Influence of Temperature and Predation on *Salmonella enterica* serovar Typhimurium Survival and *invA* Expression in Soil and Manure-Amended Soil

R García[1+2], J. Bælum[1], L. Fredslund[1], P. Santorum[2], C.S. Jacobsen[1+3]

Geological Survey of Denmark and Greenland (GEUS) DK-1350, Copenhagen, Denmark[1], Centro de Investigación y Formación Agrarias. C[a] de Desarrollo Rural, Ganadería, Pesca y Biodiversidad. Gobierno de Cantabria. 39600 Muriedas, Cantabria, Spain[2]. University of Copenhagen, faculty of Life Sciences DK-1958, Frederiksbergc C, Denmark

Corresponding author. Mailing address: Department of Geochemistry, Geological Survey of Denmark and Greenland (GEUS) Øster Voldgade 10, DK-1350 Copenhagen, Denmark. Phone +45 38142313. E-mail: csj@geus.dk.

ABSTRACT

The effects of three temperatures (5, 15 and 25 ºC) on the survival of *Salmonella enterica* serovar Typhimurium in topsoil were investigated in small microcosms by three different techniques: (i) plate counting, (ii) *invA* gene quantification, and (iii) *invA* mRNA quantification. Differences in survival were related with the effect of protozoan predation. 1.5 x 10^8 cells g^{-1} soil of the tetracycline resistant *Salmonella* serovar Typhimurium was inoculated into the soil and manure amended soil. Population densities were determined by plate counting and by molecular methods and followed during a period of 42 days. Simultaneous extraction of RNA and DNA followed by quantitative PCR (qPCR) was used to investigate *invA* gene levels and expression. Analysis with these three techniques showed that *Salmonella* serovar Typhimurium

survived better at 5 ºC. Comparing DNA and CFU levels, significantly higher values were determined by DNA based techniques. *invA* mRNA levels showed a fast decrease in activity, with no detectable mRNA after an incubation period of less than 4 days in any of the soil scenarios. A negative correlation was found between CFU *Salmonell*a serovar Typhimurium levels and protozoan Most Probable Numbers (MPNs) and we propose the role of the predator-prey interaction as a factor to explain the die-off of the introduced strain both by culture- and DNA quantification based methods. The results indicate that temperature, manure, and protozoan predation are important factors influencing the survival of *Salmonella* serovar Typhimurium in soil.

INTRODUCTION

Salmonella bacteria excreted in the faeces of asymptomatic animals may constitute an important source for fresh water and food contamination when manure is spread directly on land (43). The bacteria can be shed from manure and are reported to survive in soil for 160-200 days (23, 25).

Numerous detection methods have been developed for detection and quantification of *Salmonella* in different matrices (11, 14, 35). Especially, the introduction of molecular techniques has become an important advance to reduce the time required for detection of *Salmonella* and to detect active bacteria in environmental samples through their DNA and RNA (10, 18, 51).

Due to lack of sensitivity and problems with PCR inhibitors in environmental samples, qPCR of *Salmonella* using DNA isolated directly from soil or manure without enrichment has not yet been widely applied (34). Another problem with DNA based detection assays may also be the possible detection of DNA from inactive or non-viable bacteria arising ambiguous positive results (31). Quantification of mRNA, however, allows analysis of which genes are being expressed and thereby a quantitative measure

of the activity levels of specific functional traits of interest. As mRNA, in general, is considered an extremely labile molecule, it is commonly accepted that analysis of mRNA is a better measure of microbial activity than DNA analysis (46).

A sequence of the *invA* gene (10) has been utilized as target for detection of *Salmonella* nucleic acids in soil samples. This gene is highly conserved in almost all *Salmonella* serotypes (7, 17, 44) and detection of *Salmonella* based on the presence of this gene has previously been reported (11, 12, 24). *invA* mRNA has been used as a biomarker for active cells (13, 18, 26), but it has been discussed, however, whether transcription of the *invA* gene may differ during different physiological states of the cell, which may affect assay specificity (13). Jacobsen and Holben (26) showed detection limits of 5×10^4 seeded *Salmonella* serovar Typhimurium cells per gram of soil. Based on these data, *invA* mRNA seems to be a feasible candidate for reverse transcriptase PCR assays to specifically identify living *Salmonella* cells.

The survival of *Salmonella* in environmental habitats can be influenced by different factors. In several studies, survival of *Salmonella* in soils has been examined by culture dependent methods testing the influence of different factors like manure addition, temperature and interaction with other microorganisms (23, 28, 39, 48). *Salmonella* spp. spread with manure has been reported to survive for up to 300 days in soils (4, 29) but duration of survival depends on several factors such as for example the incubation temperature (20, 50). Another important factor influencing survival of *Salmonella* in soil is predation by protozoa. The role of protozoa in food-borne pathogens' survival in the environment is often a neglected factor within microbial ecology, and therefore this has only been investigated in few studies (5). Brandl et al. (8) investigated the viable form of *Salmonella enterica* in vesicles of the protozoa *Tetrahymena*. They showed that this protozoa releases vesicles containing high density

of *Salmonella enterica* leading to an underestimation of actual population sizes of the pathogen during predation studies.

The primary objective of the present study was to evaluate the survival of tetracycline resistant *Salmonella* serovar Typhimurium in soil and manure-amended soil at three different temperatures (5, 15 and 25 ºC), using three different techniques: plate counting, *invA* DNA quantitative PCR and direct quantification of *invA* mRNA. Furthermore, the role of predation has been evaluated to relate the survival of *Salmonella* by different methods with the estimated most probable number (MPN) of protozoa present in soil and manure-amended soil.

MATERIALS AND METHODS

Soils. Soil samples were collected from Sjællands Odde, Denmark. Topsoil was obtained from the 0-30 cm layer of an agricultural field containing 19 % clay, 18 % silt, 62 % sand and 1.2 % carbon, and with a pH of 7.2. Approximately 100 kg of soil were obtained in total using a manual composite sampling technique in which subsamples were taken from scattered locations within a 10 m^2 area, mixed thoroughly and stored frozen at -20 ºC in aliquots of ~2 kg. Prior to experiments, aliquots were thawed and acclimatized in the dark at 10 ºC for 10 days, as described by Mortensen and Jacobsen (38). Three subsamples of soil were tested by qPCR presenting negative values for *Salmonella* and any colony forming unit per gram of soil (CFU g^{-1}) of tetracycline resistant bacteria were not detected according to the procedure described below (detection limit 100 CFU g^{-1}).

Manure. Fresh manure was obtained from dairy cows. It was stored at 5 ºC during one week before the beginning of the assay. Physical/chemical analysis of subsamples indicated moisture content of 90.4%, ammonia-N of 1.94 g kg^{-1}, and total N, phosphorous, potassium, copper and magnesium of 3.94, 0.77, 3.63, 0.00995, 1.284 g

101 kg^{-1} respectively (wet basis). qPCR negative values were determined for *Salmonella* and
102 no CFU g^{-1} manure of tetracycline resistant bacteria were detected in three subsamples.
103 **Bacteria.** *Salmonella* serovar Typhimurium tetracycline resistant DSM554 was
104 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,
105 Braunschweig, Germany, and grown in Müller broth (Oxoid) containing 25 µg/ml
106 tetracycline (Sigma-Aldrich) at 37 ºC in the dark for 24 hours at 100 rpm. Then, 10 ml
107 of the culture was placed into 100 ml Müller broth with tetracycline and incubated again
108 at 37 ºC for at least 4 hours until obtaining an OD_{600} value of ~0.6. Subsequently, the
109 culture was centrifuged (6000 × g, 10 min) and resuspended in 100 mM sterile
110 phosphate buffer (PB) pH 7, resulting in an inoculum of 2×10^9 CFU ml^{-1}, based on
111 direct plate counts (25 µg/ml tetracycline Müller agar plates, 24 h, 37 ºC).
112 **Microcosm set up and *Salmonella* serovar Typhimurium inoculation.** Triplicate
113 microcosms of topsoil and manure-amended topsoil were set up in 100 ml glass flasks
114 with airtight glass stoppers (Schott Glaswerke, Mainz, Germany).
115 Prior to inoculation, 30 g of wet weight soil was placed in each flask and sterilized PB
116 or liquid manure was added to bring soils to 85% of the field water holding capacity.
117 The amount of manure in the manure-amended topsoil corresponded to a manure
118 application rate of 3 kg m^{-2} which is commonly practiced on farms in the region.
119 At the onset of the experiment 1 ml *Salmonella* serovar Typhimurium (Tet^+)
120 culture (described above) was inoculated to the microcosm soil surface without mixing
121 obtaining 1.5×10^8 CFU g^{-1} soil in each microcosm. In manure-amended topsoil
122 microcosms, manure amended with the bacteria was applied on the soil surface,
123 obtaining the same levels of CFU g^{-1} soil. The two different soil scenarios (with/without
124 manure) were incubated in triplicates at three different temperatures; 5, 15 and 25 ºC.

125 Direct plating counts of *Salmonella* serovar Typhimurium were done at days 0,
126 1, 2, 4, 7, 11, 15, 19, 24 after inoculation and thereafter once a week until pathogen
127 concentration dropped below the detection limit (100 CFU g^{-1} soil). Samples of 0.5 g
128 (w/w) as a composite of ~5 plugs were removed from the flasks and transferred to 50 ml
129 screw-cap glass tubes, 9.5 ml sterile PB (100 mM, pH 7) was added, and the mixture
130 was agitated on a horizontal shaker (150 rpm, 30 min). Serial 10-fold dilutions were
131 prepared in PB and 0.1 ml aliquots were surface-plated on Müller agar containing
132 tetracycline. Colonies were counted after incubation for 24 h at 37ºC.
133 At the same time points as for direct plate counting another 0.5 g of soil was
134 taken for nucleic acid extraction. In order to ensure a snap-shot freeze event, these
135 samples were frozen immediately in liquid nitrogen and stored at -80 ºC.
136 **DNA/RNA extraction and cDNA synthesis.** DNA and RNA were co-extracted as
137 described by Nicolaisen et al.(40). In short, the method involves bead beating in the
138 presence of cetyl-trimethyl ammonium bromide (CTAB) buffer, phenol and chloroform,
139 followed by phenol extraction and precipitation of nucleic acids from the aqueous phase
140 by 30% polyethylene glycol 6000 (PEG). Phenol and CTAB buffer were added to the
141 frozen samples and cell lysis was perfomed for 2 × 15 s at a speed setting of 5.0 m s^{-1}
142 with intermittent cooling to prevent overheating of the sample. The following
143 modification described by Bælum et al. (3) was applied to the protocol: 1µl of glycogen
144 (Roché, Basel, Switzerland) was added to PEG to aid in nucleic acid precipitation. After
145 the extraction procedure 7 µl aliquots of each sample were used for RNAse-free DNAse
146 I treatment (Promega, Madison, USA) according to the manufacturer's protocol,
147 modified by Jacobsen and Holben (26). Reverse transcription (RT) was performed using
148 Omniscript reverse transcription kit from Qiagen (Crawley, UK) with 2 µl of DNAse
149 treated extract as template, 40 pmol of *invA* reverse primer (described below), and a

reaction volume of 10 µl. RT reaction temperatures and time of incubation were as described previously by Bælum et al. (3).

Quantitative PCR (qPCR). qPCR was carried out in an iCycler (BioRad, Hercules, CA, USA). To include impacts of the soil matrix on extraction efficiency and co-extracted PCR enzyme inhibitors, standards for quantification were prepared by individual inoculations of 10-fold dilutions (10^1-10^7 g^{-1} soil) of *Salmonella* serovar Typhimurium to aliquots of 0.5 g soil, followed by nucleic acid extraction as described above. Primers employed were based in those described by Chiu and Ou (10) with the modifications described by Jacobsen and Holben (26). The primer sequences were *invA* forward: 5'-ACAGTGCTCGTTTACGACC-3' and *invA* reverse: 5'-ACTGGTACTGATCGATAAT-3'. All qPCR reactions were performed in a final volume of 20 µl containing 10 µl of premixed mastermix (Dynamo^{TM} HS SYBR Green qPCR kit (Finnzymes, Helsinki, Finland)), 0.4 µM forward primer, 0.4 µM reverse primer, 20 µg bovine serum albumine (New England BioLabs Inc., Ipswich, MA), 7.2 µl of deionized PCR grade water and 1 µl of DNA template (~10-50 ng). DNA extractions were diluted 10-fold prior to PCR quantification to avoid interactions from co-extracted enzyme inhibitors. All DNA and cDNA samples were quantified in triplicate including negative controls (containing all the reagents except DNA template). Another control using DNase-treated RNA samples was performed to discard possible DNA contamination which would interfere with cDNA based quantification of *invA* gene expression levels. Each qPCR reaction consisted of the following steps (6): 15 min initial denaturation and enzyme activation at 95 ºC, followed by 40 cycles of 30 s denaturation at 95 ºC, 30 s annealing at 55 ºC, 30 s elongation at 72 ºC, and 15 s at 77 ºC for quantification of the *invA* product. The procedure ended with one cycle of 6 min

174 at 72 ºC for elongation and a melting curve analysis generated by analyzing the amount
175 of double-stranded DNA after each 0.5 ºC increase in the temperature, up to 95ºC.
176 **Counting for Protozoa.** Samples for counting of protozoa were taken four times during
177 the experiment. A three-fold dilution series of soil and manure-amended soil samples
178 were performed in 96-well microtiter plates (Nunc-Thermo Scientific, Roskilde,
179 Denmark). Dilutions were made in modified Neff's amoebae saline buffer with 0.10 g
180 L^{-1} of tryptic soy broth as described by Page (41). Well dishes were incubated at 15 ºC
181 in the dark and protozoan enumeration was made by visual inspection of single wells
182 with an inverted microscope after 7 and 21 days of incubation. Most Probable Number
183 (MPN) calculations were performed using the computer-assisted method developed by
184 Briones and Reichardt (9). The method uses Microsoft Excel and its associated Solver
185 tool to generate MPNs, error estimates and 95 % confidence limits.
186 **Statistical analysis.** All amplification and plate counting experiments were performed
187 in triplicate and mean values and standard deviations are given. Statistical analyses were
188 carried out using SPSS statistics 17.0. Results were compared for statistical significant
189 differences using one way ANOVA test. Correlation between protozoa MPN and *S.*
190 serovar Typhimurium survival levels was evaluated using Pearson's coefficient. For all
191 tests a $p \leq 0.05$ was considered significant.
192 **RESULTS**
193 **Quantification of *Salmonella* serovar Typhimurium by plate counting and DNA**
194 **based methods.** Figure 1 shows levels of *Salmonella* serovar Typhimurium in soil and
195 manure-amended soil samples incubated at three temperatures: 5, 15, and 25 ºC. A
196 decrease in *Salmonella* serovar Typhimurium levels and activity was observed for all of
197 the three methods used throughout the experiment.

198 Using plate counting we observed a significantly ($p<0.001$) lower survival of
199 *Salmonella* serovar Typhimurium in soil samples incubated at 25 ºC compared to 5 ºC
200 as early as 4 days. This tendency was maintained throughout the experiment. At 5 °C
201 and 15 °C, the addition of manure reduced the survival of *Salmonella* serovar
202 Typhimurium, while at 25 °C no significant difference was observed. In manure-
203 amended soil samples, a significant difference between temperatures was found no
204 earlier than after 11 days with the highest survival at 5 ºC.
205 Using qPCR based on *invA* DNA copy numbers we observed a 1.5-2 log
206 decrease at 5°C, a 4 log decrease at 15°C, and a >6 log decrease at 25°C over the time
207 period of 42 days (Fig. 1). The generally lower *invA* gene levels detected in soil samples
208 incubated at 25 ºC compared to 5 ºC, corresponded well with the results obtained using
209 the plate counting technique. Contrary to the plate counting results, however, the
210 evolution of *invA* genes did not present significant differences between soil and manure
211 amended soil scenario, and only from 4-15 days at 15 ºC we observed slightly lower
212 levels of *invA* genes between soil with and without manure.
213 **Quantification of *Salmonella* serovar Typhimurium activity levels based on *invA***
214 **mRNA.** At 5 °C ~10^5 *invA* mRNA g^{-1} soil was detected until 48 h after inoculation,
215 while in the 15 and 25 ºC experiments we did only detect *invA* mRNA until three hours
216 after inoculation (Fig 1). For the scenarios without manure addition, the *invA* mRNA
217 levels increased until three h at the three temperatures assayed, while in manure-
218 amended soil samples the amount of mRNA decreased immediately after the
219 inoculation. After 4 days of incubation the levels of *invA* mRNA was lower than the
220 detection limit in all of the soil scenarios. *invA* mRNA levels could only be detected
221 when more than 10^6 *Salmonella* serovar Typhimurium per gram of soil were detected by

222 plate counting and the relative amount of mRNA in the samples was in general less than
223 1% of the *invA* DNA detected.
224 **Comparison of plate counting and DNA quantification methods.** A regression of
225 numbers of *invA* DNA copies compared to CFU of *Salmonella* serovar Typhimurium g^{-1}
226 of soil resulted in good correlations for both the soil scenarios performed at 15 ºC and at
227 25 ºC (R^2>0.9). For the soils incubated at 5ºC, however, a correlation coefficient of
228 R^2<0.7 was observed (Fig. 2). In the samples at this low temperature DNA
229 quantification values were very high even when *Salmonella* serovar Typhimurium was
230 not detected by plate counting. At 5ºC significant differences ($p<0.001$) were found
231 between DNA and CFU levels as early as 4 days in manure amended soil and from 21
232 days in soil samples.
233 Despite the good correlation between CFU values and DNA levels at 25 ºC, a
234 comparison of slopes of the regression using Fisher test indicated significant difference
235 between soils with and without manure. Also at 5 ºC, at high levels of CFU and *invA,*
236 significant differences were found between slopes for the soils with and without
237 manure. Addition of manure caused decreases in CFU levels especially in the beginning
238 of the assay. This trend was not observed when quantifications were based on *invA*
239 genes.
240 **Growth of protozoa and its relation with *Salmonella* serovar Typhimurium**
241 **survival.** The initial number of protozoa was 1.5 log higher in the manure-amended soil
242 compared to the soil samples without manure. After 24 days a bloom in protozoan
243 numbers was observed in manure-amended soil at all three temperatures, with the
244 highest levels (10^6) observed at 5 and 15 ºC (Fig. 3). In soils without manure a slower
245 increase in protozoan numbers was observed at 5 and 15 ºC, with the highest levels
246 observed at the end of the experiment (42 days). The differences in protozoan evolution

247 between the two soil scenarios we believe, was a determining factor for the significant
248 differences in *Salmonella* serovar Typhimurium survival. The decrease in CFU levels
249 was faster in manure-amended soil samples at 5 and 15 ºC, where a faster increase in
250 protozoan numbers was also observed. In all microcosms, prior to the bloom of
251 protozoa, significant negative correlations were found between the abundance of
252 protozoa and *Salmonella* serovar Typhimurium plate counting results (Pearson
253 coefficient <-0.5).

254 **DISCUSSION**

255 Temperature has been shown to be an important factor for the survival of
256 pathogenic bacteria in environmental samples (1, 22, 36). In our study, significant
257 differences between *Salmonella* serovar Typhimurium survival at different temperatures
258 were followed using three different detection methods. For the plate counting technique
259 the largest decline in bacteria levels was observed at the highest temperature (25 ºC),
260 which corresponds to what was published earlier (54). Semenov et al. (47), showed that
261 survival of *Salmonella* serovar Typhimurium cells in cow manure decreased faster at 23
262 ºC than at 7 ºC, and similar results were shown by Holley et al. (23). In several studies,
263 enhanced survival of allocthonous bacteria in manure-amended soils has been proposed
264 to be due to an increase in nutrient availability (16, 23, 27). In our study, however, the
265 manure addition induced a significantly lower survival of *Salmonella* serovar
266 Typhimurium, compared with non-amended soil. This was proven both at 5 ºC as well
267 as at 15 ºC. In our case, the high nutrient availability as a result of manure addition
268 might have increased activity of the native soil microbial community. Increasing the
269 general competition between bacteria could lead to decrease survival of the introduced
270 pathogen (15). The faster decrease in CFU levels in manure-amended soil can also be
271 related to evolution in protozoa levels, since predation by protozoa is another factor

affecting bacterial survival in soil. After inoculation of *Salmonella* serovar Typhimurium at 5 and 15 ºC, blooms in numbers of protozoa occurred within 24 days, while in soils without manure the highest level of protozoa was not observed till the end of the assay. In all microcosms, before the maximum levels of protozoa was reached, a significant negative correlation was found between the abundance of protozoa and *Salmonella* serovar Typhimurium plate counting results, corroborating other studies about the role of predation in pathogens survival (2, 45).

In all previous works the influence of factors like temperature, manure addition or protozoan predation on the survival of *Salmonella* in environmental samples, was determined by plate counting techniques. Herein, however, we report that in addition to survival of *Salmonella* serovar Typhimurium based on CFU levels, these factors may influence the survival of *Salmonella* serovar Typhimurium based on quantification of *invA* DNA or mRNA as well.

Since DNA can persist in dead cells and thereby may bias the number of viable bacteria, quantification based on extraction of DNA from environmental samples should be carefully evaluated (30, 42). Even though it has been argued that the half life of DNA in environmental samples may be very short because of the presence of nucleases (32, 53), also very old DNA has been shown to persist in soil (33). Levels of *Salmonella* serovar Typhimurium based on DNA quantifications were significantly higher compared to the counts obtained by plate counting especially in manure amended samples at low temperatures. Such a trend has also been confirmed by others (11, 51). As previously reported by Turpin et al. (52), detection of *Salmonella* using a culture based technique was not possible in any of the temperature scenarios after 42 days. Based on *invA* DNA levels, however, high levels of *Salmonella* serovar Typhimurium were still present at this time point, especially at 5 ºC where a difference of 4 orders was

297 observed between DNA and CFU levels at the end of the assay, showing a non
298 culturable response higher at 5 ºC than at 25 ºC as demonstrated by Gupte et al (21).
299 Due to a faster decrease in CFU levels, this difference was even more pronounced in the
300 soil with manure scenario at 5 ºC.

301 The elevated levels of *invA* genes might be related to the presence of viable but
302 non culturable *Salmonella* serovar Typhimurium cells, as also reported by Marsh et al
303 (35). Especially at 5 ºC and 15 ºC, the ratio between *invA* genes and CFU levels
304 revealed higher values in the soil with manure compared to the soil without manure.
305 The addition of manure to soil seems to decrease the culturability of *Salmonella* serovar
306 Typhimurium, and support the presence of high levels of *invA* DNA.

307 The initial numbers of protozoa in our manure amended soils was 1.5 log higher
308 than in soil samples without manure, indicating that a significant number of protozoa
309 was added along with the manure. These favourable conditions for predatory activity
310 can be related directly to the fast decrease of *Salmonella* serovar Typhimurium CFU
311 levels in the manure amended soil. Brandl et al. (8) observed that the protozoan ciliate
312 *Tetrahymena* contained intracellular feeding vesicles with high densities of ingested
313 *Salmonella enterica*. The subsequent release of these vesicles seemed to prolong
314 bacterial survival in natural environments as sites contaminated with manure, where this
315 pathogen is present. The presence of *Salmonella* within intracellular protozoan vesicles
316 likely underestimates the actual population of the pathogen because they cannot be
317 detected by plate counting. Hence, if *Salmonella* is able to survive in protozoan
318 vesicles, as other authors report (8, 19), it is in agreement with our results, where we
319 found high numbers of *invA* gene levels vs. low levels of CFU under conditions where
320 predatory activity was higher. Our results also indicate that predation can be proposed
321 to explain *Salmonella* dynamics in non-amended soils; the faster decline of cultivable

322 *Salmonella* serovar Typhimurium at 25 ºC is consistent with the increased predatory
323 levels observed in warm compared with cooler soil conditions (5).

324 With the above mentioned dilemma in mind, quantification of mRNA
325 transcribed from a genus specific gene could be the ideal biomarker for metabolic active
326 *Salmonella* cells in environmental samples and it would help to determine their viability
327 (49). In our study, we chose expression of the *invA* gene as such a biomarker. We
328 observed a dramatic decrease in *invA* mRNA levels after only 48 hours of inoculation at
329 5 ºC. At 15 and 25 ºC this decrease was even faster and *invA* mRNA could only be
330 detected in the 25 °C microcosms until 3 hours after the beginning of the experiment.
331 Fey et al. (14) has done a previous study on detection of *invA* mRNA in water samples,
332 and they found a significant decrease in *invA* mRNA levels 3 hours after the inoculation
333 coinciding with the late logarithm phase. In our study, however, this significant
334 decrease in *invA* mRNA levels was detected 48 hours after the inoculation of
335 *Salmonella* serovar Typhimurium in samples incubated at 5 ºC.

336 In the present study we were only able to detect *invA* mRNA in samples with
337 more than 10^6 cells per gram of soil (detected by plate counting), and this detection
338 limit is higher compared to what have been observed in previous studies on mRNA
339 quantification in soil samples (26, 37, 40). The fact that the number of *invA* transcripts
340 in general was lower than *invA* gene copy numbers may indicate that the expression is
341 down-regulated as a response to stress due to suboptimal conditions in the
342 environmental sample.

343 This study compared for the first time the fate of *Salmonella* serovar
344 Typhimurium in soil and manure-amended soil under different temperatures by
345 culturing and molecular methods. The *Salmonella* serovar Typhimurium levels detected
346 by the three methods indicate that this pathogen survives better at 5 ºC. However, at this

347 temperature, higher differences between *invA* DNA and CFU levels was observed. We
348 propose the role of the predator-prey interaction as a factor to explain the differences
349 between the culture and DNA quantification methods. The quantification of *invA*
350 mRNA with our method confirms that *Salmonella* serovar Typhimurium presents
351 invasive activity 48 hours after inoculation in soil samples although a high detection
352 limit is observed. More work has to be done to improve molecular detection methods to
353 evaluate the infectiousness of this strain in soils samples and how this ability may be
354 affected by the environmental conditions.

355 **ACKNOWLEDGMENTS**

356 This study was supported by Pathos project funded by the Strategic Research
357 Council of Denmark ENV 2104-07-0015. R. García's stay at Geological Survey of
358 Denmark and Greenland was financed by the Spanish Institute for Agriculture, Food
359 Research and Technology.

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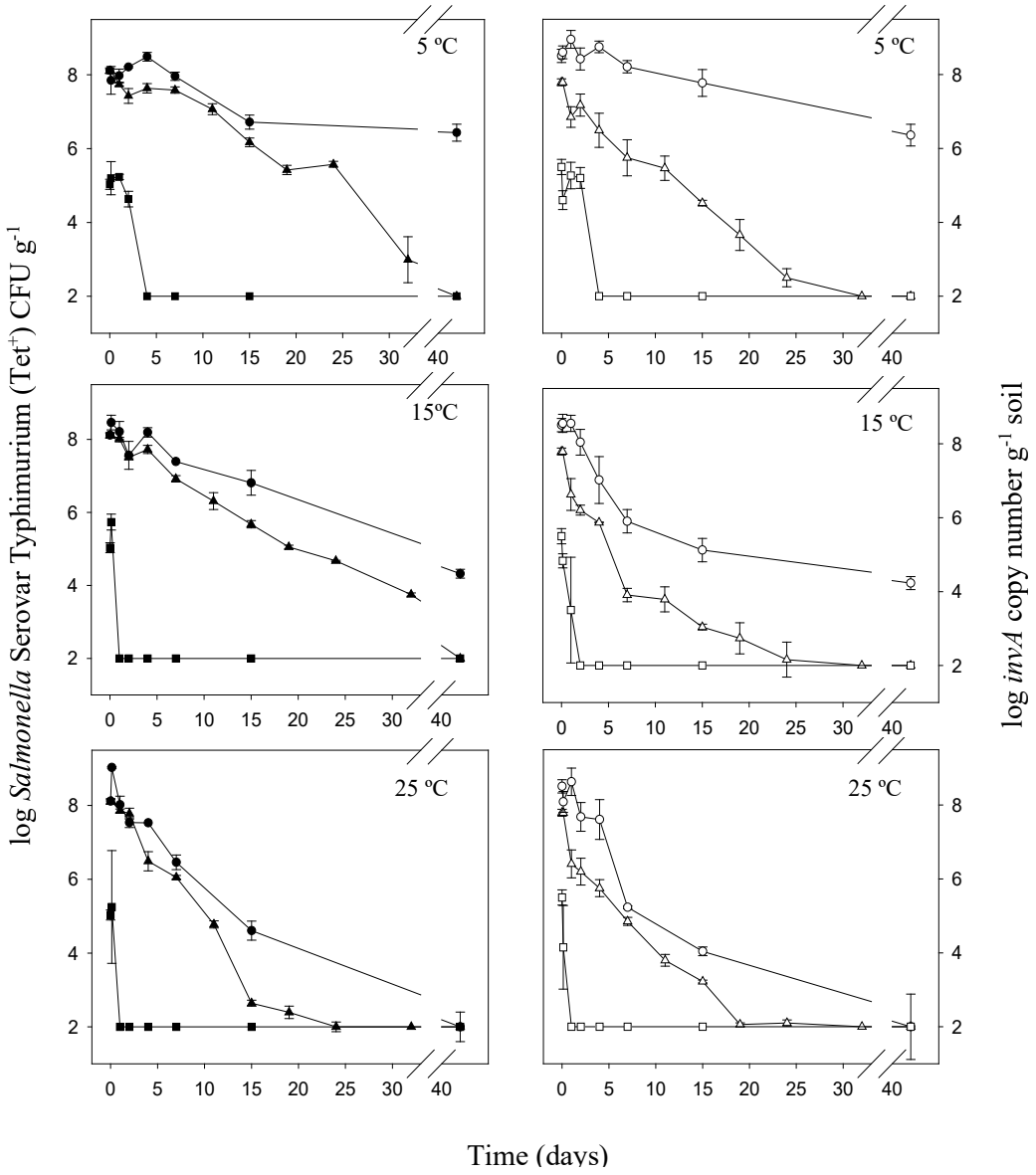

FIG. 1. Evolution of *Salmonella* serovar Typhimurium (Tet^+) measured by qPCR and plate counting in soil samples (closed symbols) and manure amended soil samples (open symbols) at three different temperatures. (●, ○) log *invA* DNA copies g^{-1} soil; (▲, Δ) log CFU g^{-1} soil; (■, □) log *invA* mRNA copies g^{-1} soil. Each point represents the mean of triplicate quantifications from one sample. Detection limit= 10^2. Error bars represent standard error of the mean. Please note the broken *x* axes.

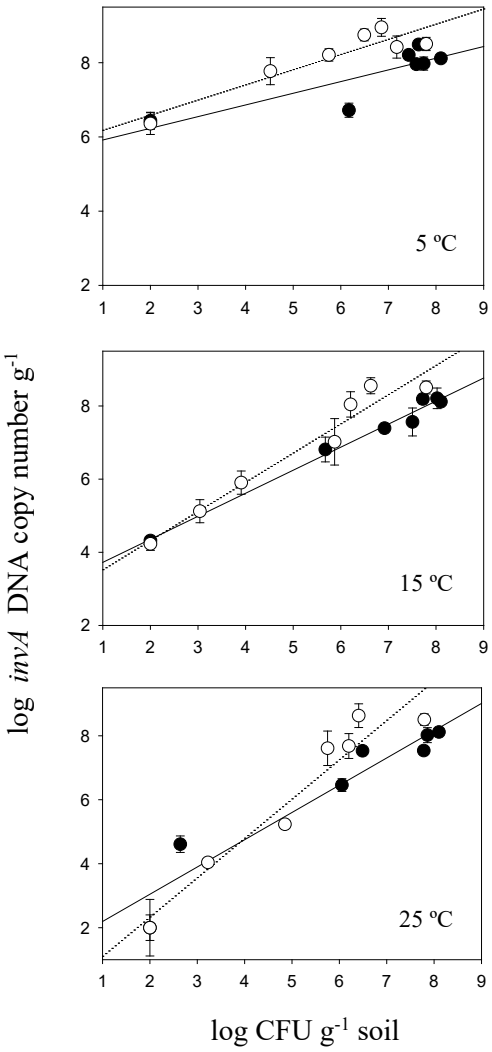

FIG. 2. Linear regression of the levels of *Salmonella* serovar Typhimurium (Tet^+) quantified by plate counting and qPCR. The logarithm of *invA* DNA copy number is plotted versus the logarithm of *Salmonella* serovar Typhimurium per gram of soil at three different temperatures: 5º C, 15 ºC and 25 ºC; (●) soil; (○) manure-amended soil. Error bars represent standard error of the mean.

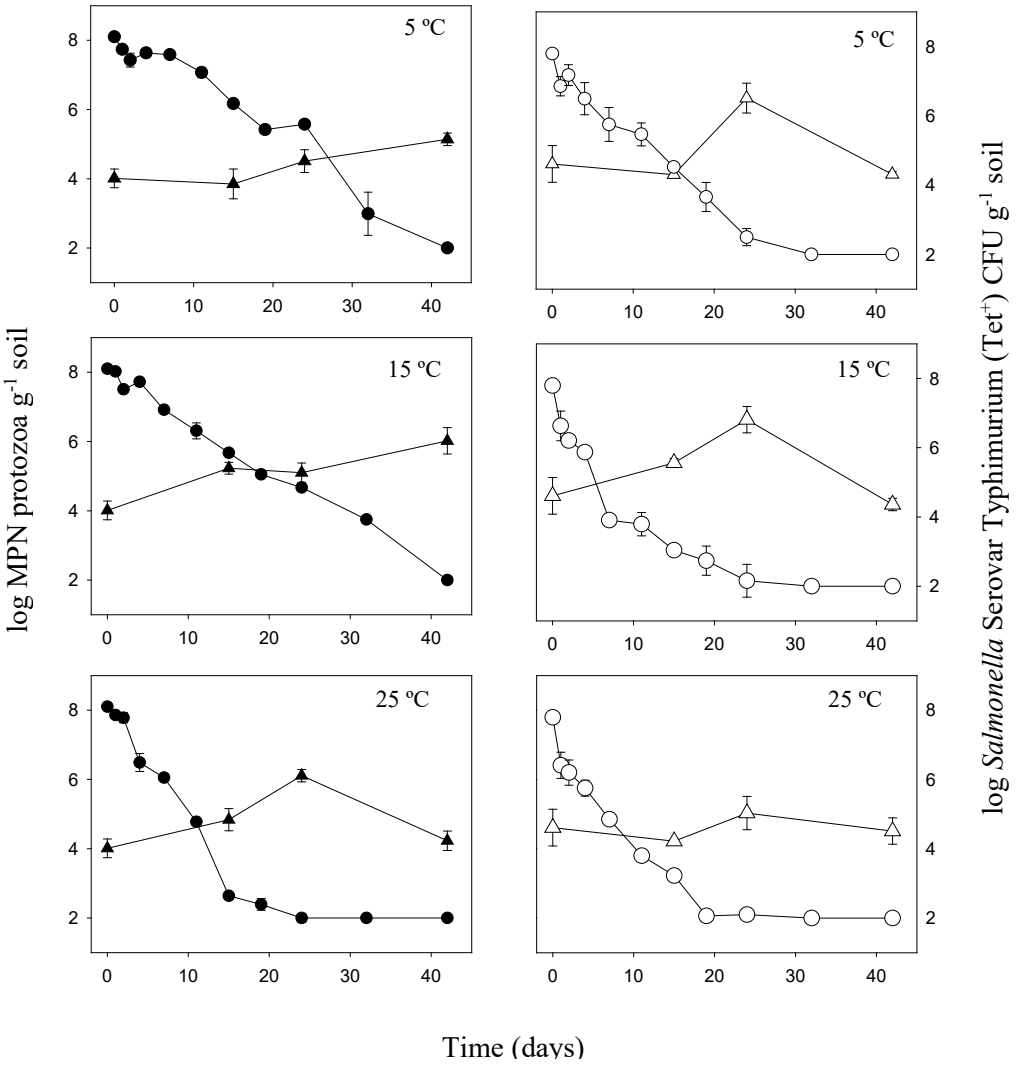

FIG. 3. Evolution of *S.*serovar Typhimurium CFU (●, ○) and protozoan levels (Δ, ▲) in soil samples (closed symbols) and manure-amended soil samples (open symbols) incubated at three different temperatures. Protozoa counting were made at 15, 24 and 42 days after the beginning of the assay. Protozoan levels at initial time were determined before the addition of the bacteria. Error bars represent standard error of the mean.