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FATE OF GENETICALLY MODIFIED MICRO- ORGANISMS DURING THERMOPHILIC COMPOSTING



Final Project Report

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EXECUTIVE SUMMARY (& REITERATION OF CONCLUSIONS)

The fate of material from genetically modified organisms (GMOs) when introduced to the environment is an important issue for the development of environmental risk assessments for sustainable waste management options. Horizontal gene transfer (HGT) from GM plants to bacteria has not been detected in the real environment despite extensive monitoring projects (Nielsen & Townsend, 2004) although this possibility has been demonstrated in laboratory settings (Gebhard & Smalla, 1998). In contrast, HGT between genetically modified and indigenous microorganisms has been observed in a field site (Peters et al., 1997). The implications of this result and other findings in laboratory systems are still being discussed in the context of bioremediation of contaminants using genetically modified microorganisms GMMO (Urgun-Demirtas, Stark & Pagilla, 2006). Composting is the enhanced natural microbial degradation and stabilization of organic waste leading to the production of a soil-like material termed compost.

During the course of this project experiments were carried to answer the following research questions using *E. coli* K12 pGLO as model GMMO:

Do GMMOs survive during thermophilic composting?

- Thermophilic in-vessel composting experiments:
 - There is no evidence of *E. coli* K12 pGLO survival during thermophilic composting when operated under optimal conditions ($T \geq 65^{\circ}\text{C}$, retention time 6 ± 2 days).
 - Potential problems might be caused by lack of feedstock, homogeneity or insufficient temperature exposure.
- Lab-based survival experiments at different temperatures:
 - Temperature is the main factor for survival of the *E. coli* K12 pGLO in non-autoclaved compost.
 - Survival rates non-autoclaved compost are:
 - $T = 65^{\circ}\text{C}$: < 5h
 - $T = 40^{\circ}\text{C}$: < 9 days
 - $T = 22^{\circ}\text{C}$: < 14 days.
 - The presence of a microbial community negatively affects the survival of GMMOs.
 - Survival rates autoclaved compost are:
 - $T = 65^{\circ}\text{C}$: < 5h
 - $T = 40^{\circ}\text{C}$: < 21 days
 - $T = 22^{\circ}\text{C}$: > 68 days.

Therefore, our results indicate that GMMOs do not survive during in-vessel thermophilic composting when temperatures are held above 65°C for periods longer than 5h.

Does transgenic DNA survive thermophilic composting?

There was no difference in survival rates of GMMO and transgenic gene in thermophilic in-vessel composting experiments. Our results suggest that transgenic DNA might survive longer periods compared to the GMMO itself in lab-based incubation experiments. This effect is greater in artificially created autoclaved environments compared to samples where an active microbial community is present.

- Thermophilic in-vessel composting experiments:
 - No evidence of marker gene survival (715bp overlapping region pBAD/*gfp*) during thermophilic composting when operated under optimal conditions ($T \geq 65^{\circ}\text{C}$, retention time 6 ± 2 days).
 - It is of great importance to control and monitor the optimal settings as irregularities might enable transgenic DNA to survive.

- Factors affecting the in-vessel compost operation: lack of feedstock homogeneity, inadequate mixture of input pre-compostable materials, insufficient feedstock addition (minimum 40l day⁻¹) leading to shortened retention and exposure times.
- Lab-based survival experiments at different temperatures:
 - Temperature is the main factor for survival of the transgenic DNA in non-autoclaved compost.
 - Survival rates non-autoclaved compost are:
 - T= 65°C: >2 days
 - T= 40°C: < 9 days
 - T= 22°C: < 14 days.
 - The presence of a pre-existing microbial community in the compostable material negatively affects the survival of the transgenic DNA.
 - Survival rates autoclaved compost are:
 - T= 65°C: >2 days
 - T= 40°C: < 28 days
 - T= 22°C: > 68 days.

Therefore, our studies suggest that transgenic DNA has the potential to survive for longer periods than the GMMOs themselves. However, such material does not survive during thermophilic in-vessel composting when temperatures are held above 65°C for periods longer than 2 days.

Does horizontal gene transfer and subsequent gene expression occur in other micro-organisms?

No evidence was found for horizontal gene transfer between E. coli K12 pGLO and indigenous microorganisms under chosen conditions during thermophilic in-vessel composting and in lab-based survival studies at 65°C, 40°C and 22°C over periods up to 68 days.

Is it possible to detect and quantify the introduced genes during thermophilic composting with novel molecular biological techniques?

This project yielded the successful development of a protocol to detect and quantify transgenic DNA from compost samples using real-time PCR. The detection limits are 3×10⁶ target copies g⁻¹ for spiked compost and feedstock samples and 8×10⁴ target copies ml⁻¹ for E. coli K12 pGLO pure culture samples.

Therefore, we suggest that the method developed for real-time PCR can be used to detect and quantify the fate of transgenic DNA during in-vessel thermophilic composting. There is potential to improve the detection limit by method development in DNA extraction and purification as well as PCR reaction mix optimisation.

INTRODUCTION AND EXPERIMENTAL APPROACH

Composting is the enhanced natural microbial degradation and stabilization of organic waste leading to the production of a soil-like material termed compost. In addition, composting is an environmentally sustainable means of waste management helping to meet national recycling targets as well as producing a beneficial product – compost. Examples for organic waste which can be treated in this way are agricultural waste including crop residues and animal manures, urban wastes like green waste and sewage sludge, wood processing waste, food processing residues, fermentation waste, human habitation waste as well as aquatic biomass (Sharma et al., 1997). Three major phases can be defined (Figure 1):

- initial mesophilic phase: degradation
 - days to week
 - temperature: 10-40°C
 - degradation of low-molecular weight compounds like sugars and organic acids
- thermophilic phase: degradation
 - months
 - temperature: 40-80°C
 - degradation of substances with high molecular weight like cellulose, fats, and proteins
- second mesophilic phase: maturation and stabilisation
 - months
 - temperature: 40-10°C

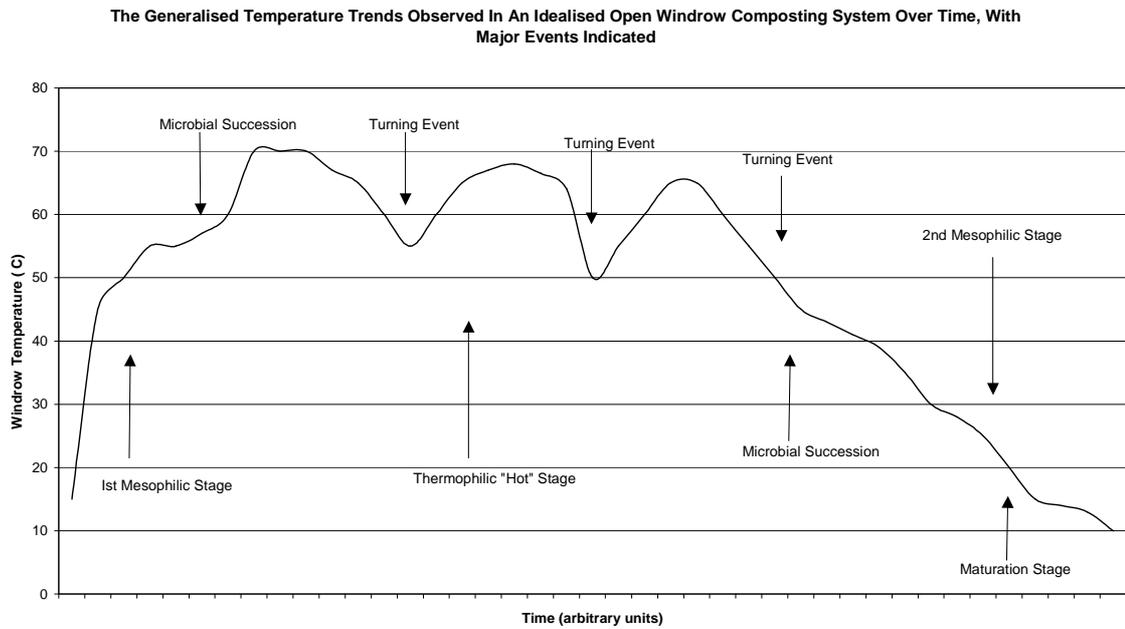


Figure 1: General temperature trends observed in an idealised open windrow composting system with major events indicated.

The experimental approach was based on the use of a specific, easy-to-monitor GMMO, *Escherichia coli* K12 pGLO, harbouring an *araC*-promoted green fluorescent protein (*gfp*) gene as well as resistance to the antibiotic ampicillin. A model GM waste (green/food/brown waste) was spiked with *E. coli* K12 pGLO, which was subject to thermophilic composting. Monitoring of the product included tests for the survival of the organism (microbiology – plate counts) as well as the survival of transgenic DNA (molecular biology – PCR, real-time-PCR). An in-vessel composter called a “Rocket” (enclosed stainless-steel system with rotating shaft, temperature and process speed control) by Accelerated Composting was chosen for conducting the GMMO survival studies, as it represented a typical small-scale commercial system available in the UK.

GENETICALLY MODIFIED ORGANISMS (GMOs) AND THE ENVIRONMENT

The fate of material from genetically modified organisms (GMOs) when introduced to the environment is an important issue for the development of environmental risk assessments for sustainable waste management options ((EC18/2001), 2001). Horizontal gene transfer (HGT) from GM plants to bacteria has not been detected in the real environment despite extensive monitoring projects (Nielsen & Townsend, 2004) although this possibility has been demonstrated in laboratory settings (Gebhard & Smalla, 1998). In contrast, HGT between genetically modified and indigenous microorganisms has been observed in a field site (Peters et al., 1997). The implications of this result and other findings in laboratory systems are still being discussed in the context of bioremediation of contaminants using genetically modified microorganisms GMMO (Urgun-Demirtas, Stark & Pagilla, 2006).

A very recent review concluded that, on the basis of scientific evidence available, composting can be considered a potentially safe process for the disposal of genetically modified organisms (Singh, Billingsley & Ward, 2006). Beside the articles included in the review (England, Lee & Trevors, 1997; Gagliardi et al., 2001; Guan et al., 2004), other studies since have been published and confirmed that thermophilic composting rapidly degrades transgenic DNA from GM plants, microorganisms and even pigs. Time periods range between few days to weeks for GMMO and GM crops to months for transgenic pigs. (Guan et al., 2007; Guan, Spencer & Ma, 2005; Murray et al., 2007; Rasmusen, Møller & Magid, 2004).

As yet, however, no comprehensive research on the survivability or otherwise of GMMOs during composting have been conducted. This is an important question in assessing environmental risks, especially in the processing of animal by-products (ABPs), catering waste, and source-segregated municipal solid waste (SSMSW) where the presence of genetically modified material may be anticipated. ABPs and catering waste composting in the UK are both regulated by the Animal-by-products Regulations (2003a; 2003b; 2003c) implementing European Union legislation ((EC1774/2002), 2002), typically requiring

amongst other features, core temperatures of $\geq 70^{\circ}\text{C}$ and a residence time of at least 60 minutes during treatment.

This report details research conducted to investigate the potential use of composting as an effective and safe means of treating biowastes which may contain GMMOs.

OBJECTIVES OF THIS STUDY

The main objectives of the project were to investigate the potential for:

- GMMOs to survive thermophilic composting
- Functional gene expression in surviving GMMOs
- Horizontal gene transfer to and functional gene expression indigenous microorganisms
- Use of novel molecular biological techniques for monitoring of GMMO DNA during composting

MATERIALS AND METHODS

Microbiology

Preparation of agar plates

Plate count agar (PCA), chromogenic *E. coli*/ coliform medium (CECM), tryptone, technical agar, yeast extract and peptone buffered water (PBW) were ready-made and obtained from Oxoid. Luria-Bertani (LB) agar contained 10g l⁻¹ tryptone, 5g l⁻¹ yeast extract, 10g l⁻¹ NaCl and 15 g l⁻¹ technical agar. The relevant amount of ready-made medium was dissolved in demineralised water prior to autoclaving for 15min at 121°C, cooling to 50°C and pouring into 90mm, single vent, disposable Petri dishes (Sterilin). Filter sterilised ampicillin and arabinose (*Biochemika*, Sigma) were added prior to pouring to final concentrations of 100µg ml⁻¹ and 1.8mg ml⁻¹, respectively, when desired

Cultivation of *E. coli* K12 pGLO

Frozen stocks of *E. coli* K12 pGLO were maintained at -80°C in 2ml cryo-vials containing 0.9ml overnight culture and 0.9ml glycerol (50%). In preparation of spiking experiments CECM with ampicillin and arabinose (CECM+AA) plates were inoculated with *E. coli* K12 pGLO and incubated at 28-32°C for 48h. One single colony served as inoculum for liquid cultures in LB medium with ampicillin and arabinose (LB+AA) as prepared as described above except no agar was added, which were incubated at 32°C at 150rpm overnight and subsequently used for spiking.

Plate counts

Pure culture samples were serial diluted in phosphate buffered saline (PBS, 10mM Na₂HPO₄/ NaH₂PO₄, 8 gl⁻¹ NaCl) to 10⁻⁸ (or less depending on experiment) before a volume of 100µl was spread onto two or three agar plates per dilution. Initially six different media (CECM, CECM+AA, LB, LB+AA, PCA, PCA+AA) were tested. Plates were counted after incubation at 28°C for 48h. All plates containing arabinose and ampicillin were checked and counted under UV using a transilluminator (312nm, Vilber Lourmat, TF 20M). CECM+AA

was chosen as the standard medium for viable *E. coli* pGLO plate counts as high and specific recovery was achieved. Three replicates of compost and feedstock samples were initially suspended at a 1:10 ratio using PBW. Samples of 1g and 10g were used for lab-based and Rocket-based experiments, respectively. PBS was used for all further dilutions before plating, incubation and counting as described before.

Molecular biology

DNA extraction

Three different methods were initially tested (Blanc et al., 1999; Dong et al., 2006; Miller et al., 1999). Modified method 3 (Dong et al., 2006) was eventually chosen as standard protocol as DNA of satisfactory quality and quantity could be obtained. 100mg sample was weighed into a 2ml screw-cap micro-tube (Sarstedt, Germany) containing about 400mg autoclaved glass beads (<106µm, acid-washed, Sigma). After addition 300µl sodium phosphate (100mM, pH=6.6) and 200µl Al₂(SO₄)₃ (100mM) samples were mixed by shaking for 30s (Minibeadbeater-8, Biospec). Next, 300µl lysis buffer (10%SDS, 100mM NaCl, 500mM Tris (pH=8)) and 150µl NaOH (1M) were included before the second bead beating step at maximum speed for 2min. After centrifugation (GenFuge 24D, Progene) for 2min at 15500×g the supernatant was transferred to a new 2ml eppendorf tube, mixed with 60µl CTAB (10%) and 75µl NaCl (5M) and incubated at 65°C for 10min. Following the addition of 250µl isoamyl alcohol/chloroform (1:24 v/v) samples were vortexed for 10s, incubated at room temperature for 5min and centrifuged at 15500×g for 2min. The aqueous phase was moved to new eppendorf tubes, mixed with 500µl PEG8000 (30% in 1.6M NaCl) and stored at 4°C for a period of >5h (overnight). Precipitated DNA was collected by centrifugation for 30min at 15500×g (at 4°C), washed once with 300µl ice-cold ethanol (70%), dried and dissolved in 100µl TE buffer (Promega).

For DNA extractions of liquid samples (pure cultures, compost and feedstock suspensions) a total volume of 300µl was added to a bead-containing micro-tube (as described

before) and the same procedure was followed. After further tests for optimising liquid sample extractions the following protocol was established as standard procedure. 300µl sample was used in a micro-tube containing about 400mg autoclaved glass beads. Next, 300µl lysis buffer was included before beating at maximum speed for 2min. After centrifugation for 2min at 15500×g the supernatant was transferred to a new 2ml eppendorf tube, mixed with 250µl isoamyl alcohol/chloroform (1:24 v/v) and vortexed for 10s. Following incubation at room temperature for 5min, samples were centrifuged at 15500×g for 2min. The aqueous phase was moved to a new eppendorf tube, mixed with 500µl PEG8000 and stored at 4°C for a period of >5h (overnight). Precipitated DNA was collected by centrifugation for 30min at 15500×g (at 4°C), washed once with 300µl ice-cold ethanol, dried and dissolved in 100µl TE buffer (Promega).

DNA sample clean-up

In total four different commercially available kits were tested for purification of DNA:

- Wizard[®] DNA clean-up kit (Promega)
- QIAQuick PCR purification kit (QIAGene)
- Q-Spin Gel Extraction/PCR Purification Kit (Genflow)
- DNA clean-up and concentrator kit (Zymo).

In all cases the manufactures protocol was followed.

Conventional PCR

All primers were purchased at vhbio (www.vhbio.com). Stock solutions were prepared at 20µM before use. GoTaq[®] DNA polymerase, GoTaq[®] Green reaction buffer and dNTP mix were obtained from Promega. Reactions (total volume 25µl) were set-up in 500µl thin-walled PCR tubes (Axygen) containing 5µl 5× *GoTaq* reaction buffer, 0.5µl dNTP mix (10mM each), 0.125µl DNA *GoTaq* polymerase ((5U µl⁻¹); all Promega), 0.5µl forward and reverse primer (20µM), 17.375µl H₂O and 1µl purified DNA as template. After optimisation the composition was modified as stated in Table 5. Reactions were carried out using a Techne thermocycler

(Progene) using the following program: Initial cycle (1×): denaturation: 94°C (4 min), annealing 60°C (1min), elongation 72°C (1.5min); further amplification (40×): denaturation: 94°C (4 min), annealing 60°C (1min), elongation 72°C (1.5min); last cycle (1×): denaturation: 94°C (4 min), annealing 60°C (1min), elongation 72°C (10min).

Amplification products were visually checked for size and purity in ethidium bromide stained 1% agarose gels after electrophoresis (90V, 60 – 90min) with size maker Hyperladder I (Bioline) using a transilluminator (Vilber Lourmat, TF 20M).

Real-time PCR

Same primers were used for conventional and real-time PCR (see above). Brilliant[®] SYBR[®] Green Mastermix was obtained from Strategene. Reactions (total volume 25µl) were set-up in 500µl thin-walled PCR stripes (8-tubes) containing 12.5µl 2× Brilliant[®] SYBR[®] Green Mastermix, 5µl forward and reverse primer (20µM), 0.375µl ROX reference dye (20µM), 10.125µl H₂O and 1µl purified DNA as template. After optimisation the composition was modified as stated in Table 6. Reactions were carried out using a MX3000P Real-Time PCR system (Strategene) using the following program: Initial cycle (1×): denaturation: 94°C (10 min); further amplification (40×): denaturation: 94°C (30 min), annealing 60°C (1min), elongation 72°C (0.5min); last cycle (1×): denaturation: 94°C (4 min), annealing 60°C (1min), elongation 72°C (10min). Melting curve analysis was carried out at the end of each program. Fluorescence data were collected for FAM (excavation 492nm, emission 516nm) and ROX (excavation 585nm, emission 610nm) for each cycle. For data analysis, including determination of threshold cycle Ct and melting temperature MX3000P software was used.

Operation of Rocket

Operation of the in-vessel composter was performed according to the manufacturer's guidelines. After optimisation temperature and speed settings of 12 and 7 were routinely used. Mass and volume of in and outgoing material were monitored daily. Feedstock was obtained

by mixed 20l of green and brown waste (if available) with 3 – 5l of water. 10 – 15 sub-samples (about 10g each) of pre-mixed feedstock and product were randomly taken and mixed to obtain a representative sample (total 100 – 200g) for moisture content, and physical-chemical analysis when desired. Moisture content was determined by drying 3 replicate samples (about 5g each) at 105°C until a constant weight was reached. Temperatures were recorded by four built-in probes. Additionally three manual temperature reading were taken each day.

Detection limits for microbiology and molecular biology

An overnight culture of *E. coli* K12 pGLO ($OD_{470nm} \approx 0.5$ for 1:10 dilution in water, $1.0 \pm 0.5 \times 10^9$ cfu ml⁻¹) was serial diluted in PBS to 10⁻⁶. 100µl of each dilution was plated on at least two CECM+AA plates for pure culture cell counts. Seven autoclaved 300ml autoclaved glass jars containing 40g compost product each were prepared. Spiking solutions consisted of 1ml of overnight culture or serial dilution (see above) and 9ml PBS. After vortexing they were added to the appropriate jars (one jar per spiking solution) in 1ml portions by mixing with a sterile inoculation loop. Jars were shaken vigorously and left at room temperature for 1h. Three 1g samples were taken out of each jar into a sterile 15ml centrifuge tube, suspended in 9ml PBW and vortexed. Again, serial dilutions of those suspensions were prepared in PBS and 100µl of appropriate dilutions were subsequently plated on CECM+AA agar plates. Colonies were counted at UV after incubation at 28°C for 48h.

The same procedure was used for determining detection limit in spiked feedstock with the difference that 50g freshly prepared feedstock was used instead of compost product. For molecular biology pure culture detection limits 300µl of each serial dilution was extracted using the standard protocol describe above. Real-time PCR was performed as described above. Detection limits for spiked compost and feedstock 300µl samples of the original suspensions (1g sample in 9ml PBW) were extracted using the optimised protocol and subject to amplification by real-time PCR.

Lab-based *E. coli* K12 pGLO survival experiments

Lab-based survival experiments were performed in autoclaved 300ml glass jars, using three jars per treatment. For the autoclaved treatment compost was sterilised (121°C, 20min) three times with 48h breaks between autoclaving. 50g of compost product was weighed into each jar. 10ml spiking solution, 15µl *E. coli* K12 pGLO overnight culture ($1.0 \pm 0.5 \times 10^9$ cfu ml⁻¹) in 10ml PBS) was added to each jar as described above. Plate counts of the spiking solution were determined to ascertain the calculated number of cells added per g of compost or feedstock. All initial samples (time point 0) were taken after incubation for 1h at room temperature by removing 1g per replicate and suspending it in 9ml PBW. After 30s vortexing, a 1ml suspension sub-sample was frozen at -20°C for molecular biology testing. Microbiological testing was performed on the day of sampling. All subsequent steps for analysis of cell counts and marker gene were the same as described above for determination of the detection limit.

Two sets of jars were incubated at water baths set at 65°C and 40°C. A third set was left at room temperature (20 – 24°C). Experiments were sampled at specific days in order to establish a reasonable time course for each data set. Two independent experiments were performed for each temperature.

***E. coli* K12 pGLO survival during Rocket-based thermophilic composting**

Before starting GM experiments a stable thermophilic in-vessel composting process was established over a period of 10 – 20 days. After the addition of spiked material (day 0), the product was sampled daily as described above for a two week period. According to GM regulations all remaining material produced was subsequently autoclaved before disposal. 40l of mixed waste, 40l compost product and 44l of green waste/ product mixture were used for spiking in experiments 1, 2 and 3. Spiking solutions containing 15ml *E. coli* K12 pGLO overnight culture (about 10^{10} cfu) in 500ml PBS were mixed with the in-going material by

hand. A 100-150g sample was prepared by mixing 10 – 15 randomly taken sub-samples (about 10g each) to establish initial numbers for cell counts and marker gene. Plate counts of the spiking solution were determined to ascertain the calculated number of cells added per g of feedstock.

Three replicates of spiked material or compost product (10g each) were weighed into autoclaved glass jars and suspended in 90ml PBW. 30s vortexing, a 1ml suspension sub-sample was frozen at -20°C for molecular biology testing. Microbiological testing was performed on the day of sampling. All subsequent steps for analysis of cell counts and marker gene were the same as described above for determination of the detection limit.

Statistical Analysis

_____All experiments were designed to facilitate statistical analysis appropriate to the nature of the work. All error bars are calculated and plotted from the Standard Error of the individual data mean.

RESULTS

Microbiology - method development

***Gfp* insertion into and expression in *E. coli* K12**

Parent *E. coli* K12 was used as a host for the pGLO plasmid (see Figure 7) which contains genes for expression of the green fluorescent protein (*gfp*) under the regulation of the P_{BAD} promoter and ampicillin resistance (commercially available as *Biotechnology Explorer*[™] pGLO[™] Bacterial Transformation Kit). Successful transformation was confirmed by checking colonies of *E. coli* K12 pGLO on LB+AA agar plates (Figure 2) under UV light.



Figure 2: Fluorescent colonies of *E. coli* K12 pGLO growing on LB+AA agar when exposed to UV light [312nm].

Media selection

Three different bacteriological media: chromogenic *E. coli* medium, standard plate count agar and LB agar were tested for their suitability to grow *E. coli* K12 pGLO. In order to verify the presence and functioning of the *gfp* gene a duplicate set of the above media were supplemented with ampicillin and arabinose (CECM+AA, PCA+AA, LB+AA). Resistance to ampicillin is encoded in the pGLO plasmid and therefore, can be used to select for *E. coli* K12 pGLO. The presence of arabinose is necessary for the expression of the *gfp* gene which can be used for specific identification (Figure 2).

The highest number of colonies was recovered from CECM ($2.6 \pm 0.4 \times 10^9$ cfu ml⁻¹) which significantly exceeded ($p < 0.05$) results obtained for LB (2 fold) and PCA (3.9 fold), respectively (Figure 3). Differences in cell counts between plates with and without ampicillin and arabinose were not significant. CECM+AA agar was chosen for further experiments as it showed highest recovery rates by providing optimal growth conditions for *E. coli*. At the same time chromogenic components within the medium allowed the identification of *E. coli* as purple colonies, as a result of a reaction catalysed by two specific enzymes glucuronidase and galactosidase (Figure 4). Furthermore, presence and expression of *gfp* can be checked using a UV light source as demonstrated in Figure 2 using the same agar plate. This unique combination provides a strong basis for the detection of *E. coli* K12 pGLO in compost samples and prevents the need for replicate plating.

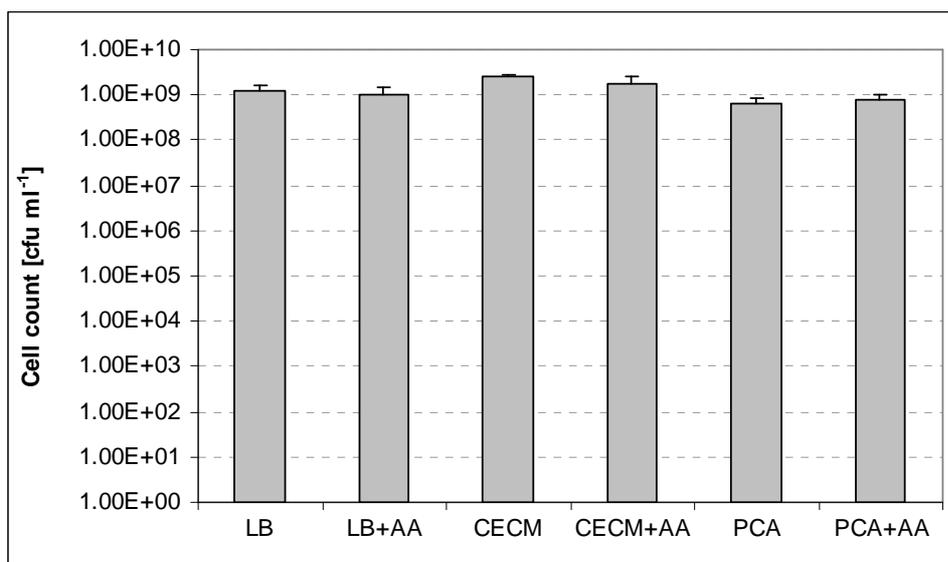


Figure 3: Cell counts of *E. coli* K12 pGLO on different culture media

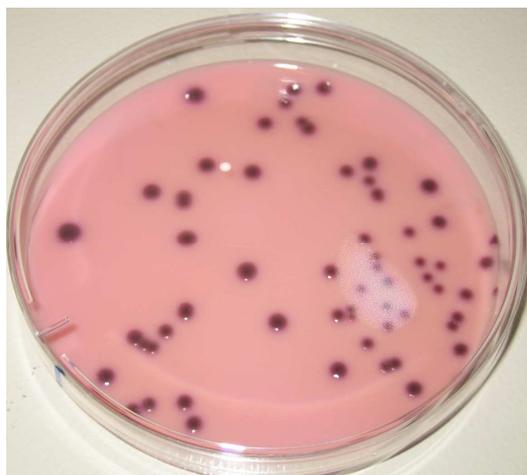


Figure 4: Formation of purple *E. coli* K12 pGLO colonies on CECM agar plates

Detection limit for viable plate counts

Lab-based experiments in glass jars were carried out to determine the detection limit for the recovery of *E. coli* K12 pGLO from deliberately spiked compost and feedstock samples. When compost was spiked with seven different levels of *E. coli* K12 pGLO ($1.28 \pm 0.1 \times 10^1$ to $1.28 \pm 0.1 \times 10^7$ cfu g⁻¹) colonies were only recovered in samples where $\geq 1.28 \pm 0.1 \times 10^4$ cfu g⁻¹ were added (Figure 5). In all other cases individual *E. coli* K12 pGLO colonies could not be identified as they were overgrown by other members of the microbial community. It should be noted that in at the lowest detectable level the number of countable colonies per plate (10 to 30) were lower than the range recommended for statistical valid data (30 to 300). In all positive treatments the number of recovered cells was in the same order of magnitude as the calculated (theoretically expected) number indication that *E. coli* K12 pGLO could successfully be recovered. The experimental detection limit was 1.28×10^4 cfu g⁻¹. Considering the fact that for spiked compost only plates at dilutions $>10^{-1}$ could be counted reliably and 30 being the minimum number of colonies, a theoretical detection limit of 3×10^4 cfu g⁻¹ can be calculated (sample volume 100μl) which is in good agreement with the experimental value. The corresponding number for pure culture samples under the same conditions is 3×10^2 cfu ml⁻¹.

E. coli K12 pGLO recovery in feedstock spiked at the same levels as above ($1.66 \pm 0.25 \times 10^1$ to 1.66×10^8 cfu g⁻¹) was only possible in samples $\geq 1.66 \times 10^5$ cfu g⁻¹ (Figure 6), one order of magnitude lower compared to compost samples.

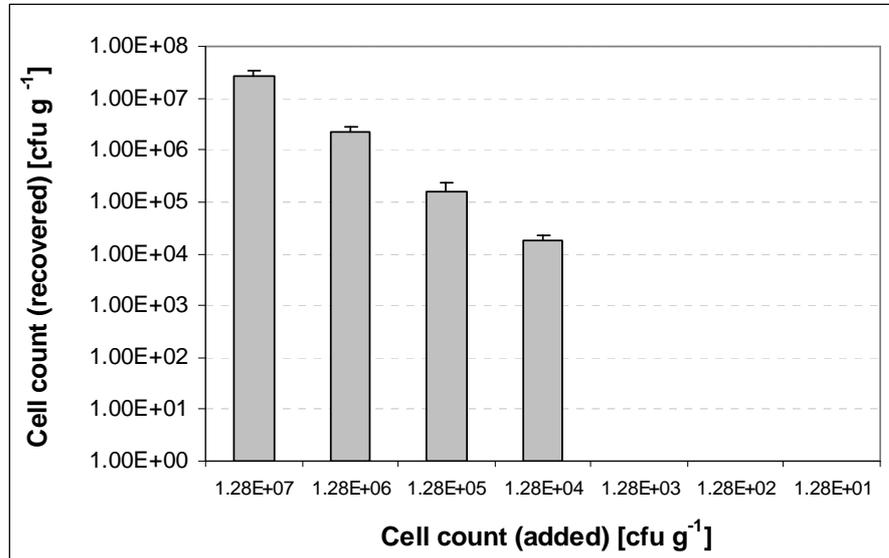


Figure 5: Recovery of *E. coli* K12 pGLO from spiked compost samples on CECM+AA agar plates

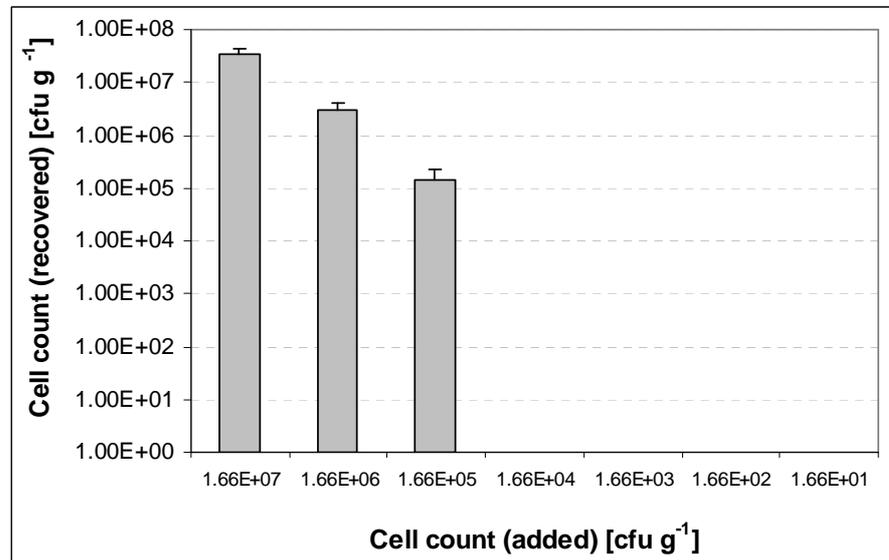


Figure 6: Recovery of *E. coli* K12 pGLO from spiked feedstock samples on CECM+AA agar plates

Molecular Biology – method development

Primer design

Initial trials were performed using a range of primer pairs designed for the detection of two regions in the pGLO plasmid: *gfp* (position 1341-2059) and promoter pBAD within *araC* (1003-1280) (Figure 7, Table 1). Detailed information about all primers tested can be found in Tables 1 and 2. *E. coli* K12 pGLO and pGLO plasmid DNA served as positive controls whereas two native *E. coli* strains and non-GM plant DNA (elm) were used as negatives. Several checks returned false-positive results for negative controls when tested with *gfp* primers (data not shown). This also was the case for two pBAD primers pairs. Initially pBAD_1F/8R and pBAD_1F/10R delivered the expected specific results (Figure 8). Product sizes between 100 and 200bp were considered to be ideal for the real-time PCR reaction therefore it was checked whether the actual sizes of 861 and 420bp were suitable for quantification. Linear relationships between initial DNA concentration and Ct value (inversely proportional to log of the initial copy number) exist in both cases over a range of 6 orders of magnitude with efficiencies of 88.3 and 101.3% (Figure 9). As pBAD_1F/10R created inconclusive outcomes in subsequent tests it was excluded.

Finally, two primers pBAD_1F and *gfp*_6R were combined to probe for the overlapping pBAD-*gfp* region which is a highly specific marker for *E. coli* K12 pGLO. The latter were chosen for the standard molecular biology tests.

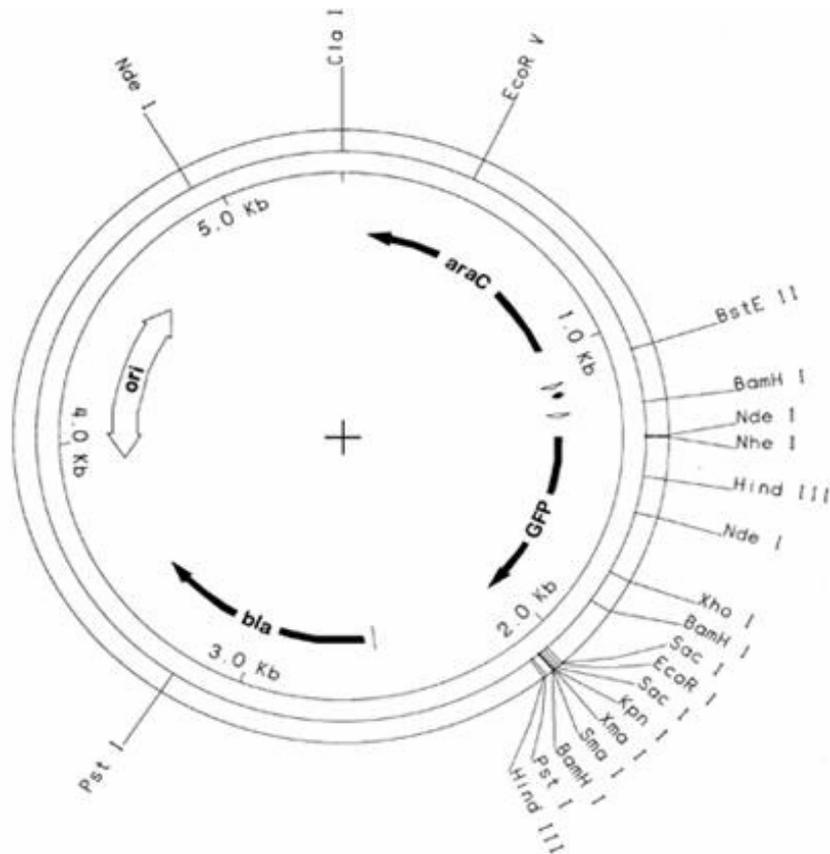


Figure 7: Plasmid map and nuclease digestion sites for plasmid pGLO (Bio-Rad, 2002)

| Table 1: Properties of primers for detection of partial pGLO sequences | | | |
|---|-------------|---------------------------------|-------------------------|
| Direction | Name | Sequence (5' → 3') | Position in pGLO |
| F | GFP_1F | ACT TTC TCT TAT GGT GTT CAA TGC | 1530 |
| R | GFP_2R | TGT AGT TCC CGT CGT CCT TGG | N/A |
| F | GFP_3F | TAC AAG ACG CGT GCT GAA GT | 1659 |
| R | GFP_4R | CAA TGT TGT GGC GAA TTT TG | 1858 |
| R | GFP_6R | CTT TTG TTT GTC TGC CGT GA | 1818 |
| F | GFP_7F | ACC TGT TCC ATG GCC AAC ACT T | 1502 |
| R | GFP_8R | TTC GGG CAT GGC ACT CTT GAA A | 1614 |
| R | GFP_10R | AAG GAC CAT GTG GTC ACG CTT T | 2004 |
| F | pBAD_1F | GTA ACA AAG CGG GAC CAA AGC CAT | 1103 |
| R | pBAD_2R | AAA GCG TCA GGT AGG ATC CGC TAA | 1257 |
| R | pBAD_4R | GCA AAG TGT GAC GCC GTG CAA ATA | 1206 |
| R | pBAD_8R | AGG GCA GAT TGT GTC GAC AGG TAA | 1964 |
| R | pBAD_10R | AGT GTT GGC CAT GGA ACA GGT AGT | 1523 |
| F | pBAD_5F | TCG CTA ACC AAA CCG GTA AC | 1062 |
| R | pBAD_6R | CGT CAG GTA GGA TCC GCT AA | 1253 |

| Table 2: Product sizes and concentration of primer pairs used for detecting of partial pGLO sequences | | | |
|--|------------------|------------------|-----------------------------------|
| Combination | Region | Size (bp) | Optimal concentration (nM) |
| GFP_1F/2R | <i>gfp</i> | 250 | N.D. |
| GFP_3F/4R | <i>gfp</i> | 199 | N.D. |
| GFP_3F/6R | <i>gfp</i> | 159 | N.D. |
| GFP_7F/8R | <i>gfp</i> | 112 | N.D. |
| GFP_7F/10R | <i>gfp</i> | 502 | N.D. |
| pBAD_1F/2R | pBAD | 154 | N.D. |
| pBAD_1F/4R | pBAD | 103 | N.D. |
| pBAD_1F/8R | pBAD | 861 | 300nM (1F)/ 100nM (8R) |
| pBAD_1F/10R | pBAD | 420 | N.D. |
| pBAD_5F/6R | pBAD | 191 | N.D. |
| pBAD_1F/ GFP_6R | pBAD/ <i>gfp</i> | 715 | 600nM (1F)/ 300nM (6R) |

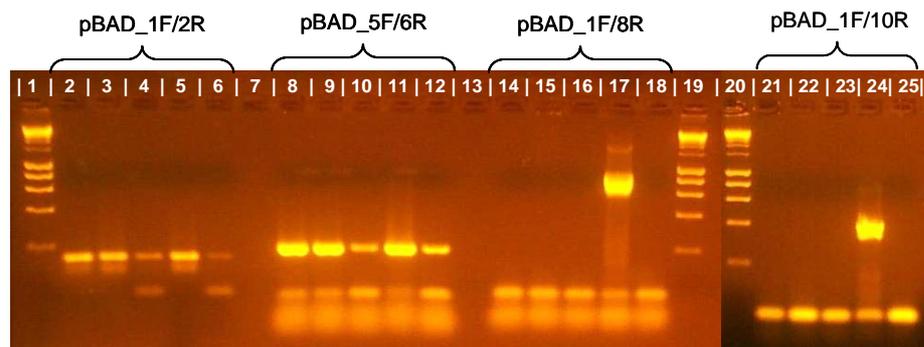
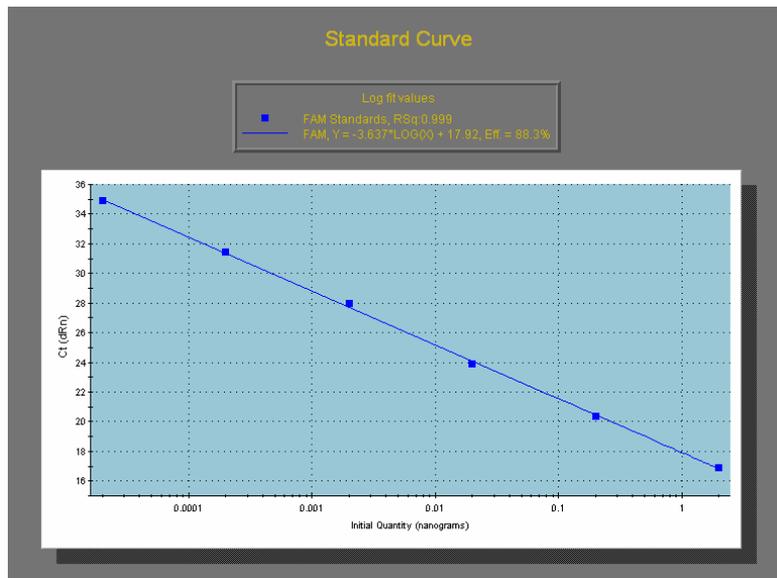
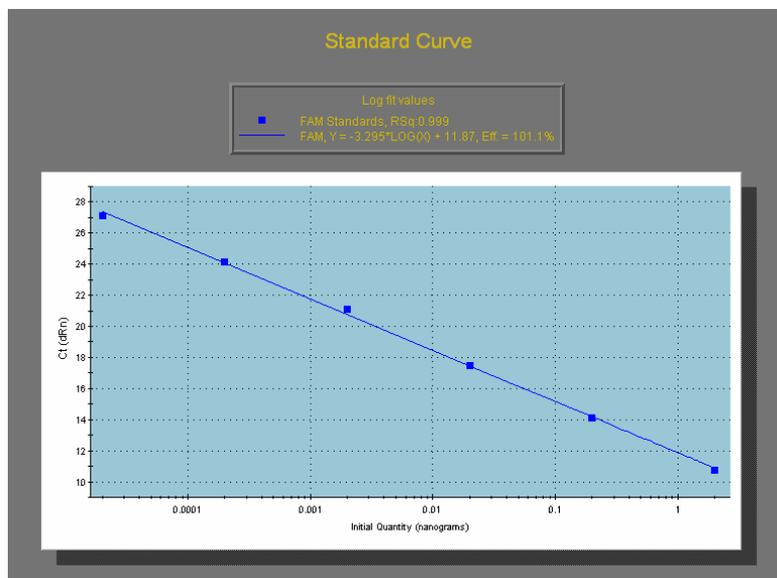


Figure 8: Ethidium bromide stained agarose gel showing products of PCR reaction with different primer pairs (Table 2).



A



B

Figure 9: Linear relationship in real-time PCR between initial copy number and C_t value for primer pair pBAD_1F/8R (A) and pBAD_1F/10R (B)

DNA extraction from solid samples

Initial method development was conducted using samples taken from compost produced with the Rocket in-vessel composter. Different extraction protocols for compost have been published of which three were tested for their suitability (Blanc et al., 1999; Dong et al., 2006; Miller et al., 1999). The basic differences between methods are, ways of cell disruption (freeze/thawing or bead-beating), composition of lysis buffer as well the inclusion of washing and pre-treatment steps.

Major problems occurred with method 1 (Miller et al., 1999) resulting in low DNA recovery due to co-extraction of major amounts of humic acids (Figure 10A, 11A). Method 2 (Blanc et al., 1999) involved pre-treatment and washing steps as well as the addition of CTAB. It resulted in improved DNA yield and better quality (Figure 10B) but proved to be time-consuming and labour intensive. Method 3 (Dong et al., 2006) included an additional approach for humic acid removal by precipitation with $Al_3(SO_4)_2$ (Figure 11B). When combined with CTAB addition it delivered the best results and was adapted as the standard protocol for solid compost and feedstock samples (Table 3, Figure 10C, 11C).

| Table 3: Improved standard protocol for DNA extraction from compost (method 3) | | |
|---|-----------------------------------|-------------------|
| Step | Chemical | Procedure |
| Humic acid removal (1) | $Al_2(SO_4)_3$ / NaOH | Bead beating |
| Cell disruption | Lysis buffer (SDS, Tris, NaCl) | Bead beating |
| Humic acid removal (2) | CTAB | Incubation @ 65°C |
| Protein removal | Chloroform | Incubation @ 25°C |
| Precipitation of DNA | PEG 8000 | Incubation @ 4°C |
| Washing step | Ethanol | |
| Dissolve DNA | TE buffer | |

Initial tests showed that crude DNA obtained with the improved protocol could not be amplified with PCR or real-time PCR unless diluted 100fold (data not shown).

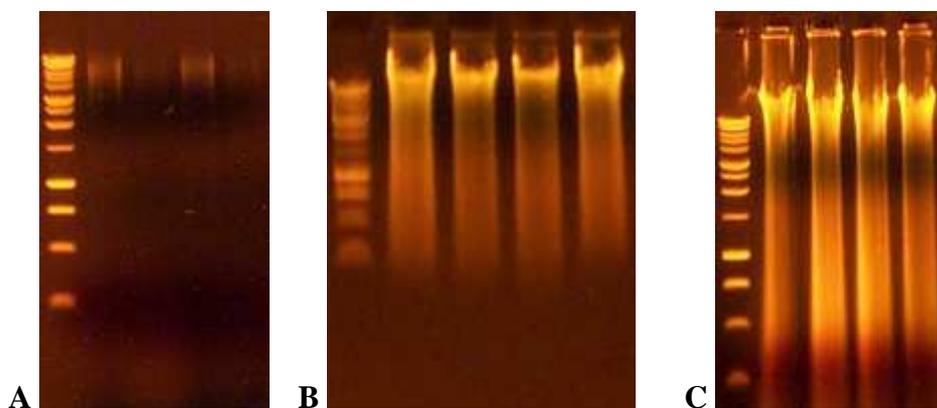


Figure 10: Ethidium bromide stained agarose gel showing total DNA extracted from compost with method 1 (A), method 2 (B) and method 3 (C) as described above.

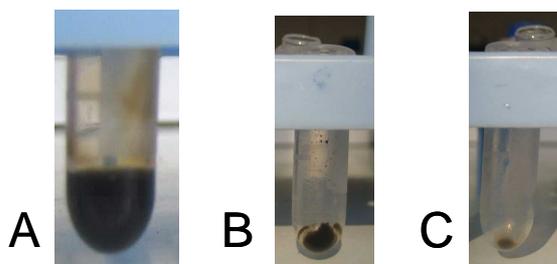


Figure 11: Formation of black, humic acids containing pellets during DNA extraction from compost using method 1 (A), unmodified method 3 (B), modified method 3 (C).

DNA extractions from liquid samples

Liquid compost suspensions were created as the first step in the process of microbiological testing by mixing solid samples (compost or feedstock) with peptone buffered water (ratio 1:10). By using those samples as source material for DNA extractions and subsequent molecular biology analysis, results should be directly comparable. Samples obtained from *E. coli* K12 pGLO liquid cultures were also included in the method development. Initial tests using the standard protocol developed for compost produced some DNA but, separation problems occurred at the chloroform step for both pure culture and compost suspensions. Therefore, further trials were carried out to solve this problem (Table 4). The results summarised in Table 4 show that the addition of CTAB might have triggered separation problems (method 3-I, 3-III) whereas, the aluminium sulphate treatment caused formation of a precipitate which additionally hampered separation (method 3-I, 3-II). There was no difference in handling pure culture samples and compost suspensions. DNA was successfully extracted in all cases with best results in treatments containing no $\text{Al}_2(\text{SO}_4)_3$ (Figure 12, lanes 6-9 and 14-17).

Initial tests were carried out to test whether the crude DNA could be amplified in PCR and real-time PCR reactions. Six and three out of eight samples from pure culture and compost suspensions, respectively, produced PCR products (Figure 13). Positive results using real-time PCR were obtained for methods 3-I, 3-III, 3-IV with pure culture extracts only and were in accordance with those of conventional PCR (Figure 14). Method 3-IV was chosen as

the standard extraction protocol for liquid samples as it consistently delivered the best results in terms of DNA quantity and quality.

| Method | Set-up | | Results | |
|--------|---|------|------------|----------------------|
| | Al ₂ (SO ₄) ₃ | CTAB | Separation | Unwanted precipitate |
| 3-I | ✓ | ✓ | bad | yes |
| 3-II | ✓ | | good | yes |
| 3-III | | ✓ | very bad | no |
| 3-IV | | | very good | no |

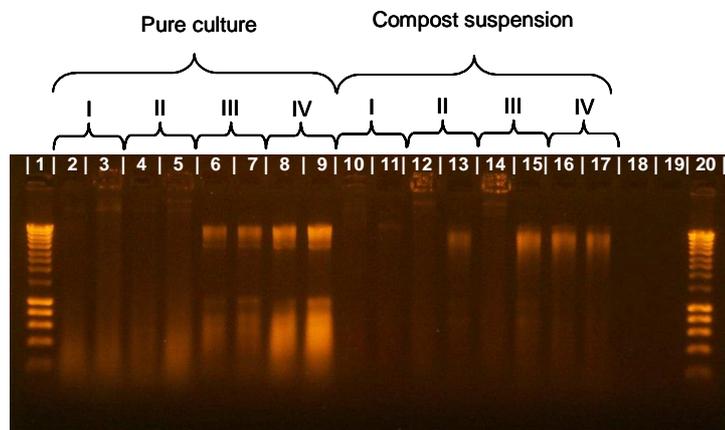


Figure 12: Ethidium bromide stained agarose gel showing total DNA extracted from pure culture and compost suspensions using methods 3-I to 3-IV (see Table 4).

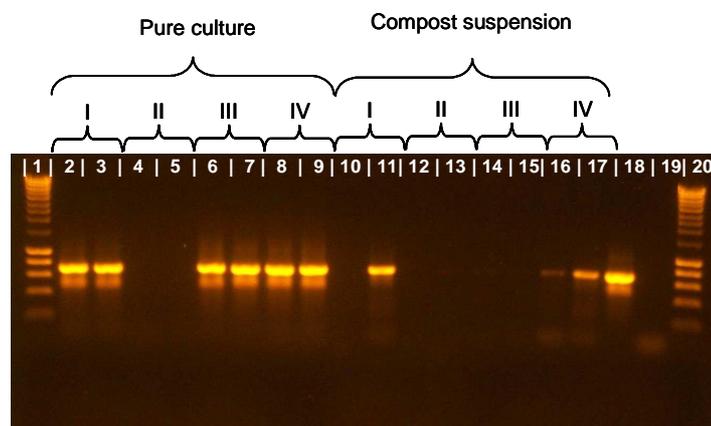


Figure 13: Ethidium bromide stained agarose gel showing results of conventional PCR with primer pair pBAD_1F/gfp_6R (715bp) testing DNA extracted from *E. coli* pGLO pure culture and spiked compost suspension using methods 3-I to 3-IV.

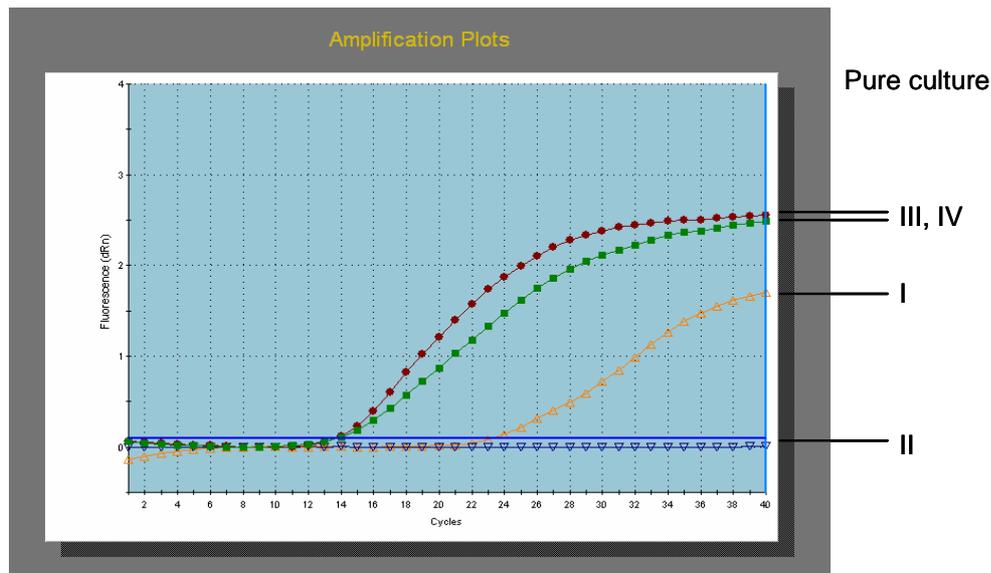


Figure 14: Amplification plot showing results of real-time PCR with primer pair pBAD_1F/gfp_6R (715bp) testing DNA extracted from *E. coli* pGLO pure culture using methods 3-I to 3-IV.

Clean-up of crude DNA

After the establishment of a DNA extraction protocol it was possible to qualitatively test compost samples for the presence of the *gfp* gene using conventional PCR. However, it was still not possible to achieve amplification in real-time PCR. As pure culture extracts could easily be amplified using this method, it seems likely that inhibitory substances affecting real-time PCR reactions were co-extracted from compost and feedstock suspensions.

One option to overcome the above problem is sample dilution. Compost suspensions had to be diluted at least 100 fold to produce real-time PCR products (Figure 15). The major drawback of the approach is the substantial loss of sensitivity by lowering the initial number of targets within the samples.

Another possibility is the removal of inhibitory substances using commercially available kits or individually developed clean-up procedures. Within the time limits of this project four different kits were tested on crude DNA obtained using method 3-IV: Wizard DNA clean-up kit (Promega), QIAQuick PCR purification kit (QIAGEN), Q-spin PCR purification kit (Genflow) and DNA clean-up and concentrator kit (Zymo). Whereas,

amplification of a 715bp product with no differences in intensity was observed in conventional PCR reactions (Figure 16), again no amplification was achieved when real-time PCR was used (data not shown). Therefore, all commercial kits are equally suitable for the clean-up of compost suspension samples for use in conventional PCR reactions.

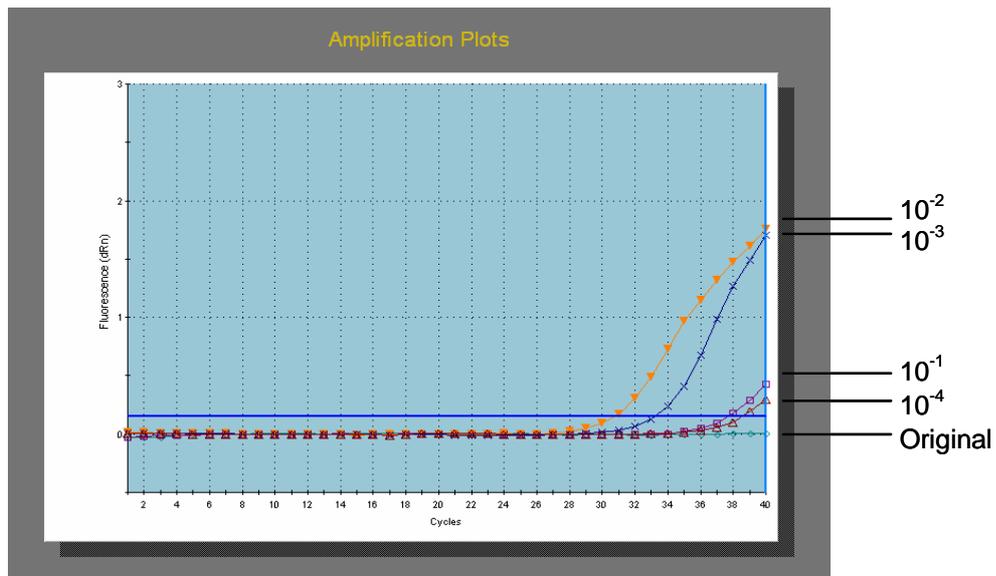


Figure 15: Amplification plot showing real-time PCR products (pBAD_1F/*gfp*_6R) of serial diluted crude DNA extracted from compost suspensions using method 3-IV.

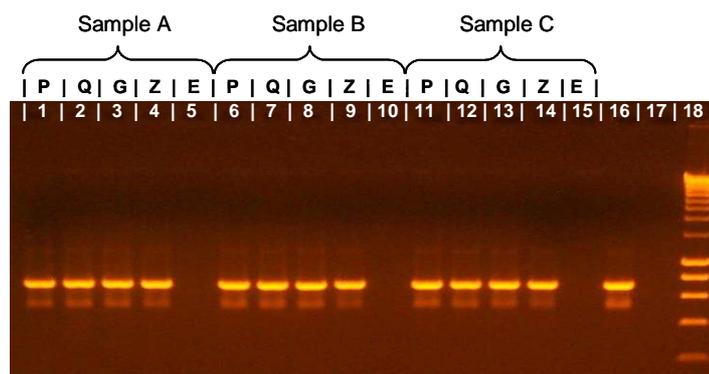


Figure 16: Ethidium bromide stained agarose gel showing results of conventional PCR using primer pair pBAD_1F/*gfp*_6R (715bp) testing DNA extracted from three replicate samples of spiked compost suspension (method 3-IV) after clean-up with kits from Promega (P), QIAGene (Q), Genflow (G), Zymo (Z), no clean-up (E).

Optimisation of PCR and real-time PCR reactions

Effective protocols were developed for DNA extraction from solid and liquid samples and crude DNA clean-up using commercial kits. The initial composition of reaction mixes used in PCR and QPCR were chosen according to the manufacturer's recommendations (Tables 5, 6). Optimised primer concentrations were subsequently determined by primer titration. Pure culture samples could be amplified without clean-up using both PCR and real-time PCR. Purified DNA obtained from compost or feedstock suspensions was suitable for PCR reactions but not real-time PCR. One of the reasons might be that the two DNA polymerases used for PCR (*GoTaq* DNA polymerase, Promega) and real-time PCR (SureStart *Taq* DNA polymerase, Strategene) have different sensitivities towards inhibitory substances. Successful amplification was achieved when *GoTaq* polymerase was included in the real-time PCR reaction mix. Due to time limitations this mixture was not further optimised as it produced satisfactory results.

| Table 5: Initial and optimised composition for conventional PCR reactions (total volume 25µl) | | | | |
|--|----------------------------|-------------------------------|------------------------------|-------------------------------|
| | Initial composition | | Optimised composition | |
| | Volume (µl) | Final concentration | Volume (µl) | Final concentration |
| 5× <i>Gotaq</i> reaction buffer | 5 | 1× (1.5mM MgCl ₂) | 5 | 1× (1.5mM MgCl ₂) |
| dNTP mix (10mM each) | 0.5 | 0.2mM each | 0.5 | 0.2mM each |
| <i>GoTaq</i> DNA polymerase (5U µl ⁻¹) | 0.125 | 1.25U | 0.125 | 1.25U |
| Primer (20µM) | 0.5 each | 400nM | 0.75 (B1F) 0.375 (G6R) | 600nM (B1F) 300nM (G6R) |
| H ₂ O | 17.375 | N/A | 17.25 | N/A |
| Template DNA | 1 | N/A | 1 | N/A |

| Table 6: Initial and optimised composition for real-time PCR reactions (total volume 25µl) (* contains reaction buffer, dNTP, SureStart<i>Taq</i> DNA polymerase) | | | | |
|--|----------------------------|----------------------------|------------------------------|----------------------------|
| | Initial composition | | Optimised composition | |
| | Volume (µl) | Final concentration | Volume (µl) | Final concentration |
| 2× Brilliant SYBR Green QPCR Mastermix * | 12.5 | 1× 2.5mM MgCl ₂ | 12.5 | 1× 2.5mM MgCl ₂ |
| ROX reference dye (2µM) | 0.375 | 30nM | 0.375 | 30nM |
| Primer (20µM) | 0.5 each | 400nM | 0.75 (G1F) 0.375 (G6R) | 600nM (B1F) 300nM (G6R) |
| H ₂ O | 10.125 | N/A | 10 | N/A |
| Template DNA | 1 | N/A | 1 | N/A |
| Go <i>Taq</i> DNA polymerase (5U µl ⁻¹) | N/A | N/A | 0.125 | 1.25U |

Detection limits for molecular biology

A dilution series using an *E. coli* K12 pGLO overnight culture at levels ranging from 6.4×10^8 to 6.4×10^2 cfu ml⁻¹ was used to evaluate the real-time PCR detection limit in samples containing no inhibitory substances originating from compost or feedstock. When a sample volume of 100 µl was extracted a positive signal could be detected down to 6.4×10^3 cfu ml⁻¹ (data not shown). When 300 µl were used the detection limit increased about 10 fold to 4.1×10^2 cfu ml⁻¹ and 8.2×10^4 copies ml⁻¹, respectively, based on an average number of 200 copies of pGLO plasmid (Bio-Rad, 2007) and *gfp* per cell (Figure 17). This is equivalent to about 80 copies of *gfp* per real-time PCR reaction. There was a linear relationship between the number of added copies in the sample and the Ct value of the real-time PCR reaction for all except the lowest concentration (Figure 17B). Conventional PCR confirmed the above findings with products generated at all tested levels (Figure 18). For all subsequent experiments a sample volume of 300 µl for DNA extractions was defined.

For spiked compost samples detection was possible at levels of 1.28×10^4 cfu g⁻¹ or 2.56×10^6 copies g⁻¹ (Figure 19) which was at the same order of magnitude compared to

testing by plate count (Figure 5). Similar results were achieved for spiked rocket feedstock samples, 1.6×10^4 cfu g^{-1} equivalent to 3.3×10^6 copies g^{-1} could be positively identified (Figure 20). For those samples real-time PCR was about 100 fold more sensitive than plate counts (Figure 6).

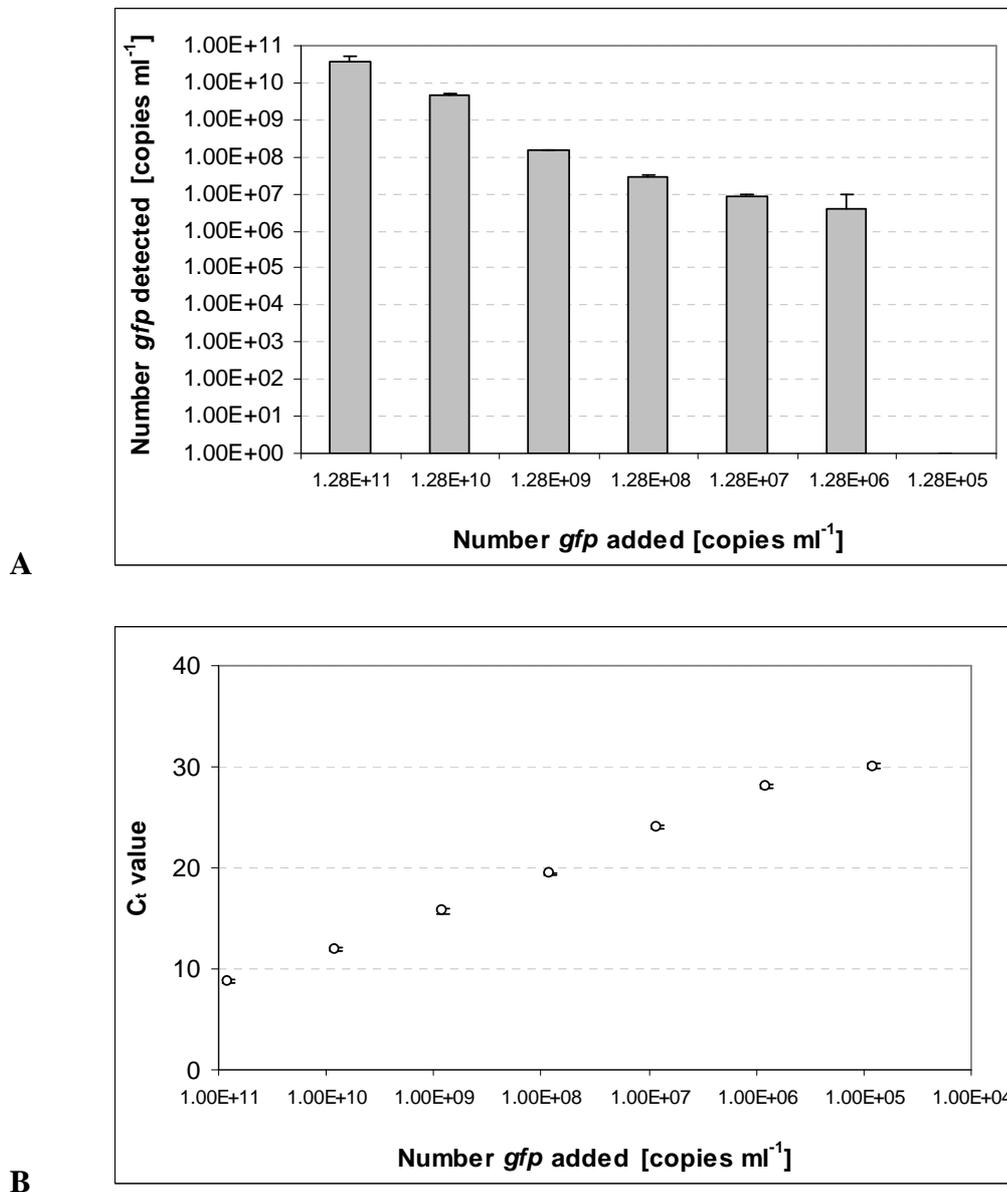


Figure 17: (A) Detection of *gfp* in *E. coli* pGLO pure cultures using real-time PCR (pBAD_1F/*gfp*_6R). (B) Relationship between Ct value and added number of *gfp*.

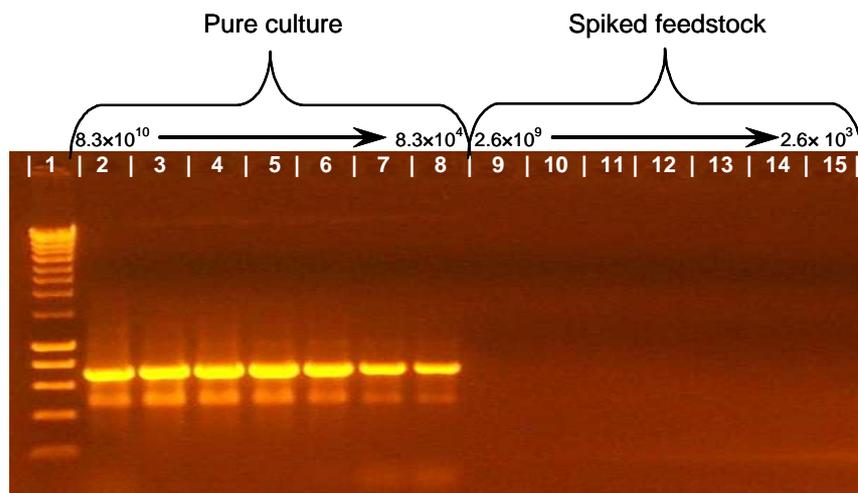


Figure 18: Ethidium bromide stained agarose gel showing results of conventional PCR using primer pair pBAD_1F/*gfp*_6R (715bp) testing purified DNA extracted from an *E. coli* pGLO pure culture and spiked compost suspension.

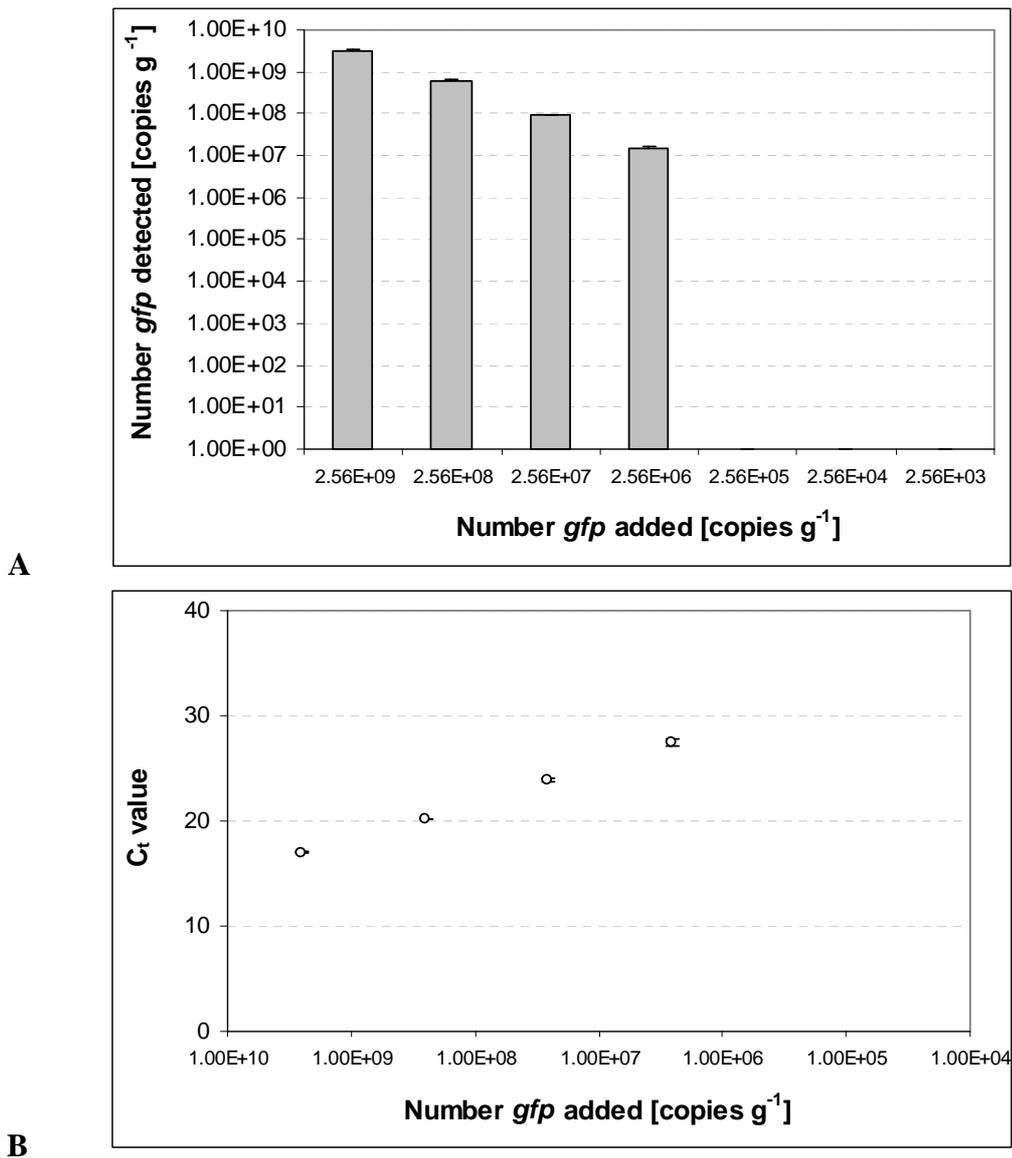


Figure 19: (A) Detection of *gfp* in compost samples spiked with *E. coli* pGLO using real-time PCR (pBAD_1F/*gfp*_6R). (B) Relationship between C_t value and added number of *gfp*.

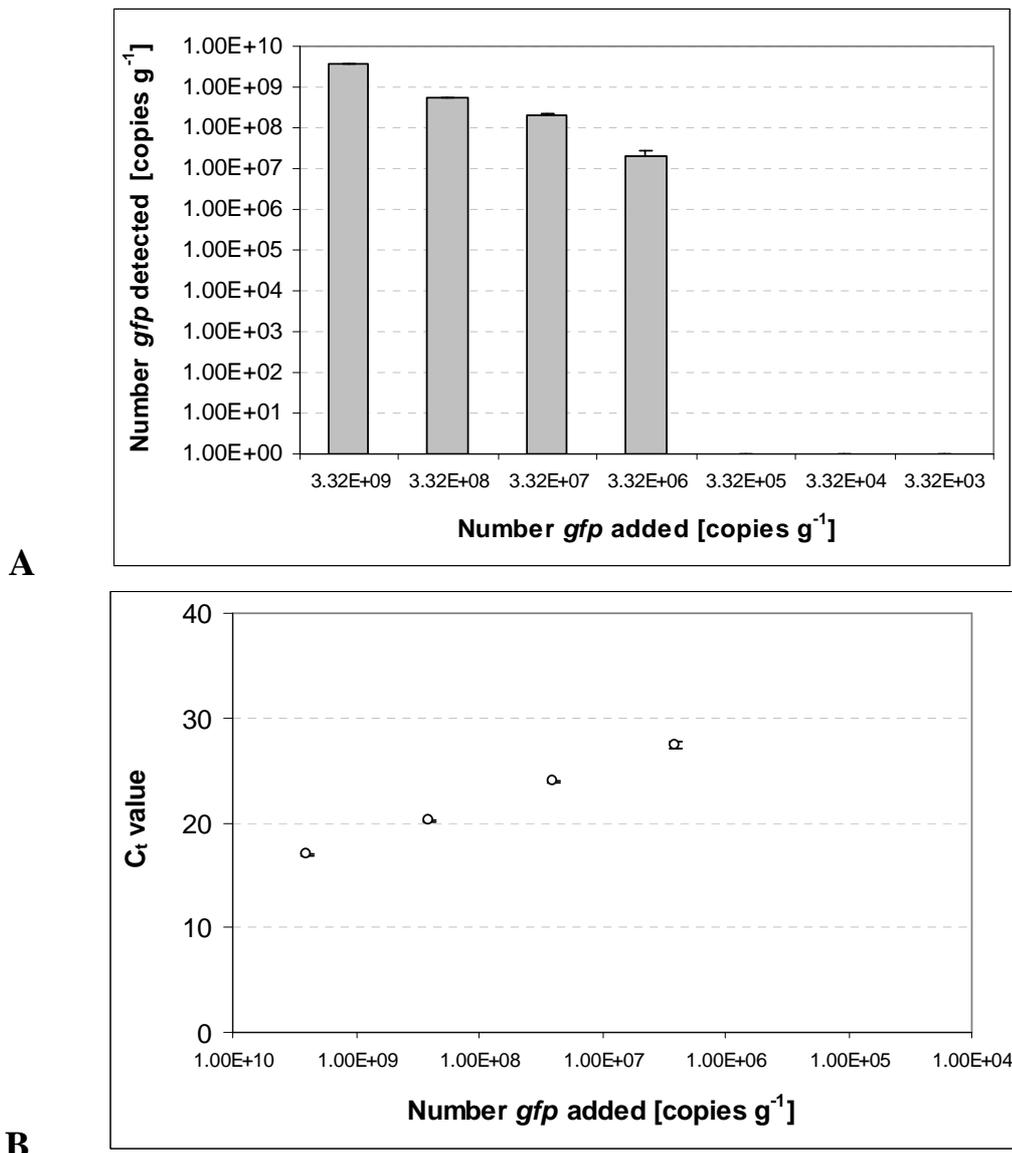


Figure 20: (A) Detection of *gfp* in feedstock samples spiked *E. coli* pGLO using real-time PCR (pBAD_1F/*gfp*_6R). (B) Relationship between C_t value and added number of *gfp*.

Rocket installation and optimisation

Temperature and speed settings

The in-vessel compostor (the Rocket™) was installed and put into operation according to the manufacturer's instructions. The Rocket consists of a covered stainless steel tube with a rotating internal shaft with five blades for moving waste from the inlet to the outlet openings. There is also insulation and a booster heating facility. It is operated in a sloped position to allow excess liquid to leave the system at the inlet end only (Figure 21). Two main parameters

can be controlled to achieve optimal composting conditions: temperature and process speed. Initial parameters were chosen based on the manual and personal recommendations.

Day-to-day feedstock was provided by the Dundee City Council as green waste (grass cuttings, hedge cuttings (broadleaf and conifer), branches, autumn foliage, weeds), brown waste (bark, wood chips, material taken from mature windrows) or mixed waste (material taken from freshly prepared windrows). At times when feedstock was not available, material which had undergone one cycle of composting was re-introduced into the Rocket.

The standard daily input of feedstock, a total volume 40l, consisted of equal parts of green and brown waste. This was equivalent to a total weight of 7 – 8kg with a ratio of green to brown waste of about 40:60. Depending on the particle size and kind of feedstock the material was shredded before mixing to ensure a homogeneous distribution of green and brown waste. Depending on the nature of raw material water was added to adjust the moisture content. A summary of target and actual values is given in Table 7.

First, the optimal parameters for temperature and process speed were determined (Figure 22). A target temperature of 65°C in the heated part of the machine (T2, T3) was aspired to, which could be achieved with setting 12 (Table 8). The process speed was set at the lowest level (7) for several reasons; first volume and mass reduction did not differ between setting 7 and 12 (data not shown) and second, a lower speed ensures a longer contact period within the relevant temperature zone. The average retention time in the Rocket at this level was determined to be 6.3 ± 2.9 days. Average values for mass and volume reduction under standard conditions (temperature setting 12, speed setting 7) were 61.2% and 55.6%, respectively.

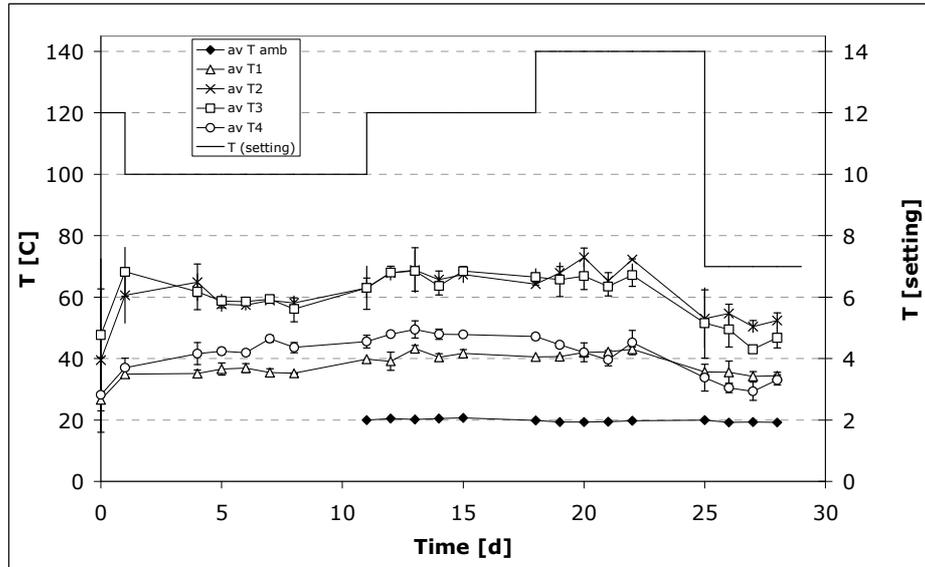
The day-to-day operation on weekdays included three general checks and temperature recordings per day including measuring, mixing, sampling and addition of feedstock as well as measuring, sampling and removing of the product.



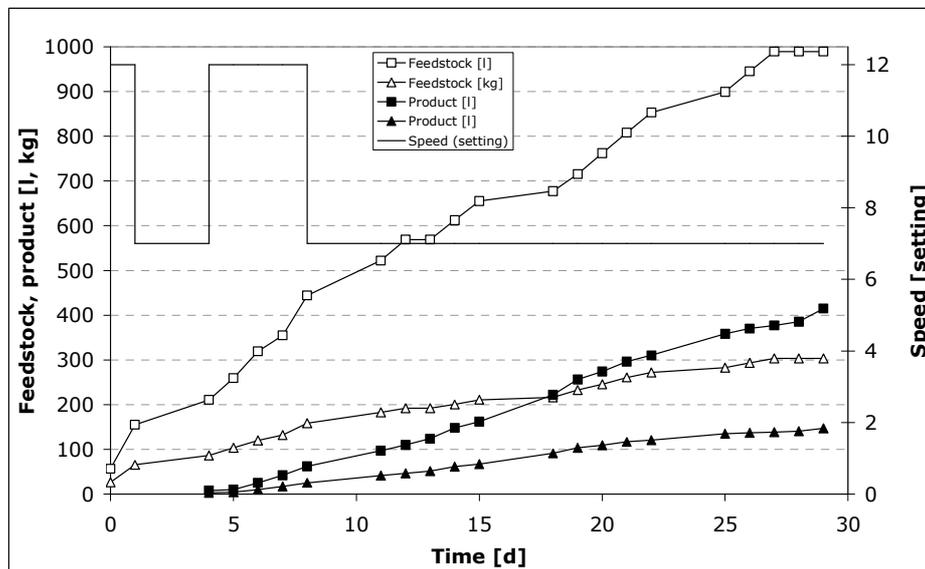
Figure 21: Rocket in-vessel composter

Table 7: Target and actual parameters thermophilic composting using in-vessel composter

| | | Volume [l] | | Weight [kg] | | Moisture content [%] | |
|-----------|-------------|------------|------------|-------------|-----------|----------------------|------------|
| | | Target | Actual | Target | Actual | Target | Actual |
| Feedstock | Green waste | 20 | 18.9 ± 6.2 | 3 | 3.1 ± 1.4 | 60 | 61.2 ± 6.2 |
| | Brown waste | 20 | 20.1 ± 8.3 | 4 | 5.1 ± 2.5 | | |
| | Mixed waste | 40 | 37.2 ± 6.2 | 7 | 7.7 ± 1.7 | | |
| | Water | 4 | 3.5 ± 1.4 | 4 | 3.5 ± 1.4 | | |
| Product | Product | N/A | 18.5 ± 8.3 | N/A | 4.4 ± 2.2 | N/A | 35.7 ± 8.2 |



A



B

Figure 22: (A) Temperature profile. (B) Mass and volumes of feedstock and product during Rocket optimisation

| Table 8: Average temperatures measured at different Rocket temperature settings | | | | |
|--|----------------|----------------|----------------|----------------|
| Setting | T1 [°C] | T2 [°C] | T3 [°C] | T4 [°C] |
| 7 | 34.5 | 51.6 | 46.2 | 31.0 |
| 10 | 35.8 | 59.7 | 59.1 | 43.1 |
| 12 | 40.8 | 66.5 | 66.3 | 47.7 |
| 14 | 41.7 | 68.9 | 65.5 | 42.9 |

Addition of food waste

Initially food waste was planned to be included into the GMMO survival experiments. A trial was carried out using general catering waste (sandwich filler, jacket potatoes, salad, bread, chicken, sausages) obtained from the University of Abertay Student Union. Between 6 – 18 l food waste mixed with 30 – 40 l green and brown waste as was added on three consecutive days. The main outcomes were:

- Considerable temperature increase (T4, Figure 23)
- Odour formation
- Part of meat products were still visible in the product.

Due to the formation of bad smells which caused problems within the wider surroundings of the Rocket it was decided to stop the experiment and exclude catering food waste from further trials.

However, in some of the later experiments vegetable based food waste was included which did not have any negative effect on the operation of the Rocket.

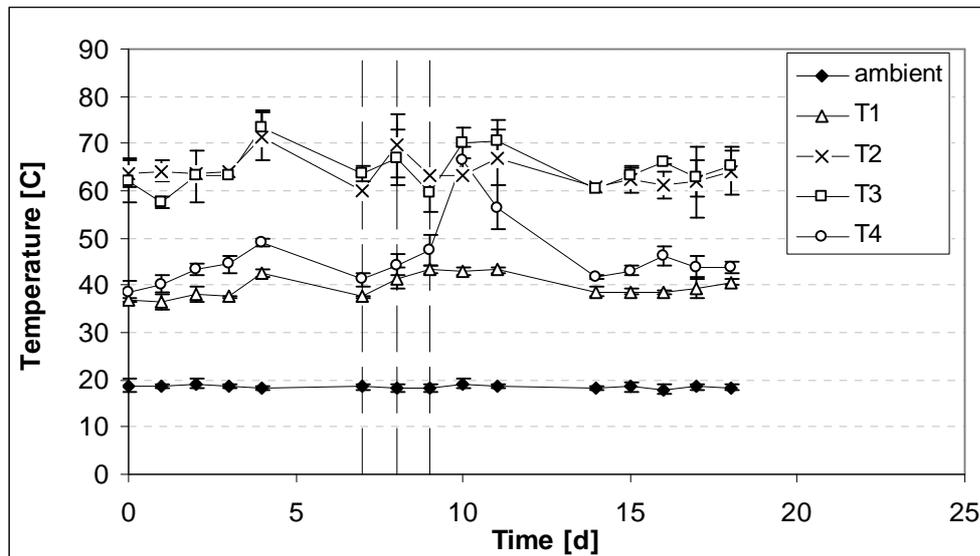


Figure 23: Temperature profile at Rocket composter during food addition (dashed lines indicate the addition of food waste).

Addition of non-GM *E. coli*

An experiment was carried out to investigate whether the addition of (non-GM) *E. coli* generally affects the composting process in the Rocket. A preparation containing 10^{10} cfu was used to spike one batch of feedstock resulting in a calculated concentration of 6×10^6 cfu g^{-1} . Temperature and moisture content was not affected (Figure 24) and product samples analysed for the presence of *E. coli* by plate counting showed no change compared to coliform background levels (data not shown).

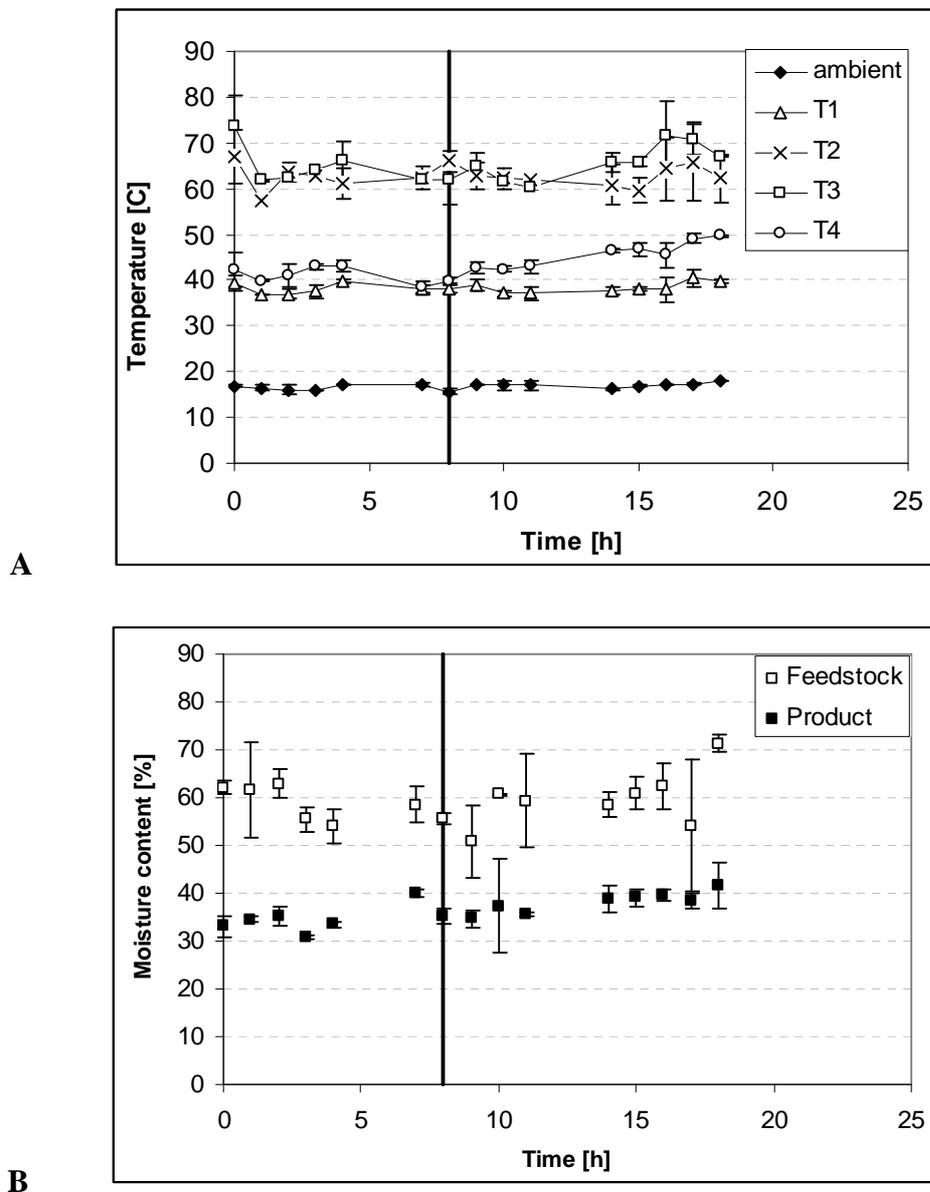


Figure 24: Time course of temperature profiles (A) and moisture content (B) during trial experiment testing the addition of non-GM *E. coli* (solid line indicates the addition of non-GM *E. coli*).

Product properties

Key parameters of product samples were analysed on a random basis. Average values were determined:

- pH-value = 8.4 ± 0.5
- Conductivity = $788 \pm 210 \mu\text{S cm}^{-1}$
- Organic matter content = $71.3 \pm 12.6 \%$

GMMO survival experiments

Lab-based *E. coli* K12 pGLO survival experiments

Studies to investigate the fate of *E. coli* K12 pGLO and the genes of interest were conducted in 300ml glass jars using 25g compost and feedstock, respectively (Figure 25) at three different temperatures chosen on the basis of Rocket temperature profile (level 7): 22°C (ambient temperature), 40°C (temperature in inlet (T1) and outlet zone (T4)) and 65°C (temperature in inner zone (T2, T3)). In order to determine whether the presence of a biological community affect *E. coli* and gene survival, autoclaved compost was investigated alongside untreated samples. Spiking was performed at starting levels (target $1 \times 10^6 \text{ cfu g}^{-1}$) similar to those planned for Rocket studies. A maximum of 10^{10} cfu (as defined by GM regulations at the University of Abertay Dundee) could be used for each Rocket-spiking experiment. Therefore, when mixed with approximately 11kg of feedstock an initial concentration of about $8.7 \times 10^5 \text{ cfu g}^{-1}$ could be expected.



Figure 25: Set-up for lab-based *E. coli* K12 pGLO survival experiments.

Samples incubated at 65°C were spiked with $9.7 \pm 3.4 \times 10^5$ cfu g⁻¹ equivalent to $1.9 \pm 0.7 \times 10^8$ copies g⁻¹. Plate counts showed equal levels of $8.0 \pm 0.3 \times 10^5$ cfu g⁻¹ and $7.9 \pm 1.0 \times 10^5$ cfu g⁻¹ at time 0 (Figure 23A). In both sets of samples no viable *E. coli* pGLO could be detected after 5h, 2, 7, 9 and 14 days (Figure 26A, B).

Tests for the survival of the selected marker gene revealed initial concentrations about one order of magnitude lower ($3 \pm 1.1 \times 10^7$ copies g⁻¹ and $2.9 \pm 0.7 \times 10^7$ copies g⁻¹) than the calculated level ($1.9 \pm 0.7 \times 10^8$ copies g⁻¹) for both treatments which remained unchanged after 5h (Figure 27A). In contrast to plate count results after 2 days 9.2 ± 7.8 and $9.3 \pm 0.5 \times 10^5$ copies g⁻¹ were still detectable for autoclaved and non-autoclaved samples but fell below the detection limit at days 7, 9 and 14 (Figure 27A, B).

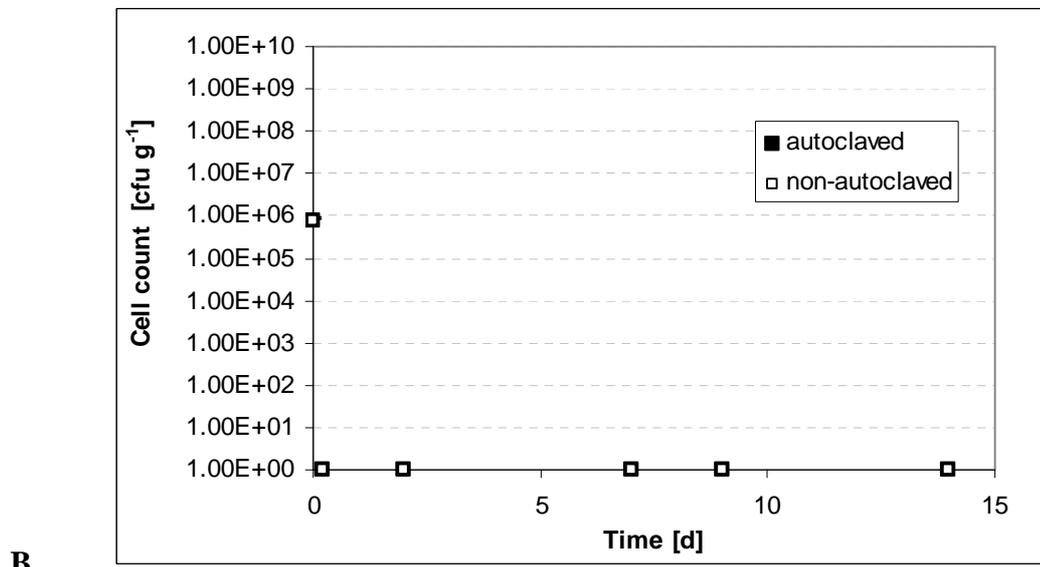
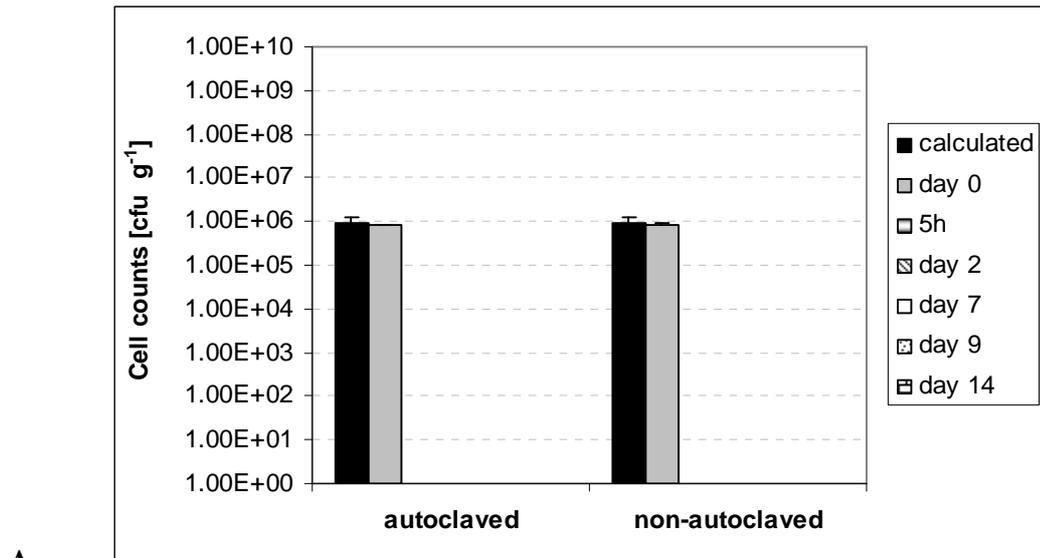


Figure 26: (A) Overview - plate counts for survival of *E. coli* K12 pGLO in spiked autoclaved and non-autoclaved compost incubated at 65°C. (B) Detailed time course.

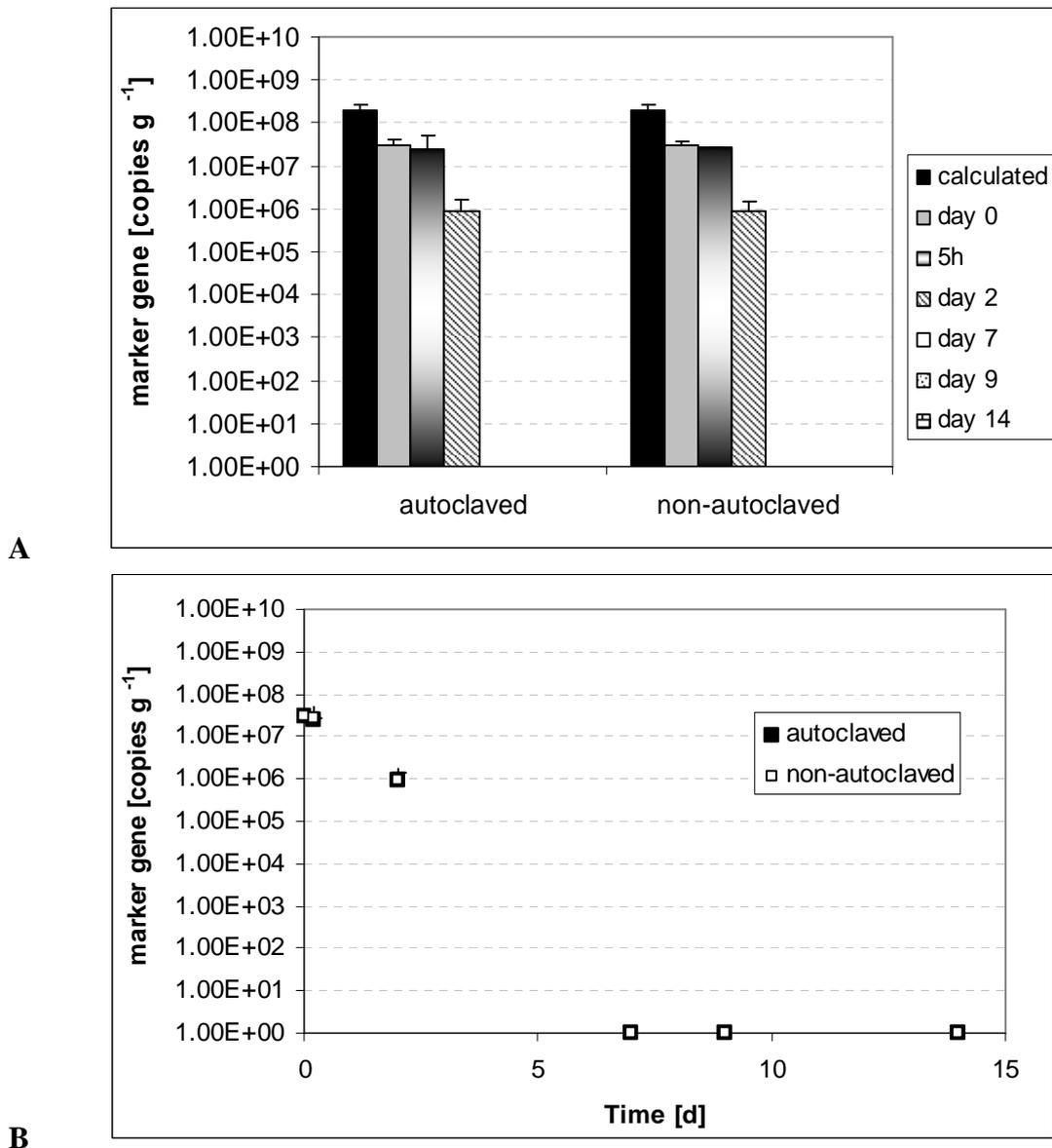


Figure 27: (A) Overview – real-time PCR results for survival of *gfp* gene in spiked autoclaved and non-autoclaved compost incubated at 65°C. (B) Detailed time course.

Spiking of samples for incubation at 40°C resulted in calculated concentrations of $8.1 \pm 1.65 \times 10^5$ cfu g⁻¹ and $1.6 \pm 0.3 \times 10^8$ copies g⁻¹, respectively.

Initial plate counts were at the same level for both treatments ($8.0 \pm 1.7 \times 10^5$ cfu g⁻¹ and $7.4 \pm 2.4 \times 10^5$ cfu g⁻¹) and equivalent to the calculated number. In the autoclaved samples cell numbers increased within the first two days by almost one order of magnitude to $3.6 \pm 2.2 \times 10^6$ cfu g⁻¹ but decreased afterwards back to the starting level after 7 days and by two and three orders of magnitude after 9 and 14 days, respectively (Figure 28A, B). No cells counts were detected at 21 and 28 days of incubation. For the non-autoclaved compost samples cell

numbers fell continuously from the start of the experiment and reached concentrations below the detection limit at day 9.

Copy numbers at time 0 were similar for autoclaved and non-autoclaved samples (1.8 ± 1.1 and $2.6 \pm 0.3 \times 10^8$ copies g^{-1}). The time course of the experiment agrees in principle with the plate counts results with the exception that at day 21 still $3.9 \pm 0.5 \times 10^6$ copies g^{-1} were detected in the autoclaved treatment (Figure 29).

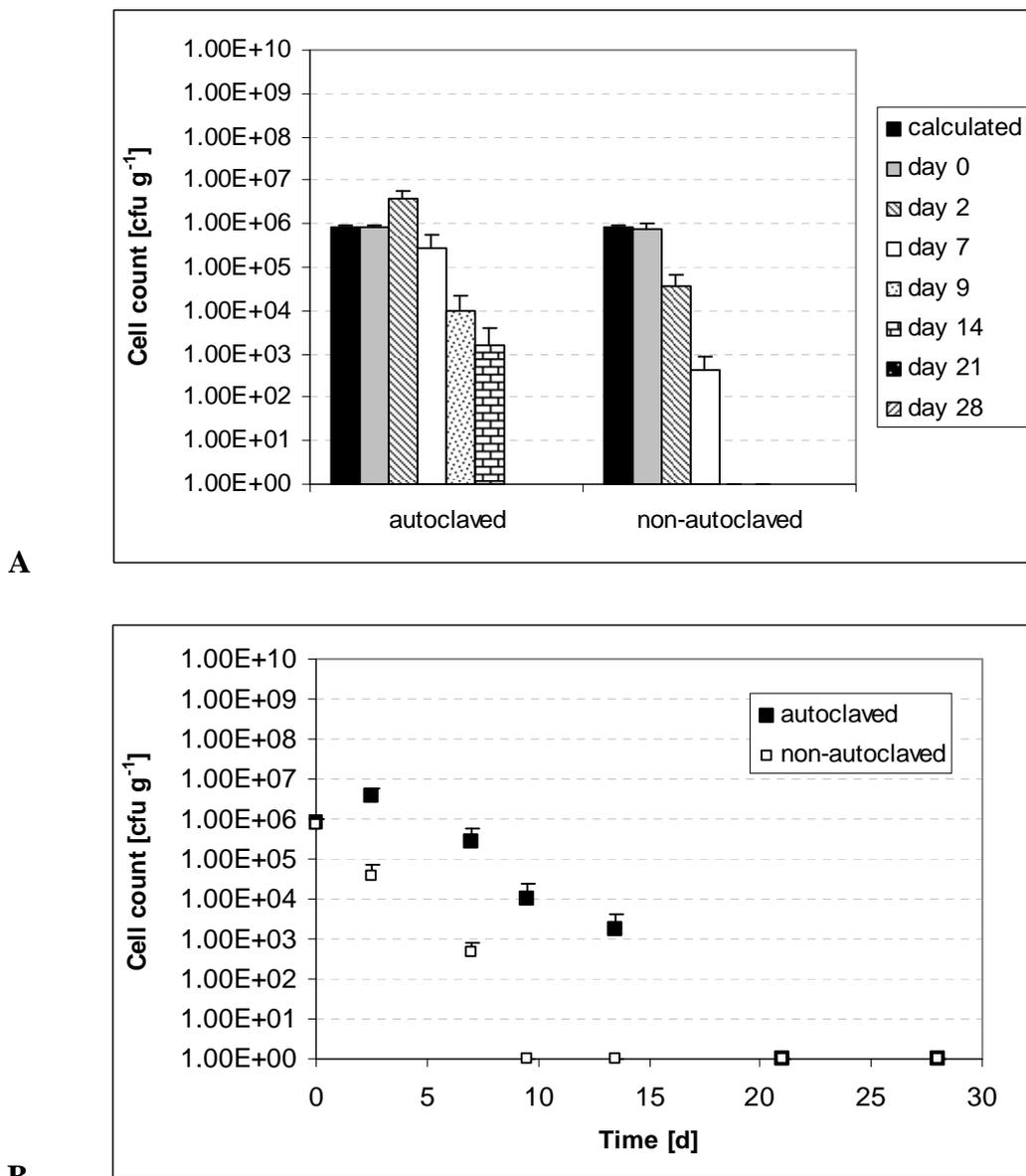


Figure 28: (A) Overview - plate counts for survival of *E. coli* K12 pGLO in spiked autoclaved and non-autoclaved compost incubated at 40°C. (B) Detailed time course.

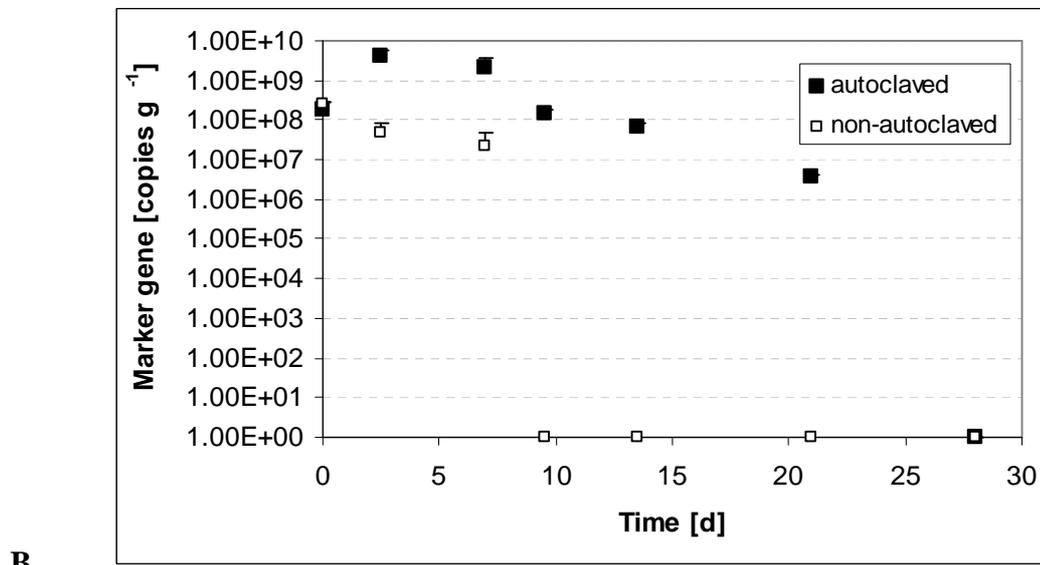
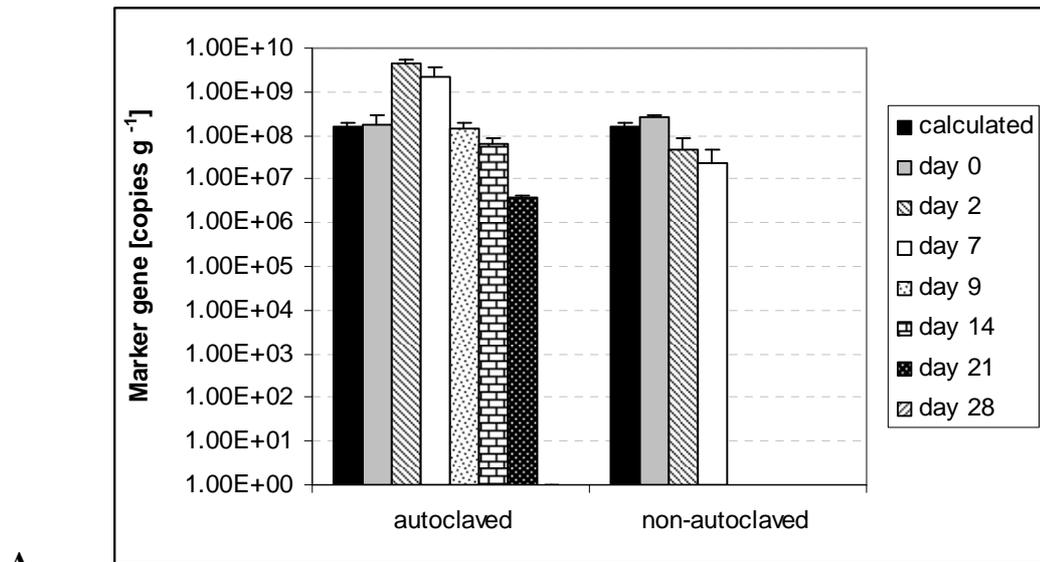


Figure 29: (A) Overview – real-time PCR results for survival of *gfp* gene in spiked autoclaved and non-autoclaved compost incubated at 40°C. (B) Detailed time course.

The third set of samples, incubated at room temperature 23 ± 2 °C, were spiked with $7.9 \pm 1.9 \times 10^5$ cfu g⁻¹ and $1.6 \pm 0.4 \times 10^8$ copies g⁻¹, respectively. At time point 0 cell numbers of $1.1 \pm 0.6 \times 10^6$ and $6.8 \pm 0.3 \times 10^5$ cfu g⁻¹ were determined for autoclaved and non-autoclaved treatments (Figure 30A). For autoclaved samples an initial increase to $4.2 \pm 3.8 \times 10^7$ cfu g⁻¹ was observed within the first week which was followed by a steady decline at all subsequent sampling days down to levels at the detection limit (4.5×10^5 cfu g⁻¹). Again

for untreated compost, cell numbers fell rapidly from the start and within 14 days no *E. coli* pGLO could be identified (Figure 30A, B).

Initial copy numbers ranged from $8.2 \pm 1.0 \times 10^7$ copies g^{-1} for the autoclaved to $1.1 \pm 0.6 \times 10^8$ copies g^{-1} for the non-autoclaved treatment (Figure 31A). As seen for the experiments at 65°C and 40°C the results for marker gene survival are in good agreement with those for the whole organisms. In the autoclaved set-up the initial increase in copy numbers by about two orders of magnitude was confirmed so was the fast decline in non-autoclaved samples (Figure 31A, B).

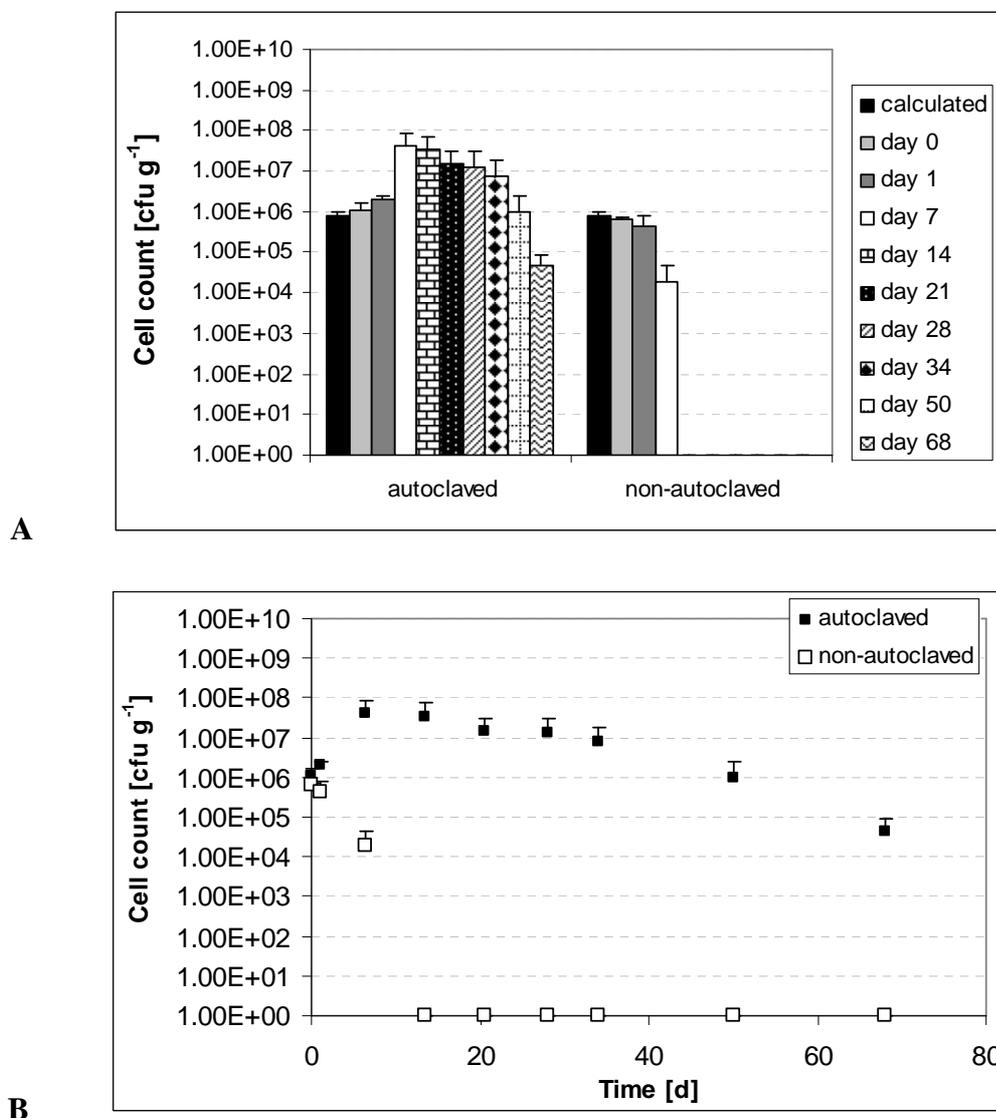


Figure 30: (A) Overview - plate counts for survival of *E. coli* K12 pGLO in spiked autoclaved and non-autoclaved compost incubated at 22°C. (B) Detailed time course.

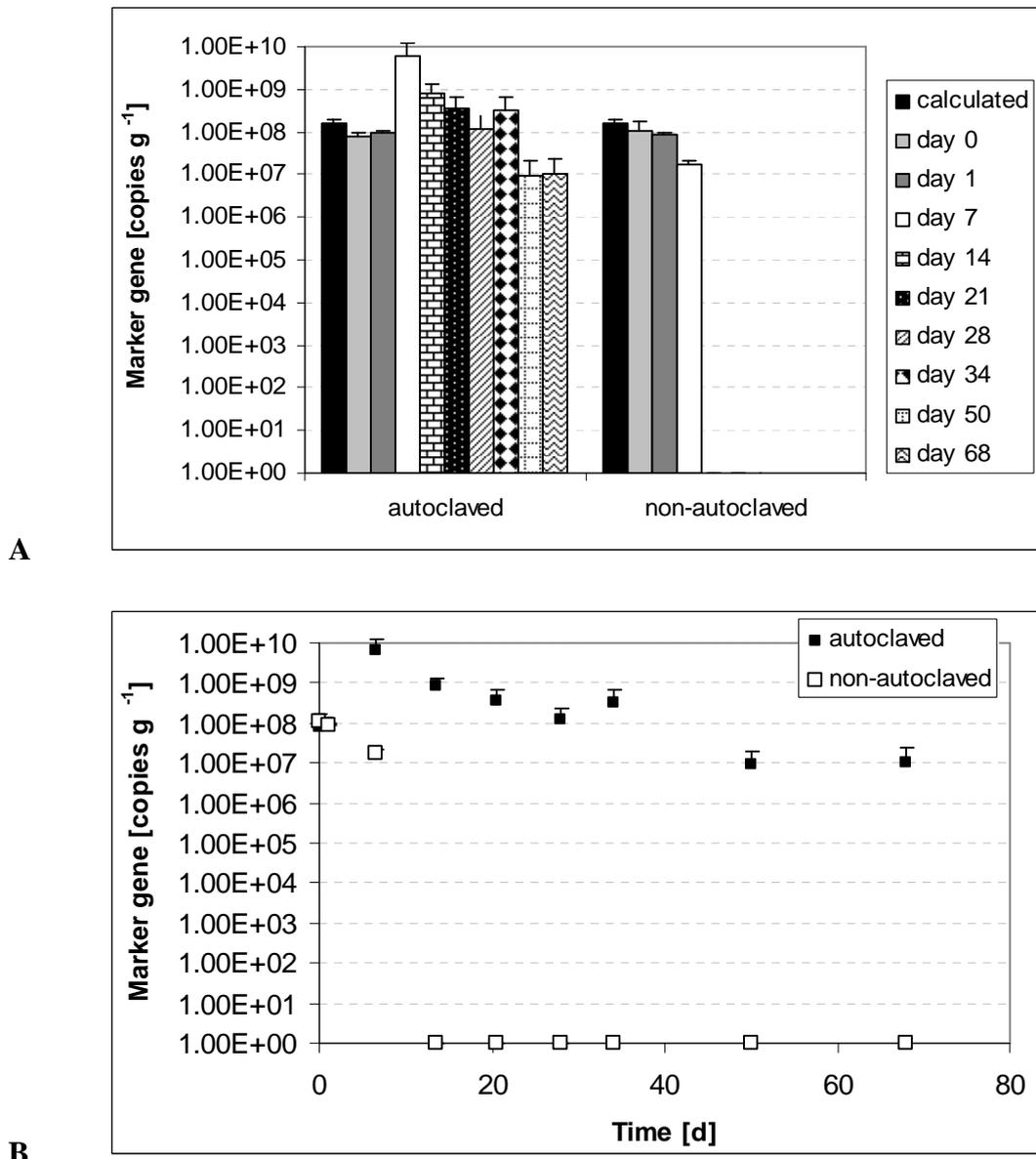


Figure 31: (A) Overview – real-time PCR results for survival of *gfp* gene in spiked autoclaved and non-autoclaved compost incubated at 22°C. (B) Detailed time course.

Rocket-based thermophilic composting experiments

Three Rocket-based experiments were carried out to test the fate of *E. coli* pGLO and the marker gene during actual thermophilic composting processes. Initial priming of the machine was carried out for 10 – 21 days with daily feedstock additions. Once stable conditions had been achieved a target number of 10¹⁰ cells were mixed with feedstock and added to the Rocket. Samples were taken daily for microbiological and molecular biological analysis for a period of two weeks.

The four built-in temperature sensors showed that there are two zones present within the Rocket. Sensors T1 and T4 are located close to the in and out-let areas with temperature ranging between 30 and 45°C. T2 and T3 are placed in the central, heated area resulting in temperatures from 60 to 75 °C. Mean values were not significantly different between experiments with overall average temperatures of 35.9, 63.5, 64.4 and 39.6 °C (Table 9). Time courses for the individual runs showed that the conditions were stable and temperature T2 and T3 were within the targeted range of >60°C (Figures 32, 33, 34) except for experiment 2 where a power cut at day 9 caused a steep fall in temperature for 24h (Figure 33).

| Table 9: Average temperatures during Rocket-based <i>E. coli</i> K12 pGLO survival experiments | | | | |
|---|----------------------------|------------------------------|-----------------------------|------------------------------|
| | Experiment1 [°C] | Experiment 2 [°C] | Experiment 3 [°C] | Average 1 – 3 [°C] |
| T ambient | 14.2 ± 0.8 | 12.7 ± 1.0 | 14.2 ± 1.5 | 13.6 ± 1.4 |
| T1 | 36.0 ± 1.2 | 34.8 ± 1.8 * | 35.3 ± 1.5 | 34.9 ± 3.2 |
| T2 | 63.7 ± 4.1 | 64.5 ± 3.4 * | 65.1 ± 3.3 | 63.5 ± 6.4 |
| T3 | 64.9 ± 3.5 | 64.6 ± 4.2 * | 65.6 ± 4.1 | 64.4 ± 6.4 |
| T4 | 41.4 ± 2.8 | 40.0 ± 3.0 * | 38.9 ± 2.9 | 39.6 ± 3.7 |
| | | * excluding power cut period | | |

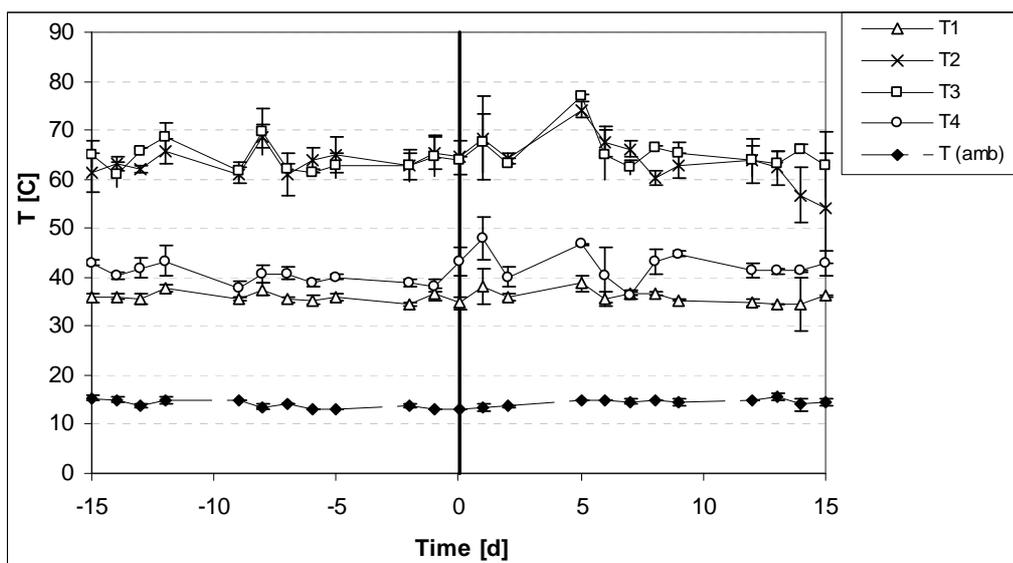


Figure 32: Time course of temperature profile during Rocket-based *E. coli* K12 pGLO survival experiment 1 (temperature setting 12, solid line indicates GMMO addition).

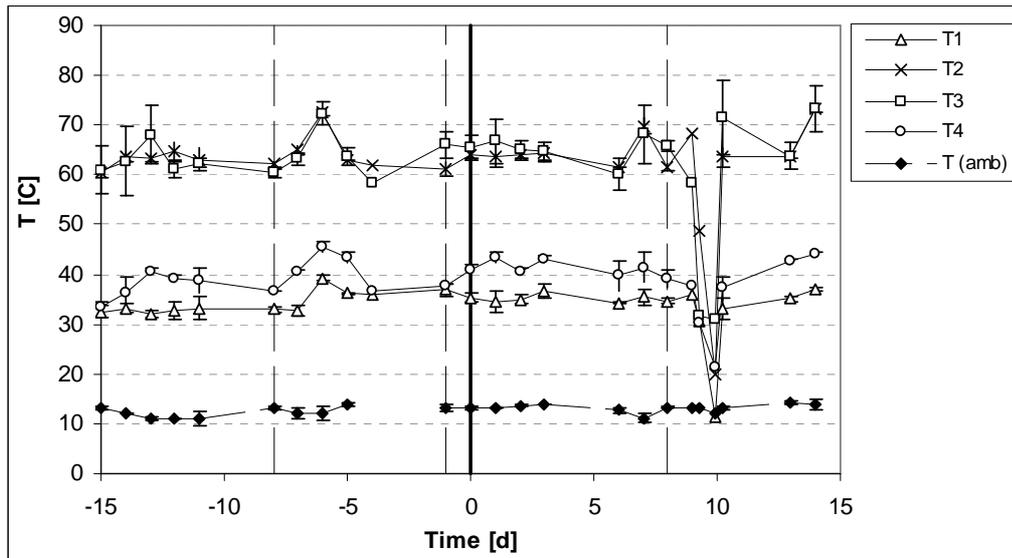


Figure 33: Time course of temperature profile during Rocket-based *E. coli* K12 pGLO survival experiment 2 (temperature setting 12, solid line indicates GMMO addition, dashed lines indicate addition of green vegetable waste).

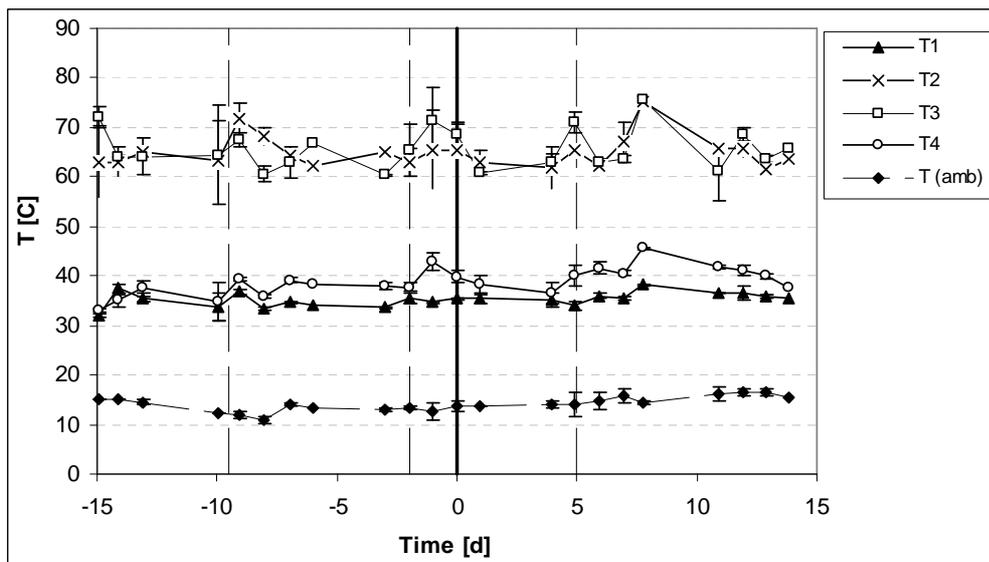


Figure 34: Time course of temperature profile during Rocket-based *E. coli* K12 pGLO survival experiment 3 (temperature setting 12, solid line indicates GMMO addition, dashed lines indicate addition of green vegetable waste).

During the composting process a loss in weight and volume is anticipated as two major products CO_2 and H_2O which will be created during the degradation of organics can discharge from the system as gas or vapour. The daily input of material was 40l (about 8kg) with similar values for volume and mass reduction achieved as in experiments 1 – 3 resulting in an average

numbers of 30.4 and 41.9% (Table 10, Figures 35, 36, 37). Due to problems in feedstock supply and the nature of material added, those values were lower than in the trial runs. In experiments 2 and 3, green vegetable waste (mainly lettuce) was included into the feedstock once a week; days of addition are marked with a dashed line (Figures 36, 37).

| Table 10: Average values for mass and volume reduction during Rocket-based <i>E. coli</i> K12 pGLO survival experiments | | | | |
|--|---------------------|---------------------|---------------------|----------------------|
| | Experiment 1 | Experiment 2 | Experiment 3 | Average 1 – 3 |
| Mass reduction [%] | 27.1 | 33.7 | 30.4 | 30.4 ± 3.4 |
| Volume reduction [%] | 37.9 | 43.8 | 44.0 | 41.9 ± 3.5 |

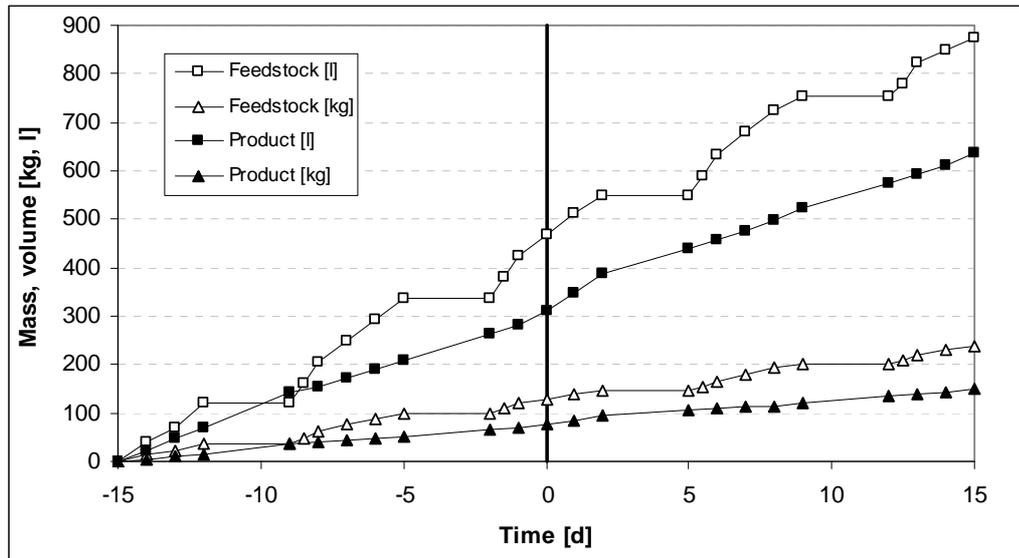


Figure 35: Time course of mass and volume values for feedstock and product during Rocket-based *E. coli* K12 pGLO survival experiment 1 (speed setting 7, solid line indicates GMMO addition).

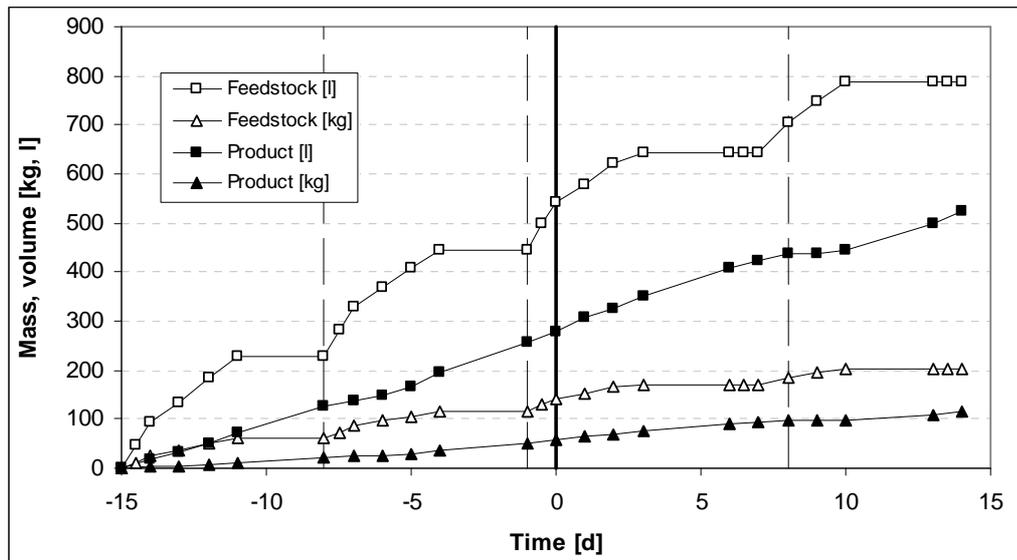


Figure 36: Time course of mass and volume values for feedstock and product during Rocket-based *E. coli* K12 pGLO survival experiment 2 (speed setting 7, solid line indicates GMMO addition, dashed lines indicate addition of green vegetable waste).

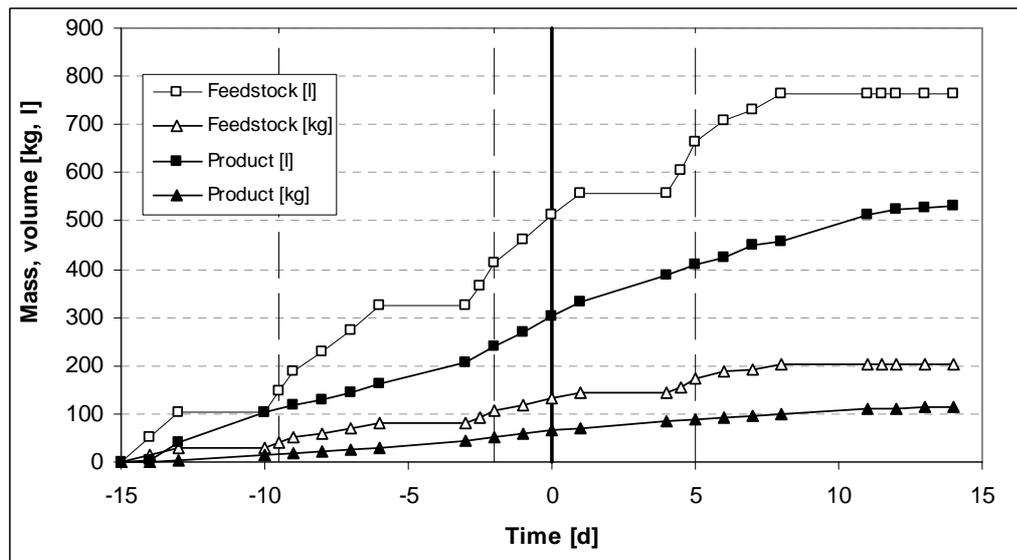


Figure 37: Time course of mass and volume values for feedstock and product during Rocket-based *E. coli* K12 pGLO survival experiment 3 (speed setting 7, temperature setting 12, solid line indicates GMMO addition, dashed lines indicate addition of green vegetable waste).

The moisture content of ingoing material is one of the key parameters for the successful running of a composting process. By addition of water it can be easily adjusted to the target value of 60%. During all three runs the moisture content of the feedstock was within

the desired range (Table 11, Figures 38, 39, 40) whereas some problems occurred with product being too dry after passing through the Rocket (Figure 38 day 8, Figure 40 day 14). The main reasons for this were problems with feedstock supply which resulted in the formation of air pockets within the composter when less material was added, and the type of feedstock. During winter months the time all three experiments were performed, the supplied material often contained large proportions of autumn leaves which tend to dry out quickly before an effective composting was possible. Average values for moisture content were 62.4 and 37.1% for feedstock and product, respectively.

| Table 11: Average values for moisture contents during Rocket-based <i>E. coli</i> K12 pGLO survival experiments | | | | |
|--|---------------------|---------------------|---------------------|----------------------|
| Moisture content [%] | Experiment 1 | Experiment 2 | Experiment 3 | Average 1 – 3 |
| Feedstock | 60.3 ± 5.7 | 65.5 ± 6.1 | 61.7 ± 4.2 | 62.4 ± 5.7 |
| Product | 36.8 ± 6.8 | 42.1 ± 7.2 | 32.5 ± 9.6 | 37.1 ± 8.8 |

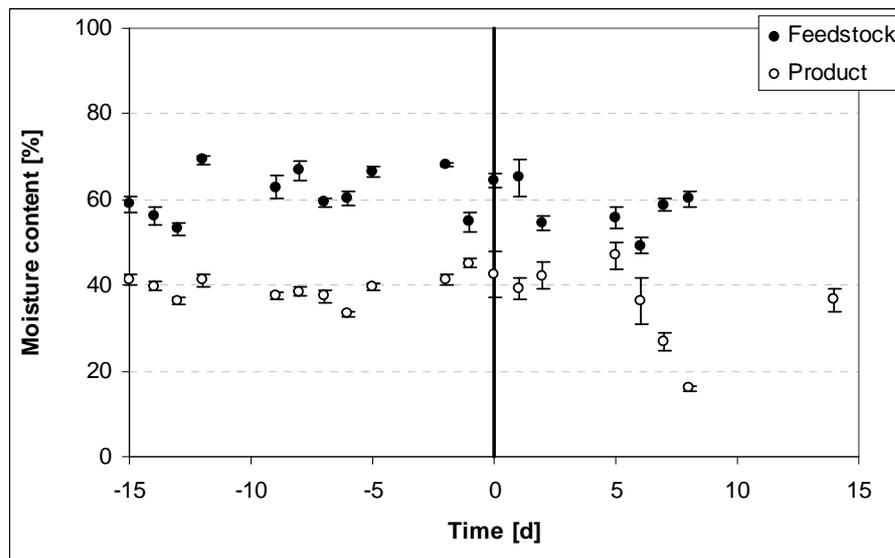


Figure 38: Time course of moisture content for feedstock and product samples during Rocket-based *E. coli* K12 pGLO survival experiment 1 (solid line indicates GMMO addition).

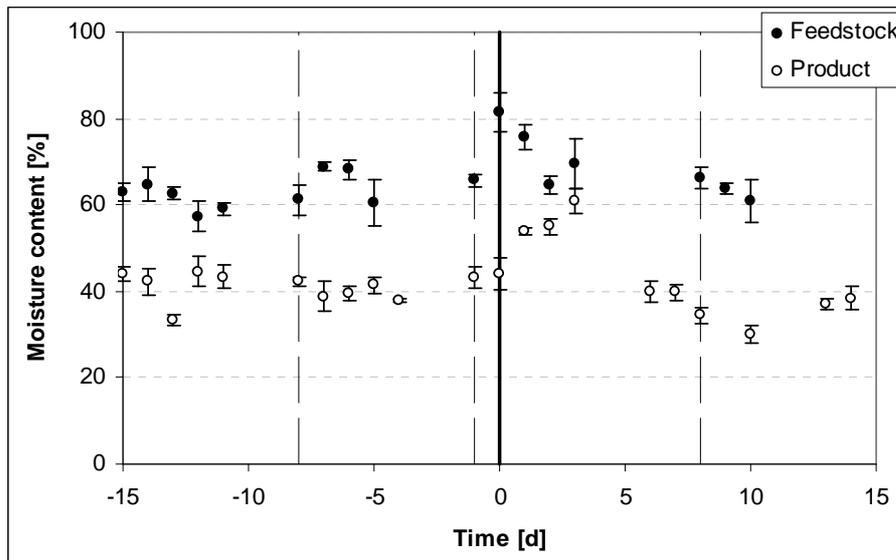


Figure 39: Time course of moisture content for feedstock and product samples during Rocket-based *E. coli* K12 pGLO survival experiment 2 (solid line indicates GMMO addition, dashed lines indicate addition of green vegetable waste).

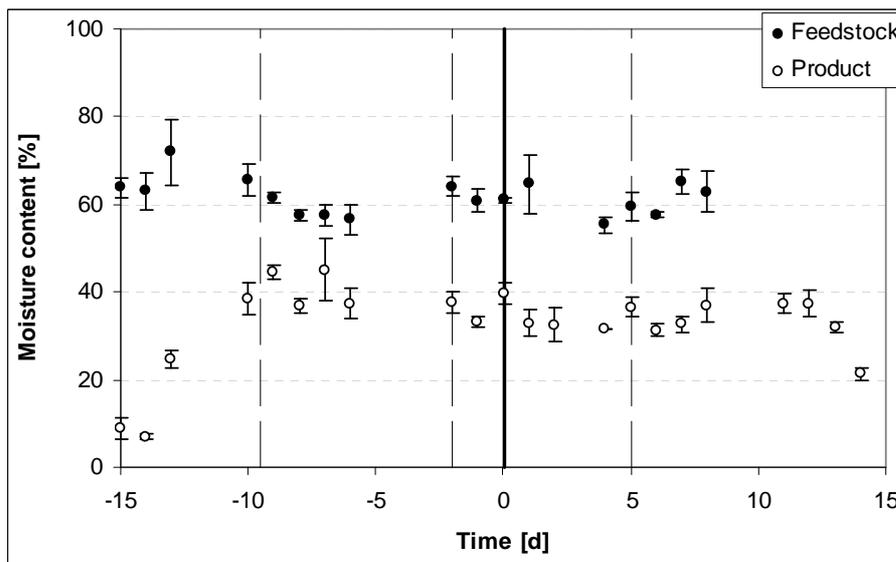


Figure 40: Time course of moisture content for feedstock and product samples during Rocket-based *E. coli* K12 pGLO survival experiment 3 (solid line indicates GMMO addition, dashed lines indicate addition of green vegetable waste).

The fate of *E. coli* pGLO and the *gfp* marker gene was tested using plate counts and real-time PCR. The spiking of the three experiments resulted in calculated concentrations of $5.0 \pm 1.4 \times 10^5$, $2.2 \pm 0.5 \times 10^6$ and $1.2 \pm 0.3 \times 10^6$ cfu g⁻¹ equivalent to $1.0 \pm 0.3 \times 10^8$, $4.4 \pm 0.9 \times 10^8$ and $2.4 \pm 0.5 \times 10^8$ copies g⁻¹ (Figures 41 – 43). At time 0 detection of *E. coli* pGLO

was only possible in experiment 2 ($9.6 \pm 1.7 \times 10^7$ cfu g⁻¹, Figure 42). Due to problems in feedstock supply the ingoing material used for this study consisted of 40l Rocket product whereas green and mixed waste (feedstock) was added in experiments 1 and 3. The one order of magnitude difference in detection limit for real feedstock samples and Rocket product might offer an explanation why no positive results were returned for experiment 1 and 3. In the course of the experiments cell counts for *E. coli* pGLO were below the detection limit for all samples tested (Figure 41 – 43).

Using real-time PCR the presence of the marker gene at levels of $3.3 \pm 0.6 \times 10^7$, $1.2 \pm 0.3 \times 10^8$ and $1.0 \pm 0.2 \times 10^7$ copies g⁻¹ could be demonstrated at time 0. Only sample, experiment 1 day 2, was tested positive ($2.5 \pm 2.6 \times 10^5$ copies g⁻¹). It should be noticed that just one out of three replicates of this sample returned results above the detection limit. At all other occasions the presence of the marker gene could not be detected. In addition, this result (taken on day 1) is an indication that this particular sample did not undergo the desired thermophilic composting process in the Rocket which should take a period of about six days. Quite likely the material found a shortcut through the machine therefore was not treated correctly. When determining the average retention time markers were occasionally found already two days after addition.

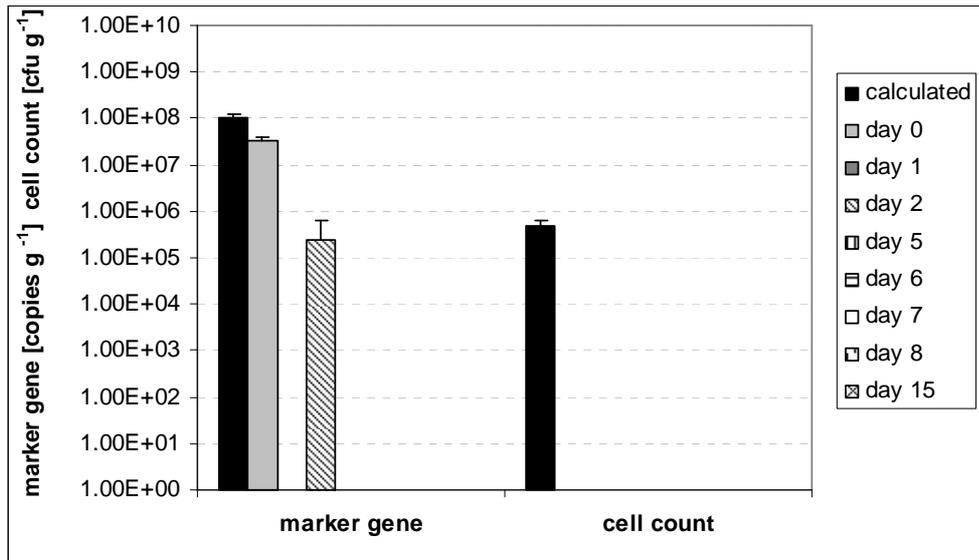


Figure 41: Plate counts for survival of *E. coli* K12 pGLO and real-time PCR results for survival of *gfp* gene during Rocket-based survival experiment 1.

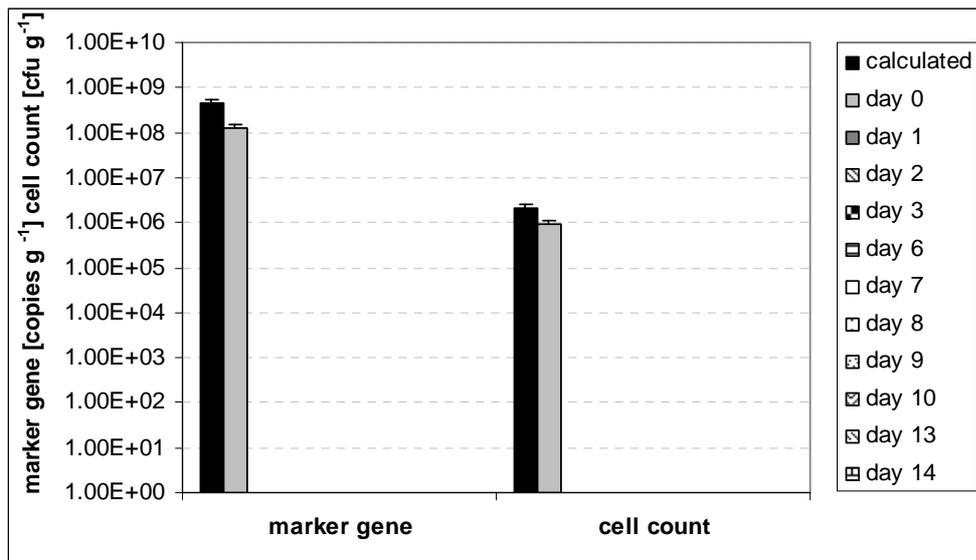


Figure 42: Plate counts for survival of *E. coli* K12 pGLO and real-time PCR results for survival of *gfp* gene during Rocket-based survival experiment 2.

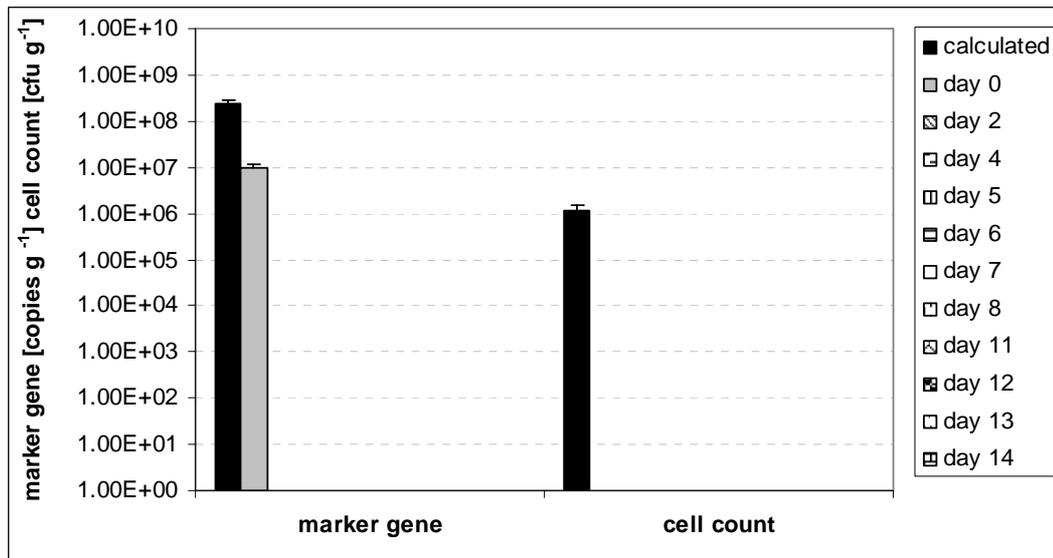


Figure 43: Plate counts for survival of *E. coli* K12 pGLO and real-time PCR results for survival of *gfp* gene during Rocket-based survival experiment 3.

Horizontal gene transfer

Possible horizontal transfer of the functional pGLO plasmid from *E. coli* pGLO to other members of the composting community was monitored by selective plate counting. Growth of a sample on CECM+AA plates would indicate that cells were resistance to ampicillin. These plates were also checked and counted under UV. Only colonies actively expressing the genes encoded in the pGLO plasmid would appear fluorescent. In addition, *E. coli* colonies could be distinguished by their unique purple colour on the chosen chromogenic medium (Figure 44A). Within a sample collected from lab-based survival studies at room temperature at day 32 days (autoclaved treatment) one glowing colony was detected which did not show the *E. coli* specific colour indicating a potential transfer of pGLO. The colony (called isolate 1) was transferred and grown on CECM+AA and PCA agar plates whereby the absence of the characteristic colour was confirmed on CECM+AA as well as the expression of ampicillin resistance and *gfp* gene (Figure 44A). On PCA no obvious differences in appearance between *E. coli* pGLO and isolate 1 could be seen (Figure 44B).

A general microbiological test for classification showed both strains were gram-negative.

Further enzymatic tests using the API strip system revealed that out of 20 tested enzymes only one was different: β -galactosidase was not present in isolate 1. According to the API classification both were subsequently identified as representatives of *E. coli*. As β -galactosidase is one of the two enzymes responsible for the chromogenic reaction's characteristic purple appearance of *E. coli* on CECM it can be concluded that isolate 1 was a β -galactosidase-negative mutant *E. coli* pGLO. Therefore, no evidence of horizontal gene transfer was detected.

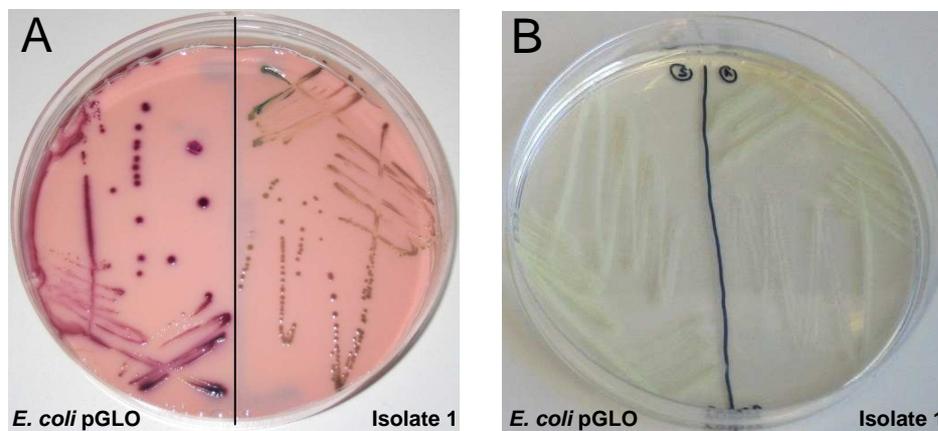


Figure 44: Growth of *E. coli* K12 pGLO and isolate 1 on CECM+AA (A) and PCA (B).

DISCUSSION

Microbiology – Method Development

During the course of the project effective microbiological methods were developed to selectively detect *E. coli* K12 pGLO in pure culture, compost and feedstock samples. The successful insertion of plasmid pGLO harbouring an *araC*-promoted green fluorescent protein (*gfp*) as well as resistance to the antibiotic ampicillin into *E. coli* K12 resulted in the creation of a stable, easy-to-monitor GMMO, non pathogenic *E. coli* K12 pGLO. By adding ampicillin and arabinose to a commercially available chromogenic *E. coli*/coliform medium and visualising under UV light a very specific detection of three GMMO characteristics (colour formation, expression of ampicillin resistance and *gfp*) was possible on agar plates at the same time without the need of further transfers. This innovative approach allowed the reliable microbiological detection of the model GMMO within 48 – 50h of sampling.

Limits for the detection of *E. coli* K12 pGLO recovered from spiked compost and feedstock samples in this study were in the order of 10^4 and 10^5 cfu g⁻¹, respectively. Due to the relative widespread natural occurring ampicillin resistance (up to 10% in bacteria (Ramessar et al., 2007)) extensive growth was observed on plates inoculated with the original suspension (1 or 10g compost or feedstock in 9 or 90ml PBW). Therefore, the reliable detection and counting of *E. coli* K12 pGLO was only possible on plates incubated with dilutions $\geq 10^{-1}$. It has been reported that different microbial communities are present at different stages during the composting process (Ryckeboer et al., 2003; Stenbro-Olsen, 1998). This fact might offer an explanation why for feedstock samples which were not yet subject to higher temperatures and therefore host a wider range of microorganisms, a tenfold lower detection limit compared to compost product was determined.

For other bacteria used in GMMO survival studies detection limits between 10^2 cfu g⁻¹ for *Bacillus subtilis* and *Pseudomonas chlororaphis* 3732RN-L11 and 10^3 cfu g⁻¹ for *Pseudomonas aureofaciens* have been reported (England, Lee & Trevors, 1997; Guan et al., 2004; McDonald et al., 1998). Different soil types and mushroom compost which host

indigenous microbial communities different compared to the one in our material, were employed in those studies. Also, the media used might offer a greater sensitivity for the growth of *Bacillus* and *Pseudomonas* strains.

Several possibilities could be investigated to improve the levels of microbiological detection of *E. coli* K12 pGLO, for example:

- Increase of initial sample weight
- Perform specific enrichment before plating
- Develop improved solid growth media.

Molecular Biology – Method Development

Primer design was initially focussed on probing the *gfp* gene of the pGLO plasmid as it should not be present in conventional household green waste (Haseloff et al., 1997). Several attempts were made to explore reasons for false-positive results which were obtained with primers probing for different parts of *gfp* (Table 1) for negative controls like non-GM *E. coli* and plant DNA as well as several unspiked compost and feedstock samples. Those problems preferentially occurred during real-time PCR analysis but were also observed in conventional PCR reactions. Formation of unspecific products is one of the major reasons for false-positive results in real-time PCR using the SYBR Green I detection system. Therefore dissociation curve analysis was carried out in order to recognise those by differences in melting temperatures between the target and false-positive product (Sharma et al., 2007). In our case, melting temperatures indicated that a genuine product was amplified. Sequencing of the (false-positive) real-time PCR products would potentially provide an explanation for the experienced difficulties. In addition, a second PCR test using the initial product as template (*nested* PCR) could be performed to confirm or reject the presence of the marker gene.

The rationale of probing for a region overlapping between the pBAD promoter and *gfp* region was that it should be even more specific as the combination between those two genes is

unique for the pGLO plasmid. As expected primer pair pBAD_1F and gfp_6R was highly specific in detection of the marker region and no false-positives were observed.

Successful DNA extraction from feedstock and compost samples poses a great challenge as it is the major requirement for carrying out subsequent tests using molecular biology tools. Several protocols for nucleic acid extraction from compost, soil and sediment have been described (Blanc et al., 1999; Dong et al., 2006; Griffiths et al., 2000; Howeler, Ghiorse & Walker, 2003; LaMontagne et al., 2002; Lloyd-Jones & Hunter, 2001; Malik et al., 1994; Miller et al., 1999). A standard protocol based on work by Dong and co-workers (Dong et al., 2006) was modified, as best as possible considering time and financial issues, to produce DNA of satisfactory yield and quality from feedstock and compost suspensions. In agreement with most published methods it was necessary to first purify crude DNA before amplification by PCR could be achieved (Howeler, Ghiorse & Walker, 2003; Juen & Traugott, 2006; LaMontagne et al., 2002; Miller, 2001; Miller et al., 1999) this could easily and quickly be achieved by using a commercial clean-up kit.

Real-time PCR proved to be a sensitive tool for quantification and detection of *E. coli* K12 pGLO at levels as low as 4×10^2 cfu ml⁻¹ equal to 8.4×10^4 pGLO copies ml⁻¹ in pure culture (Figure 17). The detection limit for spiked compost and feedstock samples was about two orders of magnitude worse (about 10^4 cfu g⁻¹ and 10^6 copies g⁻¹).

Our results are in the same range (10^2 to 10^6 target copies g⁻¹) as those reported for other GMMO used in environmental survival studies (England, Lee & Trevors, 1997; England et al., 2004; Guan et al., 2004; Ibekwe & Grieve, 2003; Ibekwe et al., 2002; Murray et al., 2007; Sharma et al., 2007). Future work should address two main issues: first, the sub-optimal real-time PCR reaction mix due to presence of inhibitory substances and second, the large product size of the amplicon (715pb).

As the Taq DNA polymerase used in conventional PCR was more robust to the presence of inhibitors it was included in real-time PCR mixtures even though the ready-made mix already contained a similar enzyme. Therefore an increased formation of unspecific

products and primer dimers occurred in samples with no DNA. Even though those could be identified by melting curve analysis it led to the creation of samples where low amounts of specific products could not be distinguished from the unspecific background.

The sensitivity of real-time PCR greatly depends on the length of the amplicon with a recommended size $\leq 250\text{bp}$ (Livak et al., 1995). Therefore, the product size of 715bp might have a negative impact on the detection limit even though the linearity of real-time PCR detection was shown over six orders of magnitude for products of 861 and 420bp (Figure 9).

Possibilities to improve performance of molecular biology methods include:

- Improvement of DNA extraction protocol
- Increase of sample volume (within limits)
- Design of an optimal real-time PCR mix
- Selection of robust Taq DNA polymerase
- Development of specific primers probing for a shorter region ($\leq 250\text{bp}$)

Rocket installation and optimisation

The in-vessel composter Rocket was a suitable system for the GMMO survival experiments. Daily standard input of green and brown of waste (40l/10-12kg, moisture content 60%) was subject to a thermophilic composting process resulting in the formation of un-matured compost-like product (18l/4.4kg, moisture content 35%). At optimised conditions, the process was characterised by volume and mass reduction of 56 and 61% and average temperatures in the heated zone between 60 – 66°C which are within the target range (Sharma et al., 1997). This was also true for product characteristics like pH, conductivity and organic matter content.

It can be concluded that thermophilic composting under well controlled and monitored conditions can be achieved using the Rocket system. In-vessel systems have also been recommended for the composting of GMO (Singh, Billingsley & Ward, 2006).

Lab-based *E. coli* K12 pGLO survival experiments

Survival of *E. coli* K12 pGLO in lab-based experiments depended on the incubation temperature as well as the presence of an active microbial community. At 65°C viable plate counts for *E. coli* K12 pGLO fell below the detection limit after 5h of incubation independent of the pre-treatment. This in agreement with previous research stating that incubation times for complete *E. coli* inactivation in model agricultural waste of 10 min at 65°C (Ugwuanyi, Harvey & McNeil, 1999). In contrast, McDonald and co-workers found viable cells of *Bacillus subtilis* released into mushroom compost at 65°C for more than 14 days; but again no difference between sterile and untreated compost (McDonald et al., 1998). *B. subtilis* can form spores to adapt to high temperatures, which were still identified after 28 days (Amner, McCarthy & Edwards, 1991).

The 715pb pBAD-*gfp* fragment was still detectable in both treatments after two days but had decreased by two orders of magnitudes. This indicates that plasmids like pGLO can survive certain periods after cell death until they have undergone complete degradation. In addition, it shows that molecular biology methods based on DNA samples can distinguish between DNA originated from living or dead cell (Urgun-Demirtas, Stark & Pagilla, 2006).

At 40°C no survival of *E. coli* pGLO was detected after day 9 and 21 for non-autoclaved and autoclaved set-ups, respectively. In the untreated system viable numbers decreased from the start at a rate of 10^5 cfu per day whereas in autoclaved samples initial growth was observed within the first two days after which a constant decline at 5×10^5 cfu per day was observed. Competition for carbon source and nutrient is the main reasons for the disappearance of introduced strains in natural environments (Singh, Billingsley & Ward, 2006; Urgun-Demirtas, Stark & Pagilla, 2006) and references therein. In addition, plasmid-bearing strains often have slower growth rates as a result of higher metabolic activity (Tiedje et al., 1989). Both facts offer an explanation for the observed differences in our study and the following: no change in viable cell counts was observed with *Bacillus subtilis* at 37°C in

autoclaved compost but a 100fold decline was noted within 7 days for untreated samples (McDonald et al., 1998).

Test for the survival of partial pGLO sequence at 40°C confirmed the microbiological findings: pcr products were detected longer in the autoclaved samples (21d) compared with the untreated system (7d). In addition, the general time-course was mirrored with one difference, the last day for the detection of the marker gene was day 21 whereas viable cells were only present until day 14. Beside the reasons mentioned above it has also been shown that the survival of free plasmid DNA within the environment varies widely (Dale, Clarke & Fontes, 2002; Singh, Billingsley & Ward, 2006; Urgun-Demirtas, Stark & Pagilla, 2006). The presence of endo-and exo-nucleases responsible for the degradation of nucleic acids is bound to be very low in the autoclaved treatment and therefore a longer survival of DNA can be expected.

Survival of *E. coli* K12 pGLO at room temperature (22°C) showed the greatest difference between non-autoclaved and autoclaved samples: viable cells were present until day 7 and day 68, respectively. Again, in the untreated system cell counts fell from the beginning at a rate of 1.4×10^5 cfu per day similar to that seen at 40°C. In the autoclaved samples an initial increase by two orders of magnitude was detected followed by a slow decrease to starting level. Also for this set of experiments a good agreement between molecular biology and microbiology results was observed. The pBDA-gfp marker gene was detected until day 7 and 68, respectively, with similar time courses for both treatments.

Comparing the results at 40°C and 22°C reveals that *E. coli* K12 pGLO was out-competed by indigenous compost community at both temperatures. The survival of both, viable cells and transgene was extended in the autoclaved system with lower temperatures favouring this process.

Few studies have been published on the GMMO survival in soil or compost at ambient temperatures. Generally, both GMMOs and their transgenes were identified for periods of several weeks and months under those conditions. In soil, a longer survival of *Pseudomonas*

aureofaciens RNL-11 in autoclaved (44 weeks) compared to non-autoclaved samples (30 weeks) was found when a low inoculum size (10^4 cfu g^{-1}) was used. When a higher inoculum size was used (10^8 cfu g^{-1}) an even greater difference was found for the persistence of the marker gene (4 and 24 weeks, respectively) (England, Lee & Trevors, 1997). These results are in good agreement with those presented in this report. The same organism was used in field trials and soil-core microcosms where viable cells survived for periods of 38 – 192 and 64 – 241 days, respectively (Gagliardi et al., 2001). Guan *et al.* found a decrease in cell numbers of *Pseudomonas chlororaphis* 3732 RN-L11 in soil at 23°C at rate of one log per day with no detection after day 7. However, there was evidence that transgenic DNA was present until day 45 (Guan et al., 2004). When soil was spiked with 1×10^9 cfu g^{-1} *Pseudomonas tolaasii*, cell counts decreased by three orders of magnitude within the first 9 days and further to 5×10^3 cfu g^{-1} during the following 14 days (Wu, Palmer & Cole, 1998).

From the results of this project and those available from scientific literature it can be concluded that temperature is the main factor for the survival of GMMO in lab-based spiked soil and compost. Temperature has a stronger effect on the existence of viable cells than on free transgenic DNA as each species has a different mechanism to adapt in order to survive whereas certain conditions affect DNA degradation in the same way. The presence of an active indigenous community can considerably prevent the long-term survival of introduced GM or non-GM micro-organisms.

Rocket-based thermophilic composting experiments

Three in-vessel thermophilic composting experiments were carried out investigating the fate of *E. coli* K12 pGLO and its transgene. Temperature sensors located in two positions within the heated zone of the machine showed that average temperatures of 63.5 ± 6.4 °C and 64.4 ± 6.4 °C were reached. These figures as well as values for mass and volume reduction (30.4 ± 3.4 % and 41.9 ± 3.5 %) and moisture content for feedstock and product (62.4 ± 5.7 % and 37.1 ± 8.8 %) indicate that a good-quality thermophilic composting process took place.

The addition of green vegetable waste in experiments 2 and 3 did not have an effect on the performance of the process.

Viable *E. coli* K12 pGLO cells were not detected at any time whereas the 715pb product probing for an overlapping pBAD/*gfp* region within pGLO was identified once (at day 1). As the average retention time is 6 days it can be assumed that the material passed through a short-cut within the Rocket and therefore, did not undergo sufficient thermophilic composting. In addition, positive results were only found in one of the three replicates and therefore the average number $2.5 \pm 2.6 \times 10^5$ copies g^{-1} was below the detection limit. Nevertheless this observation emphasises the fact that strict control of operation procedures is necessary to ensure a reliable running of the in-vessel composter.

So far, only two studies on the fate of GMMO during composting have been published. Guan and co-workers found that neither viable *E. coli* J5 RP4 and *E. coli* C600 pIE723 cells nor their respective plasmids survived in chicken manure compost microcosms at temperatures above 50°C (Guan et al., 2007). Earlier work using compost bins spiked with *Pseudomonas chlororaphis* 3732-RN-L11 showed no detection of living cells for 29 days, independent of the temperature zone sampled. In addition, the transgene was not identified in zones of compost microcosms which reached temperatures above 45°C (Guan et al., 2004).

Both studies come to the same conclusion which also in agreement with our findings: temperature controlled composting can be used as a safe method for the disposal of GMMO.

Two studies investigated the fate of GM plants, transgenic corn (*Zea mays* L.) and *Arabidopsis*, during composting (Guan, Spencer & Ma, 2005; Rasmusen, Møller & Magid, 2004). For transgenic corn the complete degradation during the thermophilic phase of composting (>50°C) was observed for plants material and seeds with two weeks and 12 months, respectively (Guan, Spencer & Ma, 2005). Transgenic DNA from *Arabidopsis* only detected until day 10 at 68°C and day 14 at 58°C, respectively (Rasmusen, Møller & Magid, 2004).

Recently the fate of transgenic pig DNA during composting has been studied. During a large scale composting process (2500kg transgenic pig carcasses and 1000kg of wood shavings) temperatures reached levels between 50°C and 70°C and transgenic pig DNA was not detected at days 89 and 180 (Murray et al., 2007).

It therefore can be summarised that all published scientific evidence as well as the results presented in this report indicate that thermophilic composting under controlled conditions can be considered as a safe method for the disposal of genetically modified microorganisms, plants and animals. This is also in agreement with a recently published review which highlights that conditions during composting (temperatures, pH, presence of nucleases) are not only leading to the elimination of plant, animal and human pathogens but also to the safe elimination of genetically modified organisms (Singh, Billingsley & Ward, 2006).

Horizontal gene transfer

No evidence was found for horizontal gene transfer (HGT) between *E. coli* K12 pGLO and indigenous microorganisms under chosen conditions during thermophilic in-vessel composting and in lab-based survival studies at 65°C, 40°C and 22°C over periods up to 66 days.

The use of genetically modified organisms has been in public discussion due to the potential risks associated with the release of alien DNA into the environment and potential uptake by indigenous species (plants, microorganisms). HGT from GM plants to bacteria has not been detected in the real environment despite extensive monitoring projects (Nielsen & Townsend, 2004) although this possibility has been demonstrated in laboratory settings (Gebhard & Smalla, 1998). The impact of GM plants on soil ecology has also been discussed (Dale, Clarke & Fontes, 2002; Lilley et al., 2006). Most scientific studies suggest the use of GM crops does not pose a safety risks (Moran & Fink, 2005; Ramessar et al., 2007; Snow et al., 2005). In addition, a recent study could not detect HGT transfer from GM food to food microorganisms

(Shin et al., 2004). HGT between genetically modified and indigenous microorganisms has been observed in a field site (Peters et al., 1997) and in compost microcosms at ambient temperature (Byzov et al., 1999; Guan et al., 2007). The implications of these results and other findings were also discussed in the context of using genetically modified microorganisms for bioremediation of contaminants (Urgun-Demirtas, Stark & Pagilla, 2006). Furthermore, Thomas and Nielsen highlight the fact that HGT occurs between indigenous bacteria and therefore, has been a major factor in natural evolution (Thomas & Nielsen, 2005).

CONCLUSIONS

During the course of this project experiments were carried to answer the following research questions using *E. coli* K12 pGLO as model GMMO:

Do GMMOs survive during thermophilic composting?

- Thermophilic in-vessel composting experiments:
 - There is no evidence of *E. coli* K12 pGLO survival during thermophilic composting when operated under optimal conditions ($T \geq 65^\circ\text{C}$, retention time 6 ± 2 days).
 - Potential problems might be caused by lack of feedstock, homogeneity or insufficient temperature exposure.
- Lab-based survival experiments at different temperatures:
 - Temperature is the main factor for survival of the *E. coli* K12 pGLO in non-autoclaved compost.
 - Survival rates non-autoclaved compost are:
 - $T = 65^\circ\text{C}$: < 5h
 - $T = 40^\circ\text{C}$: < 9 days
 - $T = 22^\circ\text{C}$: < 14 days.
 - The presence of a microbial community negatively affects the survival of GMMOs.
 - Survival rates autoclaved compost are:
 - $T = 65^\circ\text{C}$: < 5h
 - $T = 40^\circ\text{C}$: < 21 days
 - $T = 22^\circ\text{C}$: > 68 days.

Therefore, our results indicate that GMMOs do not survive during in-vessel thermophilic composting when temperatures are held above 65°C for periods longer than 5h.

Does transgenic DNA survive thermophilic composting?

There was no difference in survival rates of GMMO and transgenic gene in thermophilic in-vessel composting experiments. Our results suggest that transgenic DNA might survive longer periods compared to the GMMO itself in lab-based incubation experiments. This effect is greater in artificially created autoclaved environments compared to samples where an active microbial community is present.

- Thermophilic in-vessel composting experiments:
 - No evidence of marker gene survival (715bp overlapping region pBAD/*gfp*) during thermophilic composting when operated under optimal conditions ($T \geq 65^\circ\text{C}$, retention time 6 ± 2 days).
 - It is of great importance to control and monitor the optimal settings as irregularities might enable transgenic DNA to survive.
 - Factors affecting the in-vessel compost operation: lack of feedstock homogeneity, inadequate mixture of input pre-compostable materials, insufficient feedstock addition (minimum 40l day^{-1}) leading to shortened retention and exposure times.
- Lab-based survival experiments at different temperatures:
 - Temperature is the main factor for survival of the transgenic DNA in non-autoclaved compost.
 - Survival rates non-autoclaved compost are:
 - $T = 65^\circ\text{C}$: >2 days
 - $T = 40^\circ\text{C}$: < 9 days
 - $T = 22^\circ\text{C}$: < 14 days.
 - The presence of a pre-existing microbial community in the compostable material negatively affects the survival of the transgenic DNA.
 - Survival rates autoclaved compost are:
 - $T = 65^\circ\text{C}$: >2 days

- T= 40°C: < 28 days
- T= 22°C: > 68 days.

Therefore, our studies suggest that transgenic DNA has the potential to survive for longer periods than the GMMOs themselves. However, such material does not survive during thermophilic in-vessel composting when temperatures are held above 65°C for periods longer than 2 days.

Does horizontal gene transfer and subsequent gene expression occur in other micro-organisms?

No evidence was found for horizontal gene transfer between E. coli K12 pGLO and indigenous microorganisms under chosen conditions during thermophilic in-vessel composting and in lab-based survival studies at 65°C, 40°C and 22°C over periods up to 68 days.

Is it possible to detect and quantify the introduced genes during thermophilic composting with novel molecular biological techniques?

This project yielded the successful development of a protocol to detect and quantify transgenic DNA from compost samples using real-time PCR. The detection limits are 3×10^6 target copies g^{-1} for spiked compost and feedstock samples and 8×10^4 target copies ml^{-1} for *E. coli* K12 pGLO pure culture samples.

Therefore, we suggest that the method developed for real-time PCR can be used to detect and quantify the fate of transgenic DNA during in-vessel thermophilic composting. There is potential to improve the detection limit by method development in DNA extraction and purification as well as PCR reaction mix optimisation.

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