FINAL REPORT

HEAT AND COMPOSTING EFFECTS ON THE VIABILITY OF *PHYTOPHTHORA RAMORUM*, WHEN GROWN ON A VARIETY OF ARTIFICIAL AND PLANT SUBSTRATES

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Aims: To determine the effects of heat treatment and composting upon the viability of the plant pathogen *Phytophthora ramorum* grown on both artificial and a variety of natural substrates.
Methods and Results: *P. ramorum* was grown on V-8 agar, on the leaves of bay laurel, *Umbellularia californica*, and on woody tissues of coast live oak, *Quercus agrifolia*. Inhibition of growth was measured as a result of treatment in ovens and compost piles. Direct plating onto PARP media and pear baiting techniques were used to determine post treatment viability. No viable *P. ramorum* was ever detected at the end of the composting process, regardless of the isolation technique used. By using a PCR assay designed to exclusively detect the DNA *of P. ramorum*, we were able to conclude the pathogen was absent from mature compost, and not merely suppressed.
Conclusions: Composting for a period of two weeks at 55 ^{o}C eliminates *P. ramorum* to lower than detectable levels on all substrates tested.
Significance and Impact of the Study: Composting is a viable treatment option for sanitization of infected plant material.

INTRODUCTION

Phytophthora ramorum, the causal agent of the disease commonly referred to as Sudden Oak Death, is presumed to be an introduced pathogen (Rizzo and Garbelotto, 2003, Ivors et al. 2004). This oomycete can infect not only oaks and the related tanoaks, but also a number of other plant species, including huckleberry, bay laurel, pacific madrone, toyon, manzanita, big leaf maple, and buckeye (Rizzo and Garbelotto, 2003). The host range also includes several plant species widely used in the nursery trade (Davidson *et al* 2003). Leaves of California bay laurel (*Umbellularia californica)* can remain infectious for several weeks, even when dry (Dave Rizzo, personal communication), and viable cultures can be obtained from dead and down logs and from fire wood (Everett Hansen and Jenny Davidson, personal communications), thus, sanitation of infected material remains a serious issue for extended periods of time.

The number of infected plants growing in landscaped and wild-land areas of California is difficult to estimate, but is likely to be in excess of tens of thousands. New plant species, displaying a variety of symptoms, continue to be added to the host list (Garbelotto, 2003, Murphy and Rizzo, 2003, Hüberli *et al* 2003, Hüberli et al 2004). Furthermore, the disease is spreading to new locations on a routine basis, both through infected nursery stock and perhaps other unknown means (Werres *et al* 2001, Davidson *et al* 2001, Orlikowski and Szkuta 2002, COMTF 2004). Given the broad host range of this pathogen and its ability to infect not only the stems, but also the leaves and twigs of susceptible hosts, it is likely the pathogen may be carried into composting facilities via infected green waste. Whether the sale and transport of the compost produced in these facilities should be restricted depends on the ability of the composting process to eliminate all *P. ramorum* inoculum (Paswater 2003).

While several studies have focused on the suppressive effects of compost on plant pathogens, i.e. the inhibition of pathogen growth and sporulation (e.g. Hoitink *et al* 1976, Spring *et al* 1980, Burge *et al* 1981, Bollen 1985, Lopez-Real and Foster 1985, Hoitink and Fahy 1986, Bollen 1993, Déportes *et al* 1998, Labrie *et al* 2001, Suarez-Estrella *et al* 2003), few have actually addressed pathogen eradication in the compost substrate (Hoitink *et al* 1976, Hardy and Sivasithamparam 1991). In studies on bacteria pathogenic to humans, eradication through composting of sewage sludge and municipal solid waste (EPA 1989, Hay 1996) was regarded as successful if pathogen levels were reduced to certain threshold levels (EPA 1989, Hay 1996) Since *P. ramorum* is an extremely aggressive plant pathogen (Linderman *et al,* 2002) with limited geographic distribution, regulations have been designed to avoid its introduction into new areas (http://www.aphis.usda.gov/lpa/issues/sod/sod.html). In order for any product to be regarded as

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free of *P. ramorum*, the pathogen must be undetectable or at a minimum, pathogen concentration should be more than 11 log below the original concentration (Burge *et al* 1981).

At least three main processes are involved in pathogen suppression while composting plant material: (1) high temperatures (typically 40-70 ^{o}C) reached during the thermophilic composting phase (Burge *et al* 1981, Yuen and Raabe 1984, Bollen 1985); (2) colonization of compost by a variety of different microorganisms that either prey on or out compete the target microbes(s); and, 3) the production of antibiotic compounds by organisms involved in the composting process (Hoitink *et al.* 1997, Hoitink and Boehm 1999, McKellar and Nelson 2003). However, because of the extreme complexity and variability of the microbial populations involved in these processes, "temperature and time" monitoring is the most practical and standardized monitoring variables (Burge *et al* 1981). It is also likely that heat may be the most relevant of the three processes mentioned above (Hoitink *et al* 1976, Bollen 1985). In our experiments, composting temperature parameters always met the EPA PFRP standards designated for commercial composting (EPA 1989).

In this study, we describe several experiments designed to test both heat treatments and composting as sanitation tools for plant material infected by *P. ramorum*. The first experiment is a "time x temperature" study conducted on *P. ramorum* grown on V-8 agar in order to determine conditions necessary to kill colonies of this pathogen in vitro. The second and third experiments investigate the effects of heat-treatments and composting on plant material infected by *P. ramorum,* by placing limited amounts of infected plant material in incubators or in compost piles. We finally present a case study in which a turned windrow compost pile was created using large amounts of plant material infected by *P. ramorum*. Questions addressed by the last study include the contagion risk posed by heavily infected compost piles, the variability of sanitization within a heavily infected, and whether or not composting eliminates the pathogen when infection levels are extremely high.

MATERIALS AND METHODS

Preparation and comparison of different plant substrates colonized by *P. ramorum*

Wood chips. Wood chips were excised from cankers on living *Q. agrifolia* trees in Marin County. Chips were taken from both naturally and artificially infected trees. Chips from each tree were bagged separately and cultured for *P. ramorum* prior to use. Experiments were started within 48 hours of collection.

Stem sections. Coast live oak saplings (2-4 cm diameter) were inoculated by using a cork borer to excise a section of bark, and by placing a plug of inoculum in contact with the cambium and the outer xylem. The bark plug was then replaced on top of the inoculation point, and the wound was sealed with grafting wax before being wrapped in foil. Stems were inoculated at 30 cm-intervals: two inoculations were performed on opposite sides of the stem on each 30-cm segment. After 12-15 weeks, individual segments were cut from the stem and kept with one end in water in the laboratory until the beginning of each experiment. These inoculations will hereafter be referred to as cankers.

Laurel leaves. Bay laurel leaves were collected from ten different trees on the Berkeley U.C. campus, in order to account for potential differential susceptibility and/or resistance of different individuals. A slurry of water and V-8 agar (Erwin & Ribeiro, 1996) inoculated with a mixture of *P. ramorum* isolates was poured over the leaves. In experiment 1, leaves were scratched prior to inoculation. In experiments 2-4, leaves were unscratched, as scratching seemed to make little difference in the ultimate infection levels found on the leaves. Leaves were layered with moist paper towels and high humidity was maintained by keeping them in a sealed plastic container. Lesions started to develop after a few days, and were allowed to proceed for about 10-14 days before leaves were ready for the experiment.

Experiment 1, The effect of dry heat on colony growth and sporulation of *P. ramorum*

Three different isolates of P *ramorum* were used for this study: O4 (CBS 110534), O7 (not submitted), and O13 (ATCC MYA-2434). Each isolate was subcultured on 5% V8 growth media and allowed to grow at 18-20^{o}C until the colony diameter had reached approximately 1 cm. Three replicates of each isolate were used. Once colonies had reached the 1 cm diameter threshold they were placed in incubators whose temperatures had been set at the following temperatures: 22, 35, 45, or 55 ^{o}C. Exposure times for each temperature were as follows: 30 min, 1, 2, 4, and 24 h. Growth was monitored at 5, 8, and 13 days from exposure. A random subset of plates was also observed at the microscope for presence of reproductive

and/or resting structures such as sporangia and chlamydospores. This was done by randomly analyzing three 1 cm^2 areas on each plate. If sporangia or chlamydospores were present, plates were flooded with sterile water and the suspended structures were plated on clean V8 plates and germination was checked. A second experiment (experiment 1b) was performed by using an identical approach but testing growth at 22 ^oC and 40 ^oC only.

Experiments 2 and 3, The effect of dry heat and composting on infected plant material

Inoculation substrate Laurel leaves infected with two different strains of *P. ramorum* were used for this study, as described previously. The *P. ramorum* strains used for infection were: Pr52 (CBS110537; ATCC MYA-2436) and Pr102 (ATCC MYA-2949).

Oven Trials The infected plant material was placed in an oven and held at 55 ^oC for two weeks. After treatment, the material was kept for a week at 20 ^oC before attempting isolation of *P. ramorum* (isolation technique outlined in *Sampling,* below).

Treatment for turned windrow compost Two locations in Marin County, California were selected, and a single compost pile with four replications was used in each location. Each replicate was represented by an experimental unit consisting of ten infected *Quercus agrifolia* wood chips contained in a nylon mesh bag, three stem sections of *Q. agrifolia* saplings bearing active cankers, ten infected *Umbellularia californica* leaves contained in a nylon mesh bag, and a digital temperature recorder. The stems were drilled through their tops and held together with the mesh bags and temperature recorder with gardening wire. The wood chips were excised from infected cankers on living *Q. agrifolia* trees in Marin county, while the infected *U. californica* leaves and *Q. agrifolia* stem cankers were produced by artificial inoculation as previously described. This design resulted in four experimental units per pile, each of which contained three subunits: leaves, chips, or stems. Four additional experimental units were used as a control, and kept at 20 ^oC for the duration of the experiment. Experimental units were placed at 10m from the end of the pile, at 50-100 cm beneath the pile surface, and approximately 2/3 the way up each side. The compost piles were turned a minimum of five times during this experiment, and the experimental units were carefully removed and replaced every time the pile was turned. The compost pile at Site 1 was approximately 18 m long, 4 m wide and 1.5 m tall, and was a mix of un-ground landscape debris amended with 25% horse stall tailings (manure mixed with wood chips), 28% cow manure, and a very small percentage of olive waste. The pile at Site 2 was approximately 20 m long, 8 m wide, and 2.5 m tall, and was composed of a mix of ground green waste. The experiment took place during April 2002 and was replicated in May of the same year.

Treatment for forced-air static pile compost Two trials were carried out at a single location in Sonoma County, California, in September and October of 2002. Two compost piles each containing four replications were employed in the first trial, while only a single compost pile, again containing four replicates, was employed for the second trial. Each replicate and the corresponding controls were represented by experimental units, which were assembled as described above for turned windrow composting, and inserted similarly into the compost piles. Each compost pile was approximately 25 m long, 6 m wide and 2.5 m tall. The piles were a mix of ground landscape debris subsequently augmented with either manure or chemical fertilizers in order to achieve the correct nutrient and pH balance for composting. Air was mechanically blown into perforated pipes, which were placed beneath each compost pile as needed to maintain adequate aeration and temperature, initially for several hours and subsequently for about two hours per day. Isolations were performed from all substrates before placing the experimental units into the piles. All plant material from each experimental unit was directly plated after two weeks as described above. Untreated experimental units (controls) were stored at 12 C rather than at 20 ^oC as described above for trials on windrow piles.

Sampling Viability of the pathogen was determined by counting how many sample fragments (normally about 1 square mm in size) formed colonies, when plated on P_{10}ARP (PARP) selective media (Erwin & Ribeiro, 1996, modified to 25µg/ml PCNB). If colony morphology was dubious, a polymerase chain reaction (PCR) test was done using selective primers (Hayden et al. 2004) to confirm the species identification. Each wood chip, leaf, or stem was sampled directly onto PARP selective media just before the experimental units were placed into the piles, and then were sampled by the same method again at the end of the experiment. PARP plates were incubated at 20 ^oC and scored at two weeks from the time of inoculation. Additionally, each bag of material or group of stems was also baited with green, un-ripe D'Anjou pears at the end of the experiment. Pears were placed in water for four days and then left to dry

on paper towels for an additional four to five days, all at 12 °C. Lesions developing on pears were then plated onto PARP, and plates were scored in two weeks. For each experimental unit, 10 leaves, 10 chips and 6 stem samples were plated. Each substrate from each EU was baited separately with a pear. Five isolations per pear were attempted and baiting was considered successful if when at least one P. ramorum colony was formed on the plate. All plates were re-inspected after 8 weeks.

Statistical analysis Two analyses were performed on the data.

1)- Overall effectiveness of composting, using the difference of means test: The effect of each treatment was determined by comparing culturing and baiting data for all substrates combined, before and after treatment. Identical data for untreated controls was used as a term of comparison. Data from replicated trials was also combined together, but analyses for each site and treatment type were performed separately.. Mean survival rates were calculated for each experimental unit, and the differences of means between treatments and controls calculated. Such differences express the average change in the number of surviving subsamples per experimental unit, per treatment. 99% confidence intervals were also calculated based on standard error values for each mean .

2)- Effectiveness of composting by substrate, using a difference of means test: Isolation success and baiting success were pooled for each substrate, and forced air and turned windrow treatments were combined for each substrate. This was done for both pre-treatment and post-treatment data. All substrates were considered as equal to one another, as the question being investigated in this case is not whether there are differences between substrates, but whether heat treatment and/or composting is effective against typically infected substrate types. This data was then analyzed using a difference of means approach, as in (1), above.

Experiment 4, Direct measurement of survival rates after composting

Inoculation substrate More than one cubic meter of *U. californica* leaves were collected from multiple trees, and placed into plastic greenhouse planting flats. The flats were flooded with de-ionized water and inoculated as described for experiments 2 and 3 using isolates Pr52 and Pr102. Resulting lesions were approximately three times larger than those typically seen on naturally infected leaves (Figure 1).

Initial inoculum levels Viability of the pathogen was determined by counting how many plated leaf fragments (normally about 4 square mm in size) gave rise to a viable colony on PARP selective media after two weeks. Sample size was 35 leaves, randomly selected.

Compost pile The leaves were thoroughly mixed with 9 cubic meters of raw material from Sonoma Compost, resulting in a compost pile composition of at least 10 percent highly infectious material. Because the artificially inoculated leaves are more thoroughly colonized than naturally infected leaves, we estimate the level of infection in the pile was comparable to at least 30% using naturally infected bay leaves (figure 1). The uncovered pile was wetted heavily upon assembly on April 3, 2003 (figure 5), and then irrigated on April 4 to produce enough runoff for sampling. After runoff data had been collected (see *Runoff collection,* below) the pile was turned on April 8th and 12th in an attempt to dry it, as moisture content was above optimal the 50% moisture content optimum (Laisin 2002). On April 14th the pile was subject to heavy rains, from which we again took runoff samples. The cold weather and excess moisture caused pile temperatures to fall, and required turnings on April 15th and 22nd to dry. Thereafter, the nutrients in the pile appear to have been exhausted, as pile temperatures did not adequately recover by May 7th, in spite of pile moisture contents in the vicinity of 50%. A small amount of fresh, high nitrogen material was added on May 11th, and the windrow was turned again May 16th and June 4th.

Temperature probes Hobo digital temperature probes were placed in 24 pre-determined locations within the pile, in three layers. The bottom layer consisted of twelve temperature probes, in a 4x3 array, the middle layer consisted of eight probes in a 4x2 array, and the top layer consisted of a 4x1 array of probes (Figure 2). The temperature probes were removed each time the pile was turned, and then returned to their location as the pile was re-assembled during turning.

Runoff collection Three water traps measuring 30cm x 30cm x 5cm depth were placed on pier blocks on a longitudinal line within the pile, and connected by hoses to collection bins on the outside of the pile (Figure 2). Runoff collected was pear baited as outlined in *Sampling* for experiments 2 and 3, above. The wetted pile was irrigated thoroughly, and we collected the runoff the morning following its initial

assembly. Ten days after the compost pile was assembled, enough rain fell to cause further runoff (figure 5), and this was collected and sampled as well. A small portion of the runoff from each basin was transferred into a cup, and zoospores of strains Pr 52 and Pr 102 were introduced to the cups as positive controls. The cups were then pear baited as above. The pile was then allowed to dry to moisture levels more conducive to the composting process (approximately 50%).

Direct plating Viability of the pathogen was determined by immersing various substrates in PARP media. For sentinel *Rhododendron* plants, a sample fragment (normally about 4 square mm in size) of any leaf tissue that appeared to be infected was collected and plated. The PARP plates were then allowed to incubate for two weeks, and were checked for growth of *P. ramorum.*

Compost dilution plate method For direct assessment of compost viability, sieved (1mm) compost was mixed into PARP media at a 1:4 ratio while it media was still molten (less than 40º C) and allowed to cool. Plates were checked for growth of *P. ramorum* after 2 and 8 weeks.

Pear baiting Two compost samples, of approximately one quarter-liter volume each, were taken within 10-15 cm of each digital temperature recorder location, for a total of 48 direct samples. The compost samples were then placed into one-gallon sealable plastic bags and flooded with de-ionized water at a 4:1 water to compost ratio as described by Tsao (1983). A single green, un-ripe D'Anjou pear was placed into each bag. The pears were allowed to sit in the water for four days and then placed to dry on paper towels for an additional four to five days, all at 12 ^{o}C. The resulting pear lesions, if found, were plated as in *Direct plating,* above.

Flood sampling Half of the compost pile was transferred into six plastic pools that had been raised up on palettes. Each pool was equipped with a filter made of wire mesh and cheese cloth enclosed in PVC pipe sections. Each filter ran through the bottom of the pool and connected to hose on the outside of the pool, which was then connected to one other pool before running through a fine mesh irrigation filter, and finally to 120 20 liter collection buckets. The pools were filled with enough untreated well water to just cover the compost, and allowed to sit for two days. The water was then drained through the filters into the buckets. Pilot studies had indicated that the wire mesh and cheese cloth filters would not trap *P. ramorum* hyphae or spores to a significant extent, but the fine mesh (100 micron) irrigation filters might potentially catch some hyphae and attached chlamydospores (data not shown). Once the pools were drained, the fine mesh irrigation filters were cut up and plated onto PARP media, and all compost drainage was baited with three unripe D'Anjou pears per bucket at 12 ^{o}C. The pears were removed from the buckets after four days and then treated as in *Pear baiting,* above. Positive controls consisted of 12 20 liter buckets filled halfway with compost leachate into which 10 to 15 x 10^4 zoospores were introduced, and 9 20 liter buckets into which we emptied a single 84mm Petri dish each of strains Pr102 and Pr52 grown on 10% V-8 agar.

Sentinel plants Thirty (30) *Rhododendron* cv 'Cunningham's White' were used to test for the possibility of infection of plants nearby the composting facility. A total of 30 plants were placed downwind from the compost pile, in groups of ten (10) at distances of one (1), five (5), and ten (10) meters respectively, as well as a further five (5) plants placed approximately 50 meters upwind from the compost pile to act as negative controls.

Prior to use in the field, every plant was tested for susceptibility by removing two leaves from each plant and taping them to the side of a one gallon plastic bag, which was then filled with enough de-ionized water to bathe the leaf tips. A combination of *P. ramorum* cultures Pr 102 and Pr52 was then introduced to the bag, and the leaves were allowed to steep in the dark at 12 ^{o}C for four days before being removed and allowed to dry for a day. A portion of the resulting lesions was then excised from the leaf and plated directly onto PARP media, and as an extra precaution the remainder of the leaves were then pear baited as described in *flood sampling* above, with the exception that the compost was simply replaced by the infected leaves, and enough water added to cover approximately the bottom third of the pear.

Once the plants were installed into the field, the plants were inspected on a bi-weekly basis. Any leaves containing lesions were removed, and the lesions both directly plated and pear baited as described above.

At the conclusion of the composting phase of the experiment, these same *Rhododendron* plants were planted into the resulting compost, in a shade house. The plants received regular overhead watering using a combination of large and small droplet sizes, to facilitate splash dispersal onto the leaves of any spores that might be present. The plants were checked on a monthly basis, and symptomatic leaves were tested as described above.

Starting in December 2003, five compost samples were taken on a monthly basis from around the rhododendrons, and these were pear baited as described in *Direct Sampling,* above.

DNA Testing In February- April 2004, 495 samples were taken from the compost pile and analyzed for the presence of DNA of *P. ramorum.* The samples were extracted using the Qiagen QIAmp DNA-stool Mini Kit, and nested (2 round) PCR run using the protocol of Hayden *et al* (2004). Additionally, samples were spiked with 80 picograms of *P. ramorum* DNA and processed through the PCR assay, to ensure detection was not limited by the presence of PCR inhibitors in the compost substrate.

RESULTS

Experiment 1. The effect of dry heat on colony growth of *P. ramorum*

. Mild temperatures and low treatment times (e.g. 35˚C and 40˚C for periods of 2 hours or less) had little effect on pathogen growth rates. Combinations of milder temperatures with long treatment times (e.g. 35˚C for 24 hours, or 40˚C for 4 hours), or higher temperatures with shorter treatment times (e.g. 45˚C for 1 hour, 55˚C for 0.5 hours), appeared to temporarily suppress the pathogen, allowing for vigorous re-growth after treatment. Longer treatment times combined with higher temperatures (e.g. 40˚C for 24 hours or more, or 55˚C for 1 hour or more) killed the pathogen, resulting in no recovery. When fungal growth was completely arrested, so were the production and/or viability of any type of reproductive structure. We identified seven lethal treatments: 1, 2, 4, and 24 hours at 55˚C; 4 and 24 hours at 45˚C; 24 hours at 40˚C. Flash heat treatments at 55˚C were not effective and even prolonged exposure (4 hours) at temperature as high as 40˚C were not lethal to *P. ramorum* (Figure 3).

Experiments 2 and 3, The effect of dry heat and composting on infected plant material

No viable *Phytophthora ramorum* colonies were obtained from any substrate after exposure to 55˚C for two weeks in any of the oven trials or compost trials. In contrast, control infected plant material was always viable at the end of each experiment (Table 1). A comprehensive analysis of the effects of composting was possible by pooling results from all trials. Results indicated that treatments were equally effective on all three tested substrates (Table 2). When dealing with untreated controls, cankers on artificially inoculated stems provided the highest percentage of successful isolations by direct plating (87-88%), followed by isolations from artificially inoculated bay leaves (55-56%), and from wood chips (31%). Results obtained by baiting placed the substrates in a different order: the highest level of baiting success was obtained with bay leaves (83%), very limited baiting success was obtained with stems (17%), and no success was obtained when baiting wood chips. Independently of ease or method of isolation, all substrates were always negative for the growth of *P. ramorum* after treatments. Viability of the pathogen by substrate did not change significantly in the controls, during the length of our experiments (Tables 1 and 2).

The compost temperature level profile shown in figure 4 indicates the required 55˚C threshold was reached for longer than required by EPA and CCR guidelines for commercial composting, i.e. two weeks with five turnings for turned windrow composting, and three consecutive days for forced air static pile composting (California Code of Regulations, 2005). Forced air composting resulted in extremely high and sustained temperatures during the composting process.

Experiment 4, Survival and contagion of *P. ramorum* within a turned windrow compost pile

This experiment was conducted in the spring of 2003, a time when weather conditions should have been favorable for the survival and spread of *P. ramorum.* The weather was cool and rainy for the majority of the time at the beginning of the experiment, warming and drying toward the end of the experiment in May.

Results:

- All 48 pear baited samples from the pile were negative for viability of *P. ramorum*.
    - Positive controls: 0 of 20 tested positive for *P. ramorum*
- All 160 compost samples directly plated with PARP tested negative for viability of *P. ramorum.*

    - Positive controls: 0 of 160 tested positive for *P. ramorum*
- All leachate baited from runoff tested negative for viability of *P. ramorum.*
    - Positive control: 3 of 3 catch basins tested positive for *P. ramorum*
- All 120 flood samples tested negative for viability of *P. ramorum.*
    - Positive control: 7 of 12 zoospore buckets tested positive for *P. ramorum.*
    - Positive control: 5 of 9 hyphal colony buckets tested positive for *P. ramorum*.
- All 248 direct plating and 36 pear baitings of sentinel plant leaves tested negative for infection by *P. ramorum.*
    - Positive control: 29 of 30 sentinel plant leaves infected by *P. ramorum*
- After one year of continuous monitoring, no symptoms and/or isolates of *P. ramorum* have been obtained from the sentinel plants.
    - After eight months from the end of the experiment, all 430 PCR samples tested negative for DNA of *P. ramorum* PCR-based detection was successful when *P. ramorum* DNA was added to samples

Regardless of which isolation technique was used, we were unable to isolate *P. ramorum* from the compost pile at the end of the composting process. The artificially infected leaves used to infect the compost pile had an initial recovery rate of over 70% (n=30), and the positive controls (n=30) had a recovery rate of 50% after ten weeks.

The temperature profile shown in Figure 5 reveals that heat loss is greatest in the bottom corners, or "toes", of a compost pile. The best chances for getting the toe areas up to temperature with the rest of the pile appear to be immediately after turning, while the component material is still fresh. Internal pile temperatures were consistently higher than toe temperatures (data not shown), which is consistent with Burge *et al* (1981). Composting was extended until 55˚C was reached and maintained for a sufficiently long period of time in order to meet EPA and CCR guidelines for commercial composting in California.

DISCUSSION

Experiment 1, The effect of dry heat on colony growth and sporulation of *P. ramorum*

Results from Experiment 1 indicate that temperatures above 35 ^{o}C will reduce the growth of *P. ramorum*, but the pathogen will survive even after exposure at 40 ^{o}C for 4 hours. The ability to withstand exposure to such high temperature suggests the pathogen may be able to become established and survive in warmer regions. Heat tolerance of *P. ramorum* in planta has been recently shown by Harnik et al. (2004). For sanitation purposes three treatments were found to kill the pathogen: 24 hours at 40^{o}C, 2 h at 45 ^{o}C and 1h at 55 ^{o}C. Because temperature fluctuations are hard to avoid in most field situations, it is preferable to choose the highest temperature for the shortest period of time. Fifty-five degrees Celsius (55 ^{o}C) is also the minimum required temperature that needs to be obtained (and maintained for at least 5 pile turns) in commercial turned windrow composting facilities (Hay, 1996). It may be extrapolated that prolonged exposure to 45 ^{o}C may be as effective at eliminating *P. ramorum* as 55 ^{o}C. Conversely, flash-heat treatments of 55 C for 30 minutes are not likely to successfully eliminate the pathogen.

It should be noted that these results were obtained on cultures growing in Petri dishes. Survival of the pathogen in infected plant tissue may vary significantly depending on plant species, type of substrate (e.g. wood vs. cambium vs. leaves), and propagules produced by the pathogen on each host or host part.

Experiments 2 and 3, The effect of dry heat and composting on infected plant material

All treatments reduced the viability of the pathogen to zero, and their efficacy in terms of reduction of viability was statistically significant ($P<0.01$). This was true regardless of treatment (oven, windrow or forced air composting, see Table 1) or substrate (stem sections, leaves, or wood chips, see Table 2). The viability of the pathogen in the controls at the end of the treatments was undistinguishable ($P<0.01$) from

viability recorded before the treatments. Direct plating results were used for the analysis, but pear baiting provided identical results.

Table 2 provides some useful information on the different nature of the various substrates tested. Wood chips represented the most variable and least favorable substrate for *P. ramorum*. Baiting always failed from wood chips, suggesting the pathogen does not sporulate on this substrate. Isolations from cankers artificially caused by inoculations of coast live oak saplings yielded the largest percentage of successful isolations, but only limited baiting success. We hypothesize sporangia production on these stems occurred solely at the inoculation site, likely enhanced by wounding and by the placement of the agar plug of inoculum. Isolations from bay leaves were moderately successful, but baiting was significantly more successful than with the two other substrates. This is not surprising as bay leaves are known to support prolific sporulation of *P. ramorum*.

Both turned windrow systems maintained temperatures above 55ºC for the time mandated by EPA guidelines for commercial composting in California (figures 4 & 5), as did the forced air static pile system (figure 6). However it should be noted that in these studies the experimental units were placed in comparatively "ideal" locations within each pile. Experiments 2 and 3 fall into the category of direct process evaluation studies (Christensen et al. 2002). While results from these experiments clearly indicate that the composting process is capable of sanitizing infected green waste under the conditions tested, they do not necessarily prove that composting as a whole achieves identical results. Deviations from the conditions tested may affect the outcome. The frequency and intensity of potential deviations from the conditions tested here will define the reliability of composting as a tool to eliminate *P. ramorum* from infected plant material. Based on our results from experiments 3 and 4, it can be claimed that if conditions met in our sampling points (Figure 4) are met throughout the pile, composting will be successful in eliminating *P. ramorum* from infected plant material.

Experiment 4, Survival and spread of *P. ramorum* within a turned windrow compost pile, characterized by high levels of pathogenic inoculum.

Our last experiment was designed to test the validity of the composting process by monitoring viability and presence of the pathogen in a compost pile largely composed of infected plant material. Sampling was either complete or very intensive, to maximize our chances of detecting even limited survival by the pathogen. A complex sampling strategy was adopted including baiting, direct plating, PCR-based detection, and the use of sentinel plants, i.e. plants extremely sensitive to infection by *P. ramorum,* which serve as bioindicators for the presence of the pathogen.

Each test has individual strengths and weaknesses. The use of baiting techniques allowed us to sample large volumes of substrate. Because baiting relies on active infection of the bait by motile zoospores, this method also indicates active sporulation (i.e. asexual production of sporangia) on the tested substrate. Zoospores are generally regarded as the primary infection propagules for *Phytophthora* species, including *P. ramorum* (Werres et al. 2001) and *P. cinnamomi* (Zentmeyer 1980). When using baiting as a diagnostic detection assay, negative results have to be interpreted with caution, as it has been shown that pear baiting may be insensitive enough to miss *Phytophthora* spore densities that are high enough to cause disease (Yamak *et al* 2002). Composts can suppress sporangial production below detectable limits without eradicating the pathogen (Hardy and Sivasithamparam, 1991), and suppressed and undetected pathogens may recover sufficiently to become a threat under more favorable conditions (Hardy and Sivasithamparam, 1991, Sidhu *et al* 1999).

The positive controls used for our baiting trials failed to produce any lesions on the pear bait, which indicates that *P. ramorum* is suppressed. The positive controls used for the flood sampling involved introduction of zoospores or fully formed colonies on nutritive media to compost leachate. In contrast to the pear baiting positive controls, the flood sampling controls succeeded in infecting the pear bait, suggesting that the leachate itself is not suppressing pathogen growth, but that suppression requires either intimate contact with the compost substrate, or that suppression of *P. ramorum* in compost is severe enough that sporangia are not formed on *U. californica* leaves once they have been subjected to the biotic or chemical environment of a compost pile. Some evidence for the latter comes from a related side study in which we failed to bait *P. ramorum* from infected *U. californica* leaves that were soaking in bags with kiln dried (but not sterile) chipped lumber. It does not appear to take much time to induce suppression. We were unable to retrieve *P. ramorum* from 24 infected *U. californica* leaves six hours after placing them into

a cool (approx 15ºC) pile of ground and wetted green waste, while we were able to retrieve *P. ramorum* from ten of twelve leaves placed on the surface of the pile.

The compost dilution plate method offers the advantages of detection without the requirement for reproduction, but suffers from a much smaller volume of potential sample sizes, and shares the liability that negative results may be achieved through suppression and not eradication. We poured 160 plates of a mixture of sieved compost and PARP, but failed to *find P. ramorum*. However, our positive controls, simultaneously inoculated with *P. ramorum*, failed to grow out as well, which suggests that the compost is suppressive enough to keep *P. ramorum* from producing significant mycelial growth.

The use of highly susceptible sentinel plants at various distances downwind from the pile is a relatively good indicator that infected green waste is non contagious once in a compost pile. The closest plants were a mere one meter from the pile, and were routinely showered with debris as the pile was turned. This occurred under conditions and times of year that should have been highly conducive to infection (Davidson *et al* 2001), particularly the turning done in the cold, wet, windy weather of April 10-24, 2003. It is possible that foliar application of compost caused a suppression of infection due to induced resistance (Elad and Shtienberg 1994) during this phase of the experiment. However, the rhododendrons were later planted in this compost, pre-watered with a fine mist, and then heavily watered with large droplets allowing splash dispersal of any propagules in the compost. After a year of this treatment, not a single infected leaf was found, in spite of a 97% infection rate of control leaves. In light of the recent nursery infections that have occurred on *rhododendron*, *camellia*, and other nursery stock, it seems clear that *P. ramorum* will readily spread given adequate conditions to do so (COMTF 2004). If *P. ramorum* was present in any significant quantity under these conditions, certainly some infection should have been found. The lack of infection strongly suggests that *P. ramorum* is at least heavily suppressed under composting conditions, and perhaps not present at all.

Detection by PCR, a method of analyzing compost samples for the presence of the DNA of a given organism, offers us the ability to detect *P. ramorum* regardless of dormancy or reproductive status. Since our above results seem to corroborate Malajczuk's (1983) statement that *Phytophthora* species are easily suppressed by competing fungi and bacteria, PCR as a detection tool compliments the others nicely. Since this method detects DNA and not necessarily a living organism, it is possible to get positive results from dead cells. Positive PCR results may thus pose a complex issue from a regulatory perspective. Although the amount of substrate testable by PCR is smaller than baiting or direct plating, our PCR test was run with enough samples (425) to give us a better than 99 percent confidence that if *P. ramorum* DNA were still present at only half the amount originally introduced, we should have found it. Unlike the other tests, this would allow for the detection of dormant chlamydospores or any other resting structure and vegetative mycelium. We found no positive samples.

The composting temperatures reached in Experiment 4 were at first lower than those required by US EPA 40 CFR Part 503. Limited heating was due to the excessive moisture level in the pile, due initially to a watering regime designed to produce collectable leachate and subsequently to the heavy rains occurring in that period. Turning of the pile caused a significant reduction in moisture level, but heating did not occur after this point (especially in the toe of the pile), until further nitrogen-rich material was incorporated in the pile. It is interesting to note that the toes of the pile heated up as quickly as the rest of the pile, but cooled down faster than the rest of the pile. Maximum exposure to high temperatures can thus be obtained by shifting composting material in different locations in the pile through turnings (EPA 1989).

In spite of the fact that only moderate temperatures were reached in the first part of the experiment, no *P. ramorum* was detected in the leachate from the pile. This, along with the other results presented here suggests that the pathogen is at least effectively suppressed during the composting process due to biologically or chemically mediated processes.

It should be noted that the compost piles used in this study were both quite large and carefully monitored, as should be typical of commercially run facilities. Smaller composting piles, or those not turned and/or monitored on a rigorous schedule as might be found in "back yard" composting, frequently do not meet the temperature requirements for effective sanitization (Yuen and Raabe 1984, Ryckeboer *et al* 2002), and therefore should not be construed to meet phytosanitary conditions outlined in this report. The tests presented in this report all support the use of composting as a sanitation tool for plant material infected by *P. ramorum*. We conclude that even when this pathogen is present and sporulating on the leaves of *Umbellularia californica,* its most resilient plant host, the host material can be effectively sanitized by composting following the guidelines described by US EPA 40 CFR Part 503, which also

represent the accepted industry standard for composting (Hay, 1996). *Phytophthora ramorum* was not detected in direct sampling or baiting from compost piles in all experiments presented in this study. In the last experiment no *P. ramorum* was detected in the leachate from the compost pile, and the pathogen did not infect any of the sentinel rhododendrons, either when they were grown adjacent to the pile during the composting phase, or when they were planted in the pile during the later curing phase. Finally, after a period of eight months, we were unable to detect any DNA of *P. ramorum* when we ran PCR-based diagnostics.

The combined results from the four experiments conclusively show that composting will suppress and eliminate *P. ramorum* from a variety of infected plant substrates. Whether compost may serve as a route of spread for this aggressive plant pathogen will depend on other factors such as the ability of the pathogen to survive or colonize mature compost. *Phytophthora ramorum* has been recovered from dust samples immediately surrounding tub-grinders used to process green waste (Shelly *et al* 2004), and preliminary data suggests that *P. ramorum* is capable of surviving in finished compost if introduced, so although compost appears to eliminate the pathogen when processed correctly, this should not be construed to mean that all compost originating from infected green waste is safe by simple virtue of the fact that it has been processed in accordance with US EPA guidelines.

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Figures and Tables

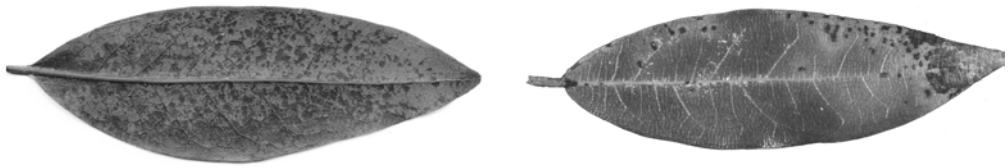

Figure 1: Active lesion area on artificially infected *U. californica* leaves (left) is larger than on naturally infected leaves (right).

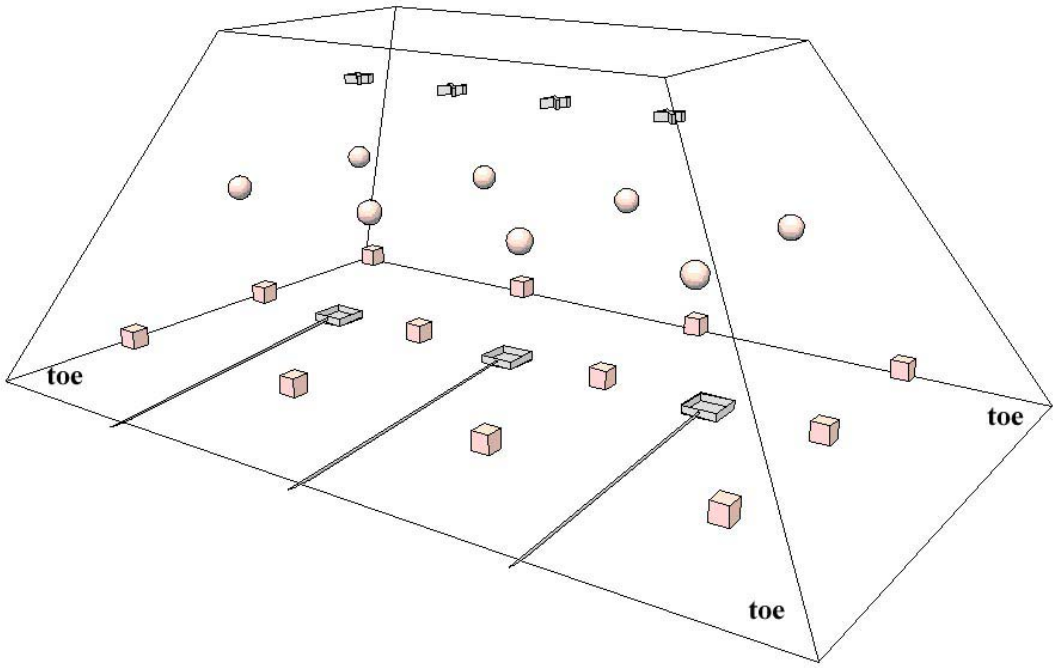

Figure 2: Perspective view schematic for compost pile. Temperature probes and sampling locations are represented here by cubes (bottom level), spheres (mid level), and X's (top level). Leachate collection traps are on the bottom level, and are represented by shallow boxes connected to hoses running to the outside of the pile. The bottom outside corners (marked "toe") have consistently been the area in which temperature values have been lowest (Burge *et al* 1981).

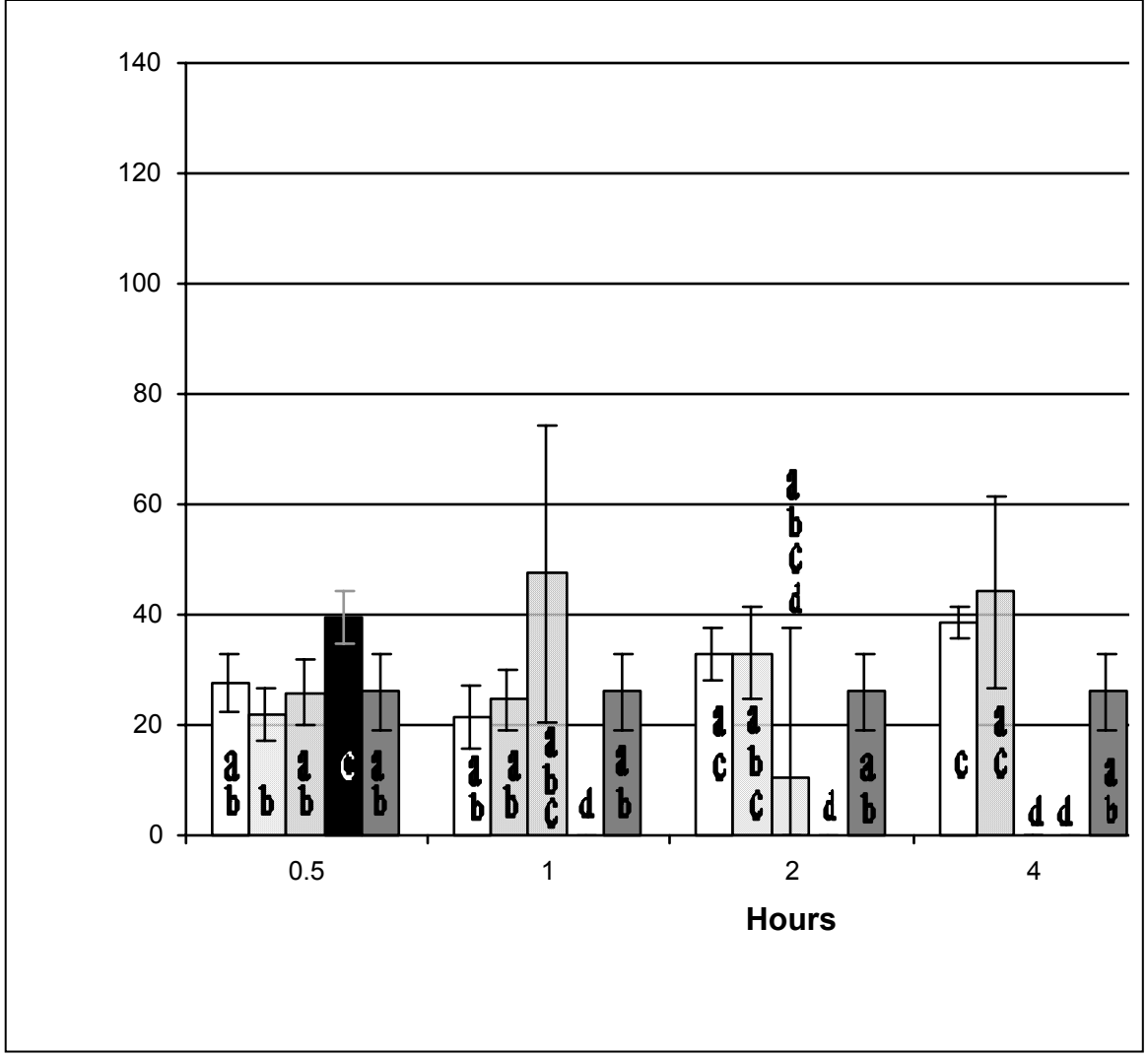

Figure 3: Average daily percentage colony growth values on the Y-axis are the means of 9 growth measurements (3 replicates for 3 isolates) 5-8 days from treatment. Different shading patterns indicate exposure to different temperatures for X hours. Error bars represent the standard errors for the means. Letter designations represent statistical significance groupings for P>99%.

Table 1: Number of viable (+) *P. ramorum* colonies before and after turned windrow composting and heat treatment (experiment 2), forced air composting (experiment 3).

Type	Site	n	Pre	% Pre	SE	Post	% Post	SE	Δ mean	SEdiff	99% CI for 0
Trn Wndrw	1	8	66/180	37%	3.85%	0/180	0%	0.00%	-37%	3.85%	0 +/- 13%
Trn Wndrw	2	8	61/180	34%	4.49%	0/180	0%	0.00%	-34%	4.49%	0 +/- 16%
Trn Wndrw	C	8	63/180	35%	3.08%	50/180	28%	1.38%	-7%	3.38%	0 +/- 11%
Oven	O	8	52/168	31%	4.03%	0/168	0%	0.00%	-31%	4.03%	0 +/- 14%
Forced Air	1	8	125/184	68%	4.45%	0/184	0%	0.00%	-68%	4.45%	0 +/- 16%
Forced Air	2	4	63/92	68%	5.95%	0/184	0%	0.00%	-68%	5.95%	0 +/- 35%
Forced Air	C	8	123/184	67%	2.40%	92/184	50%	7.79%	-17%	8.15%	0 +/- 27%

C: control replicates, O: oven replicates Pre: pre-treatment inoculum levels, SE: standard error, Post: post-treatment inoculum levels, Δ mean: change in inoculum levels from Pre to Post, SEdiff: standard error of Δ

mean, 99% CI for 0: the 99% confidence interval that the difference of the means is statistically different from zero.
Difference of means test: Delta mean values for controls fall within the bounds of the predicted possible data range for no difference from 0, (99% CI for 0), while the delta mean for the oven trials and compost piles at sites 1 and 2 do not. This indicates that both heat treatment and turned windrow composting produced an effect that was statistically significant at the 99% confidence level.

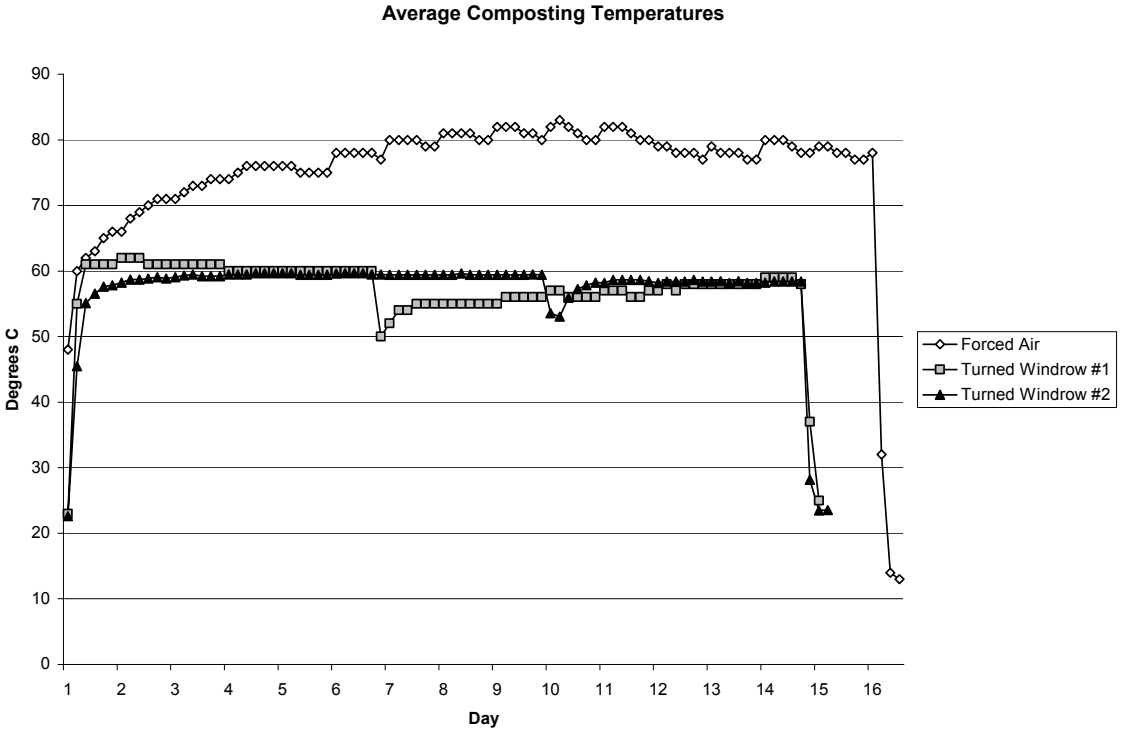

Figure 4. Average temperature profiles for composting trials in experiments 2 and 3, where the dashed line represents the critical 55 degree C temperature value.

Table 2: Number of viable (+) *P. ramorum* colonies before and after composting treatment (turned windrow and forced air data combined)

Substrate	n	Pre	% Pre	SE	Post	% Post	SE	Δ mean	SEdiff	99% CI for 0
Leaves	36	20/36	55%	4.3%	0/36	0%	0.0%	-55%	4.3%	0 +/- 12%
Control	16	9/16	56%	6.5%	8/16	50%	5.3%	-6%	8.4%	0 +/- 25%
Stems	34	30/34	87%	1.9%	0/34	0%	0.0%	-87%	1.9%	0 +/- 5%
Control	16	14/16	88%	2.8%	14/16	88%	2.7%	0%	3.9%	0 +/- 11%
Chips	36	11/36	31%	5.6%	0/36	0%	0.0%	-31%	5.6%	0 +/- 15%
Control	16	5/16	31%	8.4%	6/16	38%	8.4%	-7%	11.9%	0 +/- 35%

Pre: pre-treatment inoculum levels, SE: standard error, Post: post-treatment inoculum levels, Δ mean: change in inoculum levels from Pre to Post, SEdiff: standard error of Δ mean, 99% CI for 0: the 99% confidence interval that the difference of the means is statistically different from zero.
Difference of means test: Delta mean values for controls fall within the bounds for no difference from 0, (99% CI for 0), while the delta mean for the compost trials compost piles do not. This indicates that both heat treatment and turned windrow composting produced an effect that was statistically significant at the 99% confidence level. For all substrates, viability was always zero after treatment

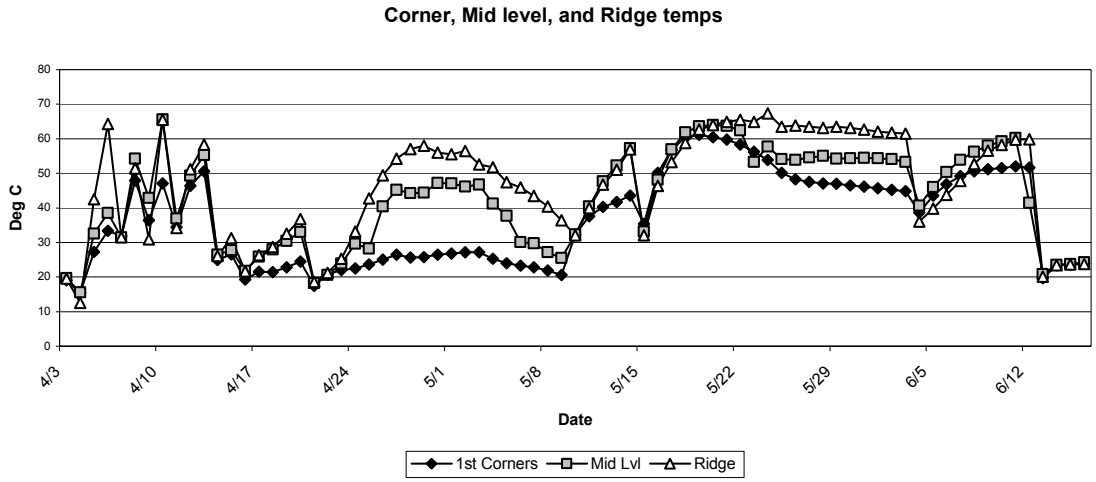

Figure 5: Temperature variation within the turned windrow compost pile in experiment 4. 1st Corners represents the average temperature of all of the bottom level outside corners, or "toe", of the pile, Mid Lvl represents the average temperature halfway up the pile from the toe, and Ridge represents the temperatures along the top of the pile (see figure 2 for these locations). All data points pictured here represent the mean of four values. Intermediate data points within a given line omitted for clarity.