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

Running title: Inactivation of parasites and bacteria in slurry

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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 enumerated every second day for two weeks in cattle slurry amended with 60 mmol l^{-1} aq. ammonia and compared with untreated slurry at three temperatures. Regardless of temperature, the proportion of non-viable *C. parvum* oocysts increased significantly faster over time in slurry with added ammonia than raw slurry ($p=0.021$) corresponding to 62.0% higher inactivation ($P=0.001$) at day 14. Additionally, 91.8% fewer *E. coli* and 27.3% fewer enterococci was observed slurry added ammonia at day 14 compared raw slurry.

Conclusion: The addition of aqueous ammonia to raw slurry significantly reduced the viability of *C. parvum* oocysts and numbers of bacterial indicators. Hence, ammonia is usable to lower pathogen concentrations 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

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1. INTRODUCTION

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

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 application of slurry in a wet winter (Pelly et al., 2007). In Walkerton, Canada, *E. coli* O157:H7 and *Campylobacter jejuni* infections resulted in 2,300 cases of illness and seven deaths among individuals who consumed drinking water from a municipal water supply contaminated with pathogens originating from a nearby farm, where cattle manure was applied to agricultural fields and subsequently washed into the water reservoir (Clark et al., 2003; Hrudey et al., 2003). Due to expected increased heavy rain events related to climate changes, slurry-contaminated surface runoff water from fields will probably be a growing source of pathogen contamination in certain parts of the world.

Abu-Ashour and Lee (2000) studied *E. coli* overland transport at field sites with 2% and 6% slopes during surface water runoff events in which grass-covered plots were contaminated by pouring water containing *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, from the initial location (Abu-Ashour and Lee, 2000). Furthermore, oocysts was detected in runoff water from manure containing *C. parvum* oocysts and applied to agricultural land (Thurston-Enriquez et al., 2005). In addition, rain events can cause vertical transport of slurry-borne microorganisms, resulting in shallow groundwater contamination (Forslund et al., 2011; Mawdsley et al., 1996; Petersen et al., 2012). Compounding this issue, *Cryptosporidium* is infectious at low dosages (Okhuysen et al., 1999) and oocysts possess very robust multi-layered structures (Harris and Petry, 1999) resistant to e.g. periods of freezing (Robertson et al., 1992), and commonly utilized water disinfectants such as chlorine (Korich et al., 1990; Shields et al., 2008; Venczel et al., 1997). Therefore, pre-treating slurry can be useful to inactivating oocysts prior to application to agricultural land, thereby preventing contamination of water and food sources. High temperature and exposure to ammonia are some of few stressors that can inactivate oocysts (Jenkins et al.,

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

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

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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.

2. MATERIALS AND METHODS

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 with a combination tester HI 98130 (Th Geyer, Roskilde, Denmark). Dry matter content was determined by oven drying at 105 °C. *Cryptosporidium* oocysts were determined in 2 g of slurry as described in Petersen et al. (2012). Briefly, *Cryptosporidium*-positive faecal samples were suspended in tap water, filtered through gauze, centrifuged and the supernatant discarded. This washing procedure was repeated 2–3 times. Tap water was added to increase the volume to 5–10 ml. The faecal solution was underlayered with a gradient consisting of 1.09/1.05/1.01 Percoll (Amersham Biosciences, Australia), centrifuged at 1,540 x g for 10 min, and oocysts were collected between the 1.09/1.05 Percoll layers. Purified oocysts were washed three times to remove Percoll, and the oocysts were then enumerated by immunofluorescence microscopy as described in 2.3. The concentrations of *E. coli* and enterococci, and total ammoniacal nitrogen (TAN) were 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).

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% NH_3 by weight) (Table 2). These temperatures correspond to the typical variation in ambient temperatures in Denmark.

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

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(Table 2) leaving approx. 30-40 ml headspace in the 50 ml tubes and approx. 5 ml headspace in the 15 ml tubes. The tubes were then stored in incubators at 4, 10, or 20 °C overnight for the slurry to reach the required temperature prior to start of the experiment. Slurry in the 15 ml blue cap centrifugation tubes was used for *E. coli* and enterococci measurements (n=144), while slurry in the 50 ml blue cap centrifugation tubes were spiked with approx. 2.2×10^5 *C. parvum* oocysts (n=144). *Escherichia coli* and enterococci were present in sufficient numbers in the slurry (Table 1) and spiking was unnecessary. Aqueous ammonia was added to 50% of both 15 ml (n=72) and 50 ml (n=72) tubes (Table 2). The tubes were immediately sealed with a lid to avoid vaporization, mixed well by shaking and placed in incubators at 4, 10 or 20 °C together with the remaining (n=72x2) of the tubes which acted as controls and contained raw slurry with no added aq. ammonia (Table 2). Inactivation of *C. parvum* oocysts, reduction of *E. coli* and enterococci, pH and Total 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.

To achieve identical uncharged NH_3 concentrations in slurry samples incubated at different temperatures, the volume of aq. ammonia added to reach a final uncharged NH_3 concentration of approx. 60 mmol l^{-1} was determined for each incubation temperature prior to start of the experiment. The target concentration of 60 mmol l^{-1} NH_3 was chosen since it has previously been demonstrated to inactivate 99.9% of the oocysts within 14 days in distilled water (Jenkins et al., 1998). The specific concentration was reached by incremental addition of aq. ammonia to a predefined volume of slurry and measurement of pH, to create an ammonia standard curve. In brief, 100 ml slurry were placed in a beaker on a magnetic stirrer, 250 µl aq. ammonia were added at once, the slurry was stirred for 1 min, and the pH was measured. The procedure was repeated until 5,000 µL aq. ammonia was added. Uncharged NH_3 along the standard curve was then calculated for each incubation temperature according to the following formula (Vinnerås, 2013):

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

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

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

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

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Based on this, 125 µl (4 °C), 100 µl (10 °C), or 88 µl (20 °C) aq. ammonia, corresponding to uncharged NH_3 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 NH_3 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 NH_3 will be equilibrated in the headspace during incubation.

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

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

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

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

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

201 **2.4. Bacteriological analysis**

202 For the *E. coli* and enterococci analysis, 10-fold dilutions of slurry were prepared in Maximum Recovery
203 Dilution (MRD) (Oxoid, Basingstoke,UK) and surface-spread onto Brilliant *E. coli*/coliform Selective Agar
204 (Oxoid, Basingstoke,UK) and Slanetz & Bartley Agar plates (Oxoid, Basingstoke,UK), respectively. For the *E.*
205 *coli* analysis, the Brilliant *E. coli*/coliform Selective Agar plates were incubated at 37 °C for 24 h and purple
206 colonies were counted. Enterococci were determined as typical red-maroon colonies on Slanetz & Bartley
207 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 l^{-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 NH_3 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 (ln) 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
222 variables were analysed as potential explanatory variables: treatment (raw slurry and slurry with added aq.
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 ln (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

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temperature*time. All mean ± Standard error of the mean (SEM) values are the geometric mean and geometric standard error of the mean. The percentage of non-viable oocysts was calculated based on the best-fitted line shown in Fig. 2.

The statistical analyses were conducted using SAS v. 9.3 [1].

3. RESULTS

3.1. Ammonia and pH

Figures 1A and 1B show the geometric mean and 95% CI of pH and calculated uncharged NH_3 concentration in cattle slurry over time for the various treatments.

The mean ± SEM pH at day 0 independent of temperature was 8.5 ± 0.03 in the raw slurry, while the addition of ammonia to slurry increased the pH to 9.7 ± 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).

The mean ± SEM uncharged NH_3 concentration at day 0 was 3.2 ± 0.2, 6.7 ± 0.6 and 16.5 ± 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.

In the ammonia treated slurry, the uncharged NH_3 concentration was constant over time and almost unaffected by incubation temperature (Fig. 1B). Hence, the desired uncharged NH_3 concentration was achieved throughout the study in slurry with added ammonia, regardless of temperature.

3.2. *Cryptosporidium* oocysts

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).

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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 day 14 (mean concentration 95.3 ± 4.54 mmol l^{-1} ammonia).

The correlations between uncharged NH_3 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).

3.3. Bacterial indicators

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 (P=0.004) in the slurry with added ammonia (Fig. 3A). The enterococci concentrations following incubation at 4 °C and 10 °C were not significantly different, and no differences in reduction was correlated to incubation temperature in raw slurry (Fig. 3B). When the incubation temperature was disregarded, the addition of ammonia to slurry resulted in a significantly faster reduction of enterococci over time (P=0.006) compared with raw slurry, corresponding to 27.3% fewer enterococci in slurry added ammonia than in raw slurry at day 14. Based on the standard linear regression model, the estimated mean time for a 1 log reduction (T_{90}) ranged from 33.9 to 77.8 days in the slurry with added ammonia, compared with 66.6–175.3 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 (T_{90}) for *E. coli* when disregarding

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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.

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

4. DISCUSSION

The reached ammonia levels of the material was 71.6 ± 5.1, 88.0 ± 2.3 and 121.0 ± 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 the temperature changing the effect on pH by the added ammonia.

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 non-viable oocysts (DAPI+ PI+) compared with untreated raw slurry.

This loss of oocyst viability due to increased ammonia concentration in the slurry agrees with results from other studies (Fayer et al., 1996; Jenkins et al., 1998; Reinoso et al., 2008), and confirms the vulnerability of the otherwise very robust *Cryptosporidium* oocysts to ammonia. For example, ammonia concentration and 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} NH_3 and 10.2 days at 60 mmol l^{-1} NH_3 in distilled water (Jenkins et al., 1998). However, in our study, an estimated proportion of 40.4% non-viable (DAPI+ PI+) *C. parvum* oocysts was present in the slurry with added ammonia (mean concentration 95.3 ± 4.54 mmol l^{-1} ammonia) at day 14. This result was unexpected as 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 aqueous ammonia solution and can differ substantially in composition due to animal species, age and diet 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 slurry may seemingly influence oocyst viability, complicating the attempts to predict survival. The organic matter also seems to act as a protector towards the inactivation effect of ammonia by providing micro-environment with lower concentration of uncharged ammonia (Kohn et al., 2017). Ammonia is only toxic in its uncharged form (NH_3), while the ammonium ion (NH_4^+) is harmless to most microorganisms (Warren, 1962). The mechanisms of ammonia on the survival of *Cryptosporidium* spp. is still unclear. There is several

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explanations for the effect upon cell inactivation. As all cells with active respiration, gasses needs to be transferred over the cell membrane. Ammonia is a small molecule (kinetic diameter 260 pm) comparable to water (265 pm), in comparison to the larger O_2 (346 pm) and is therefore expected to easily get into the cells through existing pores without active transport. Most organisms maintain a stable internal cell pH of about 7.4-7.9, but can survive over a larger external pH range of 5.5-9 (Padan et al., 2005). High ammonia concentrations in the media leads to increase cellular ammonia concentrations as well due to basic chemical forces. To decrease the internal pH, H+ needs to be imported via cation/proton antiporters in exchange for sodium or potassium. This is an energy consuming action that the cell can continue for a set time, giving a lag in the reduction. When reaching an increased cellular ammonia concentration, 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 faecal material might provide the oocysts some protection from direct exposure to the chemical activity of uncharged NH_3, while oocysts stored in water will be directly exposed to the toxic effect of ammonia. 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 by Kearney et al. (1993), who proposed that high levels of organic matter might be conducive to pathogen adherence, initiating increased survival. Unfortunately, our study did not include oocysts stored in water and added ammonia, preventing us from concluding whether slurry protects oocysts from the inactivating effect of ammonia.

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

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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.

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

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4.2 Bacterial indicators

Addition of aq. ammonia to the cattle slurry in our study significantly affected the numbers of indigenous *E. coli* and enterococci. In particular, *E. coli* appeared sensitive to treatment, as evidenced by a reduction 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} 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., 1987). Moreover, Kudva, Blanch and Hovde (1998) reported a 3.0–3.5 log reduction over 30 days at 4 °C in an *E. coli* O157:H7 strain spiked to bovine slurry, while (McGee et al., 2001) reported an approximate 3.5–5.5 log reduction of *E. coli* O157:H7 over 12 weeks at 10 °C in cattle slurry. In contrast, a rapid *E. coli* O157:H7 decline from 1.2×10^8 CFU ml^{-1} to undetectable numbers was observed within 9 days when spiked to cattle slurry and stored at 18 °C (Maule, 1997). However, in contrast to the present study, slurry samples were aerated during storage, which seemingly resulted in faster pathogen inactivation (Munch et al., 1987). These data indicate a tendency for longer survival of indigenous bacteria compared with spiked bacterial strains, possibly due to adaptations to the environmental conditions in the slurry. Consequently, results based solely on spiked bacterial strains might underestimate the decimal reduction rate. Additionally the above-mentioned studies do not keep track of mechanisms for inactivation, i.e. pH and uncharged ammonia that heavily affect the inactivation rate of *E.coli*.

Addition of aq. ammonia to slurry reduced survival of enterococci less than *E. coli*. These findings agree with those of earlier studies in which enterococci displayed lower ammonia sensitivity, were more heat-resistant, and survived longer than *E. coli*, *Salmonella* spp., and several other bacterial pathogens (Bitton, 2011; Nordin et al., 2013, 2009; Ottoson et al., 2008b; Vinnerås et al., 2008; Watcharasukarn et al., 2009). Enterococci are Gram-positive bacteria whose cell wall offers more resistance to mesophilic temperature, disinfection, and desiccation compared with Gram-negative bacteria such as *E. coli* (Bitton, 2011; Ottoson et al., 2008b; WHO, 2004). In a field study of human urine with an ammonia concentration of 4.2 g l^{-1}, 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) (Nordin et al., 2013). Similarly to *E. coli*, enterococci added to urine, and sewage- and faecal sludge samples appeared more sensitive to ammonia than indigenous enterococci (Fidjeland et al., 2013a, 2013b; Nordin et al., 2013). In addition, some studies have demonstrated that enterococci exposed to low concentrations of uncharged NH_3 display a biphasic reduction where the length of the lag phase depends on incubation temperature (Fidjeland et al., 2013b, 2013a). An approximate 20-day lag phase for enterococci was observed at 14 °C in bovine manure with 2% urea added, while the linear die-off phase was absent at 4 °C (Ottoson et al., 2008a). In the present study, enterococci lacked a biphasic die-off rate both in raw slurry

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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.

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

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 × 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.

Our findings indicate that adding ammonia to slurry prior to application to agricultural land could be used to reduce pathogen concentrations in slurry lowering the contamination of water and food sources with the zoonotic *Cryptosporidium*. However, the ammonia concentration tested where not high enough for all *Cryptosporidium* oocysts and enterococci to be inactivated within the study period to eliminate the public health risk. Hence, further experimental work is warranted to explore the full potential of ammonia treatment and to determine ammonia concentration and exposure time needed to abolish the risk of *Cryptosporidium* transmission from cattle slurry. It should also be investigated whether ammonia treatment of animal slurries increases the risk of ammonia loss to the atmosphere during slurry storage, because this would have a highly undesirable impact on the environment; ammonia emissions from animal production 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 ammonia/ammonium to waterways through potential runoff needs to be investigated, though the risk will most likely be minimal when soils are not overloaded with slurry in excess of crop nitrogen demand.

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Conflict of interest

No conflict of interest declared.

Ethics approval and consent to participate

This study did not require official or institutional ethical approval.

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References

Abu-Ashour, J., Lee, H., 2000. Transport of bacteria on sloping soil surfaces by runoff. Environ. Toxicol. 15, 149–153. https://doi.org/10.1002/(SICI)1522-7278(2000)15:2<149::AID-TOX11>3.0.CO;2-O

Bitton, G., 2011. Wastewater Microbiology. Wiley-Blackwell, Hoboken, NJ, USA.

Bodley-Tickell, A.T., Kitchen, S.E., Sturdee, A.P., 2002. Occurrence of *Crypstosporidium* in agricultural surface waters during an annual farming cycle in lowland UK. Water Res. 36, 1880–1886. https://doi.org/10.1016/S0043-1354(01)00398-0

Christensen, M.L., Sommer, S.G., 2013. Manure Characterisation and Inorganic Chemistry, in: Sommer, S.G., Christensen, M.L., Schmidt, T., Jensen, L.S. (Eds.), Animal Manure Recycling - Treatment and Management. John Wiley & Sons Ltd, Ltd, Chichester, UK, pp. 41–65.

Clark, C.G., Price, L., Ahmed, R., Woodward, D.L., Melito, P.L., Rodgers, F.G., Jamieson, F., Ciebin, B., Li, A., Ellis, A., 2003. Characterization of waterborne outbreak–associated *Campylobacter jejuni* , Walkerton, Ontario. Emerg. Infect. Dis. 9, 1232–1241. https://doi.org/10.3201/eid0910.020584

Fayer, R., 1994. Effect of high temperature on infectivity of *Cryptosporidium parvum* oocysts in water. Appl. Environ. Microbiol. 60, 2732–2735. https://doi.org/10.1128/AEM.60.8.2732-2735.1994

Fayer, R., Graczyk, T.K., Cranfield, M.R., Trout, J.M., 1996. Gaseous disinfection of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 62, 3908–3909. https://doi.org/10.1128/AEM.62.10.3908-3909.1996

Fayer, R., Nerad, T., 1996. Effects of low temperatures on viability of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 62, 1431–1433. https://doi.org/10.1128/AEM.62.4.1431-1433.1996

Fidjeland, J., Lalander, C., Jonsson, H., Vinnerås, B., 2013a. Ammonia sanitisation of sewage sludge using urea. Water Sci. Technol. 68, 1866–1872. https://doi.org/10.2166/wst.2013.443

Fidjeland, J., Magri, M.E., Jönsson, H., Albihn, A., Vinnerås, B., 2013b. The potential for self-sanitisation of faecal sludge by intrinsic ammonia. Water Res. 47, 6014–6023. https://doi.org/10.1016/j.watres.2013.07.024

Fidjeland, J., Nordin, A., Pecson, B.M., Nelson, K.L., Vinnerås, B., 2015. Modeling the inactivation of *Ascaris* eggs as a function of ammonia concentration and temperature. Water Res. 83, 153–160. https://doi.org/10.1016/j.watres.2015.06.030

Forslund, A., Markussen, B., Toenner-Klank, L., Bech, T.B., Jacobsen, O.S., Dalsgaard, A., 2011. Leaching of *Cryptosporidium parvum* oocysts, *Escherichia coli*, and a *Salmonella enterica* serovar Typhimurium

This article is protected by copyright. All rights reserved

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bacteriophage through intact soil cores following surface application and injection of slurry. Appl. Environ. Microbiol. 77, 8129–8138. https://doi.org/10.1128/AEM.05675-11

Harris, J.R., Petry, F., 1999. *Cryptosporidium parvum*: Structural components of the oocyst wall. J. Parasitol. 85, 839. https://doi.org/10.2307/3285819

Henriksen, S.A., Pohlenz, J.F.L., 1981. Staining of Cryptosporidia by a modified Ziehl-Neelsen technique. Acta Vet. Scand. 22, 594–596.

Himathongkham, S., Riemann, H., 1999. Destruction of *Salmonella typhimurium*, *Escherichia coli* O157:H7 and Listeria monocytogenes in chicken manure by drying and/or gassing with ammonia. FEMS Microbiol. Lett. 171, 179–182. https://doi.org/10.1111/j.1574-6968.1999.tb13430.x

Hrudey, S.E., Payment, P., Huck, P.M., Gillham, R.W., Hrudey, E.J., 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. Water Sci. Technol. 47, 7–14. https://doi.org/10.2166/wst.2003.0146

Hutchison, M.L., Walters, L.D., Avery, S.M., Munro, F., Moore, A., 2005a. Analyses of livestock production, waste storage, and pathogen levels and prevalences in farm manures. Appl. Environ. Microbiol. 71, 1231–1236. https://doi.org/10.1128/AEM.71.3.1231-1236.2005

Hutchison, M.L., Walters, L.D., Moore, A., Avery, S.M., 2005b. Declines of zoonotic agents in liquid livestock wastes stored in batches on-farm. J. Appl. Microbiol. 99, 58–65. https://doi.org/10.1111/j.1365-2672.2005.02585.x

Islam, M., Morgan, J., Doyle, M.P., Phatak, S.C., Millner, P., Jiang, X., 2004. Fate of *Salmonella enterica* Serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. Appl. Environ. Microbiol. 70, 2497–2502. https://doi.org/10.1128/AEM.70.4.2497-2502.2004

Jenkins, M.B., Bowman, D.D., Ghiorse, W.C., 1998. Inactivation of *Cryptosporidium parvum o*ocysts by ammonia. Appl. Environ. Microbiol. 64, 784–788. https://doi.org/10.1128/AEM.64.2.784-788.1998

Jenkins, M.B., Walker, M.J., Bowman, D.D., Anthony, L.C., Ghiorse, W.C., 1999. Use of a sentinel system for field measurements of *Cryptosporidium parvum* oocyst inactivation in soil and animal waste. Appl. Environ. Microbiol. 65, 1998–2005. https://doi.org/10.1128/AEM.65.5.1998-2005.1999

Kearney, T.E., Larkin, M.J., Levett, P.N., 1993. The effect of slurry storage and anaerobic digestion on survival of pathogenic bacteria. J. Appl. Bacteriol. 74, 86–93. https://doi.org/10.1111/j.1365-2672.1993.tb03000.x

This article is protected by copyright. All rights reserved

517 Kohn, T., Decrey, L., Vinnerås, B., 2017. Chemical Disinfectants, in: Rose, J.B., Jiménez-Cisneros, B., Haas, C.
518 (Eds.), Global Water Pathogen Project. Global Water Pathogens Projekct.

519 Korich, D.G., Mead, J.R., Madore, M.S., Sinclair, N.A., Sterling, C.R., 1990. Effects of ozone, chlorine dioxide,
520 chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol.
521 56, 1423–1428. https://doi.org/10.1128/AEM.56.5.1423-1428.1990

522 Kudva, I.T., Blanch, K., Hovde, C.J., 1998. Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine
523 manure and manure slurry. Appl. Environ. Microbiol. 64, 3166–3174.
524 https://doi.org/10.1128/AEM.64.9.3166-3174.1998

525 Langkjær, R.B., Vigre, H., Enemark, H.L., Maddox-Hyttel, C., 2007. Molecular and phylogenetic
526 characterization of *Cryptosporidium* and *Giardia* from pigs and cattle in Denmark. Parasitology 134,
527 339. https://doi.org/10.1017/S0031182006001533

528 Li, X., Atwill, E.R., Dunbar, L.A., Tate, K.W., 2010. Effect of daily temperature fluctuation during the cool
529 season on the infectivity of *Cryptosporidium parvum*. Appl. Environ. Microbiol. 76, 989–993.
530 https://doi.org/10.1128/AEM.02103-09

531 Martinelle, K., Haggstrom, L., 1993. Mechanisms of ammonia and ammonium ion toxicity in animal-cells -
532 transport across cell-membranes. J. Biotechnol. 30, 339–350. https://doi.org/10.1016/0168-
533 1656(93)90148-G

534 Maule, A., 1997. Survival of the verotoxigenic strain E. coli O157:H7 in laboratory-scale microcosms, in: Kay,
535 D., Fricker, C.R. (Eds.), Coliforms and E. Coli: Problem or Solution? Athenaeum Press Ltd, Gateshead,
536 UK, pp. 61–65.

537 Mawdsley, J.L., Brooks, A.E., Merry, R.J., 1996. Movement of the protozoan pathogen *Cryptosporidium*
538 *parvum* through three contrasting soil types. Biol. Fertil. Soils 21, 30–36.
539 https://doi.org/10.1007/BF00335990

540 McGee, P., Bolton, D.J., Sheridan, J.J., Earley, B., Leonard, N., 2001. The survival of *Escherichia coli* O157:H7
541 in slurry from cattle fed different diets. Lett. Appl. Microbiol. 32, 152–155.
542 https://doi.org/10.1046/j.1472-765x.2001.00877.x

543 Mendez, J.M., Jimenez, B., Maya, C., 2004. Disinfection kinetics of pathogens in physicochemical sludge
544 treated with ammonia. Water Sci. Technol. 50, 67–74. https://doi.org/10.2166/wst.2004.0536

545 Munch, B., Larsen, H.E., Aalbaek, B., 1987. Experimental studies on the survival of pathogenic and indicator
546 bacteria in aerated and non-aerated cattle and pig slurry. Biol. Wastes 22, 49–65.

This article is protected by copyright. All rights reserved

547 Nordin, A., 2010. Ammonia sanitation of human excreta – treatment technology for production of fertilizer.
548 Doctoral thesis. Swedish Univ. Agric. Sci. Uppsala, Sweden.

549 Nordin, A., Niwagaba, C., Jönsson, H., Vinnerås, B., 2013. Pathogen and indicator inactivation in source-
550 separated human urine heated by the sun. J. Water Sanit. Hyg. Dev. 3, 181–188.
551 https://doi.org/10.2166/washdev.2013.174

552 Nordin, A., Nyberg, K., Vinnerås, B., 2009. Inactivation of *Ascaris* eggs in source-separated urine and feces
553 by ammonia at ambient temperatures. Appl. Environ. Microbiol. 75, 662–667.
554 https://doi.org/10.1128/AEM.01250-08

555 Okhuysen, P.C., Chappell, C.L., Crabb, J.H., Sterling, C.R., DuPont, H.L., 1999. Virulence of three distinct
556 *Cryptosporidium parvum* isolates for healthy adults. J. Infect. Dis. 180, 1275–1281.
557 https://doi.org/10.1086/315033

558 Olson, M.E., Goh, J., Phillips, M., Guselle, N., McAllister, T.A., 1999. *Giardia* cyst and *Cryptosporidium* oocyst
559 survival in water, soil, and cattle feces. J. Environ. Qual. 28, 1991.
560 https://doi.org/10.2134/jeq1999.00472425002800060040x

561 Ong, C., Moorehead, W., Ross, A., Isaac-Renton, J., 1996. Studies of *Giardia* spp. and *Cryptosporidium* spp.
562 in two adjacent watersheds. Appl. Environ. Microbiol. 62, 2798–2805.
563 https://doi.org/10.1128/AEM.62.8.2798-2805.1996

564 Ottoson, J., Nordin, A., von Rosen, D., Vinnerås, B., 2008a. *Salmonella* reduction in manure by the addition
565 of urea and ammonia. Bioresour. Technol. 99, 1610–1615.
566 https://doi.org/10.1016/j.biortech.2007.04.009

567 Ottoson, J., Schnürer, A., Vinnerås, B., 2008b. In situ ammonia production as a sanitation agent during
568 anaerobic digestion at mesophilic temperature. Lett. Appl. Microbiol. 46, 325–330.
569 https://doi.org/10.1111/j.1472-765X.2007.02317.x

570 Padan, E., Bibi, E., Ito, M., Krulwich, T.A., 2005. Alkaline pH homeostasis in bacteria: New insights. Biochim.
571 Biophys. Acta - Biomembr. 1717, 67–88. https://doi.org/10.1016/j.bbamem.2005.09.010

572 Pelly, H., Cormican, M., O'Donovan, D., Chalmers, R., Hanahoe, B., Cloughley, R., McKeown, P., Corbett-
573 Feeney, G., 2007. A large outbreak of cryptosporidiosis in western Ireland linked to public water
574 supply: a preliminary report. Wkly. releases 12, 3187. https://doi.org/10.2807/esw.12.18.03187-en

575 Petersen, H.H., Enemark, H.L., Olsen, A., Amin, M.G., Dalsgaard, A., 2012. Transport of *Cryptosporidium*
576 *parvum* oocysts in soil columns following applications of raw and separated liquid slurries. Appl.

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577 Environ. Microbiol. 78, 5994–6000. https://doi.org/10.1128/AEM.07829-11

578 Pokorny, N.J., Weir, S.C., Carreno, R.A., Trevors, J.T., Lee, H., 2002. Influence of temperature on
579 *Cryptosporidium parvum* oocyst infectivity in river water samples as detected by tissue culture assay.
580 J. Parasitol. 88, 641. https://doi.org/10.2307/3285469

581 Reinoso, R., Becares, E., Smith, H. V, 2008. Effect of various environmental factors on the viability of
582 *Cryptosporidium parvum* oocysts. J. Appl. Microbiol. 104, 980–986. https://doi.org/10.1111/j.1365-
583 2672.2007.03620.x

584 Robertson, L.J., Campbell, A.T., Smith, H. V, 1992. Survival of *Cryptosporidium parvum* oocysts under
585 various environmental pressures. Appl. Environ. Microbiol. 58, 3494–3500.
586 https://doi.org/10.1128/AEM.58.11.3494-3500.1992

587 Senecal, J., Nordin, A., Simha, P., Vinnerås, B., 2018. Hygiene aspect of treating human urine by alkaline
588 dehydration. Water Res. 144, 474–481. https://doi.org/10.1016/j.watres.2018.07.030

589 Senecal, J., Nordin, A., Vinnerås, B., 2020. Fate of *Ascaris* at various pH, temperature and moisture levels. J.
590 Water Health 18, 375–382. https://doi.org/10.2166/wh.2020.264

591 Shields, J.M., Hill, V.R., Arrowood, M.J., Beach, M.J., 2008. Inactivation of *Cryptosporidium parvum* under
592 chlorinated recreational water conditions. J. Water Health 6, 513–520.
593 https://doi.org/10.2166/wh.2008.068

594 Sommer, S.G., Christensen, M.L., Schmidt, T., Jensen, L.S., 2013. Animal Manure Recycling – Treatment and
595 Management. John Wiley & Sons Ltd, Chichester, UK.

596 Thurston-Enriquez, J.A., Gilley, J.E., Eghball, B., 2005. Microbial quality of runoff following land application
597 of cattle manure and swine slurry. J. Water Health 3, 157–171. https://doi.org/10.2166/wh.2005.0015

598 Venczel, L. V, Arrowood, M., Hurd, M., Sobsey, M.D., 1997. Inactivation of *Cryptosporidium parvum* oocysts
599 and *Clostridium perfringens* spores by a mixed-oxidant disinfectant and by free chlorine. Appl.
600 Environ. Microbiol. 63, 4625.

601 Vinnerås, B., 2013. Sanitation and Hygiene in Manure Management, in: Sommer ML Schmidt, T Jensen,LS,
602 S.G.C. (Ed.), Animal Manure Recycling - Treatment and Management. John Wiley & Sons Ltd,
603 Chichester, UK, p. 104.

604 Vinnerås, B., Nordin, A., Niwagaba, C., Nyberg, K., 2008. Inactivation of bacteria and viruses in human urine
605 depending on temperature and dilution rate. Water Res. 42, 4067–4074.
606 https://doi.org/10.1016/j.watres.2008.06.014

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607 Warren, K.S., 1962. Ammonia toxicity and pH. Nature 195, 47–49. https://doi.org/10.1038/195047a0

608 Watcharasukarn, M., Kaparaju, P., Steyer, J.-P., Krogfelt, K.A., Angelidaki, I., 2009. Screening *Escherichia coli*,
609 *Enterococcus faecalis*, and *Clostridium perfringens* as indicator organisms in evaluating pathogen-
610 reducing capacity in biogas plants. Microb. Ecol. 58, 221–230. https://doi.org/10.1007/s00248-009-
611 9497-9

612 WHO, 2004. Water sanitation and hygiene links to health. Facts and figures, World Health Organization,
613 Geneva, Switzerland.

614

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

Parameter measured		**Mean ± S.D.**
pH		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 l^{-1}		8 ± 1.20[a]
E. coli (CFU ml^{-1})		68 ± 28
Enterococci (CFU ml^{-1})		$1.6\times10^4 \pm 2.5\times10^3$

[a]All oocysts found in the slurry prior to spiking were categorized as DAPI+ PI+.

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

Tube size and content (n)	**Treatment (n)**	**Analysis**	**Slurry volume used (g)**	**n**	**Sampling day for tubes incubated at 4, 10 or 20°C[a]**							
					0	**2**	**4**	**6**	**8**	**10**	**12**	**14**
50 ml with 10 g slurry	NH_3 (72)	Oocyst inactivation	1.5	63	÷	x	x	x	x	x	x	x
(144)		pH [b]	10	72	x	x	x	x	x	x	x	x
		NH_3	5	72	x	x	x	x	x	x	x	x
	Control (72)	Oocyst inactivation	1.5	63	÷	x	x	x	x	x	x	x
		pH [b]	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)	*E. coli* count	10[c]	72	x	x	x	x	x	x	x	x
(144)		Enterococci count	10[c]	72	x	x	x	x	x	x	x	x
	Control (72)	*E. coli* count	10[c]	72	x	x	x	x	x	x	x	x
		Enterococci count	10[c]	72	x	x	x	x	x	x	x	x

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50 ml with 10 ml buffer (18) pH=7 (9)	Oocyst inactivation	10	9	÷	÷	÷	÷	÷	÷	÷	x
pH=9 (9)	Oocyst inactivation	10	9	÷	÷	÷	÷	÷	÷	÷	x

[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 dilution

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FIGURE LEGENDS

Figure 1. Mean with 95% CI of A) pH and B) calculated uncharged NH_3 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 10 °C (dashed line, white 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 (● (A), striped (B)) and raw slurry (○ (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) e*nterococci*. ○: cattle slurry with added aq. ammonia; ●: 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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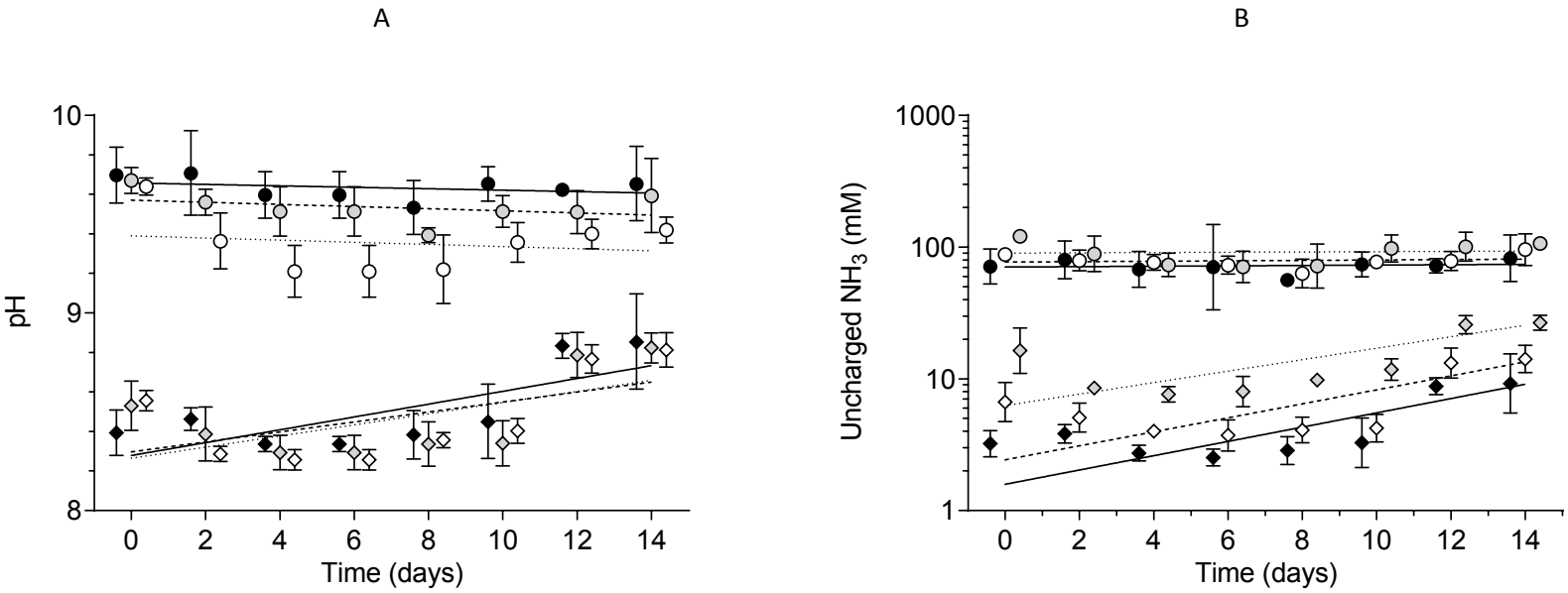

Figure 1.

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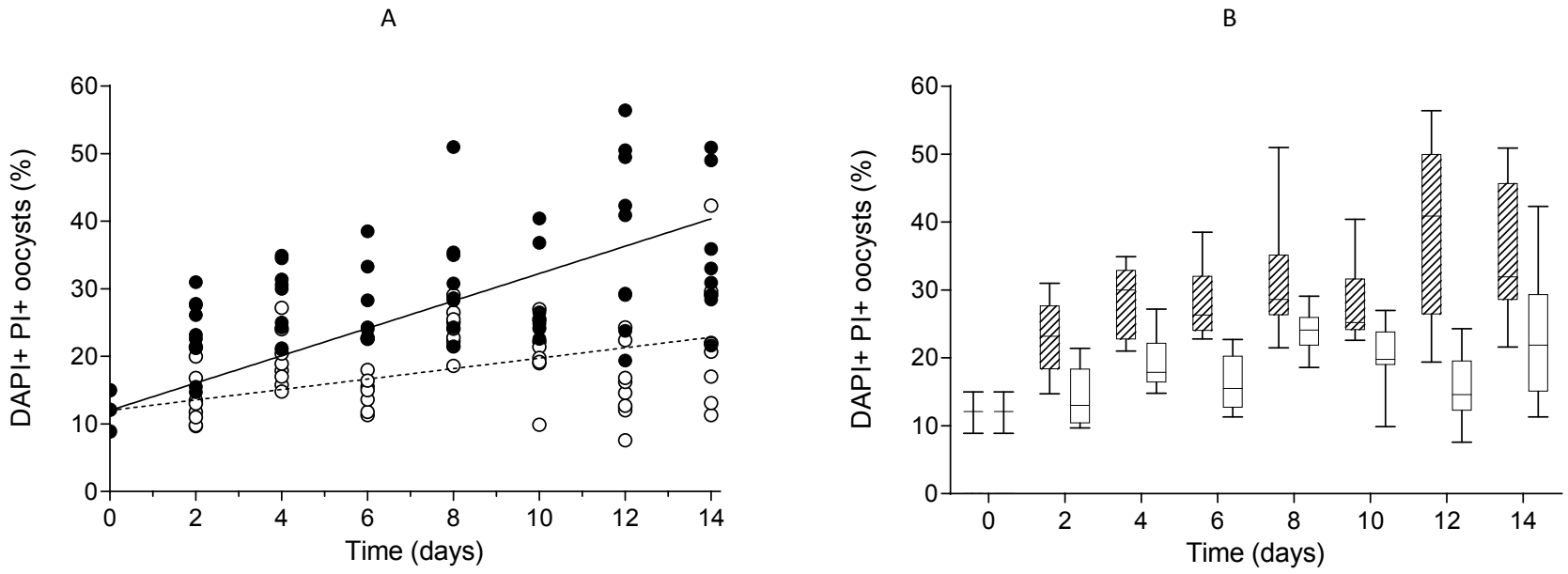

Figure 2.

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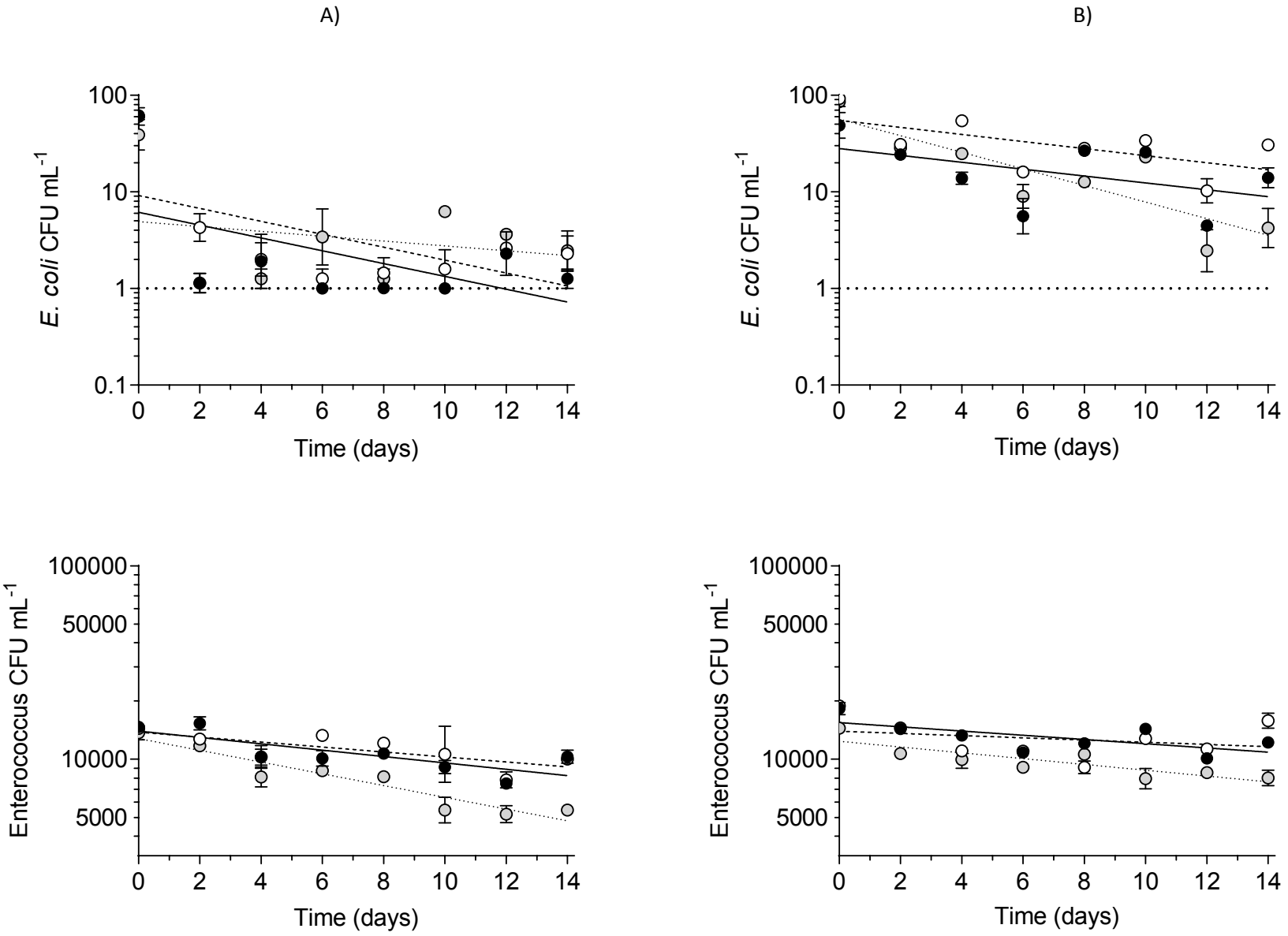

Figure 3

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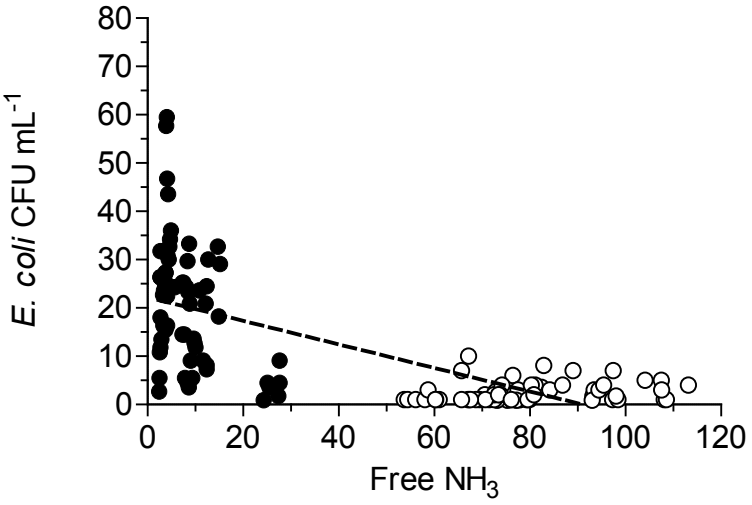
A)



B)

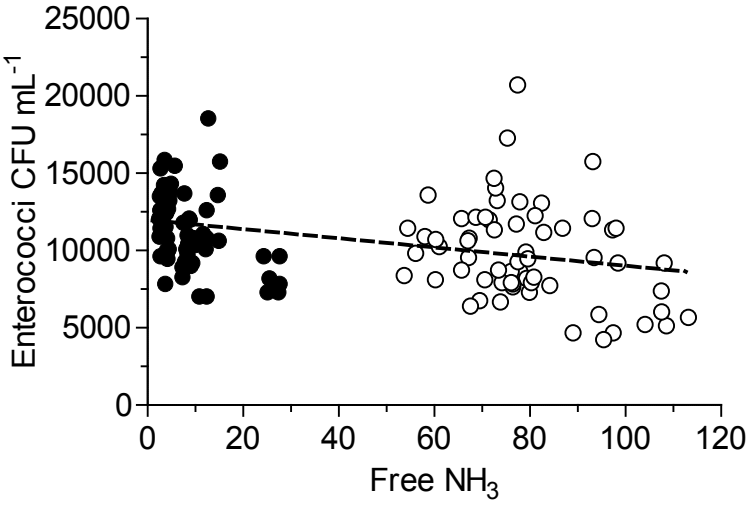


Figure 4

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