

Inactivation of Cryptosporidium parvum oocysts and faecal indicator bacteria in cattle slurry by addition of ammonia

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1 2 MRS. HEIDI HUUS PETERSEN (Orcid ID : 0000-0002-7208-7614) PROF. LARS STOUMANN JENSEN (Orcid ID : 0000-0002-1446-2084) 3 4 Article type : Original Article 7 8 Inactivation of Cryptosporidium parvum oocysts and faecal 9 indicator bacteria in cattle slurry by addition of ammonia 10 Running title: Inactivation of parasites and bacteria in slurry 11 Petersen, H.H.^{a,b*}, Dalsgaard, A.^b, Vinneras, B.^c, Jensen, L.S.^d Le, T.T.A.^b, Petersen, M.A.^f, Enemark, H.L.^{a, g} and 12 13 Forslund, A.^b 14 A National Veterinary Institute, Technical University of Denmark, Kemitorvet, 2800 Kgs. Lyngby, Denmark 15 b Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of 16 Copenhagen, Frederiksberg, Denmark 17 c Swedish University of Agricultural Sciences, Department of Energy and Technology, SE-750 07 Uppsala, 18 Sweden 19 d Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, 20 Frederiksberg, Denmark 21 f Department for Palliative Medicine, The Research Unit, Bispebjerg Hospital, Copenhagen NV, Denmark 22 g present address: Department of Animal Health and Food Safety, Norwegian Veterinary Institute, P.O. Box 23 750 Sentrum, NO-0106 Oslo, Norway This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to

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24

26 ABSTRACT

Aims: To determine inactivation of *Cryptosporidium parvum* oocysts and reduction of *Escherichia coli* and
enterococci in cattle slurry added aqueous ammonia.

Methods and Results: Escherichia coli, enterococci and non-viable C. parvum oocysts (DAPI+ PI+) were 29 30 enumerated every second day for two weeks in cattle slurry amended with 60 mmol l⁻¹ aq. ammonia and 31 compared with untreated slurry at three temperatures. Regardless of temperature, the proportion of non-32 viable C. parvum oocysts increased significantly faster over time in slurry with added ammonia than raw 33 slurry (p=0.021) corresponding to 62.0% higher inactivation (P=0.001) at day 14. Additionally, 91.8% fewer 34 E. coli and 27.3% fewer enterococci was observed slurry added ammonia at day 14 compared raw slurry. 35 **Conclusion**: The addition of aqueous ammonia to raw slurry significantly reduced the viability of *C. parvum* 36 oocysts and numbers of bacterial indicators. Hence, ammonia is usable to lower pathogen concentrations 37 in slurry prior before application to agricultural land.

Significance and Impact of Study: Livestock waste is a valuable source of plant nutrients and organic matter, but may contain high concentrations of pathogens like *Escherichia coli* and *Cryptosporidium* spp. that can be spread in the environment, and cause disease outbreaks. However, die-off rates of pathogens in organic waste can increase following increasing ammonia concentrations.

Keywords: *Cryptosporidium parvum*; *Escherichia coli;* Enterococci; faecal indicator bacteria; Cattle slurry;
Ammonia

44 **1. INTRODUCTION**

45 Livestock waste is a valuable source of plant nutrients and organic matter and is used to fertilize 46 agricultural land. Unfortunately, livestock waste may contain large quantities of microorganisms pathogenic 47 to humans (Hutchison et al., 2005b), including the zoonotic protozoan parasite Cryptosporidium parvum, 48 Salmonella spp. and Escherichia coli. Following livestock waste application to agricultural land, pathogens 49 can contaminate crops or the aquatic environment (Bodley-Tickell et al., 2002; Islam et al., 2004; Ong et al., 50 1996; Pelly et al., 2007) potentially constituting a public health risk. Dispersal of pathogens from animal 51 faeces relies on rain to release the pathogens from the faecal matrix or slurry, and surface runoff water 52 then contaminates water bodies serving as recreational, irrigation, or drinking water sources.

53 In 2007 in Galway, Ireland, approx. 182 people were infected with Cryptosporidium oocysts contracted from a lake, which was apparently contaminated by water runoff from agricultural land following 54 55 application of slurry in a wet winter (Pelly et al., 2007). In Walkerton, Canada, E. coli O157:H7 and 56 Campylobacter jejuni infections resulted in 2,300 cases of illness and seven deaths among individuals who 57 consumed drinking water from a municipal water supply contaminated with pathogens originating from a 58 nearby farm, where cattle manure was applied to agricultural fields and subsequently washed into the 59 water reservoir (Clark et al., 2003; Hrudey et al., 2003). Due to expected increased heavy rain events 60 related to climate changes, slurry-contaminated surface runoff water from fields will probably be a growing 61 source of pathogen contamination in certain parts of the world.

62 Abu-Ashour and Lee (2000) studied E. coli overland transport at field sites with 2% and 6% slopes during 63 surface water runoff events in which grass-covered plots were contaminated by pouring water containing 64 E. coli on the surface using watering cans. Escherichia coli was released from the slurry by this simulated rainfall event and 15 and 16 CFU E. coli mL⁻¹ were isolated in runoff water 20 m and 30 m, respectively, 65 from the initial location (Abu-Ashour and Lee, 2000). Furthermore, oocysts was detected in runoff water 66 67 from manure containing C. parvum oocysts and applied to agricultural land (Thurston-Enriquez et al., 2005). 68 In addition, rain events can cause vertical transport of slurry-borne microorganisms, resulting in shallow 69 groundwater contamination (Forslund et al., 2011; Mawdsley et al., 1996; Petersen et al., 2012). 70 Compounding this issue, Cryptosporidium is infectious at low dosages (Okhuysen et al., 1999) and oocysts 71 possess very robust multi-layered structures (Harris and Petry, 1999) resistant to e.g. periods of freezing 72 (Robertson et al., 1992), and commonly utilized water disinfectants such as chlorine (Korich et al., 1990; 73 Shields et al., 2008; Venczel et al., 1997). Therefore, pre-treating slurry can be useful to inactivating oocysts 74 prior to application to agricultural land, thereby preventing contamination of water and food sources. High 75 temperature and exposure to ammonia are some of few stressors that can inactivate oocysts (Jenkins et al.,

76 1998; Li et al., 2010; Reinoso et al., 2008), stressors, which potentially are available in slurry tanks. In 77 Denmark, slurry is usually stored in large tanks for approx. six months and then applied to fields during 78 spring and autumn (Vinnerås, 2013). However, during storage, fresh slurry is most often added 79 continuously to the tanks, resulting in short storage time for fractions of the slurry and limited time for 80 pathogen reduction and a corresponding risk of transmission to the external environment (Hutchison et al., 81 2005a; Vinnerås, 2013). By adding a known concentration of ammonia to the slurry tank, it is guaranteed 82 that all oocysts are exposed to equal ammonia concentrations. However, reduction rates are lacking for 83 Cryptosporidium in slurry, since previous studies have focused on oocyst survival in faecal pats, soil and 84 water. Slurry contains much more native ammoniacal N than most other products studied. Previous studies 85 on Cryptosporidium oocysts stored in distilled water or Hank's balanced salt solution (HBSS), and exposed oocysts were increasingly inactivated with increasing ammonia 86 to ammonia have showed that 87 concentration (Jenkins et al., 1998; Reinoso et al., 2008), e.g., 99.9% oocysts were inactivated after an estimated 10.2 days when exposed to 60 mmol l⁻¹ ammonia, compared with 17.8 days when exposed to 26 88 89 mmol l⁻¹ ammonia (Jenkins et al., 1998). Moreover, a good sanitization effect of ammonia in reducing other 90 pathogens have been documented in faecal wastes (Mendez et al., 2004; Nordin et al., 2013; 91 Watcharasukarn et al., 2009). The effect of ammonia is considerably stronger than of OH⁻, and a recent 92 study of Senecal et al. (2020) showed that even at pH 12 additional factors such as ammonia or heat were 93 required for inactivation of Ascaris eggs. In a study with 20% w/w ammonia added to slurry, concentrations 94 of faecal coliforms were reduced by 7 log, Salmonella spp. by 6 log, and 83% of viable helminth eggs were 95 reduced within a contact time of 2 h (Mendez et al., 2004). Moreover, the decimal reduction time (T_{90}) equivalent to 1-log reduction of Salmonella inoculated in bovine manure added 0.5% aqueous (aq.) 96 97 ammonia and stored at 4 °C was 1.1 day, compared with 34 days in raw slurry (Ottoson et al., 2008a). There 98 is an apparent linear relationship between Salmonella and E. coli inactivation rates following ammonia 99 treatment, while enterococci appear more resistant to ammonia treatment (Himathongkham and Riemann, 100 1999; Nordin, 2010; Ottoson et al., 2008a).

101 The ammonia addition serves two purposes, firstly it is an efficient treatment method for pathogen 102 inactivation. Secondly, as the ammonia is not consumed during the treatment, the treatment needs to be 103 performed in systems with roof to assure that the ammonia is not ventilated away, the ammonia will then 104 add upon the nutrient content when the slurry is applied in the field. Thereby, no additional N fertilisation 105 will be required during the growing season. However, in areas with high N overload from agriculture due to 106 too high animal density, this method will not be applicable as it will add to the total nitrogen overload. The 107 method is more applicable in areas with moderate animal density where mineral fertilisers complement the 108 use of animal manure in food and feed production.

The objective of the present study was to determine if *C. parvum* oocysts could be inactivated and *E. coli* and enterococci reduced below the detection limit in cattle slurry prior to land application by adding 60 mmol l⁻¹ aq. ammonia, and storing it at temperatures corresponding to the typical variation in ambient temperatures in Denmark.

113 2. MATERIALS AND METHODS

114 Slurry originated from a slurry tank on a conventional dairy farm in Store Heddinge, Denmark. Properties of the slurry are described in Table 1. The pH and electrical conductivity were measured directly in the slurry 115 with a combination tester HI 98130 (Th Geyer, Roskilde, Denmark). Dry matter content was determined by 116 117 oven drying at 105 °C. Cryptosporidium oocysts were determined in 2 g of slurry as described in Petersen et 118 al. (2012). Briefly, Cryptosporidium-positive faecal samples were suspended in tap water, filtered through 119 gauze, centrifuged and the supernatant discarded. This washing procedure was repeated 2-3 times. Tap 120 water was added to increase the volume to 5–10 ml. The faecal solution was underlayered with a gradient 121 consisting of 1.09/1.05/1.01 Percoll (Amersham Biosciences, Australia), centrifuged at 1,540 x g for 10 min, 122 and oocysts were collected between the 1.09/1.05 Percoll layers. Purified oocysts were washed three times 123 to remove Percoll, and the oocysts were then enumerated by immunofluorescence microscopy as 124 described in 2.3. The concentrations of *E. coli* and enterococci, and total ammoniacal nitrogen (TAN) were 125 determined as described in sections 2.4 and 2.5, respectively.

The collected slurry only contained 8 ± 1.2 oocysts L⁻¹ (Table 1) and was therefore spiked with *C. parvum* oocysts. These oocysts originated from a field isolate from a Danish Holstein calve naturally infected with *C. parvum*. The calve was diagnosed by the modified Ziehl-Neelsen technique (Henriksen and Pohlenz, 1981) and oocysts subsequently concentrated as previously described by Petersen et al. (2012). The viability of the concentrated oocysts was analysed as described in section 2.3 and the oocysts were identified to the species level as described by Langkjær et al. (2007).

132 2.1. Experimental design

Every second day throughout 14 days, inactivation of *C. parvum* oocysts and reduction of *E. coli* and enterococci in cattle slurry was determined at various temperatures (4 °C, 10 °C and 20 °C) following the addition of concentrated aq. ammonia (25% NH₃ by weight) (Table 2). These temperatures correspond to the typical variation in ambient temperatures in Denmark.

137The cattle slurry was placed in a 5 L plastic container on a magnetic stirrer and 10 g portions of slurry were138added to either 15 ml blue cap centrifugation tubes (n=144) or 50 ml blue cap centrifugation tubes (n=144)

139 (Table 2) leaving approx. 30-40 ml headspace in the 50 ml tubes and approx. 5 ml headspace in the 15 ml 140 tubes. The tubes were then stored in incubators at 4, 10, or 20 °C overnight for the slurry to reach the 141 required temperature prior to start of the experiment. Slurry in the 15 ml blue cap centrifugation tubes was 142 used for E. coli and enterococci measurements (n=144), while slurry in the 50 ml blue cap centrifugation 143 tubes were spiked with approx. 2.2 \times 10⁵ C. parvum oocysts (n=144). Escherichia coli and enterococci were 144 present in sufficient numbers in the slurry (Table 1) and spiking was unnecessary. Aqueous ammonia was 145 added to 50% of both 15 ml (n=72) and 50 ml (n=72) tubes (Table 2). The tubes were immediately sealed 146 with a lid to avoid vaporization, mixed well by shaking and placed in incubators at 4, 10 or 20 °C together 147 with the remaining (n=72x2) of the tubes which acted as controls and contained raw slurry with no added 148 aq. ammonia (Table 2). Inactivation of *C. parvum* oocysts, reduction of *E. coli* and enterococci, pH and Total 149 ammonium N (TAN) were determined in triplicate samples every second day for 14 days (Table 2).

To determine if increased pH values affected oocyst inactivation in the slurry, 10 ml buffer with pH of seven and nine (Th. Geyer, Roskilde, Denmark) were spiked with approx. 2.2×10^5 *C. parvum* oocysts and incubated together with the slurry.

153 To achieve identical uncharged NH₃ concentrations in slurry samples incubated at different temperatures, 154 the volume of aq. ammonia added to reach a final uncharged NH₃ concentration of approx. 60 mmol l⁻¹ was 155 determined for each incubation temperature prior to start of the experiment. The target concentration of 156 60 mmol I⁻¹ NH₃ was chosen since it has previously been demonstrated to inactivate 99.9% of the oocysts 157 within 14 days in distilled water (Jenkins et al., 1998). The specific concentration was reached by 158 incremental addition of aq. ammonia to a predefined volume of slurry and measurement of pH, to create 159 an ammonia standard curve. In brief, 100 ml slurry were placed in a beaker on a magnetic stirrer, 250 μ l aq. 160 ammonia were added at once, the slurry was stirred for 1 min, and the pH was measured. The procedure 161 was repeated until 5,000 μL aq. ammonia was added. Uncharged NH₃ along the standard curve was then 162 calculated for each incubation temperature according to the following formula (Vinnerås, 2013):

$$[NH_3] = \frac{[TAN] \times K_a}{K_a + [H^+]}$$
(1)

163 Where TAN is total ammoniacal N and the dissociation constant K_{a} , varies with temperature (T, Kelvin), and 164 pKa (defined as $-\log_{10}[Ka]$) which is related to T by:

$$pK_a = \frac{2728.9}{T} + 0.090181$$
 2)

165 The theoretical requirement of TAN to be added as aq. ammonia per 10 ml of slurry to reach the same 166 uncharged calculated NH_3 concentration was estimated.

Based on this, 125 μ l (4 °C), 100 μ l (10 °C), or 88 μ l (20 °C) aq. ammonia, corresponding to uncharged NH₃ concentrations of 67, 64, and 57 mM (as close as possible to the target concentration of 60 mM), respectively, were added at day 0. It was assumed that due to the high solubility of NH₃ in water (Henry's constant, K_H of 60 mol l_{aq}⁻¹ atm⁻¹ at 25 °C and higher at lower temperatures (Sommer et al., 2013)), very little of the added NH₃ will be equilibrated in the headspace during incubation.

172 **2.2.** Isolation of *C. parvum* oocysts from slurry

173 Cryptosporidium parvum oocysts were isolated from slurry samples (n=63; Table 2) by immunomagnetic 174 separation (IMS) with modifications (Dynabeads[®] Anti-Cryptosporidium Kit, Life technologies, Nærum, 175 Denmark). Briefly, 1.5 ml slurry from each sample was added to a L10 tube, diluted to 10 ml with MQ water 176 and mixed well. Hereafter, 1 ml 10X SL[™]-Buffer A, 1 mL 10X SL[™]-Buffer B, and 100 µL Dynabeads[®] anti-177 Cryptosporidium were added. The subsequent oocyst isolation was carried out according to the 178 manufacturer's instructions.

179 **2.3.** Assessment of *C. parvum* oocyst inactivation in cattle slurry

180 Oocyst inactivation was assessed by scoring oocysts according to inclusion or exclusion of the vital dyes 181 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI). In brief, purified oocysts obtained by IMS 182 was incubated simultaneously with 10 μ l DAPI working solution (2 mg ml⁻¹ in absolute methanol) (Sigma, 183 Sigma-Aldrich Denmark ApS) and 10 μ I PI working solution (1 mg ml⁻¹ in 0.1M phosphate buffered saline 184 (PBS, pH 7.2); Sigma, Sigma-Aldrich Denmark ApS). Following 3 h incubation at 37 °C, each sample was 185 washed twice in MQ water by centrifugation at $3500 \times q$ for 5 min and removal of the supernatant down to 186 approx. 100 μ l. The entire sample was then placed in a well on a three-well (12 mm) teflon printed 187 diagnostic slide (Immono-Cell, Mechelen, Belgium). The slide was air-dried, fixed for 5 min with acetone, 188 and 25 µl anti-Cryptosporidium fluorescein isothiocyanate (FITC)-labelled antibody mix (Crypto-Cell IF test, 189 Cellabs, Australia) were added according to the manufacturer's instructions. Characterization of inactivated 190 oocysts was based on inclusion and exclusion of vital dyes. Oocysts that included both dyes (DAPI-positive, 191 Pl-positive (DAPI+ PI+)) were characterized as non-viable (inactivated). Approx. 100 oocysts from each 192 sample were quantified using an epifluorescence microscope at 400× magnification (Leica DMRA2) and 193 subsequently the percentage of non-viable oocysts was calculated. Assuming a total population of 10,000 194 oocysts: If repeatedly, randomly sampling 100 oocysts out of the overall population, 80% of the samples 195 will have an inactivation estimate that differs <=6% from the true level, i.e., there is an 80% probability of 196 obtaining an estimate within then range 54-66%, if the true level is 60%. In 96% of the cases, the deviation 197 will be <10%.

The microscope was equipped with the following filter blocks: 350 nm excitation, 450 nm emissions for DAPI; 500 nm excitation, 630 nm emissions for PI; and 495 nm excitation, 519 nm emission wavelengths for FITC.

201 **2.4. Bacteriological analysis**

For the *E. coli* and enterococci analysis, 10-fold dilutions of slurry were prepared in Maximum Recovery Dilution (MRD) (Oxoid, Basingstoke,UK) and surface-spread onto Brilliant *E. coli*/coliform Selective Agar (Oxoid, Basingstoke,UK) and Slanetz & Bartley Agar plates (Oxoid, Basingstoke,UK), respectively. For the *E. coli* analysis, the Brilliant *E. coli*/coliform Selective Agar plates were incubated at 37 °C for 24 h and purple colonies were counted. Enterococci were determined as typical red-maroon colonies on Slanetz & Bartley Agar following incubation at 44 °C for 48 h. Detection limit for both indicator bacteria was 1 CFU ml⁻¹.

208 2.5. Physico-chemical analysis

209 The pH was measured directly in all replicate slurry samples on each sampling day (Table 2) with a 210 combination tester HI 98130 (Th Geyer, Roskilde, Denmark). The pH was then lowered in 5 g slurry in all 211 replicate slurry samples (Table 2) by diluting it 1:20 in 1 mol I⁻¹ KCl (74.56 g mol-1). The diluted slurry was 212 shaken on a shaker table at 170 rpm in 45 min, left to sediment for 45 min, and 15 ml of the supernatant 213 filtered through a filter paper (Advantec 5A, Frisennette Aps, Denmark). The filtrate was stored at -20°C for 214 later ammonium analysis. Total ammonium N (TAN) was measured spectrophotometrically on a Foss 215 FIAstar 5000 flow injection analysis system. The concentration of uncharged NH₃ in each individual tube 216 was then calculated according to equation (1) and (2) (Vinnerås, 2013), based on TAN, pH and incubation 217 temperature.

218 2.6. Data analysis

219 In order to obtain variance homogeneity, the natural logarithm (In) was used to transform the following 220 data as outcome variables in standard multiple linear regression models: calculated uncharged NH3 221 concentration, pH, proportion of non-viable (DAPI+ PI+) oocysts, E. coli, and enterococci. The following variables were analysed as potential explanatory variables: treatment (raw slurry and slurry with added aq. 222 223 ammonia), incubation temperature (4 °C, 10 °C, 20 °C), and time (modelled as a continuous variable). The 224 normality assumption was validated by quantile-quantile plots, and variance homogeneity was validated by 225 residual plot. To improve fit, pathogen levels were normalized by transforming to In (x) and the normality 226 assumption was validated by quantile-quantile plots, and variance homogeneity was validated by residual 227 plot. To fit *E. coli* to a standard linear regression model, time was transformed using ln (x + 1). For all 228 outcomes, non-significant effects were removed by stepwise backward model reduction using a 5% 229 significance level starting with the model with the three-factor interaction of treatment*incubation This article is protected by copyright. All rights reserved

- 230 temperature*time. All mean ± Standard error of the mean (SEM) values are the geometric mean and
- 231 geometric standard error of the mean. The percentage of non-viable oocysts was calculated based on the
- 232 best-fitted line shown in Fig. 2.
- 233 The statistical analyses were conducted using SAS v. 9.3 [1].

234 **3. RESULTS**

235 3.1. Ammonia and pH

- Figures 1A and 1B show the geometric mean and 95% CI of pH and calculated uncharged NH₃ concentration
 in cattle slurry over time for the various treatments.
- The mean \pm SEM pH at day 0 independent of temperature was 8.5 \pm 0.03 in the raw slurry, while the addition of ammonia to slurry increased the pH to 9.7 \pm 0.01. The pH in raw slurry increased significantly over time (p<0.0001), while there was no change in pH in slurry with added ammonia. The changes in pH over time were unaffected by incubation temperature in raw slurry as well as slurry with added ammonia (Fig. 1A).
- 243 The mean \pm SEM uncharged NH₃ concentration at day 0 was 3.2 \pm 0.2, 6.7 \pm 0.6 and 16.5 \pm 1.6 mmol l⁻¹ at 4,
- 10 and 20 °C in raw slurry, and 71.6 ± 5.1, 88.0 ± 2.3 and 121.0 ± 1.5 mmol l⁻¹ at 4, 10 and 20 °C in ammonia
 treated slurry, corresponding to 68.4–104.5 higher mmol l⁻¹ in ammonia treated slurry than in raw slurry at
 day 0.
- In raw slurry, the uncharged NH_3 concentration increased significantly over time (p<0.0001), but the increase was unaffected by the incubation temperature.
- 249 In the ammonia treated slurry, the uncharged NH_3 concentration was constant over time and almost 250 unaffected by incubation temperature (Fig. 1B). Hence, the desired uncharged NH_3 concentration was 251 achieved throughout the study in slurry with added ammonia, regardless of temperature.

252 **3.2.** Cryptosporidium oocysts

- 253 Of the oocysts used for spiking, 12.0 % were categorized as non-viable (DAPI+ PI+) at day 0.
- Both in slurry with added ammonia and in raw slurry, the proportion of non-viable oocysts significantly (P<0.0001; P=0.0003) increased over time (Fig. 2). However, incubation temperatures did not affect the inactivation of oocysts neither in raw slurry nor in slurry added ammonia (data not shown).

- 257 When disregarding the incubation temperature, the proportion of non-viable oocysts increased significantly
- within 14 days in slurry added ammonia (increased by 28.5%) than in raw slurry (increased 10.8%) (Fig. 2),
- resulting in 40.4% non-viable oocysts in slurry added ammonia and 22.8% non-viable oocysts in raw slurry
- at day 14. This correspond to 62.0% higher inactivation (P=0.001) of oocysts in slurry added ammonia at
- 261 day 14 (mean concentration 95.3 ± 4.54 mmol l⁻¹ ammonia).
- The correlations between uncharged NH₃ and non-viable oocysts were moderate (r = 0.58 and r = 0.56) (data not shown).
- No significant differences were observed in the proportion of non-viable oocyst following 14 days of storage in buffer with pH 7 or 9 (data not shown).

266 **3.3. Bacterial indicators**

- 267 Enterococci were naturally present in slurry, with a mean initial concentration of $1.6 \times 10^4 \pm 2.5 \times 10^3$ CFU ml⁻¹ (Table 1). Enterococci concentration was reduced faster at 20 °C than at 4 °C (P=0.019) or 10 °C 268 (P=0.004) in the slurry with added ammonia (Fig. 3A). The enterococci concentrations following incubation 269 270 at 4 °C and 10 °C were not significantly different, and no differences in reduction was correlated to 271 incubation temperature in raw slurry (Fig. 3B). When the incubation temperature was disregarded, the 272 addition of ammonia to slurry resulted in a significantly faster reduction of enterococci over time (P=0.006) 273 compared with raw slurry, corresponding to 27.3% fewer enterococci in slurry added ammonia than in raw 274 slurry at day 14. Based on the standard linear regression model, the estimated mean time for a 1 log 275 reduction (T_{90}) ranged from 33.9 to 77.8 days in the slurry with added ammonia, compared with 66.6–175.3 276 days in the raw slurry.
- Likewise, *E. coli* was naturally present in the slurry with an initial concentration of 68 ± 28 CFU ml⁻¹ (Table 1). Since *E. coli* concentration in the slurry was reduced rapidly within the first couple of days, time was ln (x+1) transformed to enable linear modelling of the correlation between *E. coli* and time by a standard linear regression model. Based on this model, the concentration of *E. coli* CFU ml⁻¹ was significantly reduced over time in both raw slurry and slurry with added ammonia. No difference in reduction was noted in correlation to incubation temperature in slurry with added ammonia (P=0.176) (Fig. 3A), while in raw slurry *E. coli* was reduced faster at 20 °C than at 4 °C (P=0.033) or 10 °C (P=0.014) (Fig. 3B).
- In slurry samples with added ammonia, the concentration of *E. coli* was already below 10 CFU ml⁻¹ at day 2
 and at 20 °C reduced to below the detection limit within 6 days (Fig. 3A). In contrast, 12.2 ± 2.6 CFU *E. coli*ml⁻¹ was detachable at day 14 in raw slurry incubated at 20 °C (Fig. 3B), corresponding to 91.8% fewer *E. coli*in slurry added ammonia. The estimated mean decimal reduction time (T₉₀) for *E. coli* when disregarding

the temperature in raw slurry and slurry with added ammonia was 25.8 days and 9.8 days, respectively,
with less reduction in the raw slurry.

- 290 A negative correlation was observed between the uncharged NH₃ concentration in slurry and E. coli (r = -
- 291 0.70) (Fig. 4A), while the correlation between uncharged NH_3 and enterococci was weak (r = 0.36) (Fig. 4B).
- 292 Moreover, the correlation between enterococci and *E. coli* was weak (*r* = 0.39) (data not shown).

293 4. DISCUSSION

The reached ammonia levels of the material was 71.6 \pm 5.1, 88.0 \pm 2.3 and 121.0 \pm 1.5 mmol l⁻¹ at 4, 10 and 20°C, which is higher than expected. This is due to the changing buffer capacity of the slurry in relation to 296 the temperature changing the effect on pH by the added ammonia.

297 4.1 Cryptosporidium oocysts

We showed that the addition of ammonia (approx. 60 mmol l⁻¹) to cattle slurry significantly affected the inactivation of *C. parvum* oocysts within 14 days of exposure resulting in 62.0% higher inactivation of nonviable oocysts (DAPI+ PI+) compared with untreated raw slurry.

301 This loss of oocyst viability due to increased ammonia concentration in the slurry agrees with results from 302 other studies (Fayer et al., 1996; Jenkins et al., 1998; Reinoso et al., 2008), and confirms the vulnerability of 303 the otherwise very robust Cryptosporidium oocysts to ammonia. For example, ammonia concentration and 304 the number of inactivated oocysts correlate in Hank's balanced salt solution (HBSS) (Reinoso et al., 2008) and distilled water with an estimated 17.8 days to reach 99.9% inactivation at 30 mmol l^{-1} NH₃ and 10.2 305 306 days at 60 mmol l⁻¹ NH₃ in distilled water (Jenkins et al., 1998). However, in our study, an estimated 307 proportion of 40.4% non-viable (DAPI+ PI+) C. parvum oocysts was present in the slurry with added 308 ammonia (mean concentration 95.3 ± 4.54 mmol l⁻¹ ammonia) at day 14. This result was unexpected as 309 Jenkins et al. (1998) reported an estimated 8.1 days to reach 99.9% oocyst inactivation in distilled water when exposed to 104 mmol l⁻¹ ammonia at 24 °C. However, manure is a more complex matter than a simple 310 311 aqueous ammonia solution and can differ substantially in composition due to animal species, age and diet 312 of the animals (Fayer, 1994), and therefore also harder to predict the end pH and thereby the concentration of uncharged ammonia (Kohn et al., 2017). Furthermore, stressors such as organic matter in 313 314 slurry may seemingly influence oocyst viability, complicating the attempts to predict survival. The organic 315 matter also seems to act as a protector towards the inactivation effect of ammonia by providing micro-316 environment with lower concentration of uncharged ammonia (Kohn et al., 2017). Ammonia is only toxic in its uncharged form (NH₃), while the ammonium ion (NH₄⁺) is harmless to most microorganisms (Warren, 317 318 1962). The mechanisms of ammonia on the survival of *Cryptosporidium* spp. is still unclear. There is several This article is protected by copyright. All rights reserved

319 explanations for the effect upon cell inactivation. As all cells with active respiration, gasses needs to be 320 transferred over the cell membrane. Ammonia is a small molecule (kinetic diameter 260 pm) comparable to 321 water (265 pm), in comparison to the larger O_2 (346 pm) and is therefore expected to easily get into the 322 cells through existing pores without active transport. Most organisms maintain a stable internal cell pH of 323 about 7.4-7.9, but can survive over a larger external pH range of 5.5-9 (Padan et al., 2005). High ammonia 324 concentrations in the media leads to increase cellular ammonia concentrations as well due to basic 325 chemical forces. To decrease the internal pH, H+ needs to be imported via cation/proton antiporters in 326 exchange for sodium or potassium. This is an energy consuming action that the cell can continue for a set 327 time, giving a lag in the reduction. When reaching an increased cellular ammonia concentration, 328 uncompensated it leads to changes in the intracellular pH and followed by stress such as disturbances of the electrochemical gradient and inhibition of enzymatic reactions (Martinelle and Haggstrom, 1993). The 329 330 faecal material might provide the oocysts some protection from direct exposure to the chemical activity of 331 uncharged NH₃, while oocysts stored in water will be directly exposed to the toxic effect of ammonia. 332 Mucopolysaccharides in faeces may be incorporated into the oocyst wall to provide the oocysts with protection from environmental stress as suggested by Robertson et al. (1992). This hypothesis is supported 333 334 by Kearney et al. (1993), who proposed that high levels of organic matter might be conducive to pathogen 335 adherence, initiating increased survival. Unfortunately, our study did not include oocysts stored in water 336 and added ammonia, preventing us from concluding whether slurry protects oocysts from the inactivating 337 effect of ammonia.

338 Our results is in line with a previous study that found approx. 60% viable Cryptosporidium oocysts at day 94 339 in slurry generated by breeding pigs during summer and stored at ambient temperature without additional 340 ammonia added (Hutchison et al., 2005b). Robertson et al. (1992) observed that at 4 °C, an oocyst fraction 341 in stool samples from individuals with cryptosporidiosis remained viable for at least 178 days, while others 342 found that oocysts remained infective to mice for 10 weeks when stored in faeces at 10 °C (Li et al., 2010; 343 Olson et al., 1999). However, contradictory results have been presented in other studies demonstrating 344 faster oocyst degradation in faeces compared with water (Jenkins et al., 1999; Olson et al., 1999), and 345 similar survival rates for oocysts placed in containers buried in cow faeces or in laboratory-grade water 346 (Robertson et al., 1992). The discrepancies in survival rates between studies might be explained by species 347 and isolate variations in ability of the oocysts to withstand environmental pressures or by differences in the 348 methods used to determine oocysts inactivation.

The proportion of non-variable oocyst were unaffected when stored in buffers at pH 7 and 9, indicating that within the examined pH range, the pH is not important for oocyst inactivation. This is in agreement with results obtained by Jenkins et al. (1998), who found minimal changes in the frequency of DAPI+ PI+ (non-This article is protected by copyright. All rights reserved viable) oocysts at pH between 7 and 11. Jenkins et al. (1998) hypothesize that the inability of pH alone to inactive *Cryptosporidium* oocysts is because the charged components of pH (H⁺ and OH⁻) remain external to the oocyst wall. Nevertheless, other studies have shown that high and low pH can affect oocyst viability significantly (Reinoso et al., 2008; Robertson et al., 1992). A recent study by Senecal *et al.* (2018) looking at the effect of pH on *Ascaris suum* showed that there were actually no effect upon the viability over 180 days at pH up to >12. This indicates that effects reported from inactivation of parasites with elevated pH often have been due to combined effect of pH and supporting molecules such as ammonia.

359 In our study, changes in the proportion of non-viable oocysts over time were unaffected by incubation 360 temperature in both ammonia treated slurry and raw slurry. This lack of any effect of temperature was 361 unexpected because stressors such as temperature are widely reported to be particularly effective in 362 influencing oocyst inactivation (Fayer, 1994; Fayer and Nerad, 1996; Li et al., 2010; Olson et al., 1999; 363 Pokorny et al., 2002). For example, Pokorny et al. (2002) showed decreased C. parvum infectivity in a 364 mouse model when the temperature of river water increased from 4 °C to 23 °C. Additionally, the 365 incubation temperature usually affects the ammonium-ammonia equilibrium, initiating a rise in uncharged 366 NH₃ concentration with increasing temperature (Christensen and Sommer, 2013), as observed in our study 367 with raw slurry. This theoretically enhances the effect of temperature, because increased uncharged NH₃ 368 concentration can escalate oocyst inactivation (Jenkins et al., 1998). However, aq. ammonia added to the 369 slurry in this study was adjusted to achieve the same uncharged NH₃ concentration regardless of 370 temperature, in order to examine whether the effectiveness of ammonia changes with temperatures as 371 reported elsewhere (Jenkins et al., 1998; Vinnerås et al., 2008). No increased effect of ammonia with 372 changing the temperature on oocyst inactivation was observed. This contradicts results obtained by Jenkins 373 et al. (1998), who found higher oocyst inactivation in distilled water at temperatures around 25 °C 374 compared with 4 °C when exposed to the same NH₃ concentration. Fidjeland et al. (2013) reported a similar 375 trend for Ascaris suum egg viability; at equal NH_3 concentration, the time to reach a 3 log reduction in 376 faecal sludge was shorter at higher temperatures. The proposed reason for increased effect of ammonia 377 with increased temperature are related to the increase of the permeability of the outer lipid layers of the 378 cells in combination to increased chemical mobility at increased temperatures (Kohn et al., 2017). For 379 Ascaris spp. a drop in ammonia sensitivity has been noticed when decreasing the temperature from 20 °C 380 to 10 °C, this was assumed to be caused of a change in permeability (Fidjeland et al., 2015), while for 381 *Cryptosporidium* oocysts it appears to be at a higher temperature.

382 **4.2 Bacterial indicators**

383 Addition of aq. ammonia to the cattle slurry in our study significantly affected the numbers of indigenous E. 384 coli and enterococci. In particular, E. coli appeared sensitive to treatment, as evidenced by a reduction 385 below the detection limit of 1 CFU ml⁻¹ within 6 days. In contrast, in raw slurry 12.2 ± 2.6 CFU E. coli ml⁻¹ 386 remained culturable at day 14, with a mean estimated decimal reduction time (T_{90}) of 25.8 days. Similar T_{90} values have been reported for indigenous E. coli in non-aerated cattle slurry at 7 °C and 20 °C (Munch et al., 387 388 1987). Moreover, Kudva, Blanch and Hovde (1998) reported a 3.0–3.5 log reduction over 30 days at 4 °C in 389 an *E. coli* O157:H7 strain spiked to bovine slurry, while (McGee et al., 2001) reported an approximate 3.5– 390 5.5 log reduction of E. coli O157:H7 over 12 weeks at 10 °C in cattle slurry. In contrast, a rapid E. coli 391 O157:H7 decline from 1.2 × 10⁸ CFU ml⁻¹ to undetectable numbers was observed within 9 days when spiked 392 to cattle slurry and stored at 18 °C (Maule, 1997). However, in contrast to the present study, slurry samples 393 were aerated during storage, which seemingly resulted in faster pathogen inactivation (Munch et al., 1987). 394 These data indicate a tendency for longer survival of indigenous bacteria compared with spiked bacterial 395 strains, possibly due to adaptations to the environmental conditions in the slurry. Consequently, results 396 based solely on spiked bacterial strains might underestimate the decimal reduction rate. Additionally the 397 above-mentioned studies do not keep track of mechanisms for inactivation, i.e. pH and uncharged 398 ammonia that heavily affect the inactivation rate of *E.coli*.

399 Addition of aq. ammonia to slurry reduced survival of enterococci less than E. coli. These findings agree 400 with those of earlier studies in which enterococci displayed lower ammonia sensitivity, were more heat-401 resistant, and survived longer than E. coli, Salmonella spp., and several other bacterial pathogens (Bitton, 402 2011; Nordin et al., 2013, 2009; Ottoson et al., 2008b; Vinnerås et al., 2008; Watcharasukarn et al., 2009). 403 Enterococci are Gram-positive bacteria whose cell wall offers more resistance to mesophilic temperature, 404 disinfection, and desiccation compared with Gram-negative bacteria such as E. coli (Bitton, 2011; Ottoson 405 et al., 2008b; WHO, 2004). In a field study of human urine with an ammonia concentration of 4.2 g l^{-1} , 406 stored at 19–31 °C, *E. coli* was reduced more rapidly ($T_{90} = 0.1$ day) than enterococci ($T_{90} = 7.6-7.7$ days) 407 (Nordin et al., 2013). Similarly to E. coli, enterococci added to urine, and sewage- and faecal sludge samples 408 appeared more sensitive to ammonia than indigenous enterococci (Fidjeland et al., 2013a, 2013b; Nordin et 409 al., 2013). In addition, some studies have demonstrated that enterococci exposed to low concentrations of 410 uncharged NH₃ display a biphasic reduction where the length of the lag phase depends on incubation 411 temperature (Fidjeland et al., 2013b, 2013a). An approximate 20-day lag phase for enterococci was 412 observed at 14 °C in bovine manure with 2% urea added, while the linear die-off phase was absent at 4 °C 413 (Ottoson et al., 2008a). In the present study, enterococci lacked a biphasic die-off rate both in raw slurry

and in slurry with added ammonia, probably due to the short duration of the study (14 days). The decimal
reduction rate was estimated based on a linear regression model, with risks overestimating the rate.

416 In general, we did not observe any temperature dependent major reductions of *E. coli* and enterococci over 417 time. However, enterococci in slurry with added ammonia and E. coli in raw slurry incubated at 20 °C 418 required a shorter storage time to achieve a 1 log reduction compared with 4 °C and 10 °C. This was 419 probably attributable to the significant rise in uncharged NH_3 concentration with higher temperature (20) 420 °C) (Christensen and Sommer, 2013) measured in raw slurry and slurry with added ammonia, rather than 421 being solely a temperature effect. Similarly, Fidjeland et al. (2013b) observed corresponding die-off for 422 enterococci treated with 0.5–2.0% urea in sewage sludge at similar temperatures within the first 14 days of 423 their study. As described for Cryptosporidium oocysts, a significant effect of temperature on bacteria die-off 424 rate has been reported (Kudva et al., 1998; Ottoson et al., 2008a).

425 **4.3 Conclusion**

Overall, addition of ammonia to cattle slurry significantly increased the inactivation of pathogenic *C. parvum* oocysts, and reduced the numbers of *E. coli* and enterococci compared with raw, untreated slurry. However, only *E. coli* was reduced to below the detection limit within the study period of 14 days, while 7.2 $\times 10^3$ CFU ml⁻¹ enterococci were still present at day 14 in ammonia treated slurry. Interestingly, the effectiveness of ammonia in inactivating microorganisms was not noted with increasing temperature. To be able to notice this for *E. coli* a higher sampling interval would be required and for the enterococcus and *Cryptosporidium* oocysts a longer sampling period would be required.

433 Our findings indicate that adding ammonia to slurry prior to application to agricultural land could be used 434 to reduce pathogen concentrations in slurry lowering the contamination of water and food sources with 435 the zoonotic Cryptosporidium. However, the ammonia concentration tested where not high enough for all 436 *Cryptosporidium* oocysts and enterococci to be inactivated within the study period to eliminate the public 437 health risk. Hence, further experimental work is warranted to explore the full potential of ammonia 438 treatment and to determine ammonia concentration and exposure time needed to abolish the risk of 439 *Cryptosporidium* transmission from cattle slurry. It should also be investigated whether ammonia treatment 440 of animal slurries increases the risk of ammonia loss to the atmosphere during slurry storage, because this 441 would have a highly undesirable impact on the environment; ammonia emissions from animal production 442 systems are being restricted in many countries through strict environmental regulations (Sommer et al., 2013). Furthermore, also potential impacts of the ammonia treatment on the risk of discharge of 443 444 ammonia/ammonium to waterways through potential runoff needs to be investigated, though the risk will 445 most likely be minimal when soils are not overloaded with slurry in excess of crop nitrogen demand.

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453 **Conflict of interest**

454 No conflict of interest declared.

455 Ethics approval and consent to participate

456 This study did not require official or institutional ethical approval.

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Table 1. Properties of the cattle slurry used in the experiment

Parameter measured	Mean ± S.D.						
рН		8.48 ± 0.08					
Electric conductivity (mS)		13.8 ± 1.7					
Dry matter (% of wet weight)		3.55 ± 0.03					
Total ammoniacal nitrogen:	(TAN, mg l ⁻¹)	2050 ± 256					
	(mmol l ⁻¹)	145 ± 10					
Oocysts I-1		8 ± 1.20 ^a					
<i>E. coli</i> (CFU ml ⁻¹)		68 ± 28					
Enterococci (CFU ml ⁻¹)		$1.6 \times 10^4 \pm 2.5 \times 10^3$					
	Parameter measured pH Electric conductivity (mS) Dry matter (% of wet weight) Total ammoniacal nitrogen: Oocysts I ⁻¹ <i>E. coli</i> (CFU ml ⁻¹) Enterococci (CFU ml ⁻¹)	Parameter measured pH Electric conductivity (mS) Dry matter (% of wet weight) Total ammoniacal nitrogen: (TAN, mg l ⁻¹) (mmol l ⁻¹) Oocysts l ⁻¹ <i>E. coli</i> (CFU ml ⁻¹) Enterococci (CFU ml ⁻¹)					

^aAll oocysts found in the slurry prior to spiking were categorized as DAPI+ PI+.

Tube size and content			Slurry volume			Sampling day for tubes incubated at 4, 10 or 20									
(n)	Treatment (n)	Analysis	used (g)	n	0	2	4	6	8	10	12	14			
50 ml with 10 g slurry	NH ₃ (72)	Oocyst inactivation	1.5	63	÷	х	х	х	х	х	х	x			
(144)		рНь	10	72	х	x	x	x	х	х	х	х			
		NH ₃	5	72	x	x	x	x	х	х	x	x			
	Control (72)	Oocyst inactivation	1.5	63	÷	x	x	x	x	x	x	x			
		рН ^ь	10	72	x	x	x	x	x	x	x	x			
		NH ₃	5	72	x	x	x	x	x	x	x	x			
15 ml with 10 g slurry	NH ₃ (72)	<i>E. coli</i> count	10 ^c	72	х	х	х	х	х	х	х	х			
(144)		Enterococci count	10 ^c	72	х	х	х	х	х	х	х	x			
	Control (72)	<i>E. coli</i> count	10 ^c	72	x	x	х	х	x	x	x	х			
		Enterococci count	10 ^c	72	x	x	x	x	x	x	x	x			

Table 2. Schematic overview of the experimental design. A "x" means that sampling has been done this day, while "÷" indicate no sampling.

	50 ml with 10 ml buffer (18) pH=7 (9)	Oocyst inactivation	10	9	÷	÷	÷	÷	÷	÷	÷	х
	pH=9 (9)	Oocyst inactivation	10	9	÷	÷	÷	÷	÷	÷	÷	х
	^a Three replicate samples each day											
	^b The slurry used for pH measurement was afterwards used for measuring NH ₃ and assessment of oocyst inactivation.											
	c	Serial									d	lilution
-												

FIGURE LEGENDS

Figure 1. Mean with 95% CI of A) pH and B) calculated uncharged NH₃ concentration at three temperatures in cattle slurry with added aqueous (aq.) ammonia and in raw slurry as a function of time. The 95% CI values not indicated are smaller than the symbols. The symbols represents: slurry added aq. ammonia at 4 °C (solid line, black circle); slurry added aq. ammonia at 10 °C (dashed line, white circle); slurry added aq. ammonia at 20 °C (dotted line, grey circle); raw slurry at 4 °C (solid line, black diamond); raw slurry at 20 °C (dotted line, grey diamond).

Figure 2. A) Individual plots (n=9, triplicate samples for each of three temperatures) and B) Box and whiskers plot of percentage of non-viable (DAPI+ PI+) *Cryptosporidium parvum* oocysts spiked to cattle slurry with added aqueous ammonia (\bullet (A), striped (B)) and raw slurry (\circ (A), white (B)) as a function of time, irrespective of incubation temperature.

Figure 3. Mean *E. coli* and enterococci concentrations (CFU ml⁻¹) with Standard error of the mean (SEM) as a function of time in A) cattle slurry with added aqueous (aq.) ammonia and B) raw slurry at the temperatures 4 °C (solid line, black circle), 10 °C (dashed line, white circle) and 20 °C (dotted line, grey circle). SSEM values not indicated are smaller than the symbols.

Figure 4. Correlation between calculated uncharged NH3 concentration in the slurry samples and A) *Escherichia coli*; and B) *enterococci*. \bigcirc : cattle slurry with added aq. ammonia; \bullet : raw slurry. Dashed line: linear regression line with correlation coefficients (*r*) = -0.80 for *C. parvum* oocysts, -0.70 for *E. coli*, and - 0.36 for enterococci.

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Figure 2.

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В

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Figure 3

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A)

B)





A)