Vermicomposting: from microbial and earthworm induced effects in bacterial sanitation to the chemistry of biodegradation under batch or continuous operation

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ABSTRACT

Vermicomposting: From microbial and earthworm induced effects in bacterial sanitation to the chemistry of biodegradation under batch or continuous operation

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Vermicomposting is a mesophilic organic waste management practice in which earthworms interact with the microbiota to drive the stabilization of organic matter. Earthworms influence the microbial populations both directly and indirectly through grazing, ingestion and digestion of organic matter and deposition of castings and modifications of the physico-chemical parameters of the substrate. Under continuous operations, distinct patterns in the dynamics of pH, organic carbon, total nitrogen, total and labile sugars content of the vermicompost were distinguished. At the beginning, there was an accumulation of organic matter whose degradation rate increased after the initiation phase. No equilibrium state with stabilizing physico-chemical parameters could be distinguished during continuous operations, but interruption of feeding led to a decrease in total sugars, organic carbon, total nitrogen and pH. Elimination of bacterial pathogens from the vermicompost is primarily attributed to the indigenous microbiota and in a minor proportion to earthworm mediated effects; abiotic factors may modulate survival patterns but they are thought to play an indirect or secondary role. Different operational modes (batch *vs.* continuous) and varying earthworm densities did not affect *E. coli* survival. We showed that vermicompost contained both opportunistic bacterial pathogens and antagonists. Vermicompost is an organic waste management technology which can safely be conducted in domestic settings, provided that recommended management guidelines are followed. Further research is necessary to ensure that different species of bacterial pathogens are reduced to safe levels during vermicomposting, and future research efforts should be directed towards sanitation mechanisms.

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List of abbreviations

Amp	Ampicillin
CCF	Coelomic Cytolytic Factor 1
CFU	Colony Forming Units
COD	Chemical Oxygen Demand
GFP	Green Fluorescent Protein
kDa	Kilo Dalton
LB	Luria-Bertani Miller media
LPS	Lipopolysaccharide
PBS	Phosphate Suffered Saline
PSRP	Process to Significantly Reduce Pathogens
PFRP	Process to Further Reduce Pathogens
Mt	Million metric tons
$OD_{600\ nm}$	Optical Density at 600nm
Pro-PO	Prophenoloxidase
rpm	Rotations per minute
US EPA	American Environmental Protection Agency
VC	Vermicompost
VFA	Volatile Fatty Acids

Introduction

The impact of anthropogenic activities on Earth is far more disturbing than anything else in the history of life, not necessarily in terms of importance or magnitude, but because we understand the extent, consequence and significance of our acts. For decades, we have practised unsustainable landfilling of organic waste with mixed refuse despite our secular knowledge of composting. The urgency to take action to protect our health and the planet's ecosystems now stimulates the application of carefully planned initiatives. We now have more than enough scientific and technical knowledge to promote safe application of several environmental protection measures, such as vermicomposting, but it is crucial that science enriches our understanding of promising environmental protection technologies. At this time, it is essential to focus interdisciplinary research efforts on unravelling complex interactions between biotic and abiotic factors operating under different time and spatial scales in order to intelligently design and apply environmental impact mitigation practices and it is crucial to facilitate the transfer of knowledge between researchers and decision makers or end-users. The work described in this thesis was planned with the above goal in mind.

1.1 Goal of the thesis

The main goal of this research was to deepen our understanding of vermicomposting, a sustainable technology for the management of organic waste. We wanted to expand the current knowledge on interactions between the abiotic parameters of vermicomposting, the indigenous microbiota and the earthworms, focusing specifically

on the presence and persistence of bacterial pathogens in the vermicompost, and suggesting possible sanitation mechanisms.

1.2 Specific objectives and hypotheses

1) To determine if continuously operated vermicomposting could reach a relatively stable equilibrium phase, we set up 24 identical vermicomposting units and fed them weekly with organic waste. Several physico-chemical parameters of the feed and vermicompost were monitored and correlated to bacterial populations study over the course of 500 days. We hypothesized that continuous vermicompost could reach an equilibrium phase characterized by a stabilization of major physico-chemical parameters and bacterial abundance and diversity shortly after an initiation phase, and that this dynamic equilibrium would be disrupted by feeding interruption.

2) To determine the influence of the earthworm *Eisenia fetida* and the indigenous microbial community on the survival of *Escherichia coli* under batch and continuous vermicomposting systems, we conducted three different survival experiments with varying earthworm density and microbial abundances (fresh *vs.* sterilized substrates), monitored changes in the major physico-chemical parameters and screened for potential bacterial pathogens and antagonist microorganisms in vermicompost systems similar to domestic units. In batch experiments, we hypothesized that the presence of earthworms and a rich microbiota of the vermicompost or feed would lead to faster *E. coli* death rate. In the continuous experiment, we hypothesized that the decline in *E. coli* abundance would be correlated with an increase in earthworm abundance. We had also hypothesized

that the nutrients regularly added to continuously operated vermicomposting system would allow longer survival of *E. coli* compared to batch systems.

1.3 Organisation of the thesis

The first chapter provides a comprehensive literature review assembled on the interaction between microbes and earthworms during vermicomposting. The second chapter aims at correlating major physico-chemical parameters to the microbial ecology of continuous vermicomposting. Finally, the third chapter addresses the issue of pathogen eradication during vermicomposting and argues that a safe application of this technology is possible when adequate precautions are taken. Appendix one describes relevant background earthworm biology, to better understand the discussion of chapter one. Appendix two describes a series of experiments testing the impact of volatile substances emitted by vermicompost on the growth of *Escherichia coli*. Appendix three complements the discussion on continuous vermicomposting from chapter two with aspects related to the impact of humidity and initial C/N ratio of the feed. Finally, Appendix four is a complete description of all the bacteria isolated from the continuous vermicomposting experiment with culture-based method and morphotyping as well as pertinent information regarding their phylogeny and identification based on 16S rDNA sequencing. The chapters of this thesis are formatted as manuscripts to be submitted in journals reaching a wide audience because disseminating scientific knowledge encourages intelligent legislations and actions. Thus, each section is introduced and contextualized independently but they follow a logical sequence from describing microbial ecology in vermicomposting systems, explaining how environmental parameters affect the microbiota, and finally culminating with a description of sanitation mechanisms.

Chapter 1 - How earthworms influence microbes during vermicomposting: A review unraveling ecological, health and environmental issues

1.1 Abstract

Vermicomposting is a sustainable organic waste management technology whereby organic matter is stabilized and oxidized in a non-thermophilic process by the joint action of earthworms and microbes under aerobic conditions. Vermicompost has a greater microbial diversity and activity than thermophilic compost. The decomposition of organic wastes by composting (thermal sanitation) and vermicomposting (homogenous product) can give an end product that is relatively free of pathogens and at a faster rate than either of the technologies alone. Earthworms are responsible for grinding and aerating organic waste, grazing upon and dispersing microorganisms, and forming aggregates. Earthworms ingest organic matter selectively and can kill, have no effect or promote the growth of the bacteria they ingest, depending on the species. The bacterial flora of ingested food materials changes qualitatively and quantitatively during gut transit. Vermicompost has a lower density of fungus but a greater fungal biodiversity than regular thermophilic compost. Intense heat generally decrease pathogen loads in traditional compost but pathogens of plants and animals are reduced during mesophilic vermicomposting, due to biological factors like microbial competition and antagonism.

1.2 Introduction

With a rapidly increasing world population, humans are faced with the critical need to produce food and manage waste in a sustainable fashion. Intensive agriculture relies on enormous chemical and energy inputs and causes a decline in soil organic matter level, which eventually leads to soil degradation. Degraded soils have lower water-holding capacity which in turn leads to excessive deep percolation losses of both indigenous nutrients and synthetic fertilizers resulting in surface and ground water contamination (2,3). Long-term monoculture on degraded soils also decreases the density of the soil macrofauna and microfauna, which are both indexes of soil productivity (2). In natural ecosystems, most of the organic matter is recycled by soil microbes and mineral elements become available again to primary producers. Harnessing the natural process of biodegradation is a sustainable way to ensure efficient agro-production and long-term soil conservation while diverting organic waste from landfills, especially in regions where agricultural and urban territories close enough to minimize compost transportation. Composting consists of transforming organic material from vegetal and animal origin in a finished product similar to natural humus (4). Organic amendments can rehabilitate degraded soils and help to restore productivity (5) by increasing water-holding capacity, aggregate stability, saturated hydraulic conductivity, water-infiltration rate, and biochemical activity (6). Adding organic matter to the soil does not only improve soil properties with respect to the quality of the organic matter itself but it also increases the activity of the microfauna and flora, which contributes further to soil quality (7). In fact, the abundance and diversity of soil invertebrates, including earthworms, is an index of soil quality in agro-ecosystems (8).

Production of organic waste is intricately linked to our essential biological needs (feeding and defecating) and concerned with important industrial processes (textile fibers, pulp and paper, wood) or management of the environment in which we live (park and garden waste) (9). The putrescible fraction of our waste deserves special attention because it is responsible for most of the leachate and landfill gas problems (10). On average, 40 % by mass of Canadian domestic refuse consists of organic waste (11). In 2000, 5.3 Mt of organic waste were landfilled in Canada (10). Landfilling is responsible for nearly 3.8 % of all greenhouse gases production in Canada, corresponding to 29 Mt of CO_2-equivalents (11).

Composting of the organic waste instead of landfilling is a global warming mitigation practice that is increasingly recommended in sustainable waste management plans (12). Adding compost or vermicompost to soils can help to replenish soil organic carbon (short-term carbon sequestration) which can help to improve soil health and promote further primary productivity (carbon fixation) (13). However, our current understanding of greenhouse gases emissions from different composting systems and stages is somewhat limited. Mechanically turned traditional compost is characterised by high emissions of CH_4, most likely due to development of anaerobic zones in the pile (14). On the other hand, vermicomposting systems emit only trace amounts of CH_4 but significant fluxes of N_2O (15). Nitrous oxide has a much greater global warming potential than CH_4, 310 compared to 21 CO_2-equivalents over 100 years, respectively, according to the International Panel on Climate Change (16)). The high N_2O emission from vermicompost have been ascribed nitrification in the processing beds combined

with the presence of denitrifying bacteria within the worm gut (14). This example emphasizes why a global understanding of the microbiology of vermicompost is essential to ensure that its applications truly benefit the environment and the human population.

Many articles have been published on vermicomposting in the past 50 years. A recent publication by Senesi and Plaza (17) reviews the humification process in vermicomposting and compares it to other organic waste management technologies. To our knowledge, no comprehensive review on the relationship between microbes and earthworms, including a review of the sanitization mechanisms of vermicomposting has been published recently. This introduction aims at critically reviewing the state of knowledge on the microbiology and ecology of vermicompost in the broader scope of core issues pertaining to organic waste management and safe agricultural practices. It is our intention to critically review the most relevant past research on vermicomposting and to suggest orientations for future research based on knowledge gathered in the related fields of earthworm biology, soil and compost microbiology and ecology. Throughout this review, vermicomposting will be compared and contrasted with traditional composting and the interactions between the microbiota and the earthworms in the context of vermicomposting will be discussed. A short summary of earthworm biology relevant to vermicomposting is provided in Appendix 1. For a complete review of the effect of earthworms in the *drilosphere* (the soil volume and microbial fauna and flora they influence in natural ecosystems) consult Brown (1).

1.2.1 Defining, comparing and combining vermicomposting and traditional composting

Vermicomposting (from *Vermis*, worm in latin) is a special type of composting during which organic matter is stabilized and oxidized in a non-thermophilic process by the interactions between earthworms and microorganisms under aerobic conditions (4,18-21). The resulting vermicast is a finely granulated peat-like material that possesses excellent soil amendment characteristics in terms of structure, porosity, aeration, drainage and moisture-holding capacity (19). Vermicomposting is a rapid, simple and low cost technology (22-25) that has a broad range of applications, ranging from waste management to soil fertility (24). The use of earthworms as agents of turning and aeration is the main economical advantage of vermicomposting over traditional thermophilic composting which relies on heavy and costly machinery in large-scale facilities (22). Vermicomposting can be practiced at a small or larger scale but as opposed to traditional composting, scaling up of vermicomposting usually leads to slower composting, more intensive handling (19) and the need for very large surface area to thinly spread the vermicompost and avoid excess heat which is lethal to the earthworms (26).

The process of vermicomposting and the resulting end-product differ from traditional composting with respect to microbial diversity, process rate and quality of the end product. The major differences between compost and vermicompost are presented in Table 1. Overall, bacterial diversity drops considerably (bottleneck) during the thermophilic phase of traditional composting (27,28), as most species of microorganisms cannot survive at temperatures higher than 60-65°C (29). Thus, because mesophilic vermicompost does not have this diversity bottleneck, it is not surprising that

vermicompost has a greater microbial biodiversity (26,30,31) and activity than mature thermophilic compost (18,32,33). However, the functional significance of this higher diversity is debated as some authors correlate it with stimulation of soil microbial activity (30,31), while others report no difference (34) or caution generalizations concerning compost and vermicompost produced using different raw materials (26).

In terms of plant growth, yield and health, vermicompost has been shown superior to inorganic fertilizers (23,30,35,36) and traditional compost made from the same source material (26). The superiority of vermicompost can partly be explained by the greater concentration of nutrients in plant available forms (26) like nitrates, phosphates, calcium and soluble potassium compared to traditional compost (37-39). However, vermicompost also promotes biological activity causing plants to germinate, flower and grow and yield better than commercial planting media independent of nutrient concentration (40,41) even at low application rate in container media or in field soil (40-48). For example, ryegrass yields were significantly higher when grown with vermicompost than with compost produced with the same source material (municipal solid waste), and both types of compost led to significantly greater yields than in the absence of organic amendment (26). The nutrient-independent effect of vermicompost has been attributed to the presence of plant growth regulators (hormones such as cytokinins and auxins (49)) and other plant growth influencing substances (contained in the humic fraction) produced by microorganisms (38,50-55) or earthworms (56). Phytohormones are present in all decaying organic matter because of the action of microbes (51). Even though we know that phytohormones present in vermicompost promote the growth of plants, we cannot say that phytohormones are responsible for the increased growth of plants fertilized with vermicompost (*vs.* traditional compost) because could not find any studies comparing

phytohormone concentrations in vermicompost versus traditional compost. However, the extent of humification is greater (higher yields) and the process is faster during the maturation stage of vermicomposting compared to traditional composting (57) and this might be of agricultural importance as the hormone-like activity of vermicompost was detected in the humic fraction (58). In terms of rate, sludge is decomposed and stabilized three times as fast when ingested by *E. fetida* than without worm ingestion, i.e. traditional composting (59). Odour also disappear quicker in vermicomposted sludge compared to composted sludge (59) and the absence of unpleasant odours during vermicomposting has been attributed to the earthworms that create aerobic conditions in the waste materials, inhibiting the action of anaerobic micro-organisms which release foul-smelling hydrogen sulfide and mercaptans (thiols) (60). Thus, from an agricultural (available nutrients and phytohormones) and practical (stabilization and odour) perspective, vermicomposting has some advantages over traditional composting.

Vermicomposting and composting are not to be considered as mutually exclusive and they can be coupled to harvest the benefits relative to each method. Integrating both the advantages of composting (thermophilic sanitation) and vermicomposting (homogenous product) can give an end product relatively free of pathogens and at a faster rate than either of the technologies alone (22,61). Several studies have pointed out that combining composting and vermicomposting (in any order) led to faster rates of curing (period beyond the active biodegradation phase characterized by slower rate of decomposition but dominated by transformation processes occurring at mesophilic temperatures and leading to maturity (62,63)) and stabilization (eliminating the potential for putrefaction and reducing odours, pathogens and vector attraction (64)) (22,65,66). A combination of vermicomposting and composting also leads to greater stability of the

end-product based on reduced particle size and heterogeneity, the presence of lower amounts of total solids (TS) and volatile solids (VS) (22), lower final C/N and NH_4/NO_3 ratios, and lower final coliform counts (67). Since fresh organic waste can contain toxic levels of ammonia (i.e. in manure), salts, tannins (i.e. in fruits, woody or green waste) and acids (i.e. in fruit and vegetable waste), pre-composting could help to decrease earthworm mortality (68) as well as allow softening of the waste and thermal stabilisation before feeding to worms (69). Additional benefits of preceding vermicomposting by a short composting phase concerning pathogen stabilization will be discussed below.

1.2.2 Earthworms interactions with the substrate and the microbes

Earthworms act on their substrates both physically and biochemically. Because vermicompost is an artificial environment maintained by man, the earthworms may not have exactly the same ecological role as in soil, but knowledge gathered from soil biology may help us to understand better the effect of earthworms in vermicompost. By burrowing and depositing castings, they influence the porosity, aggregation, pedogenesis and litter breakdown of the drilosphere (70). Earthworms affect soil microbes directly or indirectly as agents of grinding, aeration, inoculum dispersal, grazing, litter comminution and aggregate formation (1,71,72), as summarized in Figure 1. Earthworms cohabit intimately, sometimes symbiotically (73), with numerous microorganisms found in the soil and in decomposing organic matter (74).

To isolate the effect of earthworms on the substrate and on the microbial community, the arduous task of rearing axenic earthworms has been attempted (75-77), but studying earthworms without their normal associated microbes and symbionts appears ecologically irrelevant because it influences the normal activities of the earthworms. This

emphasizes a great challenge in understanding the microbiology of vermicompost: Isolating individual interactions between the earthworms and the microbes or between the microbes themselves is difficult. A reductionist approach to soil science has confined the study of individual organisms to isolated subdisciplines and for a long time, little was known about the interactions between soil organisms and their effects on community diversity and soil processes (1,78). Multidisciplinary and ecological studies have shed light on these interactions and their importance in soil processes (78), which now benefits the characterization of vermicomposting. Commonly studied interactions between microbes and earthworms concern fate during gut transit (74,79), presence in nephridia (80,81) and immunity mediation (82).

Vermicompost harbors a diverse and abundant microbial community which includes fungi, bacteria and actinomycetes (32,83). The fate of microbes introduced in soils is influenced by physical, chemical and biological factors (84), such as interaction with indigenous microbial communities and soil animals and this may be similar in vermicompost. Several invertebrates, including earthworms, influence the biogeochemical conditions of soil microenvironments which in turn influence the soil microbes (85), once again, there may be a parallel between earthworm effect on soil and vermicompost microbiota. There are controversial issues concerning the effects of earthworms on microbial diversity (1,86) and biomass (87,88) and examples are given below.

1.2.2.1 Selective grazing and digestion

Earthworms ingest fresh and decaying organic matter, living or dead small invertebrates, protozoa, algae, nematodes, bacteria and fungi (24,89). In natural settings,

earthworms such as *Eisenia andrei* are believed to ingest organic matter selectively, for example, favoring soil fractions with higher organic content than the surrounding soil (90). This selectivity may extend beyond favouring organic content, and include selectivity for certain type of plant debris varying with earthworm species (91). Selective grazing on specific microbes may reduce the numbers of a preferred species and, by the mechanism of relaxation, allow multiplication of normally competitively excluded species (92) and possibly leading to monospecific enrichment of the cast (93). Alternatively, physico-chemical or bacteriological factors in the worms' gut may stimulate bacterial growth or impose strong survival selection on fast growing bacteria (94). Food selectivity is reviewed in Curry and Schmidt (95). Earthworms were shown to survive and grow on pure cultures of bacteria and fungus (96). Zhang and co-workers (97) have observed that soil ingestion by *E. fetida* led to a decrease in microbial biomass (fumigation extraction method), a higher bacterial-to-fungal ratio, an increase in available N and P, and an elevation in the respiration coefficient. These observations led them to the conclusion that micro-organisms are a food resource to the earthworms, and that total soil microbial biomass decreases upon passage through the gut while microbial activity increased.

Oligochaete species have different abilities to digest different bacterial species (98,99). Some bacteria increase, are unaffected or decrease (inactivation by lysis (100,101)) during gut passage (99). Therefore, generalization about the fate of ingested organisms is difficult, which may explain contradictory results from different studies.

1.2.2.2 Litter comminution, burrowing and aggregate formation

Worms ingest various types of organic matter as they "eat their way through the soil" (24). While the earthworm ingests food, excretes castings and creates burrows, it increases the available surface area of soil aggregates and organic matter (32), influences nutrient availability, pre-conditions the food for further microbial activity (1,89,102) and aerates the substrate (1,74,89). Earthworms consume whole organic materials and grind it down to 2-4 microns in their gizzard (24,32) but the majority of the bacteria and organic matter still passes through undigested (89). Only 5 to 10 percent of the ingested and chemically digested material is absorbed into the body and the rest is excreted out in the form of mucus coated granular aggregates called 'vermicastings' which are rich in metabolite wastes such as ammonium, urea, proteins, nitrates, phosphates and potassium salts (89,103,104). Additional secretions include a mucus rich in polysaccharides, proteins and other nitrogenous compounds (89). By ingesting the soil, earthworms stimulate soil microbial activity (105) and it is likely that by ingesting vermicompost, earthworms may also stimulate its microbial activity.

1.2.2.3 Digestive enzymes

The worms (and/or their associated gut flora) secrete enzymes such as proteases, lipases, amylases, cellulases and chitinases in their gizzard and intestine which bring about rapid biochemical conversion of the cellulosic and the proteinaceous materials in the organic wastes (106). The gut of *E. fetida* is characterized by high cellulase activity (97), probably of microbiological origin (97,107-109), that could increase the water soluble organic carbon (OC) and water soluble carbohydrates in soils (87). Epigeic species also appear to have a greater cellulolytic activity than anecic (97) or endogeic

species (1). Earthworms enhance mineralization of N, P, and K (110) because of higher microbial and enzymatic activity in their guts (111) and intestine (112).

1.2.2.4 Dispersal

Epigeic worms used in vermicomposting are some of the most important dispersing surface dwelling organisms (1). Microbes are dispersed by the earthworms by adhesion on their body surface or by surviving gut passage (1). Much of the soil microflora is in resting stages, able to withstand adverse and harsh conditions, awaiting favourable conditions for development (113) and responding rapidly to nutrient flushes (114), this has been described as the "Sleeping Beauty" hypothesis (73). In that context, dispersal by earthworms is of crucial importance in vermicomposting. Several factors influence dispersal by earthworms: Microbial community in the substrate, soil consumption rate and selective ingestion, microbicidal and enzymatic effects in the intestine, survival rate upon gut passage and casting deposition rate, spores, cysts, and resting stages viability and deposition, the mobility of the earthworm, and other environmental parameters of the drilosphere, such as temperature (1).

1.3 Microbiota of vermicompost

1.3.1 Ecological role of microbes in vermicompost and decomposition

The ecological role of the microbiota during thermophilic composting is well understood, and it is likely that the microbiota of the vermicompost fulfills equivalent ecological roles that drive the decomposition process forward with the key players being the bacteria and actinomycetes as well as the yeasts and fungi. On the other hand, it is well known that there is a succession of microorganisms during thermophilic composting

that matches the changing temperature regimes of the process (from mesophilic to thermophilic returning back to mesophilic communities) (4,115) and it is likely that the succession of microorganisms may be less drastic as the major selective force, temperature, remains relatively constant during the vermicomposting process.

Fruit and vegetables waste, which are slightly acidic (4.5-5.0), favour the growth of fungi and yeasts, that prefer low pH (116) but can tolerate a wide range of pH (117). At the beginning of composting, yeasts and acid forming bacteria break down the soluble and easily degradable carbon source leading to pH drop from organic acids production (116). Fungal species may be important at the start of vermicomposting and then become less favored as pH increases and available nutrients become more abundant, favoring the competition by bacteria (1). In thermophilic composting, bacteria are the initial heat generators. The ammonification, linked with protein degradation, leads to an increase in pH which results in bacteria out-competing fungi (115,117). Bacteria are most active under 50-60 % humidity and near neutral pH (115).

Actinomycetes are ineffective competitors at high nutrient levels and they develop more slowly than other bacteria and fungi in general. They are responsible for the earthy smell of compost through the production of the geosmine sesquiterpenoid. Actinomycetes often inhibit microbial growth by production of antibiotics, lytic enzymes and parasitism. They degrade hard debris (wood, bark, newspaper) and use cellulose, hemicellulose, lignin and humus as C and N sources. They form spores under adverse conditions, are most active under pH 7-8 and develop poorly in too wet or too dry material (115). In thermophilic composting, actinomycetes are thought to become

important only in the later stages of the process since most of them are killed during the thermophilic phase (above 60°C) (115).

Fungi are better cellulose and lignin degraders than bacteria and actinomycetes in general (115). Because of their extending hyphal network, fungi can degrade materials that are too dry, too acidic or too low in nitrogen for bacterial decomposition (27). Most fungi are mesophilic (5-37°C) and have optimum growth temperatures between 25-30°C, which corresponds to the working temperature of vermicomposting. In thermophilic composting, thermophilic fungi can flourish at 40-55°C up to a maximum of 60-62°C. They are usually killed above 60°C or survive as spores (27,118). Yeasts also normally disappear during the thermophilic stage of composting but can be found again when temperatures cool down below 54°C (115). Because regular compost is thermophilic and vermicompost is mesophilic, it implies that the fungal community composition is not the same in both (119,120) and thus that the degradation processes and the main key players may differ between thermophilic composting and vermicomposting. For instance, an important fraction of the organic waste is composed of lignocellulose which is optimally degraded at elevated temperatures (40-50°C) by thermophilic fungi in thermophilic compost (117). Lignin is generally considered to be the starting material in humus (117). If vermicomposting generally leads to greater levels of humification than thermophilic composting (57) but that lignin is best degraded under thermophilic temperatures (in traditional composting), it suggests that a different set of mesophilic microbes process lignocellulosic matter in vermicompost systems. The microbiota responsible of lignocellulosic degradation in vermicomposting has not yet been extensively studied.

Ryckboer *et al.* (115) surveyed the bacteria and fungi reported during different types of composting. Their inventory contains 155 species of prokaryotes (including 33 actinomycetes) belonging to 66 genera and 408 fungal species belonging to 160 genera. A total of 121 fungal species were isolated from vermicompost (115). A comprehensive survey of the fungi isolated from vermicompost has been done (120,121) but an equivalent survey of bacteria and actinomycetes has yet to be conducted.

1.3.2 Earthworm gut flora

The gut of earthworms is populated by millions of decomposer microbes (24). Certain bacteria and actinomycetes are found in greater concentration in the earthworm guts than in the surrounding soil, perhaps due to selective ingestion and greater culturability of these cells in the worm guts (99), or due to specific inhibitory effect of bacteria and actinomycetes antibiotic secretions (122). In the foregut, worms grind and homogenize organic matter and secrete mucus (rich in soluble C). Bacteria can then assimilate the soluble nutrients and further metabolize the organic matter under ideal pH and humidity in the midgut. Then in the hindgut, metabolites released by the microbes are absorbed by the earthworm, along with most of the mucus and water secreted by the worm itself (123). Interestingly, the epigeic earthworms used in vermicomposting seem to have lower microbial activity in their hindgut compared to endogeic earthworms, probably underlining a less intense mutualistic relationships with microbes (105) explained by the higher quality and digestibility of the food consumed by litter-feeding epigeic earthworms (1) or perhaps because epigeic earthworms absorb nutrients better (124), leaving less food available for microbes.

Using scanning electron microscopy, two morphologies of bacteria were seen attached to the gut epithelia of *Onychochaeta borincana* (125) and three with *Eisenia fetida andrei* (126), *Lumbricus terrestris* and *Octolasion cyaneum* (127), by socket-like structures (filamentous segmented bacteria) or glued with a mucopolysaccharide-like substance (cocci and bacilli) (125-127). Molecular profiling analyses have also helped to better describe the diversity of the gut associated microflora. For instance, previously uncultured bacteria, tightly associated with the gut of *Lumbricus rubellus* and rarely or not found in the feces, were identified using 16srRNA gene clone library and observed with FISH (128). The apparently opportunistic symbiosis was dominated by a few taxa (Acidobacteria, Firmicutes, b-Proteobacteria, and one phylogenetically unclassified group) varying with earthworm sampled (128). Enumeration studies revealed a greater proportion of facultative anaerobes (*vs.* obligate aerobes) in the gut of *Lumbricus rubellus* than in surrounding soils. In addition, there is a difference in substrate consumption of worm gut homogenate versus that of the surrounding soil under anaerobic or aerobic conditions (129). Under anaerobic conditions (normally prevailing in the worm gut), glucose, cellobiose, or ferulate are consumed more readily by the worm gut homogenate than by the surrounding soil microbiota and under aerobic conditions, cellobiose and oxygen are consumed more readily by the worm gut homogenate, but this later observation is biologically irrelevant sainces the earthworm gut is anaerobic (129). These results reinforce the idea that the earthworm gut microbiota is different from that of soil, and that the earthworm gut can favour anaerobic growth and activity (129). The possibility of a specific autochthonous earthworm microbial flora has yet to be resolved, but it is strongly suggested by research results so far.

1.3.3 Earthworm symbiosis with bacteria

The association between earthworms and bacteria is not limited to the gut. Earthworm nephridia (osmoregulatory and excretory organ) harbor Gram-negative rod shaped bacteria. Based on FISH and 16S rDNA sequencing studies, each earthworm species seems to host a distinct cluster of Acidovorax bacteria that are transmitted on the surface of the developing embryo or egg capsule between generations (81). Direct plating of egg capsule homogenates revealed high concentrations of these seemingly mutualistic bacteria (up to 10^8 colony forming units (CFU) per g cocoon) (130). It is thought that the albumen exerts a bacteriostatic or bacteriolytic influence on the entrapped bacterial cells (130). For example, some bacteria remain viable or even free themselves from the bacteriostatic inhibition while other bacteria, such as *E. coli*, are selected against (131). Juvenile earthworms need to be colonized during development in the egg capsule as they do not readily acquire this bacteria directly from the soil upon hatching (81). This symbiosis described in *E. fetida* is apparently ubiquitous among the Lumbricidae and may have a functional significance for the host and possibly influences soil chemistry (81). Further research on mutualistic and symbiotic associations between bacteria and earthworms will shed light on its functional and ecological importance in soils and vermicomposts.

1.3.4 Nitrogen fixation and denitrification

There is evidence of both nitrogen fixation and denitrification in vermicompost. Vermicomposting has been shown to increase total (132,133) and plant available (134) nitrogen due to be enhanced nitrogen fixation (134,135) and increase in ammonium-nitrogen to nitrate conversion induced by the earthworm (42). On the other hand,

materials with an initially low C/N ratio can experience a decrease in total nitrogen content during vermicomposting (136). Inoculation with the nitrogen-fixing bacteria *Azotobacter* sp. and *Azospirrilum* sp. was shown to significantly increase the nitrogen content of vermicompost (137). Earthworms stimulate non-symbiotic nitrogen fixation in the substrate by modulating the microbial community. First of all, the organic waste initially processed in vermicompost is relatively low in nitrogen easily accessible for microorganisms, but in addition, earthworms favor nitrogen-fixing bacteria over spore-forming *Bacillus* (which are their main competitors for carbon nourishment sources) (135).

The earthworm gut seems to be a favourable environment for denitrification mainly due to anoxia, high osmolarity, as well as high nitrite and nitrate concentrations (138). Concomitantly with N_2O emissions, earthworms also emit N_2, which is the end product of complete denitrification (139). Earthworms are thought to stimulate N_2O-producing soil bacteria during gut transit (138). The earthworm gut is anoxic and contains numerous compounds indicative of anaerobic metabolism (formate, acetate, lactate and succinate) as well as glucose in the aqueous phase (138). The concentration of nitrite and ammonium is 10 and 100 times higher, respectively, in the earthworm gut than in the surrounding soil (138). In laboratory experiment, nitrous oxide emissions are correlated with earthworm density but such relationship does not seem to exist for larger outdoor beds (140). While nitrous oxide emissions during vermicomposting appear to be comparable to other waste processing operations, further research is required to find ways to minimize emissions from vermicomposting systems operating at high earthworm densities and high waste processing rates (140), especially if vermicompost is to be

promoted as a sustainable waste management solution and as a global warming mitigation practice.

1.3.5 Effects of earthworms on bacteria and actinomycetes

The fate of bacteria and actinomycetes during gut transit is not well understood (1,141,142). The effects of gut passage on prokaryotes is influenced by the total number of bacteria and actinomycete and their metabolic state (active or spores) and results are influenced by differences in counting procedures, earthworms species, soil and food used (1). There is evidence that earthworms digest bacteria (143). *E. fetida* juveniles were shown to grow on 22 species of bacteria, with no differences between Gram-negative versus Gram-positive bacteria (143). In fact, Doube and Brown (144) highlighted that earthworms have minimal capacity to digest organic residues and may instead obtain most nutrients by digestion of microorganisms associated with organic matter and/or may be competing directly with soil microorganisms for labile carbon sources since both are limited by this resource (145). Despite this knowledge, the exact role of bacteria in the diet of earthworms and the extent of selective feeding by the earthworms is still not precisely defined (146).

Nevertheless, we know that the bacterial flora of ingested food materials changes qualitatively and quantitatively during gut transit (99). Twenty-eight percent of heterotrophic bacteria from the earthworm pharynx (observed by epifluorescence direct counts) are culturable while 82 % of the heterotrophic bacteria in one-day old casting are culturable (99). Microbial biomass carbon does not change during transit through earthworm gut, but the casting have greater total bacterial plate counts and increased

respiration (CO_2 production) (99). Microbial biomass carbon may not be a good estimate of bacterial abundance as it may yield inconsistent results with plate counts in studies analyzing worm worked soil *vs.* gut content *vs.* cast (1). Some studies have shown that the number of microorganisms in earthworm excreta is greater than that of soil (79,147). In *Lumbricus* sp. earthworm cast, the number of heterotrophs per gram of dry matter (2.1×10^9) is higher than that of soil (1.7×10^8) but lower than in the dung that was used as initial feed (1.5×10^{10}) (99). Total bacterial plate counts (148,149), total actinomycetes (148) and some *Bacillus* spp. (99) were shown to increase in the hindgut compared to the foregut. On the other hand, the abundance of some enterobacteriaceae (99) decreased in the hindgut compared to the foregut. In *L. terrestris*, the numbers of bacteria changed in a very uniform pattern during the gut passage irrespective of food materials and season; they increased from the crop/gizzard to the foregut and then declined or remained constant in the hindgut. Bacterial population size was at a maximum in the fore or hindgut (141). Increases in microbial population during gut transit were attributed to microbial growth rather than preferential ingestion of microbial-rich food (148). Microbial populations were shown to increase in the gut of *L. terrestris*, and *Aporrectodea longa* (148) and *Lumbricus rubellus* (150), but the numbers of microbes were shown to both increase, (plate counts (148)) and decrease (epifluorescence microscopy and plate count (150)) in *Aporrectodea caliginosa*. These results underline why the fate of bacteria during gut transit is not well understood. Bacterial population growth in earthworm gut may be influenced by retention time, which is affected by food quality (151), temperature (123) and worm species. Species with short gut retention times (for example 2.5 h in *Eisenia fetida* (1)) may retain bacteria long enough in their gut to allow component of the noticeable bacterial multiplication whereas species with a

longer retention time (such as 6-8 h for *L. rubellus* (149), 9-15 h for *L. festivus* (151), and 8-20 h for *L. terrestris* ((152),(148)) would allow sufficient time for bacterial multiplication. Noteworthy, *E. fetida* is a preferred species in vermicomposting; *Lumbricus rubellus* has also been used but *L. terrestris* is not suitable for vermicomposting and the latter has a much longer retention time than the former species. Microorganisms are a major diet of *L. terrestris.* Wolter and Scheu (141) assumed that this also applies to other earthworm species particularly those with shorter gut transit times.

Bacterial survival in worm gut is obviously dependent on the variety of gut enzymes present, either secreted by the worms or other microbes (1) and on the secretion of mucus (73), which can contain bacteriostatic or bacteriocidal substances like microbial antibiotics, bacteriolysin, peroxidases, or worm phagocytic bodies (1). Each of these substances can increase, decrease or not affect different bacteria, depending on the species. Bacterial survival in the earthworm guts is species specific (99). The abundances of *E. coli* BJ18 and *Pseudomonas putida* MM1 and MM11 decreased during gut passage while those of *Enterobacter cloacae* A107 and *Aeromonas hydrophila* remained the same in the excreta and feed material. Interestingly, *E. cloacae* and *E. coli* numbers decreased by several orders of magnitude upon passage through the pharynx and/or crop but the abundance of *E. cloacae* increased back to the same levels as the dung ingested before excretion while *E. coli* numbers remained low. Beside *E. coli*, the abundances of other Enterobacteriaceae, like *Salmonella* spp. as well as *Serratia marcescens*, were shown to decrease upon passage in the earthworm gut (99). Concentrations of *Pseudomonas stutzeri* (pLV1913) decreased upon passage through

millipede, another important member of the soil macrofauna (*Pachyulus flavipes* - Diplopoda), or earthworm (*Aporrectodea caliginosa* - endogeic Oligochaeta) gut passage but numbers of *P. putida* (pVL1017) were not affected or increased slightly during this process (100). This suggests a selective killing through the animal midgut followed by a rapid regrowth of surviving bacteria in the hindgut. Apparently, *E. fetida* has no substantial effect on populations of *Bacillus* introduced in vermicomposting units (153). While the population of *Bacillus sphaericus* decreased one hundred fold within the first three days after inoculation, that of *Bacillus thurigiensis* remained constant (153). Survival of *Bacillus* sp. in vermicomposting is a crucial issue as some bacillus species may be pathogenic.

1.3.6 Effect of earthworm on fungal abundance and diversity

Vermicompost has a lower density of fungus but a greater biodiversity than regular thermophilic compost (120). In small continuous feeding experiments using pre-composted cattle manure as litter and feeding weekly with mycelium suspension, 151 species and seven varieties of microscopic fungi were isolated (132 from vermiculture substrate and 123 from intestines). In general, the fungal community is more diverse in worm-processed substrates than in fresh substrates and earthworm guts. Mycelial biomass was greater in worm-processed substrates than in fresh material and fungal biomass decreased significantly during vermicomposting (86). Anastasi *et al.* (120) suggested that selective grazing by earthworms on fast growing fungi could reduce their ability to compete and favour the K-selected species (slower growing; some ascomycetes and basidiomycetes). The specific mechanisms by which earthworms enhance or

decrease fungal abundance, through spore dispersion and favoured germination for example, are reviewed in Brown (1).

Earthworm seem to favourably ingest fungi based on abundance and not primarily on species present (154). Certain fungi, such as *Aspergillus spp.*, *Fusarium spp.* and *Penicillium spp.*, were detrimental to some species of earthworms because they secrete antibiotics or toxins that may inhibit earthworm growth (144,146,147); it is possible that these species are not ingested or digested. Other species, such as *Fusarium oxysporum* and *Mucor hiemalis* may be preferably ingested by *L. terrestris* (1) and yet others are randomly ingested. There is evidence that earthworm digest some fungi (143,154), although some fungal species seem less favourable for growth than bacterial species in general and some fungal species cannot be used as a nutrient source at all, or at least not without an additional source of grit, such as sand or ashed loam (143). Fungal hyphae abundance is lower in hindgut compared to the foregut (99,155) and fungal spores may be unaffected by ingestion (156). Fungal hyphal lengths in the crop and gizzard of *L. terrestris* generally exceed that of surrounding soil indicating selective feeding on microorganisms rich microsites (141). In each section of the gut of *L. terrestris*, the diameter of fungal hyphae is similar but exceeded that of surrounding soil considerably (141). The length of hyphae is generally at a maximum in the crop and gizzard, decreases during passage in the gut and is lower in the cast than in the hindgut (141).

The numbers of fungal species and propagules of fungi (per gram of soil on a dry weight basis) is higher in earthworm casts than in soil and this has been attributed to selective feeding by the earthworm or to enhanced germination of fungal spores after passing through the digestive tract of earthworms (in the soil these spores might have

been present but could not be isolated by culture without germinating) (154). This highlights a methodological difficulty in studying the effect of earthworm on fungi (1): Plate counts, most often used, can difficultly describe abundance changes (due to spore germination and growth potential of broken hyphae) and direct counts (microscopic observations) are relatively difficult to perform.

Earthworms selectively ingest organic matter, and therefore can influence fungal spore germination and dispersal in microsites that can be favourable or unfavourable to fungal development (1,157). The role of the vermicompost in plant symbiosis with fungi varies depending on the initial substrate, the species of fungi present and the type of plant used. While a study reveals that vermicompost significantly inhibits the activity of arbuscular mycorrhizal fungi (AMF) (21), another highlights that vermicompost stimulates plant colonization by AMF (158). Earthworms are important in dispersal of vesicular arbuscular mycorrhizal (VAM) fungi and VAM propagules can survive several months in air dried vermicompost (produced by *Eudrilus eugeniae*) such that use of vermicompost on plants could favor inoculation (1).

1.4 Environmental and health issues of vermicomposting

1.4.1 Pathogens occurrence and legislation

The presence of pathogens in vermicompost is a topic of concern. *E. coli* and *Salmonella* are common indicator organisms whose survival has been studied in vermicompost. *E. coli* is an endogenous intestinal microbiota of warm-blooded animals

whereas *Salmonella* sp. and *Enterobacter* sp. are typical inhabitant of soil invertebrates (100) and vertebrates (74). Some organic materials, such as sewage sludge and manure, are potentially highly contaminated with enteric bacteria, viruses and protozoa. In domestic settings, vermicomposting organic waste potentially contaminated with pathogen, such as animal by-products, is not recommended; but even raw fruits and vegetables, which do not typically contain high loads of pathogens, can harbor some pathogenic microorganisms. Fruits and vegetables can become contaminated with pathogenic microorganisms while growing in the field, orchards, vineyards or greenhouses, or during harvesting, post-harvest handling, processing, distribution and preparation in food services or home settings (159). Non-composted or improperly composted manure used on the farm or in gardens may lead to food contamination (159). For instance, many outbreaks of the enterohemorrhagic *E. coli* O157:H7 wereassociated with food fertilized with cattle manure on the farm (160). *E. coli* O157:H7 survival in bovine feces is temperature dependent: It can survive 42-49 days at 37°C, 49-56 days at 22°C and 63-70 days at 5°C (160). The fact that vermicomposting is a strictly mesophilic process highlights the importance of pathogens survival studies in the specific case of vermicomposting. The American Environmental Protection Agency (US EPA) legislation concerning Class A compost (unrestricted use) requires sustained high temperature (40-55°C) for proper sanitization of the compost, but vermicomposting has a maximum of 35°C (22).

1.4.2 Pathogens elimination in vermicompost

Some studies have shown a decrease in human pathogen loads during vermicomposting (161-163), faster reduction in pathogens during vermicomposting

compared to traditional thermophilic composting (25) or no difference in pathogen reduction between composting and vermicomposting (164). Unfortunately, some of these research articles relate preliminary studies with no supporting numbers (164). According to Lotzof (161), pathogens such as enteric viruses, parasite eggs and bacteria such as *E. coli* are reduced to safe levels for use in gardening during vermicomposting. Vermicomposting causes a marked reduction in pathogenic microorganisms *Salmonella enteriditis*, *Escherichia coli* and other Enterobacteriaceae (59). Vigueros and Camperos (162) observed a 100 % removal efficiency of helminth ova and a 90 % removal efficiency of fecal coliforms after 45 days in a mixture of 70 % sewage sludge and 30 % water hyacinth (88 % humidity) processed by *E. fetida*. Eastman *et al.* (25) observed that pathogenic indicators inoculated at a very high density all declined below legislation levels within a short period of time. This occurred faster in the vermicompost than in the control (where the mechanism involved is depicted as the natural die-off of organisms) using biosolids (15-20 % solids) and 1:1.5 earthworm to biosolid ratio (wet weight). They concluded that vermicomposting was a Class A stabilization method for biosolids after air-drying the material below 75 % humidity to meet vector attraction requirements (methods to reduce attractiveness of degraded organic material to flies, rodents or birds which are potential disease vectors (165)). Fecal coliform levels reduced by between 1 and 3.5 orders of magnitude are indicating effective primary treatment (25,164). Eastman *et al.* (25) observed that vermicomposting with *E. fetida* decreased human pathogens 3- to 4-fold within 144 hours after inoculating with fecal coliforms, *Salmonella* spp., enteric viruses and helminth ova. Therefore, based on this study, vermicomposting could obtain class A stabilization according to US EPA regulations on composting (fecal coliforms <1000 MPN/g dry solid and *Salmonella* <3 MPN/g dry solid). *Salmonella* sp. bacteria

are often associated with poultry and involved in salmonellosis, a type of gastroenteritis. Levels of *Salmonella* sp. were shown to be reduced in vermicomposting (25,163), but they are known to recolonize compost that is not perfectly biologically stabilized (4). A study by Eastman *et al.* (25) has often been cited as a proof of pathogen reduction in vermicompost (68,121,166-168), but their research focused only on indicators organisms and did not discuss the mechanisms involved in pathogen reduction.

1.4.3 Fungal pathogens

The persistence of different species of fungal pathogens varies in compost and in vermicompost. For example, vermicompost harbour lower numbers of *Pseudallescheria boydii* (agents of opportunistic mycoses in humans leading to invasive pseudallescheriasis that is often lethal) than thermophilic compost but *Aspergillus fumigatus* var. *fumigatus* (spores inhaled cause allergy, asthma, bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis) was found in the same abundances in both types of compost (120). Vermicompost may be particularly rich in *Chrysosporium*, *Myceliophthora* and *Scopulariopsis* species (120) that can parasitize cornified tissues (120), despite the observation that *Chrysosporium* species are normally detrimentally affected by passage in earthworm gut (157). Thus, as with bacterial pathogens, different species of fungal pathogens occur and survive variably in distinct types of compost, and the results gathered in the microbially complex compost communities where earthworms are present *(in vivo)* may differ from those gathered *in vitro* where specific microorganisms are isolated.

Both compost and vermicompost contain few phytopathogenic fungi, but it appears that vermicompost contains fewer plant pathogens than thermophilic compost (120). For example, compared to thermophilic compost made with plant debris, vermicompost made with plant debris and animal waste has a smaller load of *Fusarium* (120), an economically important plant pathogen in agriculture, which causes plant wilt. Suppression of plant disease by inclusion of compost in soil was shown to be dependent on the decomposition level of the organic matter, most likely because this affects the populations of antagonistic microbes (169). Different antagonism mechanisms may predominate in compost of different ages. For example, more advanced decomposition levels may be correlated to greater pathogen suppression efficiencies in the case of *Rhizoctonia* (damping-off of radish) (170,171). On the other hand, younger compost produces more volatile substances (as opposed to older maturing compost) which inhibit the fungal pathogen *Fusarium culmorum* (172). In the presence of earthworms, fungal phytopathogens may be controlled by physical disturbance of substrate, ingestion and digestion of spores and hyphae, acceleration of residue decomposition (competing with fungus for substrate) and other still unknown mechanisms (173).

1.5 Mechanisms of sanitation in vermicompost

Although Edwards (30) stated that we have "considerable scientific evidence that human pathogens do not survive the vermicomposting process" more studies are required to shed light on the mechanisms involved in pathogen reduction. Several sanitation mechanisms have been recognized in the context of composting (174,175): (1) Heat during the thermophilic stage of traditional composting, (2) production of toxic compounds such as organic acids and ammonia, (3) lytic enzymes present in the compost,

(4) microbial antagonism, including antibiotics and parasitism, (5) competition for nutrients, (6) natural loss of viability over time, and (7) premature germination of resting stages induced by nutrients. Here we review some mechanisms that are most relevant to vermicomposting and that should be the focus of further research. The mechanisms reviewed are environmental abiotic factors, competition for resources and antagonism or inhibition (including production of antitiotics, enzymes or other inhibitory compounds).

1.5.1 Abiotic factors

Abiotic factors, such as soil composition, pH, water activity and oxidation-reduction potential, can play a major role in modulating microbial survival (160). pH and heat are two major abiotic factors that are important for compost sanitization. First, the acidity of most food, including fruits and vegetables, normally prevents the multiplication of most bacterial pathogens (159,176). During biodegradation, the pH of the food gradually increases as acid tolerant fungi, molds and bacterial soft rots release ammonia and other alkaline by-products (159,177). The resulting pH gradient that develops around the mycelia of molds on bruised tissues or on biofilms at the surface of the substrate can yield conditions favouring the growth of bacterial pathogens such as *Salmonella* (159). Thus, during vermicomposting, the alkaline shift in pH may lead to conditions that can favour bacterial pathogens whose growth was initially inhibited by the low pH of the substrate.

We mentioned earlier that vermicomposting does not pass US EPA rules for pathogen reduction (67). In compost, "thermal inactivation of pathogens is required to obtain safe products, both in terms of phytohygiene and human disease" according to

Ryckboer *et al.* (115). Despite the recognized importance of heat sanitation, few domestic or small community composts achieve the required temperatures. Ryckboer and colleages (115) reported that pathogen inactivation is usually more efficient when higher temperatures are reached, but suppression of *Salmonella spp.* may be more efficient at 55°C than at 70°C, suggesting that antagonistic effects of other microorganisms, probably actinomycetes, may be more important than high temperatures alone (115). At temperatures between 45-55°C, actinomycetes flourish but above 60°C, the number and diversity of actinomycetes decrease (115). Even at lower temperature ranges, the survival of pathogenic bacteria is modulated in response to changing temperature. For example, *E. coli* O157:H7 survived 77, 226 and 231 days in manure-amended autoclaved soil held at 5, 15, 21°C, respectively (160), with the latter temperature being common in vermicomposting.

Composting and vermicomposting can be combined to satisfy the US EPA thermal requirements concerning composting sanitation. When microbes degrade organic matter there is an important release of unharnessed energy in the form of heat (exothermic reactions). When the organic waste is fresh, microbes find enough available energy in the substrates to sustain high rates of multiplication and metabolic activity, thus releasing massive amounts of heat, leading to heating of the compost. During vermicomposting, the earthworms graze on the microbes, transiently reducing their active population and dispersing them more evenly throughout the substrate and thus slowing down exothermic fluxes. If vermicomposting precedes composting, the easily degradable organic matter is stabilized in the presence of the worms. Thus, in a subsequent composting phase (exclusion of the earthworms), the microbes have lower metabolic activities than those in

fresh organic matter and thus the absence of a thermophilic phase. Therefore, initial vermicomposting followed by composting does not reach high enough temperatures to meet the US EPA thermal requirement (22). Despite this legislative requirements, Eastman *et al.* (25) showed that pre-composting was not absolutely necessary in order to reduce pathogens (i.e. indicator organisms) to safe levels in vermicompost.

Overall, traditional composting achieves pathogen reduction through both an increase in temperatures and competition between species (29). Biological factors were shown to be involved in the suppression of both animal (160) and plant pathogens (178) at mesophilic temperatures, as shown by decreased suppressiveness of sterilized, pasteurized or heat treated substrates, and it appears that the abiotic factors may only modulate biological effects. For extensive reviews on phytopathogen suppression by compost and biocontrol in relation to soil microbial communities, consult Noble and Coventry (170) and Hoitink and Boehm (169).

1.5.2 Competition for resources

Competition between indigenous microflora and pathogenic organisms can be brought about in two ways: Pre-emption of resources and direct competition. According to Said *et al.* (172), soil microflora pre-empt space and resources (carbon, nitrogen and minerals) such that pathogens cannot establish a viable population. Several other authors imply direct competition for nutrients between pathogenic organisms and indigenous microflora in the worm casting whereby pathogens were less efficient competitors and their numbers decreased (25,29). Competition with soil microorganisms influences the survival of *E. coli* O157:H7, as shown by the faster die-off rate observed in soil amended

with fresh manure compared to sterilized (autoclaved) manure (160). On the other hand, growth of *E. coli* O157:H7 has also been reported in soil treated with manure (179). Thus, the inactivation of *E. coli* O157:H7 may be linked to several factors whose understanding still limit extrapolation of the results. Finally, distinguishing true competition from antagonism is confounding in the context of complex microbial communities since it is arduous to isolate individual interactions between different microbial populations, especially when the studies do not report the types of organisms present in the studied substrate, as it is often the case.

1.5.3 Antagonism or inhibition

It is well known that some naturally occurring microorganisms have a lethal or antagonistic effect on bacteria capable of causing human disease (159,172). Antagonism with indigenous microbes has been evoked in the rapid die-off of *E. coli* O157:H7 in soil amended with manure (160). Production of antibiotics by vermicompost indigenous microflora is one possible mechanism of inhibition (180,181). Bacteriosins are bacterial peptides that can inhibit closely related species (182). Three *Bacillus* species isolated from vermicompost (*B. cereus*, *B. thuringiensis* and *B. licheniformis*; see Chapter 3) are known to produce bacteriocins. Actinomycetes, often present in mesophilic composts (115), are also known to produce a great variety of antibiotics (181), but this ability remains to be shown in vermicompost. In addition to antibiotics, other growth inhibiting compounds, such as H_2O_2 and organic acids produced by lactic acid bacteria (isolated from compost) can be inhibitory to *Salmonella sp.*, *Listeria sp.*, *E. coli* and *B. cereus* (183). Earthworms were also shown to harbor antibacterial producing strains of microbes. For instance, a high proportion of *Streptomycete* strains isolated from

earthworm guts (*Lumbricus rubellus* and *Octolasion montanum*) produced antibiotics active against *B. subtilis* and/or *Saccharomyces cerevisiae* but none were active against *E. coli* (122).

Some antagonist microbes can grow on or in pathogenic bacteria and this mechanism, called hyperparasitism (181), is often associated with the production of cell-wall degrading enzymes (184) such as glucanases and chitinases. Interestingly, inhibition may not necessarily require direct contact between both protagonists as it was shown that compost could produce volatile substances that were inhibiting the fungal pathogen *Fusarium culmorum* (172). However, inhibition by volatile substances has never been discussed in the specific context of vermicomposting (see Appendix 2 for information and preliminary data on the topic).

Pathogen suppression may occur in the vermicompost although antagonists may also play a role in preventing pathogenic invasion of plants. Pathogen suppression requires that the antagonist/indigenous microbes already be established in the vermicompost to be fully effective (pre-emption of resources). Production of siderophores involved in iron-competition is an example of direct competition by *P. aeruginosa* (185). In the case of plant diseases, antagonists present in vermicompost could promote growth and health of the plant via production of growth regulators (49) or other growth promoting compounds (38,50-55), or could establish mutualistic relationships that enhance the ability of the plant to obtain nutrients (1,158).

In agricultural sciences, biocontrol agents are microbes that decrease plant susceptibility to phytopathogens (186). Different species of bacterial or fungal biocontrol agents were isolated from compost including the bacteria *Flavobacterium balustinum*,

Bacillus subtilis, *Bacillus cereus*, *Pseudomonas fluorescens* and *Enterobacter spp.* and the fungi *Paecilomyces spp.*, *Streptomyces spp.*, *Penicillium spp.*, *Gliocladium virens*, and several *Trichoderma spp.* (115,169,170,186). Some biocontrol agents, such as *Trichoderma spp.*, can even be inoculated in compost to enhance phytopathogen disease suppressive effects (186).

1.5.4 The effect of earthworms on reducing pathogens

Pathogen reduction in vermicompost may also be linked to the defense mechanisms of the earthworms. Earthworms were shown to secrete certain fluids with antibacterial factors (79) from their pharyngeal gland and/or crop, gizzard and anterior intestine at the same time as digestive enzymes (147). The presence of antibacterial factors has been demonstrated in the coelomic fluid of *Eisenia fetida andrei* (187,188) and of *Lumbricus terrestris* (99). No antibacterial factors have yet been demonstrated in the intestine, but it is believed that they could act as selective factor that modulate the composition of the worm gut flora (99). Reductions in number of pathogens in vermicompost may be due to the release of antibacterial compounds from the earthworm coelomic fluid (24,60). Further research to isolate and identify earthworm antibacterial factors and selective inhibition and/or growth of different bacteria in the earthworm gut is necessary (99), but earthworm biology has already revealed certain secrets of the earthworm natural defense mechanisms. The putative antibiotics in the earthworm gut have only been studied using plate counts on selected test organisms (1,122) but *in vivo* systematic testing of the antibiotics would be desirable to determine the spectrum of action of these antibiotics (1).

Bilej *et al.* (82) have identified a 42 kDa lectin they called coelomic cytolytic factor 1 (CCF) from the coelomic fluid of *Eisenia fetida*. This protein has significant amino acid sequence homology with bacterial and animal β-1,3-glucanase but it does not have its enzymatic activity (189). CCF also has sequence homology with insect Gram-negative bacteria binding proteins (190), with the β-1,3-glucan sensitive factor of the horseshoe crab (*Tachyplesus tridentatus*) (191) and with microbial lipopolysaccharide (LPS) and β-1,3-glucan recognition proteins from arthropods (192). These invertebrate holomogs were suggested to play a role in the recognition of bacterial or yeast invaders by the immune system. Similarly, the CCF factor of *E. fetida* has been shown to bind to cell wall components of Gram-negative bacteria or yeast, initiating the prophenoloxidase (pro-PO) cascade which leads to the formation of cytotoxic and antimicrobial compounds involved in the defense mechanism of invertebrates (189). The CCF factor binds to the O-antigen of the LPS, β-1,3-glucans and N', N'-diacetylchitobiose (189). Within six hours of exposure to Gram-negative bacteria, the pro-PO cascade is activated and then the coelomic fluid exhibits antimicrobial properties against Gram-negative bacteria (193). Gram-positive bacteria can also initiate the pro-PO cascade in vitro but only upon digestion of the Gram-positive bacteria (or the isolated peptidoglycan) by lysozyme (82). Different lysozymes, including muramidase and mucopeptide N-acetylemuramoylhydrolase are involved in the innate immune reaction of annelids and other animals (194). The CCF isolated from *Lumbricus terrestris* is also involved in triggering the pro-PO cascade but it has a different recognition domain and has a narrower pattern of recognition than that of *E. fetida*. *E. fetida* has one more recognition domain on the CCF than *L. terrestris*. In addition, the coelomic fluid from *E. fetida* has greater proteolytic and hemolytic activity levels than those of *L. terrestris*. All of these

factors seem to reflect the antigenicity of the biotope of where they normally live (compost *vs.* soil).

Williams *et al.* (195) recently studied the fate of *E. coli* O157:H7 in the presence of two species of worms used in vermicomposting. The anecic worm *Lumbricus terrestris* contributed to vertical dispersion of the bacteria and the epigeic worm *Dendrobaena venata* contributed to lateral dispersion of the bacteria, which corresponds to their respective burrowing habits. They also found that *E. coli* O157:H7 was present in the cast, on the epidermis and in the internal tissues of *D. venata* in higher ($>10^6$ CFU/g), lower ($>10^4$ CFU/g) and much lower (10^3 CFU/g) abundances, respectively, that in the initial soil inoculum ($<10^6$ CFU/g). This means that *E. coli* O157:H7 could survive gut passage, but is affected negatively by secretions on the surface of the earthworm skin and is probably eliminated from the earthworm tissue by the immune system. *Lumbricus rubellus* was shown to synthesize and secrete immunoprotective proteins (196), including Lumbricin I, which is effective against one serotype of *E. coli*. On the other hand, Lumbricin I seems ineffective at controlling the number/density of *E. coli* O157:H7, at least over a short period of time. The lower epidermal concentrations described above may be linked to the localized effect of the antimicrobial worms secretions (197).

Expanding the scope of pathogen survival studies is crucial. Several researches have shown that densities of fecal coliforms were good predictors of the effectiveness of thermophilic composting in destroying enteric pathogens like *Salmonella* sp. (see Hay (198) for a review), but there is yet no evidence to confirm that the survival of fecal coliform in mesophilic vermicompost is a good predictor of pathogen survival. For instance, past research has revealed information on the survival of common indicator

organisms, such as *E. coli* and *Salmonella* sp., but it is probable that different specis of microbes survive to a varying extent and that model organisms do not necessarily reflect the specific behavior of individual species of pathogen that can be found in vermicompost. Research on spore forming bacteria, such as *Clostridium botulinum*, survival in compost is scarce, and practically inexistent for vermicompost, despite evidence that they survive the composting process (199) and persist a long time (up to three years) in soils fertilized with contaminated compost (200). *C. botulinum* has been shown to be inhibited by *Bacillus licheniformis* (201), which is a common compost and vermicompost bacteria (see Chapter 3), and perhaps the overall role of antagonists in controlling spore forming bacteria during vermicomposting could lead to some interesting findings. In addition, in some instances, the pathogenicity of certain organisms is due to the production of toxins, i.e. *Staphylococcus aureus* and *Clostridium botulinum*. These toxins may remain active after the death of the bacteria and their persistence in vermicomposts has not yet been studied. Understanding the survival of model organisms in vermicompost is a critical first step, but being able to generalize to different types of pathogens and being able to understand exactly how pathogenicity is controlled during vermicomposting is of crucial importance.

Antagonism appears as a dominant sanitization mechanism during vermicompostingm and this may be promoted by the environmental conditions, the rich microbial communities of vermicompost, and the defense mechanism of earthworms. Jiang *et al.* (160) recommended further studies to identify and characterize the soil microbes involved in the inactivation of *E. coli* O157:H7 and to better understand what environmental conditions are optimal to kill the pathogens in manure and soil. Several

microorganisms are common to soil, compost and vermicompost, and these research areas would benefit from cross-linking discoveries concerning antagonistic interactions between microbes.

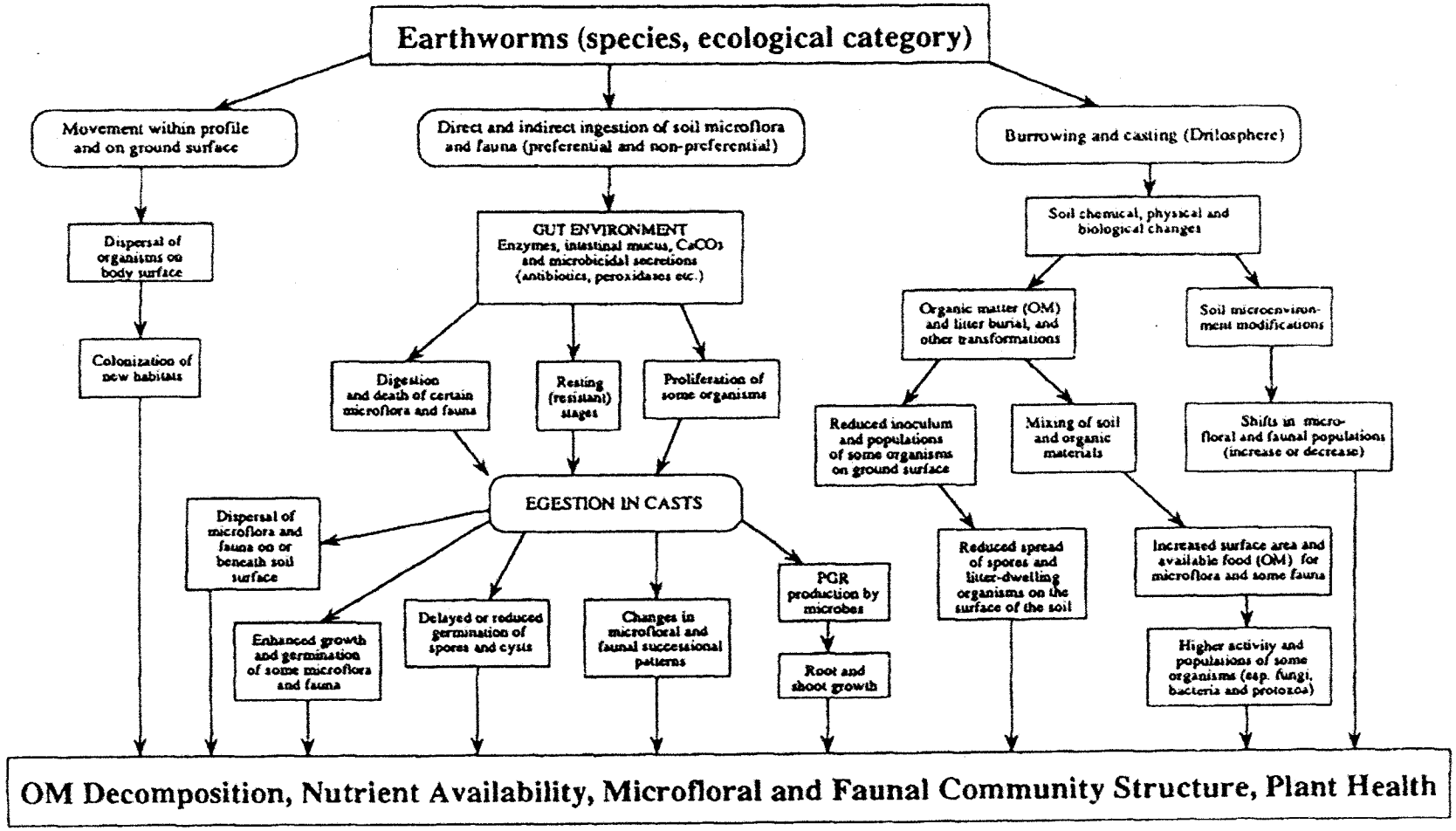


Figure 1. Earthworm effects on soil properties and microbial communities. Schematic representation of the effects of worms on the soil microbiota, leading to changes in soil properties, processes, microbial communities structure and plant health (from Brown (1)). Briefly, earthworms affect the decomposition of organic matter, nutrient availability, microbial communities and plant health via direct and indirect effects of their movements, burrowing, ingestion and cast deposition habits. Earthworms have an impact on the dispersal, survival and proliferation of microbes and they contribute to the homogenization and decomposition of organic matter. All of these natural processes may influence degradation and microbioal communities in vermicompost.

Table 1. Comparing thermophilic composting to vermicomposting

Category	Thermophilic composting	Vermicomposting
Temperature regime	Temperature succession phases: Mesophilic initiation followed by thermophilic phase (>60°C) and by mesophilic curing.	Mesophilic process (15-25°C)
Cost, process rate and technological requirements	High capital cost due to heavy machinery requirement for mixing, but high process rate at a large-scale	Lower capital cost and lower process rate at larger scale but faster rate than thermophilic composting at a smaller scale
Microbial diversity	Drops dramatically during thermophilic stage (only few thermophiles survive) and then recolonization occurs	Greater diversity and abundance than compost, all throughout the process and in the finished product
Process driven by	Bacteria and fungi	Earthworms interacting with bacteria and fungi
Plant growth	Improve plant growth and yields compared to equivalent inorganic fertilizer amendments	Vermicompost increases plant growth and yield (more than compost) directly through higher availability of nutrients and indirectly through stimulation of microbial activity (phytohormone synthesis and antagonism against phytopathogens).
Pathogen suppression	Mainly thermal with some antagonism	Mainly microbial competition or antagonism (antibiotics, etc.) modulated by earthworms
GHG emissions	Mainly CO_2 and CH_4 (due to localised anaerobic microenvironments) and traces of N_2O	Mainly CO_2 and N_2O (due to favourable earthworm gut conditions and endosymbionts metabolism) and traces of CH_4.

Chapter 2 - Do the physico-chemical properties, bacterial abundance and diversity of vermicomposting stabilize under continuous operations?

2.1 Abstract

Vermicomposting is a biooxidation process where epigeic earthworms act in synergy with microbial populations to stabilize organic matter. We hypothesized that the continuous (fed-batch) operation of model vermicomposting systems would reveal a stabilization of major physico-chemical and biological parameters of the vermicompost after a short initiation phase. Initially, we observed a gradual increase in pH, elevation of the vermicompost temperature above that of the room temperature and an accumulation of total sugars (including cellulose). The active biodegradation phase was characterized by a decrease in the C/N ratio of the vermicompost, brought about by an increasing rate of carbon volatilization paralleled by an accumulation of total nitrogen in the vermicompost. The total sugars concentration remained relatively constant after the initiation phase, but dropped rapidly after feeding interruption, while labile sugars remained relatively constant throughout the process, suggesting a rapid turnover of these sugars. Finally, the pH of the vermicompost decreased from 8 to 6 within one hundred days after feeding interruption. The different physico-chemical parameters were correlated with changes in bacterial diversity and abundance both prior to and after interruption of feeding. Several *Bacillaceae*, *Xanthomonadaceae*, *Aeromonadaceae*, *Enterobacteriaceae* and *Pseudomonadaceae* were observed in both active and maturing vermicompost, but *Actinobacteria* were only detected during the maturation phase. Finally, the practice of vermicomposting is rather simple but the interactions between the fauna and flora of the system are extremely complex. Continuous vermicomposting systems both differ (temperature and pH) and resemble (C/N ratio) batch operated composting systems. Here we have shown that despite continuous operations for over 200 days, no equilibrium

phase, where major physico-chemical parameters remain somewhat constant, could be distinguished. The three phases observed during continuous vermicomposting are the initiation, the active composting phase and finally the maturation phase after feeding interruption.

2.2 Introduction

Composting is a biotechnology that man has successfully adapted for soil conditioning, food production and organic waste management. Behind the apparent simplicity of the system, a precisely organized network of interactions between the numerous actors of composting is necessary in order to obtain a quality end-product and minimize physical or biological problems, such as odour or incomplete stabilization. Despite the long history of composting and the intensive research on the topic, we are still puzzled by poor experimental reproducibility linked to the complex nature of the process. Nowadays, composting technologies are increasingly promoted to address global issues related to food production and soil health, sustainable waste management and greenhouse gas emission mitigation practices. In this context, it is crucial to better understand the decomposition mechanism and take a holistic approach to maximize nutrients cycling and avoid further water and atmospheric pollution caused by unsustainable organic waste management practices.

Composting is the bio-oxidation of organic material catalyzed by micro-organisms and yielding stabilized humic compounds and inorganic nutrients (NO_3^-, PO_4^{3-}, K^+, Ca^{2+}) in a form readily available for plant uptake (4,22). Vermicomposting is similar to composting except for the presence of a keystone species, the earthworm. Vermicomposting is fast and odourless because worms continuously mix and aerate the compost. Compared to traditional compost, vermicompost contains higher concentrations of nitrogen (owing to bacterial N_2 fixation), more putative plant hormones that improve crop growth rate and yield, and a greater microbial community which can suppress plant pathogens (4,22). One of the main benefits of vermicomposting is that it is relatively

simple to manage and it is suitable for dense urban settings because it can be practiced indoor.

Composting systems are often operated in a batch mode where organic materials are added only at the beginning of the process and harvested once the decomposition process is completed. To maintain dense worm populations, vermicomposting is often conducted in a continuous mode. This mode of operation consists of adding organic matter and harvesting compost regularly such that the worms have a stable diet and live in a balanced environment. Despite the common use of continuous vermicomposting in domestic settings and larger scale installations, most research to date has focused on batch vermicomposting because it is simpler to study (20).

Continuous vermicomposting can further be subdivided into truly continuous and semi-continuous systems (fed-batch). A truly continuous system is operated with regular inputs and outputs, normally in a one way reactor. In a semi-continuous system, inputs and outputs do not necessarily have the same frequency and there is no directional flow, but rather the use of a box unit in which food is buried where space is available and compost is harvested where it is ready. The latter description represents better domestic vermicomposting. The three operational modes are illustrated in Figure 2.

Batch composting systems have been well characterized (4). Their physico-chemical parameters have been studied extensively and different phases have been distinguished during the process. These include: the initiation phase, the active composting or thermophilic phase and the maturation phase. Composting is an exothermic process because of the extensive amount of energy released during the oxidation of organic molecules. Upon initiation of composting, temperature rises as

bacteria multiply and the rate of degradation increases. When the temperature reaches 40-60°C (thermophilic stage) the overall microbial diversity decreases while thermophilic fungi and bacteria thrive. As most of the easily degradable matter gets oxidized, the rate of degradation slows down and the temperature decreases and remains low throughout the maturation phase (115).

During thermophilic batch composting, the substrate goes through acidogenesis, ammonification, stabilization and the maturation phases. Acidogenesis occurs early in the decomposition process when organic acids and carbon dioxide are produced, leading to an initial decrease of the pH. Once the composting process is well initiated (thermophilic phase), degradation of nitrogenous waste leads to the production of ammonia (NH_3) with a corresponding increase in pH (ammonification phase). When the more labile material is degraded, the microbial activity decreases. Then, most of the ammonia is lost by volatilization, and much of the remaining available nitrogen is fixed within microorganisms and humic molecules. During the maturation phase, the pH becomes stable close to neutrality and this stability is imputable to slow reaction kinetics and to the buffering capacity of humic substances.

Continuous vermicomposting systems have not yet been extensively characterized and are not expected to behave similarly to batch composting systems. Earthworms require temperatures between 16-26°C for activity; the gradual addition of food prevents the onset of a sharp thermophilic stage and thus, vermicomposting is considered strictly mesophilic. In addition, pH fluctuation of continuous vermicomposting are expected to be smoother than those of batch composting. This is because the regular addition of food, rapidly releasing organic acids, likely neutralizes the alkalinity generated during the less

intense ammonification step coinciding with protein degradation, which is normally facilitated by the elevated temperatures of batch composting. The decrease of the C/N ratio during continuous vermicomposting is also expected to be rather smooth (as opposed to rapid) early in the process of biodegradation because fresh organic matter is constantly replenished thus keeping the total carbon content high. The physico-chemical conditions characterizing vermicomposting can be influenced by a variety of factors including the number of earthworms as well as the abundance and diversity of the microbial communities. Predicting the evolution of these conditions is thus complex and should be based on the observation of model systems.

Although thermophilic composting microbial communities have been studied extensively, still today very little is known on the microbial composition of the mesophilic phase of traditional composting (202), and even less on the bacterial diversity of vermicompost. It has been shown that the bacterial communities of semi-continuous thermophilic composting take approximately one month to stabilize after the start of the reactors (203), and it is likely that vermicomposting communities also take some time before they stabilize. The composting process is highly variable and is influenced by source materials, scale and physico-chemical conditions. As a result, microbial communities analysis often depict distinct bacterial populations behaviors and little reproducibility (204). For those reasons, it is important to characterize the variations of major physico-chemical parameters during the vermicomposting process itself before trying to understand the microbial abundance and diversity in vermicomposting systems.

The major aim of this study was to distinguish different phases in the evolution of the physico-chemical conditions during vermicomposting operated in a continuous mode

and to contrast them with those of thermophilic batch composting. We hypothesized that continuous vermicomposting would lead to an equilibrium in the physico-chemical parameters of the vermicompost. A synthesis of the phases from initiation to maturation and the stabilization of the major physico-chemical parameters (establishment of a dynamic equilibrium) are discussed. Total mass, worm density, temperature, pH, humidity, organic carbon and total nitrogen contents, C/N ratio as well as total and labile sugars and cellulose concentrations were monitored in 24 controlled bins over a period of 500 days. An alternate goal of the study was to valorize the organic wastes produced on the Concordia University campus on a small-scale basis before scaling up vermicomposting units on a campus-wide basis. Our approach truly reflects the natural variability of organic wastes produced in this setting, and such variability in food wastes is thus *de facto* introduced as an intrinsic variable of the experiment. The ultimate objectives of this research was to lay a foundation for further research on the impact of variations in physico-chemical or biological conditions on the efficacy and innocuity of continuous vermicomposting.

2.3 Methods

2.3.1 Experimental conditions

Continuous, or more precisely semi-continuous, vermicomposting was conducted in 24 domestic-type units made of opaque plastic (40 cm × 60 cm × 35 cm high, Rubbermaid®, surface area of 2400 cm^2; Figure 3). Each bin was fitted with a drainage tube and lined with a geotextile pouch containing 7 kg of clean gravel. The lids were pierced with 16 aeration holes (2.5 cm in diameter) covered by mesh to prevent

circulation of flies. Exactly 2.8 kg of compost made from organic refuse, wood chips and bark (Ferti-Mix^{MC}, Ferti-Val®) were placed directly on the geotextile pouch and served as the initial litter. The bins were kept at constant temperature (27.7 ± 0.3°C) and ambient humidity (40.3 ± 3.2 %) throughout the experiment. Any collected leachate was immediately redistributed on top of the vermicompost.

2.3.2 Worms

Five kilograms of *Eisenia fetida* worms were purchased from *La Ferme Eugénia*, (Le Bic, Québec) and adapted to their new diet by being fed *ad libitum* for two weeks. Worms were distributed equally in the experimental bins by homogenous mixing with the litter and balancing the weight. The worms of three bins were counted, cleaned by crawling on moist paper towel and weighed.

2.3.3 Feed

Organic waste consisting of fruits, vegetables and used coffee grounds was collected daily on the Loyola campus of Concordia University (Montréal, Québec). The mixed fruits and vegetables were homogenized manually (<1 cm^3). The food waste and the coffee grounds were provided in a 1:1 ratio by mass (wet weight), supplemented with two coffee filter papers as a source of carbon. Worms were fed once a week. The feeding rate ranged from 80-300 mg food/g worm/day (30 % of body weight as per (205) and (206)). To prevent smell and problems with flies, food was buried just below the surface of the compost

2.3.4 Feed and vermicompost sampling and characterization

Over five weeks, 10 samples of food and five samples of coffee (200 g each) were collected. The pH of the feed was determined on 10 % aqueous suspensions (10 g sample in 100 mL distilled water pH 7, homogenized for one minute in a Waring blender; SympHony probe, VWR®, Mississauga, Ontario, Canada). Percent humidity was calculated by weight loss after freeze-drying 100 g samples (-50°C and 500 mbar, ModulyoD, Termo Savant®, Waltham, MA, USA). Organic carbon and total nitrogen were determined on ~4 mg samples in silver capsules in an elemental analyzer (Series II CHNS/O System model 2400, Perkin-Elmer®, Waltham, MA, USA). Before analysis, the pre-weighed litter samples were exposed to concentrated hydrochloric acid (vapour phase) for 12 hours to remove any trace of inorganic carbon (carbonates).

The physico-chemical parameters of the vermicomposters were monitored weekly. Bins were weighed and air temperature (inside the room and bins) and compost temperature (four random sites per bin) were recorded. A moisture meter (Mantis Tiller®), allowed qualitative assessment of humidity and the vermicompost was sprinkled with distilled water when the average humidity (n = 4) was below 60 %. Five grab samples of 25 mL were pooled for analysis. The pH was determined on 3 g samples (vortexed for one minute as described above). Humidity content was determined on 10-g samples (see above). The remaining compost was frozen at -80°C for further analysis. The organic carbon and total nitrogen contents on lyophilized and homogenized vermicompost were measured on 10 mg samples (see above).

Total and labile sugars extraction and measurement were carried according to the modified protocols of Lowe (207) and Scott and Melvin (208), using anthrone (Acros

Organics) in sulphuric acid (J.T. Baker) as the complexing reagent. Briefly, 0.1 g of homogenized dried compost reacted with 10 mL 0.5 M H_2SO_4 for 2 h at 95°C. The solution was filtered (2-cm glass fiber filters model GF-B, Whatman®) and adjusted to 25 mL. For total sugars, the hydrolysis was preceded by cellulose digestion using 0.4 mL of 12 M H_2SO_4 at room temperature for 4 hours. Sugar quantification was performed using a 1-mL aliquot of the suspension mixed with 2 mL of a 2 g L^{-1} solution of the Anthrone's reagent and incubated at 85°C for 15 minutes. The solution was then cooled 30 minutes and the absorbance was read at 660 nm (WinCary UV/VIS spectrophotometer, Varian®). Calibration curves consisting of eight standards ranging from 10 to 120 mg mL^{-1} were constructed for each series of samples. Cellulose concentrations were obtained by subtracting the concentration measured for labile sugars from that for total sugars. Results were transformed in mg sugar per gram dry compost.

2.3.5 Bacterial sampling and isolation

On experimental days 218, 266, 329 and 378, the vermicompost was screened for bacteria using six different selective and differential growth media. Briefly, 10 g of vermicompost was harvested from four randomly selected bins and then suspended in 100 mL of sterile phosphate buffered saline (PBS, pH 7.4) using a Waring blender. The solution was stirred at maximal intensity for 60 seconds three times, separated by 60 seconds pause to avoid heating of the solution. Then the vermicompost suspension was serially diluted and 100 µL was plated on six solid media (MacConkey Agar, Levine EMB Agar, LB Miller Agar, m-Enterococcus agar, m-Staphylococcus Agar and Yersinia Agar, all by Difco ™, BD®, USA; see Table 5 in Chapter 3 for further details). The plates were incubated for 18 to 48 hours at 25 or 35°C according to the manufacturer's

recommendations. The bacterial colonies isolated on each medium were characterized morphologically (size, shape, elevation, contour, color, optical characteristics, consistency and texture) and then counted. Samples representing the different morphologies were then restreaked for isolation on the same media and the selected colonies were separated into two aliquots. The first aliquot was stored in 30 % glycerol at -80°C for re-growth in case of need. The second aliquot was suspended, vortexed and centrifuged (16,1g) twice in 500 µL of sterile PBS prior to suspension in 250 µL sterile distilled water and three cycles of freeze-thaw using liquid nitrogen.

2.3.6 Bacteria identification by 16S rDNA sequencing

The 16s rDNA of the unknown bacterial samples was then amplified using bacterial universal primers 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). The PCR reaction was conducted in a total volume of 50 µL (PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, 2.5u Taq from Fermentas International Inc., Burlington, Canada; 10 pM of each primer and 4 µL of cell suspension and distilled H_2O). The mixture was subjected to five minutes of initial denaturing at 95°C followed by 35 cycles of one minute at 95°C for denaturing, one minute at 50°C for annealing and two minutes at 72°C for extension and finished by seven minutes of final extension at 72°C. The PCR products were visualized on a 0.8 % agarose gel stained with ethidium bromide under UV light. Samples with the expected 1.5 Kb band were directly sent for sequencing using the same bacterial universal primers (8F and 1492R) and another set of inner primers (331F (5' to 3': TCCTACGGGAGGCAGCAGT) and 1194R (5' to 3':ACGTCRTCCMCAACCTTCTC) in order to have a near complete coverage of the 16S gene. The sequences were aligned

using the Ribosomal Project Database II (Release 9.53) alignment tool (209). A consensus sequence was obtained using BioEdit Sequence Alignment Editor (210) using the CAP program (211). Sequences were compared to their closest relatives stored in GenBank. All the sequences obtained in this study will be deposited in GenBank using the Sequin software from National Center for Biotechnology Information (NCBI accession numbers not yet available).

2.4 Results and Discussion

2.4.1 Worms population: Growth and feeding rate

Worm density and mass were measured on days 0 and 194 to establish an average colony growth rate and feeding rate. On day zero, the bins were inoculated with 510 ± 18 worms (79.5 ± 6.0 g, n = 3) that on average weighed 0.16 ± 0.10 g each. On day 194, the bins contained on average 647 ± 73 worms (238 ± 24 g, n = 3) that weighed 0.37 ± 0.50 g each. Over a period of nearly 200 days, the number of worms in the present experiment increased by 25 % while their total mass tripled and their individual body weight increased 2.4 fold. The average individual weight of the earthworms on day 194 was similar to that reported by Elvira *et al.* (0.43 g) using cow manure as feed (212) but the average population growth rate of one mg earthworm/day is much lower than the 4.5-16.8 range reported by different authors using different mixtures of manure (212-215) or activated sludge (216) and bulking agents. In this experiment, the initial worm density of *E. fetida* was 0.3 kg earthworm/m^2 and reached 1.0 kg earthworm/m^2 after 194 days, which is below the optimal 1.60 kg worms/m^2 reported by Ndegwa and Thompson (22) using activated sewage sludge mixed with paper-mulch. Over the first 194 days, the

average feeding rate (>185 mg feed/g worm/day; 8.6 kg food/240 g worms was within the range of previously reported studies (0.75-300 mg feed/g worm/day) using activated sludge and mixed-paper mulch (22), unknown feed (206) or food scraps (206).

E. fetida gains weight at rates that are dependent on population density and food type (214). Perhaps the mix of fruits, vegetables and coffee was not as good as sewage sludge to sustain a large worm population growth rate and density. Biosolids and pig manure mixed with bulking agents have been reported to be the best residuals for growing earthworms (57). Nevertheless, based on the average worm weight and feeding rate, we can estimate that the worms were healthy, but the reproduction rate was below average. Increases in the C/N ratio of the feed or manure amendments to the fruits and vegetable diet could have increased population growth rate and perhaps led to a greater organic waste processing capacity.

2.4.2 Composition and physico-chemical characteristics of the organic waste

To facilitate comparisons of our conclusions with those of other studies, we characterized the typical composition of the feed, because organic waste collected in different regions and at different time of the year can vary. The overall proportion of fruits, vegetables, coffee grounds, filter papers and egg shells is presented in Figure 4a. The most abundant food by wet weight was melon (>20 %), followed by strawberry, lettuce and cabbage, banana, potato, carrot, celery and broccoli (with a proportion varying between 5-10 % each), then cucumber, cauliflower, sweet potato, pineapple, orange, brown paper, beet, apricot, egg shell, lemon and onion (with a proportion between 1-5 % each) and finally very small quantities (< 1 %) of grape, avocado, apple, prune, pepper,

tomato, pear, leaf and bean were observed. The physico-chemical parameters of the feed and litter are summarized in Table 2.

Despite the great variety of fruits and vegetables collected, the typical feed mix composition was relatively constant with respect to pH and humidity. In addition, the C/N ratios of coffee grounds and filter papers were very constant but that of the mixed fruits and vegetables were highly variable (nearly 50 % variability with 30 samples analyzed), reflecting the normal variability of domestic systems which are fed scraps of fruits and vegetables. The use of real organic waste had an additional benefit. During the realization of the present experiment, nearly 300 kg of fruits and vegetables and coffee grounds produced at Concordia University were diverted from the landfill and fed to the worms used in the continuous vermicomposting experiment.

2.4.3 Physico-chemical parameters

The differences between vermicompost and ambient temperatures ($\Delta T°$) are shown in Figure 5a. $\Delta T°$ remained around 1.4-3.5°C throughout the decomposition process and peaked around day 200. Whereas thermophilic composting leads to a thermophilic phase with temperatures above 60°C, continuous vermicomposting remained a few degrees above room temperature throughout the process (Figure 6a). Mesophilic temperatures favour the onset of a more diverse microbial community than in batch composting (most mesophilic microbes are killed during the thermophilic phase of batch composting). In fed-batch thermophilic composting systems (203) and during semi-continuous vermicomposting, the temperature fluctuated slightly over time, and this is likely due to rapid responses to the weekly organic waste additions. Massive food

additions should be avoided as they can overwhelm the processing capacity of the earthworms, and lead to sharp temperature increases which can be fatal to the worm colony.

Throughout the experiment, the humidity level was kept within the broad vital range of 60-90 % previously reported (67) (Figure 5b). Weekly water additions were necessary prior to day 194 but after, the humidity contained in food (~75 %) sufficed to keep the humidity levels relatively stable (consult Appendix 3 for details concerning the influence of temperature and humidity on the pH of the substrate). As of day 194 the humidity of the vermicompost was relatively stable and close to the optimal values of 80-90 % reported by Dominguez and Edwards (19). On the other hand, traditional compost piles with more than 70 % humidity may lead to anoxia (waterlogging), slower degradation and generation of foul smelling compounds (such as butyric and propionic acids, alcools, ketones and aldehydes, nitrogenous compounds such as ammonia, putrescine and cadaverine, as well as sulfur-containing compounds such as mercaptans, and hydrogen sulfide) (4). With earthworms in the vermicompost, the humidity of the media can reach much higher values without generation of foul smelling compounds indicative of anaerobiosis. In the present experiment, no foul smells were detected even with humidity levels above 80 %.

The moisture content of traditional batch compost decreases rapidly at the beginning of the process (leachate) due to the elevated moisture content of the organic materials, then as decomposition elevates the temperature evaporation becomes important and the final humidity content of the mature compost is dependent on ambient humidity and temperature. In typical vermicomposting bins, the lid limits excessive evaporation

such that the substrate becomes more humid with each food input. Leachate usually is not excessive if an acceptable feeding rate is observed, but free drainage of the vermicompost (by the use of drainage media and/or holes at the bottom of the bin) is important to prevent water logging.

Over the first 200 days of traditional thermophilic composting, the substrate goes through acidogenesis, ammonification, stabilization and a maturation phase. The initial pH of the vermicompost was close to that of the feed material, i.e. slightly acidic (Figure 5c). Then, the pH increased and stayed above pH 7 for over 100 days, approaching pH 8 around day 200. On day 146 and 180, the pH values were slightly lower than the previous or following days due to a slight decrease in the feeding frequency (every 11 days on average instead of 7). Beyond day 200, pH increased gradually to about 8, probably due to protein degradation. The decrease in pH beyond day 266 likely resulted from the release of acids during the die-off of the abundant decomposers community and eventually the earthworms themselves. The pH stabilized just above 6 approximately 100 days after the last feed. Overall, no sharp acidic or alkaline peaks were observed during the experiment which contrasts with traditional composting (Figure 6b). This may be due to the fact that feed input rate was low compared to total vermicompost mass. The organic waste added weekly represented an average of only 9.87 ± 4.07 % of the total compost (dry mass basis), with less food added at the beginning and a little more later in the experiment as the worm colony was growing (Figure 4b).

2.4.4 Organic carbon, total nitrogen and C/N ratio

During batch composting, the organic carbon content is expected to decrease due to the atmospheric release of carbon dioxide under aerobic conditions (217). The concentration of organic carbon was relatively stable throughout the experiment (less than 5 % variation between minimum and maximum concentrations). Nevertheless, the percent organic carbon in the vermicompost initially increased very slightly as food was added to the continuous vermicomposting system, indicating that the feeding rate were higher than the mineralization rates, and decreased back to the value of the initial litter (mature compost) when feeding was stopped (beyond day 266), indicating net carbon mineralization (Figure 5d).

The total nitrogen content of the vermicompost nearly doubled before day 266 and then decreased slightly when feeding was interrupted, although not as steeply as the carbon content (Figure 5e). Nitrogen can enter the compost with the feed material or through bacteriological N_2 fixation. Nitrogen can be lost from the vermicompost through ammonia volatilization (NH_3), denitrification by microorganisms (NO_2 and N_2O) or leaching of nitrate (NO_3^-). Nitrogen losses are especially common in systems enriched in nitrogenous compounds (i.e., low C/N ratios) (19,136).

The interpretation of the variations in carbon and nitrogen contents of the vermicompost becomes much more interesting when we compare the total nutrient inputs (including the feed and the initial litter) with the nutrients that can be accounted for in the vermicompost based on total compost weight and measured nutrient concentrations (Figure 7). The difference between the total input and nutrients present is assumed to have escaped from the system as gas (CO_2, CH_4, NO_x, NH_3, N_2O, etc.). Any leachate

produced was poured back on the vermicompost pile and thus did not exit the system. Both organic C and total N present in the earthworms biomass were negligible (< 1 % of the organic C or total N inputs on day 195), and since we did not have worm abundance beyond day 195, they were neglected from the mass balance calculations.

The mass balance behaviour of both organic carbon and total nitrogen is different during the course of the experiment. Initially, carbon accumulates in the vermicompost and gas production is low. Around day 150, the total mass of carbon that remained in the vermicompost was smaller than that which was lost as gas. As if the system had just come out of a lag phase, the carbon which had been accumulating in the vermicompost then disappeared in the gas phase more rapidly and this effect lasted until day 200. Only 62 days following the end of feeding did the organic carbon present in the vermicompost stabilize, suggesting that the vermicompost was maturing. Approximately 250 days after the last feed, the carbon volatilization rate decreased back to zero.

The total nitrogen content in the vermicompost varies in a different way. It first increases steadily until day 300. Shortly after feeding was stopped, nearly 75 % of the total-N input was still present in the vermicompost, probably locked up in the biomass. Then, within just a little more than 100 days, more than 40 % of the total N remaining in the vermicompost was released into the atmosphere, while the carbon gas emissions from the vermicompost (most likely correlated to the respiration rate) decreased during this period. This probably reveals the action of denitrifying bacteria that worked in anaerobic microsites and used up the organic N as a source of energy, while labile carbon resources were getting scarce. Indeed, nitrogen losses are especially common under nitrogen-rich conditions (low C/N ratios) (19,136). Bacterial closely related to the denitrifying *P.*

aeruginosa and *P. nitroreducens* were isolated from the vermicompost at densities below 10^2 CFU/g dry vermicompost on day 218, between 10^2-10^4 CFU/g on day 266 and above 10^6 on day 329 but they were not detected in the vermicompost 100 days after the last feed (Table 3). The abundance of potential denitrifiers was somewhat correlated with the nitrogen volatilization rates observed in the vermicompost.

Earthworms exert a major influence on the nitrogen cycling in vermicompost. Only 5–10 % of the chemically digested and ingested material is absorbed into the body of the earthworms (89), which are composed of 9.82 ± 0.83 % (n = 6) total nitrogen. Non assimilated material is excreted in the form of mucus coated granular aggregates called 'vermicastings', which are rich in metabolite wastes such as ammonium, urea, proteins, nitrates, phosphates and potassium salts (89). Additional secretions include a mucus rich in polysaccharides, proteins and other nitrogenous compounds (89). Vermicomposting has been shown to increase total N (132,133) due to the enhanced nitrogen fixation (134,135) brought about by the effect of earthworms on their environment (24) and the increase in ammonium-nitrogen to nitrate conversion induced by the earthworm (42). Earthworms stimulate non-symbiotic nitrogen fixation in the substrate by modulating the microbial community in a way that favors nitrogen fixing bacteria (135). However, earthworms are also thought to stimulate N_2O-producing soil bacteria during gut transit due to anoxia, high osmolarity, high nitrite and nitrate concentrations prevailing in their gut (138). N_2O being a powerful greenhouse gas, a better characterization of the N-cycle in vermicomposting would be important especially if vermicomposting facilities are to operate at high earthworm densities and high waste processing rates (218).

Concomitantly with N_2O emissions, earthworms also emit N_2, which is the end product of complete denitrification (139).

The decrease in C/N ratio during decomposition is a common characteristic of both batch composting and continuous vermicomposting. The C/N ratio of the litter and the feed were approximately 27 and 23, respectively and the vermicompost at the beginning of the experiment is within that range. Vermicomposting is though to have a good balance between energy available and elements for the growth of the microorganisms when the raw material has a C/N ratio of 25 (67,71). Successful vermicomposting of initial substate with a C/N ratios up to 60 were reported (36,68). The effect of the C/N ratio of the feed on the vermicompost is presented in Appendix 3. The C/N massic ratio decreased steeply and stabilized to a value near 10 just before feeding was stopped (Figure 5f). The initial rapid C/N decrease correlates with the breakdown of easily hydrolysable sugars (Figure 8) and the slowing down corresponds to the degradation of the more recalcitrant compounds and the maturation stage. The inflection point around day 231 may indicate that at this point, the bacterial community was efficiently turning over the new organic matter additions, which constituted only a small fraction of the total compost mass at this time (10.1 ± 1.4 % for days 208, 217, 242 and 266). A C/N ratio cannot alone be considered a satisfying index of maturity (219) but it can be useful to follow the degradation process. In the present experiment, the overall C/N ratio stabilized after the last feed despite ongoing changes in organic C content and pH. Maturity tests such as the germination index (indicating the presence of inhibitory compounds, such as NH_4^+, volatile fatty acids and phenolic compounds, in immature composts) (163) or respirometric tests (indicating low oxygen consumption or CO_2

emission by microbes present in the compost as a consequence of slowed metabolism on stabilized substrate) could have been performed to assess the stability of the vermicompost at the end of the experiment (220). Such tests were not performed because the main goal of the study was to assess the kinetics of continuous vermicomposting and not the maturation level of the vermicompost. Nevertheless, maturation tests would be interesting to perform to gain an understanding on the maturation stage of vermicompost when it is harvested from an active vermicompost bin. To our knowledge, respirometric tests commonly applied to traditional batch composting, have previously been used to assess loading capacity of vermicompost reactors (221), but have not yet been studied with respect to an absolute maturity threshold (as the respiration of the earthworms themselves may changes the threshold) and this methodology would probably be very interesting to develop in the future as many composting legislations are now turning to respirometric tests as index of maturity (220).

2.4.5 Carbohydrates

Because complex sugars are continuously added through weekly feeding, the abundance of total sugars, including cellulose, was expected to increase initially, as it did until day 61 (Figure 8). This indicates that the more complex sugars accumulated until the appropriate microbial fauna developed. Between days 61 and 231, constant sugars remained relatively stable, as the input was balanced by the high turnover rate (dynamic equilibrium). Total sugars (and cellulose) eventually decreased when the input was interrupted (on day 266). On the other hand, the concentration of labile sugars remained relatively constant throughout the experiment (27.9 ± 5.0 mg sugar/g vermicompost). During batch composting, the concentration of labile sugars remains relatively stable as

long as the more complex sugars are not fully degraded because the degradation of the complex sugars replenishes the pool of labile carbohydrates (4).

2.4.6 Vermicomposting phases

Three phases were distinguished during the experiment: The initiation, the active degradation and the maturation phases Figure 9. The initiation phase (~ days 1-100) is characterized by the accumulation of organic matter during which the majority of the organic carbon and total nitrogen input remained in the vermicompost while the C:N ratio, pH and temperature increased slightly. The active degradation phase is characterized by a high respiration rate (C volatilization) and cellulose degradation, as well as a sharp decline in the C:N ratio. Between days 180 and 230, the degradation rate is at a maximum, as revealed by elevated temperatures, sharp increase in pH and a maximal value of C volatilization, increased turnover of carbon and nitrogen and consumption of labile sugars, all coinciding with a stabilizing C:N ratio. Contrary to our expectations, the continuous system did not reach a well-defined equilibrium after more than 250 days. Fluctuations of all the physico-chemical parameters could reveal a dynamic process influenced by the variable abundance and composition of the feed. Nevertheless, the feeding interruption influenced markedly the observed trends with an abrupt decline in pH and carbon volatilization correlated with the death of the decomposer community. An increase in nitrogen volatilization rate may have revealed a switch from aerobic carbon compounds consumption to nitrogenous compounds consumption for energy. Two hundred and fifty days after feeding interruption, the vermicompost seemed stable, although the total sugars levels may still have been declining, suggesting an ongoing humification process.

2.4.7 Bacterial diversity and abundance

The overall total abundance of bacteria did not vary much before and after feeding ended (remaining close to 10^6 CFU/g dry vermicompost), but the diversity of bacteria, the number of both class and genus, increased gradually during the experiment (Table 3). The complete description of the bacteria phylogeny, closest GenBank relative, accession numbers and colony morphology is available in Appendix 1. Our results also suggest a community variation with time as *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* were only detected in the last sampling event, approximately 100 days after weekly feeding was interrupted. *Actinomycetes* are ineffective competitors at high nutrient levels and they develop more slowly than bacteria and fungi in general. During thermophilic composting, *actinomycetes* are thought to become key players in the later stages of the process since most are killed during the thermophilic phase (above 60°C) (115), but our results suggest that nutrient abundance may also play a key role, as a thermophilic stage is not observed in vermicomposting. Overall, the *Bacilli* and *Gammaproteobacteria* remained relatively abundant throughout the experiment, and perhaps became slightly more abundant with time. The diversity of the *Bacilli* increased slightly after the first sampling event and the diversity of the *Gammaproteobacteria* culminated 50 days after feeding ended. The *Gammaproteobacteria* are present and their abundance is relatively high and stable throughout the experiment (with a peak on day 329 for the *Pseudomonales*), except for the *Xanthomonadales* which appear in very high abundance (but low diversity) only after feeding has stopped. These results suggest that the active degradation stage is dominated by a few taxa whereas the maturation stage allows a greater diversity of bacteria to cohabit, all of this under a constant abundance of

bacteria. Indeed, a succession of microbial communities in time, already demonstrated in thermophilic composting (222), is also observed during vermicomposting.

A close examination of the species present reveals a similarity with bacteria isolated from traditional composters (Table 3). These include *Klebsiella pneumoniae* (223), *Serratia marescens* (223) and several *Bacillus* sp. (*B. cereus* (115) (223), *B. licheniformis* (115) (223), *B. pumilus* (115), *B. megaterium* (204)). *Bacillus licheniformis* is common during all phases of thermophilic composting (115). Here, there is an apparent decrease in *B. licheniformis* abundance after interruption of feeding, but this may be an artifact of incomplete identification, as the abundance of *Bacillus* sp. increased dramatically during the two last sampling events. *Bacillus* sp. are thought to dominate the thermophilic composting microbial communities (224,225) and we showed that *Bacillus* were indeed also very abundant and diverse (six species) in continuous vermicomposting.

Unfortunately, the bacterial populations described here only represent a subset of the actual community. Indeed, the heterotrophic plate counts revealed bacterial communities of approximately 10^6 CFU/g (using LB agar) while previous compost studies showed counts above 10^{10} CFU/g (using tryptic soy agar) (222). Also, we relied on culturing for isolation, and it has been suggested that most of the vermicompost bacteria are likely to be yet uncultured organisms (226). The use of culture independent methods to characterize global diversity of the complex vermicompost microbial community could thus give us a complementary portrait. Long-term characterization of both simple physico-chemical parameters and bacterial communities of continuous vermicompost showed to be an interesting approach to identify global trends of a dynamic

system. The fact that no stabilization in the physico-chemical parameters was identified in this study suggests that semi-continuous vermicompost systems never reached true equilibria due to their intrinsic variable nature or that such an equilibrium may take much longer to reach than in fed-batch thermophilic composting (203).

Table 2. Physico-chemical characterization of the organic waste used as vermicompost feed

	n*	Organic C (wt % ± SD)	Total N (wt % ± SD)	C/N (± SD)	Humidity (% ± SD)	pH
Food	30	37.9 ± 11.2	1.92 ± 0.69	22.8 ± 10.5	85.6 ± 1.6	5.13 ± 0.39
Coffee	5	53.7 ± 1.6	2.27 ± 0.10	23.7 ± 0.7	66.3 ± 2.2	6.10 ± 0.12
Filters	3	42.4 ± 0.1	0.103 ± 0.003	413.2 ± 12.9	66.3 ± 2.2	
Average feed	28	51.2 ± 0.8	2.20 ± 0.02	23.2 ± 0.2	71.8 ± 1.6	
Initial litter (compost)	3	49.9 ± 0.8	1.87 ± 0.04	26.7 ± 0.9		

* Represents grab samples of feed added to bins except for the average feed which is a arithmetic average based on weekly feed mass and average feed composition.

Table 3. Abundance and diversity of the bacterial community isolated from continuous vermicompost. Feeding was stopped after day 266.

	Weekly feeding				After feeding interruption			
	218		266		329		378	
	Abundance	(no. of genus/ no. of isolates)	Abundance	(no. of genus/ no. of isolates)	Abundance	(no. of genus/ no. of isolates)	Abundance	(no. of genus/ no. of isolates)
Class								
Actinobacteria							+++	(4/4)
Bacilli	+++	(1/6)	++++	(3/8)	++++	(3/12)	++++	(3/14)
Alphaproteobacteria							++	(1/1)
Betaproteobacteria							++++	(1/3)
Gammaproteobacteria	+++	(3/14)	+++	(4/6)	++++	(7/14)	++++	(4/21)
Order								
Xanthomonadales					++++	(1/2)	+++	(1/4)
Aeromonales	+++	(1/1)	+	(1/1)	+++	(2/2)	+++	(1/3)
Enterobacteriales	+++	(1/6)	+++	(2/3)	+++	(3/4)	+++	(1/2)
Pseudomonales	+++	(1/7)	+++	(1/2)	++++	(1/6)	+++	(1/12)
Heterotrophic count (CFU/g dry soil)	10^5	(4/20)	10^6	(7/14)	10^6	(10/26)	10^6	(13/43)

[1] Abundances of +, ++, +++ and ++++ correspond to 10^0-10^1, 10^2-10^3, 10^4-10^5 and $\geq 10^6$ CFU/g dry vermicompost respectively

[2] Diversity is expressed as the number of genus and the number of isolates characterized is also given.

[3] Obtained on LB agar

Table 4. Bacterial species isolated during continuous vermicomposting. Feeding was stopped after day 266.

Species isolated on days (no. of isolates)	218		266		329		378	
	Abundance	(no. of isolates)	Abundance	(no. of isolates)	Abundance	(no. of isolates)	Abundance	(no. of isolates)
phylum Actinobacteria								
class Actinobacteria								
Streptomyces sp. (1)							+	(1)
Brevibacterium sp. (1)							+	(1)
Agromyces sp. (1)							+++	(1)
Microbacterium sp. (1)							+	(1)
phylum Firmicutes								
class Bacilli								
Enterococcus sp. (1)					+	(1)		
Staphylococcus sp. (2)			+	(1)			+	(1)
Oceanobacillus iheyensis (2)					++	(2)		
Oceanobacillus sp. (1)							+	(1)
Halobacillus trueperi (1)			+	(1)				
Bacillus cereus (6)	+++	(2)	++++	(2)	+++	(1)	+	(1)
Bacillus firmus (1)							+	(1)
Bacillus licheniformis (3)	++	(1)	++	(2)				
Bacillus megaterium (1)							++++	(1)
Bacillus pumilus (6)			+++	(1)	++	(3)	+++	(2)

Table 4 ...*Continued*

Species isolated on days (no. of isolates)	218		266		329		378	
	Abundance	(no. of isolates)	Abundance	(no. of isolates)	Abundance	(no. of isolates)	Abundance	(no. of isolates)
phylum Proteobacteria								
class Alphaproteobacteria								
Bosea thiooxidans (1)							++	(1)
class Betaproteobacteria								
Cupriavidus sp.							+	(1)
unclassified Neisseriaceae (2)							++++	(2)
class Gammaproteobacteria								
order Xanthomonadales								
Stenotrophomonas maltophila (4)					++++	(1)	+++	(3)
Stenotrophomonas sp. (1)					+++	(1)		
unclassified Xanthomonadaceae (1)							+	(1)
order Aeromonadales								
Aeromonas hydrophila (2)							+++	(2)
Aeromonas sp. (5)	+++	(1)	+	(1)	+++	(2)	+	(1)
order Enterobacteriales								
Serratia marescens (1)					+	(1)		
Citrobacter freundii (1)			+++	(1)				
Citrobacter sp. (2)					++	(1)	+++	(1)

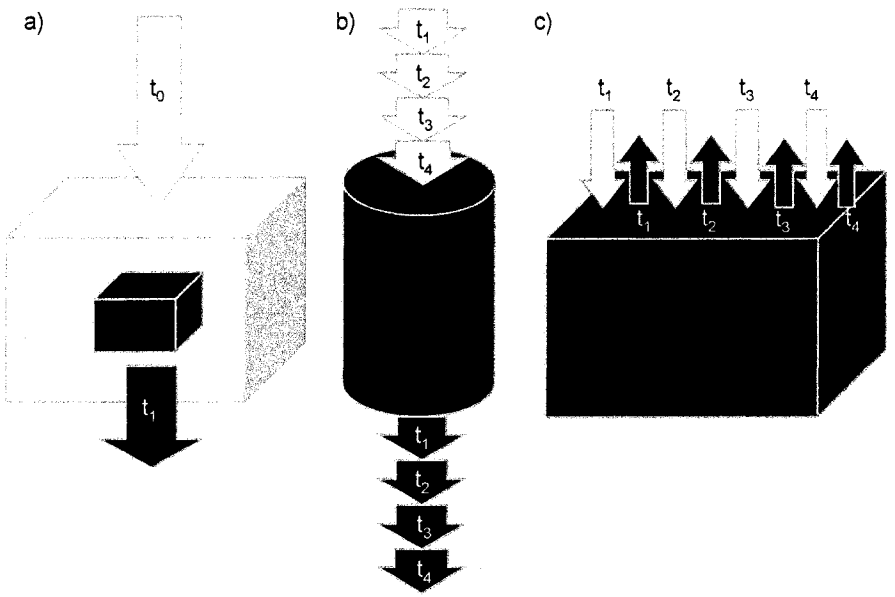


Figure 2. Schematic representation of the different operational modes for composting: a) Batch composting, b) continuous composting and c) semi-continuous composting. White arrows represent organic matter input and black arrows represent compost harvest.

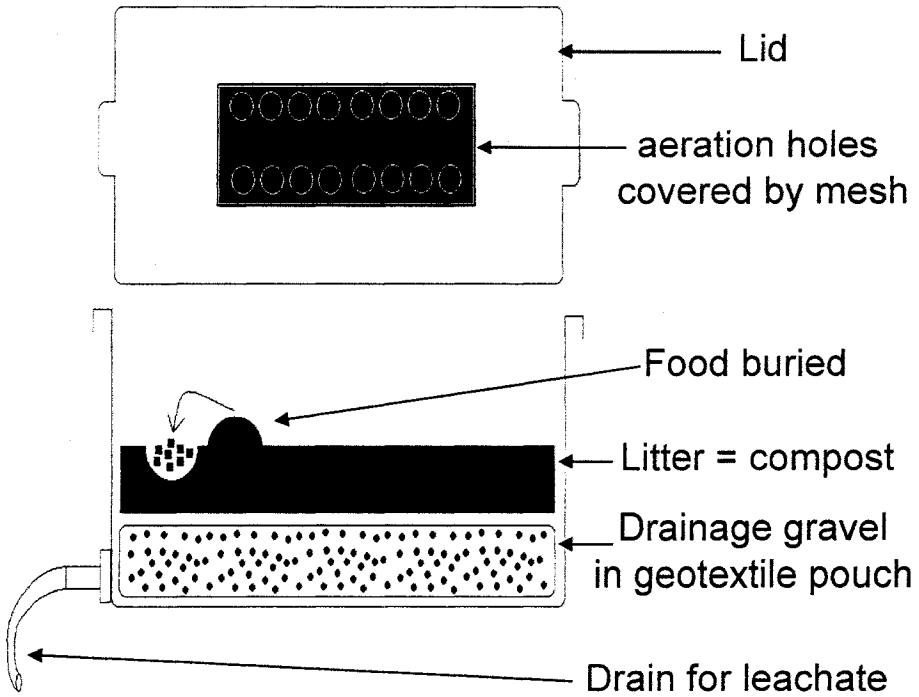
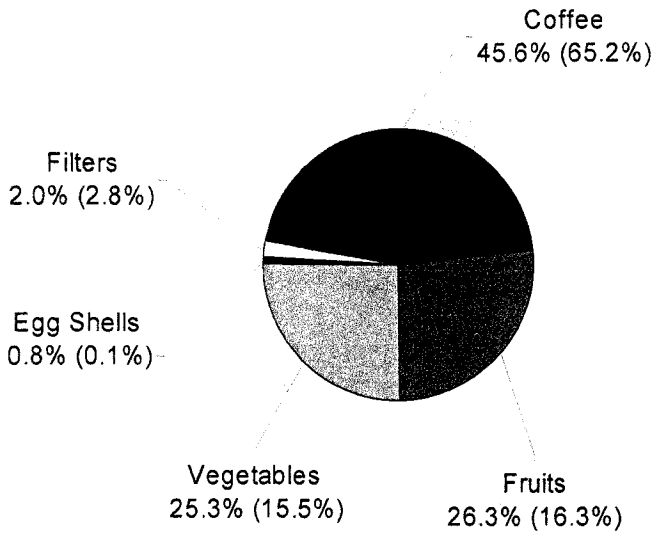


Figure 3. Schematics of the vermicomposting bins used in this study.

a)



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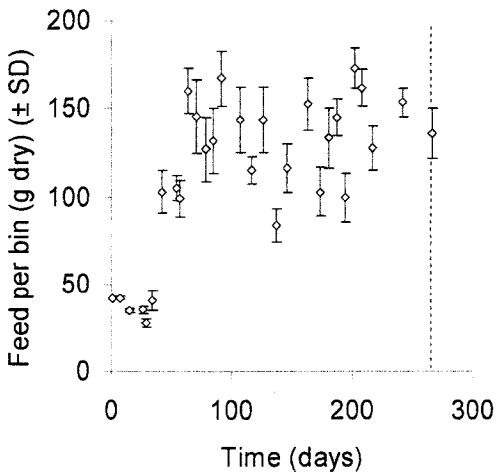


Figure 4. (a) Average composition of the worm feed based on wet weight (as given to the worms). Values in parenthesis represent the relative percentage of each category based on dry weight (as used in mass balance calculations). (b) Average worm feed per bin (n = 24) at each feeding event.

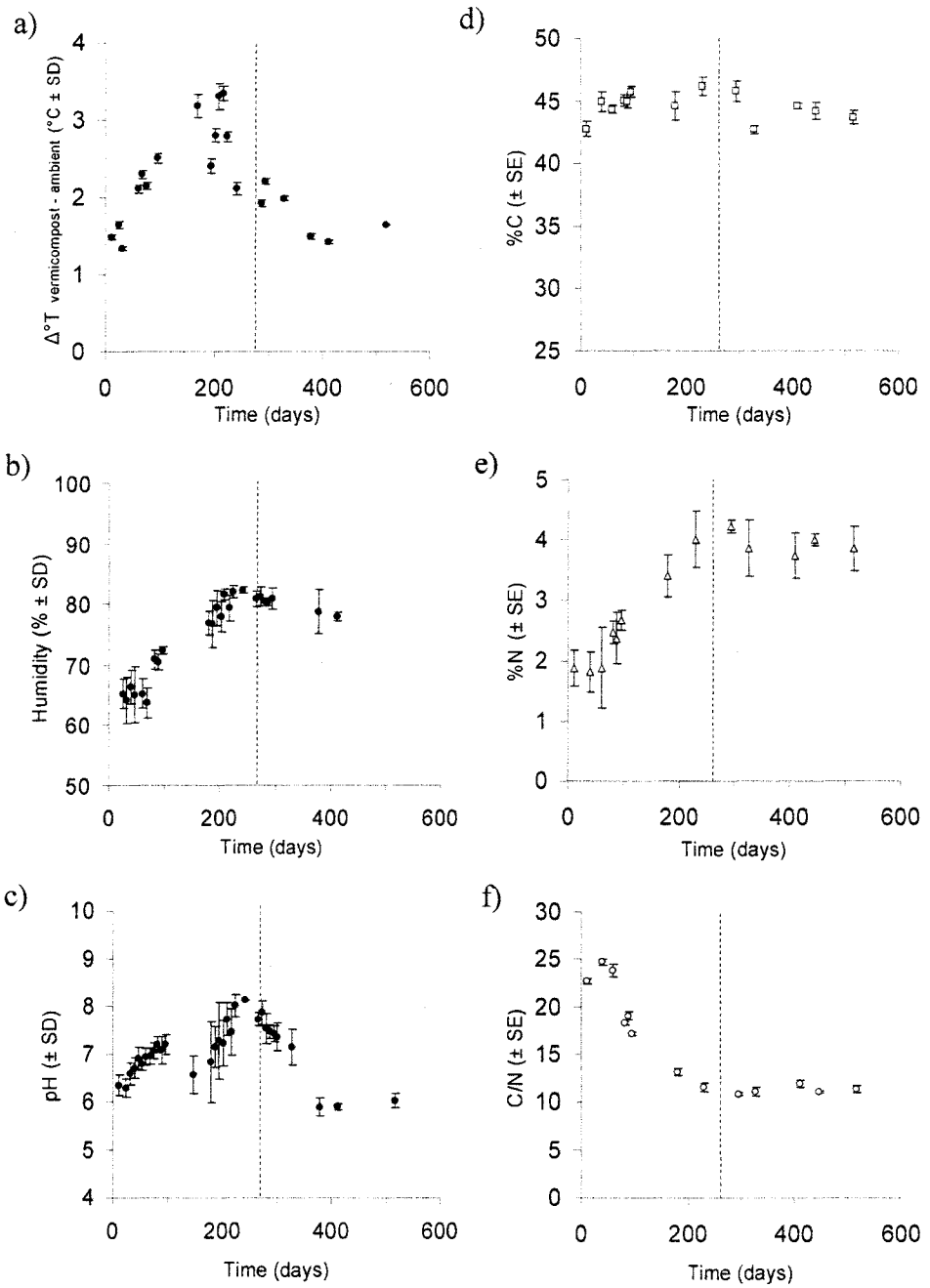
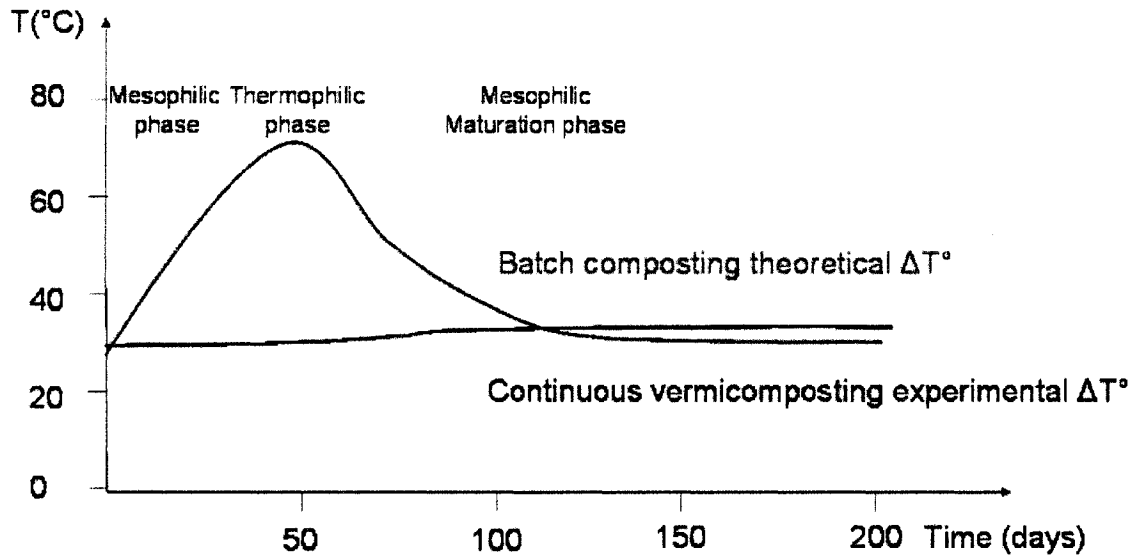


Figure 5. Variations in physico-chemical parameters during continuous vermicomposting: a) Vermicompost temperature compared to ambient room temperature ($d < 100$, $n = 24$ and $d > 100$, $n = 4$); b) vermicompost humidity ($n = 18$); c) vermicompost pH; d) concentration of (a) organic carbon, (b) total nitrogen as well as in (c) the C/N ratio. Feeding was interrupted on day 266 (dashed line).

a)



b)

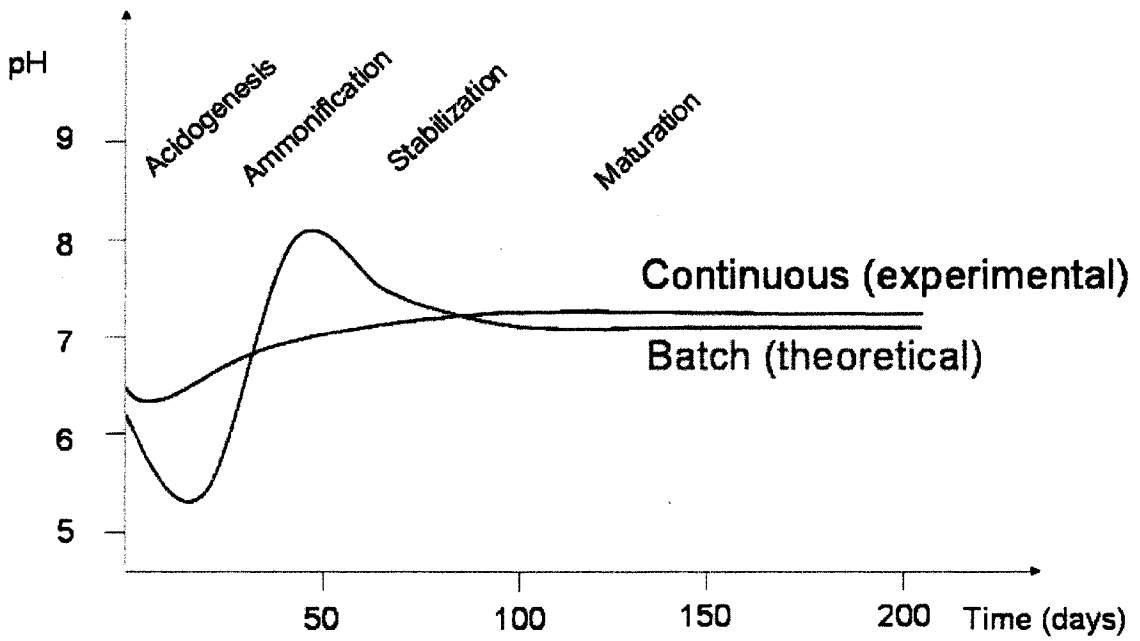


Figure 6. Comparison of the temperature (a) and pH (b) in traditional batch composting (adapted from (3)) and continuous vermicomposting (data from the present experiment). See text for explanations.

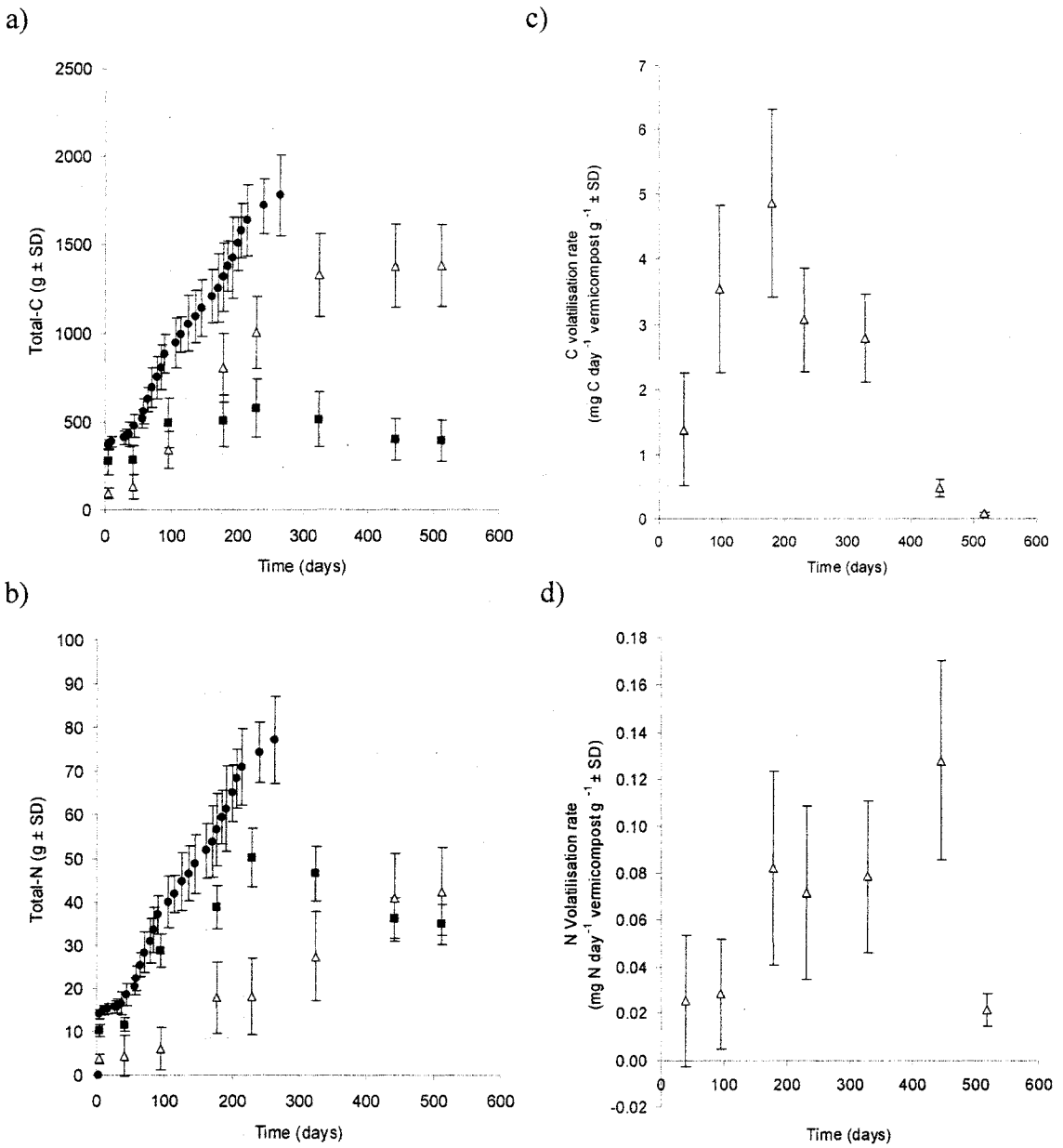


Figure 7. Variations in the average organic C (a) and total N (b) contents of the vermicompost bins. The cumulative nutrient inputs (black circles) include the feed and the initial litter. The total nutrient mass accounted for in the vermicompost (black squares) is based on total compost weight and measured nutrient concentration. The difference between the total inputs and nutrients present is assumed to have escaped from the system as gaz (empty triangles) and is represented as an estimated daily volatilization rate (calculated as an average between that point and the previous data point normalized to total vermicompost dry mass) in (c) and (d) for total N and organic C respectively. Feeding was interrupted on day 266 (dashed line).

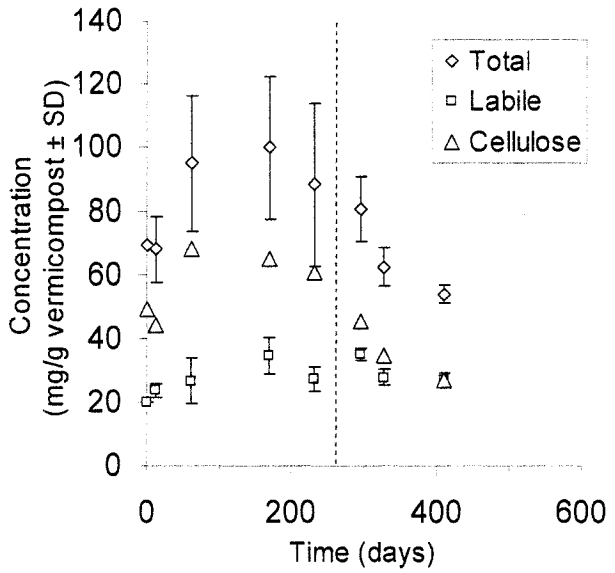


Figure 8. Variations in the concentrations of total and labile carbohydrates during the continuous vermicomposting experiment. Feeding was halted on day 266. The propagated standard deviation for cellulose is roughly equal to the sum of standard deviation of the labile and total sugars and is not shown for clarity. Feeding was interrupted on day 266 (dashed line).

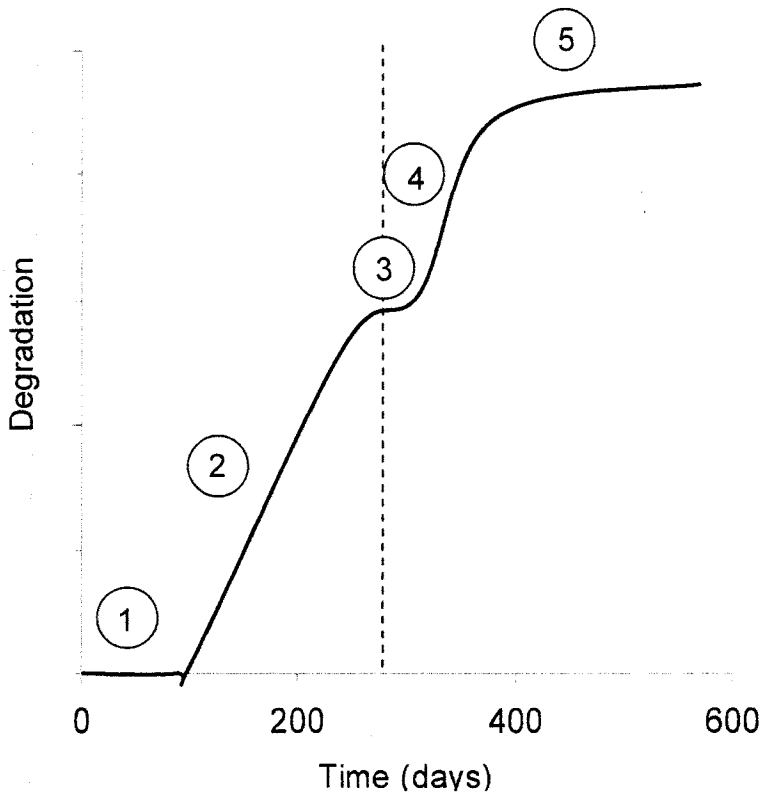


Figure 9. Schematic representation of the degradation in vermicomposting. The period before the dashed line represents continuous feeding and after feeding is interrupted. Degradation is qualitative from fresh organic matter at the bottom to stabilized materials at the top. Segment 1 represents a lag phase, where much of the organic matter accumulates. Segment 2 represents the initiation of degradation with the establishment of a process decomposer community. Segment 3 represents a break on the physico-chemical trends observed after the end of feeding. Segment 4 represents the finalization of degradation followed by maturation in segment 5.

Chapter 3 - Influence of the earthworm *Eisenia fetida* and the indigenous microbial community on the persistence of *Escherichia coli* in batch and continuous vermicomposting systems

3.1 Abstract

Vermicomposting is a biooxidation process where epigeic earthworms act in synergy with microbial populations to stabilize organic matter. Vermicomposting does not comprise a thermophilic stage as required by North American legislations for pathogen eradication. The survival of a Green Fluorescent Protein (GFP) labeled *E. coli* MG1655 in both small-scale batch and large-scale continuously operated systems was studied to discern the influence of the earthworm *Eisenia fetida* and the indigenous vermicompost microbial community. In batch systems, the microbial community had the greatest influence in the rapid decline of *E. coli* populations and the effect of earthworms was only visible in microbially reduced vermicomposts. No significant worm density-dependent relationship was observed on *E. coli* survival under continuous operation. *E. coli* numbers decreased below the US EPA compost sanitation guidelines of 10^3 CFU/g (dry weight) within 16-25 days for both small-scale batch systems and large-scale continuous system, but it took up to 64 days without worms and with a reduced microbial community to reach the legal limit. Here we showed that vermicompost hosts pool of opportunistic human pathogens, but that antagonists were also present.

3.2 Introduction

With increasing production and consumption as well as public awareness of sustainable development and global environmental issues, societies face the need to manage waste using sustainable practices. Separation of organic waste for composting is both a sustainable waste management practice and a valuable production of organic soil conditioner and fertilizer. Some organic materials such as sewage sludge and manure can contain high concentrations of pathogenic bacteria, fungi, viruses and parasites. Non-composted or improperly composted manure used on the farm may lead to food contamination which can pose a health hazard (159,227). Outbreaks of pathogenic *E. coli* O157:H7 were associated with food fertilized with cattle manure on the farm (160). Much of the research on pathogen reduction in composting was concerned with manure or sewage sludge (228), but compost from source separated organic waste (i.e. food scraps) was also shown to harbour pathogens (229) and should be the focus of further research. Human pathogens in composting must be eradicated to safely reap the benefits of compost in agricultural practices.

Vermicomposting is a mesophilic composting method that relies on epigeic earthworms to stabilize the organic waste (4,18-21). Several studies have revealed the benefits of vermicompost fertilization over thermophilic compost and chemical fertilization in terms of improved plant growth and yield (43,47,48). Vermicomposting is not yet recognized as a Class A stabilization method for organic waste, a recognition that imposes no restriction concerning pathogens tests prior to land application and sale in bags (26,195,230). Class A US EPA pathogen requirements, corresponding to unlimited use of the compost, are < 1000 Most Probable Number (MPN)/g dry solid for fecal

coliforms and < 3 MPN/g dry solid for *Salmonella*. Though we know that earthworms promote the movement of faecal indicator organisms and pathogens in soils (231,232) and vermicompost (195), few studies have specifically addressed pathogen reduction during vermicomposting. So far, the decline of bacterial pathogens during vermicomposting has only been reported for coliforms and *Salmonella* (25,233) and Williams *et al.* (195) reported that earthworms may aid in the proliferation of *E. coli* O157:H7 in the short-term but not in the long-term.

Several mechanisms for inactivation of pathogens in compost and a few more specific to vermicompost have been proposed (180,234). They include (1) inhospitable physico-chemical environment, (2) metabolic disadvantages during competition with indigenous microbes, (3) inhibition by antibiotics indigenous microbes or by (4) volatile organic compounds emitted during the composting process, (5) presence of antibiotics in the secretions of worms, (6) preferential grazing and (7) selective digestion by earthworms.

The high temperature reached during thermophilic composting is considered essential (22) or the most important factor (174,235) in pathogen eradication, but vermicomposting is strictly mesophilic. To obtain class A certification by the US EPA under the pathogen and vector attraction reduction rule (40 CFR Part 503), allowing unrestricted use and sale, the composting process must either meet thermal requirements (4 days at 40°C plus 3 days at 55°C) or be listed as a recognized Process to Significantly Reduce Pathogens (PSRP) or a Process to Further Reduce Pathogens (PFRP) (165). Otherwise, systematic bacteriological tests of the product are required. Some researchers suggested that sequential composting followed by vermicomposting would meet the

thermal criterion and still provide the benefits of vermicomposting (faster curing and more homogenous product) (22,67), but others suggested that vermicomposting alone should be recognized as a PFRP (25). Even though this issue was raised several years ago (165), and has led to some interesting research, the vermicomposting process is not yet recognized as a PFRP and much work remains to be done to ensure that pathogens cannot survive the vermicomposting process. Ensuring that proper temperature is reached is a simple way to check sanitation, as most microbes are killed above 60-65°C (22,29), but the mechanisms involved in vermicomposting are not yet fully understood and there is no easy indicator that sanitization is occurring.

Ryckboer *et al.* (115) reported that pathogen inactivation is usually more efficient when higher temperatures are reached, but suppression of *Salmonella* spp. may be more efficient at 55°C than at 70°C, suggesting that antagonistic effects of other microorganisms, probably actinomycetes (mesophilic organisms), may be more important than high temperatures alone (115). Competition with indigenous microorganisms which can cause a reduction of pathogens during composting (115) can be brought about in two ways: preemption of resources and direct competition. Indigenous microflora can pre-empt space and resources (carbon, nitrogen and minerals) such that pathogens have difficultly to establish themselves (172) or alternately, pathogenic microorganisms may be less efficient competitors for nutrients present in worms castings (25,29). Although several studies on microbial competition exist and despite the importance of such mechanisms in various ecosystems, no studies specifically related to vermicomposting were found in the literature.

Antagonists are naturally occurring organisms with traits enabling them to interfere with pathogen growth, survival or infection (236). Antagonism plays a role in compost sanitation (219,235,237) and suppression of plant diseases with the use of compost (170,238,239) and microbially-enriched compost (240). Antibiotics-producing bacteria have previously been isolated from compost (241). *Streptomycete* strains have been isolated from the guts of earthworm used in vermicomposting (122). Pathogen supressiveness effect of vermicompost is thought to be of biological origin (242), but we could not find a published a study revealing the identity of antagonists in vermicompost.

An alternate antagonistic mechanism is the production of lethal volatile substances. These may include phenolics (243,244) and volatile fatty acids (245,246), which are perhaps more abundant in younger composts (172). This contact-independent mechanism is further discussed in Appendix 2.

Even the earthworms themselves are known to synthesize and secrete various immunoprotective proteins and fluids with antimicrobial properties that can mediate lytic reactions against a variety of microorganisms (79,82,99,187,188,247,248). Antimicrobial factors can be released along with digestive enzymes from the pharyngeal glands, crop, gizzard and anterior intestine (147) thus affecting the microorganisms in the vermicompost in the presence of earthworms. A species of earthworm common to vermicomposting systems (*Eisenia fetida*) was shown to produce compounds that had greater proteolytic and hemolytic activity than the species *Lumbricus rubellus*, a soil dwelling species, reflecting the antigenicity of their normal biotope (194).

Earthworms affect soil microbes directly or indirectly by (1) comminution (fractionating), burrowing and casting, (2) grazing, and (3) dispersal (1,92,249).

Selective grazing and digestion may also play a role in controlling the survival of pathogens in vermicompost. The fate of bacteria during gut transit is variable (1,141,142) depending on the bacterial species (99), their total numbers (1), their metabolic state (active or spores) (1,250), differences in counting procedures (1), earthworms species (1), soil and food used (1). Nevertheless, there is evidence that earthworm digest bacteria (143), perhaps more than the organic waste itself (145). *E. coli* O157:H7 can survive passage through the gut of certain species of earthworms (195) even though *E. coli* numbers are generally reduced upon passage through the pharynx and/or crop (99).

The general goal of the present study is to understand to what extent pathogenic bacteria are regulated during vermicomposting. More specifically, the objective is to assess the effect of earthworms and indigenous microbes on the survival of *E. coli* during batch and continuous vermicomposting, based on the hypothesis that both must have a significant impact in limiting *E. coli* survival. We also screened normally operated continuous vermicomposters for various bacterial pathogens and antagonists using culture based methods coupled to 16S rDNA sequencing for identification.

3.3 Methods

3.3.1 Preparation of the *E. coli* inoculum

Commensal *E. coli* strains are considered good models for the survival of pathogenic *E. coli* O157 strains in soil studies (251). The strain MG1655 (ATCC 70092) was chosen because it is a non-pathogenic wild-type strain whose handling and containment requirements are less intensive than for the pathogenic strains. To facilitate enumeration in the microbial rich community, *E. coli* MG1655 was transformed with the

plasmid pGFPuv (GenBank Accession #U62636, Clontech Laboratories, Inc.), allowing synthesis of the green fluorescent protein (GFP) and conferring resistance to the antibiotic ampicillin. Briefly, competent bacterial cells were electroporated, according to the protocol of Sambrook and Russell (252), in a Gene Pulser II (Bio-Rad) in the presence of the plasmid pGFPuv in 0.2-cm cuvettes with an electrical pulse of 4-5 msec at 2.5 kV with 200Ω and 25 μF. Transformants were isolated on Luria-Bertani agar (Difco ^{TM} LB agar, Miller; BD®, USA) containing 100 μg/mL of ampicillin (ampicillin sodium salt, EMD, Germany). A colony that was glowing green under UV light (365 nm) was selected, grown in liquid LB-ampicillin broth and frozen in 15 % glycerol as a stock used for all experiments. For each independent experiment, *E. coli* cells from frozen stock cultures were grown overnight at 37°C on LB agar supplemented with ampicillin (100 μg/mL). Cells from a green-glowing colony were transferred to 10 mL of liquid LB-ampicillin medium incubated at 37°C and 250 rpm agitation overnight. The necessary volume of liquid media to achieve initial inoculation densities of 10^7-10^8 cells per gram of vermicompost was inoculated with 1 % (v/v) of the overnight liquid suspension and incubated again overnight. The cells were harvested by centrifugation (1000 g at 4°C for 10 minutes) and suspended in 10 % volume of sterile phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4) at pH 7.4 prior to inoculation in the vermicompost. The number of cells of *E. coli* MG1655 was determined by plating dilutions in triplicate on LB-ampicillin agar and counting green colonies using a UV-transilluminator.

3.3.2 Small-scale batch vermicomposting experiments

Two small-scale batch experiments were conducted in triplicate with similar set-ups and sampling. First, a small-scale batch experiment was conducted to determine if worms and the microbial community influence the survival of *E. coli* in a vermicomposting system. The first experiment aimed at better quantifying the effects of the indigenous microbial community and the earthworms on the survival of *E. coli*. The four different treatments were: (1) No worms and microbial-poor, (2) no worms and microbial-rich, (3) worms and microbial-poor and finally (4) worms and microbial-rich communities. The second small-scale batch experiment aimed at better understanding the effect of the microbial community present in the feed or in the vermicompost in reducing the numbers of *E. coli* in the absence of earthworms. The four different treatments were: (1) microbial-poor vermicompost and feed, (2) microbial-poor vermicompost and microbial-rich feed, (3) microbial-rich vermicompost and feed and (4) microbial-rich vermicompost and microbial-poor feed.

The batch experiments were done in one-litre glass jars containing 500 g of vermicompost (humidity = 75.6 ± 1.88 %, pH = 7.31 ± 0.16, organic carbon content based on dry weight = 28.83 ± 1.63 wt %; for all n = 3) with 115 g wet food/jar (pH 5.78 ± 0.46; n = 3). Microbially reduced communities were established by autoclaving the vermicompost at 121°C for 20 minutes. The worms and the vermicompost were obtained from the bins described in Chapter 2. The worms and cocoons were manually removed from the vermicompost and the worms were cleaned with distilled water and allowed to crawl on moist paper towels overnight to purge their gut content. Then, the worms were cleaned again and the 10 largest and most vigorous worms (total wet weight of 3.24 ±

0.42 g) were reintroduced in half of the jars of the first experiment. Twenty mL of PBS bacterial suspension (10^8 cells/mL) were mixed in with the compost. The jars were incubated in the dark at 25.5 ± 1.5°C for up to 81 days. For sampling, the vermicompost was mixed with a sterile metal rod (20 strokes per jar) prior to collecting 4 samples of 1 g from each jar. For microbial counts, samples were suspended in 40 mL of 0.85 % NaCl in 50 mL sterile Falcon tubes, vortexed at maximal intensity 60 sec, followed by 30 sec of settling prior to serial dilution and plating in triplicate on LB agar containing 100 μg/mL ampicillin. The plates were incubated overnight at 37°C for heterotrophic counts (on LB-ampicillin) and GFP labeled *E. coli* MG1655 counts. Humidity content was determined based on weight loss of 10 g samples after freeze-drying (at T° = -55°C and P = 500mbar). The pH was determined on the initial vermicompost suspension, after sampling for bacterial counts, using a VWR Symphony probe.

3.3.3 Large-scale continuous domestic vermicomposting experiment

The large-scale continuous vermicomposting experiment was used to evaluate if the regular addition of food, similar to domestic vermicomposting, would give similar results as those observed in the batch experiments. This experiment also aimed at establishing a quantitative relationship between the number of earthworms and the survival of *E. coli*. Three different treatments were tested in triplicate in large-scale continuous experiment: (1) No worms, (2) 35 g of worms and (3) 70 g of worms. The middle treatment represents the same initial worm density as in the small-scale batch experiment. The vermicompost and worms were collected and prepared as in the small scale experiments described in Section 2.3. The bins were filled with 5 kg of vermicompost at 80 % humidity and inoculated with 120 mL of *E. coli* suspension (at

$1.28 \times 10^9 \pm 1.53 \times 10^8$ CFU/mL (average ± SD; n = 3) giving an initial *E. coli* load of $1.50 \times 10^8 \pm 3.85 \times 10^6$ CFU/g (dry weight) (average ± SD; n = 9). In this experiment, 245 g of wet food was added weekly to the vermicomposting units. The feed preparation was similar to that presented in Chapter 2. For the 35 g worm treatment, the feeding rate (1 g food/g worm/day) was slightly above the optimal feeding rate (0.75 kg feed/kg worm/day) described by Ndegwa and Thompson (22) but slightly below in the case of the 70 g worm treatment (0.5 g feed/g worm/day). The sampling for microbial counts and physical-chemical analysis was similar to that described in the small-scale batch experiment (a 40 g composite sample taken from the total 5 kg vermicompost representing 0.8 % of total compost mass). The detection limit of the direct plating method was 500 CFU/g. When *E. coli* MG1655 was not detected on the undiluted suspension, an enrichment culture method was used to qualitatively evaluate its presence or absence. Ten mL of the initial suspension was added to 100 mL LB-ampicillin, incubated 24 hrs at 37°C with agitation at 250 rpm. Dilutions of the cultures were spread on LB-ampicillin plates and analyzed for the presence of green fluorescent colonies after 24 hrs incubation at 37°C.

3.3.4 Physico-chemical parameters of the vermicompost

Physico-chemical parameters of the vermicompost used in the batch and continuous vermicomposting experiments included % humidity, pH, organic carbon (OC), total nitrogen (N), C/N ratio, total and labile sugars as well as cellulose. The analytical methods are described in Chapter 2.

3.3.5 Bacterial community of the vermicompost

The bacterial community of the continuous vermicompost was surveyed as described in Chapter 2. Bacterial colonies distinguished by morphotyping were isolated on culture media that were specifically selected to allow the detection of potential human bacterial pathogens and the selectivity of each media is summarized in Table 5. In addition, the genus of selected pathogens that were surveyed and their potentially ill health effects are also summarized in Table 1. Amplification and sequencing of the 16S rDNA gene was favoured over classical identification methods, such as carbon-source utilization, because we expected the isolation of environmental samples that may have been misidentified using metabolic fingerprints (253). The bacteria isolated from the vermicompost were checked against the public databases of the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de) to determine their risk group.

3.3.6 Statistical analysis

The microbiological data was expressed as CFU/gram of dry substrate in order to compare it to the legislation requirements. All data was log + 1 transformed to meet normality criterion of statistical evaluation. An analysis of variance (ANOVA) for a completely randomized design with repeated measures across dates was conducted to determine if there was any general difference in the survival of *E. coli* between the treatment means. Specific comparisons between any pair of treatment means at any date were accomplished with the Fisher's least-significant difference (LSD) test. All calculations were performed using the SPSS for Windows, version 15.0 (SPSS Inc., Chicago, Illinois, USA).

3.4 Results

3.4.1 Small-scale batch experiments: The influence of earthworms and the indigenous vermicompost microbiota on *E. coli* survival

Small-scale batch experiments demonstrated that both the earthworms and the indigenous microbial populations reduced *E. coli* survival (Figure 10). The number of *E. coli* CFU per gram dry weight varied significantly with time ($F_{1,8}$ = 244.997, p = 0.000). The microbiota of the feed significantly reduced the survival of *E. coli* ($F_{1,8}$ = 119.426, p = 0.000) and so did the earthworms ($F_{1,8}$ = 24.921, p = 0.001). There was an interaction between time and microbial abundance of vermicompost ($F_{1,8}$ = 8.353, p = 0.020) and between time and the presence of earthworms ($F_{1,8}$ = 5.546, p = 0.046). Treatment with sterile vermicompost and no worms showed the highest counts of *E. coli* at all time points (statistically significant difference with $p < 0.05$ on days 9, 16, 36 and 49), establishing that either or both the indigenous microbial population or the worms influenced the survival of *E. coli*. The initial inoculation density was about 10^8 CFU/gram dry substrate and it increased to nearly 10^9 CFU/gram dry substrate on day 1 in the sterile vermicompost but remained significantly lower in the microbial-active vermicompost ($p < 0.05$). As early as day 1, treatments with an active microbial population had lower *E. coli* counts and this effect lasted until day 64 (the difference between Fresh/No Worm and Sterile/Worms is statistically significant ($p < 0.05$) on days 1, 4 and 9 and the difference between Fresh/Worms and Sterile/Worms is statistically significant ($p < 0.05$) on days 1, 4, 9, 16 and 25). The impact of earthworms was noticeable in the sterile treatments, as observed by a decreased survival of *E. coli* in the presence of earthworms (the difference between Sterile/Worms and Sterile/No Worm is statistically significant ($p < 0.05$) on days

9, 16, 36 and 64). In contrast, with the microbial-rich treatments, the presence of earthworms only correlated with a significant decrease in *E. coli* numbers on days 25 and 36 compared to the treatment with no worms present ($p < 0.05$). It took 16-25 days, 25-36 days and 49-64 days for *E. coli* counts to decrease below the legal criteria of 10^3 CFU/g substrate (dry weight) in the treatment with worms and fresh compost, no worms and fresh compost and sterile compost with or without worms, respectively (Table 6). Control experiments established that none of the starting substrate contained green fluorescent microbes and also confirmed that no contamination between jars was occurring during the experiment (comparing jars inoculated *vs.* jars not inoculated with *E. coli* sampled at the same frequency).

The humidity (average = 75.6 ± 1.9 %; n = 72) and pH (7.37 ± 0.27; n = 72) of the vermicompost was stable throughout the experiment and across treatments. On day 25, the difference in *E. coli* abundance was greatest between each treatment. On that same day, the carbohydrates concentration also varied between treatments (Figure 11). The concentration of total carbohydrates was highest in the microbial-poor/no worm treatment, followed by the microbial-poor/worm treatment, and finally, they did not differ between the microbial-rich treatments with the worms present or absent. The concentration of labile carbohydrates was greatest in the microbial-poor treatment with no worms compared to all other treatments, which did not differ from each other. On the other hand, the organic carbon, total nitrogen and C/N ratio of the different treatments did not vary through time between the different treatments (Figure 12).

3.4.2 Small-scale batch experiments: The influence of the indigenous vermicompost and feed microbiota on *E. coli* survival

The second small-scale batch experiment testing the difference between sterile and non-sterile feed of vermicompost on the survival of *E. coli* in the absence of worms showed a similar trends as that observed in the first experiment (Figure 13), that is, the indigenous microbiota influences the survival of *E. coli*. The abundance of *E. coli* significantly declined with time ($F_{3,6}$ = 65066121, p=0.000), and the microbiota of the vermicompost significantly reduced E. coli ($F_{1,8}$ = 19.387, p = 0.002) and the micriobiota of the feed did so, but less significantly ($F_{1,8}$ = 8.285, p = 0.021). There was a significant interaction between time and the microbiota of the compost ($F_{3,6}$ = 16.431, p = 0.003) and in a minor proportion between time and the microbiota of the feed ($F_{3,6}$ = 5.879, p = 0.032). After one day, the treatments where both the food and the vermicompost were sterile contained a higher abundance of *E. coli* than all the other treatments. After a week, the sterile food/sterile compost combination allowed greater survival of *E. coli* than the fresh food/sterile compost combination. In both sterile compost treatments, *E. coli* remained abundant longer than in both treatments where fresh compost was used, but there was no difference between treatments containing sterile or fresh food when microbial-rich vermicompost was used. On day 35, *E. coli* was below the detection threshold of all the treatments.

3.4.3 Large-scale continuous experiment

In the light of the results from the small scale experiments, we set out to test the effect of continuous feed addition on the survival of *E. coli*. Overall, there is a significant decline of *E. coli* over time ($F_{1,5}$ = 421,220, p = 0.000), but the abundance of earthworms

had no significant effect of *E. coli* survival ($F_{2,5}$ = 1.853, p = 0.250) and there was no interaction between abundance of worms and time on the survival of *E. coli* ($F_{2,5}$ = 1.363, p = 0.337). In the large-scale continuous experiment, *E. coli* was inoculated at an initial density of $1.50 \times 10^8 \pm 3.85 \times 10^6$ (average ± SD) CFU/g substrate dry weight (Figure 14). On day 2, *E. coli* had decreased to 10^7 CFU/g dry weight in all treatments and then continued to decrease with time. There was no difference between the abundance of *E. coli* with either 35 g or 70 g of worms in the system on any day, but the number of *E. coli* surviving in the treatment without worms was slightly (but not significantly) greater on days 9 and 16. The average number of *E. coli* remaining decreased below the legal threshold of 10^3 CFU/g dry weight after 16-25 days in all treatments.

The percent humidity remained constant throughout the experiment at 79.7 ± 1.8 % (average ± SD; n = 54). Because the pH of the substrate did not differ significantly between treatments on any day in the small-scale batch experiment, the pH of the large-scale continuous experiment was only tested twice during the experimental period. The pH did not vary between treatments, but varied with time (8.17 ± 0.04 on day 36 and 7.81 ± 0.13 on day 49; average ± SD; for each n = 9). Organic carbon and total nitrogen did not differ between treatments of the continuous experiment, and together with the C/N ratios, these parameters exhibited a greater variability in the continuous experiment than in the batch experiment (Figure 12).

3.4.4 Comparison of batch *vs.* continuous experiments

The percent survival of *E. coli* in vermicomposters operated under batch or continuous modes were compared to see if the feeding frequency has an effect on *E. coli*

survival (Figure 15). The percent survival significantly reduced with time ($F_{1,7}$ = 193.740, p = 0.000) and the presence of earthworms ($F_{1,7}$ = 12.103, p = 0.010). Contrary to our expectations, *E. coli* survival was not affected by operational mode (batch *vs.* continuous; $F_{1,7}$ = 1.178, p = 0.314). There was no interaction between the operational mode and time ($F_{1,7}$ = 0.955, p = 0.361) or worm with time ($F_{1,7}$ = 1.548, p = 0.254).

3.4.5 Opportunistic pathogens and antagonists

Seven genera and eight species (28 % of all species identified; 25 % of isolates) isolated from vermicompost, are bacteria known to have opportunistic pathogenic potential, i.e. risk group 2 (Table 7). Antagonistic bacteria were also isolated from the vermicompost. Eight genera and four species (14 % of all species identified; 50 % of isolates) could be involved in antagonistic interactions in the vermicompost ecosystem (Table 8).

3.5 Discussion

3.5.1 Effect of indigenous microbes on *E. coli* survival

The indigenous microbial community was the most important factor in determining *E. coli* survival. In both small-scale batch experiments, the number of *E. coli* CFU was lower in fresh compost or feed harbouring a microbial rich community. The lethal effect of fresh manure and soil microbiota (compared to that of autoclaved substrate) on the survival of *E. coli* O157:H7 has been reported previously (160). Also, soil amended with fresh compost *vs.* those amended with autoclaved compost corresponds to shorter *E. coli* O157:H7 survival (160). It was also shown that the

antagonistic effects of indigenous microorganisms peak in young compost and gradually decline with the maturity of the compost due to a decrease in nutrient availability that leads to a decrease in microbial diversity and abundance (219). Mature thermophilic compost, which harbours lower abundances of indigenous microbes, or sterilized compost, was shown to allow regrowth of pathogens such as *Salmonella* spp. when proper humidity and temperature conditions were restored (219). These reports, along with those presented here clearly suggest that antagonistic interactions with the indigenous soil, manure, compost or vermicompost microorganisms reduce the survival of *E. coli* and other pathogens. Indeed, continuous vermicomposting could be better at sanitation than batch composting because of the maintenance of an abundant and diverse microbial community through regular food. In addition, thermophilic composting can allow the survival of pathogens (229) (heat resistant mutants or those present in cooler parts of the pile) and the thermophilic phase drastically reduces the diversity of indigenous microbes which could have prevented the regrowth of pathogens during maturation or storage (198). Thus, while vermicomposting may not achieve the rapid pathogen removal rates observed with heat (inactivation of *E. coli* O157:H7 or O103:H2 and *Salmonella enteriditis* and *S. typhimurium* within 30 minutes at 55°C (174)), vermicomposting could be classified as a proper sanitation technique because of its abundant and diverse mesophilic community. More work needs to be done to confirm this hypothesis.

The effect of the vermicompost microbiota on *E. coli* survival appears more important than the presence of earthworm or the addition of microbial rich feed. In the first small-scale batch experiment, earthworms enhanced *E. coli* decrease in abundance in

the presence of a reduced microbial community but they did not cause further *E. coli* decrease in abundance in the presence of a rich microbial community. Similarly, the second small-scale batch experiment, suggests that some of the antagonist microorganisms are introduced in the vermicompost via the feed input, but also that the vermicompost indigenous microbial community may exert a greater influence on *E. coli* survival than the feed microbiota. The effect of the feed associated microbes was visible with sterile vermicompost, but not with fresh vermicompost, and this could have been due to concentration effects, i.e. there was a greater mass of vermicompost than food.

3.5.2 Effect of earthworms on *E. coli* survival

The effect of earthworms on decreasing *E. coli* survival was shown to be of secondary importance compared to the effect of the indigenous microbial community. Earthworms rapidly induce a decrease in *E. coli* abundance in microbial-poor vermicompost, but the effect is not observed as early and with the same magnitude when microbial-rich vermicompost is used. On the other hand, despite the observation that earthworms negatively influenced *E. coli* survival in the batch experiment, we did not observe a worm density-dependent relationship in the continuous experiment, i.e. the survival of *E. coli* did not decrease with increasing worm density. Our results contradict those of Williams *et al.* (195) who observed that earthworms (*Lumbricus terrestris* and *Dendrobaena venata*) extended the survival of *E. coli* O157:H7 in soil and compost due to earthworm predation on protozoans that feed on *E. coli* (250,254), or perhaps to earthworm predation on antagonistic microorganisms in the substrate (the difference fades out in the long-term). Overall, the results from this study reinforce the idea that earthworms contribute to shorter survival of coliforms (25) but this was only clearly

visible in small-scale batch treatments with sterile substrate. As opposed to Eastman *et al.* (25) who had observed an important difference between a control sewage sludge row and similar row inoculated with *E. fetida* (both with normal microbial rich communities) as early as day one, we only observed the effect of earthworms in fresh vermicompost later in time. A possible explanation to the apparent discrepancy is that the vermicompost we used in the experiment already had a rich established microorganisms community (previously influenced by the presence of earthworms), while the biosolids Eastman *et al.* (25) used had a different initial community that the worms modified, via microbes inoculation or other indirect effect, upon introduction into the substrate.

3.5.3 Nutrient effects

In batch systems, the organic matter is degraded with time whereas in a continuous system, fresh organic matter is constantly added in the system. This difference is interesting to investigate further because many studies suggest that nutrients have an effect in pathogen survival (although they do not all agree concerning the outcome). In addition, batch systems are commonly used in laboratory experiments, while systems are often operated continuously in real life and it is critical to understand if batch system correctly model continuous systems.

Gagliardi *et al.* (179) observed that addition of manure to undisturbed soil cores enhanced the survival of *E. coli* O157:H7 probably because of the formation of favourable microhabitats and the addition of organic nitrogen. They observed a significant positive correlation between survival rate of total coliforms as well as *E. coli* O157:H7 strain B6914 and concentrations of NO_3^- and NH_4^+. This led to the hypothesis

that with a continuous operational mode, pathogenic bacteria may be able to survive longer than under a batch mode due to changing nutrient availability in the latter. The availability of nutrients was expected to lead to longer *E. coli* survival in the continuous system versus the batch system. Surprisingly, this effect was not observed, i.e. the survival of *E. coli* was not different between batch and continuous operational modes (Figure 15).

The opposite effect is that nutrient may be most important in maintaining the indigenous community healthy as opposed to keeping *E. coli* alive. Indeed, other researchers have underlined that nutrients may stimulate indigenous microbes which could lead to greater antagonistic activity (219). More specifically the biological activity of antagonictic actinomycetes is enhanced by the addition of cellulose to the substrate (184), clearly indicating the necessity of providing a nutrient source for the antagonists. The organic carbon content was higher in the continuous vermicomposting experiment than in the batch experiment, but this did not correlate with differential survival in *E. coli* in the present experiment.

Scheu and Schaefer (145) have suggested that direct competition for labile carbon sources between earthworms and soil microorganisms could result in lower *E. coli* survival but the opposite effect was observed here as the treatments with the lower abundance in sugars were those where *E. coli* survived longer. This reinforces the hypothesis that starved antagonists may allow for survival of pathogens on longer time scales, and also agrees with the suggestion pathogens may be less efficient competitors for labile carbon sources than the indigenous microbial flora (25,29,219). We also showed that in batch vermicomposting, the microbial community has a greater

importance on reducing total and labile sugars concentrations than the earthworms. This contradicts Williams *et al.* (195) who observed a higher glucose metabolization rates in the presence of earthworms. In our experiment, the influence of earthworms on sugar concentrations was only visible in the sterile substrate, and this effect may have been due to inoculation of microbes upon introduction of earthworms to the sterilized vermicompost.

Coliforms survive longer or reproduce faster when nitrogen is more abundant (179). Earthworms are also known to increase N fixation in vermicompost (255). Thus, increased N abundance could have compensated for increased earthworm grazing when more earthworms were present in vermicompost, explaining the lack of difference in *E. coli* counts with a low or high abundance of earthworms. The total nitrogen concentrations did not correlate with earthworm abundances in either of the continuous or batch experiment treatments, as opposed to results from Ozawa *et al.* (255), but *Eisenia fetida* is perhaps not the worm species that favours the N fixation the most (256). *E. fetida* was shown to enhance nitrogen fixation by 0.3 μg N g worm^{-1} day^{-1} (256). Thus, at the end of 64 days of continuous vermicomposting, 70 g of worms would have stimulated the fixation of N and increased the vermicompost total N by approximately 0.08 % (w/w) (considering continuous feeding but neglecting earthworm growth rate), and this difference surely went undetected with the present methodology. Finally, total N concentration, as opposed to organic C and/or sugar concentrations, may not have played an obvious role in *E. coli* survival in both vermicomposting experiments, or the influence of N could be linked to the available form present (NO_3^-, NH_4^+, etc.) instead of total concentration of N.

E. coli survival sometimes varies in compost and soils that have different physico-chemical properties (257). For example, *E. coli* O157:H7 survives longer under lower pH than under higher pH (160,257), but physico-chemical properties are not always determinant (228) and they may only play a secondary role (258). Here, we observed no pH or humidity differences between the treatments that could have determined the fate of *E. coli*, but we observed a nutrient effect that could have modulated the predominant role of the indigenous microbial community.

3.5.4 Opportunistic pathogens and antagonists

Opportunistic pathogens generally do not represent a severe threat to most people, but in the last two decades, the impact of opportunistic infections on human health has increased dramatically (259). Risk group 2 bacteria, such as *Pseudomonas aeruginosa*, are commonly considered facultative or opportunistic human pathogens, only causing diseases in patients with a strong predisposition to illness, such as those who are severely debilitated, immunocompromised or suffering from cystic fibrosis or HIV-infections (259,260). Cases of nosocomial infections, caused by opportunists and sometimes leading to death, are reported worldwide stimulating research to identify potential environmental pools (259). Here, we have shown that vermicompost does harbour opportunistic pathogens. Measures to minimize contamination threat by opportunistic pathogens living in the compost include basic sanitary practices, such as washing hands after feeding the earthworms, and should include extra cautionary measures for immunocompromised people, such as wearing a mask when harvesting the compost. Beyond these recommendations, one of the worries concerning environmental opportunistic pathogens found in highly competitive environments, such as

vermicompost, is that the microbes often exhibit resistance to multiple antibiotics (this characteristic is essential to their survival ability in nature (261)). Thus, with the growing public concern about antibiotic resistance and nosocomial infections (262), it would be wise to determine the extent of antibiotic resistance in natural pools of opportunists such as vermicompost. For example, *Stenotrophomonas maltophila* is often associated with drug resistance in cases of nosocomial infections (263), and we have identified high densities of this species in maturing vermicompost (Chapter 2). Antibiosis is a common defense mechanisms in microbial rich and highly competitive environments (264,265) and it could be interesting to further research antagonists populations and antibacterial compounds found in vermicompost.

We have identified several bacterial antagonists from the vermicompost. Six of these microbes were shown to be antagonists to pathogens of humans or animals. For example, *Brevibacterium* spp. were shown to inhibit the growth of *Listeria monocytogenes* (266). *Bacillus licheniformis* was shown to inhibit the growth of *Listeria monocytogenes*, *Bacillus cereus* and clinical isolates of *Streptococcus* spp. (267). *Cupriavidus* and *Bacillus* species can inhibit the growth of the aflatoxin-producing *Aspergillus flavus* (268). *Klebsiella* spp. was shown to inhibit *Shigella flexneri* (269) and some *Pseudomonas* species were reported to inhibit the growth of *E. coli*, *Streptomyces* sp., *Bacillus megaterium*, *Cupriavidus necator* and *Saccharomyces cerevisiae (270).*

Lactic acid bacteria isolated from compost were previously shown to be effective under both aerobic and anaerobic conditions against a variety of pathogens (*E. coli*, *Listeria monocytogenes*, *L. innocua*, *B. cereus*, *Staphylococcus aureus*, *Streptococcus faecium*, *Salmonella* sp., *P. aeruginosa* and *Proteus mirabilis*) (183). The mechanisms

involved include the production of lactic acid and hydrogen peroxide under aerobic conditions and the production of bacteriocins under anaerobic conditions. The inhibitory effect of the antagonistic microbes was dependent on their source and the bacteria isolated from the compost showed lower inhibition extents than those from pure cultures.

The hypothesis that vermicompost indigenous microbes may have caused the decline observed in *E. coli* during this experiment is supported by the isolation of an antagonist genus that was previously shown inhibitory against *E. coli* (*Pseudomonas* sp.) and by reports in the literature concerning *E. coli* antagonists isolated from compost (183).

3.6 Conclusions

Research on vermicomposting still is in its infancy and significant advances must be made to fully understand the dynamics between indigenous or pathogenic microbes and earthworms. Further complementary studies would include the identification and characterization of the antagonistic microorganisms present in the vermicompost and responsible for the inactivation of pathogens. Other bacterial pathogens presently ignored by North-American composting legislations are being included in European legislation guidelines. These include *Clostridium perfringens*, *Enterococcus* spp. and *Listeria monocytogenes* (271). Further studies with *E. coli* should seek to establish if virulent strains behave similarly to model or indicator organisms under mesophilic conditions.

Methodological improvements to the present study include: (1) Adding worm gut homogenates to sterilized vermicompost to help control for the worm-associated gut flora in treatments without earthworms since the use of axenic earthworms is difficult (75,77)

and could yield ecologically irrelevant results; (2) combining the use of molecular detection and culturing techniques to reinforce the inactivation rates observed in the present study and avoid underestimates due to survival of "Viable But Non Culturable" *E. coli* under stressful conditions; and (3) using a model that has the GFP marker on its chromosome to circumvent potential long-term plasmid losses, since the stability of the pGFPuv marker in *E. coli* in the absence of the selective antibiotic has only been shown in the short-term (272).

The conclusions emerging from this study are useful in recommending legislation specific to the practice of vermicomposting and in establishing recommendations for safe domestic vermicomposting. Our results reinforce the proposition that vermicomposting is a safe organic waste management technology (25) where *E. coli* numbers drop below the legal limit of 10^3 CFU/g dry weight within 16-25 days, in both continuous or batch systems with a rich microbial community and varying worm abundance. On the other hand, the absence of worms or a reduction in the indigenous microbial community may lead to longer *E. coli* survival. Bacteriological tests of the finished product is still recommended for marketed vermicast, especially if the source organic waste was potentially contaminated, but this recommendation is impractical for domestic vermicomposting, and thus only non-contaminated organic waste should be vermicomposted. In addition, maintaining the worm population healthy and observing a curing period of three month prior to use of the vermicast, as sometimes recommended in the popular literature and in the scientific literature (61), seems like a safe and reasonable recommendation since *E. coli* did not survive longer than 64 days under any of the conditions experimented here.

Table 5. Selective and differential media used for detecting the presence of various bacteria associated with potential human health concerns. A plus sign (+) indicates that the selected bacterial genus can grow on the specified media and a negative sign (-) indicates growth inhibition according to the growth media manufacturer (Difco™).

Media		Mac[1]	Sta[2]	Lev[3]	Ent[4]	LB[5]	Yer[6]
Incubation temperature (°C)		35	35	35	35	35	25
Incubation time (hrs)		18-24	40-48	18-24	40-48	18-24	18-48
Target bacteria	**Potential health concern**	Growth supported (+) or inhibited (-)					
Bacillus	Food poisoning		+				
E. coli	O157:H7 Hemorrhagic colitis	+		+	-	+	-
Enterobacter	Nosocomial origin; urinary, pulmonary, wound and bloodstream infections	+		+			
Klebsiella	Urinary and pulmonary infections; wound infections, enterotoxic			+			
Proteus	Urinary tract infection, nosocomial infection, bacteriemia	+					-
Pseudomonas	Opportunistic, respiratory and urinary tract infections			+			-
Salmonella	Gastroenteritis	+		+			
Shigella	Bacillary dysentry	+					
Staphylococcus	Staphylococcal food poisoning	-	+	-			
Streptococcus	Septic sore throat & scarlet fever				+		-
Yersinia	Gastroenteritis						+

[1] MacConkey agar isolates and differentiates lactose fermenting gram - (crystal violet and bile salts inhibit gram+), [2] m-*Staphylococcus* agar isolates pathogenic and enterotoxigenic *Staphylococci* (inhibits most contaminating bacteria high NaCl concentration (7.5 %)), [3] Levine EMB agar isolates and differentes lactose fermenting from lactose non-fermenting gram negative bacilli, [4] m-*Enterococcus* agar isolates *Enterococci*, [5] Luria-Bertani Miller agar maintains and allows propagation of *E. coli*, [6] Yersinia agar selects for Yersinia based on mannitol fermentation and inhibits normal enteric bacteria (novobiocin and cefsulodin).

Table 6. Time needed to reach the legal limit of 10^3 *E. coli* per gram of dry substrate for different treatments in both the small-scale batch and the large-scale continuous vermicomposting experiments. Time to reach the legal limit calculated based on regression equations for each treatment (see Figures 10 and 14) and the interval in parenthesis represents the estimate based on averages at different sampling times.

Operational mode	Microbial community	Mass of vermicompost (g)	Mass of worms (g worm/ 100g vermicompost)	Time to reach $<10^3$ CFU/g (dry weight) (days)
Batch	Reduced	500	-	49 (49-64)
Batch	Reduced	500	0.648	46 (36-49)
Batch	Rich	500	-	31 (25-36)
Batch	Rich	500	0.648	23 (16-25)
Continuous	Rich	5000	-	23 (16-25)
Continuous	Rich	5000	0.700	20 (16-25)
Continuous	Rich	5000	1.400	21 (16-25)

Table 7. List of opportunistic pathogen (risk group 2) isolated from the vermicompost.

Opportunistic pathogens (no. of isolates)
Aeromonas hydrophila (2)
Bacillus cereus (6)
Citrobacter freundii (1)
Citrobacter sp. (2)
Klebsiella pneumoniae (4)
Pseudomonas aeruginosa (4)
Pseudomonas plecoglossida (2)
Serratia marescens (1)
Stenotrophomonas maltophila (4)

Table 8: List of bacteria isolated from the vermicompost that have shown antagonistic properties against microbes responsible for human and animal or plant diseases.

Antagonists (no. of isolates)	Animal	Plant	**References**
Streptomyces sp. (1)		×	(184)
Brevibacterium sp. (1)	×		(266)
Agromyces sp. (1)		×	(270)
Bacillus cereus (6)		×	(273)
Bacillus licheniformis (3)	×		(267)
Bacillus thuringiensis (1)		×	(273)
Bacillus sp. (13)	×	×	(267,268,273)
Stenotrophomonas maltophila		×	(274-277)
Klebsiella sp. (6)	×		(269)
Cupriavidus[1] sp. (1)	×		(268)
Serratia marescens (1)		×	(265,274)
Pseudomonas sp. (17)	×	×	(265,270,278-280)
Pseudomonas aeruginosa		×	(185)

[1] The genus *Ralstonia* has been reclassified as *Cupriavidus* (281)

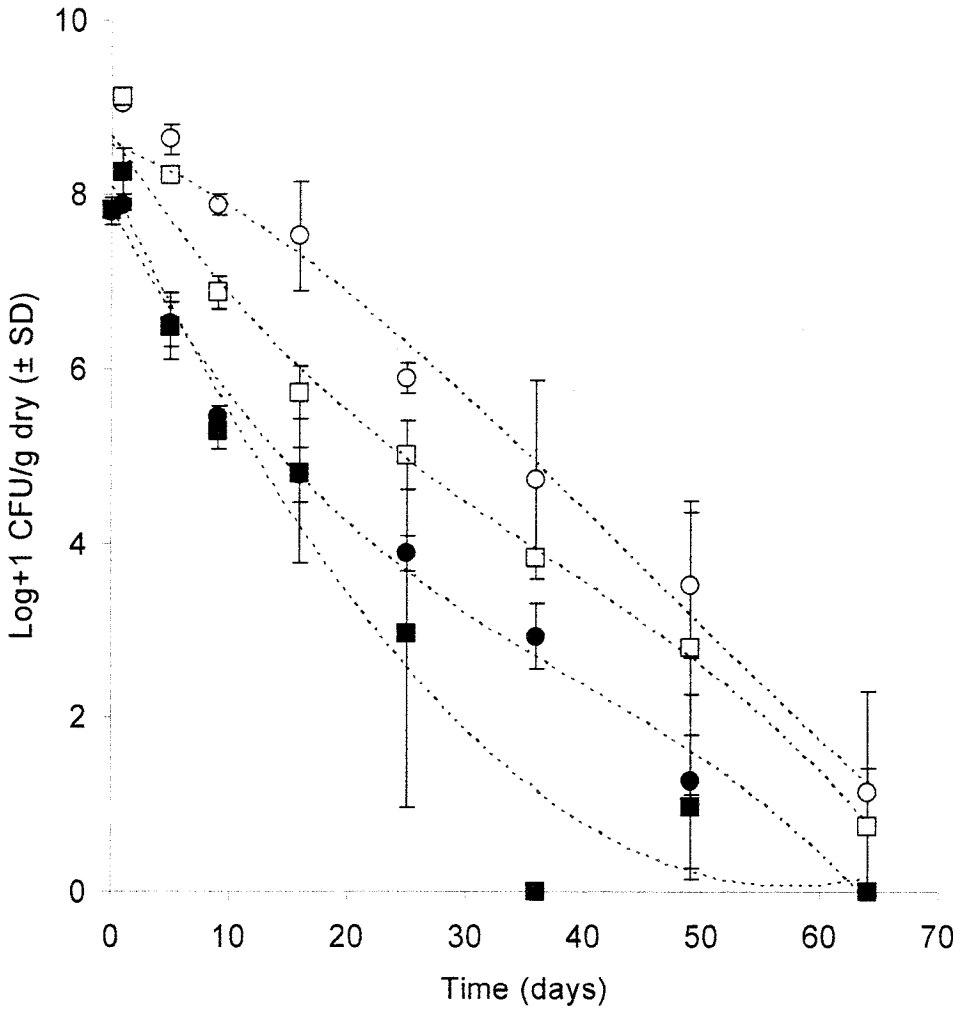


Figure 10. Abundance of *E. coli* MG1655 (pGFPuv) in the presence (P; squares) or absence (A; circles) of worms and in fresh (F; microbial-rich; black) or autoclaved (A; microbial-reduced; white) vermicompost in a small-scale batch experiment (n = 3). Regression lines from top to bottom are expressed with the following equations: AA: $y = 1\times10^{-05}x^3 - 0.0017x^2 - 0.056x + 8.5654$, $R^2 = 0.9753$; AP: $y = -3\times10^{-05}x^3 + 0.0033x^2 - 0.2125x + 8.6662$, $R^2 = 0.9742$; AF: $y = -4\times10^{-05}x^3 + 0.0043x^2 - 0.2508x + 7.8041$, $R^2 = 0.9913$ and PF: $y = -2\times10^{-06}x^3 + 0.0027x^2 - 0.2858x + 8.0767$, $R^2 = 0.9643$.

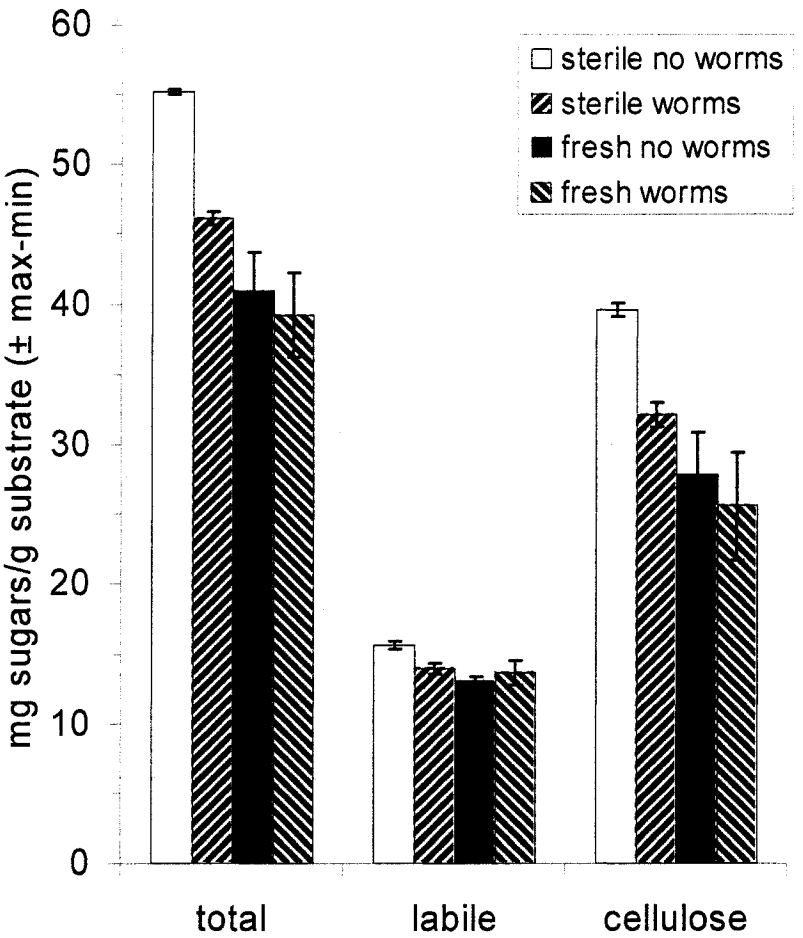


Figure 11. Concentration of carbohydrates in the presence or absence of worms in a microbial-rich (fresh) or poor (sterile) community on experimental day 25 (n = 2).

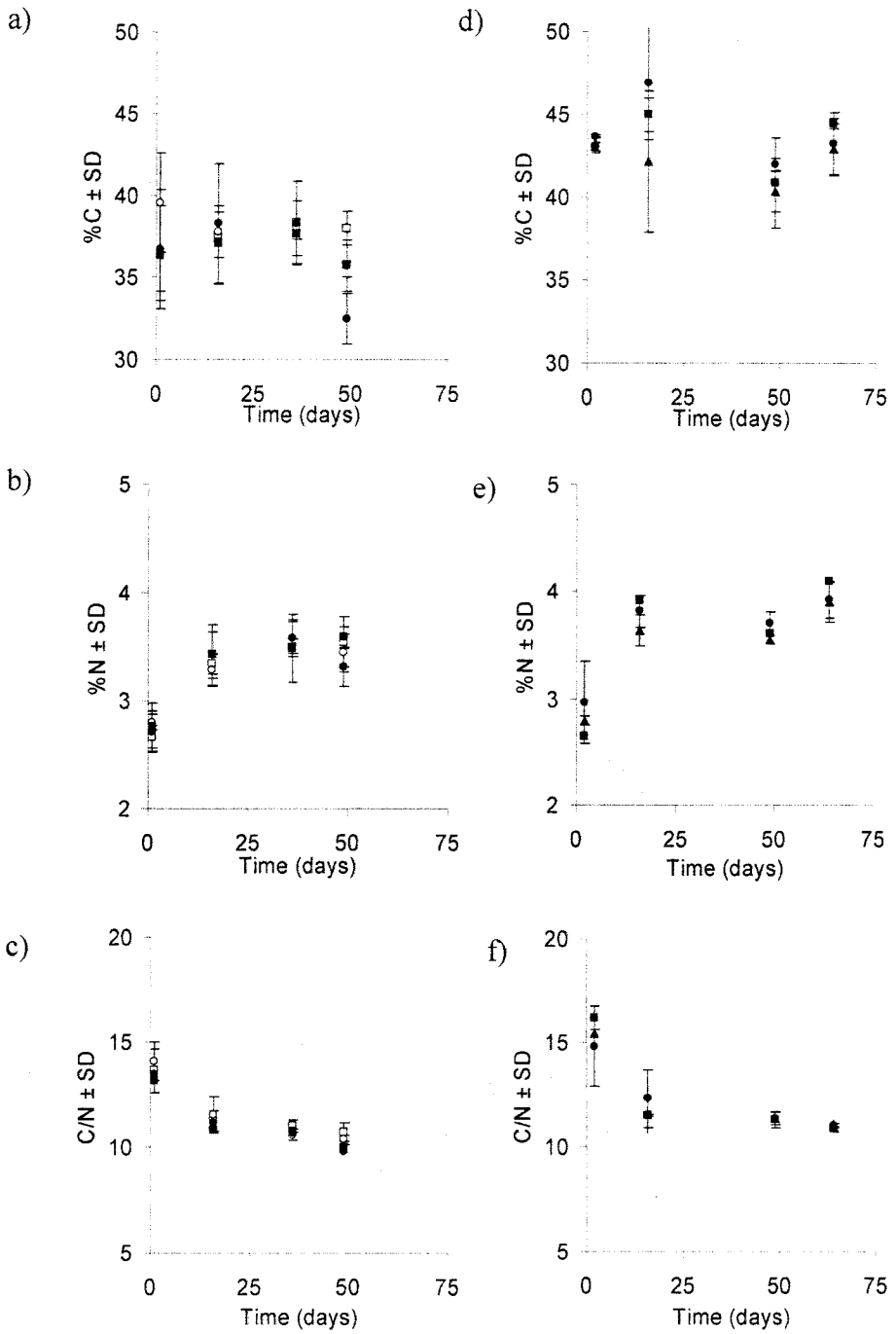


Figure 12. Organic C (a), total N (b) and C/N ratio (c) of vermicompost in a small-scale batch vermicomposting experiment with (squares) or without (circles) earthworms and in the presence of a rich (black) or a reduced (white) microbial community (n = 3). Organic C (d), total N (e) and C/N ratio (f) of vermicompost in a large-scale continuous vermicomposting experiment with 0 g (circles), 35 g (squares) or 70 g (triangles) of earthworms and in the presence of a rich microbial community (n = 3).

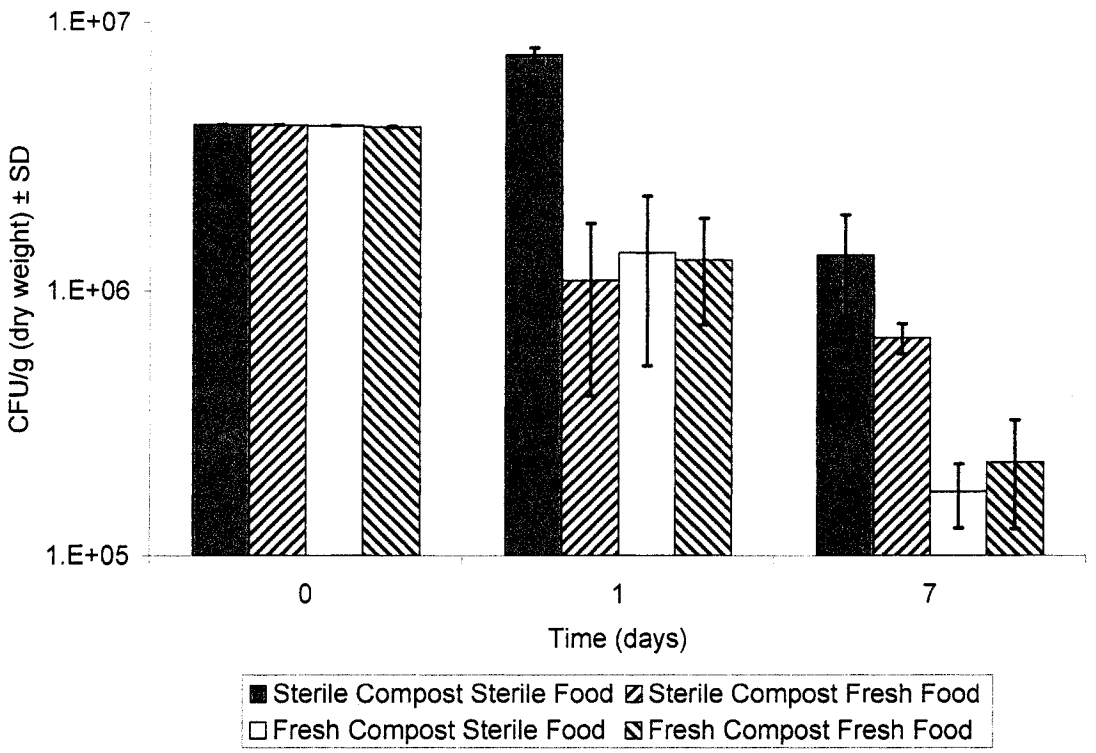


Figure 13. Abundance of *E. coli* MG1655 (pGFPuv) in small-scale batch experiment without earthworms and in the presence of sterilized or fresh feed and vermicompost (n = 3).

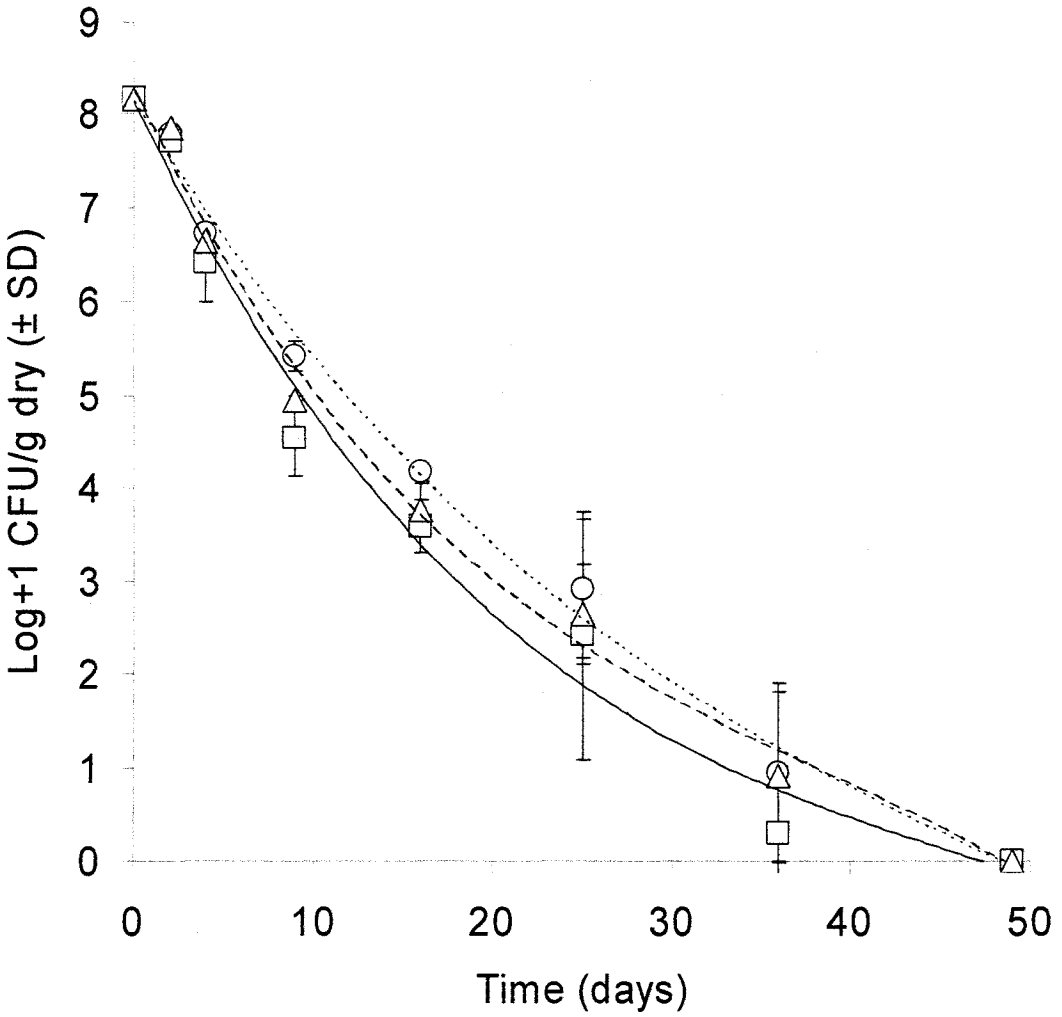


Figure 14. Abundance of *E. coli* MG1655 (pGFPuv) in continuous vermicompost with a rich microbial community with 0 g (circles, ·····), 35 g (squares, - - -) or 70 g (triangles, ——) of worms (n = 3). The regression curves from top to bottom fit the following equations: 0g: $y = -3\times10^{-05}x^3 + 0.0045x^2 - 0.3155x + 8.1465$, $R^2 = 0.9948$; 35g: $y = -5\times10^{-05}x^3 + 0.0073x^2 - 0.3993x + 8.1318$, $R^2 = 0.9853$ and 70g: $y = -7\times10^{-05}x^3 + 0.0081x^2 - 0.395x + 8.2456$, $R^2 = 0.9929$.

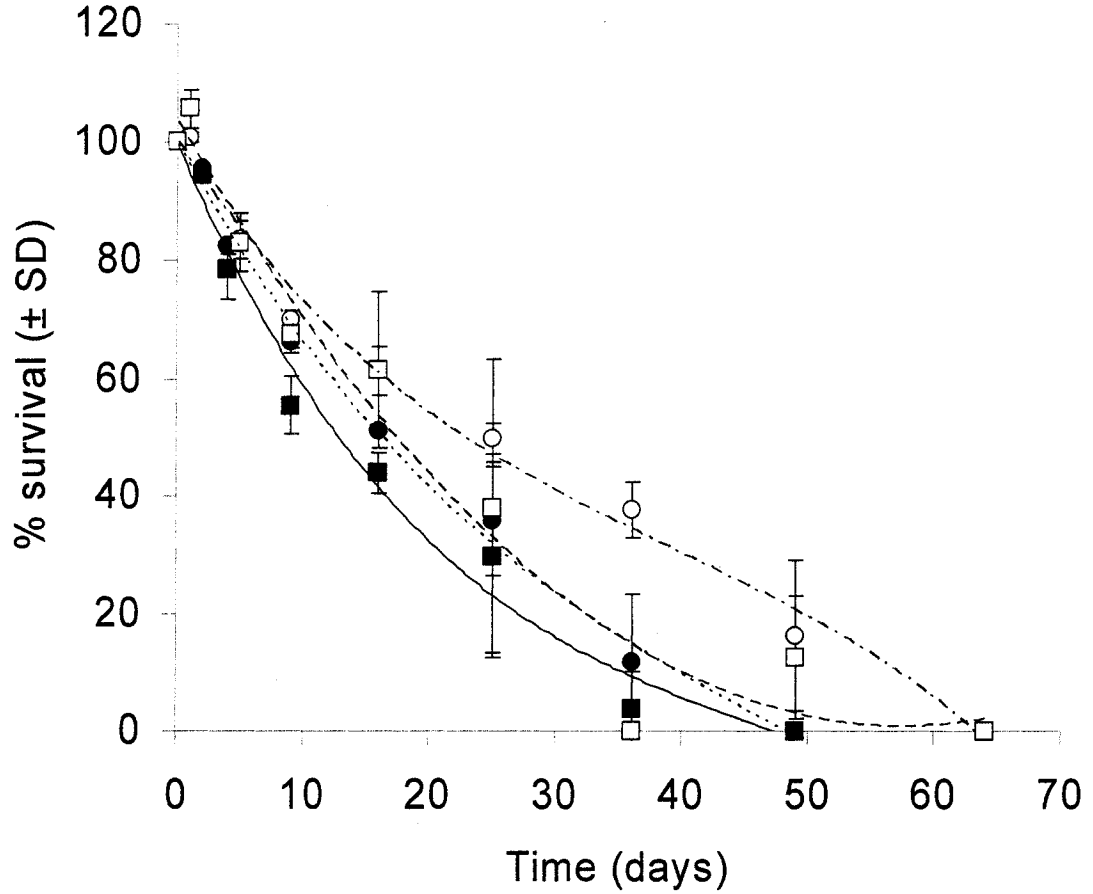


Figure 15. Percent of *E. coli* survival over time in treatments with earthworm present (P, squares) or absent (A, circles) in continuous (C, black) or batch (B, white) operational modes (n = 3). The zero line represents time at which *E. coli* was not detectable using the direct counts procedure. From top to bottom, the equation of the regression lines representing the percent survival are: AB (-·-·-): $y = -5\times10^{-05}x^3 + 0.0557x^2 - 3.2134x + 99.979$, $R^2 = 0.9913$; PB (- - -): $y = -3\times10^{-05}x^3 + 0.0343x^2 - 3.6563x + 103.32$, $R^2 = 0.9643$; AC (·····): $y = -0.0004x^3 + 0.055x^2 - 3.8608x + 99.697$, $R^2 = 0.9948$ and PC (——): $y = -6\times10^{-4}x^3 + 0.0893x^2 - 4.879x + 99.358$, $R^2 = 0.9853$.

Appendix 1 - Earthworm biology relevant to vermicomposting

A1.1 Introduction

This section aims at presenting basic earthworm biology that is relevant to the proper understanding of vermicomposting. This section could have been included in the introduction but we chose not to because this information will not be included in the publication manuscript of the literature review to avoid making it too long. The species of earthworms, their ecological niches and importance in natural soil systems is presented. The sections on vital environmental conditions, reproduction, growth rate and feeding rate are of interest for those who wonder how efficient vermicomposting can be (and who are willing to do the math: *x* earthworms take *y* days to become sexually mature and can produce *z* cocoons that each can eat *w* grams of food per day). Finally, the section on the vermicomposting diet gives a broad perspective to the variety of organic waste that can be processed by earthworms.

A1.2 Earthworm species

There are 3920 known species of worms worldwide (282), but only a few are used in vermicomposting as agents of turning, fragmentation and aeration (19). Earthworms can be classified as **epigeics** (living in litter or top soil layers where they forage primarily on fresh organic matter), **anecics** (living in permanent deep vertical burrows where organic matter, gathered at the surface, is accumulated) and **endogeics** (living in the soil and foraging on soil organic matter, sometimes in highly mineral soil) (283,284).

Epigeic earthworm used in vermicomposting are surface dwelling, humus feeder, that make shallow burrows (69). The most commonly used species for waste

management are *Eisenia fetida*, *Eisenia andrei*, *Lumbricus rubellus*, *Perionyx excavatus* and *Eudrilus eugeniae* (89), but other species such as *Lampito mauritii* and *Octochaetona surensis* have been reported (69). Different earthworm species have different resistance to environmental conditions and local waste diets, and local species should be preferred where vermicomposting is to be conducted outdoors (69). For instance, *Eudrilus eugeniae*, the African night-crawler, and *Perionyx excavatus*, an Asian species cannot withstand low temperatures (30).

Some authors state that mixed worm populations (*Eisinia foetida, Eudrilus euginae,* and *Perionyx excavatus)* are better at degrading organic waste than single species alone (24), probably because individual species prefer particular components in the food waste. On the other hand, some authors warn that mixed colonies may lead to reduced colony fitness due to reproductive isolation (285), especially when using the closely related *Eisenia fetida* and *Eisenia andrei* (taxonomically speaking they are really *E. fetida fetida* and *E. fetida andrei* respectively but they are rarely referred with this precision in the literature, blurring some variety specific discoveries). Thus, the use of mixed population may lead to complementary earthworm effects if the species have different ecological niches, for example, if epigeic earthworms are mixed with anecic earthworms, but care should be taken to avoid mixing closely related species. Interaction between different species of earthworms may be complimentary or competitive and combining different species may lead to enhanced or reduced N or organic matter mineralization (284).

E. fetida (the red wiggler or manure worm) is the most commonly used species in vermicomposting (30,286), although Dominguez *et al.* (285) recommend *E. andrei* over *E. fetida* in vermicomposting because of its higher reproductive and growth rates.

Eudrilus eugeniae (African nightcrawler) can eat between half and its own body weight in food per day and grows twice as fast as *E. fetida* (287); it has thus been referred to as a favourite vermicomposting species.

A1.3 Earthworm importance in soil ecosystem

Earthworms constitute up to 80 % of the soil invertebrate in many ecosystems (282). Aristotle philosophically described the worms as the "Instestine of Earth" (282). They are the most important soil fauna in regulating decomposition and nutrient cycling (113,288). Earthworms have also been regarded as ecosystem engineers because they actively change and ameliorate their soil environment (289) and keystone species because they regulate many nutrient recycling processes in many ecosystems (32). Earthworms are 'secondary decomposers' (24), they enhance decomposition rate, primary productivity and plant uptake of N (290). Earthworm participation enhances natural biodegradation and decomposition of wastes from 60 to 80 percent (given optimum temperature and moisture) thus significantly reducing the composting time by several weeks (291). Earthworms act as aerator, grinder, crusher, chemical degrader and biological stimulators (106). Most soil animals do not feed selectively but show a high degree of polyphagy (249). Enchytraeids, earthworms and microarthropods regularly feed on resources from various trophic levels: they are omnivorous (292,293). Enchytraeids and earthworms exert a greater influence on the soil processes than do microarthropods or nematodes (290). In field microcosm experiments using defaunated or re-faunated soil (monolith from a spruce forest), meso and macrofauna together increased N mineralization more than microbiota alone (294).

A1.4 Environmental conditions

Earthworms are sensitive to touch, light and dryness and their activity is impaired at low temperatures and under light conditions (24). Earthworms can survive in temperatures ranging from 5 to 29°C but they are most active between 20-25°C and with a humidity content of 50-60 % (106). Vermicomposting is mesophilic, and the moderate temperatures are a constraints imposed by the biology of worms. *E. fetida* tolerates 0-35°C (67,147,295) their optimal temperature is between 16-23°C (147,295), 15-20°C (38) or 25°C (69). In addition, vermicomposting efficiency is affected by temperature. Systems operated at lower temperatures (~6.3 ± 2.3°C) have lower rate of reproduction, lower number of cocoons and hatchlings and lower final worm biomass than those operated at higher temperatures (13.7 ± 0.8°C) (sp. *Dendrobaena venata*) (140). Earthworms are very sensitive to humidity content (163). They prefer humid to drier conditions but optimal moisture levels are still debated (80-90 % according to Edwards (32); 70 % according to Tripathi and Bhardwaj (69); 60-90 % according to Alidadi (67), 70-80 % according to Vigueros and Camperos (162), 70-88 % according to Kaplan *et al.* (296)). Optimal humidity may also vary with species. While *E. fetida* may reach a greater biomass at 70 % moisture content and 25°C (0.805 ± 0.132 g/worm), L. *mauritii* prefers a humidity level of 60 % at 25°C (0.779 ± 0.094 g/worm) (297). pH requirements are strict for *E. fetida.* Below 5 or above 9 (67,298) they may die within one week (296). Although Contreras-Ramos and co-workers found that *E. fetida* was not affected by high sodium concentrations (163), it is recommended to keep the salt content below 0.5 % (298) or below an ionic conductivity of about 8 mS cm-1 (143) while the ammonia content of the substrate should not be higher than 0.5 mg g substrate^{-1} (298).

A1.5 Earthworm reproduction

Time to reach sexual maturity depends on food availability and population density (214). A minimum worm weight 0.4 g is required to reach sexual maturity in *E. andrei* (299-301). Earthworms are hermaphrodites but they normally cross-fertilize in a copulation that may last approximately one hour (24,302,303). Post-copulation, a cocoon is secreted from the clitellum and it is shed from the back of the animal, as the sperm comes in contact with the eggs (24). Cocoon production rates of 3 (24) to 5.5 per worm per week or 1 mg cocoon per 100 mg adults (216) have been recorded for *E. fetida*. From each cocoon, 10-12 baby worms will emerge 2-3 weeks after deposition depending on the temperature and humidity (24). Worms can live between 3 to 7 years depending on the species and ecological situation (89).

A1.6 Growth and feeding rates

Organic matter is vital to earthworms and if physicochemical soil conditions are acceptable, earthworm numbers will increase until food resources, in the form of organic matter, become a limiting factor (304,305). For growth, worms depend on a source of carbohydrate (such as cellulose), microorganisms and grit (143). *E. fetida* gains weight depending on population density and food type provided (214). The optimal worm colony density is 1.6 kg worm/m^2 (22), while the optimal feeding rate is around 0.75 kg feed/kg worm/day (22) or 80-300 mg food/g worm/day (up to 30 % of body weight in (205), although some authors have claimed that earthworms can eat up to twice their own weight in food per day (25,216). Therefore, waste processing rates increase with increasing worm density such that a high ratio of worm to organic matter should make vermicomposting efficient and economical (25,216). In fact, processing rates double with

threefold increase in worm density (from 50 to 150 worms litre^{-1}) (306). Doubling the number of earthworms (from 50 to 100 for 500 g of organic waste) increases the rate of degradation but not necessarily by 100 % most likely because after a certain time, food become limiting at higher worm density and the overall activity of the worm colony decreases (24). Increasing beyond a certain density can even become unsustainable (>2 kg m^{-1}(71)). For *Lumbricus rubellus* upper bearing capacity is around 2kg/m^2 (anaerobically treated dried vine fruit effluents feed). At this loading capacity, chemical oxygen demand (COD) removal is around 0.10-0.15 kg COD/m^2·d (287,307). In general, earthworms living in crowded conditions grow more slowly individually and have smaller final body weights but the total weight of earthworm biomass is greater at higher initial worm densities (308) for *E. fetida* and for *E. andrei* (19,57,164).

A1.7 Diet in vermicomposting

Many types of anthropogenic wastes have already been transformed into useful compost by different species of earthworms. Earthworms feed easily on partially degraded materials like cattle dung, primarily acted upon by microbes (24). Vermicomposting has been adapted to process organic waste of different origins, such as sewage sludge, animal manure, and agro-industrial wastes (163,309,310). Cattle dung is rich in cellulosic material that has been partially degraded such that it is very efficiently degraded by earthworms (24). Sewage sludge can also be treated with earthworms but certain precautions should be taken since sewage sludge has high concentrations of pathogens (*E. coli*, cysts of protozoa, parasitic ova), heavy metals (lead and cadmium) and toxic chemicals (industrial wastes and agriculture) (168,311). Sewage sludge is a much better substrate when mixed with pine needles, paper, sawdust or cardboard than

alone or mixed with grass clippings and mixed food (59). The advantages of using vermicomposting as a means to process sewage sludge over conventional composting are that the product is more homogenous, rich in plant nutrients, the levels of organic contaminants are reduced and the resulting vermicompost appears to retain a higher proportion of the nutrients over a longer period of time (89). In baiting experiments, earthworms will slowly accept food waste if no cattle dung is available (24). Cooked waste is degraded faster by earthworms because heat break primary material into simpler compounds and softens the food (24). Brittle calcium compounds like egg shells or bone can be degraded during vermicomposting but this process is long. Sinha *et al.* (24) have observed that these material can retard the degradation of cellulosic compounds. On the other hand, addition of calcium carbonates can help to buffer the pH of very acidic feed. Single type of substrate such as fresh cattle solids, fresh young pig solids, fruit waste and vegetable waste alone can be fatal to *E. fetida* due to high electrical conductivity, high NH_4^+ or low pH (20).

Two very important parameters of feed are the carbon and nitrogen contents and their ratio. Carbon-rich material can also be used as bulking agents. Adding carbon to the system can improve the C/N ratio (giving more available energy for decomposers) at the same time as prevent N losses by ammonia volatilization (19). Examples of bulking agents include newspaper, cardboard, wood chips, dried leaves, pine needles or grass clippings. Dominguez and co-workers (59) observed that paper and cardboard as bulking agent yields highest cocoon production rate (2.82 ± 0.39 and 3.19 ± 0.30 cocoons earthworm^{-1} week^{-1}) compared to food waste, grass clippings, pine needles and sawdust mixed in 1:1 proportion with sewage sludge. On the other hand, food waste yields

highest worm weight (755 ± 18 mg) compared to paper and cardboard, grass clippings, pine needles and sawdust mixed in 1:1 proportion with sewage sludge (59).

Appendix 2 - Inhibition of *E. coli* by volatile substances emitted by vermicompost

A2.1 Introduction

Previous experiments have shown that volatiles emitted from compost could be inhibitory to certain pathogenic fungi (312) and bacteria (183). Lethal volatile substances could include phenolics (243,244), volatile fatty acids (VFA) (245,246,269) or simply CO_2 (183). For example, young compost (3-month old) emits more volatile substances than mature compost which lead to greater inhibition of the pathogen *Fusarium culmorum* (172) but the specific nature of these volatile substances was not yet identified. *Klebsiella* sp. were isolated from vermicompost (Chapter 2). Members of this genus are known to produce VFA that are inhibitory to *Salmonella typhimurium*, including formic acid and acetic acid (245). High concentrations of volatile fatty acids found in the gut of ruminants has been shown to inhibit *E. coli* and *Salmonella* rapidly (269).

We sought to determine if the reduction in *E. coli* abundance in vermicompost reported in Chapter 3 was mediated by contact-dependent factors (antibiotics, siderophores, extracellular lytic enzymes, competition for nutrients, worm preferential grazing and digestion) or by volatile factors. The goal of the two following experiments carried out during this project was to assess whether volatile substances emitted from different samples of vermicompost could inhibit the growth of *E. coli* in solid or liquid cultures. In the first experiment, fresh or autoclaved vermicompost was placed in close proximity to solid cultures (LB agar) of *E. coli* MG1655 while in the second, air blown

through fresh or mature vermicompost was injected into a liquid culture of *E. coli* MG1655 (M9 minimal media + glycerol).

A2.2 Growth of *E. coli* on solid substrate

A2.2.1 Comparing fresh or sterile vermicompost

A2.2.1.1 Experimental design and set up

The set-up of the experiment on solid substrates was tricky for several reasons. First, vermicompost had to be placed in a small container that retained most volatiles without allowing physical contact between the vericompost and the bacterial cultures. Since Petri dishes were used to grow *E. coli*, vermicompost was initially placed on the lid inside the Petri dish, and the Petri dish was placed lid-down and sealed shut with parafilm. This design was functional but only small quantities of vermicompost could be placed inside the contraption. In addition, this design favoured condensation on the solid agar substrate which allowed *E. coli* movement in a thin liquid film at the surface of the agar. To control the condensation effect, the control treatment with no vermicompost contained filter paper that was moistened with the same amount of water present in the vermicompost of the experimental treatments (5, 10 or 15 g of vermicompost at 70 % humidity).

The second challenge was to place a physical barrier between the vermicompost and the solid culture media to limit the movement of the microscopic invertebrates living in the vermicompost. Different mesh materials glued to the lid of a 10cm Petri dish pierced with an 8-cm hole were tested. Nylon mesh had pores larger than the smallest

animals but could be re-used after being sterilized with ethanol and prolonged UV radiation. Filter papers did not allow invertebrates to pass through but could not be reused as prolonged exposure to humidity and fungal spores led to rots. Nevertheless, in the preliminary experiment, no difference in *E. coli* growth was observed when comparing set-ups that used nylon or filter papers as barriers so the results were pooled together. The experimental set-up used for the preliminary experiment is presented in Figure 16.

A2.2.1.2 Methods

The growth of *E. coli* MG1655 colonies was monitored daily for two weeks. Ten isolated colonies were randomly selected. Colonies that started merging into each other or into the side of the dish were abandoned and plates that became contaminated with fungus were discarded. Colony diameter was estimated to the nearest 0.5mm using a ruler. The results presented here are taken from the active growth period (the first 5 days) and represent the average diameter of two independent set-ups where 10 colonies were measured (i.e. for each data point n = 20).

A2.2.1.3 Results

The results of this experiment are presented in Figure 17. A striking result is that the diameter of the colonies in the control plates (without vermicompost) is smaller than the colonies in the plates where vermicompost is present, especially at the beginning of the experiment. This could be due to the presence of growth enhancing volatiles in the vermicompost (perhaps volatile fatty acids or other molecules that can be used as nutrients or growth factors). On days 2 and 3, the diameter of the *E. coli* colonies was

inversely proportional to the abundance of vermicompost in the treatment, i.e. set-ups with most vermicompost had smaller colonies than those with less vermicompost and this effect was observable in both the sterile and the fresh vermicompost. On days 4 and 5, this inhibitory effect is still somewhat visible in the sterile vermicompost treatment but not in the fresh vermicompost treatment. These results suggest that a volatile substance emitted from the vermicompost is more inhibitory at higher concentrations and that this volatile substance is emitted continuously from sterile vermicompost but that it stops being emitted or eventually degrades over time in fresh (microbially active) vermicompost. Thus, if the inhibitory substance is secreted by microbes present in the vermicompost it may not be affected by sterilization (high temperature and pressure), but this would be surprising. Also, the production of the inhibitory substance may decrease as the substrate becomes more degraded after a few days in fresh vermicompost or it may eventually be degraded (perhaps consumed) by microbes present in the fresh vermicompost.

A2.2.2 Comparing different vermicompost and feed

A2.2.2.1 Experimental design and set-ups

In this experiment, three types of vermicompost were tested. Fresh vermicompost was actively degrading vermicompost obtained from the continuous vermicomposting experiment. Mature vermicompost was that taken in bins that received no fresh food for a period of three months. Sterile vermicompost obtained by autoclaving fresh vermicompost at 121°C for 15 minutes. The food tested in this experiment is a mix of fruits and vegetables and coffee described in Chapter 2. Fresh food had been collected

within three days before the start of the experiment whereas old food had been left in the refrigerator for three weeks before the start of the experiment. The experimental set-ups used in this experiment is the same as that used in the preliminary experiment except that only nylon mesh was used and that only two masses of substrate were tested (5 and 15 g). The sampling methodology is the same as the previous experiment.

A2.2.2.2 Results

The results of this experiment are presented in Figure 18. Once again, the average colony diameter in the control (with no food or vermicompost present) was slightly smaller than the colonies in the other treatments but this trend was not statistically significant. As opposed to the previous experiment, we did not observe consistent trends in colony size over time in the different treatments with 5 g or 15 g of different types of vermicompost. Young compost was expected to release more volatiles than older compost where the organic substrate is already well degraded and than sterile vermicompost where no active microbes are present. The sterile and old compost treatments were very similar throughout the experiment and both seemed slightly higher than the colonies exposed to young vermicompost although this effect is not statistically significant. Fresh food contains more sugars than old food (already colonized by bacteria, yeasts and fungi); it was thus expected to produce more volatiles. Early in the experiment (24 h and 48 h), *E. coli* colonies exposed to young food seemed slightly smaller than those exposed to old food but this trend was not statistically significant and it was not visible after 72 h. In addition, larger quantities of food (15 g *vs.* 5 g) appeared to lead to smaller *E. coli* colonies throughout the whole duration of the experiment, but once again, this trend was not significant. Perhaps the absence of significant trends in this

experiment was due to very small quantities of substrate used and to the low resolution of the measurement tool (millimetric ruler) used to measure colony diameter.

A2.3 Growth of *E. coli* in liquid media

A2.3.1 Experimental design and set-ups

The growth of *E. coli* exposed to volatiles from fresh or old vermicompost was also checked in liquid media to quantify inhibition. Measuring colony diameter and surface area on the solid substrate allowed seeing the effect of the compost volatiles on single colonies growth whereas the measurement used in the liquid experiment allowed to see how overall abundance of *E. coli* was affected upon exposure to volatiles from the vermicompost. The growth of *E. coli* is very rapid in rich LB media and to better see possible inhibitory effects, minimal media (M9) supplemented with 0.2 % glycerol and 100 mg/mL of ampicillin was used. Glucose was also tested as a carbon source but it was rejected, despite good growth of *E. coli*, since glucose inhibited the expression of the GFP gene which is on a plasmid downstream from the *lac* promoter thus preventing easy screening for contamination using GFP fluorescence under UV light.

E. coli pGFPuv was isolated from the frozen stock (Chapter 3) on a LBamp plate at 37°C. The largest fluorescing colony was used to inoculate 10 mL of liquid culture (M9amp) incubated overnight at 37°C and 250 rpm. Two mL of the M9-adapted overnight culture were used to inoculate 500 mL of M9amp used in the set-ups. The vermicompost used in this experiment was obtained from the bins described in Chapter 2. Fresh vermicompost was from regularly fed bins and mature vermicompost came from

bins that had not been fed in six months. The humidity content of the vermicompost was adjusted to 75 % after drying a sample overnight at 105°C.

Volatiles were transported through the set-ups using a compressed air outlet. The set-ups is illustrated in Figure 19. The air was initially humidified by passing through 500 mL of distilled water placed inside a flask with two bent glass pipettes as air inlets and outlets. Then the air was then blown into 500 g of vermicompost placed inside a 1-L polyethylene container equipped with a plastic divider that favored more even air movement across the compost between the inlet and the outlet. The air then passed through a coarse filter (sterile cotton fiber packed loosely in a Pasteur pipette) and a sterile 0.2 μm filter before being blown gently into the agitated (75 rpm) liquid culture (500 mL) incubated at room temperature (25°C). The optical density was checked regularly using a 1ml sample retrieved from the liquid culture using a sterile glass pipette. The optical density of the liquid cultures was checked at 600 nm (Cary 50 WinUV spectrophotometer, Varian). The experiment was conducted in duplicate.

A2.3.2 Results

The growth of *E. coli* exposed to volatiles from vermicompost using a liquid culture media is illustrated in Figure 20. The growth of *E. coli* is remarkably slow in liquid M9amp using 0.2 % glycerol. A three-day lag phase was observed before the active growth phase. The active growth phase does not yield carrying capacities normally observed for *E. coli* MG1655 (pGFPuv) in LB broth (OD_{600} > 2 within a day). Nevertheless, we can observe a greater yield of *E. coli* in both replicate where fresh vermicompost was used. The different results observed between both replicates of the

experiment may be due to the use of slightly different compost (collected after a week interval). Thus this reinforces the hypothesis that more volatiles are emitted from fresh vermicompost (as opposed to old or mature vermicompost) but it contradicts the hypothesis that more inhibitory volatiles would be produced from young vermicompost. In fact, this seems to support the observation conducted in the solid phase experiments where volatiles from active vermicompost would support the growth of *E. coli* rather than inhibit it.

A2.4 Conclusion

The experiments described here do not allow definitely ruling out or confirming the production of inhibitory volatiles from vermicompost. A recurring observation is that volatiles emitted from the vermicompost seem to enhance the growth of *E. coli* rather than to inhibit it. This observation is visible on the solid substrate (smaller colonies in the controls compared to the treatments with vermicompost present) and in the liquid media (lower *E. coli* yield in the presence of young vermicompost as opposed to old vermicompost).

VFAs are mainly effective at lower pH in their protonated form (i.e. acetic acid not acetate) (245,312) and they are mainly produced under anaerobic conditions, thus it is not very likely that these compounds would be very important in vermicompost (aerobic with high pH). Nevertheless, because of the low initial pH of the feed (116) combined with the anaerobic environment of the earthworm gut (138) we cannot completely rule out the occurrence of VFA related pathogen death in vermicompost. Other substances such as phenolics may also be found in the fresh fruits waste, and these may also play a

role in reducing the abundance of pathogens such as *E. coli* and *Salmonella* sp. through the effect of volatile substances (313). Perhaps volatile terpenoid compounds, present in plants may be present released when the organic material is degraded.

More research on the effect of volatiles emitted from vermicompost on the growth of *E. coli* could be interesting to confirm that *E. coli* decreases observed in the survival experiments (presented in Chapter 4) could be mediated by volatile substances which are independent of direct contact between the vermicompost and the bacteria of interest. The lack of strong evidence on the antagonistic effects of volatile substances on the growth of *E. coli* may suggest that this mode of inhibition may not be dominant in mediating *E. coli* reduction in the vermicompost compared to contact dependent mechanisms (antibiotics, siderophores, extracellular lytic enzymes, competition for nutrients, worm preferential grazing and digestion).

Methodological improvements to the experimental design used here include: A) Using a constant total mass of vermicompost between the different treatments (50 grams total) as opposed to the varying masses used in the previous experiments (5 to 15 g), with varying ratios of young and mature vermicompost were used would have allowed to (1) use a larger mass of vermicompost to have more concentrated volatiles in contact with the growing colonies of *E. coli*, (2) provide a continuum of ratios to assess dose-dependent relationships and (3) better control the condensation differences between the different treatments; B) incubation of the set-ups at room temperature (25°C) since this temperature better reflects the conditions of normal vermicompost (as opposed to incubation at 37°C which favour rapid *E. coli* growth); C) establishing a better colony measurement unit by photographying the set-ups daily with a digital camera, and

calculating the average colony surface area after conversion from pixels to millimetres using a software program such as ImageJ 1.37v (National Institure of Health, USA, http://rsb.info.nih.gov/ij/).

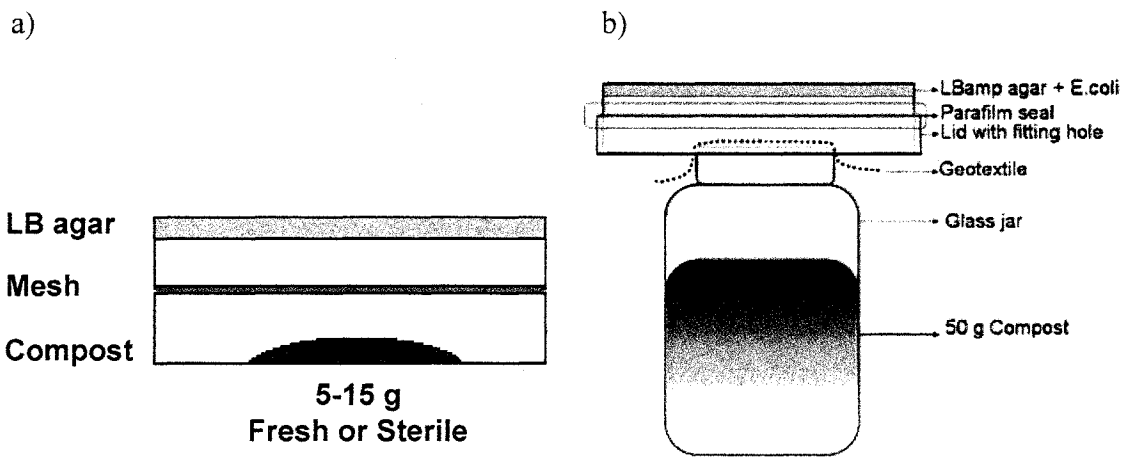


Figure 16. Experimental design of the *E. coli* inhibition experiment by vermicompost volatiles on solid substrate. The figure (a) represents the design of the first two experiments while figure (b) represents the design of the third experiment.

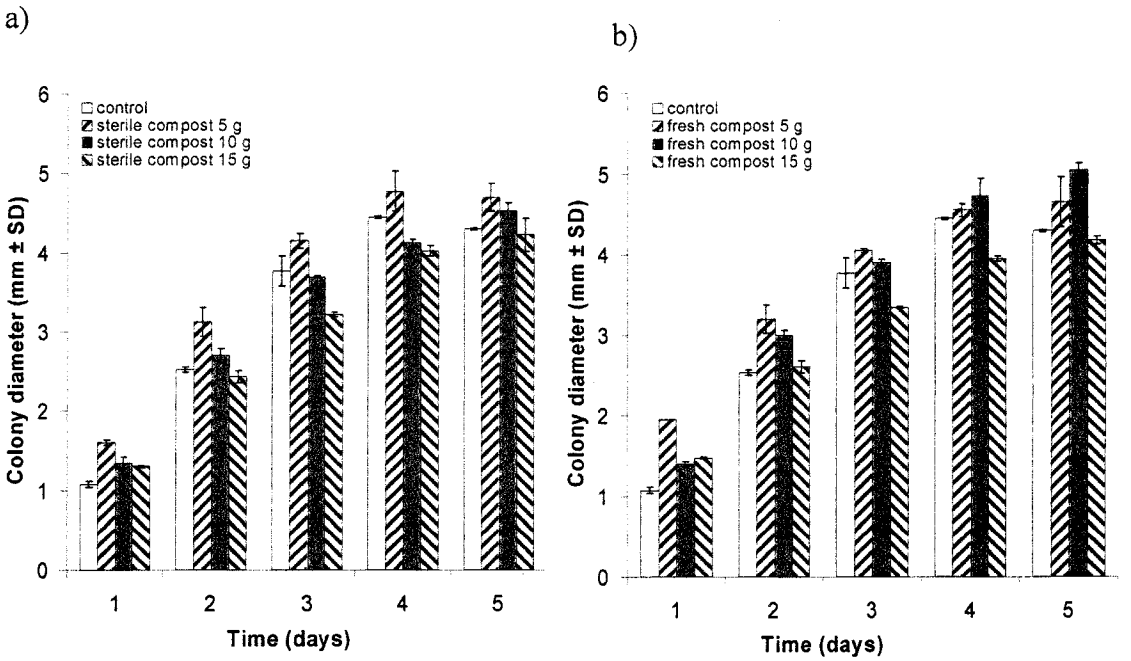


Figure 17. Growth of *E. coli* MG1655 (pGFPuv) on solid media (LBamp agar) under exposure to different quantities (0-15 g) of sterilized (a) or fresh vermicompost (b). The colony diameter is the average of two independent experiments where the growth of 10 colonies was monitored for 5 days.

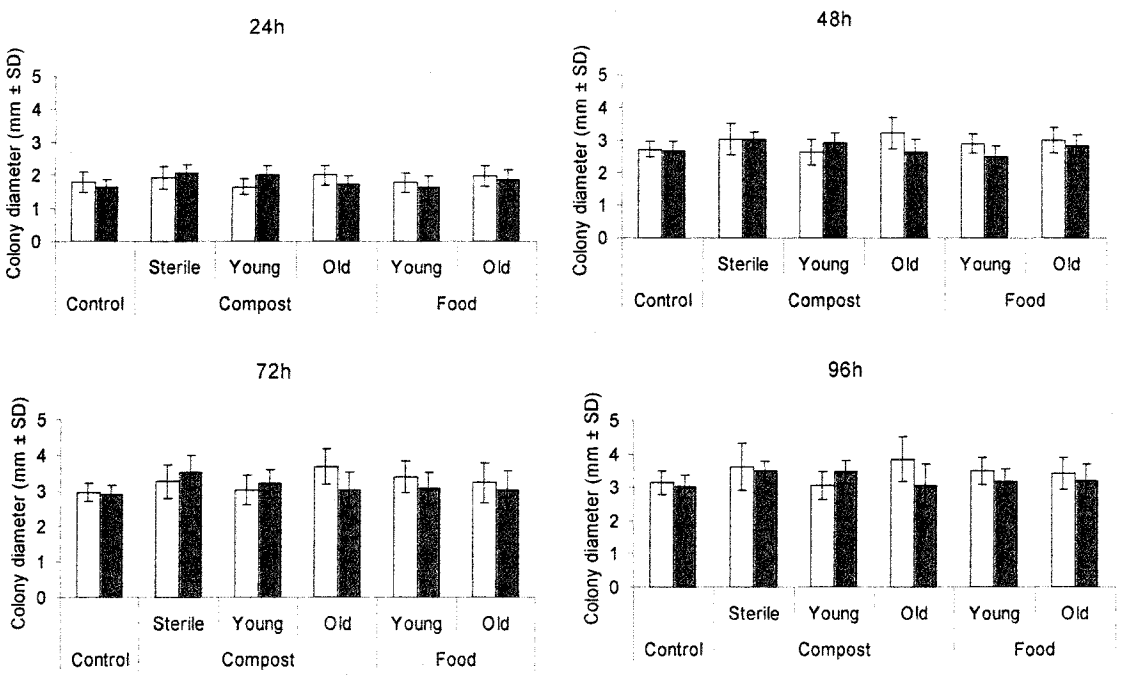


Figure 18. Growth of *E. coli* MG1655 (pGFPuv) on solid media (LBamp agar) at 37°C under exposure of volatiles from varying quantities (5 (white) or 15 (grey) g) of different vermicompost (sterile, young or old) and feed (young or old) and at different times.

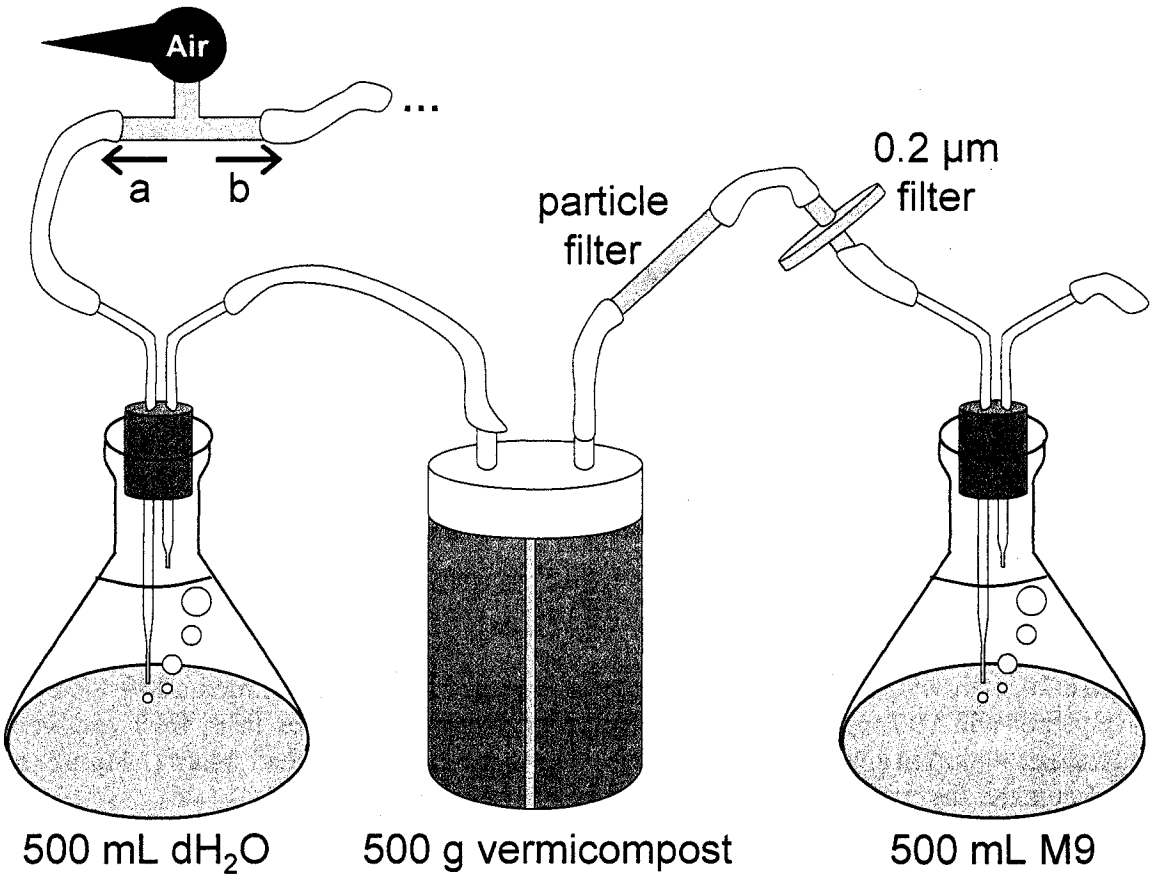


Figure 19. Experimental set-up for comparing the growth of *E. coli* MG1655 (pGFPuv) in liquid media with exposure to volatiles from fresh or mature vermicompost. The two treatments were ran in parallel (a and b) and the replicates were sequential. The compressed air is first humidified by passing through distilled water, then through a vermicompost filter, a coarse particle filter (cotton packed into a Pasteur pipette) and a 0.2-µm filter before being blown into the inoculated growth media (M9 with 0.2 % glycerol and 100 mg/mL ampicillin).

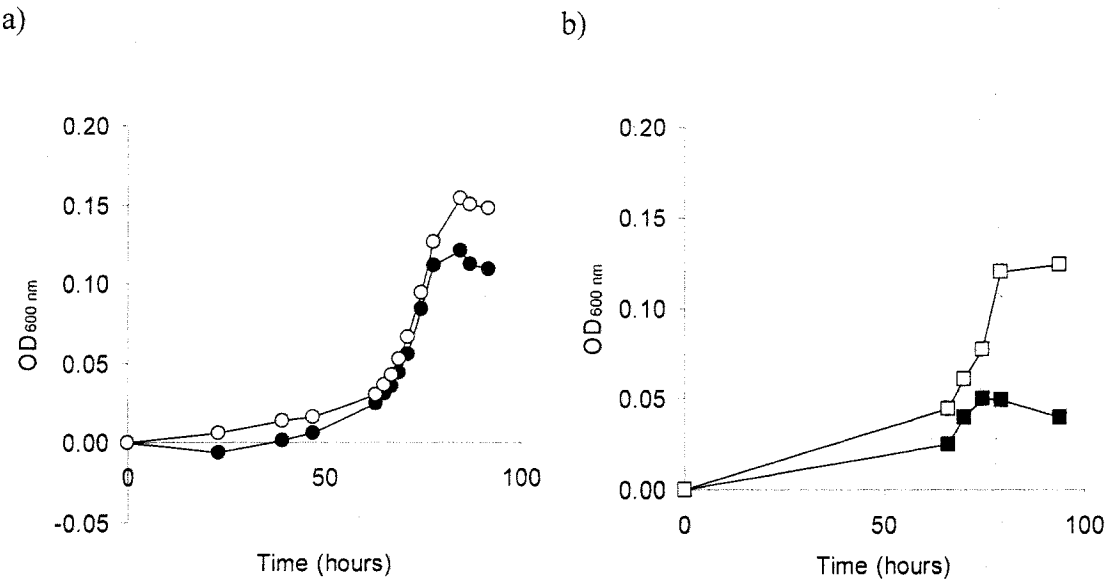


Figure 20. Growth of *E. coli* MG1655 (pGFPuv) in liquid minimal media M9 with 0.2 % glycerol and 100 mg/mL ampicillin under exposure to volatiles from fresh (actively degrading; white) or mature vermicompost (after 6 months of last feed; black). Figure (a) represents the first trial and figure (b) represents the second trial.

Appendix 3 - Continuous vermicomposting

A3.1 The effect of higher temperature and lower humidity of vermicompost on the pH

Although 24 bins were set up for the experiment, only 18 were considered in most of the analysis. This is because the bins were stored on four shelves and despite the use of a controlled environmental chamber, there was a temperature difference between the bottom and the top of the room (Figure 21). The difference between the maximal temperature observed on the top shelf and the minimal temperature observed on the bottom shelf was 5.90 ± 1.83°C. At the time of sampling, the top shelf was on average 1.84 ± 0.56°C (n = 37) warmer than the lower shelf. Despite the seemingly small difference (~2°C), this temperature gradient was enough to seriously enhance evaporation of water from the bins stored on the top shelf. As a result, the humidity of the bins stored on the top shelf could not be maintained constant, despite regular water addition and blocking of the aeration holes in the lid. Figure 22 illustrates the wide fluctuation of the top shelf bins compared to the average of the three others.

Moisture is important for degrading microbes because below a certain treshold, microorganisms become dormant and above a certain point, waterlogging leads to anoxia, slower degradation and generation of foul smelling compounds (fatty acids butyric and propionic, alcools, ketones and aldehydes, nitrogenous compounds like putrescine and cadaverine, sulfuric compounds like mercaptans, hydrogen sulfide and ammonia) (4). Earthworms are also sensitive to humidity content. They prefer humid to drier conditions

and their optimal range is located somewhere between 60 to 90 % depending on the author (Figure 23). The optimal humidity conditions are 80-90 % according to Edwards (32); 70 % according to Tripathi and Bhardwaj (69,297,314); 60-90 % according to Alidadi (67), 70-80 % according to Vigueros and Camperos (162) and 70-88 % according to Kaplan *et al.* (296).

Therefore, the bins stored on the top shelf were excluded from the analysis described in Chapter 3. Comparing data from vermicomposting units with adequate humidity content and that from vermicomposting units that are dryer gives interesting results with respect to the importance of humidity on the process of vermicomposting. Figure 24 illustrates the pH of the vermicompost operated continuously as it varies through time. Notice that the pH of the bins with a lower and more variable humidity content tends to be more acidic than that of the more humid bins, especially beyond day 223. This indicates that under dryer conditions, the biodegradation process is different than under more moist conditions. Fungus is known to tolerate lower pH (116) and lower humidity than bacteria (27), and perhaps this may have influenced the decomposition process of the dryer bins, favouring fungi over bacteria.

A3.2 The effect of carbon-rich feed on the vermicompost C/N ratio

An initial ratio of C/N of 30 is a reasonable theoretical ratio for a mixture of organic waste that is to be composted (measured optima fall between 20 and 30). This is though to balance energy (C compounds) and nutrients availability (N) necessary for the metabolic activity and growth of the decomposer microorganisms (67,71). Generally, organic waste should be balanced to have an initial C/N ratio around 25 for

vermicomposting. Successful vermicomposting of initial substrate with a C/N ratios up to 60 were reported (36,68). On the other hand, lower C/N may be linked with N losses through leaching or volatilization (19,136).

Source separated organic waste, such as fruits and vegetables, have a C/N ratio of 20-25 (see Table 2). Typically, in domestic settings, vermicompost feed is supplemented with newspapers (carbon rich) to increase the C/N ratio of the initial feed. Two continuous vermicomposting experiments with a carbon-rich diet and a carbon-poor diet were compared. The set-ups and the feed are as described in Chapter 2, except that the carbon-rich treatment had newspaper covering the surface of the vermicompost. Temperature, pH, humidity and feed were otherwise similar (data not shown).

Figure 25 shows the overall difference of the vermicompost C/N ratio over the initial 100 days of the experiments compared here. In both treatments, there is a decline in the C/N ratio of the vermicompost over time, but the carbon-rich diet has C/N values about 10 units more elevated than the carbon-poor diet. The long-term continuous vermicompost experiment described in Chapter 2 is the same as that represented here in Figure 5. Beyond day 266, when feeding was interrupted, we noticed a drastic volatilization of N and this may have been influenced by the fact that the initial diet was relatively low in C.

A3.3 Conclusion

Balancing the C/N ratio of organic matter is essential for successful vermicomposting. Adding shredded newspaper in vermicomposters can lead to the formation of compact masses of humid paper that limit oxygen diffusion. Here we have

shown that the C/N ratio of the organic waste was acceptable using only coffee filters as carbon-rich substrate. Without newspaper, there was no compact mass of slowly degrading newspaper, and fewer rots were visible on the surface of the vermicompost (qualitative observation). On the other hand, a lower initial C/N ratio led to very high nitrogen volatilization after feeding interruption (not determined for the higher C/N experiment) and it is likely that a slightly higher C/N ratio would have prevented this. Thus, using a small amount of newspaper in vermicompost bins may be beneficial for the decomposition process, but care should be taken not to add too much of it at once, and to mix it well with the substrate.

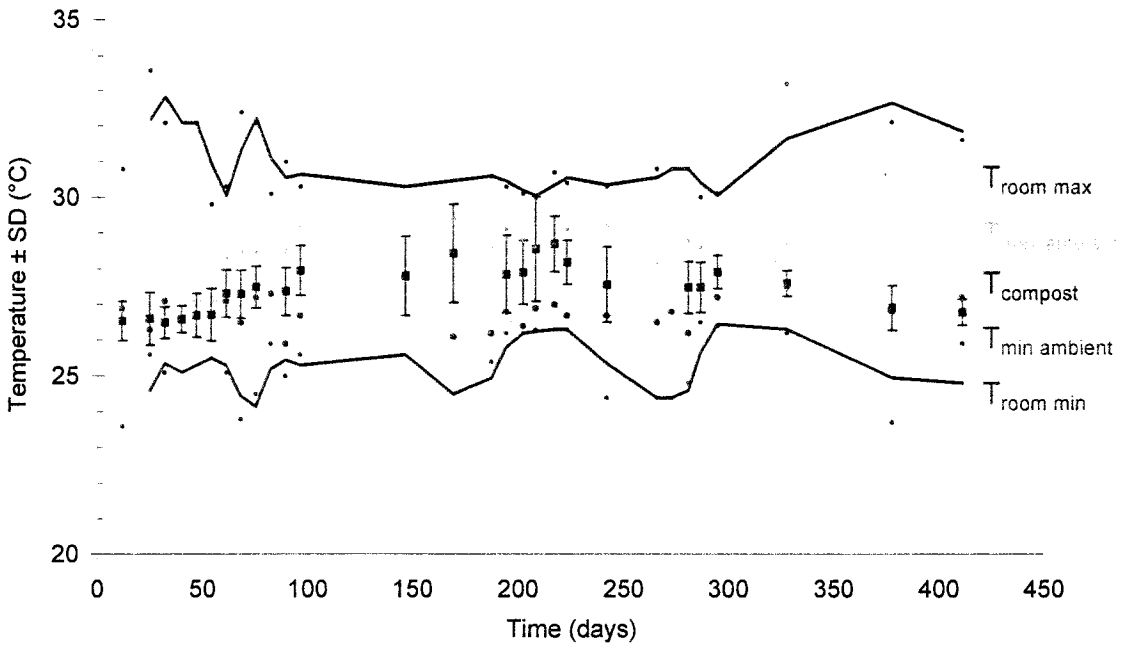


Figure 21. Temperature variation in the atmospheric chamber where the vermicompost units were maintained. The maximum temperatures were recorded on the top shelf were the vermicomposting units were stacked while the minimum temperature was observed on the lower shelf. The ambient temperature refers to that at the time of sampling and the room temperature was obtained from a weekly reset min-max thermometer.

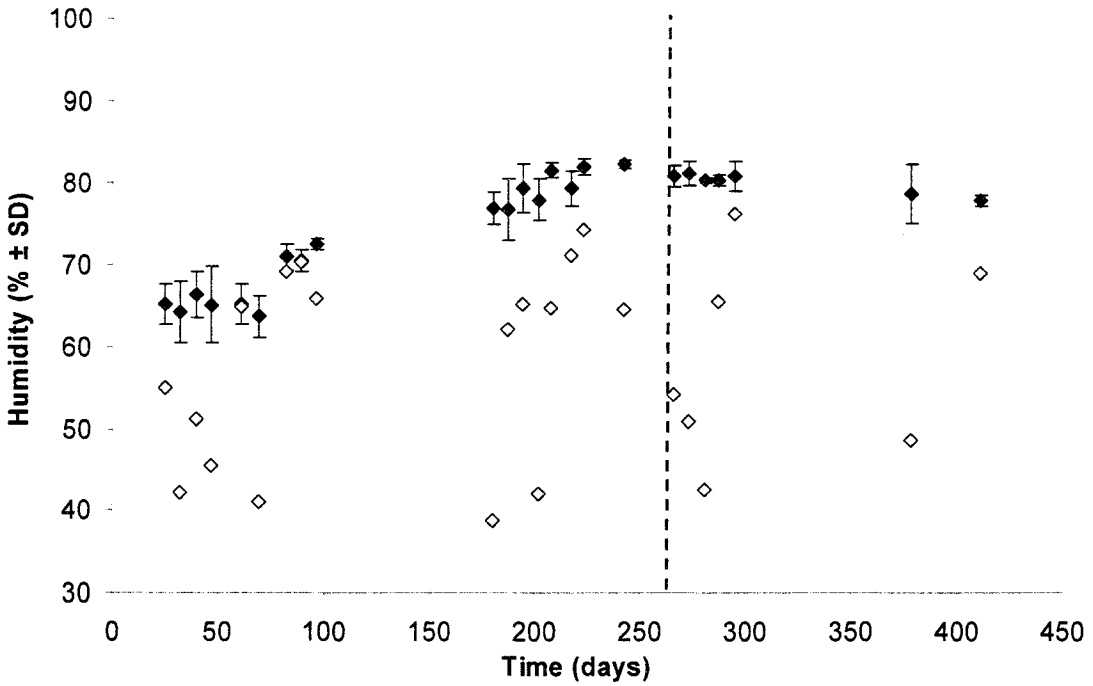


Figure 22. Humidity variation of the vermicompost over time. The full diamonds represent the average of 18 or 3 bins (before and after day 100 respectively) and the empty diamonds represent the highly variable humidity of the vermicompost in the bins stored on the top shelf.

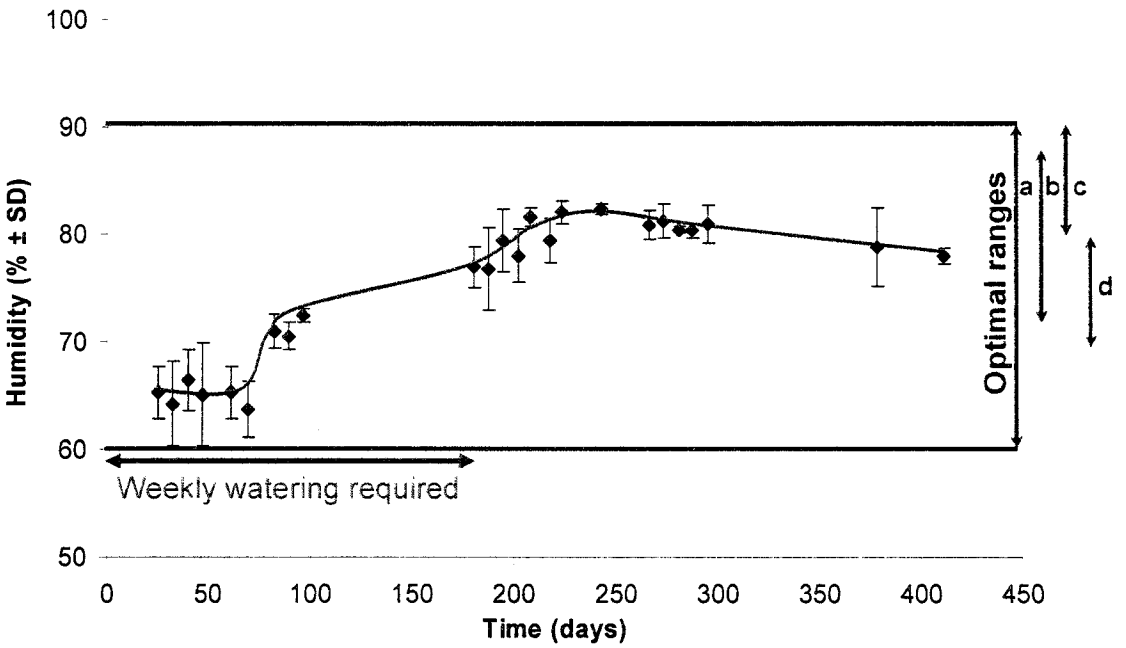


Figure 23. Humidity variation with time in the vermicomposting units (n = 18). Notice that the minimal vital range was maintained but that weekly watering was required over the initial 180 days. The arrows labelled (a) to (d) correspond to the different optimal ranges proposed by different authors (Alidadi (67), Kaplan *et al.* (296), Edwards (32) and Vigueros and Camperos (162) respectively). After that, adequate humidity levels were maintained with the addition of food (which had on average a 75 %-humidity level). Notice that beyond day 295, when feeding was halted, the humidity level did not decrease much in the bin, highlighting the water retention capacity of vermicompost.

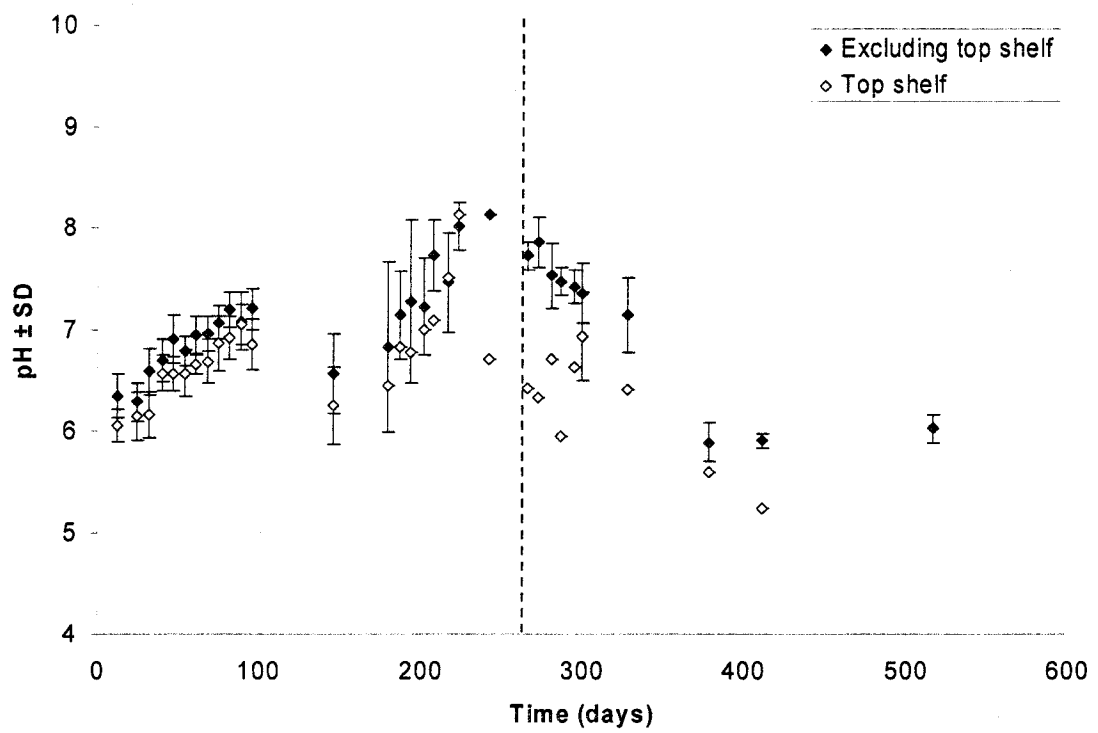


Figure 24. pH variation of the vermicompost with time. Data before day 180 is based on n = 18 for all data excluding top shelf and n = 6 for top shelf and after day 180 n = 3 for all data excluding top shelf and n = 1 for top shelf.

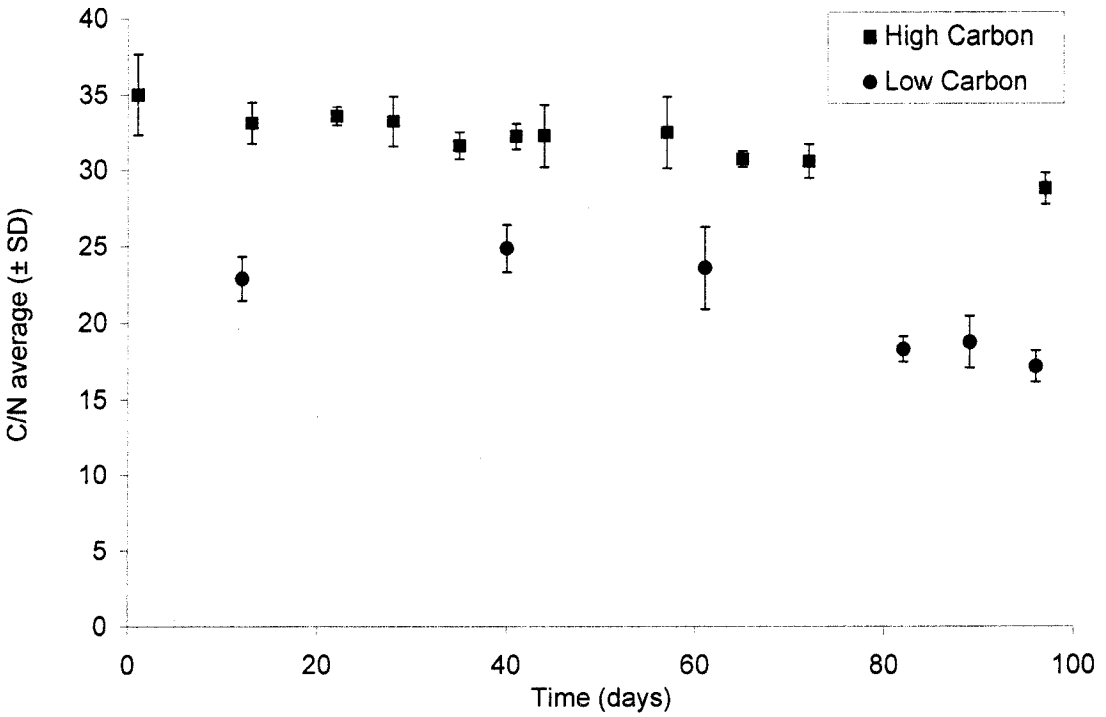


Figure 25. C/N ratio of continuous vermicomposting under a high carbon diet and under a low carbon diet.

Appendix 4 - Isolating and identifying bacteria from fed-batch vermicompost

This Appendix contains the complete morphotyping and phylogenetic assignments of the 110 bacteria isolated and identified from the vermicompost (Table 9).

The main goal for isolating and identifying vermicompost bacteria was to detect potential pathogens dwelling in vermicompost made from material unlikely to be contaminated (i.e. source-separated fruits, vegetables and coffee with only egg shells as an animal derived product), thus we used selective and differential growth media which could support potential pathogens (Chapter 3). Morphological characterization of bacterial colonies has the merit of being an effective way of selecting and isolating different bacteria but cannot be used alone for proper genus identification. Many bacterial colonies that had a slightly different morphology were indeed different bacteria. For example, isolates 80, 81 and 82 differ only by optical characteristics (transparent, opaque and semi-transparent respectively) and all other morphological characteristics were alike and they were identified as unclassified Gammaproteobacteria, *Pseudomonas* sp. and *Bacillus pumilus*, respectively. On the other hand, numerous isolates that had different morphologies, on the same growth media, were of the same genus (for example *Bacillus* sp.).

Furthermore, it is very tricky to use media that were intended for clinical isolation with environmental samples as many bacteria, uncommon in clinical isolates, could still grow on certain media, despite manufacturer's recommendation. For example, according to the manufacturer (Difco ^{TM}), *Pseudomonas* sp. should be inhibited on *Yersinia* agar

supplemented with novobiocin and cefsulodin, but in the present study we have characterized 12 isolates of that genus on *Yersinia* selective agar. Finally, we wish to underline that no *E. coli* or *Salmonella* sp. were detected in our isolates, despite the use of growth media targeting these two groups and despite the presence of egg shells in the vermicompost.

Beyond morphological characterization, biochemical or carbon-source utilization by the isolates would have been a good way to identify common isolates. On the other hand, we could have isolated some uncommon or yet uncultured environmental microbes whose identification could have been erroneous with this methodology (253). Thus the choice of molecular identification, using the common 16S gene seemed like a good choice. In addition, DNA identification allowed us to go beyond our initial pathogen targeting goal since we also identified benign microbes that are also active in vermicompost. This allowed us to notice the presence of several isolates who could be involved in antagonistic interactions in the vermicompost ecosystem (Chapter 3).

Table 9. Bacteria isolated from continuous vermicomposting on four sampling events, both before and after feeding interruption on day 266. Isolates phylogeny and comparison with closest relative on GenBank as well as isolate morphological descriptions are provided.

Table 9 (1/7)

	Phylogeny[1]												Morphology					
	Isolate						Closest relative						Isolate					
	No.[2]	ACC#[4]	Seq. lenght (bp)	%C[1]	Genus	Possible species[3]	Max ID[4]	ACC#[4]	Description[4]	Med[6]	ID[7]	Size[8] (mm)	Sha[9]	Ele[10]	Con[11]	OC[12]	Tex[13]	Colour$_{24\text{-}48h(48\text{-}96h)}$[12]
phylum Actinobacteria (4)																		
class Actinobacteria (4)																		
subclass Actinobacteridae (4)																		
order Actinomycetales (4)																		
suborder Streptomycineae (1)																		
family Streptomycetaceae (1)																		
genus Streptomyces (1)																		
	124		1337	100%	Streptomyces		100%	AY944250.1	Actinomycetales bacterium N12 [PS]	LB	F2	< 0.5	P			T(O)	S	(Reddish, concentric orange rings)
suborder Micrococcineae (3)																		
family Brevibacteriaceae (1)																		
genus Brevibacterium (1)																		
	126		1340	100%	Brevibacterium		99% *	AY299093.1	Brevibacterium aureum strain Enb17 [PS]	LB	F4	< 0.5 (2)	P		(R)	T(O)	S	Translucent (Creamy-Yellow)
family Microbacteriaceae (2)																		
genus Agromyces (1)																		
	123		1335	100%	Agromyces		98%	AY452074.1	Agromyces sp. CM01 [PS]	LB	F1	< 0.5 (1)	P			T	S	Translucent (Bright-yellow)
genus Microbacterium (1)																		
	118		1351	100%	Microbacterium sp.		98%	AY040877.1	Microbacterium sp. ASD [PS]	LB	B2	10	C	F	D	O	R	Creamy white (Yellowish)
phylum Firmicutes (47)																		
class Bacilli (46)																		
order Lactobacillales (1)																		
family Enterococcaceae (1)																		
genus Enterococcus (1)																		
	64a		1407	100%	Enterococcus sp.		99% *	AM084029.1	Enterococcus sp. R-25205 [PS]	Ent	A	0.5	C	C	R	O	S	Dark red
order Bacillales (45)																		
family Staphylococcaceae (2)																		
genus Staphylococcus (2)																		
	38		985	100%	Staphylococcus sp.		99% *	AJ969171.1	Staphylococcus sp. R-23212 strain R-23212 [PS]	LB	F	0.5	C	F	R	O	S	Pure white
	65		1387	100%	Staphylococcus sp.		99% *	AB188210.1	Staphylococcus sp. TUT1203 [PS]	Sta	A2	3	C	F	R	O	S	(Darker orange with concentric rings)
family Bacillaceae (43)																		
genus Oceanobacillus (3)																		
	43		1206	100%	Oceanobacillus *iheyensis*		99%	BA000028.3	Oceanobacillus iheyensis HTE831 [CG]	Sta	E	1	C	F	L	T	S	Grey-White
	44		1388	100%	Oceanobacillus *iheyensis*		99%	BA000028.3	Oceanobacillus iheyensis HTE831 [CG]	Sta	F	1	B	F	B	T	S	White-Grey
	78		1390	100%	Oceanobacillus sp.		97%	AY345420.1	Bacterium K2-35 [PS]	Sta	G2	< 0.5	P					(Beige-Dark-Red Bull's eye)
genus Halobacillus (1)																		
	21		1398	100%	Halobacillus *trueperi*		99% *	AY505522.1	Halobacillus trueperi strain GSP38 [PS]	Sta	A	1	C	F	R	O	S	Mat Yellow-Grey

Table 9 *Continued* (2/7)

#		Phylogeny[1]									Morphology								
		Isolate						Closest relative			Isolate								
		No.[2]	ACC#[4]	Seq. lenght (bp)	%C[1]	Genus	Possible species[3]	Max ID[4]	ACC#[4]	Description[4]	Med[6]	ID[7]	Size[8] (mm)	Sha[9]	Ele[10]	Con[11]	OC[12]	Tex[13]	Colour[12] 24-48h(48-96h)
37	genus Bacillus (37)																		
38		9		1385	100%		Bacillus *licheniformis*	99% *	AB219153.1	Bacillus licheniformis strain:SSH4 [PS]	Sta	B	3.5	C	C	R	S	S	Shiny White-Grey
39		10		1212	100%		Bacillus *cereus*	100% *	AY224388.1	Bacillus cereus strain BGSC 6A5 rrnM operon [CS]	Lev	A	4	C	F	R	O	R	Beige-Pink
40		17		1321	100%		Bacillus sp.	99%	CP000001.1	Bacillus cereus E33L [CG]	LB	D	7	I	F	R	O	S	Creamy-White mat
41		18		1350	100%		Bacillus *cereus*	100% *	AY224388.1	Bacillus cereus strain BGSC 6A5 rrnM operon [CS]	LB	B	10	C	F	D	O	R	Creamy-White
42		22		1396	100%		Bacillus *licheniformis*	99% *	AY291582.1	Bacillus licheniformis strain GXN151 [PS]	Sta	B	1-2	C	F	R	S	S	Shiny Grey-White
43		23		1350	100%		Bacillus *pumilus*	100% *	AB098578.1	Bacillus pumilus gene [PS]	Sta	C	< 0.5	P	F	R	S	S	Grey
44		24		1199	100%		Bacillus sp.	100% *	DQ167473.1	Bacillus licheniformis strain HDM02 [PS]	Sta	D	0.05	P	F	D	S	R	Grey
45		34		1147	100%		Bacillus *cereus*	100% *	AY224385.1	Bacillus cereus strain BGSC 6A5 rrnI and rrnJ operons [CS]	LB	B	10	C	F	D	O	R	Creamy-White
46		35		1374	100%		Bacillus *cereus*	100%	CP000001.1	Bacillus cereus E33L [CG]	LB	C	7	C	F	D	O	R	Mat White
47		37		1179	99%		Bacillus *licheniformis*	100% *	AB189316.1	Bacillus sp. SD-B1 [PS]	LB	D	3	C	C	R	O	S	White
48		39		1349	100%		Bacillus *pumilus*	100% *	AB098578.1	Bacillus pumilus gene [PS]	Sta	A	1	C	F	R	O	S	Mat Yellow-Grey
49		40		1199	100%		Bacillus sp.	99% *	AY162133.1	Bacillus subtilis strain BZ15 [PS]	Sta	B	1-2	C	F	R	S	S	Shiny Grey-White
50		41		1205	100%		Bacillus *pumilus*	100% *	AB098578.1	Bacillus pumilus [PS]	Sta	C	< 0.5	P	F	R	S	S	Grey
51		42		1209	100%		Bacillus sp.	100% *	AY505499.1	Bacillus aquimaris strain GSP18 [PS]	Sta	D	1	C	F	L	S	R	Yellowish
52		45		1325	100%		Bacillus *cereus*	100% *	AY224388.1	Bacillus cereus strain BGSC 6A5 rrnM operon [CS]	Lev	A	1-2	C	F	L	O	R	Beige-Pink
53		48		1204	100%		Bacillus *pumilus*	99% *	AB098578.1	Bacillus pumilus gene PS	Lev	E	1	C	F	L	O	S	
54		59		1368	100%		Bacillus sp.	99% *	AY224385.1	Bacillus cereus strain BGSC 6A5 rrnI and rrnJ operons [CS]	LB	B	10	C	F	D	O	R	Creamy-White

Table 9 *Continued* (3/7)

	Phylogeny[1]								Morphology								
	Isolate					Closest relative			Isolate								
	No.[2]	ACC#[4]	Seq. lenght (bp)	%C[1]	Genus	Possible species[3]	Max ID[4]	ACC#[4]	Description[4]	Med[6]	ID[7]	Size[8] (mm)	Sha[9]	Ele[10]	Con[11]	OC[12]	Tex[13]	Colour[24-48h(48-96h)][12]
genus Bacillus (37)																		
	61		774	100%		Bacillus sp.	100%	AJ315064.1	Bacillus sp. 19496 [PS]	LB	E	4	C	F	D	T	S	Translucent-White
	62		1203	100%		Bacillus sp.	99% *	AJ542508.1	Bacillus balaviensis strain LMG 21833 [PS]	LB	F	2	R	F	R	S	S	Creamy-White
	64b		1205	100%		Bacillus *pumilus*	100% *	AB098578.1	Bacillus pumilus gene PS	Sta	A1	2	C	F	R	O	S	Mat Yellow-Grey (lighter color)
	66		1399	100%		Bacillus sp.	100% *	DQ400916.1	Bacillus subtilis strain 3A25 [PS]	Sta	B1	1-2	Cl* (C)	F	R	S	S	Shiny Grey-White
	67		1383	100%		Bacillus sp.	98% *	AB219153.1	Bacillus licheniformis strain:SSH4 [PS]	Sta	B2	1-2	Cl* (C)	F	R	S	S	Shiny Grey-White
	68		688	100%		Bacillus *megaterium*	99% *	AY505511.1	Bacillus megaterium strain GSP55 [PS]	Sta	C1	< 0.5 (2)	P	F	R	S	S	Grey (Mat Grey)
	70		1207	100%		Bacillus sp.	100% *	AY505499.1	Bacillus aquimaris strain GSP18 [PS]	Sta	D1	2	C	F	DL	O	R	Yellow with concentric orange rings)
	71		1207	100%		Bacillus sp.	100% *	AY505499.1	Bacillus aquimaris strain GSP18 [PS]	Sta	D2	3	C	F	DL	O	R	Yellowish (Bright Yellow with concentric orange rings)
	72		412	100%		Bacillus sp.	94% *	AY224388.1	Bacillus cereus strain BGSC 6A5 rrnM operon [CS]	Sta	E1		C	F	L	O(S)	S	Grey (Center semi-translucent)
	73		554	99%		Bacillus sp.	100%	AJ315064.1	Bacillus sp. 19496 [PS]	Sta	E2		C	F	L	O	S	Grey
	77		1405	100%		Bacillus sp.	99% *	AY124766.1	Bacillus sp. FP1/2002 [PS]	Sta	G1	(< 0.5)	(P)					(Beige-Dark Red Bull's eye)
	79		1191	100%		Bacillus sp.	98%	AY904032.1	Bacillus neonatiensis SMC 4352-1 [PS]	Sta	H	2	C	F	R	O	S	Mat Yellow-Grey (Reddish)
	82		1175	100%		Bacillus *pumilus*	100% *	AB098578.1	Bacillus pumilus [PS]	Lev	A3	1-2	C	F	L	O(S)	R	Beige-Pink
	85		1374	100%		Bacillus sp.	99% *	AY224388.1	Bacillus cereus strain BGSC 6A5 rrnM operon [CS]	Lev	B2	1-2	C	F	R	S	S (R)	Translucent-Pink (Pink)
	86		760	100%		Bacillus *cereus*	100% *	AY224385.1	Bacillus cereus strain BGSC 6A5 rrnI and rrnJ operons [CS]	Lev	B3	1-2	C	F	R	S	S (R)	Translucent-Pink (Pale Pink)
	115		794	100%		Bacillus sp.	100%	AJ315064.1	Bacillus sp. 19496 [PS]	LB	A2	1-2	C	F	R(L)	O	S	Milky-White
	116		1390	100%		Bacillus *firmus*	99%	AY833571.1	Bacillus firmus [PS]	LB	A3	1-2	C	F	R(L)	O	S	with dark brown concentric rays
	117		1413	100%		Bacillus *thuringiensis*	100%	AF501348.1	Bacillus thuringiensis strain HAMBI2389 [PS]	LB	B1	10	C(I*)	F	D	O	R	Creamy-White
	119		1373	100%		Bacillus sp.	99% *	AY124766.1	Bacillus sp. FP1/2002 [PS]	LB	B3	10	C	F	D(L)	O	R	(Budding crest in center)
	120		1354	100%		Bacillus sp.	99%	AY964602.1	Bacillus sp. P01 [PS]	LB	C1	1(2)	C	F	R	T	S	Yellowish (Orangish)
unclassified Bacillaceae (2)																		
	8		1347	62%		Ornithinibacillus *pumillus*	100% *	AY112667.1	Bacillus pumilus [PS]	Sta	A	1	C	F	R	O	S	Mat Grey
	19		1161	68%		Bacillus *pumilus*	100% *	AB098578.1	Bacillus pumilus [PS]	LB	B	10	C	F	D	O	R	Creamy-White

Table 9 *Continued* (4/7)

	Phylogeny[1]									Morphology								
	Isolate						Closest relative			Isolate								
	No.[2]	ACC#[4]	Seq. lenght (bp)	%C[3]	Genus	Possible species[3]	Max ID[4]	ACC#[4]	Description[4]	Med[6]	ID[7]	Size[8] (mm)	Sha[9]	Ele[10]	Con[11]	OC[12]	Tex[13]	Colour$_{24\text{-}48h(48\text{-}96h)}$[12]
phylum Proteobacteria (60)																		
class Alphaproteobacteria (1)																		
order Rhizobiales (1)																		
family Bradyrhizobiaceae (1)																		
genus Bosea (1)																		
	91		1313	100%	Bosea	*thiooxidans*	99%	AF508803.1	Bosea thiooxidans strain BI-42 [PS]	Lev	D3	2	B	S	B	S	H	Translucent-White (Pale-Pink)
class Betaproteobacteria (3)																		
order Burkholderiales (1)																		
family Burkholderiaceae (1)																		
genus Cupriavidus (1)																		
	100		1355	100%	Cupriavidus	sp.	98%	DQ219398.1	Cupriavidus sp. MApud8.1 [PS]	Mac	C3	0.5 (1.5)	P	F	R	T(S)	S	Translucent (Pink)
order Neisseriales (2)																		
family Neisseriaceae (2)																		
unclassified_Neisseriaceae (2)																		
	84		1356	56%	Aquitalea					Lev	B1	1-2	C	F	R	S	S	(Metalic Green Sheen)
	98		1347	62%	Aquitalea		99%	AY345393.1	Bacterium G5_GreenLake [PS]	Mac	C1	0.5	P	F	R(L)	T	S	contour with brownish center)
class Gammaproteobacteria (56)																		
order Xanthomonadales (6)																		
family Xanthomonadaceae (6)																		
genus Stenotrophomonas (5)																		
	52		952	100%	Stenotrophomonas	sp.	96% *	X95923.1	S.maltophilia strain LMG 958-T [CS]	Mac	C	0.5	P	F	R	T	S	Translucent
	60		1346	100%	Stenotrophomonas	*maltophila*	99% *	DQ141193.1	Stenotrophomonas maltophilia [PS]	LB	C	1	C	F	R	T	S	Yellowish
	101		1382	100%	Stenotrophomonas	*maltophila*	99% *	DQ141193.1	Stenotrophomonas maltophilia [PS]	Mac	C4	0.5	P	F	R	T	S	Translucent
	109		1332	100%	Stenotrophomonas	*maltophila*	99% *	DQ141193.1	Stenotrophomonas maltophilia [PS]	Yer	C1	1(2)	C	C	R	T	S	Salmon-Pink (Pink-Grey)
	112		1335	100%	Stenotrophomonas	*maltophila*	100%	AY379973.1	Stenotrophomonas sp. AHL 1 [PS]	Yer	C4	1	C	C	R	T	S	Salmon-Pink
unclassified_Xanthomonadaceae (1)																		
	88		1388	52%	Stenotrophomonas	sp.	96%	CP000050.1	Xanthomonas campestris pv. campestris str. 8004 [CG]	Lev	C2	< 0.5	P	F	R	T	S	Pinkish (Pink-Orange)

Table 9 *Continued* (5/7)

	Phylogeny[1]											Morphology						
	Isolate						Closest relative					Isolate						
	No.[2]	ACC#[4]	Seq. lenght (bp)	%C[1]	Genus	Possible species[3]	Max ID[4]	ACC#[4]	Description[4]	Med[6]	ID[7]	Size[8] (mm)	Sha[9]	Ele[10]	Con[1]	OC[12]	Tex[13]	Colour[24-48h(48-96h)][12]
order Aeromonadales (7)																		
family Aeromonadaceae (7)																		
genus Aeromonas (7)																		
	20		1093	100%	Aeromonas sp.		99% *	AY345394.1	Bacterium G3_GreenLake [PS]	LB	A	2	C	C	R	O	S	Milky-White
	33		642	100%	Aeromonas sp.		100%	AF468055.1	Aeromonas hydrophila strain 45/90 [PS]	LB	A	2	C	F	R	O	S	Milky-White
	53		1344	100%	Aeromonas sp.		100%	AY345394.1	Bacterium G3_GreenLake [PS]	Yer	A	2	C	F	R	O	S	Pink-Purple
	58		1345	100%	Aeromonas sp.		98% *	AF468055.1	Aeromonas hydrophila strain 45/90 [PS]	LB	A	1-2	C	F	R	O	S	Milky-White
	96		1367	100%	Aeromonas *hydrophila*		99% *	AF468055.1	Aeromonas hydrophila strain 45/90 [PS]	Mac	B1	1-2	C	F	R	S	S	Salmon-Pink (Greyish-Pink)
	105		1366	100%	Aeromonas sp.		99%	AF099027.1	Aeromonas sp. strain T8 [PS]	Yer	A3	2	C	S	R	O	S	Pink-Purple Bull's eye
	114		1345	100%	Aeromonas *hydrophila*		99% *	AF468055.1	Aeromonas hydrophila strain 45/90 [PS]	LB	A1	1-2	C	F	R	O(S)	S	Milky-White (Orangish)
order Enterobacteriales (15)																		
family Enterobacteriaceae (15)																		
genus Serratia (1)																		
	54		1365	100%	Serratia *marcescens*		99%	AY043386.1	Serratia marcescens strain AU736 [PS]	Yer	A	2	C	F	R	O	S	Pink-Purple
genus Citrobacter (3)																		
	31		1340	100%	Citrobacter *freundii*		99%	AB210978.1	Citrobacter freundii strain: SSCT56 [PS]	Yer	A	2	C	F	R	O	S	Pink-Purple
	49		1353	99%	Citrobacter sp.		99%	DQ010114.1	Citrobacter freundii strain HQ010516B-1 [PS]	Lev	F	1-2	C	F	R	S	R	Translucent-Purple
	104		1356	98%	Citrobacter sp.		99%	DQ010114.1	Citrobacter freundii strain HQ010516B-1 [PS]	Yer	A2	2	C	S	R	O	S	eye (Thin Beige outskirt and Yellow-
genus Klebsiella (6)																		
	2		598	100%	Klebsiella sp.		100% *	AY517552.1	Klebsiella sp. YHB [PS]	Mac	A	3	C	C	R	O	S	Pink Bull's eye
	3		846	100%	Klebsiella *pneumoniae*		99% *	AY114159.1	Klebsiella pneumoniae [PS]	Mac	A	3	C	C	R	O	S	Pink Bull's eye (Purple Bull's eye)
	11		1094	100%	Klebsiella sp		99% *	U31075.1	Klebsiella sp. strain zmmo [CS]	Lev	B	2	C	C	R	O	S	Translucent-Pink
	26		1375	100%	Klebsiella *pneumoniae*		99% *	AF130981.1	Klebsiella pneumoniae PS	Lev	B	1-2	C	F	R	S	S	Translucent-Pink
	46		1379	100%	Klebsiella *pneumoniae*		99% *	AF130981.1	Klebsiella pneumoniae [PS]	Lev	B	1-2	C	F	R	S	S	Translucent-Pink
	50		1341	100%	Klebsiella *pneumoniae*		99%	AF228920.1	Klebsiella pneumoniae [PS]	Mac	A	1-3	C	F	R	O	S	rose
unclassified Enterobacteriaceae (5)																		
	12		1093	58%	Enterobacter sp		98%	AB098582.1	Enterobacter sp. TUT1014 [PS]	Lev	C	1.5	C	F	R	O	S	Beige-Pink Bull's eye
	27		366	41%	Enterobacter		100%	AY345447.1	Bacterium KA55 [PS]	Lev	C	1-2	C	F	R	S	S	Translucent-Purple
	4		621	54%	Citrobacter sp.		98%	AB210978.1	Citrobacter freundii strain: SSCT56 [PS]	Yer	A	2	C	F	R	O	S	Purple Red Bull's eye
	13		463	64%	Citrobacter sp.		98% *	AB210978.1	Citrobacter freundii strain: SSCT56 [PS]	Lev	D	2	C	F	R	O	S	Beige-Purple Bull's eye
	94		1021	92%	Citrobacter sp.		99%	AF025373.1	Citrobacter werkmanii [PS]	Mac	A1	1-3	C	F	R	O	S	Pink

Table 9 *Continued* (6/7)

	Phylogeny[1]									Morphology								
	Isolate						Closest relative			Isolate								
	No.[2]	ACC#[4]	Seq. lenght (bp)	%C[1]	Genus	Possible species[3]	Max ID[4]	ACC#[4]	Description[4]	Med[6]	ID[7]	Size[8] (mm)	Sha[9]	Ele[10]	Con[11]	OC[12]	Tex[13]	Colour$_{24\text{-}48h(48\text{-}96h)}$[12]
order Pseudomonadales (25) family Pseudomonadaceae (25) genus Pseudomonas (25)																		
	1		586	100%	Pseudomonas sp.		100% *	AY230195.1	Pseudomonas sp. strain 1131 [PS]	Mac	A	3	C	C	R	O	S	Pink Bull's eye
	5		1046	100%	Pseudomonas sp.		100%	DQ305283.1	Pseudomonas sp. BCA5-1 [PS]	Yer	B	1.5	C	F	R	S	S	Beige (Yellowish)
	6		624	100%	Pseudomonas sp.		100% *	AY536741.1	Pseudomonas sp. TS1138 [CS]	Yer	B	1.5	C	F	R	S	S	Beige
	7		995	99%	Pseudomonas *plecoglossida*		99%	DQ140381.1	Pseudomonas plecoglossicida strain R2 [PS]	Yer	A	2	C	F	R	O	S	Purple-Red Bull's eye
	14		1235	100%	Pseudomonas *aeruginosa*		99%	AY548953.1	Pseudomonas aeruginosa strain Z11 [CS]	LB	E							Green
	15		1337	100%	Pseudomonas sp.		100% *	AM084037.1	Pseudomonas sp. strain R-25209 [CS]	LB	B	10	C	F	D	O	R	Creamy-White
	16		1359	100%	Pseudomonas sp.		99%	AY515308.1	Pseudomonas sp. Y2-1-1 [PS]	LB	A	2	C	C	R	O	S	Milky-White
	25		1159	100%	Pseudomonas *aeruginosa*		100% *	AY548953.1	Pseudomonas aeruginosa strain Z11 CS	Lev	A	1-2	C	F	L	O	R	Beige-Pink
	32		1308	100%	Pseudomonas *nitroreducens*		99%	AM088473.1	Pseudomonas nitroreducens strain IAM 1439 [PS]	Yer	B	1.5	C	F	R	S	S	Beige
	47		1342	100%	Pseudomonas sp.		99%	AF039488.1	Pseudomonas sp. 273 [PS]	Lev	D	< 0.5	P	F	R	T		Pinkish
	51		1377	100%	Pseudomonas *aeruginosa*		100% *	AY162139.1	Pseudomonas aeruginosa strain BHP7-6 [PS]	Mac	B	1-2	C	F	R	S	S	Salmon-Pink
	55		1320	100%	Pseudomonas sp.		98% *	DQ079062.1	Pseudomonas sp. ONBA-17 [PS]	Yer	B	1.5	C	F	R	S	S	Beige
	56		1343	100%	Pseudomonas sp.		99% *	DQ095915.1	Pseudomonas plecoglossicida [PS]	Yer	C	1	C	C	R	T	S	Salmon-Pink
	57		1322	100%	Pseudomonas sp.		99% *	DQ141542.1	Pseudomonas putida strain OW-18 [PS]	Yer	D							
	63		1362	100%	Pseudomonas *aeruginosa*		100% *	AY548953.1	Pseudomonas aeruginosa strain Z11 CS	LB	G	2	C	F	R	S	S	Yellowish-Green

Table 9 *Continued* (7/7)

	Phylogeny[1]									Morphology								
	Isolate						Closest relative			Isolate								
	No.[2]	ACC#[4]	Seq. lenght (bp)	%C[1]	Genus	Possible species[3]	Max ID[4]	ACC#[4]	Description[4]	Med[6]	ID[7]	Size[8] (mm)	Sha[9]	Ele[10]	Con[11]	OC[12]	Tex[13]	Colour[12] 24-48h(48-96h)
unclassified_Gammaproteobacteria (3)	81		1379	100%	Pseudomonas	sp.	99%	AF039488.1	Pseudomonas sp. 273 PS	Lev	A2	1-2	C	F	L	O	R	Beige-Pink (Pinkish)
	95		1359	100%	Pseudomonas	sp.	99% *	DQ079062.1	Pseudomonas sp. ONBA-17 PS	Mac	A2	1-3	C	F	R	O	S	Pink
	97		1380	100%	Pseudomonas	sp.	99%	AF039488.1	Pseudomonas sp. 273 PS	Mac	B2	1-2	C	F	R	S(O)	S	Salmon-Pink (Dark Pink)
	99		1379	100%	Pseudomonas	sp.	100% *	AM084037.1	Pseudomonas sp. strain R-25209 CS	Mac	C2	0.5 (1.5)	P	F	R	T	S	Translucent
	107		1342	100%	Pseudomonas	sp.	99% *	DQ079062.1	Pseudomonas sp. ONBA-17 PS	Yer	B1	1.5	C	F	R	S(O)	S	Beige (Creamy-Pink)
	108		1081	100%	Pseudomonas	sp.	99% *	AY464946.1	Pseudomonas sp. DU21490/00 PS	Yer	B2	1.5	C	F	R	S(T)	S	Beige (Pink)
	110		1359	100%	Pseudomonas	sp.	99%	AY464946.1	Pseudomonas sp. DU21490/00 PS	Yer	C2	1	C	C	R	T	S	Grey with darker center)
	111		1358	100%	Pseudomonas	sp.	99%	AF039488.1	Pseudomonas sp. 273 PS	Yer	C3	1	C	C	R	T	S	Pink with darker center)
	113		1340	100%	Pseudomonas	*plecoglossida*	99%	DQ095907.1	Pseudomonas plecoglossicida strain S19 PS	Yer	C5	1(8)	C	C	R	T	S(W)	Salmon-Pink (Pink)
	129		1351	100%	Pseudomonas	sp.	99%	DQ079062.1	Pseudomonas sp. ONBA-17 PS									
	80		1343	17%	Flavimonas					Lev	A1	1-2	C	F	L	O(T)	R	Beige-Pink
	83		1366	27%	Flavimonas					Lev	A4	1-2	C	F	L	O	R	Beige-Pink
	102		1329	15%	Flavimonas					Mac	C5	0.5	P	F	R	T	S(H)	Translucent (Cark pink)

Table 9 *Notes*

[1] Phylogenetic assignment based on (209); % C, Percent confidence; [2] Isolates 1-20, 21-38, 39-64a, 64b-129 collected on experimental day 218, 266, 329 and 378 respectively; [3] If genus assignment is above the 95 % confidence treshold, a possible species is suggested based on GenBank Blastn search only if no other species have an equal maximal identity score (if Blastn confirms genus only sp. is written and if genus assignment differs between (209) and GenBank, this column is left empty); [4] If the maximum identity of the isolates is greater than 90 % to a sequence deposited in GenBank, the accession number, description (CG, complete genome; CS, complete sequence; PS, 16S rRNA gene partial sequence) and maximum identity score of the closest relative is given; [5] If more than one sequence in GenBank has an equivalent maximum identity, e-value and maximum score, an asterix highlights that there are more than one closest relative in GenBank; [6] Growth and Isolation media: LB, Luria-Bertani agar; Ent, m-enterococcus agar; Lev, Levine-Eosine Methylene Blue agar ; Mac, MacConkey agar; Sta, m-Staphylococcus agar; Yer, Yersinia cefuldionin and novobiocyn agar; [7] Independent identification letters were assigned during morphotyping on each different sampling day and isolates with a similar letter on the same sampling day have a very similar colony morphology and subsequent number denotes very slight variation from the 24-48 h morphology based on observations upon isolation after 48-96 h; [8] The approximate average sizes colonies is based on observations after 24-48 h; [9] Shape of colonies: C, circular; P, punctiforme; B, branching; I, irregular; *, invasive; R, circular with a raised center; [10] Elevation of colonies: F, flat; C, convexe; S, slightly convexe; 11 Contour of colonies: R, regular; L, lobed; O, ondulated or dented, B, branched; [12] Optical characteristics of colonies: T, translucent; O, opaque; S, semi-opaque; 13 Texture of colonies: S, smooth; R, rough; W, Wrinkled; H, Hairy; [14] Colors after 24-48h incubation.

Conclusion

In this research, we have described vermicomposting using an interdisciplinary approach to better grasp the complex ecological and physico-chemical interactions that characterize this environmental biotechnology. We have introduced the discussion by reviewing the current state of knowledge on the microbial ecology of vermicomposting and the interactions between the earthworms and the microbiota. The major highlights from this literature review are that earthworms influence the microbiota indirectly through alterations of the physico-chemical environment and directly by grazing on microbial populations. We emphasized the importance of vermicompost sanitation and exposed the major biotic factors involved, as well as the modulating abiotic effects. In the second chapter, we showed that continuous vermicomposting was characterized by an initiation phase, but we observed no clear stabilization of major physico-chemical parameters indicative of an equilibrium state. Finally, we showed that interrupting regular food input affected major physico-chemical parameters and the microbial diversity, but not the overall microbial abundance. Vermicomposting was shown to rapidly reduce *E. coli* populations below legal sanitation guidelines in both continuous and batch systems. Antagonism with the microbiota of the vermicompost was the main mechanism involved in sanitation, although earthworms had an effect in batch systems. To our knowledge, this research was the first one to reveal the composition of the vermicompost bacterial community and we showed that vermicompost harbours both opportunistic bacterial pathogens and bacterial antagonists possibly involved in reducing animal and phytopathogens during vermicomposting. We were not able to determine that volatile substances played a role in regulating the levels of *E. coli* in the vermicompost.

The main conclusions from this research are that the major mechanisms involved in controlling bacterial pathogen in vermicompost are biological, but fluctuating abiotic factors influence the composition of the bacterial community. The specific interactions between antagonistic microorganisms and pathogens in the context of vermicomposting are yet to be characterized. Further research on the sanitation process of vermicomposting are necessary, especially concerning species other than the common indicators, survival of spore-forming bacteria and persistence of bacterial toxins in the vermicompost. Nevertheless, we feel that vermicomposting can be a safe organic waste management practice, provided that operational recommendations described therein are followed.

We have entered an era where environmental consciousness is rising rapidly. Science deepens the understanding of our world and unravels complex interactions between the elements and living beings, from the infinitely small mechanisms to the global ecosystems, over the instant of a breath or over geological time scales. Social and political spheres have initiated vital debates concerning the protection of our planet and several individuals and communities are extending beyond promoting popular awakening; engaging in concrete actions to rectify and prevent neglectful behaviours. Better management of organic waste, through composting or fermentation technologies, are perfect examples of sound applications of scientific knowledge. Focusing research efforts on practical sustainable solutions should not shade the quest for pure wisdom and should be contextualized in the interdisciplinary web of knowledge. Protecting our air, water and soil is vital. We need visionaries to seek inspiration beyond imagination to ensure

that all humans and living beings can share natural resources equitably as long as our sun is shining.

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