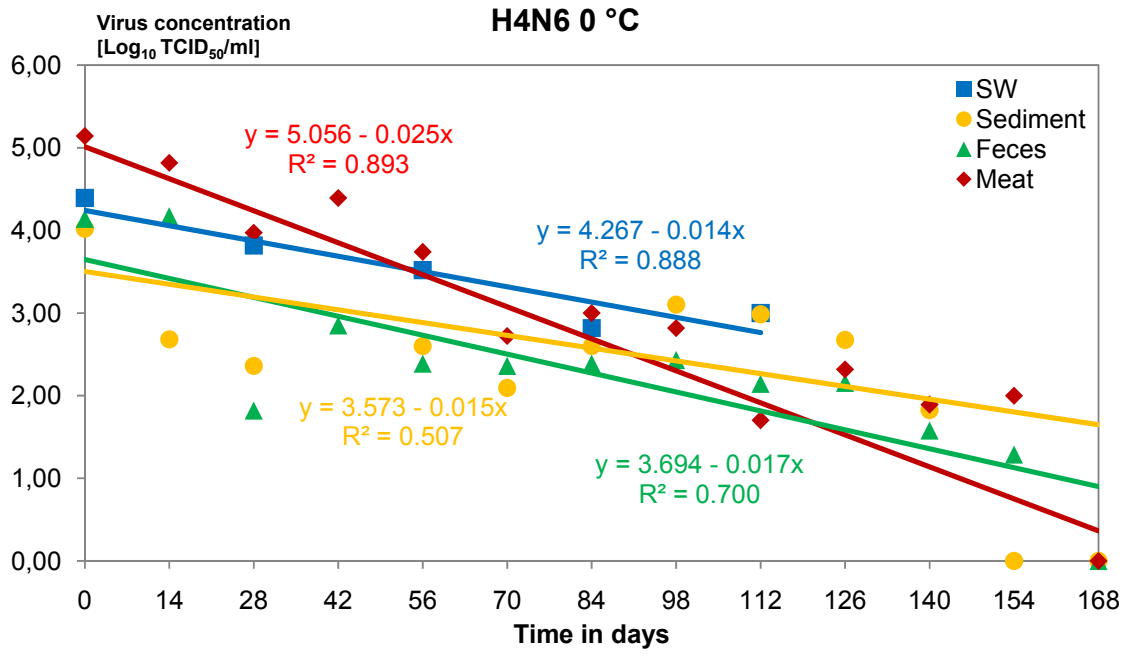


Figure 4.41: Persistence of LPAI H4N6 virus in SW, sediment, duck feces, and meat at 0 °C

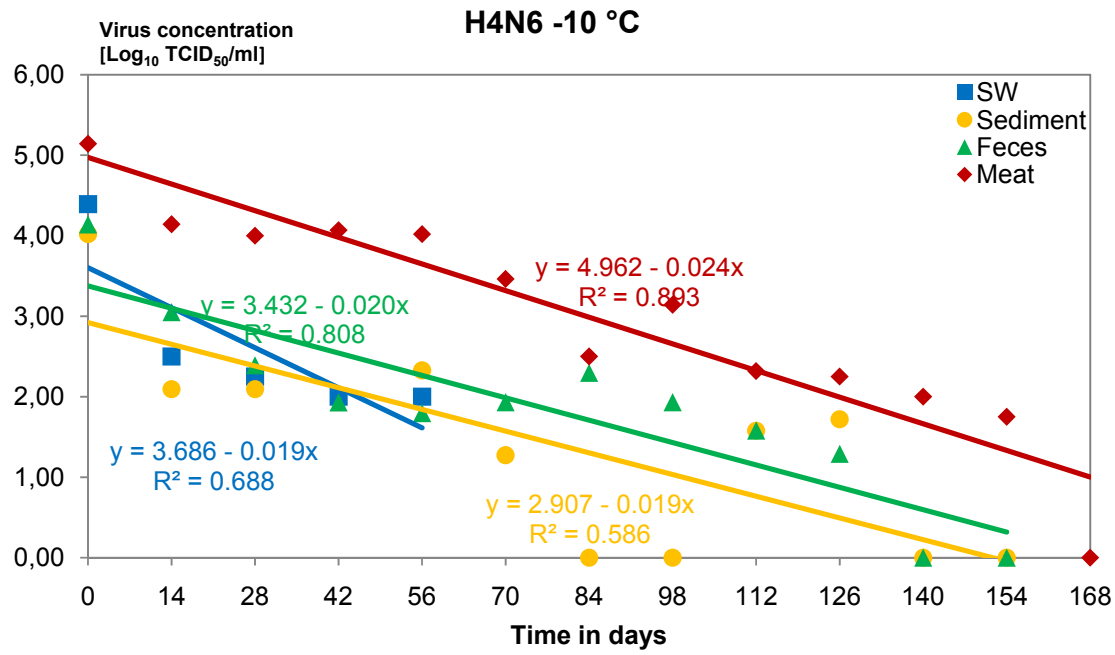


Figure 4.42: Persistence of LPAI H4N6 virus in SW, sediment, duck feces, and meat at -10 °C

4.6.2 Tenacity of LPAI H5N1 virus in SW, sediment, duck feces, and meat at various temperatures

The linear regression models showing the persistence of H5N1 AIV in SW, sediment, duck feces, and meat at various temperatures are presented in Figures 4.43 to 4.47. In SW with a starting virus titer of $10^{5.64}$ $TCID_{50}$/ml the virus was detectable for ≥ 16, ≥ 32, ≥ 56, ≥ 112, and ≥ 112 days at 30, 20, 10, 0, and -10 °C, respectively. In sediment with a starting virus titer of $10^{5.00}$ $TCID_{50}$/ml the virus was detectable for 24, 56, ≥ 98, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively. In duck feces with a starting virus titer of $10^{5.00}$ $TCID_{50}$/ml the virus was detectable for 7, 14, 70, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively, while in duck meat with a starting virus titer of $10^{5.50}$ $TCID_{50}$/ml the virus was detectable for 7, 10, 49, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively.

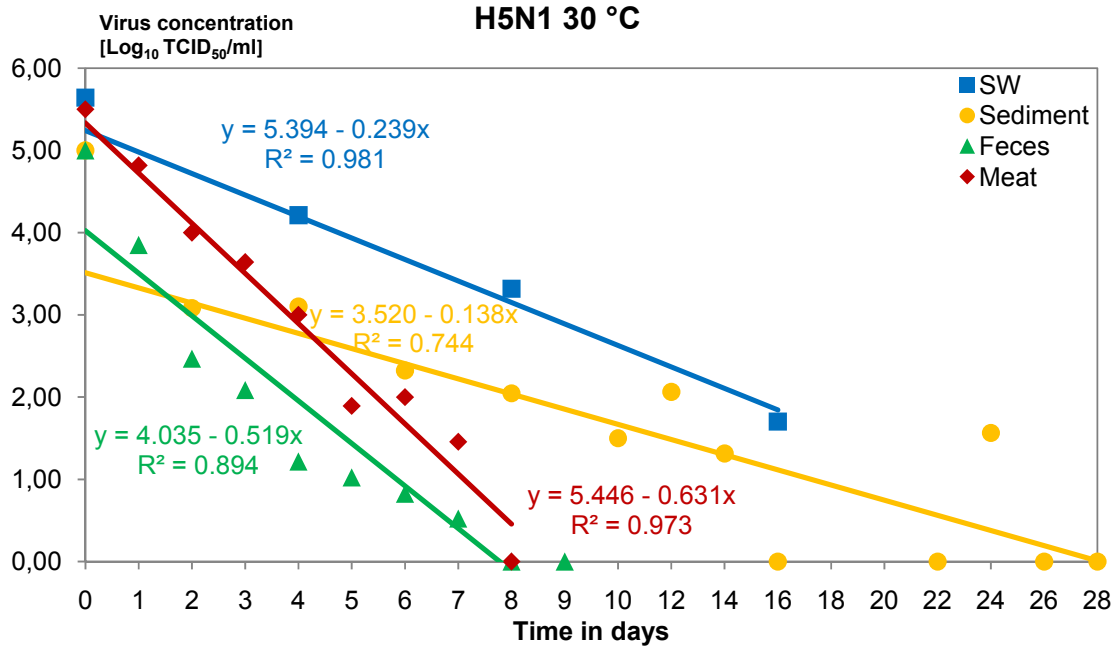


Figure 4.43: Persistence of LPAI H5N1 virus in SW, sediment, duck feces, and meat at 30 °C

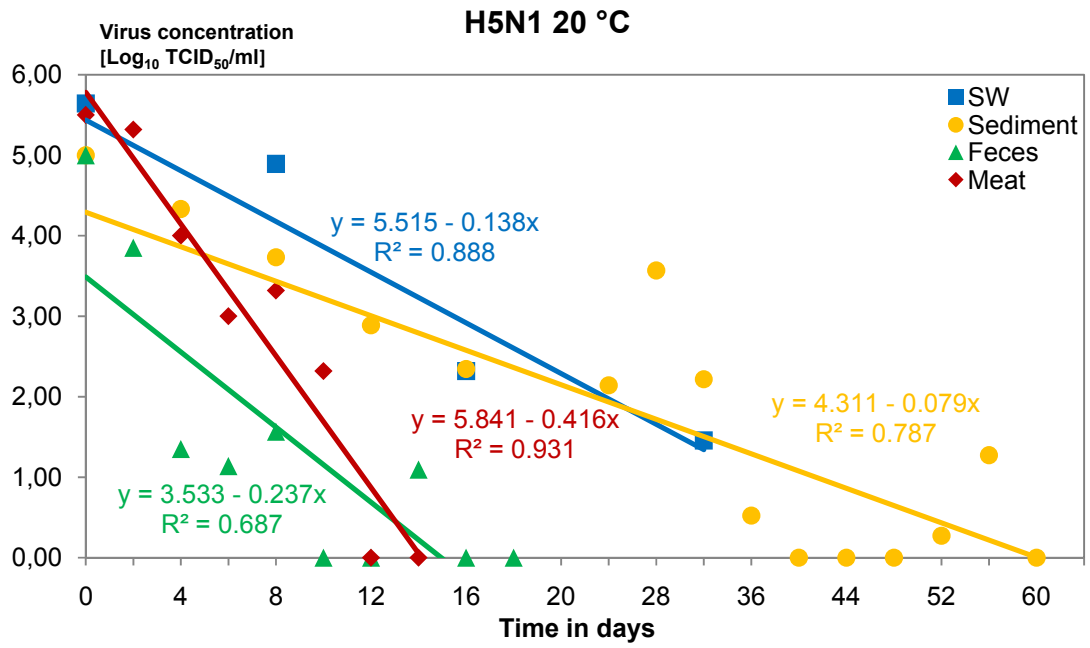


Figure 4.44: Persistence of LPAI H5N1 virus in SW, sediment, duck feces, and meat at 20 °C

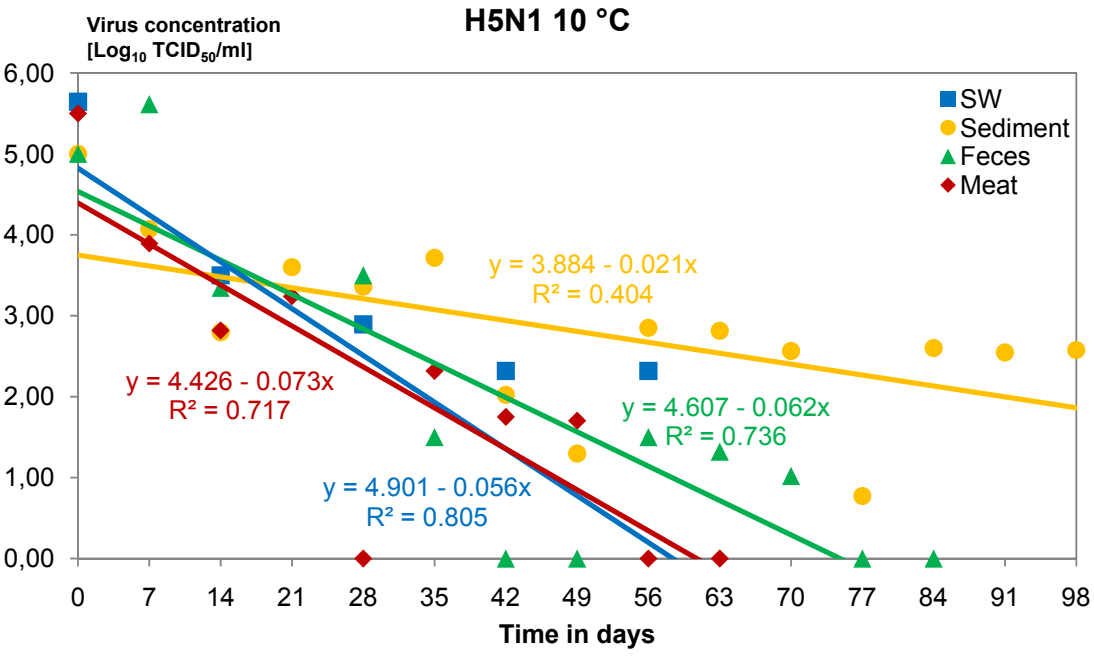


Figure 4.45: Persistence of LPAI H5N1 virus in SW, sediment, duck feces, and meat at 10 °C

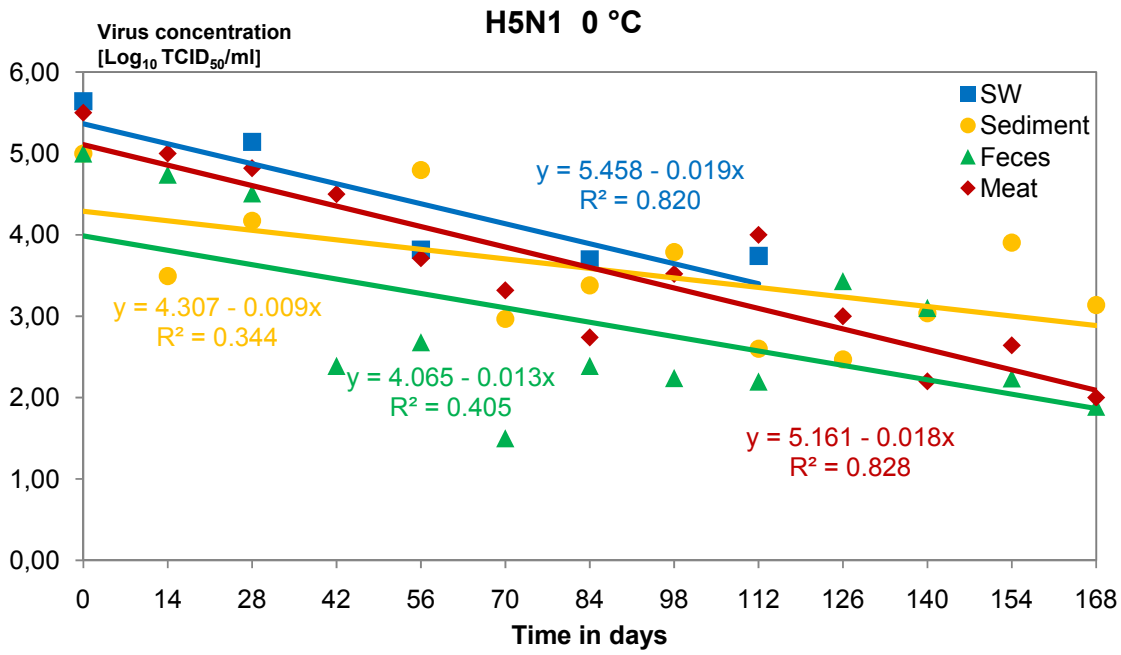


Figure 4.46: Persistence of LPAI H5N1 virus in SW, sediment, duck feces, and meat at 0 °C

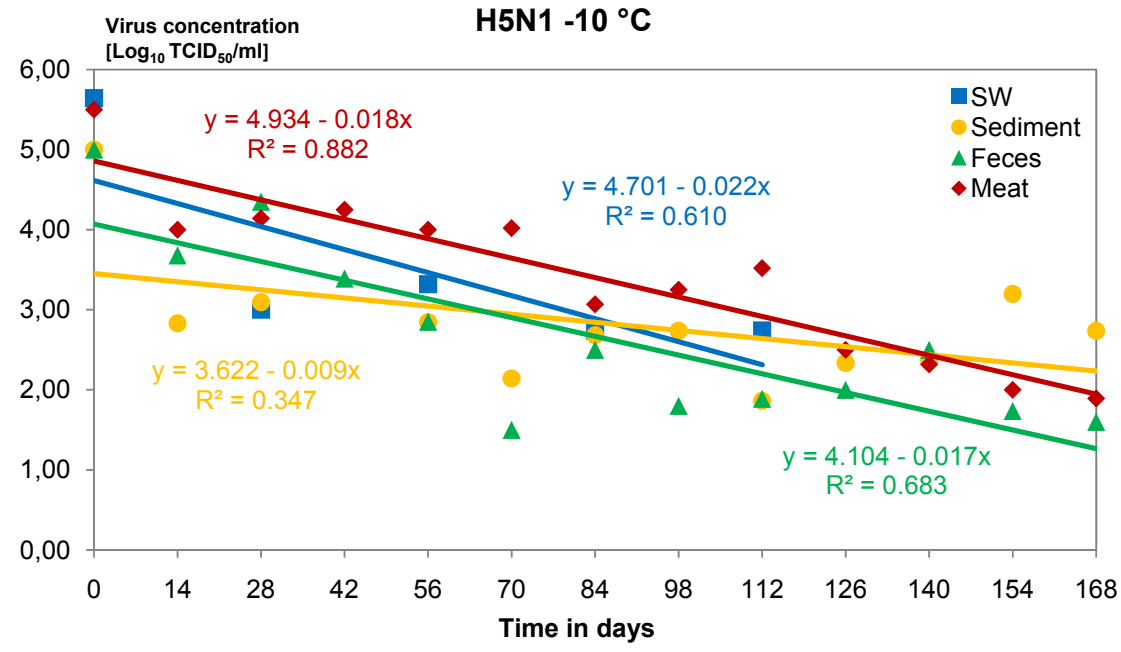


Figure 4.47: Persistence of LPAI H5N1 virus in SW, sediment, duck feces, and meat at -10 °C

4.6.3 Tenacity of LPAI H6N8 virus in SW, sediment, duck feces, and meat at various temperatures

The linear regression models showing the persistence of H5N1 AIV in SW, sediment, duck feces, and meat at various temperatures are presented in Figures 4.48 to 4.52. In SW with a starting virus titer of $10^{5.75}$ $TCID_{50}$/ml the virus was detectable for ≥ 16, ≥ 32, ≥ 56, ≥ 112, and ≥ 112 days at 30, 20, 10, 0, and -10 °C, respectively. In sediment with a starting virus titer of $10^{4.50}$ $TCID_{50}$/ml the virus was detectable for 16, 56, ≥ 98, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively. In duck feces with a starting virus titer of $10^{5.32}$ $TCID_{50}$/ml the virus was detectable for 9, 26, 56, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively, while in duck meat with a starting virus titer of $10^{5.39}$ $TCID_{50}$/ml the virus was detectable for 7, 14, 49, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively.

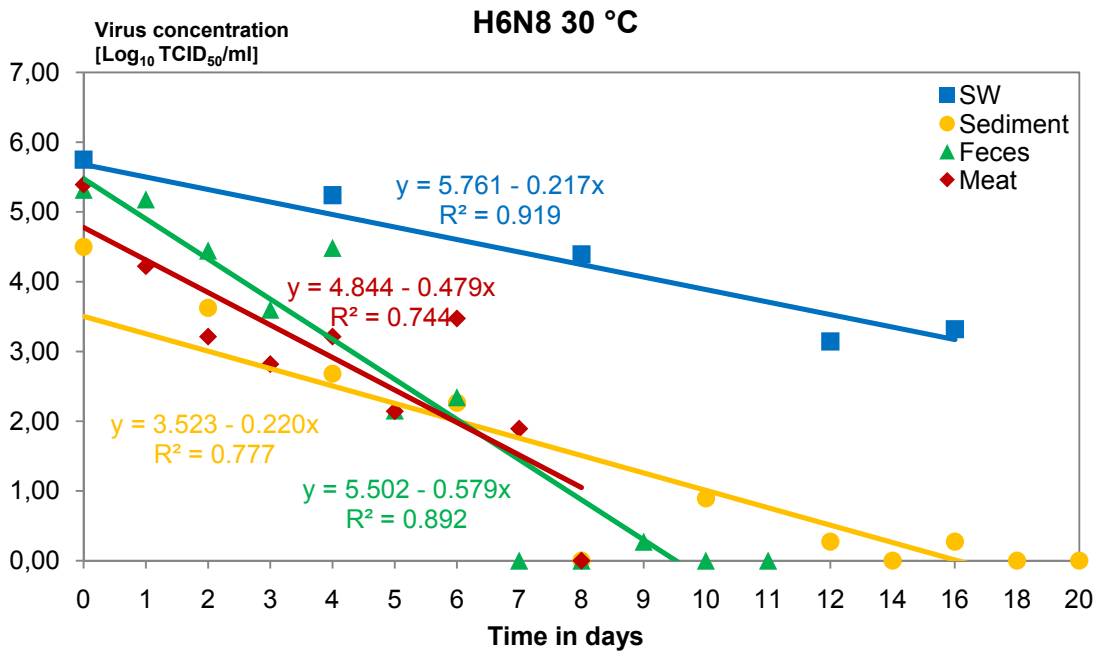


Figure 4.48: Persistence of LPAI H6N8 virus in SW, sediment, duck feces, and meat at 30 °C

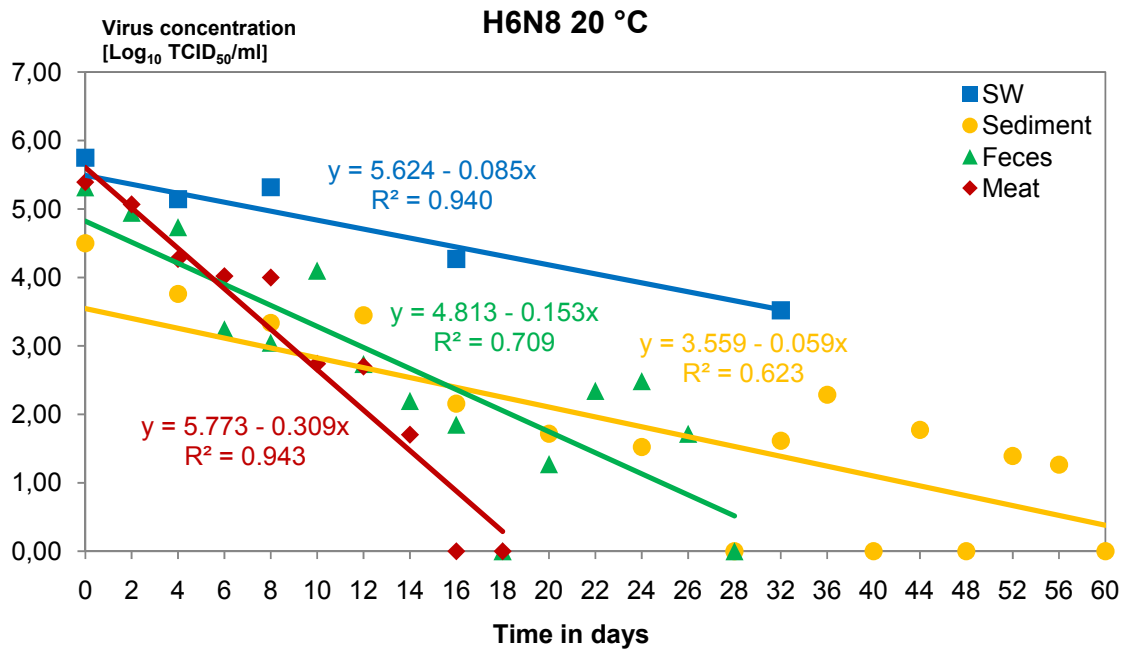


Figure 4.49: Persistence of LPAI H6N8 virus in SW, sediment, duck feces, and meat at 20 °C

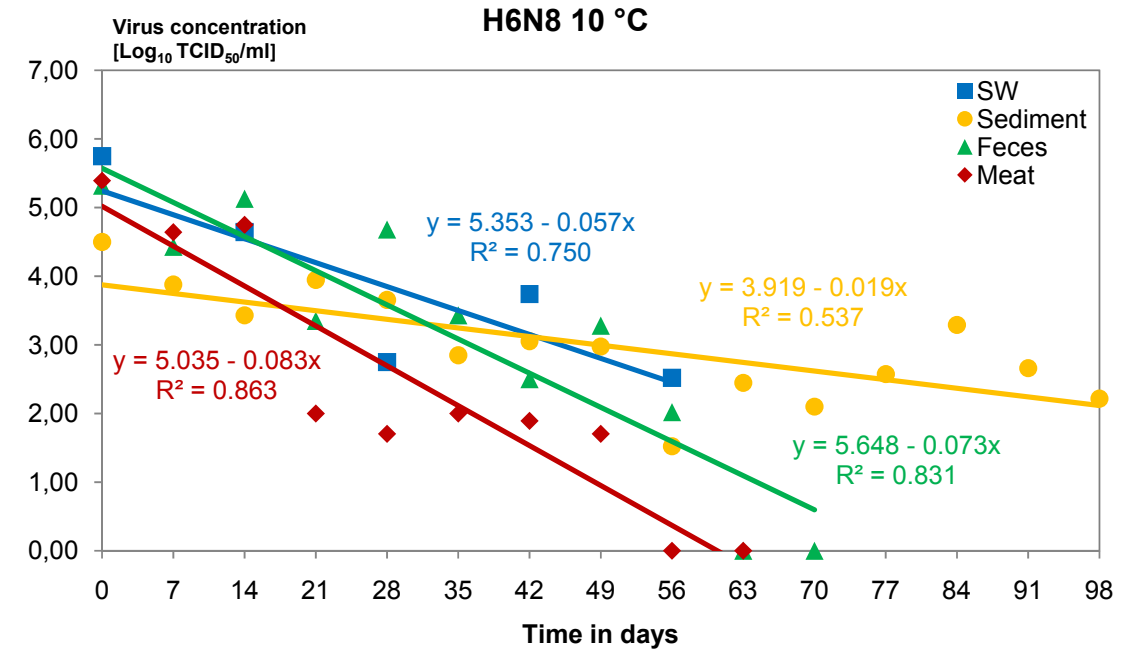


Figure 4.50: Persistence of LPAI H6N8 virus in SW, sediment, duck feces, and meat at 10 °C

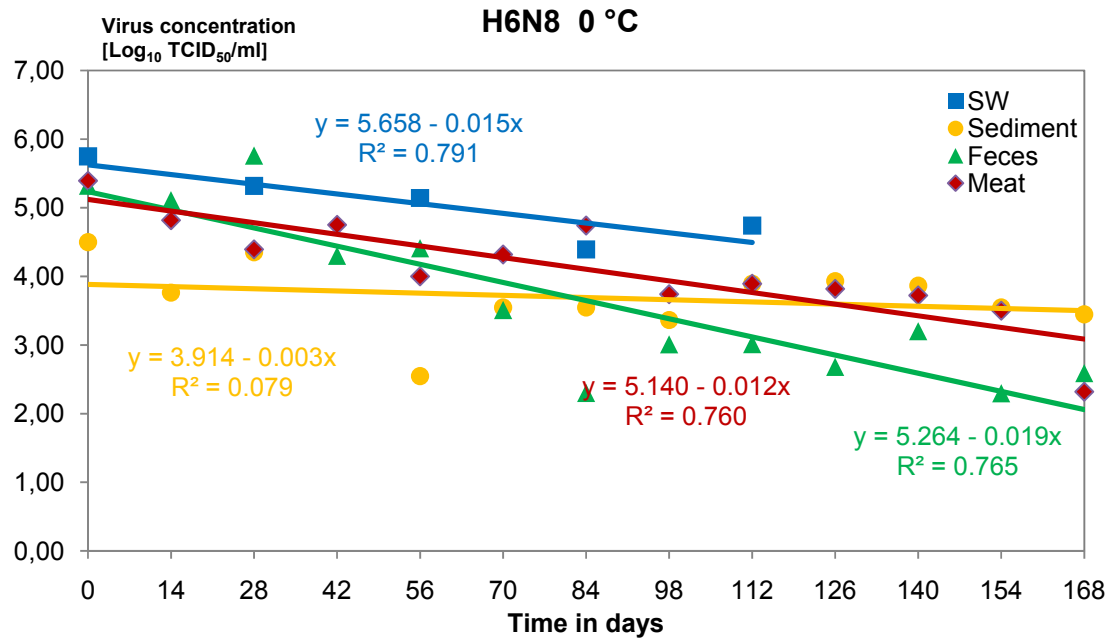


Figure 4.51: Persistence of LPAI H6N8 virus in SW, sediment, duck feces, and meat at 0 °C

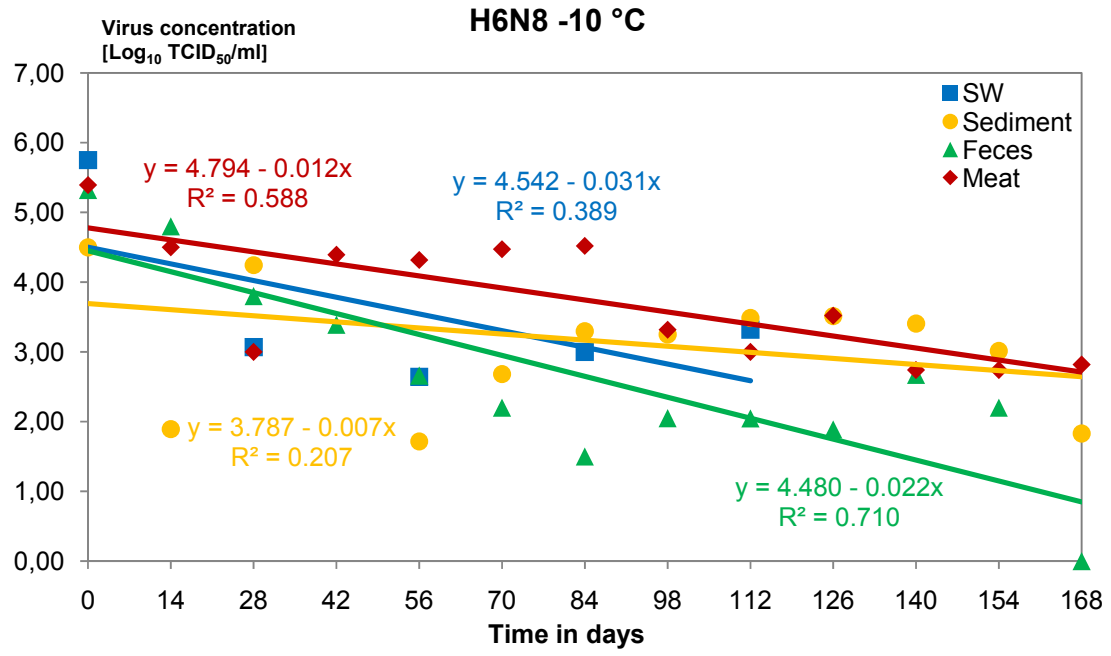


Figure 4.52: Persistence of LPAI H6N8 virus in SW, sediment, duck feces, and meat at -10 °C

4.6.4 Tenacity of human influenza virus H1N1 in SW, sediment, duck feces, and meat at various temperatures

The linear regression models showing the persistence of human influenza virus H1N1 in SW, sediment, duck feces, and meat at various temperatures are presented in Figures 4.53 to 4.57. In SW with a starting virus titer of $10^{5.50}$ $TCID_{50}$/ml the virus was detectable for 8, ≥ 16, ≥ 56, ≥ 112, and ≥ 112 days at 30, 20, 10, 0, and -10 °C, respectively. In sediment with a starting virus titer of $10^{3.50}$ $TCID_{50}$/ml the virus was detectable for 5, 32, 49, 126, and 140 days at 30, 20, 10, 0, and -10 °C, respectively. In duck feces with a starting virus titer of $10^{4.00}$ $TCID_{50}$/ml the virus was detectable for 4, 12, 49, 140, and 140 days at 30, 20, 10, 0, and -10 °C, respectively, while in duck meat with a starting virus titer of $10^{5.82}$ $TCID_{50}$/ml the virus was detectable for 6, 14, 56, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively.

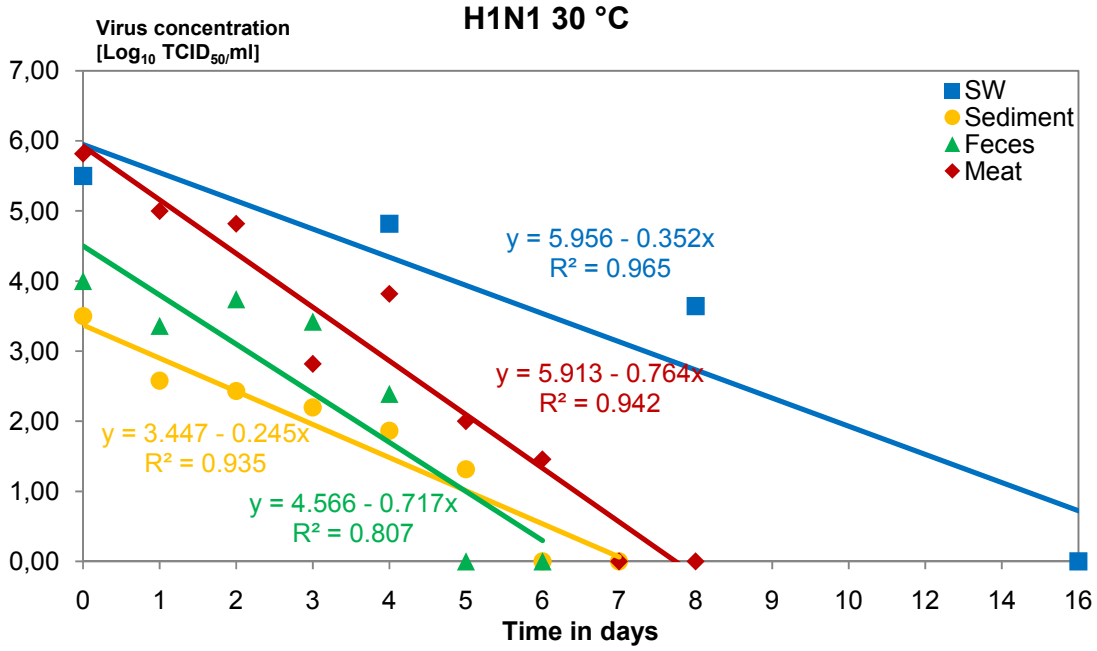


Figure 4.53: Persistence of human influenza virus H1N1 in SW, sediment, duck feces, and meat at 30 °C

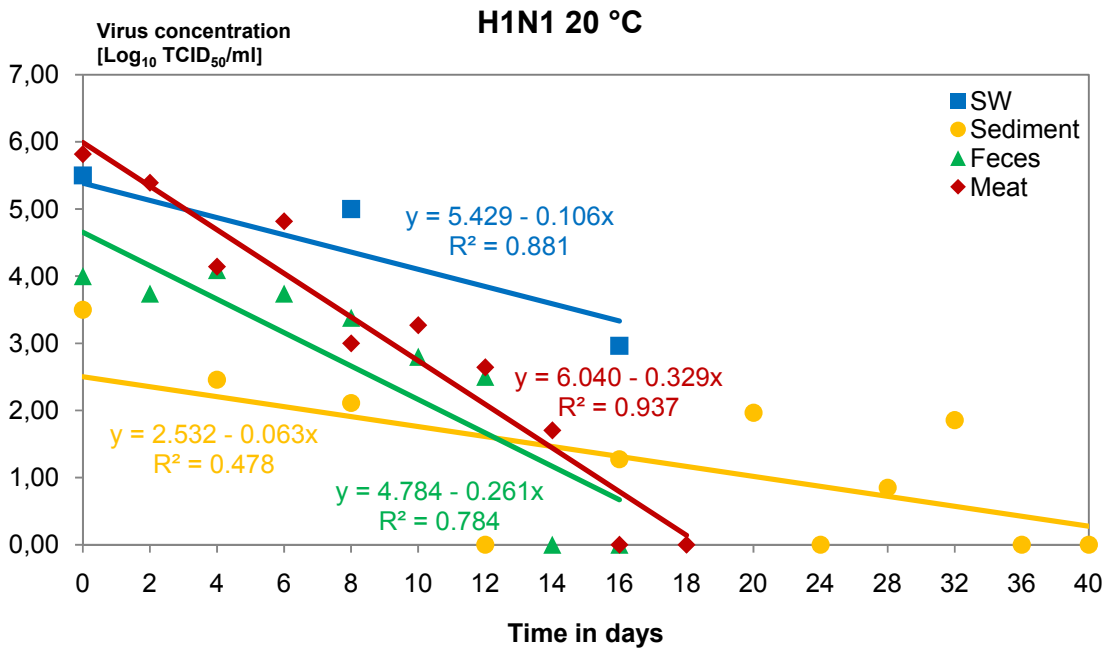


Figure 4.54: Persistence of human influenza virus H1N1 in SW, sediment, duck feces, and meat at 20 °C

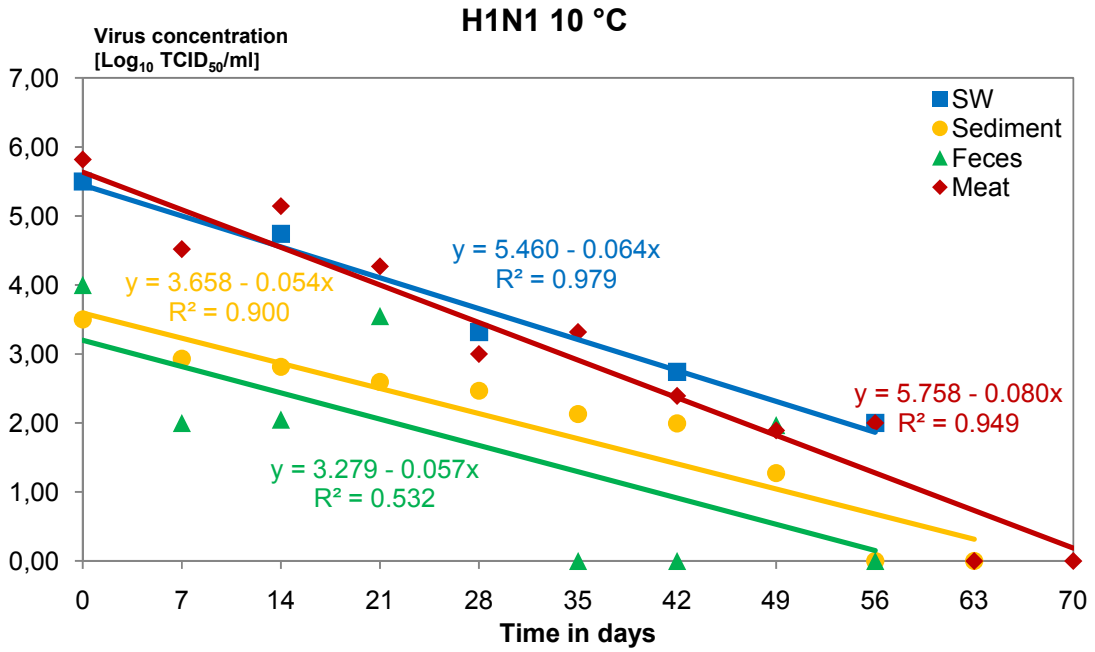


Figure 4.55: Persistence of human influenza virus H1N1 in SW, sediment, duck feces, and meat at 10 °C

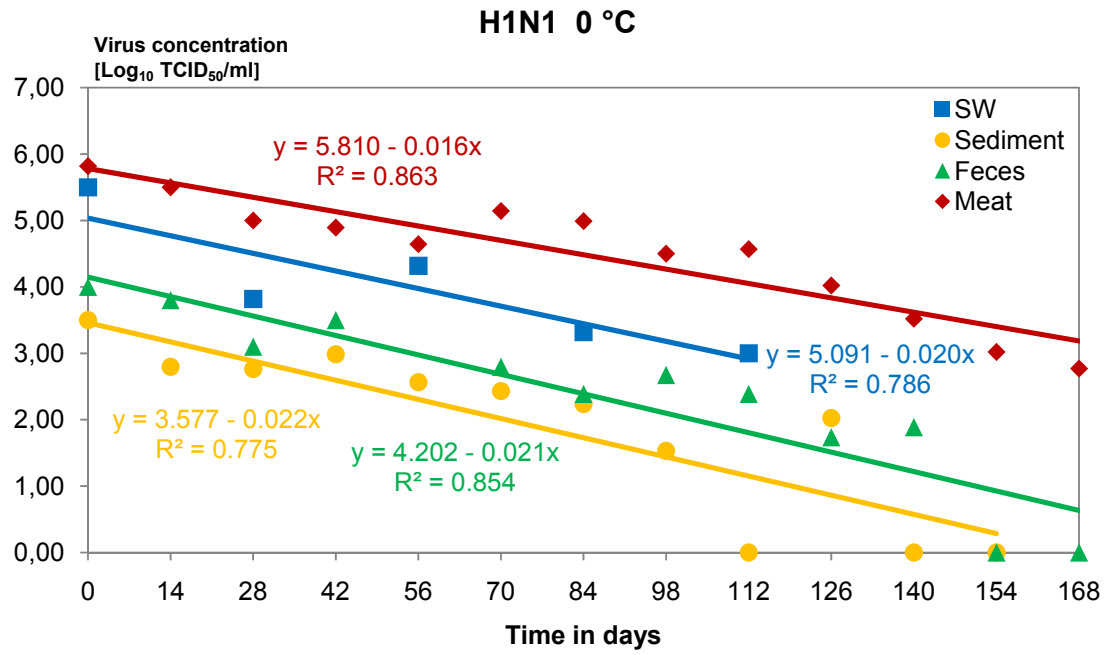


Figure 4.56: Persistence of human influenza virus H1N1 in SW, sediment, duck feces, and meat at 0 °C

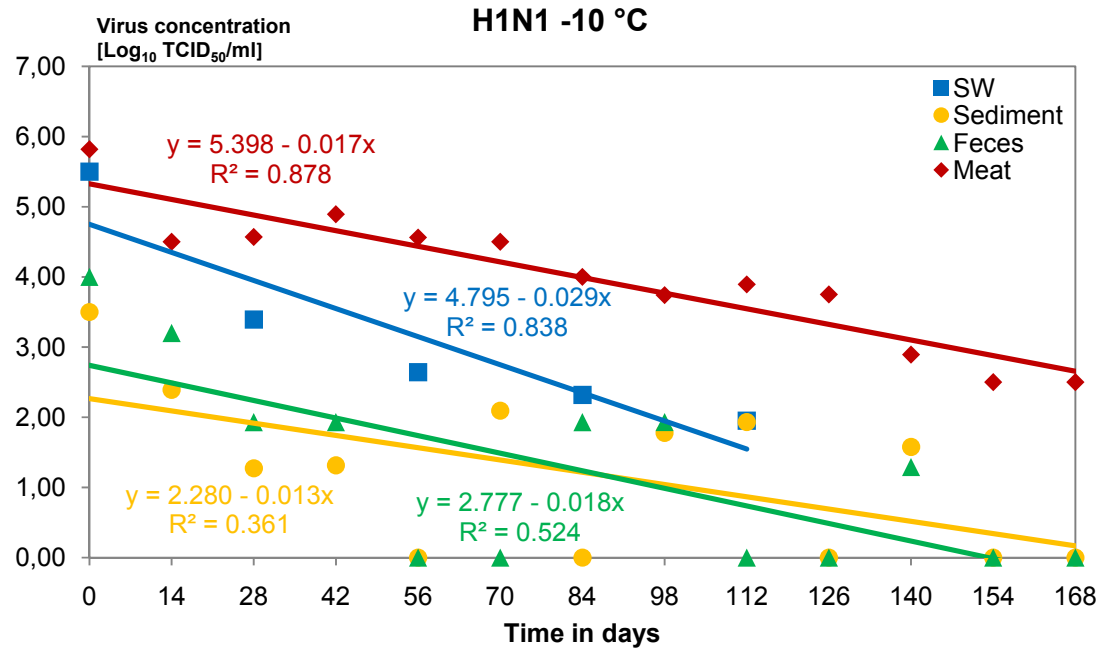


Figure 4.57: Persistence of human influenza virus H1N1 in SW, sediment, duck feces, and meat at -10 °C

4.6.5 Tenacity of NDV in SW, sediment, duck feces, and meat at various temperatures

The linear regression models showing the persistence of NDV in SW, sediment, duck feces, and meat at various temperatures are presented in Figures 4.58 to 4.62. In SW with a starting virus titer of $10^{5.89}$ $TCID_{50}$/ml the virus was detectable for ≥ 16, ≥ 32, ≥ 56, ≥ 112, and ≥ 112 days at 30, 20, 10, 0, and -10 °C, respectively. In sediment with a starting virus titer of $10^{7.20}$ $TCID_{50}$/ml the virus was detectable for ≥ 30, ≥ 60, ≥ 98, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively. In duck feces with a starting virus titer of $10^{7.50}$ $TCID_{50}$/ml the virus was detectable for ≥ 14, ≥ 28, ≥ 84, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively, while in duck meat with starting virus titer of $10^{6.32}$ $TCID_{50}$/ml the virus was detectable for ≥ 14, ≥ 28, ≥ 84, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively.

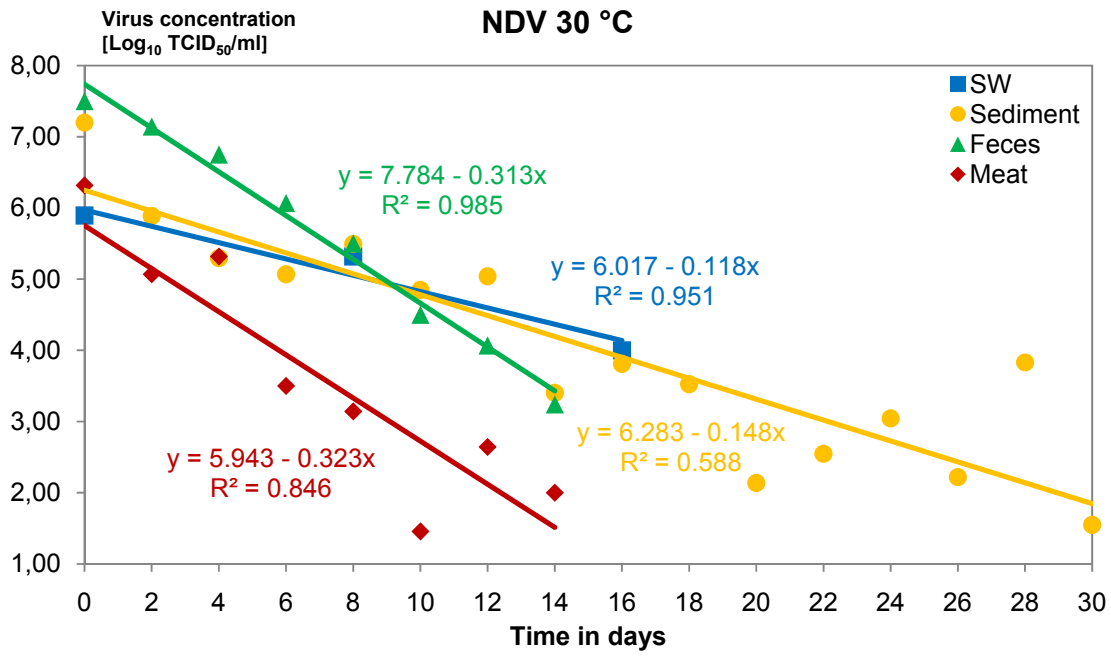


Figure 4.58: Persistence of NDV in SW, sediment, duck feces, and meat at 30 °C

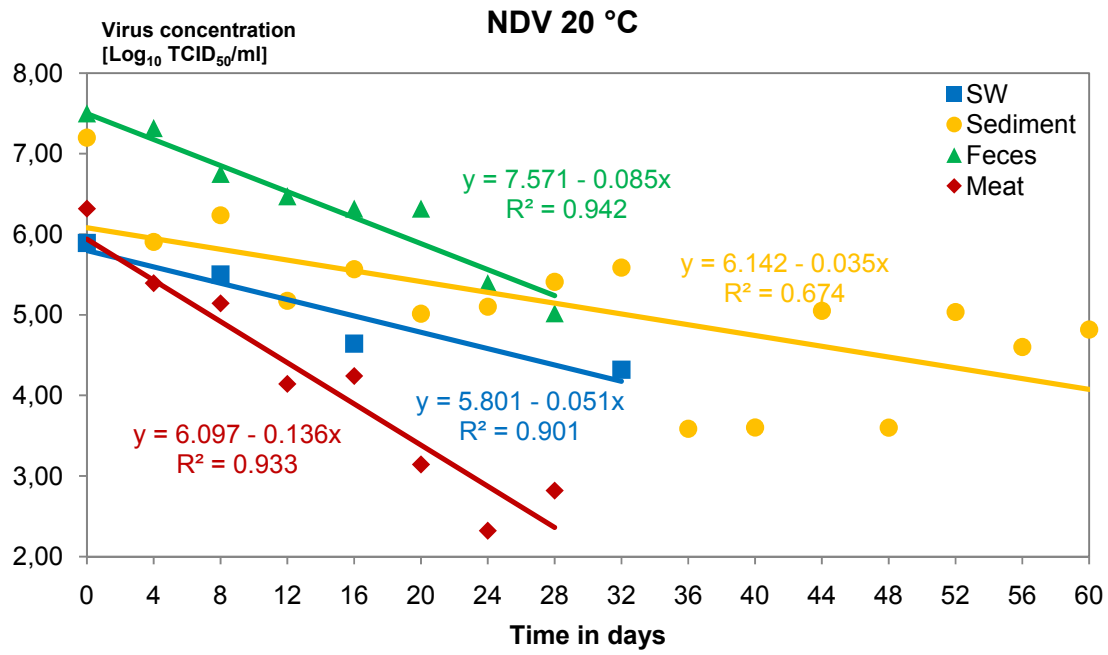


Figure 4.59: Persistence of NDV in SW, sediment, duck feces, and meat at 20 °C

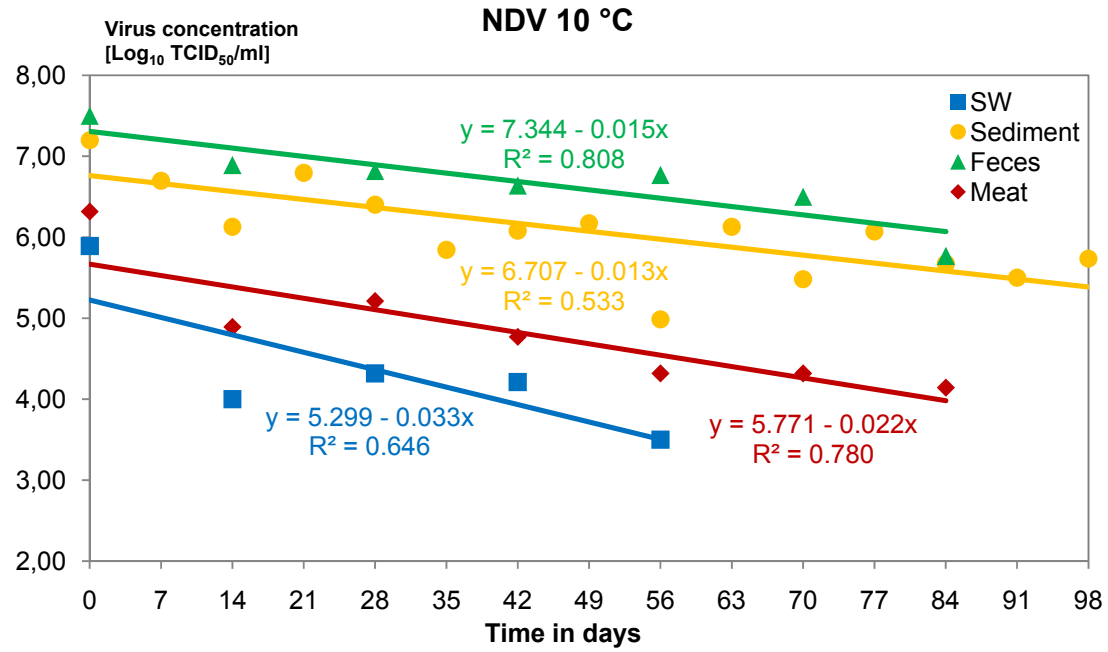


Figure 4.60: Persistence of NDV in SW, sediment, duck feces, and meat at 10 °C

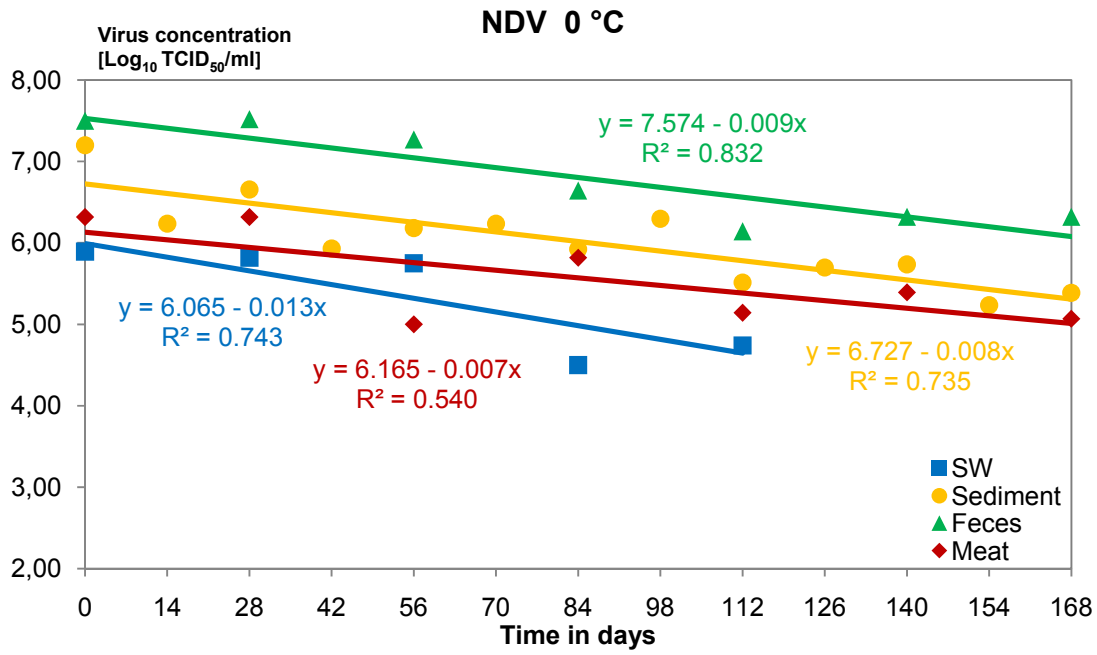


Figure 4.61: Persistence of NDV in SW, sediment, duck feces, and meat at 0 °C

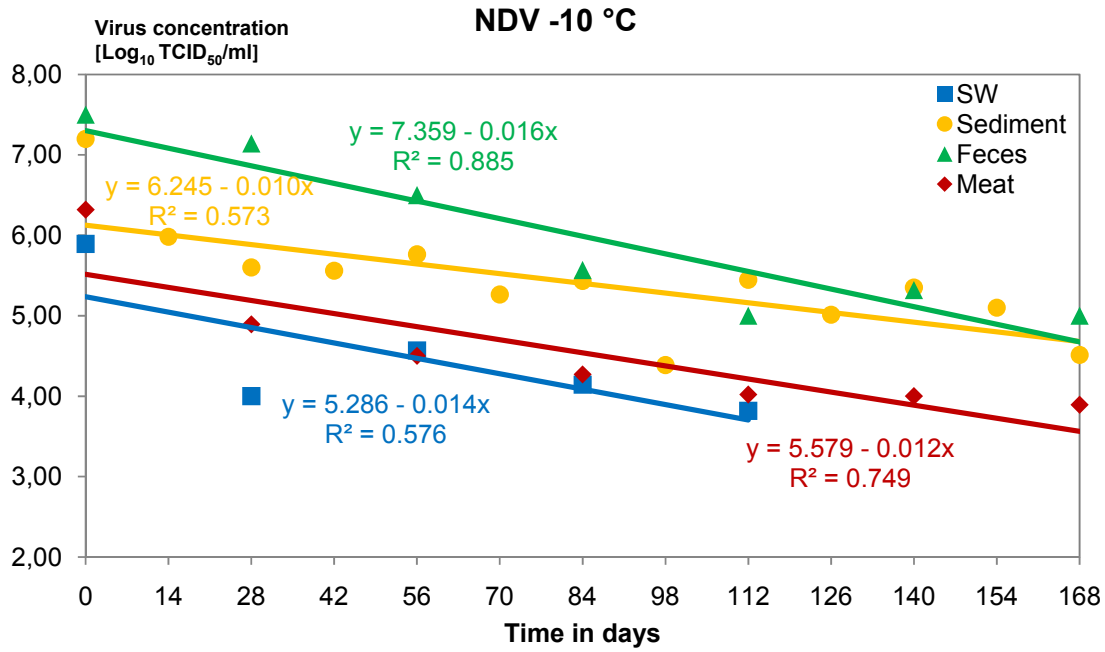


Figure 4.62: Persistence of NDV in SW, sediment, duck feces, and meat at -10 °C

4.6.6 Tenacity of ECBO in SW, sediment, duck feces, and meat at various temperatures

The linear regression models showing the persistence of ECBO in SW, sediment, duck feces, and meat at various temperatures are presented in Figures 4.63 to 4.67. In SW with a starting virus titer of $10^{6.50}$ $TCID_{50}$/ml the virus was detectable for ≥ 16, ≥ 48, ≥ 56, ≥ 112, and ≥ 112 days at 30, 20, 10, 0, and -10 °C, respectively. In sediment with a starting virus titer of $10^{5.25}$ $TCID_{50}$/ml the virus was detectable for ≥ 30, ≥ 60, ≥ 98, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively. In duck feces with a starting virus titer of $10^{6.75}$ $TCID_{50}$/ml the virus was detectable for ≥ 14, ≥ 28, ≥ 84, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively, while in duck meat with a starting virus titer of $10^{6.75}$ $TCID_{50}$/ml the virus was detectable for ≥ 14, ≥ 28, ≥ 84, ≥ 168, and ≥ 168 days at 30, 20, 10, 0, and -10 °C, respectively.

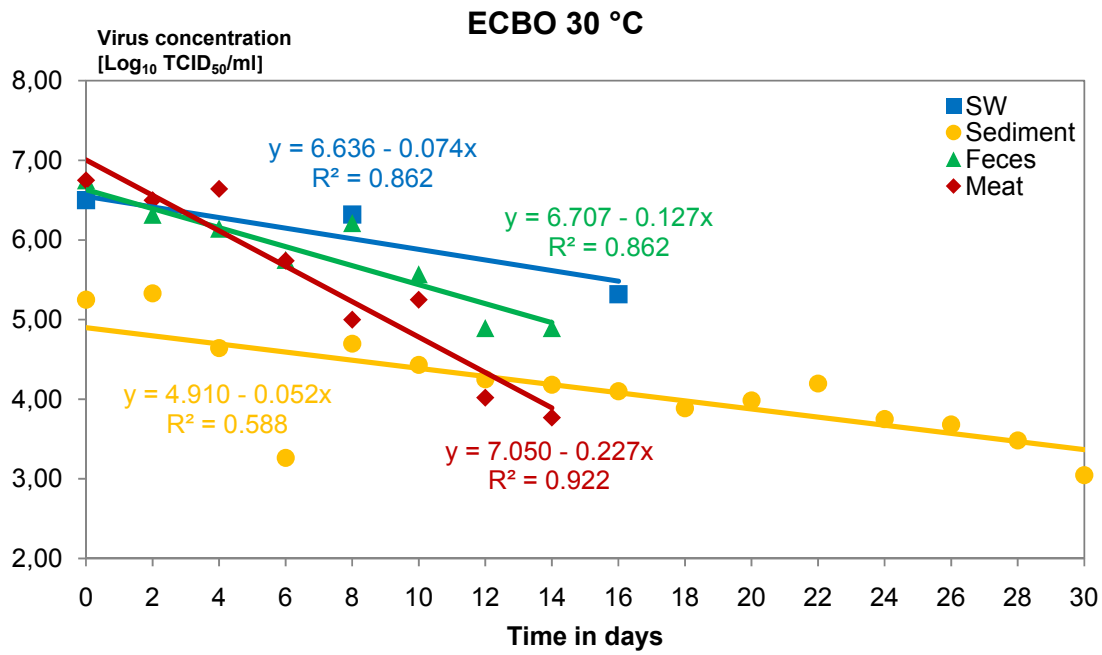


Figure 4.63: Persistence of ECBO in SW, sediment, duck feces, and meat at 30 °C

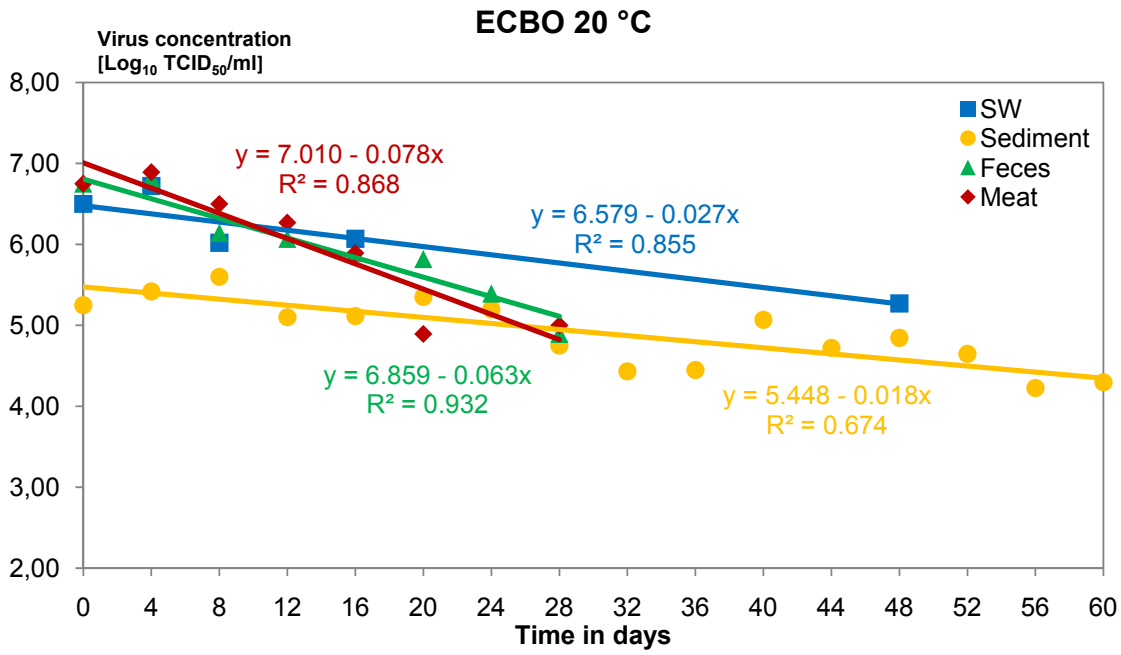


Figure 4.64: Persistence of ECBO in SW, sediment, duck feces, and meat at 20 °C

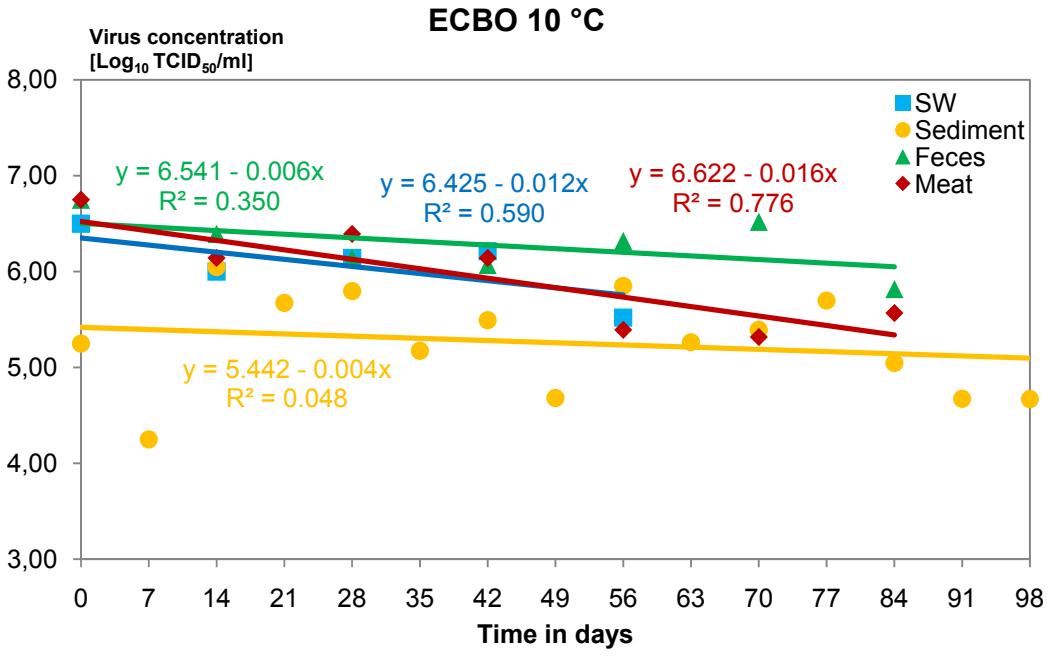


Figure 4.65: Persistence of ECBO in SW, sediment, duck feces, and meat at 10 °C

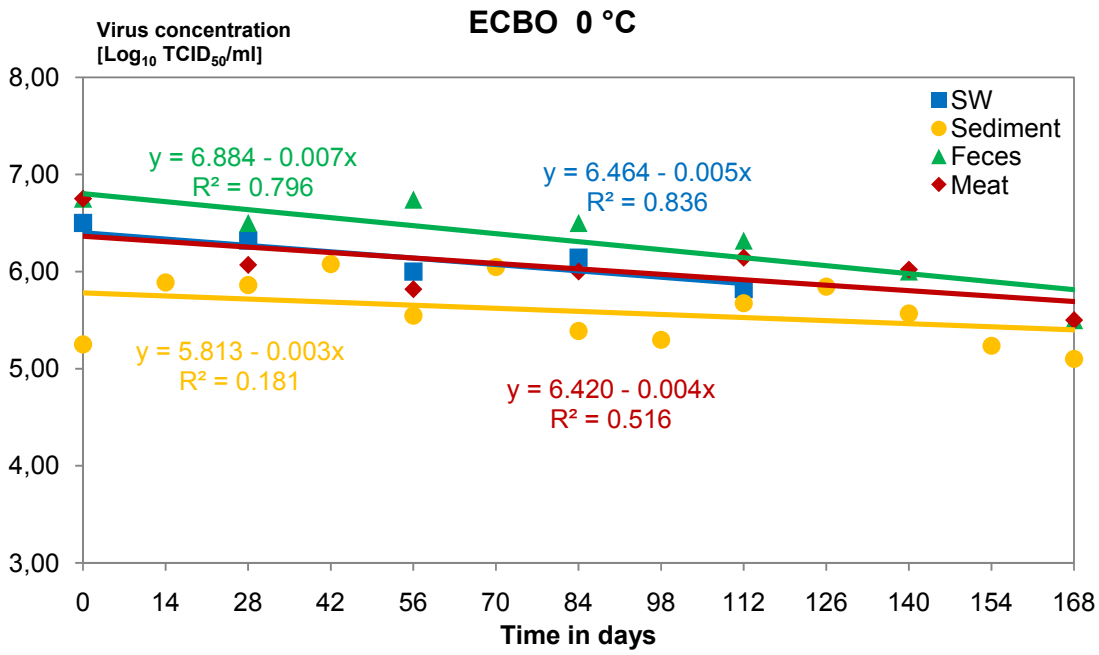


Figure 4.66: Persistence of ECBO in SW, sediment, duck feces, and meat at 0 °C

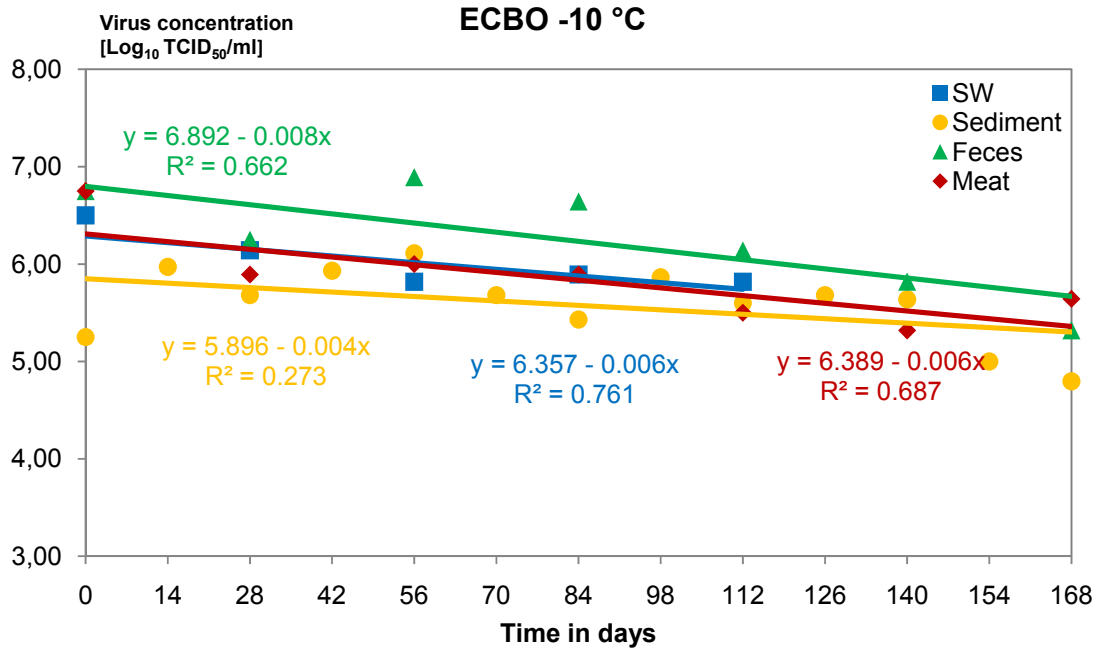
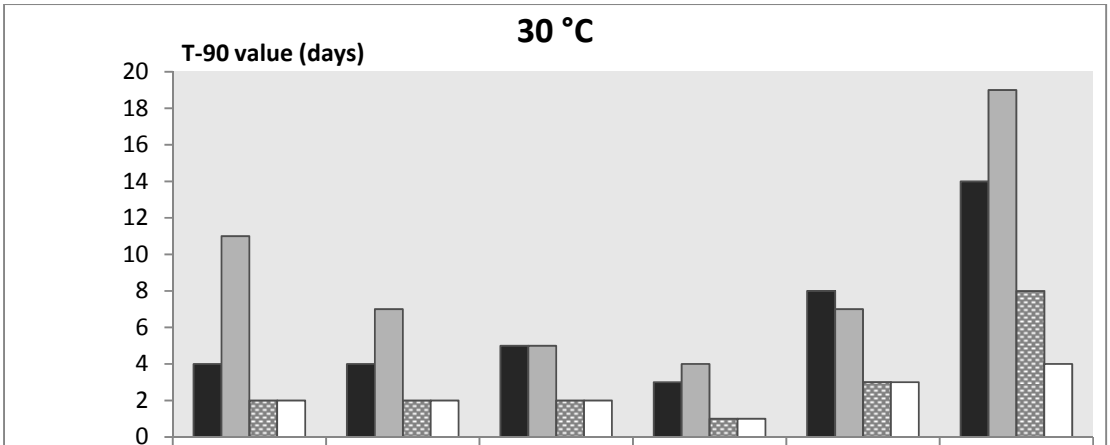


Figure 4.67: Persistence of ECBO in SW, sediment, duck feces, and meat at -10 °C

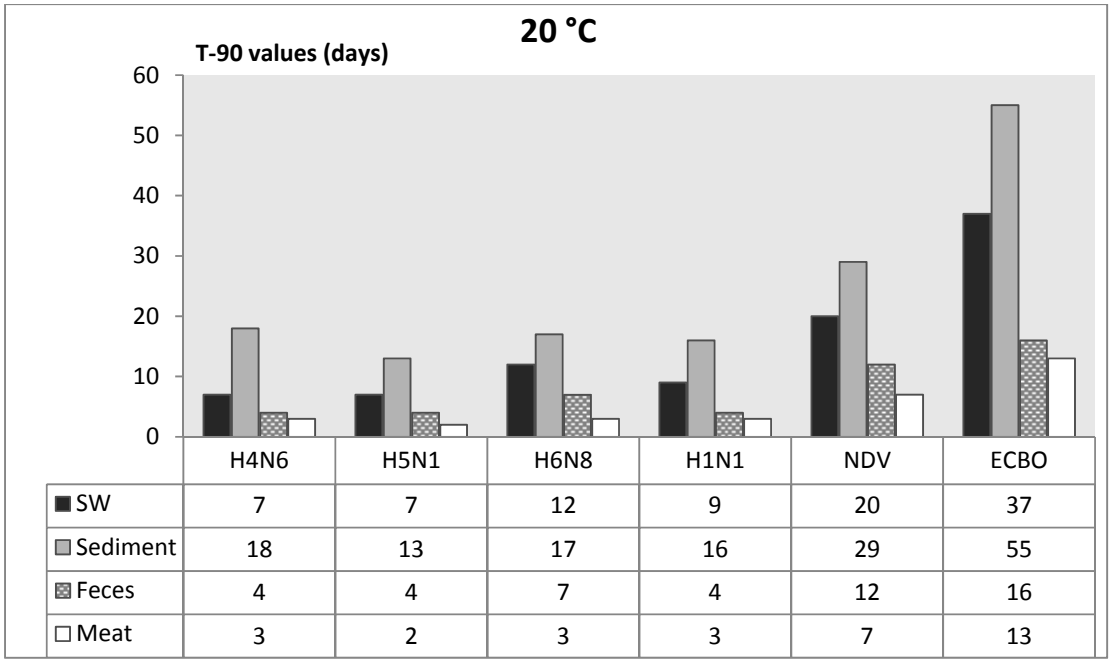
4.7 Comparison of T-90 values of the viruses in various substrates as calculated using the germ carrier technique

For a better comparison of the persistence of influenza and model viruses in various substrates the T-90 values calculated from linear regression models are presented in Figures 4.68 to 4.72. The T-90 values show that in all of the substrates the persistence of the influenza viruses is highest at -10 °C followed by 0, 10, 20, and 30 °C. T-90 values of individual influenza viruses vary in different substrates but generally the viruses survived for longest in the sediment followed by in SW, while virus survival was the shortest in both duck feces and meat. The human influenza virus has slightly lower T-90 values than those of AIV. The model viruses have higher T-90 values than the influenza viruses and within these viruses the ECBO has the highest T-90 values in all of the substrates at all temperatures.



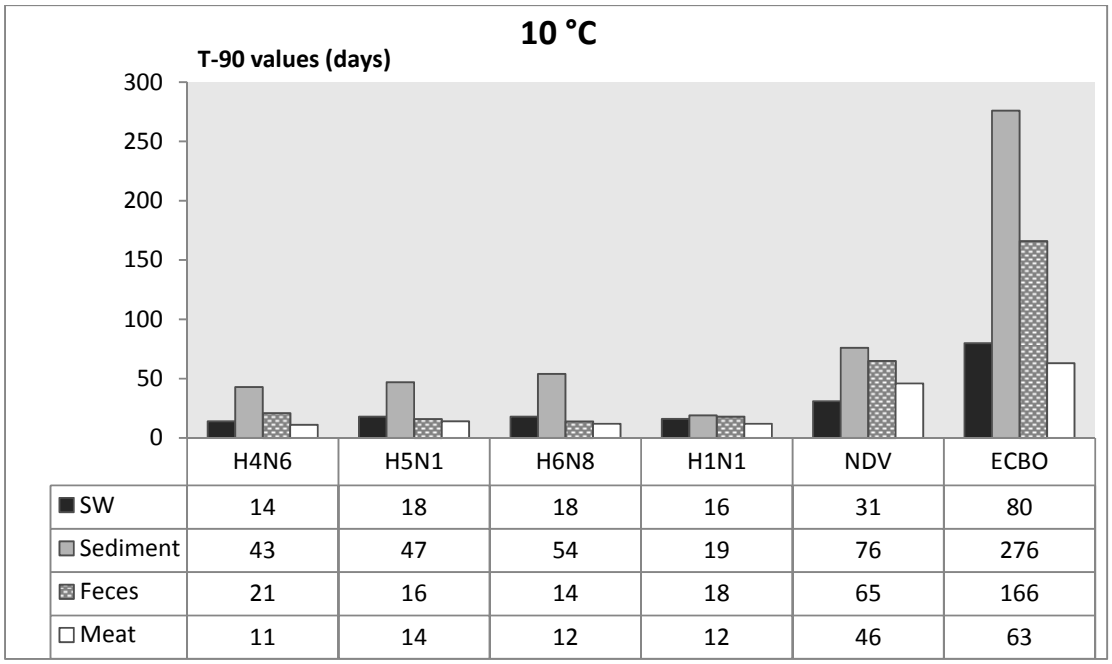
	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
SW	4	4	5	3	8	14
Sediment	11	7	5	4	7	19
Feces	2	2	2	1	3	8
Meat	2	2	2	1	3	4

Figure 4.68: Comparison of T-90 values of influenza and model viruses in SW, sediment, duck feces, and meat at 30 °C



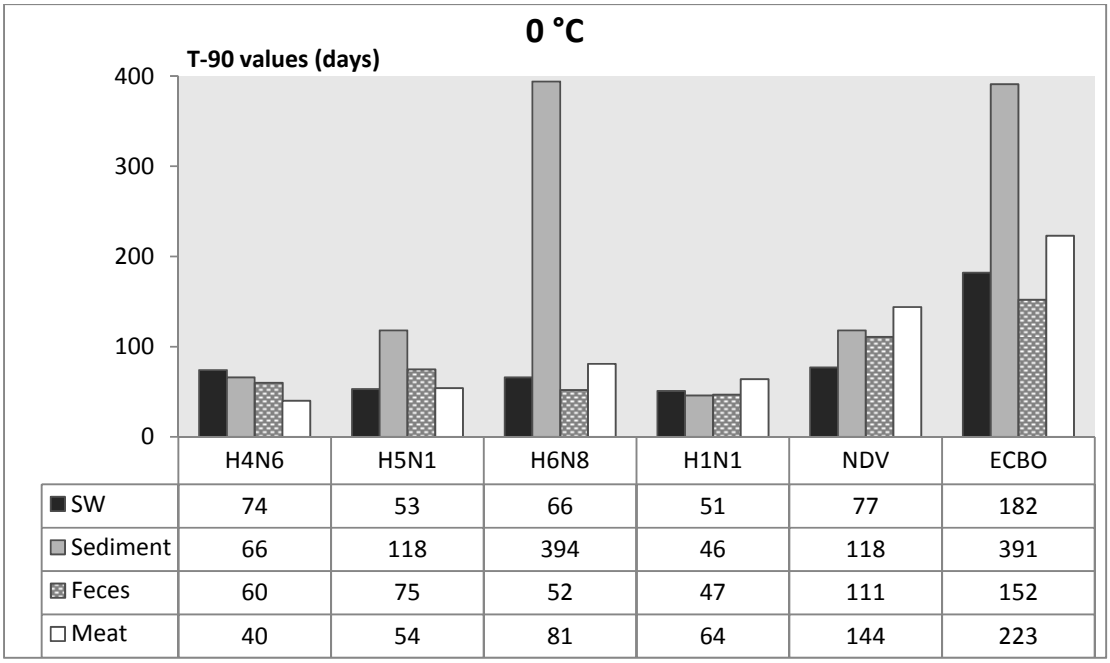
	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
SW	7	7	12	9	20	37
Sediment	18	13	17	16	29	55
Feces	4	4	7	4	12	16
Meat	3	2	3	3	7	13

Figure 4.69: Comparison of T-90 values of influenza and model viruses in SW, sediment, duck feces, and meat at 20 °C



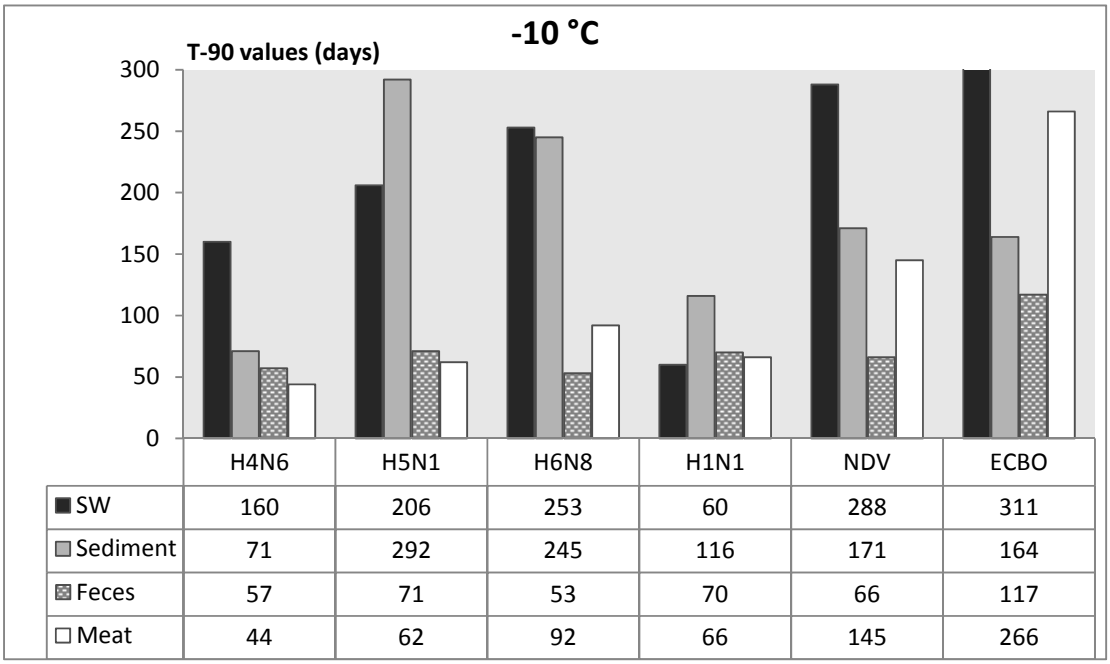
	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
SW	14	18	18	16	31	80
Sediment	43	47	54	19	76	276
Feces	21	16	14	18	65	166
Meat	11	14	12	12	46	63

Figure 4.70: Comparison of T-90 values of influenza and model viruses in SW, sediment, duck feces, and meat at 10 °C



	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
SW	74	53	66	51	77	182
Sediment	66	118	394	46	118	391
Feces	60	75	52	47	111	152
Meat	40	54	81	64	144	223

Figure 4.71: Comparison of T-90 values of influenza and model viruses in SW, sediment, duck feces, and meat at 0 °C



	H4N6	H5N1	H6N8	H1N1	NDV	ECBO
SW	160	206	253	60	288	311
Sediment	71	292	245	116	171	164
Feces	57	71	53	70	66	117
Meat	44	62	92	66	145	266

Figure 4.72: Comparison of T-90 values of influenza and model viruses in SW, sediment, duck feces, and meat at -10 °C

4.8 Comparison of T-90 values of the influenza and model viruses calculated by suspending the virus in water and using the germ carrier technique

The comparison of the T-90 values of the influenza and model viruses calculated both by adding the virus directly into the SW and by placing the inoculated germ carriers into the SW are shown in Figures 4.73 to 4.77. The linear regression analyses showing the persistence of viruses calculated after suspending the viruses directly in the SW are presented in Figures 4.1 to 4.30. Those calculated using the germ carrier technique are presented in Figures 4.38 to 4.67. In general, T-90 values of the viruses in the SW using the germ carrier technique were higher by a factor of up to 10 times as compared to suspending the viruses in the water. There were only two exceptions from this rule (H4N6 at 10 °C and ECBO at -10 °C).

Figures 4.78 and 4.79 present the results for the recovery of filter bound H4N6, N5N1, and H6N8 AIV after incubation in the lake water at 0 and -10 °C. It is clear from Figure 4.79 that a single freeze-thaw cycle at -10 °C resulted in an abrupt decline in the titers of the viruses. Due to the effect of freeze-thawing on viral infectivity, the zero day time point is an outlier in the regression analysis. Hence the regression analyses on -10 °C samples were performed beginning with day 28. Keeping in view this observation the linear regression analyses of the sequential data, for all of the viruses in various substrates at -10 °C were performed including and excluding the 0 day data point and are presented in Tables 4.6 and 4.7.

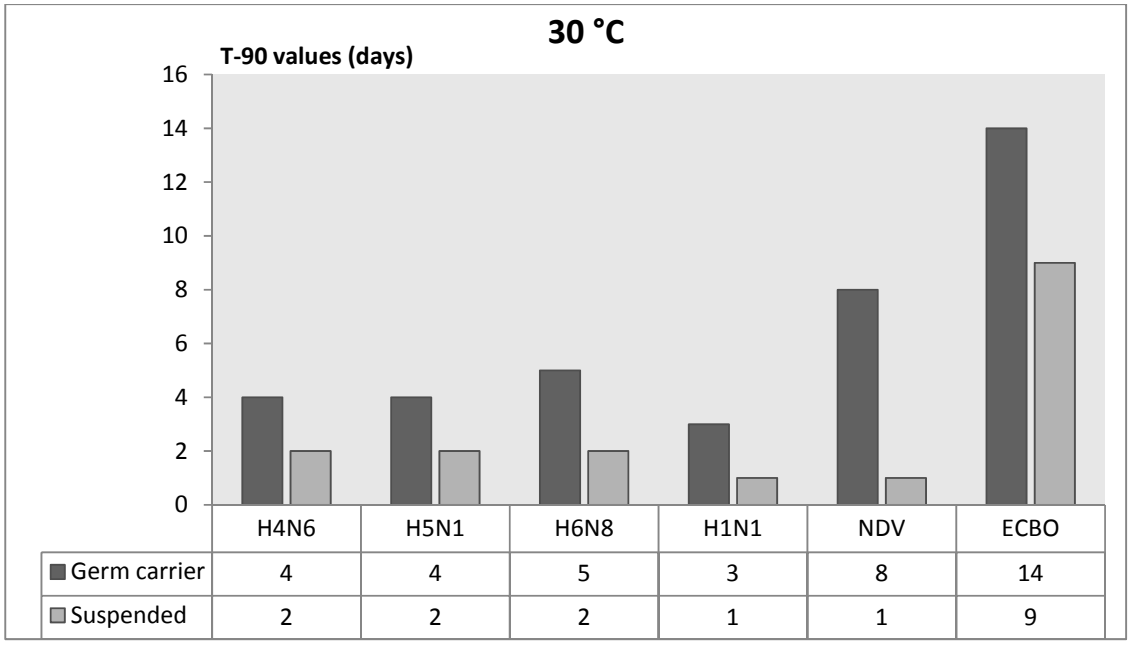


Figure 4.73: Comparison of T-90 values of the viruses in SW obtained by suspending the viruses in water and using the germ carrier technique at 30 °C

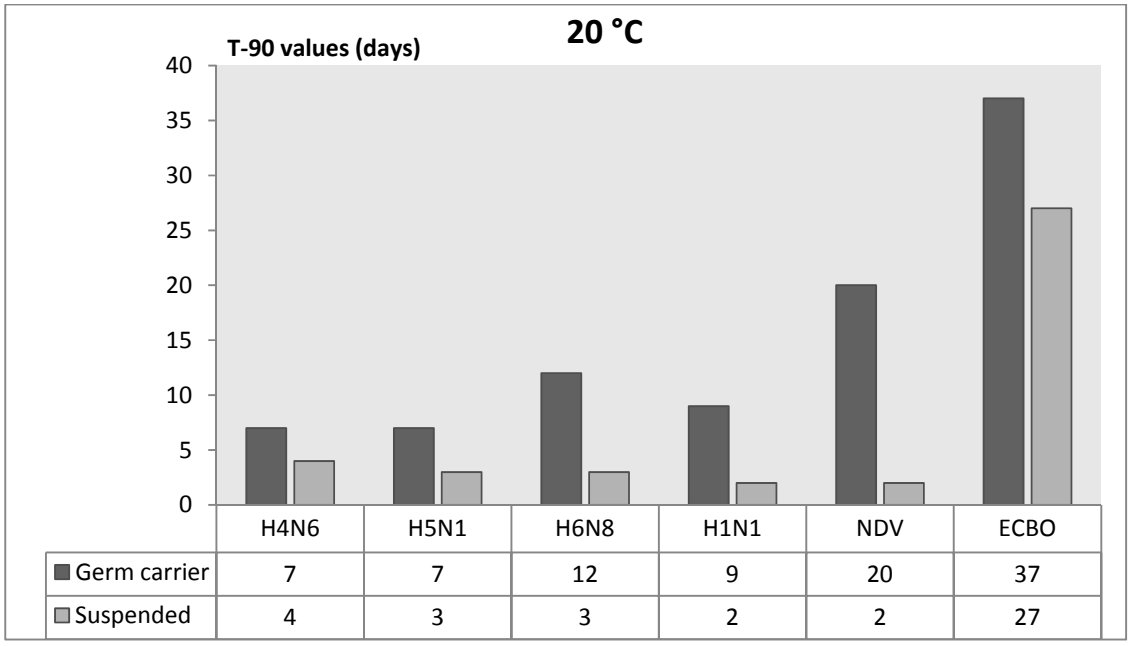


Figure 4.74: Comparison of T-90 values of the viruses in SW obtained by suspending the viruses in water and using the germ carrier technique at 20 °C

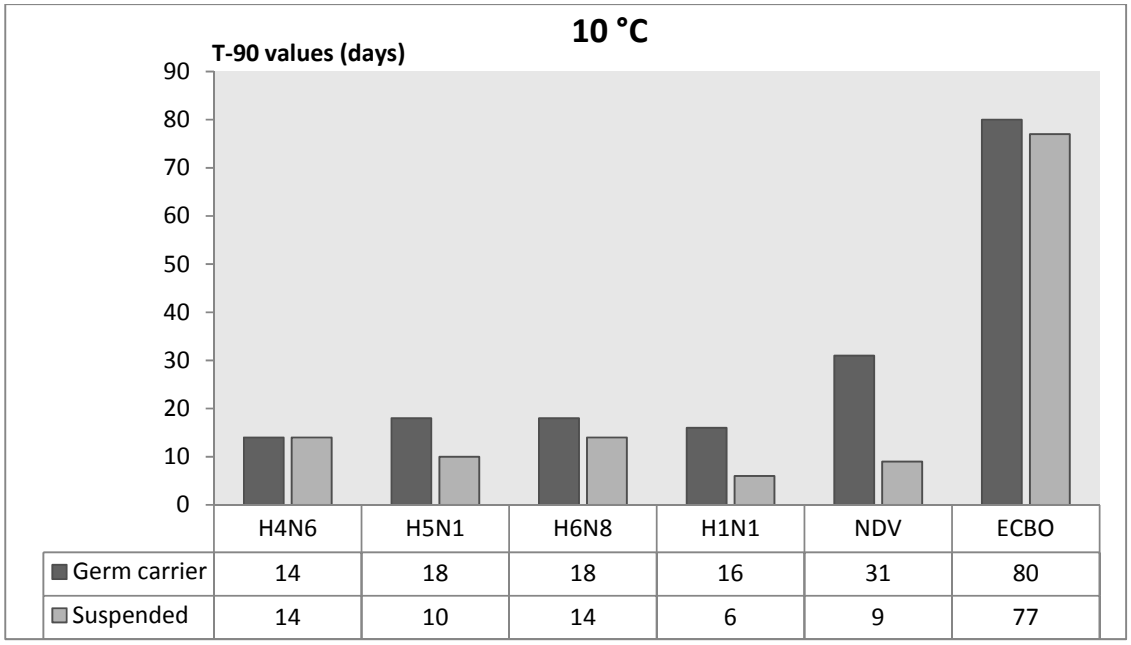


Figure 4.75: Comparison of T-90 values of the viruses in SW obtained by suspending the viruses in water and using the germ carrier technique at 10 °C

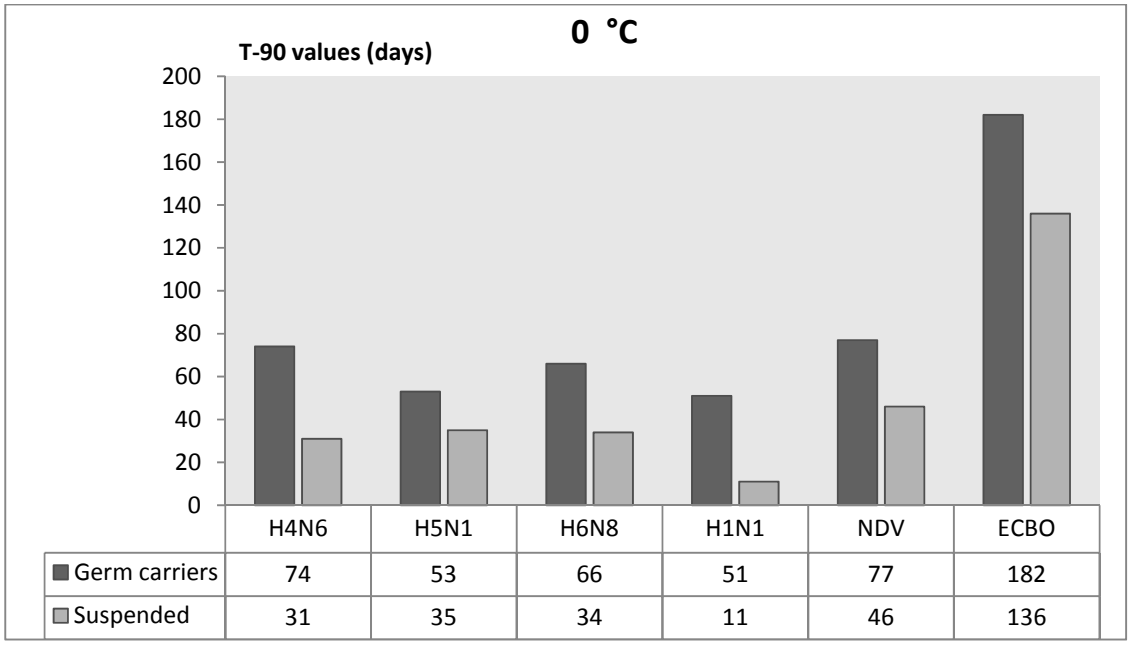


Figure 4.76: Comparison of T-90 values of the viruses in SW obtained by suspending the viruses in water and using the germ carrier technique at 0 °C

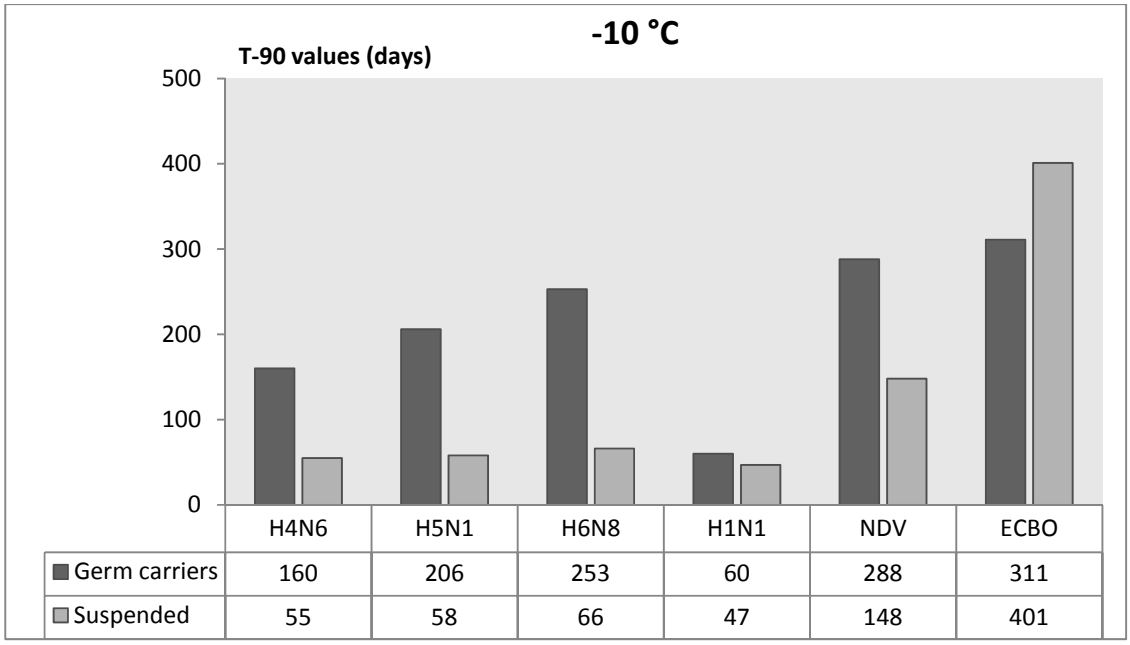


Figure 4.77: Comparison of T-90 values of the viruses in SW obtained by suspending the viruses in water and using the germ carrier technique at -10 °C

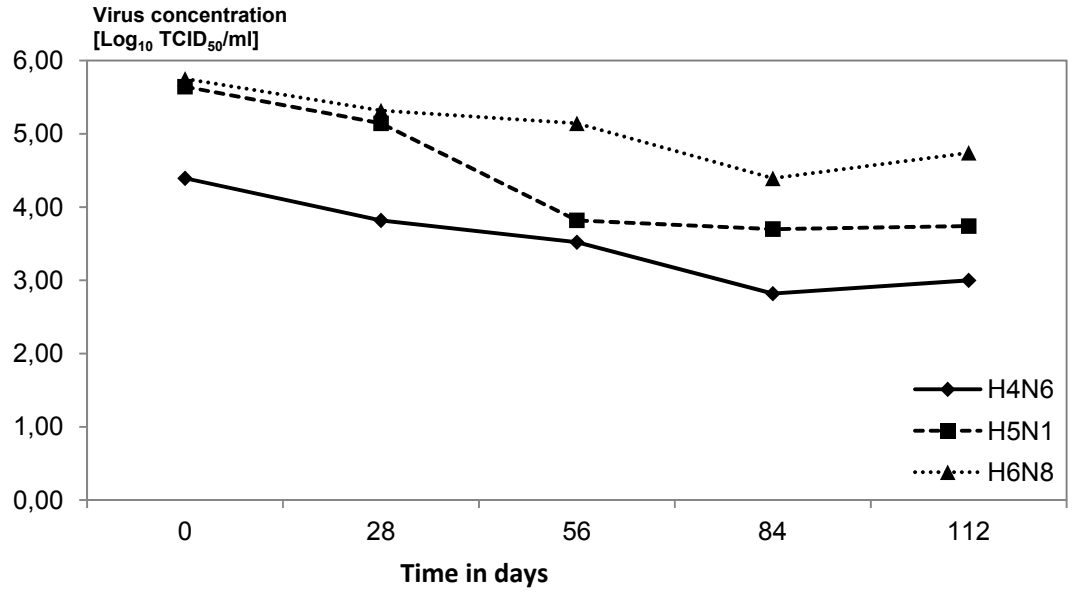


Figure 4.78: Persistence of H4N6, H5N1, and H6N8 AIV in lake water at 0 °C using the germ carrier technique

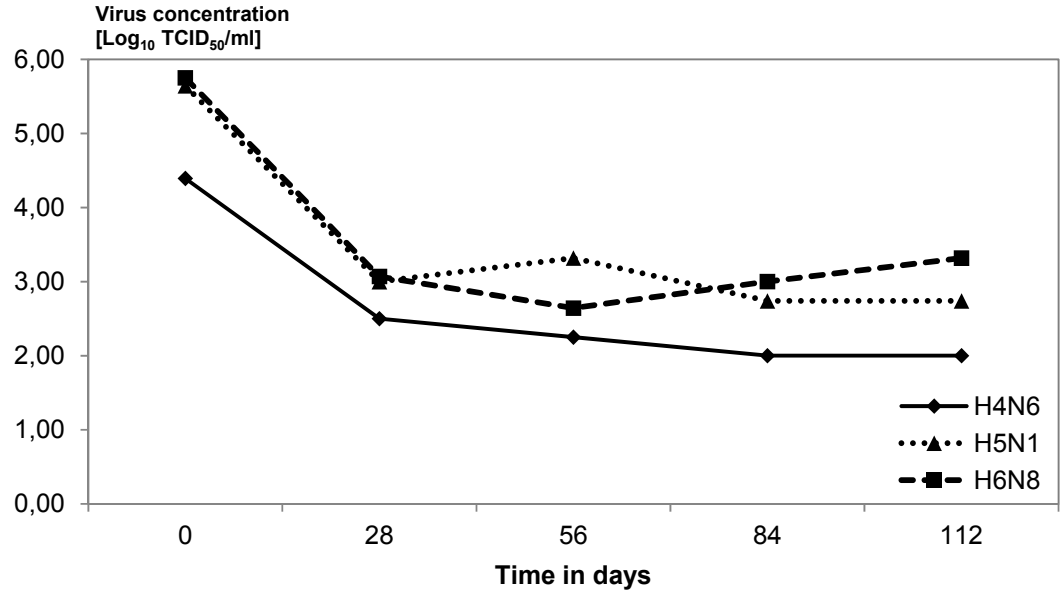


Figure 4.79: Persistence of H4N6, H5N1, and H6N8 AIV in lake water at -10 °C using the germ carrier technique

Table 4.6: Linear regression models for the persistence of influenza and model viruses in various substrates at -10 °C calculated including the initial data points

Virus Type	Surface water			Sediment			Duck feces			Duck meat		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	$y = 4.80 - 0.029x$	0.84	206 (34)	$y = 2.28 - 0.013x$	0.36	476 (79)	$y = 2.78 - 0.018x$	0.52	329 (55)	$y = 5.40 - 0.017x$	0.88	363 (60)
H4N6	$y = 3.69 - 0.019x$	0.69	318 (53)	$y = 2.91 - 0.019x$	0.59	314 (52)	$y = 3.43 - 0.020x$	0.81	294 (49)	$y = 4.96 - 0.024x$	0.89	255 (42)
H5N1	$y = 4.70 - 0.022x$	0.61	277 (46)	$y = 3.62 - 0.009x$	0.35	698 (116)	$y = 4.10 - 0.017x$	0.68	353 (59)	$y = 4.93 - 0.018x$	0.88	333 (55)
H6N8	$y = 4.54 - 0.031x$	0.39	194 (32)	$y = 3.79 - 0.007x$	0.21	888 (148)	$y = 4.48 - 0.022x$	0.71	276 (46)	$y = 4.79 - 0.012x$	0.59	482 (80)
NDV	$y = 5.29 - 0.014x$	0.58	419 (70)	$y = 6.25 - 0.010x$	0.57	620 (103)	$y = 7.36 - 0.016x$	0.89	372 (62)	$y = 5.58 - 0.012x$	0.75	493 (82)
ECBO	$y = 6.36 - 0.006x$	0.76	1041 (174)	$y = 5.90 - 0.004x$	0.27	1637 (273)	$y = 6.89 - 0.008x$	0.66	797 (133)	$y = 6.39 - 0.006x$	0.69	946 (158)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, x: days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 4.7: Linear regression models for the persistence of influenza and model viruses in various substrates at -10 °C calculated after excluding the effect of a single freeze-thaw cycle

Virus Type	Surface water			Sediment			Duck feces			Duck meat		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 3.74 - 0.017*x*	0.96	361 (60)	y = 1.82 - 0.009*x*	0.29	694 (116)	y = 2.31 - 0.014*x*	0.40	421 (70)	y = 5.24 - 0.015*x*	0.85	395 (66)
H4N6	y = 2.63 - 0.006*x*	0.89	960 (160)	y = 2.35 - 0.014*x*	0.44	425 (71)	y = 3.14 - 0.018*x*	0.77	340 (57)	y = 4.89 - 0.023*x*	0.86	261 (44)
H5N1	y = 3.29 - 0.005*x*	0.41	1238 (206)	y = 3.00 - 0.003*x*	0.13	1754 (292)	y = 3.77 - 0.014*x*	0.60	426 (71)	y = 4.72 - 0.016*x*	0.87	371 (62)
H6N8	y = 2.73 - 0.004*x*	0.26	1517 (253)	y = 3.47 - 0.004*x*	0.08	1471 (245)	y = 4.16 - 0.019*x*	0.63	316 (53)	y = 4.57 - 0.010*x*	0.48	572 (92)
NDV	y = 4.38 - 0.003*x*	0.15	1731 (288)	y = 5.88 - 0.006*x*	0.50	1027 (171)	y = 7.24 - 0.015*x*	0.82	397 (66)	y = 4.94 - 0.007*x*	0.91	871 (145)
ECBO	y = 6.14 - 0.003*x*	0.57	1869 (311)	y = 6.14 - 0.006*x*	0.57	983 (164)	y = 7.02 - 0.009*x*	0.63	702 (117)	y = 6.08 - 0.004*x*	0.55	1594 (266)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

5 Discussion

Influenza A viruses infect a wide range of animal species including humans and are of great zoonotic importance. Wild aquatic birds in the orders Anseriformes and Charadriiformes serve as primary reservoirs for AIV (Stallknecht and Brown, 2008). The influenza viruses are transmitted and maintained in wild bird populations by the fecal oral route indirectly through contaminated water (Stallknecht and Brown, 2009). After shedding, AIV are associated with organic matter, poultry waste, and certain inanimate objects in the environment (Stallknecht and Brown, 2009; Swayne and Halvorson, 2003). It is difficult to measure the persistence of influenza viruses in the environment as this can be affected by a number of physicochemical factors. Although free AIV are relatively unstable in the environment (Swayne and Halvorson, 2003), contaminated fomites in the form of frozen water and sediment may serve as a long term environmental reservoir of these viruses (Lang et al., 2008; Zhang et al., 2006). It is therefore important to measure the persistence of AIV in a variety of contaminated media and substrates at a wide range of temperatures to completely understand the ecology of these viruses.

5.1 Tenacity of influenza and model viruses in water

In the present study a remarkable difference was observed in the persistence of AIV in various water types. The individual viruses were also inconsistent in their sensitivity to inactivation under all experimental conditions. Infectivity of the viruses was preserved for a maximum period of time in DW. The role of certain physicochemical factors representing natural aquatic environments on the persistence of AIV in water has been extensively studied. The individual or interactive effect of the factors studied has shown that persistence is dependent on temperature, pH, and salinity of the waters. The viral persistence was highly variable among viruses of the same subtype or different subtypes (Brown et al., 2007 and 2009; Stallknecht et al., 1990 and 1990a). The HPAI viruses were more readily inactivated than the wild type AIV tested (Brown et al., 2007). The model DW system used in previous trials was logical for focusing on lab adjusted values of these factors but the

ecology of natural SW is quite complex and in addition to pH and salinity many other biotic and abiotic elements may also influence the survival of AIV in this medium. As evident from the results of the present study, the survival time of AIV in SW is comparatively shorter than in DW and NS at all temperatures. It is likely that the AAF containing virus added to the water promoted microbial growth at high temperatures and could have resulted in quick inactivation of viral particles either by microbial metabolites or by passive adherence of virus particles to the microbes and subsequent removal following centrifugation and filtration. However, at low temperatures at which no microbial growth was observed, the survival time was still much shorter as compared to DW, indicating the role of unidentified factors prevailing in natural SW on viral persistence. The complication produced by microbial growth after adding AAF as a virus source can be avoided by using purified virus. However, in reality infected birds excrete influenza viruses via feces or body secretions (Swayne and Halvorson, 2003) which, in addition to being highly contaminated, may also act as a nutritional source for microorganisms. Therefore, the role of biotic factors in water ecology must be considered. The experimental approach adopted in the present study is an initial attempt to mimic the influence of these biotic factors on AIV persistence under laboratory conditions.

Physiological saline was used to measure the tenacity of influenza viruses in a medium whose osmotic pressure is equivalent to body fluids and secretions. The persistence of AIV under these conditions was found to be lower as compared to DW. The differences were particularly evident at low temperatures. It appears that the high salinity level in NS of 9,000 ppm is the only factor responsible for the short survival time of the viruses in relation to DW. This is in accordance with previous reports (Brown et al., 2007; Stallknecht et al, 1990a) in which the salinity had a negative effect on viral persistence and this effect was more prominent at lower temperatures.

Testing duplicate samples during the whole study period reduced the effects of experimental errors and hence conferred high coefficients of determination (R^2) values as presented in the figures 4.1-4.30. Generally, the R^2 values calculated by

linear regression models in the present study are greater than those reported previously (Brown et al., 2007). The use of a continuous cell line in combination with extensive sampling and a long term experimental approach might have helped additionally to produce consistent results.

Viruses were more readily inactivated in SW than in DW. In a previous study, the persistence of an influenza virus checked in five different surface waters was the same as in DW except in water with high salinity (Stallknecht et al., 1990). The samples in that study were filtered prior to virus inoculation which might explain the results, as in another study an enormous difference was observed in the survival of influenza viruses between filtered and unfiltered natural water (Zarkov et al., 2006). Generally, the viral persistence in SW was shorter than in DW but this trend was variable between the different temperatures: at 30 and 20 °C the survival time in SW was 3-5 times shorter while at lower temperatures it was 8-12 times shorter than in DW as clear from Figures 4.31-4.35. As previously described (Brown et al, 2007, 2009; Stallknecht et al., 1990) one possible reason for such results could be high water salinity. However, the salinity of the water from Lake Constance was 150 ppm which should not have had a strong effect on viral persistence. This indicates that undefined factors present in the lake water have a strong effect on the virus persistence at lower temperatures. Hence much more information is required before reaching a conclusion about the viral persistence in freshwater habitats. However, AIV were able to survive in SW for a few days at 30 and 20 °C and even for a few weeks at 10 °C. Following excretion by infected birds, AIV may be readily diluted in large volumes of flowing water (Webster et al., 1978) but in the stagnant water of small ponds or lakes where a large number of birds gather, there is a clear danger of virus spread to susceptible birds. At lower temperatures (0 and -10 °C) the AIV even survived for several months. This finding explains how water basins in some frozen lakes could harbor virus over winter until the following spring as hypothesized previously (Webster et al., 1992; Zhang et al., 2006).

5.2 Persistence of viral RNA in the AIV inoculated SW

The measurement of viral RNA in the inoculated lake water samples at various time points were performed in order to gain insight into the persistence of AIV RNA in water at various temperatures. Quantitation of viral RNA was performed in pg as the RRT-PCR for M-gene amplification has a detection limit of 10 fg (Spackman et al., 2002). It is clear from the results presented in Tables 4.1-4.3 that the RNA of LPAI H4N6, H5N1, and H6N8 viruses was still detectable at all of the temperatures even after no replicating virus was detectable in cell culture. This is in line with previous findings that RRT-PCR is more sensitive than either cell culture or embryo inoculation techniques for the detection of AIV in some specimens (Atmar et al., 1996; Moresco et al., 2010; Spackman et al., 2002). Similar results were recorded in a recent study by Yamamoto et al. (2010) who measured the persistence of an H5N1 influenza virus in duck feathers, water, and duck feces and found that viral RNA was much more stable than viral infectivity in the feathers. Furthermore, viral RNA was detectable in fecal and water samples even when the samples were negative for viral infectivity by embryo inoculation. In another report, the persistence of HPAI virus (H7N1) was evaluated in various samples (muscle, skin, brain, feather pulp, and oropharyngeal and cloacal swabs) collected from infected carcasses by cell culture titration and qRRT-PCR detection. In all of the samples, viral RNA was more stable and detectable to a higher amount than infectious virus by cell culture titration (Busquets et al., 2010). Presently, RRT-PCR for the detection of viral nucleic acid is regarded as the preferred method for the identification of AIV in field samples. Although RRT-PCR is highly sensitive, this assay can detect both live and inactivated viruses and is therefore inappropriate for use in viral tenacity studies where detection of live virus is mandatory (Spackman et al., 2008).

In the present study, the RNA samples, extracted from the virus inoculated SW, were diluted tenfold as it was anticipated that environmental samples would contain inhibitory substances which might interfere with the enzymatic amplification in the RRT-PCR reaction (Haumacher et al., 2010) and dilution of samples reduces the effect of these factors. A lower amount of viral RNA was detectable in all of the H6N8

inoculated water samples as compared to the H4N6 and H5N1 inoculated samples even in the presence of a comparable infectious virus titer. The reason for these differences was unclear.

5.3 Appropriateness of the germ carrier technique

The results of the present study suggest that the filter germ carrier technique offers a good method for measuring the persistence of influenza and other desiccation sensitive viruses in the environment. Carrier tests serve as a standard method to evaluate the virucidal activity of biocides (ASTM, 2002; Sattar and Springthorpe, 2001) and as a rule of thumb highly resistant viruses are used as test organisms for such procedures. The protocols include the application of test organisms onto the carrier followed by drying and subsequent exposure to certain physical or chemical challenges. Commonly used materials for germ carriers include stainless steel, plastic, glass, and wood (Maillard, 2004). The use of such materials for studying the inactivation kinetics of influenza viruses in the environment is inappropriate because the desiccation process can result in the loss of viral infectivity as reported by Lombardi et al. (2008). These investigators used metal, plastic, and wood carriers to measure the virucidal activity of common detergents against AIV and observed a low recovery rate from the dry positive controls as compared to wet ones. The metal and plastic carriers are also unsuitable for use in moist environments as surface wash-off under wet conditions may lead to the elution of virus into the surroundings and subsequent low or no virus recovery from the carriers. Another hurdle for measuring exact viral inactivation rates in a particular environment is poor virus recovery from the inoculated substrates. In an experiment while measuring the tenacity of AIV and NDV during the composting of various wastes from poultry production, $10^{3.30}$ EID_{50}/ml of an H6N2 influenza virus was recovered right away after mixing 0.1 ml of the virus suspension with a titer of 7.8 x $10^{8.00}$ EID_{50}/ml in 10 g of liver or breast muscle while in another substrate it was impossible to recover the virus immediately after adding it to the substrate (Guan et al., 2009). These issues can be resolved by the use of carriers made up of material with a high binding capacity for viruses that can be kept

under moist conditions for long periods of time without elution of virus into the surrounding medium.

Sandwich germ carriers have been used previously to measure the inactivation rates of animal viruses during sewage sludge treatment processes (Pesaro et al., 1995; Spillmann et al., 1987; Traub et al., 1986) and adsorption of virus onto germ carriers was facilitated by the use of a loading buffer with an acidic pH (6.00). Test organisms included in prior experiments were bacteriophages and enteric viruses of humans and animals but the use of such techniques for influenza and other enveloped viruses requires modifications as influenza viruses are sensitive to acidic pH conditions (De-Benedictis et al., 2007; Stallknecht and Brown, 2009). Among three loading media tested in the present study, the one with a pH 7.40 resulted in the adsorption of more virus particles to the carrier than with pH 6.50 or 6.00 as shown in Table 4.4. The experiment was repeated independently three times and it was observed each time that the adsorption rate of virus particles onto filter discs is equivalent at the three pH values. However, lowering the pH resulted in reduced residual infectivity of the virus in the loading medium, leading to less virus adsorption on carriers, and subsequent reduced recovery (Table 4.4). This is in contrast to the findings of Goyal et al. (1980) who reported efficient adsorption of influenza viruses to positively charged filters when infectious allantoic fluid with an adjusted pH value of 6.00 was passed through it. The majority of the experimental data shown in that study, however, was based on HA test results. Most probably, high protein and salt contents in allantoic fluid were converted to insoluble precipitates at an acidic pH. Virus particles adsorbed to these precipitates might have been removed by filtration (Lukasik et al., 2000), possibly leading to positive HA results. In the present study, adsorption of about 70 % of the virus particles to the filter discs was achieved for an enveloped virus (H5N1 avian influenza virus) which is in line with previous findings where lower virus adsorption and recovery rates (70 %) were recorded for an enveloped virus (herpes virus) as compared to higher recovery rates (95 %) for non-enveloped animal viruses (Pesaro et al., 1995). Although a reasonable number of virus particles did adsorb onto the filter discs using phosphate loading buffer with a slightly alkaline pH, the adsorption rate may be further improved by altering the

loading medium (loading buffer made up of other salts and adjusted to various pH values) or adopting other means (multiple filtrations through the same carrier) of loading the viruses to the filter discs. Similarly, virus recovery from the germ carriers may also be increased by improving the elution system (using protein and salt solutions of various concentrations alone or in combination adjusted to diverse pH values).

Viruses are electro-positively charged below their isoelectric point (p*I*) and electro-negatively charged above it, whereas p*I* for a given virus is that pH value at which the virus exists in a state of zero net charge. The p*I* value for influenza A viruses is 5.3 (Gerba, 1984). This means that influenza A viruses are electro-negatively charged at pH 7.40 and have a high binding capacity to the positively charged Virosorb filter discs used in the current experiment. A loading buffer with a slightly basic pH was used in subsequent trials as firstly, it produced germ carriers with a comparatively high virus load (Table 4.4) which is a prerequisite for tenacity studies and secondly to avoid pH-based inactivation of viruses, as it is clear from past studies that acidic pH has a negative effect on the persistence of AIV (Brown et al., 2007, 2009; Stallknecht et al., 1990; Stallknecht and Brown, 2009).

It is known that desiccation reduces the infectivity of influenza and other viruses on environmental fomites (Abad et al., 1994; Lombardi et al., 2008; Tiwari et al., 2006). The present study also confirmed that drying has a drastic effect on the survival of filter bound influenza viruses. Starting with a concentration of about $10^{5.00}$ $TCID_{50}$/ml, the virus was either undetectable or was detectable at a very low titer after a period of 6 hours when kept under dry conditions while in a damp environment there was a negligible effect on the virus titer (Table 4.5). This finding underlines that filter discs can be suitable germ carriers for tenacity studies of influenza and other desiccation labile viruses in damp and wet environmental conditions like SW, lake sediments, fecal material, and meat while they are inappropriate for use in dry environments.

5.4 Comparative persistence of the viruses in SW by direct suspension and using the germ carrier technique

It is not easy to measure the persistence of viruses in the environment as they are associated naturally with particulate matter, which has a major effect on their survival (Gerba, 1984). Interestingly, the AIV persisted longer in lake water using the germ carrier technique as compared to suspending the viruses in the same surface water. The T-90 values of the filter bound AIV increased three to four folds at -10 °C, two-fold at 0, 20, and 30 °C, and slightly at 10 °C than the suspended viruses in the SW. An increase in the microbial count was recorded in germ carrier-containing water samples as well as water samples to which virus-containing AAF was added incubated at 30, 20, and 10 °C. It has been hypothesized that biotic factors present in natural water might influence the survival of AIV in that medium since high microbial counts have been associated with a reduced persistence of AIV in natural water (Domanska-Blicharz et al., 2010; Zarkov, 2006). It was, however, unclear whether viruses were inactivated by microbial enzymes or adsorbed passively onto the microbes and removed by centrifugation and filtration. The use of sandwich carriers eliminates the ambiguity, as filter discs were wrapped and sealed in PCM with a pore size of 10 nm that allows contact between soluble water contents (microbial enzymes and other chemicals) and the filter bound virus without contact between the virus and microbes. It is, however possible that this small pore size could potentially inhibit diffusion of substances present in low concentrations in the surrounding medium, leading to an over-estimation of survival times. Increased persistence of AIV in the current study could therefore be due to inaccessibility of virus within the filter to deleterious effects of the external environment. However, this scenario should better mimic natural environmental conditions where viruses can also be concealed within organic matter.

It is clear from Figure 4.79 that an abrupt decline in eluable virus titer was recorded in the first titration following incubation of samples at -10 °C which was not observed in subsequent titrations, while the samples stored at 0 °C did not show such an inconsistent pattern (Figure 4.78), indicating that freeze-thawing has a drastic effect

on the infectivity of filter-bound viruses. This is in line with the observations of Grieff et al. (1954) who demonstrated that freeze-thawing adversely affects the infectivity of influenza A virus since five freeze-thaw cycles resulted in a reduction of virus titer from $10^{8.60}$ EID_{50}/ml to $10^{0.80}$ EID_{50}/ml. The loss of virus infectivity by freeze-thaw cycles can be minimized by rapid freezing of the samples (Grieff et al., 1954) before storage at -10 °C and subsequent rapid thawing before processing for elution. However, the exact effect of freeze-thawing needs to be further evaluated to optimize the germ carrier technique for use in studies of the persistence of influenza viruses at freezing temperatures.

The viral persistence data in SW presented here show that successful recovery of eluable virus from filter discs stored at low temperatures was possible for influenza and model viruses during the whole study period and no virus was detected in the water samples in which the germ carriers were immersed. These findings confirm that Virosorb filter discs wrapped in a PCM are appropriate germ carriers for studying the persistence of influenza viruses in wet environmental conditions and do not lead to contamination of the surrounding environment. This makes them suitable for use in large scale tenacity studies.

5.5 Persistence of the influenza and model viruses in lake sediment

It is clear from the T-90 values as shown in Figures 4.31-4.35 and 4.68-4.72 that both the influenza and the model viruses can survive longer in lake sediments than in lake water either when measured by suspending the virus directly in water or by incubating germ carrier adsorbed viruses in the water. The virus recovery data (Figures 4.1-4.30 and 4.38-4.67) also show that all of the viruses were detectable for a longer period of time in the sediment than in the lake water. These findings are in agreement with Gerba et al. (1977) and LaBelle et al. (1980) who observed that enteric viruses are present in higher amounts in polluted estuarine sediments than in the overlying see water. In those studies, viruses were sometimes isolated from the sediments even when the overlying seawaters were acceptable for recreational use according to bacteriological water quality standards (LaBella et al., 1980).

AIV are excreted in large amounts in the feces of infected birds (Webster et al, 1978). Most probably, the fecal material passed by infected wild birds near the banks of water bodies does not completely dissolve in the water but is rather deposited at the bottom, preserving the virus in the sediment. In one study, a positive correlation was observed between the number of viruses and the fecal coliform counts in the sediment indicating that the presence of viruses is associated with fecal deposition in the sediments (LaBella et al., 1980). There is a possibility that diving ducks could ingest these infected materials when searching for food at the bottom of contaminated lakes. Thus the estimation of viral persistence in lake sediment can provide valuable information to comprehend the epidemiology of AIV in the environment. To date, no experimental data is available on the persistence of influenza viruses in lake sediments. Only one study, using the detection of influenza virus RNA by RRT-PCR, showed that influenza viruses were detectable in a high percentage (> 50 %) of sediment samples collected from three ponds in Alaska that are heavily used by migratory waterfowl. Characterization of the viruses by sequencing of the H-gene showed that there is a diverse collection of viruses in these sediments (Lang et al., 2008). The importance of lake sediment as a long-term environmental reservoir has been discussed by Stallknecht and Brown (2009) who argued that it is unlikely for influenza viruses to be equally distributed in the water column of the infected water bodies. It is rather more likely that the viruses are present within or at the sediment surface in association with organic matter, thus providing the best opportunity for the birds that feed at the sediment interface to become infected.

Lake sediments are largely composed of organic mud and sand (Gerba et al., 1977a) and have the capacity to readily adsorb viruses (LaBelle and Gerba, 1979). The germ carrier technique was adopted to study the persistence of AIV in lake sediments as in spiking trials a poor recovery of influenza virus was observed after addition of virus suspension to the lake sediment. After mixing the infective AAF into the lake sediment, a loss of more than 99.9 % of the virus infectivity was recorded. The experiment was repeated several times but similar results were obtained each time. It remained unclear whether the virus was inactivated or readily adsorbed to the

sediment matrix. Previous studies show that sediments can readily adsorb enteric viruses (Gerba et al., 1977a, LaBella et al., 1980). Gerba et al. (1977a) demonstrated that more than 99 % of polioviruses were adsorbed after mixing the virus suspension with the estuarine sediments and recovery of viable viruses was also possible from the sediments following elution in organic solutions. The elution process used in such studies requires the treatment of sediment with elution medium under extremely alkaline conditions (pH 11.00) that promote the elution process. However, while working with influenza viruses such treatments should be used with caution as these viruses are highly sensitive to extremes in pH (Swayne and Halvorson, 2003) and can be readily inactivated. The germ carrier technique provides the best alternative which not only exposes the virus to the sediment environment but also ensures appropriate virus recovery. The higher persistence of the influenza and model viruses in lake sediment suggests that sediment has some protecting effect on the infectivity of the viruses. In a previous study, the relatively higher survival rates of the viruses in the sediment than in the overlying water was also proposed to be due to the shielding effect of sediment from the microbial enzymes present in the aquatic habitat (LaBella and Gerba, 1980).

5.6 Persistence of influenza and model viruses in duck feces and fecal waste

It has been reported that one infected Muscovy duck can deposit 6.4 gram fecal material per hour with an infectivity titer of $10^{7.80}$ EID_{50}/g of the feces. Hence in a 24 hours period one duck would shed approximately 10^{10} EID_{50} of this virus (Webster et al., 1978) which can lead to heavy contamination of the environment including water. Such environmental contaminations may serve as a connection for the transfer of AIV from free living water birds to domesticated land poultry (Swayne, 2008). The estimation of viral persistence in fecal material is of great concern for the spread of the disease as well as to understand the ecology of the influenza viruses (CDC, 2005; WHO, 2007). It became clear from the presented experimental results (Figures 4.38-4.67) that influenza viruses may remain infectious in duck feces for periods of time ranging from a few days (at 30 and 20 °C) or a few weeks (10 °C) to several months (at 0 and -10 °C). At lower temperatures the viruses were still detectable

beyond the trial period of six months. Considerable differences exist in the stability and inactivation rates of viruses in feces and animal manure not only among viruses of different families and genera, but also among viruses of the same family, genus, and even among similar types of viruses (Sobsey and Meschke, 2003). These differences were also confirmed by the results of the present study, where virus persistence was variable not only among the influenza and model viruses but also between the various influenza virus subtypes.

Most of the previous studies conducted to determine the survival of AIV in bird feces or fecal waste (Chumpolbanchorn et al., 2006; Lu et al., 2003; Songersam et al., 2006; Webster et al., 1978; WHO, 2007) were based on quantitation of viral infectivity in the beginning and subsequent testing of residual viral infectivity after a defined period of time. The present experiments include the collection of sequential data and the calculation of a definite time (T-90 values) required for the inactivation of the viruses in fecal material at a wide range of temperatures. This is helpful to determine how long viruses can remain infectious in fecal material. In the present experimental setup, fecal material was placed in plastic tubes together with the virus loaded germ carriers. Tubes were closed and then incubated at various temperatures. By using this method, the effect of drying on the infectivity of the viruses was excluded from the studies. In a previous study, an influenza virus with a concentration of 2.25-3.75 $\log_{10}$ EID_{50} per g of fresh duck feces became undetectable after the feces were dried overnight at room temperature (20 °C) while in wet feces, the virus remained viable for 4-6 days at 37 °C (WHO, 2007). Additional studies would be necessary to incorporate the effect of drying while calculating the inactivation rates of the influenza viruses in fecal material. However, the current experimental setup examines the effect of temperature and other soluble inactivating factors including the microbial enzymes present in the feces on AIV infectivity. The microbes present in the feces and their metabolites are hypothesized to play a role in the persistence of viruses in fecal material. The quick inactivation of an H7N2 virus in field chicken manure as compared to SPF chicken manure at ambient or higher temperatures was believed to be due to the destructive action of microorganisms or their byproducts present in the field chicken manure on the inoculated influenza viruses (Lu et al., 2003).

The survival of influenza viruses in the feces is influenced by: the strain of virus, type of feces (species from which the feces were obtained), physical properties of the feces and the temperature at which the feces is incubated (De Benedictis et al., 2007). Similarly, the source of fecal manure also effects the persistence of AIV in this medium as in one study with three different sources of chicken manure, viral persistence was highest in the manure collected from SPF chickens housed under BSL-2 conditions followed by that collected from commercial layers removed from a field farm and housed separately in the BSL-2 facility, while viral persistence was shortest in the manure collected from the field commercial layer's houses (Lu et al., 2003). In the present study, duck feces was selected to study the persistence of influenza and model viruses since ducks are considered as one of the key reservoirs of AIV and play a major role in the ecology of influenza viruses (Stallknecht and Brown, 2008; Sturm-Ramirez et al., 2005). However, the estimation of viral persistence in the feces of other wild and domestic bird species is also important for comparison and to draw final conclusions on the persistence of AIV in fecal material.

5.7 Persistence of the influenza and model viruses in meat and bird carcasses

In experimentally infected chickens, LPAI viruses produce localized infections in the respiratory and GI tracts, while HPAI viruses cause systemic infections and may be detectable in various organs including the breast and thigh meat (Spickler et al., 2008; Swayne and Beck, 2005). In the case of free-ranging birds, cloacal or tracheal swabs are the preferred sampling methods for the screening of birds for AIV (Sturm-Ramirez et al., 2005). However, in some cases viruses have been isolated from the visceral organs of dead birds (Chen et al., 2005; Liu et al., 2005) while less information is available on the detection of AIV from the meat of wild bird species. Reports of the presence of AIV in the bird's meat are mostly based on the detection of virus from commercial poultry or duck meat following natural or experimental infection of birds (Harder et al., 2009; Mase et al., 2005; Serena Beato et al., 2006; Swayne and Beck, 2005; Tumpey et al., 2002). The estimation of viral persistence in the meat is significant as consumption of infected meat has been linked with HPAI disease

outbreaks in backyard poultry (Harder et al., 2009). Wild bird carcasses from animals that have died of avian influenza pose a danger of virus transmission to susceptible birds and other animals through predation or cannibalization of infected carcasses (Swayne, 2008). There are several reports of HPAI virus infections following consumption of infected poultry meat by chickens (Swayne and Beck, 2005), consumption of infected duck carcasses by poultry (Harder et al., 2009), consumption of infected chickens by cats (Kuiken et al., 2004), consumption of infected duck carcasses by dogs (Songserm et al., 2006a), and consumption of infected poultry by tigers and leopards (Keawcharoen et al., 2004). Hence, contaminated meat can be a potential source of virus transmission to many animal species.

As shown in Figures 4.38-4.67, influenza viruses can survive in duck meat for a few days at 30 and 20 °C, some weeks at 10 °C, and several months at 0 and -10 °C. This is in line with Easterday et al. (1997) who stated that AIV can survive for several days in carcasses at ambient temperatures, compared with a few weeks at refrigeration temperatures. On the other hand, Busquets et al. (2010) observed that an HPAI H7N1 virus was promptly inactivated in the pectoral muscles of infected chicken carcasses at a temperature of 30-31 °C but was detectable for 5 days at 22-23 °C. The rapid viral inactivation at higher temperatures in that study could be due to the differences in the experimental setup as muscle samples were collected from freshly dead infected carcasses. Following death or slaughtering, the carcasses undergo rigor mortis which results in a decrease of the meat pH and such acidic conditions may negatively affect the survival of viruses in the meat (Henderson and Brookyby, 1948; Panina et al., 1989). Post mortal pH decrease is delayed by lowering the storage temperature (Panina et al., 1989) which ultimately results in lower viral inactivation rates. In the current study, the pH of the duck meat was not monitored so it is difficult to draw any conclusions about the role of meat pH on the persistence of viruses. However, in the current experimental setup the virus adsorption to the germ carriers might have resulted in an extended persistence of the viruses.

Although HPAI viruses have been detected in infected chicken, duck, and quail meat to a very high titer (Antarasena et al., 2006; Thomas and Swayne, 2007), no evidence is available for the presence of LPAI viruses in the meat of infected birds (Spickler et al., 2008). On the other hand, LPAI viruses from respiratory secretions or feces could be a source of carcass surface contamination during the slaughtering process (Animal health Australia, 2008; Thomas et al., 2008). In the case of HPAI virus, the contaminated frozen meat can sufficiently retain infective AIV as evident from the detection of H5N1 in frozen duck carcasses in Germany (Harder, 2009) and the isolation of AIV from frozen duck meat that was imported from China to Korea and Japan (Mase et al., 2005; Tumpey et al., 2002). In the present study, successful recovery of the influenza viruses from meat samples stored at 0 and -10 °C during the whole experimental period and T-90 values of up to 81 days at 0 °C and 92 days at -10 °C confirm that viral infectivity can be preserved for a longer period of time in infected meat or carcasses at lower temperatures. These findings suggest that contaminated bird carcasses could remain infectious for an extended period of time at freezing temperatures in the colder climates and can play a role in the spread of disease.

5.8 Significance of model viruses included in the study

The two model viruses (NDV and ECBO) were incorporated in this study to serve as representative enveloped and non-enveloped viruses and for direct comparison with influenza viruses as these viruses serve as test organisms in viral tenacity studies and to measure the virucidal activity of disinfectants (DVG, 2007). As clear from the linear regression models of all viruses under each tested condition at all temperatures and from Figures 4.31-4.35 and 4.68-4.72, the persistence of NDV and ECBO viruses was consistently higher than that of influenza viruses. However, of the model viruses, ECBO as a non-enveloped virus had higher T-90 values while NDV, as an enveloped virus, had lower T-90 values which are much closer to those of the influenza viruses at all temperatures. This is in line with the observations of Pesaro et al. (1995) who calculated the inactivation of animal viruses in the sewage treatment process and found that enveloped viruses are less persistent than non-enveloped

viruses as the time required for one log reduction in virus titer was less than 1 week for a herpesvirus (enveloped) and more than 6 months for a rotavirus (non-enveloped) in liquid animal waste. The results of the current study indicate that NDV may be a good surrogate virus for studying the persistence and inactivation of AIV, as it has a slightly higher tenacity than those viruses, allowing for a sufficient margin of safety in interpretation of results.

5.9 Conclusions

The findings of the present study demonstrate that

- Persistence of AIV is shorter in unsterile natural lake water as compared to sterile distilled water.
- Filter-based sandwich germ carriers can be used to measure the persistence of influenza viruses in a variety of substrates under moist environmental conditions.
- Viral association with particulate matter enhances the survival of AIV in the aquatic environment.
- Persistence of AIV in aquatic habitats and contaminated feces or meat is inversely proportional to the storage temperature.
- The avian and human influenza virus subtypes were inconsistent in their sensitivity to inactivation under all experimental conditions.
- AIV can survive in surface water, lake sediments, duck feces, and meat for several months at low temperatures (0 and -10 °C), allowing persistence of the viruses in the environment over winter.

6 Summary

The present study was designed to investigate the persistence of AIV in a variety of contaminated media and substrates under diverse environmental conditions. Wild aquatic birds serve as a reservoir of influenza A viruses. The infected birds excrete a large number of viruses through their nasal secretion and feces that leads to heavy contamination of the environment. In order to determine the tenacity of these viruses under natural environmental conditions, the persistence of three LPAI viruses (H4N6, H5N1, H6N8), one human influenza virus (H1N1), and two model viruses (NDV and ECBO) was calculated in various types of waters (DW, NS, and SW), lake sediment, duck feces, and duck meat at -10, 0, 10, 20, and 30 °C for extended periods of time.

The NDV and influenza viruses were propagated in the allantoic sac of 9-11 day old SPF chicken embryos while ECBO was propagated in MDBK. For the tenacity studies in water, DW and NS were autoclaved while SW collected from Lake Constance was used without any treatment. Viral quantitation was performed in the beginning of trials and then afterwards at regular intervals by the end point serial dilution method on MDCK for influenza viruses, Vero cells for NDV, and MDBK for ECBO. The virus titers were calculated as $TCID_{50}$/ml by the Spearman-Kärber method. Duplicate samples were tested each time for all of the treatment groups for a maximum of 36 weeks. The sequential data thus obtained was analyzed by a linear regression model to calculate T-90 values (time required for one log reduction in the virus titer) and the estimated persistence of viral infectivity with a starting viral concentration of $10^{6.00}$ $TCID_{50}$/ml.

The infectivity of the influenza viruses was preserved for a maximum period of time in DW followed by in NS and SW and the individual influenza viruses were also inconsistent in their sensitivity to inactivation under all experimental conditions. T-90 values of the influenza viruses in the inoculated DW ranged between 5-13 days at 30 °C, 14-37 days at 20 °C, 62-197 days at 10 °C, 205-558 days at 0 °C, and 202-642 days at -10 °C. The viral persistence was shorter in SW than DW and this trend was variable at different temperatures: at 30 and 20 °C the persistence was 3-5 times

shorter while at lower temperatures it was 8-12 times shorter in SW than in DW. The microbiological analysis showed an increase in the bacterial counts of the virus inoculated SW samples after storage at 30, 20, and 10 °C. To check the stability of viral RNA in the SW samples, H4N6, H5N1, and H6N8 inoculated SW samples were processed for quantitation of viral RNA by qRRT-PCR reaction at the start of the experiments and after storage at various temperatures. A significant amount of viral RNA was still detectable in the contaminated water samples at all temperatures after the virus was no longer detectable by cell culture titration. The rate of viral RNA degradation was faster at high temperatures than at lower ones.

A germ carrier technique was adapted to study the persistence of influenza viruses in various substrates. Electro positively charged Zeta Plus Virosorb filters discs wrapped in a polycarbonate membrane of 10 nm pore size were used as sandwich germ carriers. To facilitate the adsorption of influenza viruses to the filter discs, PLB adjusted to three pH values (6.00, 6.50, and 7.40) was checked and the one with a pH 7.40 produced germ carriers with high virus titer. Furthermore, an enormous loss of virus titer was recorded when the filter discs inoculated with H5N1 virus were kept dry for 6 hours while a negligible loss of the virus titer was observed when loaded filter discs were kept under moist conditions. Sandwich germ carriers were first used to estimate the persistence of influenza and model viruses in SW. Persistence of all of the viruses was highest at -10 °C followed by 0, 10, 20, and 30 °C. At -10 °C, the single freeze-thaw cycle resulted in an abrupt decline in the virus titer, followed by long term persistence. Interestingly, the AIV persisted longer in SW using the germ carrier technique as compared to suspending the viruses in the water. The T-90 values of the filter bound AIV increased three to four folds at -10 °C, two-fold at 0, 20, and 30 °C, and slightly at 10 °C compared to the suspended viruses in the SW. No virus was detected in water samples in which germ carriers were incubated while successful recovery of eluable virus from filter discs stored at low temperatures was possible for influenza and model viruses during whole study period, making the technique suitable for use in large-scale tenacity studies.

The survival of influenza and model viruses was also evaluated in lake sediment, duck feces, and meat using the sandwich germ carrier technique. Among all of these substrates, viral persistence was highest in the lake sediments as compared to duck feces and meat, in which the virus persistence was quite similar. In lake sediment, the persistence of the AIV was even higher than in the SW which indicates that sediment can protect the viruses from the inactivating factors present in the surrounding environment and prolong viral survival. In duck feces, the T-90 values of influenza viruses ranged from 1-2 days at 30 °C, 4-7 days at 20 °C, 14-21 days at 10 °C, 47-75 days at 0 °C, and 53-71 days at -10 °C and equivalent T-90 values were also recorded in the duck meat.

Two model viruses were incorporated into this study to serve as representative enveloped and non-enveloped model viruses and for direct comparison with influenza viruses as these viruses serve as test organisms in viral tenacity studies. Generally, in all of the water types and within all of the substrates, the persistence of NDV and ECBO viruses was consistently higher than that of influenza viruses. However, of the model viruses, ECBO as a non-enveloped virus had higher T-90 values while NDV, as an enveloped virus, had lower T-90 values which are much closer to those of the influenza viruses at all temperatures. This indicates that NDV may be a good surrogate virus for studying the persistence and inactivation of AIV, as it has a slightly higher tenacity than those viruses, allowing for a sufficient margin of safety in interpretation of results.

The findings of the present study underline the importance of the aquatic habitat in the maintenance and spread of AIV in the environment. At lower temperatures, AIV can remain infectious in SW, lake sediments, duck feces, and meat for extended periods of time, allowing persistence of the viruses in the environment over winter.

7 Zusammenfassung

Die vorliegende Untersuchung wurde durchgeführt, um die Persistenz von AIV in einer Vielfalt von kontaminierten Medien und Substraten unter unterschiedlichen Umweltbedingungen zu untersuchen. Wildwasservögel dienen als Reservoir für Influenza-A-Viren. Infizierte Vögel scheiden eine hohe Anzahl an Viren über die nasale Sekretion und den Kot aus. Dies führt zu einer erheblichen Kontamination der Umwelt. Um die Tenazität dieser Viren unter natürlichen Bedingungen zu bestimmen, wurde die Persistenz von drei LPAI-Viren (H4N6, H5N1, H6N8), eines humanen Influenza-Viruses (H1N1) und von zwei Modellviren (NDV und ECBO) in unterschiedlichen Wasserarten (destilliertes Wasser, physiologische Kochsalzlösung und Oberflächenwasser), in Seesediment, Entenkot und Entenfleisch bei -10 °C, 0 °C, 10 °C, 20 °C und 30 °C über eine lange Zeitdauer bestimmt.

Das NDV und die Influenzaviren wurden im Allantoissack von 9-11 Tage alten SPF-Hühnerembryonen vermehrt, während das ECBO-Virus in MDBK-Zellkulturen vermehrt wurde. Für die Tenazitätsuntersuchungen in Wasser wurden das destillierte Wasser und die physiologische Kochsalzlösung autoklaviert, während das Oberflächenwasser, das aus dem Bodensee entnommen wurde, ohne Behandlung verwendet wurde. Die Virusquantifizierung wurde zu Beginn der Versuche und dann anschließend in regelmäßigen Zeitabständen mit der Endpunktverdünnungsmethode auf MDCK-Zellen für Influenzaviren, auf Vero-Zellen für NDV und auf MDBK-Zellen für ECBO-Virus durchgeführt. Die Virustiter wurden als $TCID_{50}$/ml mit der Spearman-Kärber-Formel errechnet. Aus allen Behandlungsgruppen wurden jedes Mal Doppelproben über die Dauer von maximal 36 Wochen untersucht. Die Daten, die der Reihe nach erhalten wurden, wurden mit einem linearen Regressionsmodell analysiert, um die D-90-Werte (benötigte Zeit für die Reduktion der Viruskonzentration um eine Log_{10}-Stufe) zu berechnen und damit die geschätzte Persistenz der Virusinfektiosität bei einer Virusstartkonzentration von $10^{6,00}$ $TCID_{50}$/ml.

Die Infektiosität der Influenzaviren blieb am längsten in destilliertem Wasser erhalten, gefolgt von physiologischer Kochsalzlösung und Oberflächenwasser. Die einzelnen

Influenzaviren waren bei allen Versuchsbedingungen in ihrer Sensitivität gegenüber der Inaktivierung nicht einheitlich. Die D-90-Werte der Influenzaviren in beimpftem destilliertem Wasser bewegten sich zwischen 5-13 Tagen bei 30 °C, 14-37 Tagen bei 20 °C, 62-197 Tagen bei 10 °C und 205-558 Tagen bei 0 °C und 202-642 Tagen bei -10 °C. Die virale Persistenz war kürzer in Oberflächenwasser im Vergleich zu destilliertem Wasser und dieser Trend war bei unterschiedlichen Temperaturen variabel: bei 30 °C und 20 °C war die Persistenz 3-5 mal kürzer, während sie bei niedrigeren Temperaturen in Oberflächenwasser 8-12 mal kürzer war als in destilliertem Wasser. Die mikrobiologischen Analysen zeigten eine Zunahme im Bakteriengehalt bei den mit Virus beimpften Oberflächenwasserproben bei der Lagerung bei 30 °C, 20 °C und 10 °C. Um die Stabilität von viraler RNA in Oberflächenwasserproben zu untersuchen, wurden mit H4N6-Virus, H5N1-Virus und H6N8-Virus beimpfte Oberflächenwasserproben mit der qRRT-PCR zu Beginn der Versuche und nach der Lagerung bei den verschiedenen Temperaturen untersucht. Eine signifikante Menge an viraler RNA war in den kontaminierten Wasserproben bei allen Temperaturen noch feststellbar, nachdem das Virus bei der Titration in Zellkultur nicht mehr nachweisbar war. Die Rate des viralen RNA-Abbaus war größer bei hohen als bei niedrigeren Temperaturen.

Eine Keimträgertechnik wurde angepasst, um die Persistenz von Influenzaviren in verschiedenen Substraten zu untersuchen. Elektropositiv geladene Zeta Plus Virosorb-Filterscheiben, die in einer Polycarbonat-Membran mit einer Porengröße von 10 nm eingepackt waren, wurden als Sandwich-Keimträger verwendet. Um die Adsorption von Influenzaviren an die Filterscheiben zu ermöglichen, wurde Phosphatbeladungspuffer mit 3 unterschiedlichen pH-Werten (6,00; 6,50 und 7,40) getestet. Keimträger mit hohen Virustitern wurden mit Phosphatbeladungspuffer bei einem pH-Wert von 7,40 erzeugt. Außerdem wurde ein enormer Verlust bei der Viruskonzentration beobachtet, wenn Filterscheiben, die mit H5N1 beimpft waren, über 6 h trocken aufbewahrt wurden, während der Verlust der Viruskonzentration nur geringfügig war, wenn die beladenen Filterscheiben unter feuchten Bedingungen gelagert wurden. Sandwich-Keimträger wurden zuerst verwendet, um die Persistenz der Influenzaviren und der Modellviren in

Oberflächenwasser abzuschätzen. Die Persistenz von allen Viren war bei -10 °C am höchsten, gefolgt von 0 °C, 10 °C, 20 °C und 30 °C. Bei -10 °C resultierte ein einziger Gefrier-Auftau-Zyklus unabhängig von der Lagerdauer in einer abrupten Abnahme im Virustiter, gefolgt von einer Langzeitpersistenz. Interessanterweise persistierten AIV länger in Oberflächenwasser, wenn sie an Keimträgern absorbiert waren, als wenn sie frei in Wasser suspendiert waren. Die D-90-Werte der an Filter adsorbierten AIV nahmen um das drei- bis vierfache bei -10 °C, um das zweifache bei 0 °C, 20 °C und 30 °C und nur geringfügig bei 10 °C im Vergleich zu frei suspendierten Viren zu. In Wasserproben, in denen die Keimträger gelagert wurden, wurde kein Virus nachgewiesen, während die Influenzaviren und Modellviren von den Filterscheiben, die bei niedrigen Temperaturen gelagert wurden, während des ganzen Untersuchungszeitraums erfolgreich eluiert und quantifiziert werden konnten. Dies erlaubt den Einsatz der Technik bei Tenazitätsuntersuchungen in großtechnischem Maßstab.

Das Überleben der Influenzaviren und der Modellviren wurde ebenso in Seesediment, Entenkot und Fleisch unter der Verwendung der Sandwich-Keimträgertechnik zahlenmäßig bestimmt. Unter all diesen Substraten war die virale Persistenz in Seesediment am höchsten, verglichen mit Entenkot und Fleisch, in denen die Viruspersistenz ähnlich war. In Seesediment war die Persistenz von AIV sogar höher als in Oberflächenwasser, was anzeigt, dass Sediment die Viren vor den Inaktivierungsfaktoren, die in der Umwelt vorhanden sind, schützen und somit das Überleben der Viren verlängern kann. In Entenkot schwankten die D-90-Werte der Influenzaviren von 1-2 Tage bei 30 °C, 4-7 Tage bei 20 °C, 14-21 Tage bei 10 °C, 47-75 Tage bei 0 °C und 53-71 Tage bei -10 °C. In Entenfleisch wurden sehr ähnliche D-90-Werte ermittelt.

Zwei Modellviren (NDV und ECBO-Virus) wurden in die Studie aufgenommen, stellvertretend für behüllte und unbehüllte Modellviren und für den direkten Vergleich mit Influenzaviren, da sie als Testorganismen für virale Tenazitätsuntersuchungen dienen. Im Allgemeinen war die Persistenz von NDV und ECBO-Viren in allen Wasserarten und in all den Substraten durchweg höher als die der Influenzaviren.

Von den Modellviren hatte jedoch ECBO-Virus als ein unbehülltes Virus die höheren D-90-Werte, während das behüllte NDV die niedrigeren D-90-Werte hatte, die bei allen Temperaturen denen der Influenzaviren ähnlich waren. Dies zeigt, dass NDV ein Surrogat-Virus für Persistenz- und Inaktivierungsuntersuchungen an AIV sein kann, indem es eine leicht höhere Tenazität als diese Viren hat, was einen ausreichenden Sicherheitsspielraum bei der Interpretation von Ergebnissen gewährleistet.

Die Ergebnisse der vorliegenden Studie unterstreichen die Wichtigkeit der Wasserbiotope beim Fortbestand und bei der Verbreitung von AIV in der Umwelt. Bei niedrigen Temperaturen können AIV in Oberflächenwasser, Seesediment, Entenkot und Fleisch über einen längeren Zeitraum infektiös bleiben, was die Persistenz der Viren in der Umwelt über den Winter ermöglicht.

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9 Appendix

Table 9.1: Linear regression models for the persistence of influenza and model viruses in various water types at 30 °C by suspending the viruses in the water

Virus Type	Distilled water			Normal Saline			Surface water		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 5.707 - 0.122*x*	0.95	49 (8)	y = 6.379 - 0.441*x*	0.99	14 (2)	y = 4.402 - 1.650*x*	0.88	4 (1)
H4N6	y = 5.224 - 0.129*x*	0.91	46 (8)	y = 4.426 - 0.172*x*	0.92	35 (6)	y = 3.479 - 0.434*x*	0.83	14 (2)
H5N1	y = 5.050 - 0.198*x*	0.92	30 (5)	y = 4.630 - 0.179*x*	0.94	33 (6)	y = 3.570 - 0.469*x*	0.74	13 (2)
H6N8	y = 5.587 - 0.078*x*	0.83	77 (13)	y = 5.869 - 0.419*x*	0.96	14 (2)	y = 4.037 - 0.423*x*	0.81	14 (2)
NDV	y = 7.109 - 0.064*x*	0.96	93 (16)	y = 7.390 - 0.122*x*	0.96	49 (8)	y = 5.315 - 1.119*x*	0.92	5 (1)
ECBO	y = 6.134 - 0.078*x*	0.94	77 (13)	y = 6.019 - 0.154*x*	0.97	39 (6)	y = 4.336 - 0.106*x*	0.84	57 (9)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 9.2: Linear regression models for the persistence of influenza and model viruses in various water types at 20 °C by suspending the viruses in the water

Virus Type	Distilled water			Normal Saline			Surface water		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 6.485 - 0.050*x*	0.97	119 (20)	y = 5.843 - 0.077*x*	0.96	78 (13)	y = 4.311 - 0.482*x*	0.94	12 (2)
H4N6	y = 5.764 - 0.069*x*	0.87	88 (15)	y = 5.316 - 0.084*x*	0.95	71 (12)	y = 3.522 - 0.266*x*	0.90	23 (4)
H5N1	y = 5.026 - 0.071*x*	0.86	84 (14)	y = 5.130 - 0.079*x*	0.95	76 (13)	y = 3.522 - 0.320*x*	0.82	19 (3)
H6N8	y = 5.485 - 0.027*x*	0.90	221 (37)	y = 5.312 - 0.146*x*	0.96	41 (7)	y = 4.704 - 0.305*x*	0.93	20 (3)
NDV	y = 6.937 - 0.022*x*	0.98	267 (45)	y = 7.556 - 0.050*x*	0.98	120 (20)	y = 5.208 - 0.555*x*	0.95	11 (2)
ECBO	y = 5.452 - 0.020*x*	0.84	302 (50)	y = 6.309 - 0.061*x*	0.95	98 (16)	y = 4.657 - 0.038*x*	0.83	160 (27)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 9.3: Linear regression models for the persistence of influenza and model viruses in various water types at 10 °C by suspending the viruses in the water

Virus Type	Distilled water			Normal Saline			Surface water		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 6.263 - 0.016*x*	0.89	375 (62)	y = 6.266 - 0.022*x*	0.97	269 (45)	y = 4.321 - 0.170*x*	0.95	35 (6)
H4N6	y = 4.709 - 0.008*x*	0.83	729 (121)	y = 5.014 - 0.017*x*	0.93	353 (59)	y = 3.536 - 0.070*x*	0.91	85 (14)
H5N1	y = 5.019 - 0.012*x*	0.91	508 (85)	y = 4.725 - 0.015*x*	0.87	410 (68)	y = 3.485 - 0.099*x*	0.83	61 (10)
H6N8	y = 5.512 - 0.005*x*	0.85	1185 (197)	y = 4.911 - 0.014*x*	0.91	419 (70)	y = 4.350 - 0.074*x*	0.84	82 (14)
NDV	y = 6.921 - 0.004*x*	0.84	1601 (267)	y = 6.913 - 0.005*x*	0.89	1232 (205)	y = 4.636 - 0.117*x*	0.94	51 (9)
ECBO	y = 6.386 - 0.009*x*	0.91	664 (111)	y = 6.314 - 0.009*x*	0.96	666 (111)	y = 5.080 - 0.013*x*	0.82	460 (77)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 9.4: Linear regression models for the persistence of influenza and model viruses in various water types at 0 °C by suspending the viruses in the water

Virus Type	Distilled water			Normal Saline			Surface water		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 6.182 - 0.005*x*	0.83	1227 (205)	y = 6.243 - 0.007*x*	0.85	893 (149)	y = 4.642 - 0.092*x*	0.92	65 (11)
H4N6	y = 5.059 - 0.002*x*	0.79	2656 (443)	y = 5.155 - 0.005*x*	0.96	1140 (190)	y = 3.909 - 0.032*x*	0.78	188 (31)
H5N1	y = 4.967 - 0.003*x*	0.53	2295 (383)	y = 5.244 - 0.005*x*	0.95	1112 (185)	y = 3.328 - 0.029*x*	0.69	208 (35)
H6N8	y = 5.429 - 0.002*x*	0.72	3347 (558)	y = 5.440 - 0.006*x*	0.88	1013 (169)	y = 4.374 - 0.029*x*	0.84	206 (34)
NDV	y = 6.808 - 0.001*x*	0.37	4468 (745)	y = 6.825 - 0.002*x*	0.45	2723 (454)	y = 4.732 - 0.022*x*	0.92	279 (46)
ECBO	y = 6.466 - 0.002*x*	0.26	3346 (558)	y = 6.461 - 0.005*x*	0.77	1213 (202)	y = 5.237 - 0.007*x*	0.73	814 (136)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 9.5: Linear regression models for the persistence of influenza and model viruses in various water types at -10 °C by suspending the viruses in the water

Virus Type	Distilled water			Normal Saline			Surface water		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 6.361 - 0.005*x*	0.75	1212 (202)	y = 6.274 - 0.007*x*	0.91	813 (136)	y = 4.100 - 0.021*x*	0.84	279 (47)
H4N6	y = 5.008 - 0.002*x*	0.65	3850 (642)	y = 5.120 - 0.003*x*	0.70	1929 (321)	y = 4.449 - 0.018*x*	0.90	328 (55)
H5N1	y = 4.996 - 0.002*x*	0.67	3454 (576)	y = 5.244 - 0.004*x*	0.91	1415 (236)	y = 4.376 - 0.017*x*	0.89	350 (58)
H6N8	y = 5.425 - 0.002*x*	0.80	3565 (594)	y = 5.488 - 0.004*x*	0.80	1399 (233)	y = 5.313 - 0.015*x*	0.79	395 (66)
NDV	y = 6.829 - 0.001*x*	0.29	5138 (856)	y = 6.601 - 0.001*x*	0.30	4773 (795)	y = 5.502 - 0.007*x*	0.65	885 (148)
ECBO	y = 6.558 - 0.002*x*	0.19	3928 (655)	y = 6.433 - 0.003*x*	0.73	2154 (359)	y = 5.190 - 0.002*x*	0.47	2405 (401)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 9.6: Linear regression models for the persistence of influenza and model viruses in various substrates at 30 °C using the germ carrier technique

Virus Type	Surface water			Sediment			Duck feces			Duck meat		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 5.956 - 0.352*x*	0.97	17 (3)	y = 3.447 - 0.245*x*	0.94	25 (4)	y = 4.566 - 0.717*x*	0.81	8 (1)	y = 5.913 - 0.764*x*	0.94	8 (1)
H4N6	y = 4.423 - 0.253*x*	0.83	24 (4)	y = 2.817 - 0.094*x*	0.56	64 (11)	y = 4.354 - 0.637*x*	0.91	9 (1)	y = 5.171 - 0.598*x*	0.96	10 (2)
H5N1	y = 5.394 - 0.239*x*	0.98	25 (4)	y = 3.520 - 0.138*x*	0.74	43 (7)	y = 4.035 - 0.519*x*	0.89	12 (2)	y = 5.446 - 0.631*x*	0.97	10 (2)
H6N8	y = 5.761 - 0.217*x*	0.92	28 (5)	y = 3.523 - 0.220*x*	0.78	27 (5)	y = 5.502 - 0.579*x*	0.89	10 (2)	y = 4.844 - 0.479*x*	0.74	13 (2)
NDV	y = 6.017 - 0.118*x*	0.95	51 (8)	y = 6.283 - 0.148*x*	0.59	40 (7)	y = 7.784 - 0.313*x*	0.99	19 (3)	y = 5.943- 0.323*x*	0.85	19 (3)
ECBO	y = 6.636 - 0.074*x*	0.86	81 (14)	y = 4.910 - 0.052*x*	0.59	116 (19)	y = 6.707 - 0.127*x*	0.86	47 (8)	y = 7.050 - 0.227*x*	0.92	26 (4)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 9.7: Linear regression models for the persistence of influenza and model viruses in various substrates at 20 °C using the germ carrier technique

Virus Type	Surface water			Sediment			Duck feces			Duck meat		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	$y = 5.429 - 0.106x$	0.88	57 (9)	$y = 2.532 - 0.063x$	0.48	95 (19)	$y = 4.784 - 0.261x$	0.78	23 (4)	$y = 6.040 - 0.329x$	0.94	18 (3)
H4N6	$y = 5.037 - 0.141x$	0.87	43 (7)	$y = 2.799 - 0.055x$	0.61	109 (18)	$y = 4.292 - 0.262x$	0.94	23 (4)	$y = 5.564 - 0.342x$	0.90	18 (3)
H5N1	$y = 5.515 - 0.138x$	0.89	43 (7)	$y = 4.311 - 0.079x$	0.79	76 (13)	$y = 3.533 - 0.237x$	0.69	25 (4)	$y = 5.841 - 0.416x$	0.93	14 (2)
H6N8	$y = 5.624 - 0.085x$	0.94	70 (12)	$y = 3.559 - 0.059x$	0.62	102 (17)	$y = 4.813 - 0.153x$	0.71	39 (7)	$y = 5.733 - 0.309x$	0.94	19 (3)
NDV	$y = 5.801 - 0.051x$	0.90	118 (20)	$y = 6.142 - 0.035x$	0.67	172 (29)	$y = 7.571 - 0.085x$	0.94	71 (12)	$y = 6.097 - 0.136x$	0.93	44 (7)
ECBO	$y = 6.579 - 0.027x$	0.86	224 (37)	$y = 5.448 - 0.018x$	0.67	311 (55)	$y = 6.859 - 0.063x$	0.93	95 (16)	$y = 7.010 - 0.078x$	0.87	77 (13)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, x: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 9.8: Linear regression models for the persistence of influenza and model viruses in various substrates at 10 °C using the germ carrier technique

Virus Type	Surface water			Sediment			Duck feces			Duck meat		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 5.460 - 0.064*x*	0.98	93 (16)	y = 3.658 - 0.054*x*	0.90	112 (19)	y = 3.279 - 0.057*x*	0.53	106 (53)	y = 5.758 - 0.080*x*	0.94	75 (12)
H4N6	y = 4.242 - 0.073*x*	0.96	83 (14)	y = 3.020 - 0.023*x*	0.39	259 (43)	y = 3.933 - 0.048*x*	0.76	124 (21)	y = 4.538 - 0.094*x*	0.90	64 (11)
H5N1	y = 4.901 - 0.056*x*	0.81	107 (18)	y = 3.884 - 0.021*x*	0.40	282 (47)	y = 4.607 - 0.062*x*	0.74	97 (16)	y = 4.426 - 0.073*x*	0.72	82 (14)
H6N8	y = 5.353 - 0.057*x*	0.75	106 (18)	y = 3.919 - 0.019*x*	0.54	322 (54)	y = 5.648 - 0.073*x*	0.83	83 (14)	y = 5.035 - 0.083*x*	0.86	72 (12)
NDV	y = 5.299 - 0.033*x*	0.65	184 (31)	y = 6.707 - 0.013*x*	0.53	454 (76)	y = 7.344 - 0.015*x*	0.81	390 (65)	y = 5.771 - 0.022*x*	0.78	274 (46)
ECBO	y = 6.425 - 0.012*x*	0.59	481 (80)	y = 5.442 - 0.004*x*	0.05	1656 (276)	y = 6.541 - 0.006*x*	0.35	995 (166)	y = 6.622 - 0.016*x*	0.78	380 (63)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 9.9: Linear regression models for the persistence of influenza and model viruses in various substrates at 0 °C using the germ carrier technique

Virus Type	Surface water			Sediment			Duck feces			Duck meat		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 5.091 - 0.020*x*	0.79	305 (51)	y = 3.557 - 0.022*x*	0.78	276 (46)	y = 4.202 - 0.021*x*	0.85	281 (47)	y = 5.810 - 0.016*x*	0.86	382 (64)
H4N6	y = 4.267 - 0.014*x*	0.89	444 (74)	y = 3.573 - 0.015*x*	0.51	395 (66)	y = 3.694 - 0.017*x*	0.70	358 (60)	y = 5.056 - 0.025*x*	0.89	241 (40)
H5N1	y = 5.458 - 0.019*x*	0.82	320 (53)	y = 4.307 - 0.009*x*	0.34	705 (118)	y = 4.065 - 0.013*x*	0.40	451 (75)	y = 5.161 - 0.018*x*	0.83	325 (54)
H6N8	y = 5.658 - 0.015*x*	0.79	396 (66)	y = 3.914 - 0.003*x*	0.08	2363 (394)	y = 5.264 - 0.019*x*	0.77	313 (52)	y = 5.140 - 0.012*x*	0.76	489 (81)
NDV	y = 6.065 - 0.013*x*	0.74	464 (77)	y = 6.727 - 0.008*x*	0.74	710 (118)	y = 7.574 - 0.009*x*	0.83	666 (111)	y = 6.165 - 0.007*x*	0.54	862 (144)
ECBO	y = 6.464 - 0.005*x*	0.84	1092 (182)	y = 5.813 - 0.003*x*	0.18	2345 (391)	y = 6.884 - 0.007*x*	0.80	910 (152)	y = 6.420 - 0.004*x*	0.52	1336 (223)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

Table 9.10: Linear regression models for the persistence of influenza and model viruses in various substrates at -10 °C using the germ carrier technique

Virus Type	Surface water			Sediment			Duck feces			Duck meat		
	LRM	R^2	Estimated Persistence[a] (T-90 value)[b]	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)	LRM	R^2	Estimated Persistence (T-90 value)
H1N1	y = 4.795 - 0.029*x*	0.84	206 (34)	y = 2.280 - 0.013*x*	0.36	476 (79)	y = 2.777 - 0.018*x*	0.52	329 (55)	y = 5.398 - 0.017*x*	0.88	363 (60)
H4N6	y = 3.686 - 0.019*x*	0.69	318 (53)	y = 2.907 - 0.019*x*	0.59	314 (52)	y = 3.432 - 0.020*x*	0.81	294 (49)	y = 4.962 - 0.024*x*	0.89	255 (42)
H5N1	y = 4.701- 0.022*x*	0.61	277 (46)	y = 3.622 - 0.009*x*	0.35	827 (138)	y = 4.104 - 0.017*x*	0.68	353 (59)	y = 4.934 - 0.018*x*	0.88	333 (55)
H6N8	y = 4.542 - 0.031*x*	0.39	194 (32)	y = 3.787 - 0.007*x*	0.21	793 (132)	y = 4.480 - 0.022*x*	0.71	276 (46)	y = 4.794 - 0.012*x*	0.59	482 (80)
NDV	y = 5.286 - 0.014*x*	0.58	419 (70)	y = 6.245 - 0.010*x*	0.57	620 (103)	y = 7.359 - 0.016*x*	0.89	372 (62)	y = 5.579 - 0.012*x*	0.75	493 (82)
ECBO	y = 6.357 - 0.006*x*	0.76	1041 (174)	y = 5.896 - 0.004*x*	0.27	1637 (273)	y = 6.892 - 0.008*x*	0.66	797 (133)	y = 6.389 - 0.006*x*	0.69	946 (158)

LRM: Linear regression model, R^2: Coefficient of determination

y: virus titer in $\log_{10}$ $TCID_{50}$/ml, *x*: persistence in days

[a] Estimated persistence in days with a starting virus concentration of 10^6 $TCID_{50}$/ml

[b] T-90 value: Time in days required for 90 % decrease in virus titer

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