

# Ammonia treatment of hatchery waste for elimination of avian flu and model viruses: treatment recommendations

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## Abstract

Ammonia treatment of hatchery waste (HW) was studied regarding the virus inactivation. Two enveloped viruses, avian influenza virus H5N3 (AIV) and infectious bovine rhinotracheitis virus (IBRV), a herpesvirus, was investigated. Hatchery waste was spiked with viruses and indicators (bacteriophages MS2 and  $\phi$ X174) and ammonia was added to final concentrations of 0.25, 0.5 and 0.75 % (w/w), at a temperature of 14°C. As controls hatchery waste with only deionised water was used. The results indicate that the enveloped viruses are readily inactivated at all ammonia concentrations tested. Bacteriophage  $\phi$ X174 did not show any significant decay at any of the ammonia concentrations, whereas bacteriophage MS2 was inactivated at a slower rate than the enveloped viruses. Ammonia is a good alternative for inactivation of AIV and IBRV in hatchery waste and bacteriophage MS2 can be used as a conservative indicator.

## Introduction

Hatchery waste (HW) is a by-product from the hatchery industry which may contain pathogenic microorganisms, e.g. viruses, and needs sanitation treatment in accordance with EU legislation (EU 2006). Virus inactivation methods can for example be physical or chemical. Examples of physical methods are high pressure, which proved to inactivate noroviruses in foods (Kingsley et al. 2007), and thermal processes. Thermal inactivation works mainly through denaturation of proteins. Chemicals can possess different inactivation mechanisms, such as covalent bonding to nucleic acids to disturb the replication of the virus (e.g. aldehydes), protein denaturation or resolving lipid containing viruses (alcohols or other organic solvents), cleavage of nucleic acid strands (ammonia) and diverse pH effects. The action of chemicals depends on the organic content of the material, and of the temperature (Poschetto et al. 2007). Treatment processes must be validated and, among other demands, prove a 3 log<sub>10</sub> reduction of thermoresistant viruses (EU 2006). This can for example be achieved through liming, which is a common method in Sweden to disinfect HW. However, liming leads to unsuitable working conditions and is technically complicated, e.g. due to the high pH (>12) and formation of sediments. Another option is disinfection with ammonia, which have been studied regarding human faeces (Vinnerås et al. 2003) and bovine manure (Ottoson et al. 2008). In ammonia treatment, ammonia (NH<sub>3</sub>) is the active substance and is present at pH values >8 with a pKa of 9.3. The equilibrium of NH<sub>3</sub> and its ionised form NH<sub>4</sub><sup>+</sup> depends on both pH and temperature and is shifted towards NH<sub>3</sub> when either of them is increased. Further, ammonia treatment contributes to the fertiliser value of the hatchery waste. Studies have indicated that NH<sub>3</sub> acts by cleavage of viral RNA; this has been shown regarding poliovirus, a picornavirus, at temperatures between 10°C and 40°C (Burge et al. 1983).

An environmentally safe sanitation method would be desirable in case of epizootic disease outbreaks, instead of e.g. sodium hydroxide or formaldehyde treatments presently recommended in Sweden (SJV 2006). Considering the virus inactivation effect, seen in the case of picornavirus, our objective in the present study was to investigate the virus inactivation capacity of ammonia on a selected panel of further important viral diseases of interest for the poultry industry: avian influenza, Newcastle disease and infectious laryngotracheitis. The causative agents of these diseases are avian influenza virus (AIV), Newcastle disease virus (NDV) and infectious laryngotracheitis virus (ILT). AIV and NDV

belong to the *Orthomyxoviridae*, respective *Paramyxoviridae* families of viruses (Alexander 2003). Considering the similarities between these two families, we suppose that the effects of ammonia treatment should be similar between the two viruses and results obtained with AIV be representative for NDV. ILTV, on the other hand, is a herpesvirus with segmented dsDNA (Minson et al. 2000).

The aims of the present study were to: 1) determine the inactivation rates for viruses in ammonia treated hatchery waste at 15°C, 2) examine the possibility to use bacteriophages as indicators of virus reduction in hatchery waste and 3) suggest treatment of hatchery waste during normal and outbreak situations.

## Materials and Methods

### Hatchery waste

The material used for the disinfection studies was untreated hatchery waste (HW) consisting of eggshells and non-hatched eggs, obtained from a company in the south of Sweden. The batch used had a dry matter content of 60% and was kept at -70°C until the experiment started.

### Microorganisms

Disinfection of the HW was monitored by analysis of viable added microorganisms. Viruses used were avian influenza virus (AIV), strain A/duck/Sweden/Eskilstuna/2005(H5N3), isolated at the Department of Virology, SVA (Zohari et al. 2008); infectious bovine rhinotracheitis virus (IBRV), strain Jura; Enterobacteria phage MS2, (ATCC 15597-B1) and Enterobacteria phage  $\phi$ X174 (ATCC 13706-B1). Table 1 presents an overview of viral properties. *Salmonella enterica* strain WG49 (ATCC 700730) and *Escherichia coli* strain C (ATCC 13706™) was used as host strains for  $\phi$ X174 and MS2 respectively.

Table 1. Physico-chemical properties of the viruses used in the study

Organism	Shape	Virus family	Size nm	Envelope	Genome	Genome size kb
AIV	Spherical/pleomorphic	Orthomyxoviridae	80-120	Yes	segm –ssRNA	13.6
MS2	Icosahedral/spherical	Leviviridae	26	No	+ssRNA	3.6
$\phi$ X174	Icosahedral	Microviridae	26-32	No	circular +ssDNA	5.4
IBRV	Spherical/pleomorphic	Herpesviridae	150-200	Yes	dsDNA	125-240

-ss = negative sense single stranded; segm. = segmented; +ss = positive sense single stranded; ds = double stranded

### Cell lines and cell culture media

For AIV cultivation, the cell line Madin Darby Canine Kidney (MDCK) (ATCC CCL-34) was used, using as cell culture medium (CCM) Eagle's minimal essential media (EMEM; SVA production, Uppsala, Sweden) (Eagle 1959) containing trypsin (Worthington Biochemical Corporation, Lakewood, NJ) at a concentration of 2.5 µg/ml. For IBRV cultivation, bovine turbinate (BT) cells were used cultivated in EMEM containing 3 g/l tricine and 2%(v/v) fetal bovine serum.

### Analysis of microorganisms

Viruses were analysed by an end-point titration method through cell culture cytopathic effect using 96-well plates, and the virus titres were calculated according to the Spearman Kärber formula (Kärber 1931) and expressed as log<sub>10</sub> tissue culture infectious dose (TCID)<sub>50</sub> values per gram HW. The virus reduction factors where no virus was found were calculated

according to (Anon 1995). The double agar layer method (Adams 1959) was used to determine the number of the bacteriophages. The bacteriophage titres were expressed as  $\log_{10}$  plaque forming units (PFU) per gram HW. Bacteriophage and virus titres were plotted against time and linear regression was used to determine the inactivation rates.

### Experimental design laboratory trials

HW in 0.9 g portions was spiked with 0.1 ml of the respective microorganism to an initial concentration of 5-7  $\log_{10}$  per gram. Ammonia was added in 0.1 ml portions to final concentrations of 0.25, 0.5 or 0.75% (w/w) by thorough vortexing for complete homogenisation. As controls, spiked HW with only deionised water was used, to determine the inactivating effect of the HW and temperature alone. The resulting HW had a dry matter content of about 49%. For comparison, virus in cell culture medium was kept at the actual temperature and time periods. All mixtures were treated at  $14 \pm 0.5^\circ\text{C}$ . Sampling was performed 3-6 times for each ammonia inactivation trial, depending on the inactivation rate, up to 72 hours. Two samples of HW were taken in repeated experiments, diluted 10-fold in cell culture medium, and extracted by vigorous shaking. After centrifugation at 3000 g for 10 min, the supernatant was gel filtrated through Sephadex G-25 columns in cell culture medium, to remove ammonia and other cytotoxic low molecular weight substances, and subjected to filtration through 0.45  $\mu\text{m}$  filters Filtropur S.

### Planned design large scale trials (only MS2)

HW in about 100 kg portions will be spiked with the microorganism to an initial concentration of approximately  $10^7$  PFU  $\text{g}^{-1}$ . Ammonia will be added to a final concentration of 0.5 % w/w by thorough mixing for complete homogenisation. As controls, spiked HW with only deionised water is used and MS2 in cell culture medium will be kept at the actual temperatures and time periods. All mixtures are treated at ambient temperature. Sampling is performed about ten times for each ammonia inactivation trial, depending on the inactivation rate, up to one week. Samples are treated and analysed as above.

Table 3. Inactivation rate constants (k) of avian influenza virus and MS2 bacteriophages in hatchery waste treated with 0.25%, 0.50% and 0.75% w/w  $\text{NH}_3$  (aq). As controls, inactivation in untreated hatchery waste and cell culture medium was determined

Virus	Addition [% w/w]	k [log/h]	SE(k)	n	R <sup>2</sup>	p	Dt* [h]
AIV	Control	0.053	0.40	9	0.20	0.23	19
	0	0.11	0.022	8	0.81	0.002	9.1
	0.25	0.31	0.031	10	0.93	<0.001	3.2
	0.50	0.47	0.061	8	0.91	<0.001	2.1
	0.75	0.69	0.024	6	0.99	<0.001	1.4
MS2	Control	0.0048	0.0010	10	0.73	0.002	209
	0	0.0080	0.0012	14	0.78	<0.001	125
	0.25	0.069	0.0036	14	0.97	<0.001	14
	0.50	0.13	0.013	12	0.90	<0.001	7.9
	0.75	0.19	0.020	12	0.89	<0.001	5.4

\* Decimal reduction time, i.e. the time for 90% ( $1 \log_{10}$ ) reduction

### Cytotoxicity and viral interference assays

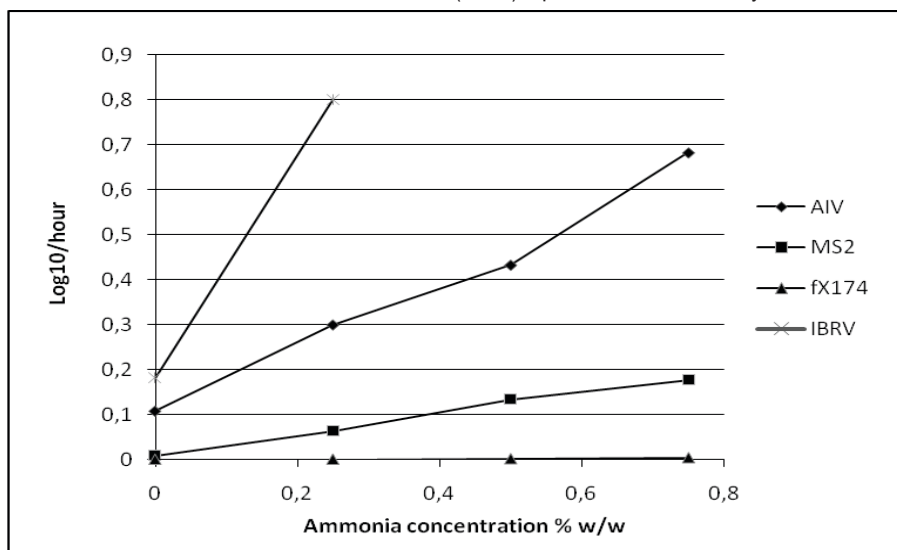
To assess the detection limits before analysis, cytotoxicity and viral interference assays were performed according to (Blumel et al. 2002): HW with the highest ammonia concentration was extracted and treated as above. The cytotoxicity of the resulting effluent

and the effluent diluted 1/5 was evaluated by inspecting the cells daily for the time used in the assays. The viral interference assays were performed to see the interfering properties of the effluent and the effluent diluted 1/5 with the infection of cells. The microorganisms were titrated in the resulting effluent and in the effluent diluted 1/5, and these virus titres were compared to the virus titres obtained using cell culture medium as titration media.

## Results

The inactivation rates for AIV and MS2 coliphages in the respective treatments are presented in Table 3. The effect of the material itself can be seen in the doubling of the inactivation rate compared to the control in cell culture medium. Addition of 0.25% increased the reduction by a factor between 2 and 3 for AIV and MS2. Somatic coliphages inactivation, as measured by reduction of  $\phi$ X174, was, however, insignificant. IBRV on the other hand was inactivated most rapidly of all organisms tested,  $>0.77 \log_{10} h^{-1}$  after 0.25% ammonia addition (Figure 1).

Figure 1. Inactivation rates from the different added ammonia concentrations for avian influenza (AIV), Enterobacteria phage MS2 (MS2), Enterobacteria phage  $\phi$ X174 ( $\phi$ X174) and Infectious bovine rhinotracheitis virus (IBRV), spiked into the hatchery waste



## Discussion

The addition of ammonia to hatchery waste is a possible means to disinfect the material and at the same time increasing its fertilizer value. AIV and IBRV, the tested viruses in the present study, were both sensitive to ammonia, as it was indicated by the rapid reduction of viral infectivity after the addition of 0.25% w/w. The Decimal reduction time at 14°C was 3.2 and <1.3 hours respectively. Bacteriophages were added as indicators for virus reduction; however, they proved to be more persistent than the viruses they were intended to indicate the inactivation of. Both AIV and MS2 are ssRNA viruses, the differences between the genomes could be seen in Table 1. AIV possesses a segmented genome 4-5 times larger than MS2, and the larger genome could be more sensitive for ammonia, since the mechanism of ammonia disinfection is probably by cleavage of nucleic RNA (Ward 1978). AIV also possesses an envelope, which could make it more sensitive to ammonia. MS2 and AIV were affected by ammonia in a similar way, and the fact that MS2 were more tolerant to all treatments makes it an excellent indicator for AIV reduction

in ammonia treatment processes. Further, MS2 is easy to propagate to high titres and is not infectious to any organisms but its host bacteria. As could be read in the material and methods section, full-scale ammonia treatments with the addition of MS2 to hatchery waste are planned.

The naked bacteriophage  $\phi$ X174, on the other hand, seems to be resistant to ammonia. It has a small circular ssDNA genome. The other studied DNA virus, IBRV, was extremely sensitive to ammonia, even though possessing dsDNA. The large genome and/or the envelope could be the reasons for the fast inactivation by ammonia. The results from the present study indicate that the envelope may play a crucial role in ammonia tolerance, enveloped viruses being more susceptible to ammonia treatment. Ideally, an enveloped bacteriophage could be used as an indicator. There are only few enveloped bacteriophages, for example *Pseudomonas* phage  $\phi$ 6 of the *Cystoviridae* family (Ackermann 2007). However,  $\phi$ 6 has proven to be much more sensitive to environmental stress than most viruses (data not shown).

Otherwise, parvovirus is the virus suggested to use when validating a treatment process for animal by-products in accordance with EU legislation (EU 2006). In this approach, we based the recommendation on the inactivation of MS2. To achieve the intended target of 3 log<sub>10</sub> reduction, addition of 0.25% ammonia should be followed by storage for 65 h at 14°C (minimum). The storage time after 0.5% addition should be 59 h (these figures represent the 95<sup>th</sup> percentile). The relatively small difference in storage times depends on the higher uncertainty in the results from the 0.5% ammonia addition (Table 3). However, before suggesting ammonia treatment, tests on the removal of other viruses and ova of *Ascaris* spp. should be performed (EU 2006). The storage time of 59 h can be compared of that of seven days recommended to achieve the reduction targets for salmonella in bovine manure (Ottoson et al. 2008).

In an outbreak situation, contaminated litter must be taken care of properly (SFS 1999). Is the causative agent AIV, NDV or ILTV, ammonia may be an effective means to treat the hatchery waste. Treatment should preferably achieve at least 12 log<sub>10</sub> reduction of the epizootic agent in question. The recommendation is to add 0.75% ammonia and store for at least 26 hours at 14°C (99<sup>th</sup> percentile) based on the data on AIV inactivation. During storage, it is important to make sure that ammonia is not lost. A way of controlling ammonia losses is by measuring the pH on-line.

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