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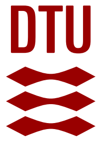

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Inactivation of Cryptosporidium parvum oocysts and faecal indicator bacteria in cattle slurry by addition of ammonia

Petersen, H.H.; Dalsgaard, A.; Vinneras, B.; Jensen, L.S.; Le, T.T.A.; Petersen, M.A.; Enemark, H.L.; Forslund, A.

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1

2 MRS. HEIDI HUUS PETERSEN (Orcid ID : 0000-0002-7208-7614)

3 PROF. LARS STOUMANN JENSEN (Orcid ID : 0000-0002-1446-2084)

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12 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

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

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24 *Corresponding author. Tel.: +45 40 83 69 35;
25 E-mail address: hhpet@vet.dtu.dk

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26 **ABSTRACT**

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

29 **Methods and Results:** *Escherichia coli*, enterococci and non-viable *C. parvum* oocysts (DAPI+ PI+) were
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.

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

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

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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
54 from a lake, which was apparently contaminated by water runoff from agricultural land following
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
65 rainfall event and 15 and 16 CFU *E. coli* mL^{-1} were isolated in runoff water 20 m and 30 m, respectively,
66 from the initial location (Abu-Ashour and Lee, 2000). Furthermore, oocysts was detected in runoff water
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.,

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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
86 to ammonia have showed that oocysts were increasingly inactivated with increasing ammonia
87 concentration (Jenkins et al., 1998; Reinoso et al., 2008), e.g., 99.9% oocysts were inactivated after an
88 estimated 10.2 days when exposed to 60 mmol l^{-1} ammonia, compared with 17.8 days when exposed to 26
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})
96 equivalent to 1-log reduction of *Salmonella* inoculated in bovine manure added 0.5% aqueous (aq.)
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.

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109 The objective of the present study was to determine if *C. parvum* oocysts could be inactivated and *E. coli*
110 and enterococci reduced below the detection limit in cattle slurry prior to land application by adding 60
111 mmol l^{-1} aq. ammonia, and storing it at temperatures corresponding to the typical variation in ambient
112 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
115 the slurry are described in Table 1. The pH and electrical conductivity were measured directly in the slurry
116 with a combination tester HI 98130 (Th Geyer, Roskilde, Denmark). Dry matter content was determined by
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.

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

132 2.1. Experimental design

133 Every second day throughout 14 days, inactivation of *C. parvum* oocysts and reduction of *E. coli* and
134 enterococci in cattle slurry was determined at various temperatures (4 °C, 10 °C and 20 °C) following the
135 addition of concentrated aq. ammonia (25% NH_3 by weight) (Table 2). These temperatures correspond to
136 the typical variation in ambient temperatures in Denmark.

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

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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. 2.2×10^5 *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).

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

153 To achieve identical uncharged NH_3 concentrations in slurry samples incubated at different temperatures,
154 the volume of aq. ammonia added to reach a final uncharged NH_3 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 l^{-1} NH_3 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 µl 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 NH_3 along the standard curve was then
162 calculated for each incubation temperature according to the following formula (Vinnerås, 2013):

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

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:

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

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.

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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 ^{o}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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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**

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

238 The mean ± SEM pH at day 0 independent of temperature was 8.5 ± 0.03 in the raw slurry, while the
239 addition of ammonia to slurry increased the pH to 9.7 ± 0.01. The pH in raw slurry increased significantly
240 over time ($p<0.0001$), while there was no change in pH in slurry with added ammonia. The changes in pH
241 over time were unaffected by incubation temperature in raw slurry as well as slurry with added ammonia
242 (Fig. 1A).

243 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,
244 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
245 treated slurry, corresponding to 68.4–104.5 higher mmol l^{-1} in ammonia treated slurry than in raw slurry at
246 day 0.

247 In raw slurry, the uncharged NH_3 concentration increased significantly over time ($p<0.0001$), but the
248 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.

254 Both in slurry with added ammonia and in raw slurry, the proportion of non-viable oocysts significantly
255 ($P<0.0001$; $P=0.0003$) increased over time (Fig. 2). However, incubation temperatures did not affect the
256 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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288 the temperature in raw slurry and slurry with added ammonia was 25.8 days and 9.8 days, respectively,
289 with less reduction in the raw slurry.

290 A negative correlation was observed between the uncharged NH_3 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**

294 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
295 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**

298 We showed that the addition of ammonia (approx. 60 mmol l^{-1}) to cattle slurry significantly affected the
299 inactivation of *C. parvum* oocysts within 14 days of exposure resulting in 62.0% higher inactivation of non-
300 viable 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)
305 and distilled water with an estimated 17.8 days to reach 99.9% inactivation at 30 mmol l^{-1} NH_3 and 10.2
306 days at 60 mmol l^{-1} NH_3 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
310 when exposed to 104 mmol l^{-1} ammonia at 24 °C. However, manure is a more complex matter than a simple
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
313 concentration of uncharged ammonia (Kohn et al., 2017). Furthermore, stressors such as organic matter in
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
317 its uncharged form (NH_3), while the ammonium ion (NH_4^+) is harmless to most microorganisms (Warren,
318 1962). The mechanisms of ammonia on the survival of *Cryptosporidium* spp. is still unclear. There is several

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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
329 the electrochemical gradient and inhibition of enzymatic reactions (Martinelle and Haggstrom, 1993). The
330 faecal material might provide the oocysts some protection from direct exposure to the chemical activity of
331 uncharged NH_3, 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
333 protection from environmental stress as suggested by Robertson et al. (1992). This hypothesis is supported
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.

349 The proportion of non-variable oocyst were unaffected when stored in buffers at pH 7 and 9, indicating that
350 within the examined pH range, the pH is not important for oocyst inactivation. This is in agreement with
351 results obtained by Jenkins et al. (1998), who found minimal changes in the frequency of DAPI+ PI+ (non-

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352 viable) oocysts at pH between 7 and 11. Jenkins et al. (1998) hypothesize that the inability of pH alone to
353 inactive *Cryptosporidium* oocysts is because the charged components of pH (H^+ and OH^-) remain external to
354 the oocyst wall. Nevertheless, other studies have shown that high and low pH can affect oocyst viability
355 significantly (Reinoso et al., 2008; Robertson et al., 1992). A recent study by Senecal *et al.* (2018) looking at
356 the effect of pH on *Ascaris suum* showed that there were actually no effect upon the viability over 180 days
357 at pH up to >12. This indicates that effects reported from inactivation of parasites with elevated pH often
358 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 NH_3 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 NH_3
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 NH_3 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 NH_3 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.

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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}
387 values have been reported for indigenous *E. coli* in non-aerated cattle slurry at 7 °C and 20 °C (Munch et al.,
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 1.2×10^8 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 NH_3 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

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414 and in slurry with added ammonia, probably due to the short duration of the study (14 days). The decimal
415 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**

426 Overall, addition of ammonia to cattle slurry significantly increased the inactivation of pathogenic *C.*
427 *parvum* oocysts, and reduced the numbers of *E. coli* and enterococci compared with raw, untreated slurry.
428 However, only *E. coli* was reduced to below the detection limit within the study period of 14 days, while 7.2
429 $\times 10^3$ CFU ml^{-1} enterococci were still present at day 14 in ammonia treated slurry. Interestingly, the
430 effectiveness of ammonia in inactivating microorganisms was not noted with increasing temperature. To be
431 able to notice this for *E.coli* a higher sampling interval would be required and for the enterococcus and
432 *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.,
443 2013). Furthermore, also potential impacts of the ammonia treatment on the risk of discharge of
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.**
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 (144)	NH_3 (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
	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 (144)	NH_3 (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
	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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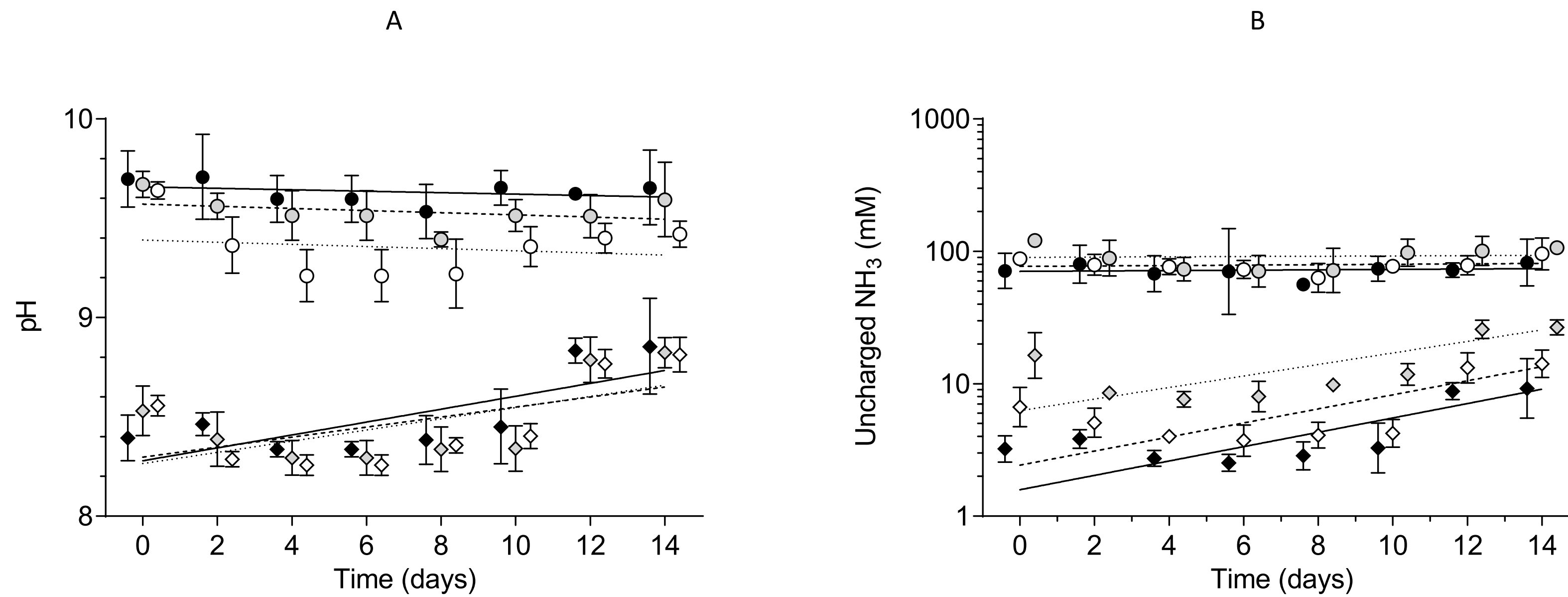

Figure 1.

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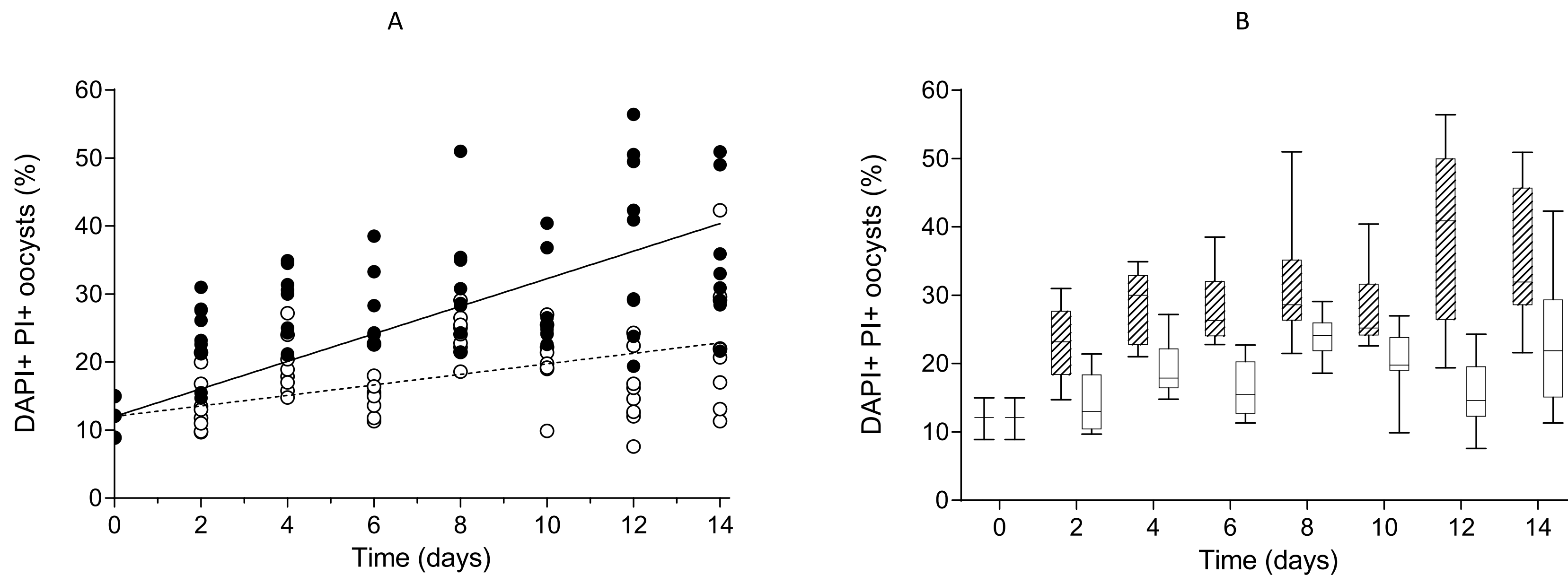

Figure 2.

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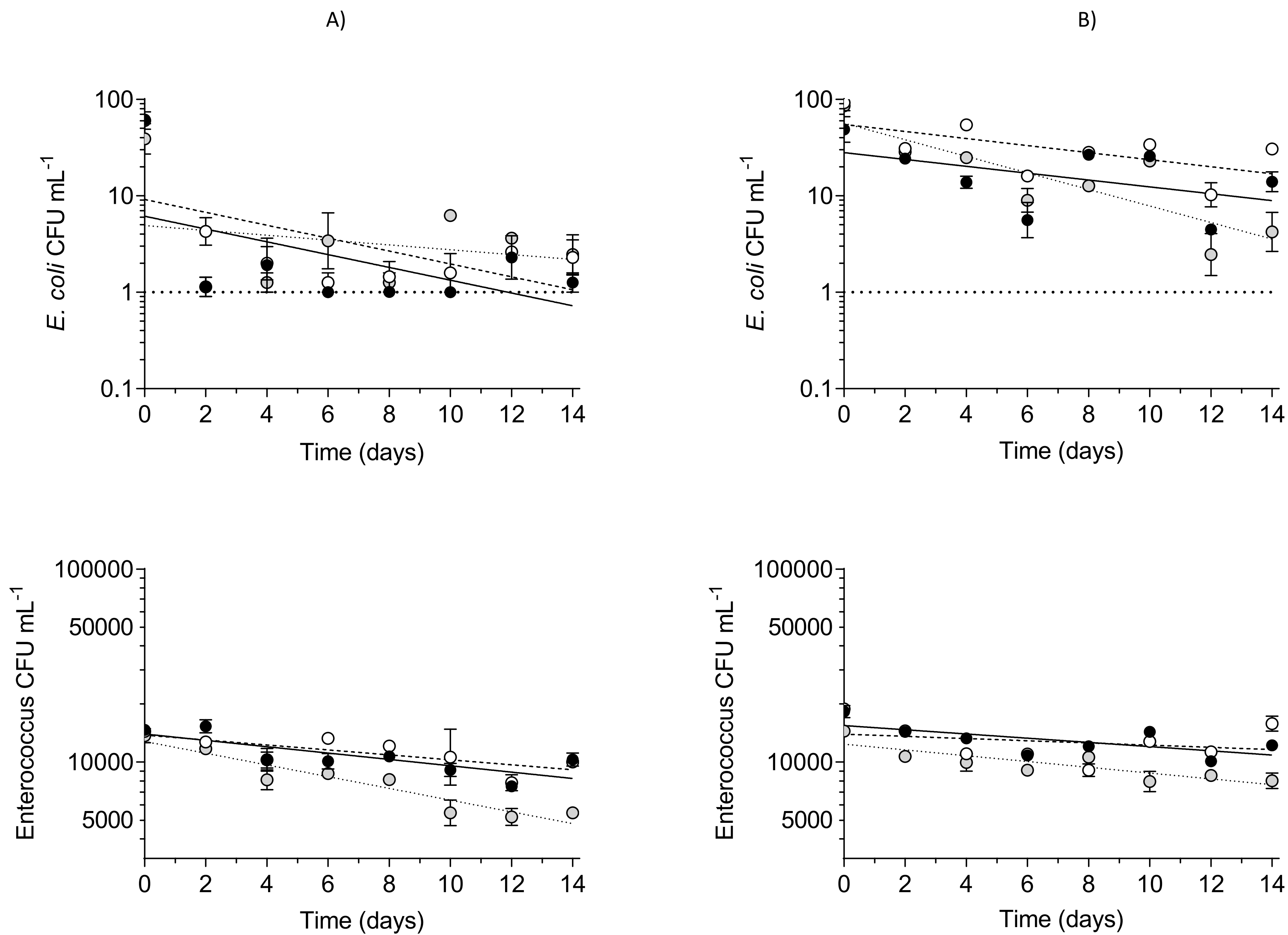

Figure 3

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A)

B)

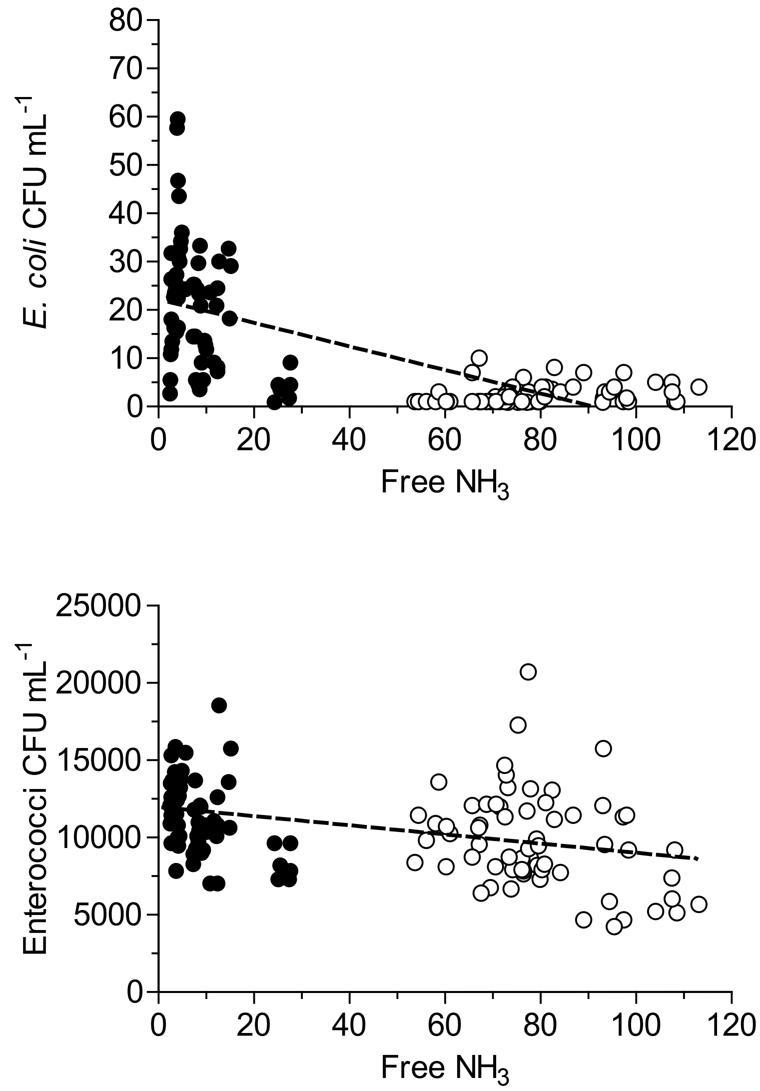

Figure 4

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