EU No 1774/2002; experiences with process validation of Biowaste composting & digestion in The Netherlands

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EXECUTIVE SUMMARY

The Netherlands implemented source separation of municipal garden and kitchen waste by law in 1994, resulting in 1,5 million tons each year. This is processed in 23 facilities. These plants can be classified, using 5 technologies; Bühler/GECO, Tunnel, VAR, PACOM, Biocel. All systems have extensive temperature measurements and quality systems. Regulation (EC) No 208/2006 allows process validation to comply with Regulation (EC) No 1774/2002. This study is focussing on three microbiological requirements: 1. a 5log10 reduction for Enterococcus Faecalis 2. Enterococcus or E.Coli < 1000 cfu/g after sanitation, 3. compost: Salmonella not detected in 25 g. Two validation systems were discussed, spot test analysis and direct process evaluation. We decided to use the spot test analysis which can produce information about requirements 1, 2 and 3 and is cheaper. Enterococcus Faecalis is estimated as Enteroccocus, which is a wider group but the same as mentioned in requirement 2. Direct process evaluation can use specific microbiological strains such as E. Faecalis and viruses and seems to show higher reduction levels (about 2log10 higher) but conditions in the probes can differ from surrounding biowaste in the real process.
Three studies using spot test analysis were carried out in 2006:
First: A pilot study in week 7 and 12 (winter), covering the 5 systems used in The Netherlands, covering biowaste from ± 100 municipalities. In all the untreated biowaste the same level of E.Coli and Enterococcus was found (6,2 log units/gr). This is somewhat low for demonstrating a 5log10 reduction. 67% of the samples after sanitation or from fresh compost could meet the standards for E.Coli and Enterococcus (<1000 cfu/g). In plant number 10 (VAR) a 5log10 reduction for Enterococcus was demonstrated. To reduce the risk of recontamination during the sampling, the sampling strategy was simplified.
Second: A study, covering 21 Dutch facilities was carried out in week 22, 25 and 32 (summer). Now the level of Enterococcus in the untreated biowaste was a bit higher although not significant, for all facilities between 6,6 and 7,7 (mean value 7,1 log units/g). This confirms that all Dutch biowaste has the same level of Enterococcus. Over all, the 21 plants demonstrated a 4,7 log units reduction for Enterococcus (7,1→2,4). 15 of the 21 plants showed a reduction of almost 5 log units or more. There was no clear coherence between the used technology (system) and reduction levels. 4 of the 5 technologies were represented in the group of 6 with < 5 log units reduction. Plant number 10 (the best performer in the pilot with > 5 log units reduction) was now in this group of 6. On the other hand, a poor performing facility number 6 (a tunnel system) in the pilot was in this second study one of the best performers. Over all results from 21 plants show lowest Enterococcus values after sanitation before screening (67% of the samples < 1000 cfu/g). Values of Enterococcus in fresh compost are over all higher and inconsistent, indicating regrowth. Results for E.Coli are significant better (after sanitation 76% and in fresh compost 81% < 1000 cfu/g). In all plants (except 1) Salmonella was not detected in the compost.
Third: In addition, at facility number 12 (lowest performer in study 2) a study was carried out with the aim to clarify the inconsistencies in Enterococcus values. When sampling at the right spot during sanitation, 5log10 reduction was demonstrated at this plant. Although temperatures during the first and second stage were > 65 ^{o}C, even 3-6 days 75-80 ^{o}C, Enterococcus was demonstrated on these temperature levels ranging from 1,40 to 3,11 log units/g. The compost, both 1 and 14 days after screening, showed levels ranging from 5,6 to 6,7 log units/g. We assume regrowth and recontamination both take place because of remoistening the compost with leachate. Evaluation of the sampling strategy and cooled transport to the laboratory proved that the used practice in the studies 1 and 2 was solid.
Discussion: applying spot test analysis we showed over all satisfying sanitation in Dutch facilities. But inconsistencies in results show that the performance of facilities can be different when validation according to spot test analysis is repeated. An additional problem is that Enterococcus was demonstrated to survive ±6 days > 70 ^{o}C and there are strong indications for regrowth after sanitation. Results indicate no regrowth for E.Coli.

Additional work 2007-2008: based on the gathered experience and making use of the improved sampling strategy, the 9 lowest performers from the second study in 2006 have been evaluated, but also 3 plants which were not in the 1th and 2nd study. Spot test analysis was in most cases repeated 3 times at these plants. Enterococcus, E.Coli and Salmonella were analysed. In all cases (except one) 5log10 reduction of Enterococcus was demonstrated as mean value of the 3 measurements and there was compliance with the microbiological requirements 2 and 3. Salmonella was demonstrated in nearly all incoming Municipal Biowaste.

1. Introduction:

In 1994, The Netherlands implemented source separation of municipal biowaste (garden- and kitchen waste) by law, resulting in around 1,5 million ton/year of bio waste. This waste is treated in 24 plants, most of it in the 21 plants which are covered in this paper, all of them members of the Dutch Waste Management Association (Vereniging Afvalbedrijven). This association conducted this study and the Dutch Food and Consumer Product Safety Authority (VWA) was consulted before, during and after it. Table 1 shows the type of technology that is used in Dutch plants:

Table 1 System (all composting systems with forced aeration)	Number of systems	Capacity (kton/year)	Share in Capacity (%)
Bühler or GECO system (composting in closed hall)	7	704	41%
Tunnel composting system	11	590	35%
VAR-system (open air composting covered with layer of composted oversize)	2	229	13%
PACOM-system (composting in closed hall)	3	100	6%
BIOCEL (anaerobic digestion followed by tunnel composting)	1	85	5%
Total	24	1.708	100%

(EC) No 208/2006 amending Annexes VI and VIII at the beginning of 2006 was a new possibility to comply with EU 1774/2002. We have to find out how the Dutch plants can comply with the new requirements and how validation can be conducted. A first approach of the problem is to take a closer look at the 5 used technologies. To start with, all systems have up to date temperature registration systems in place (table 2):

Table 2	First stage		Second stage	
	Days	Temp (^{o}C)	Days	Temp (^{o}C)
Bühler/GECO	7	55-65	14	45-55
Tunnel	1	60	7	45-55
VAR	14	55	28	40
			With ≥10	55
PACOM	7	55-65	17	45-55
BIOCEL	15	35-40	7	40-45

The most important 3 requirements of (EC) No 208/2006 with respect to validation and microbiological testing are:

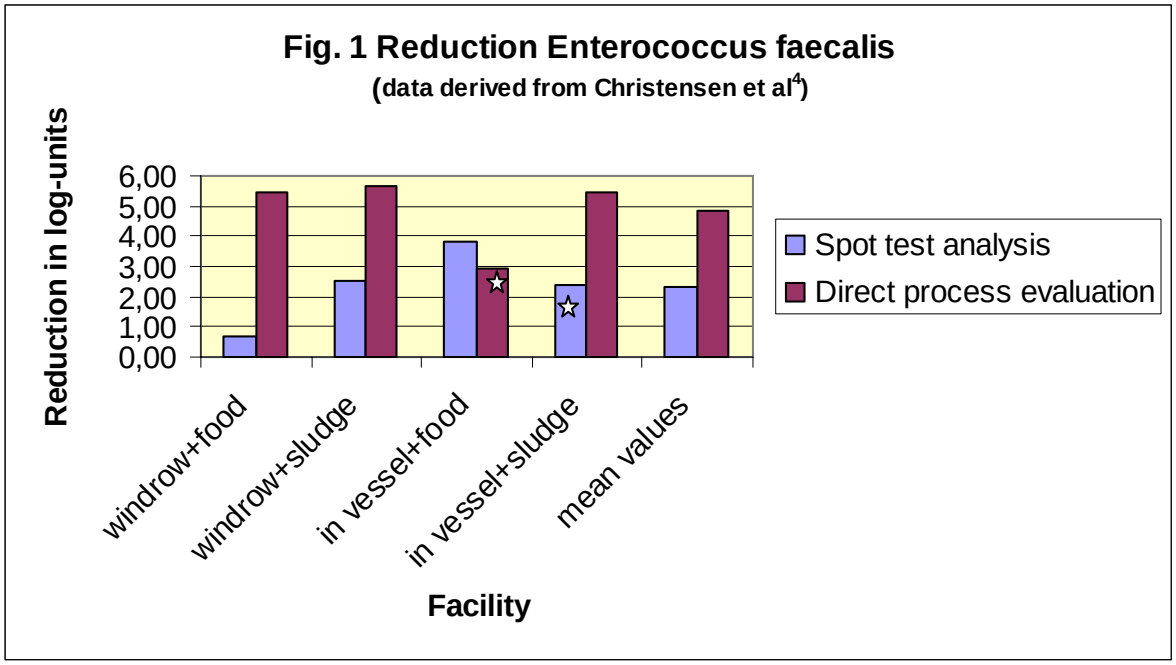
1. Reduction of 5 log10 of Enterococcus faecalis or Salmonella Senftenberg (775W, H2S negative); reduction of infectivity titre of thermo resistant viruses such as *parvovirus* by at least 3 log10, whenever they are identified as a relevant hazard;
2. Representative samples of the digestion residues or compost taken during or immediately after processing at the biogas or composting plant in order to monitor the process must comply with the following standards: *Escherichia coli*: n = 5, c = 1, m = 1000, M = 5000 in 1 g; or *Enterococcaceae*: n = 5, c = 1, m = 1000, M = 5000 in 1 g;
3. Representative samples of the digestion residues or compost taken during or on withdrawal from storage at the biogas or composting plant must comply with the following standards: *Salmonella*: absence in 25 g: n = 5; c = 0; m = 0; M = 0

where: n = number of samples to be tested, m = treshold value for number of bacteria; the result is considered satisfactory if the number of bacteria in all samples does not exceed m; M = maximum value for the number of bacteria; the result is considered unsatisfactory if the number of bacteria in one or more samples is M or more; c = number of samples the bacterial count of which may be between m and M, the sample still being considered acceptable if the bacterial count of the other samples is m or less.

With regard to requirement 1, we discussed the meaning and impact of '*whenever they are identified as a relevant hazard'.* We feel that with respect to thermo resistant viruses such as *parvovirus,* first attention should be directed to facilities (co-) digesting manure, which is not the case in the 21 plants of our study. The Danish Environmental Agency[3] said no viral pathogens survive composting, so we restricted ourselves first to the 5log10 reduction of Enterococcus faecalis or Salmonella Senftenberg (775W, H2S negative).

2. Spot test analysis and direct process evaluation:

The study of Christensen et al[4] compares two validation systems: the spot test analysis and direct process evaluation. Spot test analysis is less expensive and combining the set-up of the sampling and analysis, the spot test analysis will give you data with respect to requirements 1 (5log10 reduction), 2 (E.Coli or Enterococceae levels during/after processing) and 3 (Salmonella in compost; not detected in 25 gram) at the same time.
Direct process evaluation makes it possible to study the reduction of selected microbes/pathogens separately by using inoculated probes or bags, but the reduction conditions in the probes/bags are not always the same as in the surrounding material in the process. The differences in results are very important. From the study of Christensen et al[4] we get the impression that with direct process evaluation estimated reductions are 2,4log10 higher (mean reduction value around 4,8log10) compared with spot test analysis (mean reduction value 2,4log10). Figure 1 summarizes results from Christensen et al[4]:



(EC) No 208/2006 does not prescribe one of these methodologies. We decided to start with spot test analysis, giving us more data and covering requirements 1, 2 and 3 at the same time. Sampling and micro-biological analysis were carried out in the same way as were carried out by Christensen et al[4]. Sampling and micro-biological methods: samples were drawn from the untreated and sanitized bio-waste and from the fresh compost and stored at 4 °C until micro-biological analysis were started within 30 hours after sampling.
Escherichia coli was counted on MacConkey agar after 20-24 hours of incubation at 44 °C as described in the Bacteriological Analytical Manual van de USDA.
Enterococcus (earlier described as *Faecal Streptococci*) among which *Enterococcus Faecalis* was counted on Slanetz and Bartley agar after 44-48 hours of incubation at 44 °C as described in NMKL 68. Both for Enterococcus and E.Coli the counted cfu/g are expressed as log units/g.
Salmonella in 25 g is estimated in a simplified version of ISO 6579; pre-growth in buffered peptonwater, 16-20 hours at 37°C, selection in tetrathionate bouillon according to Müller Kauffmann, 24-48 hours at 43°C, demonstration at briljant green agar, 24 hours at 37°C and conformation agglutination and biochemical reactions at three sugar ferro agar, lysine decarboxylase bouillon and ureum agar.

3. Pilot study 5 technologies (including review methodology):

To get experience with the spot test analysis we started with a pilot study in February/ March, covering the 5 technologies that are used in The Netherlands. Sampling was carried out by Blgg Oosterbeek, an independent laboratory with a certified sampling service in the area of soils, animal feedstuffs and materials such as compost. Micro-biological analyses was carried out by CCL Research in Veghel, a leading micro-biological laboratory in the field of foods for animal and human consumption. It is a 'Sterlab', certified pursuant to the ISO 17025 standards. The results of the pilot study, conducted in week 7 and week 12 of 2006 are summarized as 34 mean values in Table 3 (counted cfu/g expressed as log units/gram):

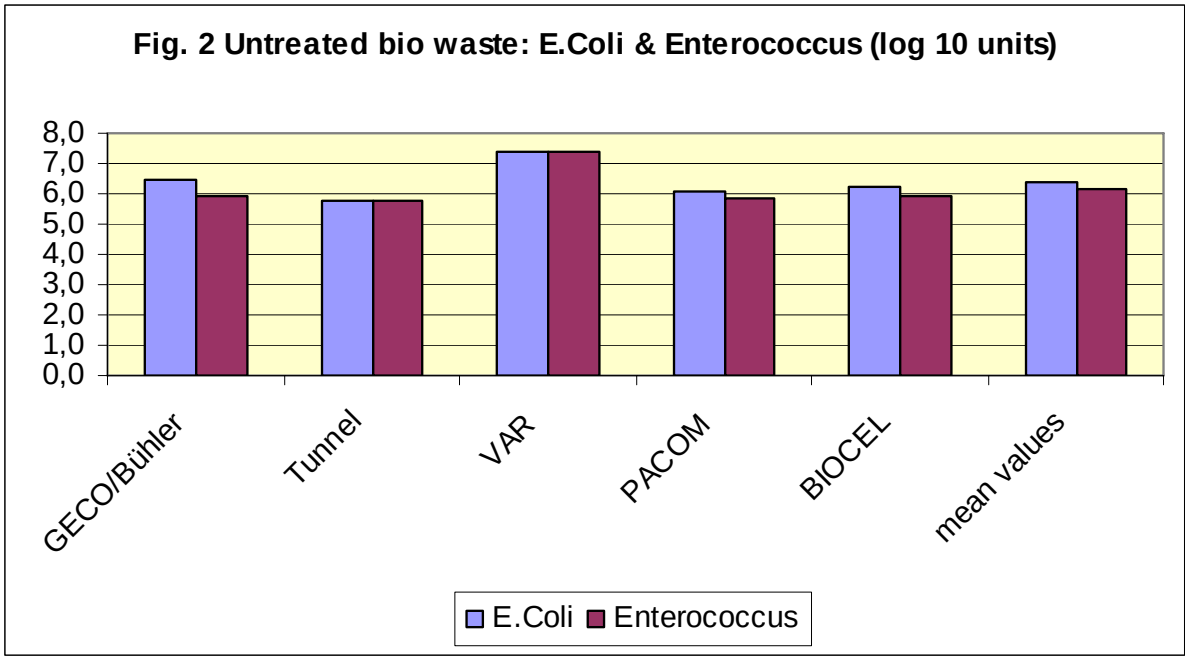
Table 3 (for all 34 x 4 = 136 basic results: see table 3.1 Annex 1)

Nr.	System	Untreated Bio waste		After sanitation Before screening		Fresh compost		Reductions of Enterococcus	
		E.Coli	Ent.coc	E.Coli	Ent.coc	E.Coli	Ent.coc	After Sanit.	Fresh compost
5	Bühler/GECO	6,45	5,90	2,29	<2			3,90	
17	Bühler/GECO			*2*	*2,82*	*2*	*2*		
6	Tunnel	5,80	5,79	5,23	4,35	*3,41*	*3,03*	1,44	2,76
10	VAR	7,38	7,40	*2,99*	*2,76*	6,61	5,50	4,64	1,90
						2	*2*		5,40
11	PACOM	6,08	5,83	*6,08*	*4,38*	*2*	*2,1*	1,45	3,73
15	BIOCEL	6,23	5,91	2,73	2,97			2,94	
				2	*6,67*			-0,76	

Remarks by table 3:

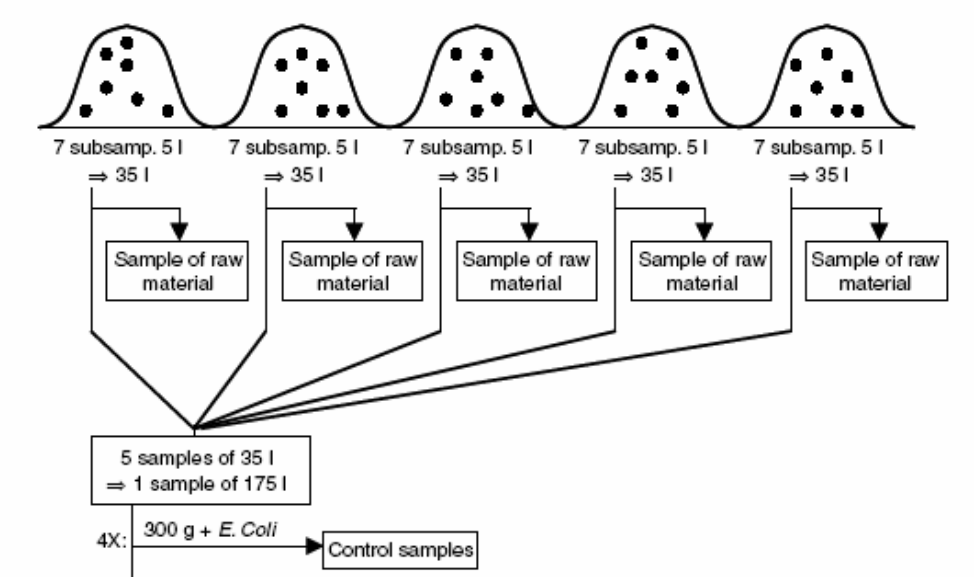
1. Samples were collected in week number 7 and in week number 12 *(cursive values)*
2. <2 means below detection level.
3. After sanitation means: samples were collected after composting before the first screening
4. In the case of BIOCEL, the samples after sanitation in this pilot were samples collected after digestion, but before composting (see also table 1 and 2)

Results and discussion: the untreated bio waste that has been sampled at 5 plants is collected from around 100 municipalities. In addition to that, all plants process smaller amounts of catering waste, waste from the food industry or retail waste. This sampling of fresh biowaste is expected to represent Dutch biowaste fairly well. The results show that in the untreated biowaste E. Coli and Enterococcus are at the same level. All the untreated bio waste samples have almost the same contamination with Enterococcus.



This could be very helpful. When all the untreated bio- waste has about the same level of contamination, then sampling of the product after sanitation and of the end product is sufficient to give also an impression of the reduction level and the performance of the process. In that case, it would not be needed to follow the same

untreated material exactly during and after the process. But to be sure of this, we decided that more data would be needed to support this assumption.
From the treated biowaste (after sanitation before screening or fresh compost, table 3) 67 % of the samples can meet the requirements for E.Coli and Enterococcus. Values in the fresh compost are always lower then compared with after sanitation before screening (except in the case of VAR). Christensen et al[4] noticed the same phenomenon as was found in the compost samples of week 7 by VAR and mentioned regrowth or recontamination as explanations. Requirement 2 says: *'[..] digestion residues or compost taken during or immediately after processing[..]'* . It is not unusual to consider a short storage for some weeks as a part of the processing, because it is needed for the maturing of the compost. Therefore both sampling spots (after sanitation or fresh compost) could be considered. We concluded that more data is needed, from the sanitation process before the screening, but also from the fresh compost.
Reduction levels: the initial contamination level of the fresh compost (mean value Enterococcus 6,17) is too low to demonstrate a 5log10 reduction, when 100 or 200 cfu/g (2 log units/g) is the detection limit. Therefore, the methodology should be improved to a detection limit of 10 cfu/g (1 log unit/g). And we would like the initial contamination to be higher. These samples were collected in the winter (week 7) and due to the low temperatures the growth level could be low. We decided that summer would be the best season to measure reduction levels, higher temperatures will support growth in the collection bins. The VAR-plant had the highest initial contamination level. Samples after sanitation indicate a 4,64 log units reduction for Enterococcus. The mean reduction value of the 5 plants in table 3 is 2,7 log units for Enterococcus (to compare with 2,6 log units found by Christensen et al[4]).
The problem of sampling and handling of the samples: a major concern during the pilot was how to prevent recontamination during the handling of the material which is needed to do the sampling. We started with the sampling as was proposed by Christenen et al[4]:

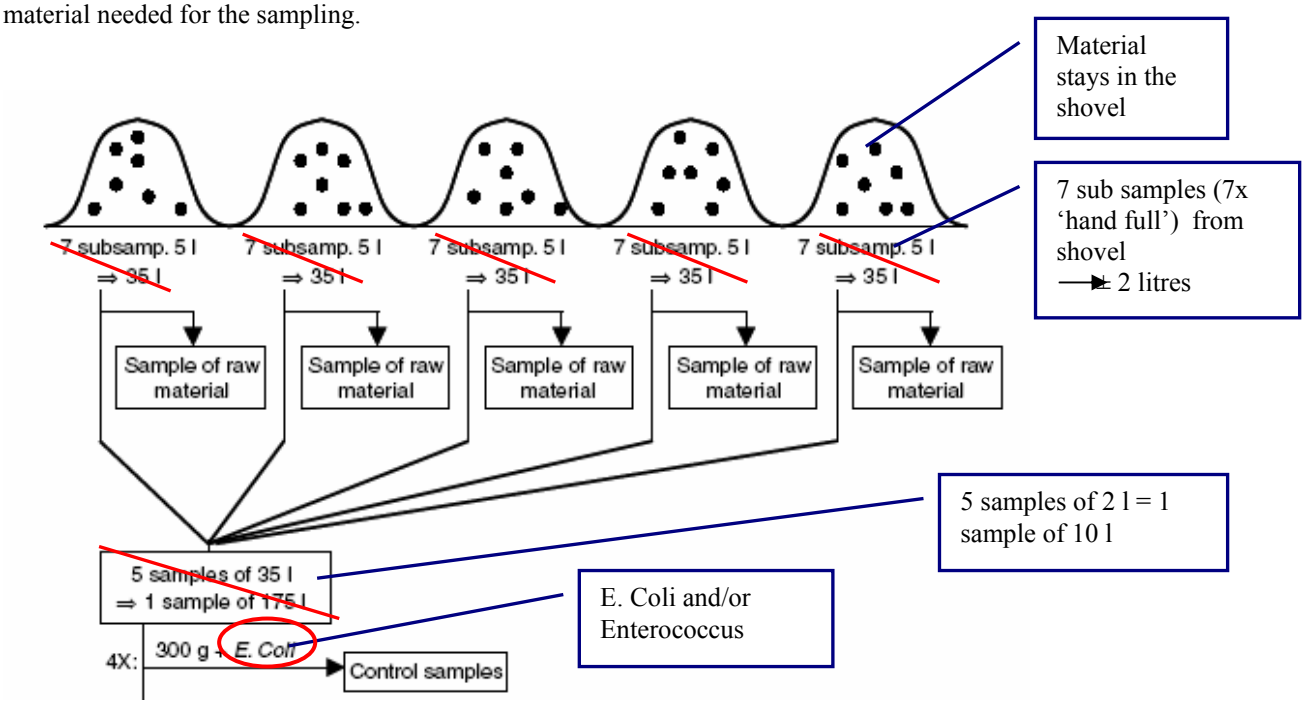


We conducted the sampling as was proposed by Christenen et al[4] with the following instructions:

1. A cleaned shovel takes one shovel load from 5 different places from the compost that should be sampled.
2. From each of these 5 shovel loads we take 7 samples of 5 litres, together 35 litres.
3. The 5 samples of 35 litres are mixed to get 1 homogeneous sample of 175 litres.

4. From this 175 litres sample we take 4 samples to be analysed for Enterococcus and E. Coli.

BLGG had practical objections to work with these instructions. In practice the risk of contamination during the sampling in the circumstances of a composting plant is very high.
This is demonstrated by the photo during the sampling at VAR in week 7. Buckets should be sterile, the same counts for the scoop. Looking at the wet surrounding, one should realize that one drop of contaminated moisture from the floor with 10^7 units/gram will bring a 100 gram sample of sterile compost at a level of 10^5 units/gram.
At request of BLGG the sampling strategy was adapted to prevent recontamination due to the handling of the material needed for the sampling.



According to this adapted sampling schedule, the pilot was conducted. Nevertheless, the impression was that the adaptations could be insufficient. The photo below shows the sampling of the digested material from the shovel at the BIOCEL plant.
This photo demonstrates that it is not easy to get the samples without contamination in the bucket. For the next study after the pilot, we decided that buckets should be replaced by new sterile plastic bags for each sampling. 7 x 7 x 1 'hand full' = 10 litres should be collected immediately in one bag and also mixing could be done in this same bag, keeping it closed during the mixing.

Another problem is how to transfer the collected material (10 litres sample) after mixing in a sterile way in 4 separate pots. We used pots which are closed with a cover that fits in the opening. On top of that a lid is screwed (red on the photo). An other point of discussion was the cooling and transportation of the samples. According to BLGG-practice, collected samples are transported in a cooling box in the car and are kept in a cooler overnight, following by cooled transportation next morning to the laboratory. Samples are processed in the laboratory within 24 hours. We decided that this practice should be checked during the next study.

4. Study covering 21 facilities (including second review methodology):

With the mentioned adaptations in the sampling strategy we took samples in week 22, week 25 and week 32 in 21 Dutch facilities, which process over 95% of source separated municipal garden and kitchen waste collected at the Dutch households (see table 4).
We focused on the 3 requirements of (EC) No 208/2006, so the untreated bio waste was only analyzed on Enterococcus (requirement 1). The product after sanitation, before screening was analyzed for E. Coli and Enterococcus (requirement 2). The fresh compost produced from the bio waste of week 22 was kept at stock and sampled in week 32 and was analyzed for E. Coli, Enterococcus and Salmonella (requirements 2 and 3). For E.Coli and Enterococcus the detection level was improved to 10 cfu/g. To achieve this, the application of the pre-treated sample to the agar was adapted. Inspectors of the Dutch Food and Consumer Product Safety Authority (VWA) were invited to all facilities to be present during the sampling in week 22 and week 32.

Table 4 (for all 168 x 4 = 672 basic results: see table 4.1 Annex 1)

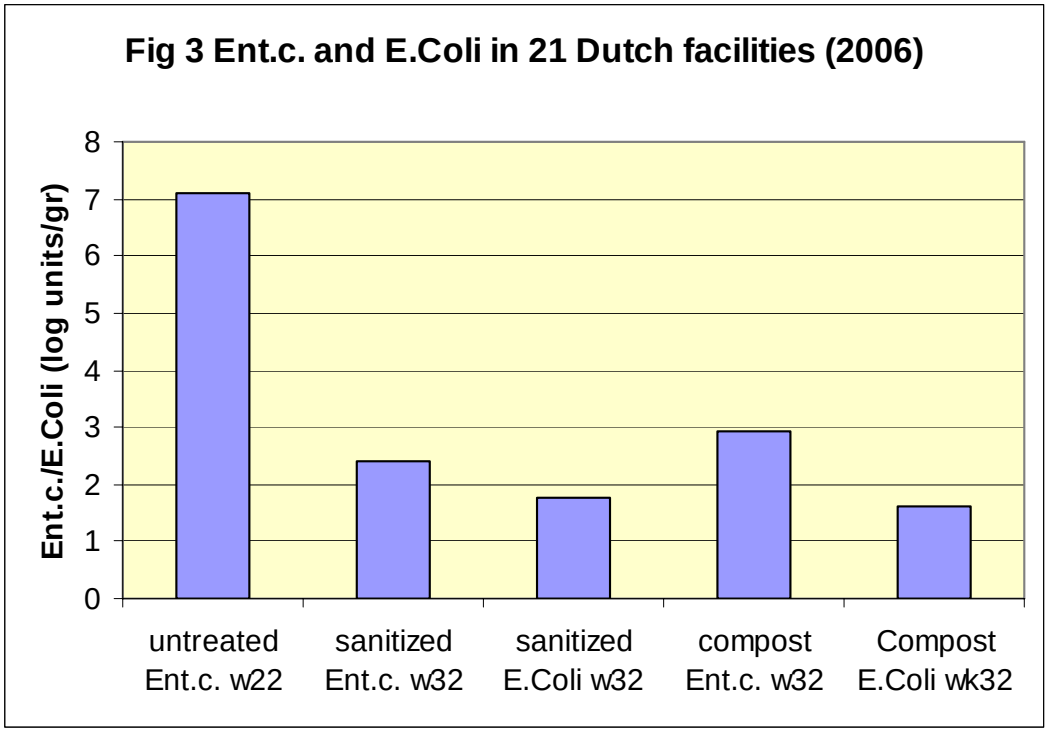
Counted numbers (cfu/g) are expressed as log units/g			Untreated Bio waste Week 22	After sanitation Before screening Week 32		Fresh compost				
						Week 32		Week 25	Week 22	week 32
nr		system	Ent.c.	E.Col	Ent.c	E.Col	Ent.c	Ent.c	Ent.c	Salm.
1		Tunnel	6,68	<1,00	1,18	1,29	3,30	4,53	5,23	neg
2		Tunnel	7,03	<1,00	1,49	0,95	1,35	4,57	2,23	neg
3		Tunnel	7,09	<1,00	1,44	3,52	5,09	5,68	5,23	neg
4		Bü/GE	7,36	<1,00	2,43	1,75	3,25	4,79	3,17	neg
5		Bü/GE	7,25	<1,00	0,78	1,14	3,10	0,70	1,36	neg
6		Tunnel	6,98	<1,00	2,07	<1,00	0,97	1,17	0,78	neg
7		Tunnel	7,69	2,13	2,16	<1,00	<1,00	3,19	2,14	neg
8		Bü/GE	6,81	<1,00**	3,82**	<1,00	4,77	3,86	6,23	neg
9		Bü/GE	7,69	1,50	1,89	<1,00	<1,00	3,61	4,71	neg
10		VAR	7,23	4,40	3,91	<1,00	4,53	3,16	0,70	neg
11		PACOM	6,95	3,47	2,75	<1,00	2,13	5,30	5,66	neg
12		VAR	6,92	4,29	4,42	<1,00	5,19	5,57	6,50	neg
13		Tunnel	7,22	2,27	2,37	1,69	2,19	3,41	1,38	neg
14		Tunnel	6,60	<1,00	3,08	4,39	3,90	5,10	3,85	neg
15		Biocel	7,47	0,85	2,94	1,18	1,55	4,93	4,71	neg
16		PACOM	6,82	3,90	3,40	<1,00	4,67	4,29	5,85	neg
17		Bü/GE	7,60	<1,00	1,06	<1,00	1,04	3,18	4,76	neg
18		PACOM	7,06	<1,00	1,21	2,65	3,88	4,27	4,38	neg
19		Bü/GE	7,22	<1,00	0,89	3,62	4,60	2,95	3,11	pos
20		Bü/GE	7,03	2,86	3,15	4,88	4,06	5,47	5,42	neg
21		Tunnel	7,11	4,79	3,75	<1,00	<1,00	6,83	4,52	neg
Mean values			**7,1**	**1,8***	**2,4**	**1,6***	**2,9**	**4,1**	**3,9**	

Systems that (almost) meet the required reduction level of 5log10 for Enterococcus are marked green. <1,00 means: all basic data were below detection level (see table 4.1). When a value of <1,00 is needed to calculate a mean value, it is valuated as 0,7 log units/g (5 cfu/g). For plant number 15, Biocel, 'after sanitation' means: after anaerobic digestion followed by tunnel composting (see table 1 and 2). * *E.Coli significant lower (p < 0,05)* ** *according to BLGG, due to process disturbance in plant 8 sampling was carried out after 50% of the normal sanitation time. New sampling is planned but results are not yet available.*

The untreated biowaste samples of all 21 plants have almost the same level of *Enterococcus*: lowest value 6,6, mean value 7,1 and highest value 7,7 log units/g. This supports the mentioned assumption that all the source separated municipal biowaste in The Netherlands has the same initial level of contamination. This is very important, because now we can assume that each sampling after sanitation gives a good indication of the achieved reduction in the process, without the necessity to follow the untreated biowaste exactly when it passes through each facility. As we expected, the contamination level in summer was a little bit higher (although not significant) than it was in winter in the pilot: lowest value 5,8, mean value 6,2 and highest value 7,4 log units/g.
Reduction levels (requirement 1): Overall, *after sanitation* Enterococcus was reduced from 7,1 to 2,4, a reduction of 4,7 log units. When we look at requirement 1 (5log10 reduction of Enterococcus), 15 of the 21 facilities have a reduction of almost 5 log units or more. In the case of 11, 15 and 21 the lower values of the fresh compost from week 32 were taken into account. The mean value for Enterococcus in the *fresh compost* (2,9 log units/g) is higher, compared with *after sanitation*. The mean values for *E.Coli* are significant lower than for

Enterococcus (table 4 and fig.3). In the case of E.Coli the level in the *fresh compost* is a little bit lower than *after sanitation*.

Regrowth or recontamination of Enterococcus: the lower values for E.Coli in fresh compost suggest that recontamination of fresh compost after sanitation is of minor importance. We assume that one of the explanations is that in composting facilities, Enterococcus levels seem to increase after sanitation because of regrowth, as one of the organisms that normally belongs to the composting microbes. Perhaps recontamination plays its role, but we think that regrowth, first mentioned by Christenen et al[4] is an important factor.



<u>*Requirement 2 (E.Coli or Enterococceae levels during/after processing):*</u> table 4.2 summarizes the results from table 4 with respect to this requirement:

Table 4.2 compliance with requirement 2	After sanitation		Fresh compost			
	E.Col wk 32	Ent.c wk 32	E.Col wk32	Ent.c wk32	Ent.c wk25	Ent.c wk22
Plants that do comply	16	14	17	10	3	6
% of plants that do comply	76%	67%	81%	48%	14%	29%

Requirement 2 holds also the numbers for n=5, c=1, m=1000, M=5000. Table 4.2 contains the results of 1 sample that has been analyzed 4 times. When we would take 5 independent samples in a year (n=5) and we apply the values c=1, m=1000, M=5000 we expect that even less plants can comply.
Results for Salmonella: Christenen et al[4] showed that Salmonella was present in the raw materials at all 4 investigated facilities in their study. We could expect the same in the Dutch situation, considering that initial contamination levels with Enterococcus and E.Coli are somewhat higher then was found in their study. Table 4 shows only 1 of the 21 plants Salmonella positive in fresh compost. This indicates that the over all sanitation at these 21 plants is satisfying. Normally, we would expect 10-20% of the samples to be Salmonella positive. In the study of Christenen et al[4] 1 of the 4 plants showed Salmonella in the sanitized product. In table 5, we summarize data that were collected by the members of the Dutch Waste Management Association (21 plants mentioned above) in 2003. These data were collected to be used for discussions because of the decision making concerning Regulation (EC) No 1774/2002:

Table 5 *historical data (compost samples from these Dutch plants in 2003):*

salmonella in 25 gr	**negative**		**positive**		**total**		
number of samples	98		12		110		
%	89%		11%		100%		
E. Coli 2003							
E. Coli in 1 gr	**<100**	**x*100**	**x*1.000**	**x*10.000**	**x*100.000**	**x*1.000.000**	**total**
number of samples	12	7	10	7	6	4	46
%	26%	15%	22%	15%	13%	9%	100%

In general, compost data from table 4 for Salmonella and E.Coli is better. We don't know in how far this is a structural improvement.
Looking to the 5 technologies/systems, we conclude that all 4 composting systems (there is only 1 Biocel digestion facility followed by composting) are represented in the 6 cases that do not achieve the required 5 log 10 reduction. We conclude that there is no clear coherence between the applied technology and the measured reduction performance.
It is interesting to notice that in the pilot from the 5 technologies the VAR-technology showed the best performance (plant number 10, see table 3 and table 4). It was the only one that showed more than 5 log 10 reduction of Enterococcus (4,64 log units reduction after sanitation, 5,4 log units reduction in fresh compost). But it means also that when a validation has been carried out very carefully and is repeated, you could expect another result. This is also illustrated by the results of plant 6 (tunnel system). This facility showed the lowest reductions in the pilot (see table 3), but it is one of the best and most constant performers in table 4.
Looking for explanations: some important questions were raised, we tried to resolve some of them in an additional study:

1. Plants performing good (plant 10) in the pilot (table 3) can perform poor when validation is repeated (table 4) and also the reverse can take place (plant 6). To get more information, we decided to take a closer look at plant number 12, that showed over all the lowest reductions.
2. There is no consistency for Enterococcus between results of repeated sampling of the compost of the same plants. We would like to make sure that we don't make a mistake with the sampling strategy.

5. Looking for explanations: study at plant number 12, VAR system: place of sampling, sampling system and transportation of the samples

Table 6 is an abstract from table 4:

Table 6	Town/city	system	Untreated Bio waste Week 22	After sanitation Before screening Week 32		Fresh compost				
						Week 32		Week 25	Week 22	week 32
			Ent.c.	E.Col	Ent.c	E.Col	Ent.c	Ent.c	Ent.c	Salm.
12	Rijssenhout	VAR	6,92	4,29	4,42	<1,00	5,19	5,57	6,50	neg
Mean values 21 plants			7,1	2,0	2,4	1,8	3,0	4,1	3,9	

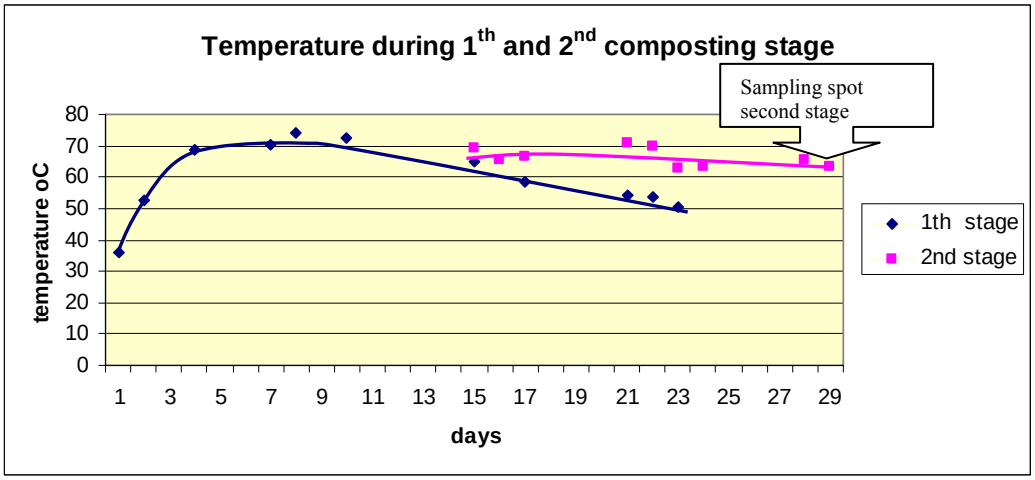
We decided to see if we could determine the place in the process where we would expect the most effective sanitation. At the same time, we wanted to check the used sampling system by using another system at the same time. Sampling was carried out at July 5th between 9.00 and 12.00 am. See table 7:

Table 7: Sampling spot in process		Second stage (at 80 °C)		Fresh compost 1 day old (46 °C)		Compost 2 weeks old (48 °C)	
Sampling system		4 pots from 10 l. bag 'from new 'crack'	4 pots straight from new 'crack'	4 pots from 10 l. bag 'from new 'crack'	4 pots straight from new 'crack'	4 pots from 10 l. bag 'from new 'crack'	4 pots straight from new 'crack'
Transport of samples	Cooled	1,2,3,4	9,10,11,12	17,18,19,20	25,26,27,28	33,34,35,36	41,42,43,44
	warm	5,6,7,8	13,14,15,16	21,22,23,24	29,30,31,32	37,38,39,40	45,46,47,48

Further explanation of table 7 (place in the process or sampling spot, sampling system and transport of samples) is given below.

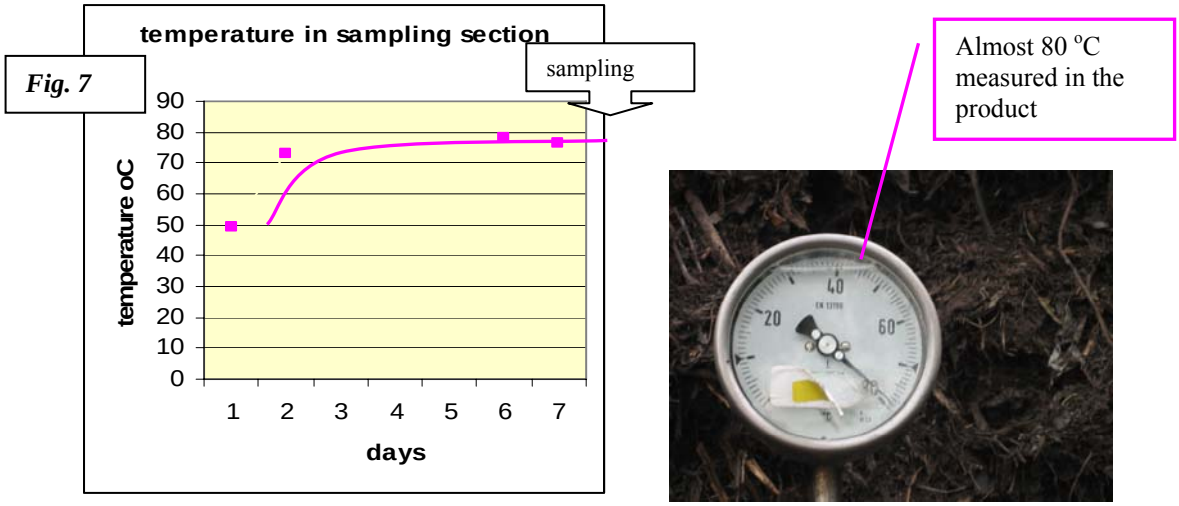
Place in the process: the facility in Rijssenhout is a VAR system (open air composting covered with a layer of composted oversize and forced aeration). The facility is working with an extensive temperature registration system (Annex I table 5.1). The mean temperature values are shown in figure 6:

Fig 6



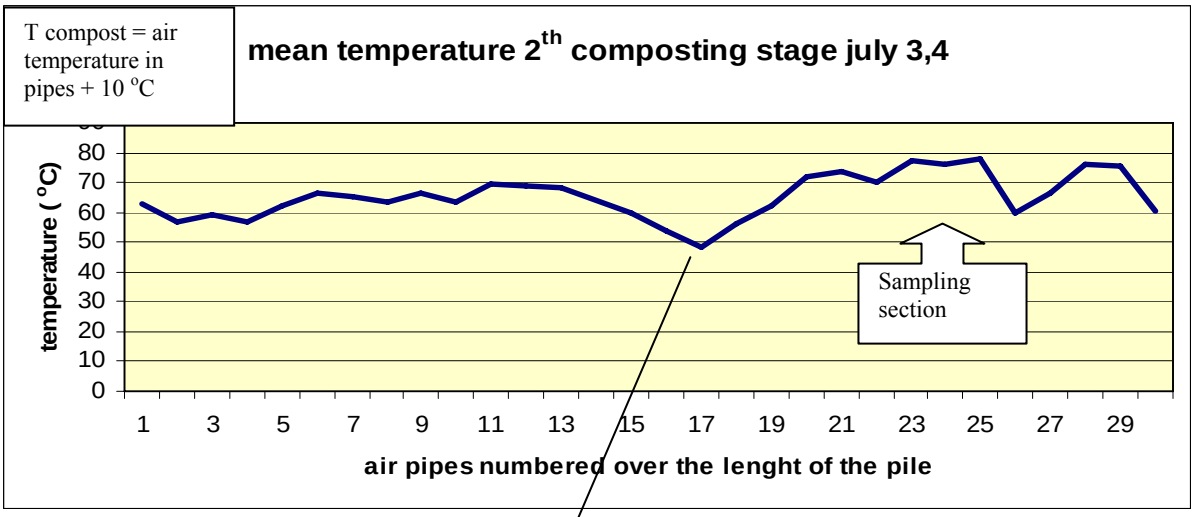
In the first stage, mean temperature rises within 3 days above 70 °C. For around 10 days, temperature is maintained > 65 °C and after that it drops gradually to 50 °C.
After day 15 the composting biowaste is turned and transferred to the second stage. There, the material typically will stay between 62 °C and 70 °C. Sampling took place at day 29. The material at the spot of sampling had been for 7 days at that place in the windrow. From the registration system we could collect the historical temperature values at that specific spot. Results are shown in figure 7. We found a very high temperature at the spot (section in windrow of 6 meters x 20 meters), so we carried out a manual check (see photo).

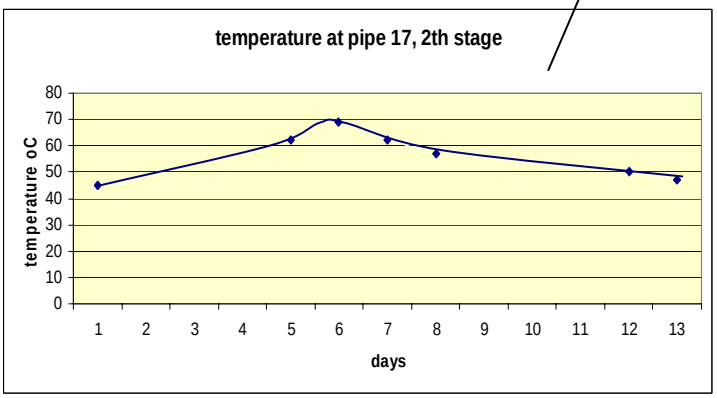
Fig. 7



From experience the management of the plant knows that normally the temperature in the composting bio waste is around 10 °C higher than is measured in the aeration pipes under the windrow (aeration is carried out at a constant flow with under pressure in the aeration pipes). This was checked and confirmed. From the data of the registration system, we can present fig. 8. Here we see the mean temperature values over the total length of the windrow, including our sampling section:

Fig 8





We can see that the temperature at the level of pipe 17 is during these 2 days 50 °C. But when we consider the temperatures of the last 13 days at the place of pipe 17, temperatures were > 60 °C for 3 days and for 1 day 70 °C. We found this to be a typical pattern. So, over the whole length of the second stage windrow, temperatures are >70 °C for at least 1 day.
The photo shows the spot (section) in the second stage windrow that was sampled. The two other places of sampling (Fresh compost 1 day old at 46 °C and fresh compost 2 weeks old 48 °C) do not need further clarification.

Sampling system: 4 pots from 10 l. bag from new 'crack' or 4 pots straight from new 'crack';
In chapter 3. 'pilot study 5 technologies (including review methodology)' we explained the system that we call '4 pots from 10 l. bag'. This system was used, also during the study of the 21 facilities. Now we have made a further adaptation to the system. The aim is to lower the possible occurrence of recontamination during the sampling at the same time eliminating an unwanted source of variation. Because of this, we did not take samples from the shovel. Instead of that, before the sampling we created a new crack (clean uncontaminated surface area) of ± 6 meters wide. The windrow and compost stock are ± 3-4 meters high, so an uncontaminated surface of 20 m^2 was created. The photo shows the creation of this surface in the compost stock.
Sampling according to *4 pots from 10 l. bag from new 'crack'*: 16 to 20 hands filled with compost (hand with new surgery gloves) were put in 1 new plastic bag. After that the sample was mixed in the closed bag by shaking the bag. From that, 4 pots were filled to be analyzed for Enterococcus.

Sampling according to *4 pots straight from new 'crack':* a pot is opened and with the pot we drew the samples straight from the uncontaminated surface.

Photos method 1: 16 to 20 hands full in a plasic bag, then mixing the bag by shaking, and after that 4 pots are filled from this bag.

Photo method 2:
A new pot is opened and with the pot we draw from ± 7-10 places the samples straight from the uncontaminated surface. In this way 4 pots are filled from the surface area.

Transport of samples, cooled and analyzed after 24 hours (4 °C) or warm (39 °C) and analyzed within 5 hours: the first system is normally used. Samples are put in a cooling box (the cooling box type from the photo was used) by BLGG with electrical supply from the car.

The sampler takes the samples home and puts them over night in a bigger cooling unit at home. The next day samples are transported with cooled transport to the laboratory.

Warm and analyzed within 5 hours: the camping gas boxes can also operate as warm keeping units. And because all the samples are warm when they are drawn, we decided that we could keep them more or less at the same temperature, avoiding condensation of moisture. Temperatures of the boxes were set at 55 °C. In addition to that, these samples were brought to the laboratory were immediately after the sampling. All these samples During all processed within 4 hours after they were drawn. measured. stages of sampling and processing temperatures were The results of the temperature measurements are presented in table 8:

Temperatures measured during transport to and when arriving at the laboratory of CCL

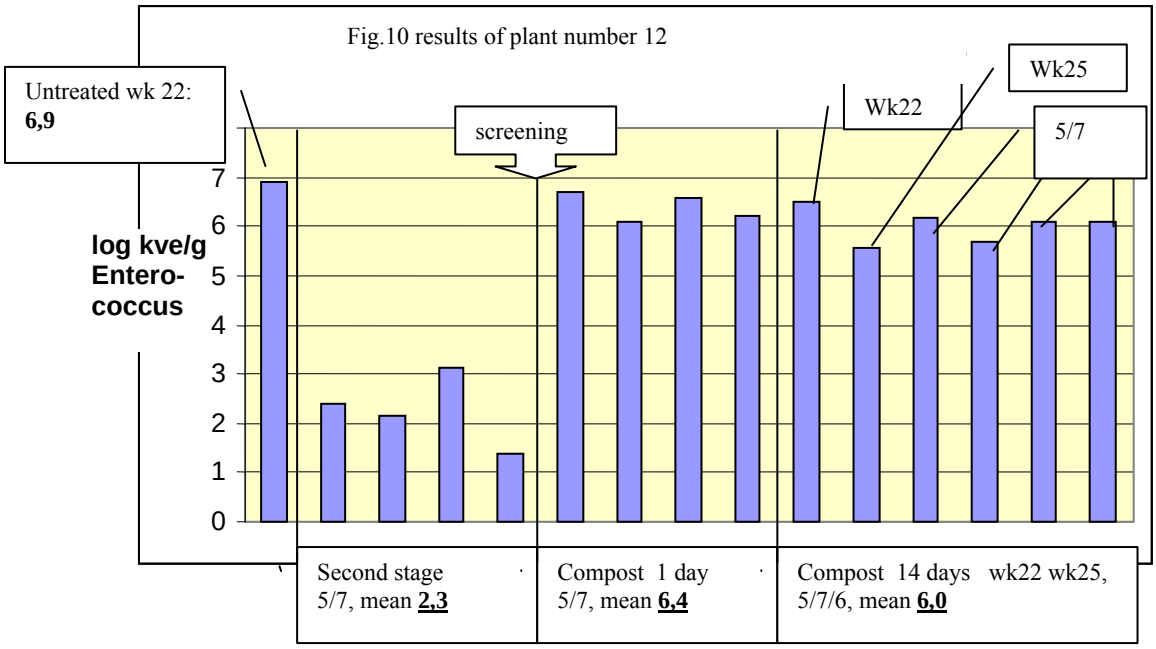
Table 8	T during sampling 9.30 hrs -10.30 hrs am	T cooling boxes after sampling 10.30 hrs am	T cooling boxes when leaving at 10.45 hrs am	T measured at arrival at laboratory of j CCL	T during incubation of the samples
Transport cold	between 45 °C and 80 °C	20 °C	23 °C	< 4 °C	± 14 °C
Transport warm		55 °C	55 °C	± 39 °C	± 31 °C

Results of micro-biological analysis for Enterococcus:

Table 9: Counted numbers (cfu/g) are expressed as log units/g

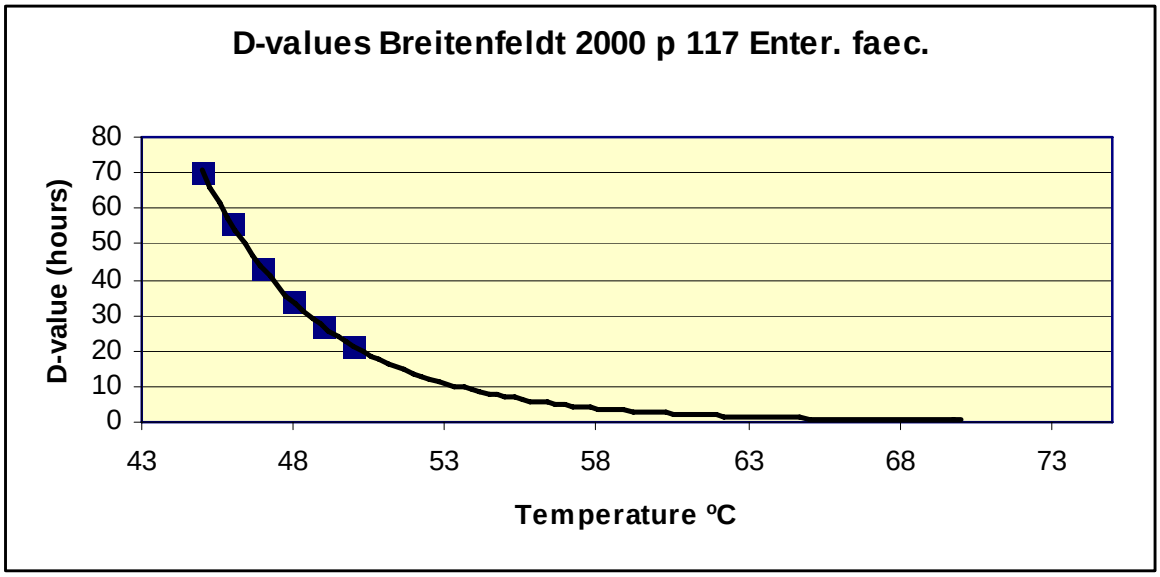
Place in the process	Untreated wk 22	Compost wk22	Compost wk25	Second stage 5/7/6		Compost 1 day old Temp 46 °C 5/7/6		Compost 2 weeks old Temp 48 °C 5/7/6		mean
Sampling system	See chapter 3	See chapt3	See chapt3	4 pots from bag	4 pots straight from crack	4 pots from bag	4 pots straight from crack	4 pots from bag	4 pots straight from crack	
cooled	6,30	6,49	5,87	2,18	2,15	6,83	6,18	6,18	5,60	
	6,79	6,46	5,41	2,32	2,23	6,83	5,74	6,26	5,73	
	7,53	6,46	5,54	2,56	2,26	6,83	6,15	6,15	5,74	
	7,04	6,58	5,46	2,56	1,98	6,32	6,36	6,15	5,71	
	6,92	**6,50**	**5,57**	**2,40**	**2,15**	**6,70**	**6,11**	**6,18**	**5,70**	**4,9**
warm				2,89	1,70	6,32	6,26	6,00	6,15	
				3,68	0,70	6,83	6,83	6,15	6,20	
				3,11	2,51	6,83	6,23	6,15	6,15	
				2,76	1,70	6,32	5,53	6,15	5,95	
				3,11	**1,40**	**6,58**	**6,21**	**6,11**	**6,11**	**4,9**
Mean values	**6,9**	**6,5**	**5,6**	**2,8**	**1,8**	**6,6**	**6,2**	**6,1**	**5,9**	

Reductions: data are collected in fig 10, which shows clearly the reduction of ± 5 log values in the second stage.



We see that compost of one day old and compost of 2 weeks old have almost the same values for Enterococcus. Of course we tried to find an explanation for the sudden regrowth after screening. In this plant, after screening the compost is sprayed with leachate, which could mean recontamination or cause regrowth. Nevertheless, it is surprising that levels of 1,4 to 3,1 log units/g can be demonstrated in composted biowaste from this spot in the second stage that was for 6 days > 70 °C, almost 80 °C! Fig, 11 is extrapolated from the data collected by Dr. Petra Breitenfeldt[5] (Hohenheim) for compost at a moisture level of 50%. At a moisture level of 30% D-values are higher. But from these estimated D-values (time in hours to achieve 1 log unit reduction) we would expect that Enterococcus Faecalis can not survive a 6 days period > 70 °C, almost 80 °C. This would perhaps mean that Enterococcus – like we estimated it [6] – is far more stable then a purified Enterococcus Faecalis strain used by Dr. Breitenfeldt.

Fig. 11



Sampling system: although the values from '4 pots from 1 bag' are in all cases higher, there is no significant difference with '4 pots straight from crack'. So no further explanation for inconsistent Enterococcus results in chapter 4 could be found here.
Transport of the samples: with both systems we obtained exactly the same over all mean values (4,9 log values).

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[2] Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption OJ L 273, 10.10.2002, p. 1-95 and Commission Regulation (EC) No 208/2006 of 7 February 2006 amending Annexes VI and VIII to Regulation (EC) No 1774/2002 of the European Parliament and of the Council as regards processingstandards for biogas and composting plants and requirements for manure (Text with EEA relevance) OJ L 36, 8.2.2006, p.25-31
[3] The Danish Environmental Agency 2000: 'Occurence and survival of viruses in composted human feaces'
[4] K.K. Christensen, M. Carlsbaek and E. Kron, 'Strategies for evaluating the sanitary quality of composting', Journal of Applied Microbiology 2002, 92, 1143-1158
[5] Petra Breitenfeldt, 'Untersuchungen zur Human- und Veterinärhygiene der Bioabfallkompostierung', Dissertation, 2000, Universität Hohenheim, page 117
[6] Additional remarks on Enterococci: ISO (and NEN) have only standards for estimation of Enterococcus in (waste-)water, but not for other matrices like compost. The present ISO for water starts with culturing on Slanetz & Bartley agar at 37°C followed by conformation reactions like growing on gal esculine azide agar at 44°C. For compost we used NMKL 68 (Enterococcus. Determination in food) as starting point. This standard prescribes counting on Slanetz & Bartley agar incubated at 44°C. Conformation is only prescribed for weak coloured colonies (gram colouring, katalase test and growtests at pH 9,6 and 6,5% salt). This NMKL does not prescribe esculine verification. We only counted colonies with a clear colouring or with a pink core. In cases of doubt the degree of overgrowth was considered. We are convinced that the followed procedure is fairly accurate for the estimation of enterococci in compost, considering the use of enterococci as an indicator for faecal pathogens. According to Dr. Jacob Ottoson (research from Statens Veterinärmedicinska Anstalt SVA, Sweden) in Swedish studies in compost SVA has noticed growth on Slanetz & Bartley agar with colonies typical in colour but significantly smaller than the added Ent. faecalis. These were Esculine positive (which Dr Ottoson did not know but just found out) and thus per method definition Enterococci, however not of faecal origin.

SVA noticed also a slowgrowing commencal Enterococci microbiota in high ammonia anaerobic treatment (35 degrees C). Perhaps we need to type them in the future. Another problem with Enterococci is their higher tolerance to many treatments than gram negative rods like Salmonella and VTEC which may be the main bacterial pathogens of concern.

Annex I table ***3.1*** (mean values are printed in **bold**): Counted numbers (cfu/g) are expressed as log units/g

Nr.	System	Untreated Bio waste		After sanitation Before screening		Fresh compost	
		E.Coli	Ent.coc	E.Coli	Ent.coc	E.Coli	Ent.coc
5	Bühler/GECO	**6,45**	**5,90**	**2,29**	**<2**		
		6,53	7,26*	2,85	<2		
		6,23	5,79	2,60	<2		
		6,76	5,96	2,00	<2		
		6,28	5,93	<2,00	<2		
17	Bühler/GECO			***2***	***2,82***	***2,2***	***2,1***
				2	*2,30*	*2*	*2,3*
				2	*2,78*	*2*	*2*
				2	*2,78*	*2,9*	*2*
				2	*3,41*	*2*	*2*
6	Tunnel	**5,80**	**5,79**	**5,23**	**4,35**	***3,41***	***3,03***
		5,82	6,11	3,23	2,00	*3,91*	*3,00*
		5,87	5,60	6,51	6,28	*3,51*	*2,90*
		5,79	5,72	4,68	5,08	*2,90*	*3,20*
		5,72	5,72	6,51	4,04	*3,30*	*3,00*
10	VAR	**7,38**	**7,40**	***2,99***	***2,76***	**6,61**	**5,50**
		7,38	7,08	*2,60*	*2,00*	3,45*	5,53
		7,58	7,38	*2,85*	*2,78*	6,54	5,34
		7,76	7,87	*3,81*	*2,48*	6,70	5,53
		6,79	7,26	*2,70*	*3,79*	6,58	5,60
						2	***2***
						2	*2*
						2	*2*
						2	*2*
						2	*2*
11	PACOM	**6,08**	**5,83**	***6,08***	***4,38***	***2***	***2,1***
		5,91	5,92	*5,82*	*4,53*	*2*	*2,3*
		6,20	5,82	*5,38*	*3,75*	*2*	*2*
		6,04	4,23*	*7,00*	*4,66*	*2*	*2*
		6,15	5,75	*6,11*	*4,58*	*2*	*2*
15	BIOCEL	**6,23**	**5,91**	**2,73**	**2,97**		
		6,04	5,88	<2,00**	2,60		
		6,51	5,95	3,32	3,30		
		6,23	5,82	2,30	2,95		
		6,15	5,98	3,58	3,04		
				2	***6,67***		
				2	*6,66*		
				2	*6,84*		
				2	*6,58*		
				2	*6,59*		

* numbers labelled with * are drop outs according to the Grubbs test (p=0,05)

**numbers labelled with ** are below detection level (100 cfu/g); for calculating means they got the value 1,70 (log units/g)

Annex I table ***4.1*** (mean values are printed in **bold**): Counted numbers (cfu/g) are expressed as log units/g

nr		system	Untreated Bio waste Week 22	After sanitation Before screening Week 32		Fresh compost				
						Week 32		Week 25	Week 22	week 32
			Ent.c.	E.Col	Ent.c	E.Col	Ent.c	Ent.c	Ent.c	Salm.
1		Tunnel	**6,68**	**<1,00**	**1,18**	**1,29**	**3,30**	**4,53**	**5,23**	**neg**
			6.56	0.70*	0.70	1.65	3.81	4.76	5.11	neg
			6.45	0.70	0.70	0.7	2.74	4.72	5.20	neg
			7.18	0.70	0.70	2.11	3.92	4.51	5.34	neg
			6.53	0.70	2.60	0.70	2.75	4.15	5.26	neg
2		Tunnel	**7,03**	**<1,00**	**1,49**	**0,95**	**1,35**	**4,57**	**2,23**	**neg**
			7.23	0.70	1.48	0.70	0.70	4.32	2.11	neg
			7.08	0.70	1.65	0.70	0.70	4.65	2.26	neg
			7.11	0.70	1.18	1.70	2.81	4.60	2.28	neg
			6.68	0.70	1.65	0.70	1.18	4.70	2.28	neg
3		Tunnel	**7,09**	**<1,00**	**1,44**	**3,52**	**5,09**	**5,68**	**5,23**	**neg**
			7.28	0.70	2.66	4.59	5.11	5.62	4.36	neg
			6.83	0.70	0.70	2.98	5.08	5.64	4.92	neg
			6.98	0.70	0.70	3.34	5.40	5.77	5.52	neg
			7.28	0.70	1.70	3.15	4.76	5.68	6.11	neg
4		Bü/GE	**7,36**	**<1,00**	**2,43**	**1,75**	**3,25**	**4,79**	**3,17**	**neg**
			7.23	0.70	2.81	1.30	3.15	4.82	3.00	neg
			6.95	0.70	2.70	0.70	2.70	4.52	4.08	neg
			7.26	0.70	2.45	2.59	4.08	4.98	3.00	neg
			8.00	0.70	1.78	2.40	3.08	4.85	2.60	neg
5		Bü/GE	**7,25**	**<1,00**	**0,78**	**1,14**	**3,10**	**0,70**	**1,36**	**neg**
			6.83	0.70	0.70	0.70	2.52	<1.00	1.54	neg
			6.88	0.70	0.70	0.70	3.64	<1.00	1.30	neg
			7.32	0.70	0.70	1.00	3.34	<1,00	1.30	neg
			7.98	0.70	1.00	2.15	2.90	<1.00	1.30	neg
6		Tunnel	**6,98**	**<1,00**	**2,07**	**<1,00**	**0,97**	**1,17**	**0,78**	**neg**
			7.04	0.70	0.70	0.70	0.70	1.30	0.70	neg
			6.60	0.70	1.30	0.70	0.70	1.70	1.00	neg
			6.91	0.70	3.08	0.70	1.78	0.70	0.70	neg
			7.36	0.70	3.20	0.70	0.70	1.00	0.70	neg
7		Tunnel	**7,69**	**2,13**	**2,16**	**<1,00**	**<1,00**	**3,19**	**2,14**	**neg**
			7.20	1.30	0.70	0.70	0.70	2.88	1.78	neg
			8.04	1.95	2.64	0.70	0.70	3.82	2.00	neg
			7.23	0.70	0.70	0.70	0.70	2.90	1.81	neg
			8.28	4.58	4.61	0.70	0.70	3.18	2.99	neg
8		Bü/GE	**6,81**	**<1,00**	**3,82**	**<1,00**	**4,77**	**3,86**	**6,23**	**neg**
			7.11	0.70	3.72	0.70	3.66	4.11	6.36	neg
			6.66	0.70	4.36	0.70	5.77	3.60	5.81	neg
			6.66	0.70	3.68	0.70	5.04	3.64	6.32	neg
			6.81	0.70	3.51	0.70	4.60	4.08	6.45	neg
9		Bü/GE	**7,69**	**1,50**	**1,89**	**<1,00**	**<1,00**	**3,61**	**4,71**	**neg**
			7.26	2.20	2.30	0.70	0.70	3.38 3.73	3.87	neg
			7.23	0.70	1.54	0.70	0.70	3.85	5.38	neg
			8.08	0.70	0.70	0.70	0.70	3.97	5.26	neg
			8.18	2.41	3.00	0.70	0.70	3.11	4.32	neg
10		VAR	**7,23**	**4,40**	**3,91**	**<1,00**	**4,53**	**3,16**	**0,70**	**neg**
			7.41	3.96	3.70	0.70	4.49	3.20	0.70	neg
			6.87	3.95	3.53	0.70	4.51	2.90	0.70	neg
			7.11	5.56	4.66	0.70	4.51	3.60	0.70	neg
			7.51	4.11	3.76	0.70	4.60	2.92	0.70	neg
11		PACOM	**6,95**	**3,47**	**2,75**	**<1,00**	**2,13**	**5,30**	**5,66**	**neg**

			6.56	1.78	2.18	0.70	2.30	5.52	5.62	neg
			6.83	5.20	2.81	0.70	1.85	5.20	5.61	neg
			6.86	4.11	3.70	0.70	1.78	5.23	6.62	neg
			7.57	2.79	2.30	0.70	2.60	5.23	5.67	neg
12		VAR	**6,92**	**4,29**	**4,42**	**<1,00**	**5,19**	**5,57**	**6,50**	**neg**
			6.30	4.87	5.34	0.70	5.23	5.87	6.49	neg
			6.79	3.04	3.38	0.70	5.26	5.41	6.46	neg
			7.53	5.00	4.79	0.70	5.08	5.54	6.46	neg
			7.04	4.23	4.18	0.70	5.20	5.46	6.58	neg
13		Tunnel	**7,22**	**2,27**	**2,37**	**1,69**	**2,19**	**3,41**	**1,38**	**neg**
			7.40	2.04	2.58	1.00	2.11	5.57	1.30	neg
			7.20	2.18	2.92	2.38	2.38	3.08	0.70	neg
			7.26	2.00	2.00	1.78	2.30	2.90	2.20	neg
			7.04	2.85	2.00	1.60	1.95	2.08	1.30	neg
14		Tunnel	**6,60**	**<1,00**	**3,08**	**4,39**	**3,90**	**5,10**	**3,85**	**neg**
			6.88	0.70	4.26	3.79	2.58	5.08	3.89	neg
			6.41	0.70	2.91	4.79	4.23	4.95	3.86	neg
			6.41	0.70	2.23	5.15	4.28	4.59	3.87	neg
			6.70	0.70	2.91	3.85	4.51	5.08	3.79	neg
15		Biocel	**7,47**	**0,85**	**2,94**	**1,18**	**1,55**	**4,93**	**4,71**	**neg**
			6.78	1.00	2.72	0.70	1.85	5.08	4.36	neg
			7.85	0.70	2.69	0.70	1.60	4.95	4.53	neg
			8.20	0.70	3.45	2.60	2.04	4.59	5.04	neg
			7.04	1.00	2.88	0.70	0.70	5.08	4.89	neg
16		PACOM	**6,82**	**3,90**	**3,40**	**<1,00**	**4,67**	**4,29**	**5,85**	**neg**
			6.82	4.94	3.68	0.70	4.89	4.53	5.81	neg
			6.38	4.83	4.32	0.70	4.11	3.20	5.86	neg
			7.11	1.00	2.00	0.70	5.04	4.90	5.88	neg
			6.97	4.83	3.58	0.70	4.62	4.51	5.83	neg
17		Bü/GE	**7,60**	**<1,00**	**1,06**	**<1,00**	**1,04**	**3,18**	**4,76**	**neg**
			8.53	0.70	2.15	0.70	1.00	3.11	4.79	neg
			6.90	0.70	0.70	0.70	0.70	3.38	4.78	neg
			5.11	0.70	0.70	0.70	0.70	3.26	4.73	neg
			7.38	0.70	0.70	0.70	1.78	2.98	4.72	neg
18		PACOM	**7,06**	**<1,00**	**1,21**	**2,65**	**3,88**	**4,27**	**4,38**	**neg**
			6.83	0.70	0.70	1.85	2.60	4.23	3.66	neg
			6.00	0.70	2.74	2.58	4.57	4.45	4.30	neg
			7.85	0.70	0.70	2.51	4.08	4.23	3.30	neg
			7.56	0.70	0.70	3.66	4.26	4.18	6.26	neg
19		Bü/GE	**7,22**	**<1,00**	**0,89**	**3,62**	**4,60**	**2,95**	**3,11**	**pos**
			7.82	0.70	1.18	3.70	4.54	2.66	2.93	pos
			7.04	0.70	0.70	3.53	4.62	3.62	3.18	pos
			7.04	0.70	0.70	3.59	4.70	3.16	3.18	pos
			6.99	0.70	1.00	3.66	4.52	2.34	3.18	pos
20		Bü/GE	**7,03**	**2,86**	**3,15**	**4,88**	**4,06**	**5,47**	**5,42**	**neg**
			7.2	3.00	3.56	6.83	5.56	5.20	5.30	neg
			7.11	0.70	2.51	3.85	3.56	5.73	5.26	neg
			6.04	3.87	3.86	5.76	4.60	5.62	5.53	neg
			7.78	3.87	2.70	3.08	2.54	5.47	5.58	neg
21		Tunnel	**7,11**	**4,79**	**3,75**	**<1,00**	**<1,00**	**6,83**	**4,52**	**neg**
			6.96	4.96	3.82	0.70	0.70	6.83	4.89	neg
			7.11	5.38	4.51	0.70	0.70	6.83	4.52	neg
			6.85	5.20	4.04	0.70	0.70	6.83	4.54	neg
			7.51	3.62	2.64	0.70	0.70	6.83	4.11	neg
Mean values			**7,1**	**1,8**	**2,4**	**1,6**	**2,9**	**4,1**	**3,9**	

*numbers labelled with * are below detection level (10 cfu/g); for calculating means they got the value 5 cfu/g (0,70 log units/g).