CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Comparison of *E. coli* and *Salmonella* spp. detection methods

4.1.1 Enumeration of pure culture

The reference *E. coli* 25922 (Lot.1896) was not significant difference when determined by comparing between plate count on L-EMB agar and RT-PCR. Their population was 9.47 and 9.10 log CFU/mL, respectively. The 3M Petrifilm method could detect the cell number of *E. coli* equal to 8.34 log CFU/mL and 7.05 log MPN/mL as detected by BAM method, which were highly significant at $P<0.01$. These results showed that using BAM and 3M Petrifilm methods gave lower population than plate count and RT-PCR. Whereas, that of *S. typhimurium* ATCC 1311 with plate count, BAM and RT-PCR, indicated there was no significant difference between plate count and RT-PCR ($P>0.05$) with 8.94±0.00 log CFU/mL and 9.40±0.32 log CFU/mL, respectively. In the case of BAM method, generally indicates occurring of microbe as positive or negative only (Table 6).

Table 6 Comparison of *E. coli* ATCC 25922 and *S. typhimuriun* ATCC 1311 population in Plate count, BAM, 3M Petrifilm, and RT-PCR cultivation techniques.

Test methods	*E. coli* ATCC 25922[1/]	*S. typhimurium* ATCC 1311[1/]
Plate count	9.47±0.77 (log CFU/mL) [a]	8.94±0.00 (log CFU/mL)
BAM	7.05±0.00 (log MPN/mL) [c]	detected
3M Petrifilm	8.34±0.07 (log CFU/mL) [b]	NA
RT-PCR	9.10±0.07 (log CFU/mL) [a]	9.40±0.32 (log CFU/mL)
F-test	**	ns
C.V.	4.72	2.43

[1/] Each value represents mean ± standard deviation of three replications. Means with the same letter within column are not significantly different at the $P\leq 0.05$ by least significant difference. The symbol "NA" is not available, "ns" is no significance and "**" is highly significantly different at the $P\leq 0.01$.

The enumeration of *E. coli* and *Salmonella* spp. from reference cultures with RT-PCR and plate count techniques were in accordance with the results of Made *et al.* (2004) who used RT-PCR and conventional method to detect *Salmonella* spp. in food samples (European standard EN 12824: 1997). They found significant correlation between the two methods. RT-PCR was applied to detect and quantify *E. coli*, *Salmonella* spp. and other pathogenic bacteria in various samples e.g., soil, food products, water, fecal slurry, etc. (Bach *et al.*, 2002b; Made *et al.*, 2004; Nam *et al.*, 2005). The specific character of RT-PCR is the fluorescence of reporter dye SYBG Green I bound on DNA. Increasing of dye emission has appeared at the end of each successive round of amplification. Further amplification in RT-PCR is analyzed by determining the melting temperature (T_m) for each sample. T_m is dependent upon the length of the sequence, as well as G/C content of sequence. Fluorescence emission spectra of SYBG Green I are 50- to 100- fold brighter when the dye is bound to double-standard DNA. As the T_m is reached, the DNA denatures and releases SYBG Green I, causing a sharp decline in fluorescence. The decrease in fluorescence is plotted (as negative slope) versus temperature, which results in a melting peak and T_m for each PCR product. Primer-dimers, which are typically shorter in length, usually melt at a much lower T_m than the intended product and are therefore easy to distinguish. Secondary or non-specific products can be of varying length range of detection and enumeration for several microbes in possible melting temperature (Bhagwat, 2003). Thus, the RT-PCR can be used for various samples by changing primer and optimum condition for each species of microbes.

Quantity of both reference cultures determined by RT-PCR technique was closed to that of plate count technique more than BAM and 3M Petrifilm methods (Table 6). This result was in agreement with experiment of Seo *et al.* (2007) who found no significant difference ($P>0.05$) between RT-PCR and traditional culture method. They compared two methods for detection *Salmonella enteritidis* in pooled eggs.

4.1.2 Enumeration of *E. coli* contaminated in agricultural samples

Contamination of *E. coli* from agricultural samples were detected by BAM method, 3M Petrifilm and RT-PCR, the value were 0 to 5.04 log MPN/g, 0 to 7.53 log CFU/g, and 5.72 to 9.03 log CFU/g, respectively. The population of *E. coli* from

agricultural samples by these three methods showed highly significant different ($P<0.01$). The observed results of RT-PCR method showed higher quantity than other methods and could detect in all samples while BAM and 3M Petrifilm could not detect in some samples (Table 7). Highly significant correlation (r^2) ($P<0.01$) of BAM and 3M Petrifilm was 0.65, BAM and the RT-PCR was 0.46, and 3M Petrifilm and RT-PCR, was 0.45, respectively (Table 8).

The over quantification of *E. coli* from RT-PCR assay (Table 7) might be due to the fact that it detected both active cells and inactive cells (Heidelberg *et al.*, 1997) while BAM and 3M Petrifilm detected only active cell. Therefore, an accurate detection limit of RT-PCR should be determined by total cell counting method, such as microscopy or flow cytometry in which permits enumeration of cells regardless of their metabolic state (An *et al.*, 2006). Akerlun *et al.* (1995) referred Grattepanch *et al.* (2005) reported that the variation of size and content of *E. coli* cells was in accordance with growth phase. During the exponential phase, they observed that the variation of ratio between cells mass and average DNA content might occur. As quantification of bacterial cells by RT-PCR was based on DNA, difference in DNA content per cell could lead to an error in bacterial population estimation. This is in agreement with study of Reichert-Schwillinsky *et al.* (2009) who compared growth curves of *Listeria monocytogenes* from RT-PCR, optical density and viable count. Its good moderate growth condition yielded good correlation of RT-PCR data and plate count data (r^2=0.96 and 0.99). When growth conditions became worse, the numbers of CFU decreased during the stationary phase, whereas RT-PCR derived bacterial cell equivalents differed in this regard; the correlation RT-PCR worsened (r^2=0.84). Accumulation of dead cells and extracellular DNA was observed, but not all extracellular DNA might have been associated with cell debris collected by centrifugation. The presence of more than one genome per bacterial cell, especially in fast-growing cells, might be another reason for over quantification by RT-PCR (Adahi *et al.*, 2008). *E. coli* and *Salmonella* spp. contaminated agricultural samples (from this experiment) could not be known of growth phases. Therefore, the amounts of both types detected by three methods were difference.

Table 7 Monitoring of *E. coli* and *Salmonella* spp. detected by BAM, 3M-Petrifilm and RT-PCR methods in agricultural samples.

No.	Name of samples	*E. coli* [1/]					*Salmonella* spp. [1/]	
		BAM log of MPN/g	3M-Petrifilm log CFU/g	RT-PCR log CFU/g	F-test	CV	BAM	RT-PCR log CFU/g
1	peppermint	2.80±0.28 a	0.00±0.00 b	0.00±0.00 b	**	17.30	ND	0.00±0.00
2	convolvulus	2.80±0.49 b	0.00±0.00 c	6.76±0.32 a	**	10.53	D	2.01±0.19
3	ceylon spinach	2.80±0.29 b	0.00±0.00 c	7.08±0.14 a	**	5.66	D	2.24±0.19
4	*Acacia pennata*	3.97±0.34 b	0.00±0.00 c	6.65±0.17 a	**	6.24	D	2.25±0.45
5	Chinese chive leave	3.43±0.51 b	0.00±0.00 c	7.06±0.07 a	**	8.47	D	3.22±0.90
6	green shallot	2.48±0.00 b	0.00±0.00 b	6.83±0.07 a	**	13.01	D	3.55±0.30
7	sweet basil	2.48±0.00 b	0.00±0.00 b	6.51±0.55 a	**	10.49	D	3.19±0.15
8	holy basil	2.48±0.00 b	0.00±0.00 b	6.60±1.42 a	**	26.98	D	0.00±0.00
9	coriander	2.48±0.00 b	0.00±0.00 b	7.34±0.44 a	**	7.74	D	3.82±0.76
10	yard long bean	2.51±0.05 b	0.00±0.00 b	8.41±0.42 a	**	6.71	D	3.62±0.07
11	ka-yang	2.48±0.00 b	0.00±0.00 b	7.58±0.91 a	**	15.59	D	2.81±0.29
12	Chinese celery	2.48±0.00 c	0.00±0.00 b	7.92±0.68 a	**	11.3	D	5.40±0.99
13	lemon glass	2.48±0.00 c	0.00±0.00 b	7.57±0.47 a	**	8.16	D	4.17±0.35
14	bergamot leaves	3.87±0.51 c	4.69±0.53 b	7.49±0.32 a	**	8.70	D	3.38±0.25
15	asparagus	3.43±0.51 c	3.00±0.00 b	7.03±0.56 a	**	9.66	D	3.36±0.06
16	centella	3.42±0.14 b	3.65±0.35 b	6.78±0.00 a	**	4.71	D	2.01±1.75
17	lettuce	2.80±0.49 b	2.43±0.75 b	4.78±0.00 a	**	15.55	D	3.31±0.11
18	stink weed	2.45±0.04 c	3.00±0.02 b	5.03±0.03 a	**	0.96	ND	3.40±0.48
19	*Piper samentosum*	2.74±0.25 b	2.00±0.00 b	7.24±0.17 a	**	4.25	ND	3.32±0.24
20	water mimosa	2.48±0.00 b	2.48±0.00 b	6.38±0.40 a	**	6.02	ND	3.27±0.10
21	bush bean	2.48±0.00 b	0.00±0.00 b	6.66±0.44 a	**	8.24	ND	3.40±0.13
22	baby corn	2.48±0.00 b	0.00±0.00 b	6.95±0.63 a	**	11.54	ND	3.60±0.16
23	Chinese chive leave	3.02±0.41 b	0.00±0.00 c	6.70±0.61 a	**	13.07	ND	3.58±0.19
24	choy sum	0.00±0.00 b	0.00±0.00 b	6.38±0.03 a	**	0.94	ND	3.17±0.24
25	green shallot	0.00±0.00 b	0.00±0.00 b	6.77±0.65 a	**	16.69	ND	6.21±0.21
26	celery	2.48±0.00 b	0.00±0.00 c	6.03±0.04 a	**	0.82	ND	6.36±0.20
27	water	0.00±0.00 b	0.00±0.00 b	6.41±0.00 a	**	19.32	ND	6.26±0.11
28	water	0.00±0.00 b	0.00±0.00 b	6.76±0.25 a	**	6.30	ND	6.24±0.11
29	water	0.00±0.00 b	0.00±0.00 b	7.21±0.07 a	**	1.70	ND	6.12±0.14
30	water	0.00±0.00 b	0.00±0.00 b	6.92±0.69 a	**	17.21	ND	6.47±0.12

Table 7 Monitoring of *E. coli* and *Salmonella* spp. detected by BAM, 3M-Petrifilm and RT-PCR methods in agricultural samples (continued).

No.	Name of samples	*E. coli* [1/]					*Salmonella* spp. [1/]	
		BAM log of MPN/g	3M-Petrifilm log CFU/g	RT-PCR log CFU/g	F-test	CV	BAM	RT-PCR log CFU/g
31	soil	2.63±0.19 b	3.00±0.00 b	7.50±0.21 a	**	3.77	ND	6.18±0.06
32	soil	3.41±0.39 c	4.14±0.00 b	6.09±0.24 a	**	5.79	ND	6.44±0.10
33	pig feces	4.37±0.36 c	6.20±0.00 b	7.37±0.05 a	**	3.47	ND	6.35±0.17
34	pig feces	0.00±0.00 b	0.00±0.00 b	6.00±0.37 a	**	9.00	D	6.29±0.05
35	cow feces	3.08±0.72 c	3.70±0.20 b	5.96±0.21 a	**	9.00	D	6.40±0.08
36	chicken feces	5.04±0.00 b	0.00±0.00 c	8.89±0.18 a	**	2.19	ND	6.27±0.05
37	chicken feces	3.22±1.02 b	0.00±0.00 c	7.39±0.20 a	**	6.00	ND	6.26±0.44
38	cow feces	2.51±0.05 b	0.00±0.00 c	6.28±0.26 a	**	5.22	ND	6.51±0.23
39	water	0.00±0.00 b	0.00±0.00 b	6.25±0.77 a	**	6.00	ND	6.55±0.26
40	water	0.00±0.00 b	0.00±0.00 b	5.95±0.06 a	**	1.77	ND	6.53±0.24
41	soil	0.00±0.00 b	0.00±0.00 b	5.87±0.12 a	**	3.74	ND	6.43±0.16
42	soil	0.00±0.00 b	0.00±0.00 b	6.03±0.27 a	**	7.65	ND	6.59±0.24
43	soil	0.00±0.00 b	0.00±0.00 b	5.79±0.17 a	**	5.16	ND	4.79±4.17
44	organic fertilizer	2.48±0.00 b	0.00±0.00 c	6.88±0.38 a	**	7.04	ND	6.06±0.05
45	water	0.00±0.00 b	0.00±0.00 b	5.87±0.12 a	**	3.57	ND	6.08±0.10
46	water	0.00±0.00 b	0.00±0.00 b	6.16±0.10 a	**	2.89	D	6.11±0.20
47	soil	2.48±0.00 b	0.00±0.00 c	5.18±0.13 a	**	2.78	ND	6.00±0.07
48	soil	2.48±0.00 b	0.00±0.00 c	5.92±0.07 a	**	1.46	ND	6.01±0.09
49	soil	2.48±0.00 b	0.00±0.00 c	6.05±0.24 a	**	4.82	ND	6.03±0.08
50	soil	0.00±0.00 b	0.00±0.00 b	5.93±0.17 a	**	4.97	ND	6.13±0.18
51	organic fertilizer	0.00±0.00 b	0.00±0.00 b	5.89±0.06 a	**	1.67	ND	5.99±0.04
52	soil	0.00±0.00 b	0.00±0.00 b	6.16±0.17 a	**	4.84	ND	6.03±0.05
53	water	0.00±0.00 b	0.00±0.00 b	5.95±0.08 a	**	2.20	ND	6.02±0.06
54	water	0.00±0.00 b	0.00±0.00 b	5.98±0.12 a	**	3.54	ND	6.13±0.21
55	soil	0.00±0.00 b	0.00±0.00 b	5.96±0.10 a	**	2.94	ND	6.08±0.13
56	soil	0.00±0.00 b	0.00±0.00 b	6.04±0.28 a	**	7.91	ND	6.01±0.07
57	soil	0.00±0.00 b	0.00±0.00 b	5.97±0.06 a	**	1.7.0	ND	6.08±0.15
58	soil	2.48±0.00 b	0.00±0.00 c	5.94±0.05 a	**	1.06	ND	6.09±0.08
59	organic fertilizer	0.00±0.00 b	0.00±0.00 b	6.05±0.23 a	**	6.70	ND	6.32±0.04
60	organic fertilizer	3.45±0.16 b	0.00±0.00 c	5.88±0.20 a	**	4.71	ND	6.09±0.06
61	organic fertilizer	3.48±0.26 b	0.00±0.00 c	5.97±0.03 a	**	4.79	ND	6.05±0.06
62	organic fertilizer	2.53±0.18 b	0.00±0.00 c	6.47±0.14 a	**	3.89	ND	6.22±0.32
63	organic fertilizer	0.00±0.00 b	0.00±0.00 b	6.55±0.08 a	**	2.00	D	6.03±0.12

Table 7 Monitoring of *E. coli* and *Salmonella* spp. detected by BAM, 3M-Petrifilm and RT-PCR methods in agricultural samples (continued).

No.	Name of samples	*E. coli* [1/]					*Salmonella* spp. [1/]	
		BAM log of MPN/g	3M-Petrifilm log CFU/g	RT-PCR log CFU/g	F-test	CV	BAM	RT-PCR log CFU/g
64	organic fertilizer	3.05±0.54 b	0.00±0.00 c	6.42±0.06 a	**	9.95	ND	6.15±0.09
65	chicken feces	5.04±0.00 c	6.48±0.00 b	7.78±0.09 a	**	0.81	ND	5.99±0.10
66	soil	2.48±0.00 c	2.10±0.17 b	6.48±0.06 a	**	2.86	ND	6.24±0.24
67	soil	2.48±0.00 c	3.60±0.26 b	6.53±0.18 a	**	4.34	ND	6.26±0.28
68	soil	2.48±0.00 b	0.00±0.00 c	6.43±0.07 a	**	1.43	ND	6.10±0.11
69	cow feces	3.92±0.64 c	5.64±0.18 b	8.22±0.06 a	**	6.50	D	6.14±0.08
70	cow feces	3.06±0.11 c	4.17±0.26 b	6.53±0.06 a	**	3.80	ND	6.31±0.08
71	cow feces	3.70±0.42 c	4.92±0.83 b	6.52±0.03 a	**	10.6	ND	6.05±0.09
72	cow feces	3.78±0.76 c	4.68±0.59 b	6.48±0.06 a	**	1.19	ND	6.34±0.11
73	pig feces	5.04±0.00 c	6.84±0.10 b	7.45±0.06 a	**	0.97	ND	6.18±0.04
74	chicken feces	5.04±0.00 c	7.16±0.09 b	7.72±0.07 a	**	1.00	ND	5.02±0.21
75	soil	0.00±0.00 b	0.00±0.00 b	6.51±0.07 a	**	1.92	ND	5.02±0.15
76	soil	2.48±0.00 b	0.00±0.00 c	6.43±0.02 a	**	0.39	ND	4.98±0.26
77	organic fertilizer	3.86±0.19 c	5.03±0.15 b	6.77±0.03 a	**	2.77	ND	4.74±0.05
78	soil	2.48±0.00 b	0.00±0.00 c	6.47±0.03 a	**	0.7	ND	4.87±0.27
79	soil	2.48±0.00 b	0.00±0.00 c	6.50±0.05 a	**	0.96	ND	4.93±0.12
80	soil	2.48±0.00 b	0.00±0.00 c	6.56±0.12 a	**	2.31	ND	5.03±0.08
81	water	0.00±0.00 b	0.00±0.00 b	6.61±0.11 a	**	2.88	ND	4.70±0.06
82	water	0.00±0.00 b	0.00±0.00 b	6.47±0.04 a	**	1.17	ND	4.89±0.16
83	soil	0.00±0.00 b	0.00±0.00 b	6.43±0.08 a	**	2.14	ND	4.88±0.10
84	water	0.00±0.00 b	0.00±0.00 b	6.24±0.27 a	**	7.58	ND	4.87±0.12
85	soil	0.00±0.00 b	5.46±0.00 b	6.09±0.03 a	**	8.84	ND	4.94±0.05
86	water	0.00±0.00 b	0.00±0.00 b	6.11±0.19 a	**	5.47	ND	4.94±0.11
87	soil	0.00±0.00 b	0.00±0.00 b	6.13±0.20 a	**	5.56	ND	4.91±0.19
88	soil	0.00±0.00 b	0.00±0.00 b	6.34±0.26 a	**	7.21	ND	5.03±0.22
89	water	0.00±0.00 b	0.00±0.00 b	6.11±0.07 a	**	1.85	ND	4.85±0.08
90	soil	2.48±0.00 b	0.00±0.00 c	6.20±0.04 a	**	0.76	ND	4.94±0.04
91	water	0.00±0.00 b	0.00±0.00 b	6.27±0.08 a	**	2.07	ND	4.91±0.12
92	soil	2.48±0.00 b	0.00±0.00 c	5.99±0.08 a	**	1.59	ND	4.97±0.00
93	soil	2.48±0.00 b	0.00±0.00 c	5.99±0.14 a	**	2.98	ND	4.90±0.07
94	water	0.00±0.00 b	0.00±0.00 b	6.04±0.05 a	**	1.43	ND	4.41±0.42
95	soil	2.48±0.00 b	0.00±0.00 c	5.94±0.14 a	**	2.88	ND	4.85±0.14
96	soil	2.48±0.00 b	0.00±0.00 c	6.05±0.16 a	**	3.15	D	4.98±0.01
97	goat feces	5.04±0.00 b	7.05±0.14 a	7.08±0.22 a	**	2.35	D	5.06±0.06

Table 7 Monitoring of *E. coli* and *Salmonella* spp. detected by BAM, 3M-Petrifilm and RT-PCR methods in agricultural samples (continued).

No.	Name of samples	*E. coli* [1/]					*Salmonella* spp. [1/]	
		BAM log of MPN/g	3M-Petrifilm log CFU/g	RT-PCR log CFU/g	F-test	CV	BAM	RT-PCR log CFU/g
98	cow feces	5.00±0.00 [b]	7.35±0.30 [a]	7.59±0.17 [a]	**	3.02	ND	3.23±2.80
99	soil	2.48±0.00 [b]	0.00±0.00 [c]	5.98±0.10 [a]	**	1.97	D	5.01±0.04
100	cow feces	4.06±0.56 [c]	5.00±1.00 [b]	6.34±0.08 [a]	**	5.00	ND	5.08±0.24

[1/] Each value represents mean ± standard deviation of three replications. Means with the same letter within row are not significantly different at the $P \leq 0.05$ by least significant difference. The symbol "**" is highly significantly different at the $P \leq 0.01$, "ND" is not detected and "D" is detected.

Table 8 Significant correlation of *E. coli* population counted by conventional, 3M Petrifilm, and RT-PCR techniques in 100 agricultural tested samples.

Test method	Conventional[1/]	3M Petrifilm[1/]	RT-PCR
conventional	1		
3M Petrifilm	0.65**	1	
RT-PCR	0.46**	0.45**	1

[1/] The symbol "**" is highly significantly different at the $P \leq 0.01$.

For *Salmonella* spp., detection with RT-PCR could detect contamination in all samples with quantity indication. But BAM method could detect contamination in only 25 samples from a total of 100 samples; the quantity could not be obtained (Table 7). The methods for detection of *Salmonella* spp. in present has several methods to detect *Salmonella* spp. such as ISO 6579 (07/2002), U 47/100(07/2002), AIASKA method, DIN EN 12824 (German and European standard), these were selective media which take longer time more than RT-PCR. Lepper *et al.* (2002) had developed method of detection of *Salmonella* spp. in food based on enrichment and isolation on selective media. AOAC International has approved a rapid automatic method, using the VIDAS® Immuno-Concentration Salmonella, which captures *Salmonella* cells for subsequent detection by the VIDAS immunofluorescent assay. The VIDAS ICS method was shown to be conventional and considerably faster than

the BAM procedure for detection, but it could not quantify and take longer time than RT-PCR. Many studies have used RT-PCR for detection of *Salmonella* in several samples. More recent studies have reported the successful application of developed RT-PCR assays for the detection of *Salmonella* in naturally-contaminated samples (Catarame *et al.*, 2006). They developed and successfully applied a RT-PCR method for the LightCycler instrument in the detection of *Salmonella* in naturally contaminated meat samples within a much shorter time than the standard culture method, i.e., within 27-28 h. Liming and Bhagwat (2004) applied RT-PCR to detected *Salmonella* species. They could detect as few as 1-4 colony-forming units (CFU) per reaction. A comparison of two commercially available kits utilizing MB-PCR (iQ-Check, Bio-Rad laboratory) and conventional (AOAC)-approved PCR was performed on artificially inoculated product. As few as 4 CFU/25g of product were detected after 16 h of enrichment in buffered peptone broth.

BAM method was using of most probable number (MPN) technique as an indirect technique which estimated the number of bacteria (not counting) by cultivating the sample and growing the microorganisms on a selective medium. This technique is based on a statistical method using serial dilutions of the sample. Estimated populations are derived from the pattern of attribute occurrence across a serial dilution from MPN tables that are based on the mathematical approaches by Halvorson and Ziegler (1993) (Gomez-Ullate *et al.*, 2008).

The MPN was particularly useful for low concentrations of organisms (<100/g), especially in milk and water, and for those foods whose particulate matter may interfered with accurate colony count (Blodgett, 2006). The bacteria are distributed randomly within the sample. The bacteria are separate, not clustered together, and they did not repel each other. Cho *et al.* (2010) compared enumeration of *E. coli* between MPN and plate count (CFU). They found one order of magnitude greater than that in CFU, which the same as a study of Gronewold and Wolpert. (2008). Moreover, MPN analysis used longer time and very complicate process. At present, MPN has a program assists in calculation and can be adjusted for inoculation volume and initial dilution (Woomer *et al.*, 1990). However, it still took longer time than RT-PCR and 3M Petrifilm.

The 3M Petrifilm *E. coli* appropriate to detected low levels of *E. coli* contamination, which results in the same as a MPN (AOAC) (Matner *et al.*, 1990). The 3M Petrifilm plate contain a beta-glucuronidase-specific indicator dye that precipitates a permanent blue halo around *E. coli* colonies, in addition to coliform selective agents found in violet red bile nutrients. Non- *E. coli* coliforms appeared as red colonies with gas bubbles. Vail *et al* (2003) enumerated *E. coli* with 3M Petrifilm Plates compared to standard methods. *E. coli* counts in environmental water samples enumerated with 3M Petrifilm were significantly correlated ($R>0.9$; slope = 0.9-1.0; $P<0.01$) with counts obtained with three commonly-used methods, m-TEC (Becton Dickin- son, Sparks, MD), m-ColiBlue (Hach, Loveland, CO), and Colilert-18/IDEXX Quanti-Tray 2000 (IDEXX, Westbrook, ME). In addition, blue colonies with gas were easily removed from 3M Petrifilm Plates and streaked on other nutrient media to isolate individual close. It is recognized by International recognition which were AFNOR (Association française de Normalisation), AOAC International Official Method of Analysis, International Dairy Federation (FID/IDF) and recognize by country were Australia, Canada, Chile, France, Germany, Japan, Korea, New Zealand, Nordic Countries, Poland, Republic of South Africa, United Kingdom, United States and Venezuela (Anonymous, 2011). Thus, this technique could detect both *E. coli* and coliform. However, 3M Petrifilm method could not be used to detect *Salmonella* spp. whereas RT-PCR could detect and enumerate of both *E. coli* and *Salmonella* spp (Table 6 and Table 7).

These reason supported results of this experiment was RT-PCR could enumeration and detection in very low, low and high contamination value of both pathogens. Besides, the RT-PCR is not specific detected sample e.g. milk, drinking water and foods, but it could be detected in soil, vegetable, feces, organic fertilizer and other natural samples. But it necessarily improved primer for specific pathogens.

4.1.3 Percentage of the sensitive and specific method of 3M Petrifilm and RT-PCR compared to BAM method

The detection of *E. coli* and *Salmonella* spp. using different test methods were BAM, 3M Petrifilm and RT-PCR. The sensitivity and specificity of various tests in the detection of *E. coli* and *Salmonella* spp. in 100 agricultural samples compared

with the BAM method (reference method) are presented in Table 9. The RT-PCR has 100% sensitivity in the detection of both *E. coli* and *Salmonella* spp. but low specificity with the value of 2.78% and 1.33%, respectively. Incontrast, 3M Petrifilm has high specificity (97.27%) but low sensitivity (37.50%). The results were consistent with the results of Harman and Hannines (2006). They evaluated detection volume of *E. coli* in 110 water samples. They used seven methods which were Lactose Tergitol-7a agar method, MF m-Endo Agar LES, Colilert.-18, Readycult Coliforms 100, Water Check 100, 3M Petrifilm E.coli/Coliform and DryCut Coli.Coliform. They used Lactose Tergitol-7a agar method as the reference method. The results showed that the 3M Petrifilm E. coli/Coliform has a low sensitivity (37.5%), but high specificity (90.90%). The high sensitivity resulted in almost false negative result while low sensitivity resulted in high false positive (Lemon and Gardner, 2008). This data showed high sensitivity (Table 9) to demonstrate that DNA extraction with this technique had no problem, DNA template were sufficient in purities and/or that not interfere with the assay (Cambell and Wrigth, 2003). The reason for low sensitivity of RT-PCR detection by the initial protocol is not clear; decreased sample volumes may increase cell lysis and DNA extraction efficiency and/or reduce concentrations of PCR inhibitors (Huijsdens *et al.*, 2002; Bhagwat 2003; Corless *et al.*, 2000). Additionally, optimum conditions of amplication, interference from vegetable compounds and method for cultural enrichment (Ibekwe *et al.*, 2002; Made *et al.*, 2004).

For the reason of low specificity, it may lead to false positive signal from closely DNA, precursors and genomic sequence (Chen *et al.*, 2005). Signatures are generally based on conserved gene regions, they often fail to take into account all of the variation within a target set of organisms. This may be because the signatures were developed using sequence data from a handful of strains, rather than a thorough study of all strains publicly available at an ever-increasing rate, there is great benefit in re-evaluating clinically-used DNA signatures regularly. When new sequence data leads to false negative predictions for a signature, one of two represent recently recognized variation that has been around since the time the signature was published, or new variation, the results of mutation and natural selection. In either case, an improved or additional signature should be designed. High false positive do not

necessarily indicate a bad DNA assay. In clinical laboratory, a signature with high sensitivity but perhaps low specificity may be preferred over a test with lower sensitivity in case where the putative pathogen requires immediate treatment or may spread quickly (Lemmon and Gardner, 2008).

Table 9 Sensitivities and specificities of various test methods for detection of *E. coli* and *Salmonella* spp. in 100 samples using BAM method as a reference.

Type of bacteria	Test method	Total outcome	Result of BAM method			Sensitivity (%)	Specificity (%)
			No. of negative	No. of positive	total		
E. coli	3M Petrifilm	negative	35	40	75		
		positive	1	24	25		
		total	36	64	100	37.50	97.22
	RT-PCR	negative	1	0	1		
		positive	35	64	99		
		total	36	64	100	100	2.78
Salmonella spp.	RT-PCR	negative	1	0	1		
		positive	75	24	99		
		total	76	24	100	100	1.33

4.1.4 Comparison of procedure steps, time consuming, and budget application among conventional, 3M Petrifilm, and RT-PCR techniques

The comparison of BAM, 3M Petrifilm and RT-PCR methods of *E. coli* enumeration and, procedures, time consuming, and budget results showed that BAM is the longest time (73-121 h), highest number of steps and each step took longer time than other methods. 3M Petrifilm has two steps, and shorter time (49 h) than BAM method, however it used longer time than RT-PCR method (4.3 h). However, to compare budget for 1 sample and 3 replications, RT-PCR cost more than other methods which was 742.01 Bahts (Table 10). However, when consider management and time-saving, the RT-PCR method could be well managed for many samples in

one day because it could be applied for detection and enumeration in 96 wells a time or about 24 samples within 4.3 h. This method also helps to reduce material, area, labor, time and device. Omicciololi *et al.* (2009) developed a multiple PCR-based platform for the simultaneous detection of the widespread milk-associated pathogens *Salmonella* spp., *Listeria monocytogenes* and *E. coli* O157. It was a detection limit of 1 CFU for each pathogen in a total of five 25 mL aliquots of raw milk and duration of two working days. The assay represents an alternative approach for the qualitative detection of the cited bacteria species, suitable for a relatively inexpensive screening of several milk samples, reducing the turnaround time and the workload.

For *Salmonella* spp., comparison of BAM and RT-PCR method, indicated that BAM method have total time for detection of 49 h which was more than the RT-PCR (4.3 h). Steps of testing in both two methods are three steps. But, budget for 1 sample and 3 replications of BAM was 338.73 Bahts which was cheaper than that of RT-PCR (724.01 baths). Catarame *et al.* (2006), they compared BAM and RT-PCR for the detection of *Salmonella* in Irish beef, chicken, pork and turkey. They found that developed 16s rRNA gen-based RT-PCR assay demonstrated comparable specificity and sensitivity to the currently used standard culture method but was considerably more rapid. This method dramatically reduces the time required to detect *Salmonella* in agricultural samples in comparison with the standard culture method (BAM). In additional, some selective agents employed are expensive, toxic and unpleasant to use (Catarame *et al.*, 2006). Cloak *et al.* (1999) compared BAW and RV medium results that BAW is the most suitable enrichment broth as it allowed the greatest rate of cell growth and is the enrichment broth of choice for use in rapid-method experiments. Other studies found that methods involving both nonselective (pre-enrichment) and selective broth-culture steps detected a significantly higher percentage of food samples contaminated with *Salmonella* than direct plating or methods involving direct selective-broth enrichment, without prior pre-enrichment (Gast, 1993). The elimination of a pre-enrichment step increases the overall speed of the detection method. For this study, time saving could be of benefit when information on the presence of *Salmonella* in vegetables export is reported rapidly because it is easily rotten.

Table 10 Working steps and time consuming of *E. coli* and *Salmonella* spp. detected by BAM and RT-PCR methods in one sample with triplicate.

Bacteria	Test methods	Steps	Time (h)	Total (h)	Budget (Bahts)
E. coli	conventional	preparation sample	1		
		presumptive test	24-48		
		confirmed test	24-48		
		completed test	24	73-121	281.89
	3M Petrifilm	preparation sample	1		
		analysis	48	49	582.94
	RT-PCR	preparation sample	1		
		DNA extraction	0.3		
		RT-PCR test	3	4.3	724.01
Salmonella spp.	conventional	preparation sample	1		
		Enrichment	24		
		Isolation	24	49	338.73
	RT-PCR	preparation sample	1		
		DNA extraction	0.3		
		RT-PCR test	3	4.3	724.01

Our study was in agreement with Schneider *et al.* (2002) who used RT-PCR for detection of the genus Salmonella contaminated in foods. They found that RT-PCR is time saving compared to the conventional method. The time to get the result is less than 24 h in case of a negative result and up to 6 days in the case of a positive result (due to the reactions necessary to confirm the positive result in conventional method). Moreover, it has a much higher sensitivity and specificity as well as greater time saving. It does not require any molecular biology skills, or the use of additional chemicals.

4.2 Quantification of the contamination of *E. coli* and *Salmonella* spp. in animal manure compost and effect on dynamic population of both species

4.2.1 Dynamic population of *E. coli* and *Salmonella* spp. in compost and non compost poultry layer and cow manures

In both composted materials, *E. coli* subsequent highly significant decreased with time ($P<0.01$), its population continually decreased until reached no contamination at 70 days and 84 days after incubation, respectively. On the other hand, non-composted poultry layer, *E. coli* increased from 2.78 to 4.02 log MPN/g but it decreased from 6.40 to 4.69 log MPN/g in those of cow feces. For *Salmonella* spp., it was not found at 56 and 70 days after incubation in poultry layer and cow feces, respectively, whereas the non-composted materials had contaminated until the end of experiment at 140 days (Table 11). Factors affecting the survival of *E. coli* in non-composted poultry layer seemed to be complex correlation of materials quality, especially the remaining nutrients which were sufficient for the growth and propagation of *E. coli*. Moreover, the composition of poultry layer was 70% husk which it might maintain sufficient moisture. Consequently, the temperature might be the most effective factor for survival of the bacteria.

Fluctuation of *Salmonella* spp. population had not appeared in both non-composted poultry layer and non-composted cow feces, for example at 14, 28 to 56 days and at 21, 42, 56, 112 and 126 days, respectively (Table 11). High temperature and dry weather might be the effects affecting reduction of *Salmonella* spp., the *Salmonella* spp. decreased until undetectable, but it can recover and increase again when the increasing of high moisture in surrounding environment from rainfall (Chandler and Craven, 1980). The survival of pathogen during composting depends upon various factors such as storage, temperature, compost amendments, moisture, redox potential, pH, physical composition and inter microbial competition (Turner, 2002). There are only a few studies on the survival of *E. coli* and *Salmonella* during composting of manure and sludge mixed with municipal waste. Himathongkham *et al.* (1999) had evaluated the survival of *E. coli* O157:H7 and *S. typhimurium* cultures stored at 4, 20, and 37°C. They observed an experimental linear destruction for *E. coli* O157:H7 and *S. typhimurium* in which decimal reduction times ranged from 6 days to 3 weeks in manure and from 2 days to 5 weeks in manure slurry. Increasing

of electrical conductivity are more likely related to the poration rate and physical damage of cell membranes, while decreasing pH is more likely to be related to changes in the cytoplasmic conditions due to the osmotic imbalance caused by the poration (Vega-Mercado *et al.*, 1996; Petric and Selibasic, 2008).

Additionally, similar report of You *et al.* (2006) supported our finding that *Salmonell*a serovar Newport in manure and manure-amended soils decreased with time. Log reduction time was 14 to 32 days for 1 log, 28 to 64 days for 2 log and 42 to 96 days for 3 log inoculated concentrations. Most-probable-number monitoring data indicated that the organic persisted for 184, 332 and 405 days in manure, manure-amended nonsterilezed soil and manure-amended sterilized, respectively. Including, Kudva *et al.* (1998) studied the survival of *E. coli* O157:H7 in ovine or bovine manure and manure slurry. Results showed that *E. coli* O157:H7 survived in manure for 21 months, and concentrations of bacteria recovered ranged from $<10^2$ to 10^6 CFU/g at different times over the course of the experiment. *E. coli* O157:H7 survived best in manure incubated without aeration at temperatures below 23°C, but it usually survived for shorter period of time than it survived in manure held in the environment. The bacterium survived at least 100 days in bovine manure frozen at -20°C or in ovine manure incubated at 4 or 10°C for 100 days, but under all other conditions, the length of time that it survived ranged from 24 h to 40 days.

The number of *E. coli* was decreased in non-composted cow feces (packed in plastic bag), might be due to dry materials because moisture is a necessity for growth, propagation activity and survival of bacterial pathogen. Pathogenic bacteria need moisture to break down solid food to the point where they can absorb it. For this reason, pathogen bacteria could not grow well in a material with a moisture content of less than 15% (Integarted, 2010). Himathongkham and Riemann (1999) reported that *E. coli* O157:H7 and *S. typhimurium* were reduced by 8 log units in chicken manure followed drying the manure to a moisture content of 10%. In addition, packing of cow feces in plastic bag influenced O_2 decrease which sustain growth for the bacteria (FDA U.S., 2001; Seafood Network Information Center, 2010). *E. coli* was increased from 2.78 log MPN/g up to 4.02 log MPN/g in non-composted poultry layer (Table 11).

Table 11 Dynamic population of *E. coli* and *Salmonella* spp. in each periodical sampling of composted and non-composted poultry layer and cow feces.

Timing (days)	Population of *E. coli* (log MPN/g)[1/]				Detection of *Salmonella* spp.[2/]			
	M1	M2	M3	M4	M1	M2	M3	M4
1	3.70±1.26	2.78±0.03	5.98±0.67	6.40±0.52	+	+	+	+
3	2.91±0.05	2.77±0.01	5.63±0.77	5.18±0.92	+	+	+	+
5	2.87±0.01	2.77±0.01	5.67±0.06	6.45±0.01	+	+	-	+
7	2.88±0.01	2.75±0.00	4.97±0.25	5.86±0.01	+	+	+	+
14	2.89±0.07	2.74±0.00	4.09±0.56	5.02±0.75	+	-	-	+
21	2.88±0.02	5.43±1.57	4.74±1.07	5.41±0.28	+	+	+	-
28	2.90±0.05	3.61±0.01	4.31±0.74	5.73±0.57	+	-	+	+
42	2.85±0.00	2.71±0.00	3.25±0.38	5.59±0.27	+	-	+	-
56	3.53±0.02	2.69±0.00	2.90±0.01	5.57±1.04	-	-	+	-
70	0.00±0.00	2.99±0.36	4.50±0.13	5.40±0.86	-	+	-	+
84	0.00±0.00	3.77±1.07	0.00±0.00	5.71±0.27	-	+	+	+
98	0.00±0.00	3.34±0.32	0.00±0.00	5.63±0.38	-	+	-	+
112	0.00±0.00	2.81±0.01	0.00±0.00	4.53±0.25	-	+	-	-
126	0.00±0.00	3.54±0.80	0.00±0.00	4.73±0.59	-	+	-	-
140	0.00±0.00	4.02±1.31	0.00±0.00	4.69±1.25	-	+	-	+

[1/] Each value represents mean± standard deviation of three replication. M1 is composted poultry layer, M2 is non-composted poultry layer, M3 is composted cow feces and M4 is non-composted cow feces.

[2/] The symbol "+" is positive test and "-" is negative test.

4.2.2 Factors affecting correlation efficient on dynamic population of *E. coli* and *Salmonella* spp. in composted and non composted poultry layer and cow feces

Carbon: nitrogen ratio (C:N)

C:N of composted poultry layer and composted cow feces significantly decreased with time of composting ($P<0.01$). The C:N ratio of composted cow feces decreased at the higher rate than poultry layer and those of non compost materials. It had decreased from 15:1 from the beginning to 8:1 at the final of composting, while

poultry layer composted decreased from 21: 1 to 12: 1. In non-composted poultry layer, C:N ratio decreased at the same rate as composted one. However, the C:N ration of non-composted cow feces was not significantly different ($P>0.05$) in all sampling periods. Although, decreasing of C:N ratio in composted poultry layer and cow feces correlated with decreasing of *E. coli* ($P<0.01$) with r^2 of 0.60 and 0.71, respectively (Table 12) but in those of non-composted materials were not have significant correlation ($P<0.05$) (Figure 3). Erickson *et al.* (2009a) reported that *E. coli* O157: H7 survived for significantly ($P<0.05$) longer periods of the time in 41:1 C:N systems than in 30:1 or 20:1 system, which *Salmonella* spp. had similarly affected (Erickson *et al.*, 2009b). The C:N ratio of 20:1 to 40:1 serve as nutrient for the types of microbes that produce the most desired form of compost. Carbon amendment varies in their availability to microorganism. Hansun *et al.* (1993) referred Haque and Vanderpopyliere (1994) reported that most of the nitrogen lost from during composting was via ammonia volatilization. Additionally, an N loss from nitrification was active (Meunchang *et al.*, 2005). They also mentioned that finished litter compost will have less nitrogen and mineralizes more slowly than the original litter nitrogen. Most of the carbon was believed to have evolved as CO_2 during composting. Ammonium is naturally generated by indigenous microorganism in moist chicken manure at appropriate temperature and can cause a significant reduction of non-spore forming pathogens in stacked manure. Himathongkham and Riemann (1999) reported that ammonia gas in an amount of 1% of the manure wet weight; *S. typhimurium* and *E. coli* O157:H7 were reduced by 8 log units, *L. monocytogenes* by 4 log units.

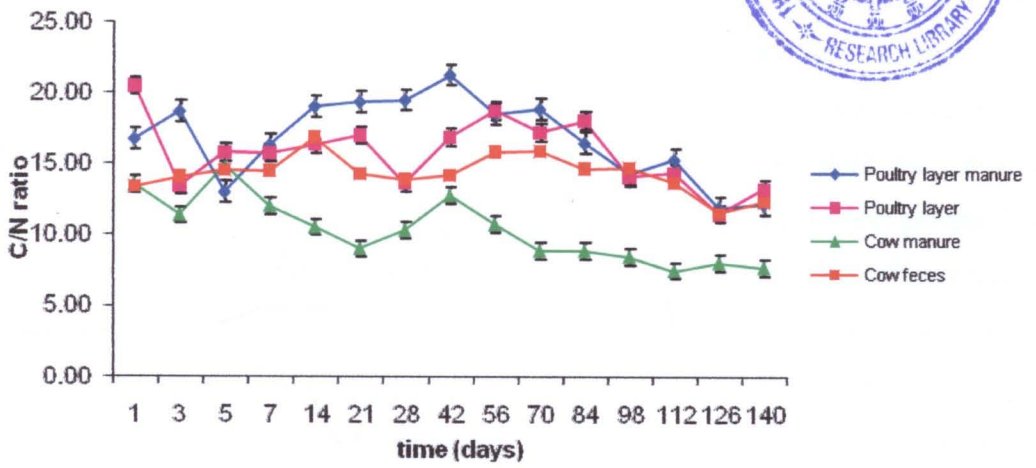

Figure 3 Changing of C:N ratio in composted and non-composted materials, the error bars represent the standard error.

Electrical conductivity (EC)

The EC value was increased from 1.63 to 2.76 dS/m at 3 to 42 days after that it was decreased to 1.98 dS/m (140 days) in composted poultry layer. For the composted cow feces, EC was increased at 1 to 112 days from 1.24 to 3.04 dS/m but after 126 days it was decreased. EC in non-composted materials, was increased at initial 14 days only, after that there was no significant increase (Figure 4). However, increasing of EC in two composted materials was not over level of standard of organic fertilizer, which is appointed by Department of Agricultural (Department of Agricultural, 2005). The r^2 between EC decreasing and population of *E. coli* showed positive correlation, thus EC deceasing affected decreasing of *E. coli*. The negative correlation between EC and population of *E. coli* in composted cow feces was found with r^2 equal to -0.53. This indicated that EC was increased while *E. coli* population decreased according with time. On the other hand, non-composted poultry layer and cow feces had no correlation between EC and *E. coli* (Table 12). The EC value were reflected the degree of salinity in compost, indicating its possible phytotoxic/phyto-inhibitory effects on the growth of plant if applied to soil. EC values increased from 3.50 to 4.31 ds/m during the composting process. These high values might be due to the effect of the concentration of salts as a consequence of the degradation of organic matter (Cambell *et al.*, 1997) Salinity has a negative effect on microbial biomass as

to slow microorganisms growth (Tokashvnad *et al.*, 2006). The EC in the compost was 28.1 ms/cm and was much greater than the EC of 4.8 dS/m obtained by Van Heerden *et al.* (2002) referred by Contreras-Ramos *et al.* (2004) in citrus supplement with calcium hydroxide composted for three months. The high EC, however, appeared not to have affected microbial activity, as witnessed by the production of CO_2. Large EC in compost might lyses microbial cells (Brock *et al.*, 1994 referred by Contreras-Ramos *et al.* (2004)). Santaria-Romero and Ferrera-Cerrato (2001) referred by Contreras-Ramos *et al.* (2004) reported that salt concentration above 8 dS/m negatively affected the microorganisms' population as well as biotransformation of organic matter. Salt causes the high osmotic pressure, which has a detrimental effect on a cell by causing the passage of water from within a cell to the outside. For a cell that is in a hypertonic solution, cellular water pressure through the plasma membrane to the high solution concentration. The result of the osmotic loss of water is plasmolysis in which the plasma membrane shrinks and pulls away from the cell wall (Tsong, 1990)

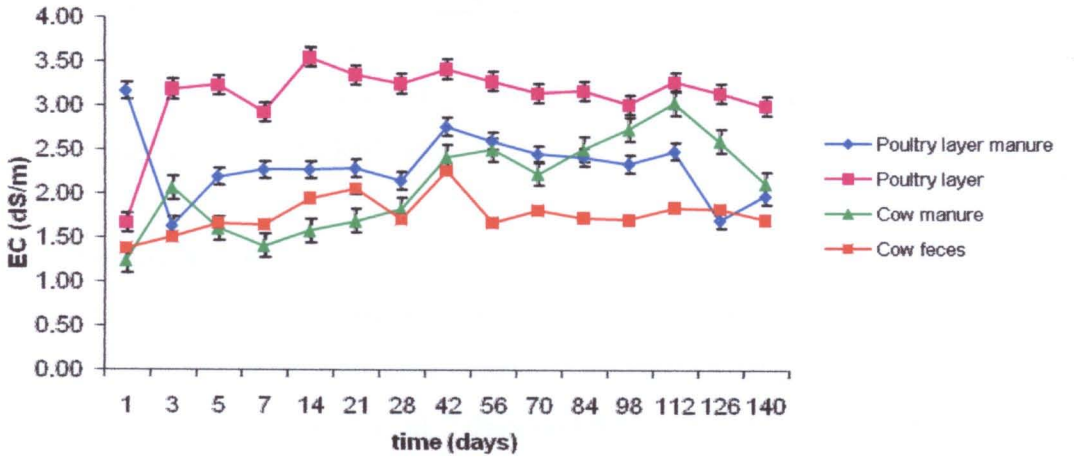

Figure 4 Changing of EC of composted and non-composted materials, the error bars represent the standard error.

Organic matter (OM)

OM was decreased from 48.47% to 40.50% in composted poultry layer, and 30.09% to 26.43% in composted cow feces. Although, the OM in both non-composted materials were decreased but these reduction values were more than

composted materials (Figure 5). Decreasing of OM in composted materials showed a significant correlation with decreasing of *E. coli* population with r^2 of 0.59% (Table 12). Reduction of OM caused of mesophilic and thermophilic microorganism to decompose such as *Thermoactinomycetes* sp., *Aspergillus* sp., *Thermospora* sp., *Streptomyces* sp., and *Bacillus* sp. (Gaur, 1987).

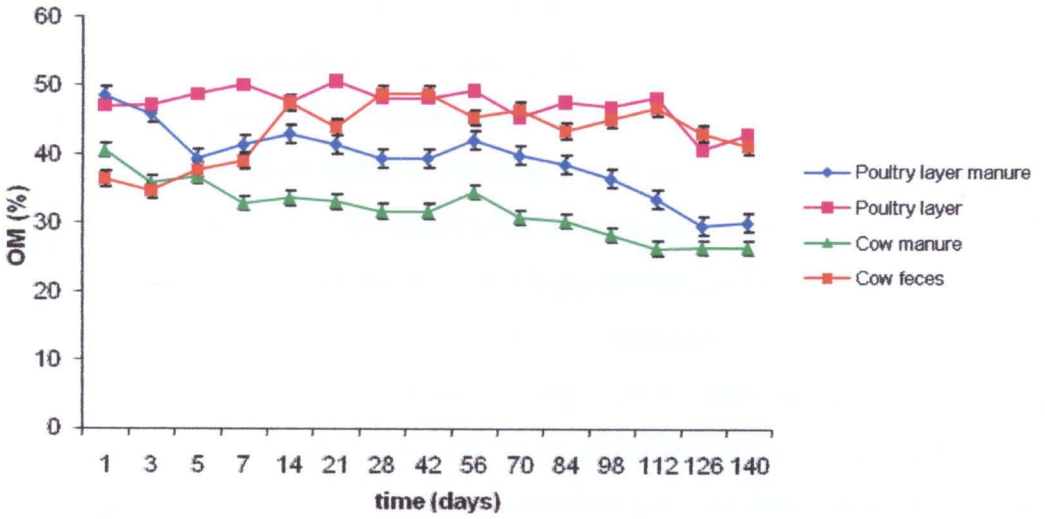

Figure 5 Changing of OM in composted and non-composted materials, the error bars represent the standard error.

Temperature

Changing of temperature showed significantly different ($P<0.01$) in composted poultry layer and cow feces. The highest temperature in composted poultry layer went up to 66.7°C at 7 days and 60.3°C at 14 days for composted cow feces. Thereafter, the temperature had continuously declined until stable at 30.1 to 37.5°C after 42 days of piling. Temperature in both non-composted materials was not significant variation and did not have a peak of high temperature. It was 37.4 to 25.4°C and 30.8 to 25.6°C, respectively (Figure 6). Highly significant correlation between increasing temperature and reduction of *E. coli* population in both composted materials and non-composted cow feces were 0.36, 0.79, and 0.41, respectively (Table 12). The raising temperature within the compost pile is decomposable activities of mesophilic bacteria and thermophilic bacteria. The mesophilic bacteria required

optimum temperature at 10 to 45°C for growth and propagation while that of thermophilic bacteria was at 45 to 70°C (Jundendoung, 2010. *E. coli* and *Salmonella* spp. could grow between 6.5 to 49.4°C, 5.2 to 46.2°C, respectively (FDA U.S., 2001; Seafood Network Information Center, 2010). The results of this experiment showed higher temperature than optimum growth temperature for *E. coli* and *Salmonella* spp. for growth. Thus, this caused both types of pathogens in compost to reduced and eventually died. Turner (2002) reported that temperatures in excess of 55°C for 2 h are required for inactivation of *E. coli*. You *et al.* (2006) studied survival of *Salmonella enteric* serovar Newport in manure and manure-amended soils and found that it decreased by up to 400% in the first 1 to 3 days following inoculation and a trend of decline followed. After that its log reduced was 14 to 32 days for $\log_{10}$, 28 to 64 days for 2 $\log_{10}$, and 42 to 96 days for 3 $\log_{10}$ declines in the organisms' population from initially inoculated concentrations. Temperature is also an important consideration, with higher temperature, e.g., 35°C reduced pathogen survival. Temperature was determined to be the most important factor influencing pathogen survival in sludge-amended soils, with increasing survival times being a function of decreasing temperature (Islam *et al.*, 2004c). The underlying mechanism for bacterial inactivation at high temperatures could be attributed to an increased fluidity of the cellular membrane, there by compounds, e.g., ammonia or organic acids into the cytoplasm (Demceyer and Henderick, 1967).

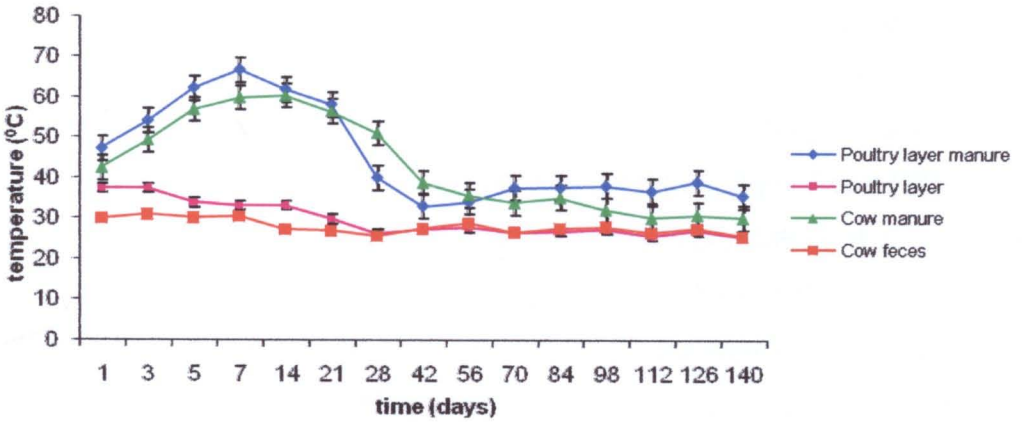

Figure 6 Changing of temperature in composted and non-composted materials, the error bars represent the standard error.

pH values

The pH values in composted poultry layer and cow feces were decreased from 7.5 to 7.0 and 7.7 to 6.2, respectively by pH in non-composted materials were lower than composted ones, thoroughly ($P<0.01$) (Figure 7). Decreasing of *E. coli* contamination was highly significant correlation with the changing of pH in composted poultry layer and cow feces by r^2 were at 0.65 and 0.77, respectively (Table 12). In contrast, non-composted of both materials showed no correlation ($P>0.05$) with pH values. A decrease in pH of composting was caused by the volatilization of ammoniacal nitrogen and the H^+-released as a result of microbial nitrification process by nitrifying bacteria (Eklind and Kirchmann, 2002). The large quantities of carbon dioxide that are given off during the composting process with sufficient aeration might also be responsible for the decrease in pH value, because it led to the acidification of the mixture once buffering effect of the bicarbonate had diminished (Caceres *et al.* (2006) referred Petric and Selimbasic, 2008). Changes in pH will induce changes in the protonation of biologically active molecules on surface of the conductivity of the cell membrane with a subsequent change in the sensitivity of organisms to environmental or processing factors (Vega-Mercada *et al.*, 1996). The role of pH in the survival of microorganisms is related to the ability of the organisms to maintain the cytoplasmic pH near neutrality. Membrane permeability increased due to formation of pores in the cell wall during the pulsed electric fields treatment and the osmotic imbalance around the cell. Thus, a reduction in the cytoplasmic pH may be observed because a higher number of H^+ are available composed with a neutral pH. The change in pH within the cell may induce chemical modifications in fundamental compounds such as DNA or ATP as discussed by Wigging (1975). However, *E. coli* could grow in condition of pH 4 to 9 (FDA. U.S., 2010), but decreasing of pH in composting pile affected population of *E. coli*. The study of Larney *et al.* (2003) reported that the pH values decreased to 7.3 on days 14 for the straw-bedded manure and pH 6.8 on day 14 for the woodchip-bedded manure, which may have enhanced the pathogen reduction effect. Franz *et al.* (2005) reported the roughage type, but not dietary crude protein level, influences the survival capabilities of both *E. coli* O157:H7 and *S.* serovar Typhimurium. Decline of *E. coli*

O157:H7 was faster in manure derived from a pure straw diet (higher pH and higher fiber content) than in manure maize silage diet (lower pH and lower fiber content). Persistence of *S.* serovar Typhimurium in manure was better than that of *E. coli* O157:H7.

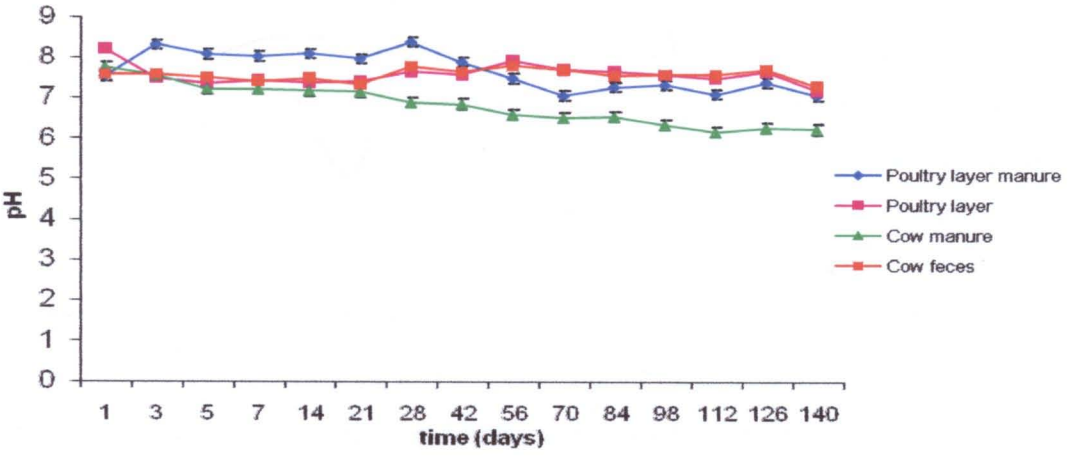

Figure 7 Changing of pH in composted and non-composted materials, the error bars represent the standard error.

Table 12 Correlation (Pearson) of *E. coli* in poultry layer and cow manure both in composting and non-composting on various factors at 1 to 140 days.

Composting methods[1/]	Factors[2/]				
	C/N ratio	$EC_{(1:10)}$	%OM	Tm (°C)	pH
M1	0.60**	0.38**	0.50**	0.36**	0.65**
M2	-0.11^{ns}	-0.45^{ns}	-0.18^{ns}	-0.12^{ns}	-0.28^{ns}
M3	0.71**	-0.53**	0.59**	0.79**	0.77**
M4	0.31*	-0.02^{ns}	0.10^{ns}	0.41**	0.12^{ns}

[1/] M1 is composted poultry layer, M2 is non composted poultry layer, M3 is composted cow feces and M4 is non composted cow feces.

[2/] The symbol "ns" is no significance, "*" is significantly different at the $P \leq 0.05$, and "**" is highly significantly different at the $P \leq 0.01$. The symbol "-" is negative correlation.

4.3 Reduction of *E. coli* and *Salmonella* spp. in export vegetables with application of cow manure compost

4.3.1 Contamination of *E. coli* in soil and vegetables under manures application

There was not *E. coli* contaminated in soil samples at 7th to 35th days after planting in composted cow manure and without manure treatments. On the other hand, application of non-composted cow manure was highly contamination of *E. coli* in soil samples at 3.80, 4.20, 4.49, and 4.26 log MPN/g in each subsequence sampling date, respectively. However, at 49th day *E. coli* was contaminated in all soil samples treatments. It was higher in treatment applied with non-composted cow manure (4.26 log MPN/g) and imperceptible of bacterial population was found in the composted and without manures. Application of non-composted cow manure resulted in *E. coli* contamination on vegetables at 2.61 log MPN/g, whereas no contamination was found in using composted cow manure and without manure treatments (Table 13).

Table 13 Effect of manures application on contamination of *E. coli* in soil and vegetables.

Organic fertilizer[1/]	*E. coli* contaminated in soil (log MPN/g)[2/]				*E. coli* contaminated in plants (log MPN/g)[2/]
	7 day	21 day	35 day	49 day	
WCCM	0.00±0.00	0.00±0.00	0.00±0.00	0.57±0.30	0.00±0.00
CCM	0.00±0.00	0.00±0.00	0.00±0.00	0.63±0.34	0.00±0.00
NCCM	3.80±0.44	4.20±0.53	4.49±0.39	4.26±0.15	2.61±1.48
F-test	**	**	**	**	**

[1/] WCCM is without composted cow manure, CCM is composted cow manure, and NCCM is non-composted cow manure.

[2/] Each value represents mean± standard deviation of three replication. The symbol "**" is highly significant difference at the $P \leq 0.01$

4.3.2 Interaction between types of vegetables and manures application on contamination of *E. coli*

There was an interaction between types of vegetable and application of non-composted cow manure. Mostly, influence of *E. coli* contamination was found in soil

samples and vegetables at all sampling periods, but that of peppermint. Contamination found on asparagus, stink weed, and peppermint had less than in the soils, 2.78, 2.78, and 0.00 log MPN/g, while kale and coriander had not significant different from soils at the population of 3.11, 4.38, and log MPN/g, respectively. Indeed, vegetables grown under treatments of composted manure and without manure, showed absolutely disinfection of *E. coli* (Table 14).

Table 14 Interaction between types of vegetables and manures application on contamination of *E. coli*.

Type of vegetables	Organic fertilizer[1/]	*E. coli* contaminated in soil (log MPN/g)[2/]				*E. coli* contaminated in plants (log MPN/g)[2/]
		7 day	21 day	35 day	49 day	
Asparagus	WCCM	0.00±0.00	0.00±0.00	0.00±0.00	3.57± 0.00	0.00±0.00
	CCM	0.00±0.00	0.00±0.00	0.00±0.00	3.14± 0.01	0.00±0.00
	NCCM	3.55±0.14	4.05±0.01	4.55±0.35	4.62±0.15	2.78±0.01
Kale	WCCM	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	CCM	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	NCCM	3.89±0.30	4.30±0.44	4.45±0.02	4.25±0.19	3.11±0.01
Coriander	WCCM	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	CCM	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	NCCM	3.56±0.14	3.64±0.14	4.93±0.00	4.45± 0.01	4.38±0.01
Stink weed	WCCM	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	CCM	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	NCCM	4.46±0.01	3.92±0.30	4.44±0.02	4.69±0.15	2.78±0.01
Peppermint	WCCM	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	CCM	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	NCCM	3.55±0.14	4.62±0.16	4.06±0.20	3.29±0.20	0.00±0.00
	F-test	**	*	*	**	**

[1/] WCCM is without composted cow manure, CCM is composted cow manure, and NCCM is non-composted cow manure.

[2/] Each value represents mean± standard deviation of three replication. The symbol "*" is significant ($P \leq 0.05$) and "**" is highly significant ($P \leq 0.01$).

4.3.3 Contamination of *Salmonella* spp. in soil samples and on vegetables

Contamination of *Salmonella* spp. exhibited similar to that of *E. coli* had appeared under vegetables grown in soil samples treated with non composted manure at every sampling date. More evidences had shown that types of vegetable grown at 7th day after planting were not ceased its contamination in the tested soils. Fluctuation of contamination was occurred thereafter, such as at 21st day, there were found the contamination in soil samples of asparagus, kale and coriander in all treatments, while soil of stink weed and peppermint was not had contamination. At 35th day, it was also found to contaminate in soil samples of asparagus, kale and stink weed treated with composted cow manure. Finally, at 49th day it was found to contaminate even in all soil samples of control. Composted cow manure treatment could be detected contamination in soil samples of asparagus, kale and peppermint, except for coriander and stink weed. The contamination of *Salmonella* spp. on vegetables at harvesting, the use of non-composted cow manure caused contamination on four types of vegetables except peppermint. Nevertheless, using of composted cow manure, contamination was not detected on all vegetables. Although in the control treatment, *Salmonella* spp. contamination had been found on coriander but there was not on the rests (Table 15).

Disinfection of *E. coli* and *Salmonella* spp., in soils and vegetables in this experiment, especially in the treatments applied with composted manure are surely due to low or not contamination of these microbes in the composted manure processing as mention above. Although, some sample of soil and vegetable had contamination, it might be came from natural water irrigation or other sources such as the drops of passing wild animals around area (Fykson, 2010; Ingham *et al.*, 2004). Mainly, decontamination of these bacteria found in composted manure and control treatments and they were not significant increased in the treatment of non composted one, indicated that the bacteria could not grow in alien environment. Naturally, they familiar with growing in appropriate high content of organic matter and suitable environment, e g; moisture, pH and temperature (FDA U.S., 2001; Seafood Network Information Center, 2010). *E. coli* and *Salmonella* spp. was found to contaminate in all vegetables of non-composted cow manure application more than other methods, excepted peppermint (not detected in all methods). This might be due to the harvest

methods of the plants are different, i.e., harvested by collecting the whole plant which can be easily contaminated and harvested by outing the shoot in the case of peppermint.

Table 15 Contamination of *Salmonella* spp. contaminated in soil and plants applied with composted cow manure and non-composted cow manure.

Type of vegetables	Organic fertilizer[1/]	*Salmonella* spp. contaminated in soil (log MPN/g)[2/]				*Salmonella* spp. contaminated in plants (log MPN/g)[2/]
		7 day	21 day	35 day	49 day	
Asparagus	WCCM	+	+	-	+	-
	CCM	+	+	+	+	-
	NCCM	+	+	+	+	+
Kale	WCCM	+	+	-	+	-
	CCM	+	+	+	+	-
	NCCM	+	+	+	+	+
Coriander	WCCM	+	+	-	+	+
	CCM	+	+	-	-	-
	NCCM	+	+	+	+	+
Stink weed	WCCM	+	-	-	-	-
	CCM	+	-	+	+	-
	NCCM	+	+	+	+	+
Peppermint	WCCM	+	-	-	+	-
	CCM	+	-	-	+	-
	NCCM	+	+	+	+	-

[1/] WCCM is without composted cow manure, CCM is composted cow manure, and UCCM is non-composted cow manure.

[2/] The symbol "+" is detected and "-" is not detected.

Thus, methods of harvesting are also influenced of contamination. It was easily contaminated in root or bulb, tuber and com (part under the soil) more than seed or pod. Differences in surface morphology and metabolic function of leaves, stems, florets, fruits, root, and tuber provide a wide range of diverse ecological niches selective for specific species or groups of microorganism. The results in this

experiment showed the contamination of both pathogens on coriander more than other vegetables. Thus, type of plant has an influence on the contamination of pathogens. Mukherjee *et al.* (2004) reported that organic lettuce had approximately 22.4% of samples positive for *E. coli* and this level was significantly higher than that of leafy greens, cabbage, tomatoes, green peppers, cucumbers, and broccoli. There were *E. coli* not detected in strawberries, apples, summer squash, raspberries, cantaloupes, carrots, beets, and kohlrabi. But *Salmonella* was isolated from one organic lettuce and one organic green pepper. Islam *et al.* (2004c) studied the fate of *E. coli* O157:H7 in manure compost-amended soil and on carrot and green onions grown in an environmentally-controlled growth chamber. *E. coli* O157:H7 cell numbers decreased within 64 days by 3 log CFU/g in soil and soil beneath the roots of green onions and by more than 2 log CFU/g on onions. *E. coli* O157:H7 survived better during the production of carrots, with a 2.3 log CFU/g reduction in soil and a 1.7 log CFU/g reduction on carrots within 84 days. This may be due to the presence of high concentrations of antimicrobial phenolic compounds in onions compared to carrot (Sofos *et al.*, 1998 referred Islam *et al.*, 2005). Burnett and Beuchat (2001) reported the number of reported produce-related out breaks per year doubled between the period 1973-1987 and 1988-1992 in the United States.

The results of non-composted cow manure application showed contamination of *E. coli* and *Salmonella* spp. on asparagus, kale, coriander and stink weed. These data were over the level of exporting standard that the Department of Agriculture issued for fresh vegetable export to Norway and EU (European Union), *E. coli* should be lower than 100 CFU/g (2 log CFU/g), *Salmonella* spp. should not be detected in 25g samples. Although units of this experiment (MPN) was not in CFU because using different analytical procedures from the Department of Agriculture. The MPN unit was estimated by statistic based on a process-related attribute while CFU was a measure of viable bacteria number. The enumerated contamination in MPN are greater than those in CFU (Cho *et al.*, 2010). The data showed that if CFU count was applied in this experiment, the value should higher than MPN. Thus, using of non-composted cow manure influenced contamination of *E. coli* and *Salmonella* spp. in vegetables which exceeded the standard of export.

4.3.4 Effect of manures on fresh and dry weight of vegetables

Harvest data (Table 16) for the crops revealed that soil fertilized with composted cow manure provided the highest fresh weight and dry weight of asparagus, kale, coriander and peppermint except stink weed. The results were the same as applied with non-composted cow manure. The lowest fresh and dry weights were obtained from without fertilizer (control). But, the stink weed was not significantly different ($P>0.05$) in all treatments for both fresh and dry weight. At day 49 when asparagus, kale, coriander and peppermint were ready for harvest, the average fresh weights of plants were 10.58, 22.61, 46.84 and 4.99 g when grown with composted cow manure, respectively. The dry weights of these were 1.07, 6.86, 4.17 and 0.74 g, respectively. The fresh weights of these vegetables applied with non-composted cow manure were 16.33, 18.22, 21.90 and 5.10 g, respectively.

These data showed that using of cow manures (both of composted and non-composted cow manure) could obtain the highest fresh weight and dry weight of vegetables. Because of compost or manure promoted growth of vegetables, they are supply many nutrients for crop production, including micronutrients and valuable sources of organic matter. Increasing soil organic matter improves soil structure or tilth, increases the water-holding capacity of coarse-textured sandy soils, improves drainage in fine-textured clay soils, provides a source of slow release nutrients, reduces wind and water erosion, and promotes growth of earthworms and other beneficial soil organisms. The nutrient content of manures varies with animal, bedding, storage, and processing. The approximate nutrient composition of various solid manures, including some composted manures, is presented in Table 17. Fresh, non-composted manure will generally have a higher N content than composted manure (Table 17).

Table 16 Application of manures on fresh and dry weight of vegetables.

Type of vegetables	Organic fertilizer[1/]	fresh weight (g) [2/]	dry weight (g) [2/]
Asparagus	WCCM	5.02±0.59	0.56±0.07
	CCM	10.58±2.40	1.07±0.20
	NCCN	16.33±5.13	1.85±0.38
Kale	WCCM	17.05±0.50	1.34±0.58
	CCM	22.61±1.85	6.86±1.84
	NCCN	18.22±0.89	2.40±0.85
Coriander	WCCM	6.13±0.80	0.60±0.03
	CCM	46.84±2.60	4.17±0.11
	NCCN	21.90±3.30	1.96±0.15
Stink weed	WCCM	27.93±2.26	3.95±0.42
	CCM	26.57±3.22	4.13±0.81
	NCCN	24.18±6.49	4.35±1.42
Peppermint	WCCM	2.23±0.36	0.33±0.05
	CCM	4.99±1.51	0.74±0.22
	NCCN	5.90±0.66	0.84±0.02

[1/] WCCM is without composted cow manure, CCM is composted cow manure and NCCM is non-composted cow manure.

[2/] Each value represents mean± standard deviation of three replication.

However, the use of composted manure will contribute more to the organic matter content of the soil. Fresh manure is high in soluble forms of N, which can lead to salt build-up and leaching losses if over applied. Fresh manure may contain high amounts of viable weed seeds, which can lead to weed problems. In addition, various pathogens such as *E. coli* may be present in fresh manure and can cause illness to individuals eating fresh produce unless proper precautions are taken. Apply and incorporate raw manure in fields where crops are intended for human consumption at least three months before the crop will be harvested (Rosen and Bierman, 2010).

Table 17 Approximate nutrient composition of various types of animal manure and compost (all values are on a fresh weight basis).

Manure type	Dry matter	NH_4^+-N	Total N [1/]	P_2O_5	K_2O
	%	----------kg/ton----------			
Swine, no bedding	18	6	10	9	8
Swine, with bedding	18	5	6	7	7
Beef, no bedding	52	7	21	14	23
Beef, with bedding	50	8	21	18	26
Dairy, no bedding	18	4	9	4	10
Dairy, with bedding	21	5	9	4	10
Sheep, no bedding	28	5	18	11	26
Sheep, with bedding	28	5	14	9	25
Poultry, no litter	45	26	33	48	34
Poultry, with litter	75	36	56	45	34
Turkey, no litter	22	17	27	20	17
Turkey, with litter	29	13	20	16	13
Horse, with bedding	46	4	14	4	14
Poultry compost	45	1	17	39	23
Dairy compost	45	<1	12	12	26
Mixed compost Dairy/Swine/Poultry	43	<1	11	11	10

[1/] Total N = Ammonium-N plus organic N

Sources: Modified of *Livestock Waste Facilities Handbook*, 2nd ed., 1985, Midwest Plan Service; *Organic SoilAmendments and Fertilizers*, 1992, Univ. of Calif. #21505 referred Rosen and Bierman (2010).