A potential energy-saving heat treatment for re-circulated irrigation water and its biological mechanisms

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Abstract

Heat pasteurization is an effective water treatment to address the emerging plant pathogen issue associated with increased water recycling practices in the ornamental horticulture industry. The current protocol that recommends treating water at 95 ℃ for 30 s, however, faces two major challenges: its energy cost and environmental footprint. We hypothesized that temperature required to inactivate major pathogens in re-circulated water may be substantially lowered from 95 ℃ with extended exposure time. The goal of this study was to test this hypothesis and make this water decontamination technology economically more attractive while reducing its environmental impact. Specific objectives were to (1) examine the effect of water temperature on the survival of *Phytophthora* and bacterial species, two major groups of plant pathogens in water recycling systems, and (2) elucidate the underlying biological mechanisms by which plant pathogens are killed at those temperatures. Lab assays were performed to determine the survival of zoospores and chlamydospores of *P. nicotianae*, and oospores of *P. pini* as well as seven bacterial species after heat treatments at given periods of time. Greenhouse experiments were conducted to determine the applicability of the lab assay data to the real world using annual vinca (*Catharanthus roseus*) and *P. nicotianae* as a model system. The results of these studies indicated that the water temperature required to eliminate *Phytophthora* and bacterial species can be lowered to 48 ℃ from 95 ℃ if treatment time extends to 24 h. Two major steps were taken to elucidate the underlying biological mechanisms. Firstly, a scheme based on the DNA fingerprint and sequence analysis was developed for characterizing bacterial species in irrigation water, after comparing two typing strategies, three sample concentration methods, and evaluating conditions in denaturing gradient gel electrophoresis (DGGE) profiling. Bacterial species detected by culture-dependent and -independent strategies were rather different. The

greater bacterial diversity was detected when water samples were concentrated by using both methods than centrifugation or filtration alone. As for DGGE profiling, 40 to 60% denaturant concentrations at 70 V for 16 h revealed the highest bacterial diversity. Secondly, water samples were taken from an irrigation reservoir in a local nursery and analyzed for bacterial diversity following heat treatments at 42 and 48 ℃. After these heat treatments α-proteobacteria, γ-proteobacteria, and Firmicutes became dominant which presents a substantial shift of bacterial community structure compared to those in the control water at 25 ℃. Among the dominant in treated water were *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Brevibacillus*, and *Lysobacter* species, which may have potential biocontrol activities against plant pathogens. This study provided the scientific basis for developing a more energy-efficient and environmentally sound heat pasteurization protocol for water decontamination.

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Attributions

Several colleagues have contributed to this dissertation.

Chuanxue Hong, Ph.D. is a professor in Department of Plant Pathology, Physiology and Weed Science and Hampton Roads Agricultural Research and Extension Center at Virginia Tech. He is the primary advisor and committee chair for this project. He advised each and every major step of this project from research planning to execution, data analyses and interpretation, and edited this dissertation.

Chapter 2. Inactivation of *Phytophthora* and bacterial species in water by a potential energy-saving heat treatment

Boris A. Vinatzer, Ph.D., an associate professor in Department of Plant Pathology, Physiology and Weed Science at Virginia Tech, serves on my graduate committee. He supported and guided the bacterial assays. He is listed as a co-author in a publication of work described in Chapter 2.

Monday O. Ahonsi, Ph.D., a former post-doctoral research associate in Dr. Hong's lab, performed the assays investigating zoosporic survival of *Phytophthora nicotianae* (Figure 2-1). He is listed as a co-author in a publication of the work described in Chapter 2.

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Chapter 1

Introduction

Literature review

Plant pathogens in irrigation systems

Numerous plant pathogens, including zoosporic oomycetes, bacteria, viruses, fungi and nematodes, have been found in irrigation water (Thomson and Allen 1974; Geldreich 1996; Hong and Moorman 2005; Cayanan et al. 2009). These plant pathogens can enter the irrigation system in several ways. They may inhabit the water source, the soil, nearby diseased resident plants. Alternatively, they may be brought in from other places via infected plants or other contaminated materials. Irrigation water sources, including ponds, lakes, rivers, streams, and manmade reservoirs, may be contaminated by underlying or surrounding soil and plant debris, thus affecting the entire irrigation system. Plant pathogens may also enter the irrigation system at several points along the distribution path. Any irrigation method in which the water comes in contact with soil or plant debris may acquire plant pathogens, for example, overhead sprinkling, furrow flooding, field flooding, and flooded floor or bench. Runoff from flooding or other methods of irrigation can re-circulate pathogens throughout the system. In outdoor production facilities, when the excess water is captured and recycled, soil, which is possibly contaminated, may be a significant source of pathogens. Also, pathogen-contaminated soil may fall or be blown into water holding tanks, and then contaminate the whole irrigation system (Hong and Moorman 2005).

Irrigation systems is a powerful means for pathogen dissemination from a single infection spot to the entire production facility and from an infested farm to all the other farms sharing the same water resources (Harrison et al. 1987; Krczal et al. 1995; Hong and Moorman 2005). Irrigation water facilitates pathogen infection by inoculating plants at every irrigation event and providing a wet environment required for pathogens to germinate, colonize, and reproduce themselves. Recycling irrigation also may allow monocyclic soilborne pathogens to complete more than one disease cycle per growing season.

Zoosporic oomycetes, particularly those belonging to the genera *Pythium* Pringsh. and *Phytophthora* de Bary, are the most common and destructive plant pathogens found in water (Baker and Matkin 1978; Stanghellini and Rasmussen 1994; Stanghellini et al. 1996a; Stanghellini et al. 1996b; Bush et al. 2003; Hong and Moorman 2005). Many species of *Phytophthora*, *Pythium*, and other plant pathogens have been reported from irrigation water (Bush et al., 2003; Neher and Duniway, 1992; Hong et al., 2003; Hong and Moorman, 2005; Thomson and Allen, 1974). Specifically, over 17 species of *Phytophthora* have been reported in irrigation reservoirs and natural waterways. These include *P. alni, P. cactorum, P. cambivora, P. capsici, P. cinnamomi, P. citricola, P. citrophthora, P. cryptogea, P. drechsleri, P. gonapodyides, P. heveae, P. insolita, P. megasperma, P. nicotianae, P. pseudosyringae, P. ramorum, P. syringae*, and numerous undescribed *Phytophthora* species (Ho et al. 2002; Hong and Moorman 2005; Ivors and Greene 2008). Twenty six *Pythium* species also have been reported in ponds, rivers, canals, streams, lakes, wells, and sewage (Hong and Moorman 2005).

Bewley and Buddin (1921) detected *P. cryptogea* and *P. parasitica* (syn. *P. nicotianae*) in greenhouse irrigation water in the early 1920's. Many species of *Phytophthora* carried in water have been reported to cause many diseases in fruit crops, vegetables, ornamentals, and

forest trees (Dukes et al. 1977; Neher and Duniway 1992; von Broembsen et al. 2001; Yamak et al. 2002; Bush et al. 2003; Watanabe et al. 2008). In Germany, ten known *Phytophthora* species and at least twelve new taxa were isolated from drains in reservoirs and wells at four commercial nurseries with water recirculation systems (Themann et al. 2002). In a water re-circulation irrigation system at a perennial container nursery in southwestern Virginia, seven *Phytophthora* species and many *Pythium* species were found (Bush et al. 2003). In Japan, *P. helicoides*, *P. aphanidermatum,* and *P. myriotylum* were recovered in ebb-and-flow and hydroponic culture systems, causing root rot of miniature rose (*Rosa hybrida* L.), kalanchoe (*Kalanchoe blossfeldiana* Poelln.), cucumber, tomato, tobacco, bell pepper, floral crops, lettuce, artichoke and many other crops (Watanabe et al. 2008).

Zoospores are a major dispersal and infective propagule in the life cycle of *Phytophthora* species. They may be dispersed by their own locomotion using flagella, or passively by water current. The latter is particularly important in greenhouse industries that employ recirculation of the irrigation water, including hydroponic and ebb-and-flow cultural systems (Neher and Duniway 1992; Stanghellini et al. 1996a). Thomson and Allen (1974) indicated that zoospores were the only propagules of *Phytophthora* spp. present in the irrigation water from 20 sites in citrus areas near Phoenix, AZ. Stanghellini et al. (1996b) concluded that zoospores of *Pythium aphanidermatum* were the propagule responsible for pathogen dissemination in hydroponic systems after efficacy trials of surfactants. Surfactants could rapidly kill fungal structures lacking a cell wall (i.e. zoospores), but not affect fungal structures having a cell wall (i.e. hyphae, encysted zoospores, sporangia, oospores, or chlamydospores). Stanghellini et al. (1996a) made the same observation with *Phytophthora capsici.* Thinggaard and Andersen (1995) also suggested that zoospores of *P. cryptogea* were strongly involved in spread of root rot in ebb-and-

flow systems. In re-circulated irrigation at a large ornamental nursery, zoospores comprised more than 94% of *Phytophthora* propagules in runoff water entering the retention basin (Charlton and von Broembsen 2000). Usually, oospores or chlamydospores are found in sediment or plant debris at the bottom of the reservoir (Pittis and Colhoun 1984).

Bacteria are also common pathogens found in irrigation water (Toze 1999, 2006b). A total of eight species of bacteria has been reported in irrigation reservoirs and natural waterways as reviewed by Hong and Moorman (2005). *Erwinia* species are among the most frequently detected bacterial pathogens in water (Harrison et al. 1987; Cappaert et al. 1988; Eayre et al. 1995). The soft rot bacterium *Erwinia carotovora* subsp. *carotovora* was detected in irrigation water from drains, ditches, streams, rivers, and lakes in southern Scotland and in Colorado, United States (McCarter-Zorner et al. 1984). *Ralstonia solanacearum* biovar 2, which causes brown rot in potato and other plants has been observed in different soil and water systems, and it may spread to and survive in local waterways following disease in potato fields (Stevens and van Elsas 2010). *Pseudomonas syringae* strains have been detected in rain, snow, alpine streams, lakes, epilithic biofilms, and irrigation water (Morris et al. 2008). *Xanthomonas* species have also been found in irrigation water (Steadman et al. 1979; Hoitink and Boehm 1999).

Twenty-seven genera of fungi have been found in irrigation water based on a review by Hong and Moorman (2005). Species in the genera *Alternaria*, *Botrytis*, *Ascochyta*, *Rhizoctonia*, and *Verticillium* are present in water because of their natural abundance but they may not survive well in water (Bewley and Buddin 1921; Shokes and McCarter 1979). Shokes and McCarter (1979) recovered thirteen species of *Fusarium*, *Rhizoctonia solani*, *R. zeae*, and *Macrophomina phaseolina* from sediment of irrigation ponds. *Fusarium oxysporum* has been reported to spread through irrigation water and cause plant diseases (Jenkins and Averre 1983; Wick and Haviland

1992), but it may not always be able to live long enough in water to reach and infect plants (Rattink 1990). *Fusarium oxysporum* f. sp. *basilici* spread via a re-circulating water system and caused fusarium wilt on basil in greenhouses (Wick and Haviland 1992). *Colletotrichum* spread via water in hydroponically grown tomatoes (Jenkins and Averre 1983).

At least ten viruses have been reported in river, lake and irrigation systems (Hong and Moorman 2005). Pelargonium flower break virus (PFBV) was transmitted via a re-circulating nutrient system from infected plants to healthy plants in 6 weeks (Krczal et al. 1995). And tomato mosaic virus (ToMV) also spread rapidly in re-circulating nutrient solution from inoculated plants and remained infective for at least 6 months (Pares et al. 1992).

Thirteen species of plant parasitic nematodes have also been reported in irrigation water (Hong and Moorman 2005). Because plant parasitic nematodes are pathogenic roundworms that live in water films in soil and plant tissues, they are essentially aquatic organisms and can be washed from soil and enter the irrigation systems. Faulkner and Bolander (1967) demonstrated that irrigation canals were sources of large numbers of plant parasitic nematodes. Simmons et al. (2008) indicated that *Ditylenchus dipsaci* and foliar nematode *Aphelenchoides ritzemabosi* may be transported across alfalfa fields via irrigation water.

Capture and reuse of surface water for irrigation is of critical importance to ensure an adequate supply of quality water in light of growing global water scarcity (Exall 2004; Hong and Moorman 2005; Toze 2006a). Many nurseries and greenhouses have implemented collection, recovery, and recirculation of irrigation water. These practices reduce water consumption and release of fertilizer and pesticide pollutants into the environment, and provide important nutrients for crops (Bush et al. 2003; Hong et al. 2003; Steele and Odumeru 2004; Hong and Moorman 2005; Cayanan et al. 2009). Re-circulation of irrigation water generally involves collecting

nursery effluent into holding tanks or outdoor reservoirs through soil lined channels then mixing with fresh water from nearby rivers or wells for subsequent irrigation (Bush et al., 2003). However, re-circulation of water may accumulate plant pathogens in irrigation systems and spread to new crops. Van Voorst et al. (1987) demonstrated that when *Phytophthora nicotianae* inoculum was added to the roots of tomatoes grown in a greenhouse by nutrient film technique in which the nutrient solution was re-circulated, the pathogen also freely circulated within the system and caused polycyclic infection to the hosts. Once pathogens enter an irrigation system, the repeated pathogen exposure due to water re-circulation increases the occurrence of diseases (Hong and Epelman 2001). Werres et al. (2007) indicated that *Phytophthora ramorum* can survive and infect *Rhododendron* species through contaminated re-circulated irrigation water

Decontamination of irrigation water

One principle of plant disease management in the ornamental industry is to ensure the health of the plant material entering production (Daughtrey and Benson 2005). Use of a clean water source and preventing plant pathogens from entering irrigation systems are the first and important steps to ensure irrigation water health. Water treatment prior to delivering to crops should be used as the last resort but it is necessary in most situations for crop health risk avoidance and mitigation (Hong and Moorman 2005).

Currently, physical, chemical, and biological means are utilized for controlling plant pathogens in water. These include filtration, heat treatment, chlorination, use of surfactants, and ultraviolet (UV) radiation. All these control methods aim at reducing microbial levels, or inactivating microorganisms (Evans 1994; Hong and Moorman 2005; Schumann and D'Arcy 2006). Several factors must be considered when selecting water treatments for a particular

production facility. For example, it may be most cost-effective to use high quality and not re-circulated water for sensitive crops, such as young plants, and use recycled water only for those less susceptible plant species. In making a decision, it is useful to first identify the target pathogens for treatment (classes, life stages) in order to design the parameters for the treatment system. Some important considerations include: 1. quantity of water to be treated and therefore the installation and operation cost; 2. water quality conditions, such as pH, turbidity, electrical conductivity, etc, and the water quality changes allowed during treatment; 3. training, education and safety of the workers; and 4. residual which may enter the environment or have potential phytotoxicity to the crops (Hong and Moorman 2005).

Each of these water decontamination technologies has their advantages and disadvantages. UV radiation, chlorination, and heat are generally effective, but may have a negative effect on benefical microorganisms in irrigation water. Slow sand filtration is less disruptive to the beneficial microorganisms, but may be less effective on plant pathogens (Ehret et al. 2001). Most of the water treatments are expensive, and a certain level of education/training is needed for the growers to actually employ these control methods. Moreover, none of the practices are simple or one-time treatments but require monitoring and repeated treatments (von Broembsen et al. 2001; Hong and Moorman 2005).

Filtration is a separation process by the flow of a liquid through a porous material, such as a bed of fine filter sand, to retain the solids and allow the liquid (filtrate) to pass through (Ellis 1985). Slow sand filtration is efficient against many plant pathogens spread in irrigation water (Ellis 1985; Wohanka 1995; Runia et al. 1997; van Os 1999). It's a low energy input technology and also the construction and operation are simple (McPherson et al. 1995). Use of slow

filtration is limited due to its limited capacity and clogging caused by high loads of particulate matter in water (Hong and Moorman 2005).

Chlorination is widely used in horticultural production to disinfest irrigation water. It is highly effective against a number of pathogens, especially the oomycetes *Pythium* and *Phytophthora*, with a dose of free residual chlorine lower than 2 ppm (Hong et al. 2003; Cayanan et al. 2009; Granke and Hausbeck 2010). This technology only needs a relatively low initial and operation cost, and the installation, operation, and maintenance is relatively simple. But chlorine efficacy depends largely upon water quality parameters and may also produce byproducts harmful to human health and the environment (Hong and Moorman 2005).

UV radiation is an effective choice for irrigation water in a nursery or greenhouse to eliminate plant pathogens and algae. Exposure to germicidal UV radiation kills microorganisms by damage to DNA in the form of strand breaks, cross links, and dimerization of adjacent pyrimidine bases in DNA, which usually is lethal or mutagenic to cells (Setlow and Setlow 1962). UV radiation adds no residual chemistry to the water after treatment, therefore, there is no residual disinfestation effect. It also destroys the beneficial microorganisms in water (Ehret et al. 2001). It is typically used in combination with filtration to remove organic matter, which increases light transmission and efficacy (Fynn et al. 2011).

Surfactants disinfest the irrigation water by disrupting the membrane of microorganisms due to their amphilic character, and rapidly killing the plant pathogens. Certain surfactants, mainly cationic, are commonly used as disinfestants in water (Prescott et al. 2005). Surfactants are highly efficient against zoosporic pathogens, such as *Phytophthora* and *Pythium* species, because the primary inoculum zoospores, which lack cell walls, are very sensitive to surfactants (Stanghellini and Miller 1997). Limitations of surfactants include the toxic residue to the

environment due to slow degradation, and potential phytotoxicity when using in irrigation systems (Uhlig and Wissemeier 2000; Irish et al. 2002).

Heat is one of the most reliable methods for treating water to eliminate many types of plant pathogens, including fungi, bacteria, and viruses. Research on the thermal inactivation of microorganisms in diverse media started as early as 1920 (Bigelow and Esty 1920; Coelho et al. 2000). For example, Bigelow and Esty (1920) showed the effect of different temperature and exposure time combinations necessary to inactivate thermophilic bacteria and Grooshevoy et al. (1941) used natural heat sources to disinfest seed bed soil.

Heat treatment is commonly used by greenhouse growers, especially in the Netherlands and the United Kingdom. This practice is one of the most commonly used water treatments in the closed soilless growing system and has been used for several years (Runia et al. 1988; Runia 1995; van Os 1999). However, this technology is used to a lesser extent in other countries, due to energy efficiency issues and high investment costs (Hong and Moorman 2005).

Extensive studies have been conducted to investigate thermal inactivation of plant pathogens in infested plants, soil, and culture media. Half of mycelium of two isolates of *Phytophthora cinnamomi* were inactivated by hot water at 39 ℃ for 26.3 and 51.7 min or 43 ℃ for 2.7 and 3.3 min on agar disks, respectively. And Fraser fir seedlings inoculated with *P. cinnamomi* in growing medium within 14 d were disinfested by water therapy at 45 ℃ for 15 min (Benson 1978). Hyphae and oospores of *Phytophthora cactorum* and *P. cinnamomi* survived only 30 min at 45 ℃ in inoculated walnut twigs or artificially infested Reiff silty clay loam soil (Juarezpalacios et al. 1991). The population of chlamydospores of *P. nicotianae* in soil, as baited with tomato seedlings, declined to very low levels after heat treatment at 47 ℃ for 2 h, or 50 and 53 ℃ for only 5 min (Coelho et al. 2000). Pullman et al. (1981) found that temperatures between

37 to 50 ℃ for different time periods can kill mycelia, spores, and resting spores of *Verticillium dahliae*, *Pythium ultimum*, and *Thielaviopsis basicola* on agar media. Twenty-three, 27, 33, and 68 min of heat treatment at 50 ℃ were required to cause 90% mortality of *V. dahliae* (strains T9 and SS4), *P. ultimum*, and *T. basicola*, respectively. There are differences in temperature response between different types of fungal propagules within the same species. For example, to inactivate *Phytophthora cinnamomi* on agar discs within 1 to 2 h, the required temperature was 38 ℃ for mycelium, and 40 ℃ for chlamydospores (Gallo et al. 2007). Mycelium of *P. capsici* was more sensitive than oospores with the former being eliminated with a heat treatment at 42.5 to 45 ℃ for 30 min and the latter still surviving a heat treatment at 50 ℃ for 30 min in soil (Bollen 1985).

Propagule suspensions have been used by researchers to study the effect of heat on inactivation of plant pathogen spores by heating to a certain temperature for a certain period of time, which provides the basis for estimating the temperature and time required to control many types of plant pathogens in irrigation water (Runia et al. 1988; van Os et al. 1988; McPherson et al. 1995; Runia 1995; Poncet et al. 2001). Dyer et al. (2007) demonstrated that oospores of the oomycete *Aphanomyces cochlioides*, the primary inoculum during growing seasons, were susceptible to moderately high temperatures. The viability of oospores in water declined to zero after exposure to 45 ℃ for 72 h or 50 ℃ for 6 h. The authors also suggested that the heat sensitivity of *Phytophthora* spp. in the laboratory closely corresponded that in soil. Oospore suspensions of *P. infestans*, which may be a long-term source of inoculum, did not germinate after treatment at or above 46 ℃ for 2 h, or at 40 ℃ for 12 h (Fay and Fry 1997).

The development of current protocols for heat treatment started with Runia et al. (1988) who studied the disinfection of recirculated water by heat treatment using tobacco mosaic virus

(TMV), *Verticillium dahliae*, and *Fusarium oxysporum* f. sp. *melongenae* in the laboratory and greenhouse. TMV was one of the main root-infecting viruses in crops in soil-less cultures in the Netherlands, and *F. oxysporum* f. sp. *melongenae* was one of the most resistant soilborne fungal pathogens (Bollen 1985). Fungal spore suspensions were dilution-plated and host plants were inoculated by dipping root systems into fungal suspensions or by rubbing leaves with TMV suspension after heat treatments. Results indicated that TMV can be inactivated after heat treatment at 95 ℃ for 10 s, and spores of *V. dahliae* were killed at 90 ℃ for 10 s. The propagules of *F. oxysporum* declined significantly after being heat-treated at 94 ℃ for 10 s. McPherson et al. (1995) studied the effect of heat treatment on *Phytophthora cryptogea* and *Pythium aphanidermatum* inoculated in re-circulated hydroponic nutrient solutions on tomato and cucumber crops. They reported that the inoculated pathogen did not spread through the re-circulating system after heat treatment at 95 ℃ for 30 s compared to non-treated nutrient solution, which transmitted diseases to most plants. Poncet et al. (2001) reported the disinfestation of drainage water in soilless cultures of roses. They counted total bacteria and *Agrobacterium tumefaciens* in drainage water before and after heat treatment. Bacteria, including *A. tumefaciens,* with an initial concentration of 10^8 cfu per liter, in the re-circulated irrigation water were eliminated after heat treatment at 95 ℃ for 30 s. Therefore, based primarily on the studies described above that were performed in the Netherlands (Runia et al. 1988) and the United Kingdom (McPherson et al. 1995), current protocols recommend raising and maintaining water temperature to 95 ℃ for 30 s.

Several studies in regard to heat disinfestation of nutrient solution systems were performed using heat exchangers. In collaboration with the Institute of Agricultural and Environmental Engineering at Wageningen, Runia et al. (1988) constructed a heat-exchange

system to treat drainwater reused for soilless cultures in the Netherlands. Basically, the drainwater returning from the plants was collected in a recatchment tank. After filtering out organic particles and leaves the drainwater was pumped into the first heat exchanger to preheat to about 80 to 90 ℃. Water was then further heated to the disinfestation temperature above 95 ℃ by a second heat exchanger. This temperature was kept for a certain exposure time. After heat treatment the disinfected water flowed back to the first heat exchanger to be cooled down and be stored in a holding tank to cool further. The heat recovered from the hot disinfested water was used in the first heat exchanger, and an external heat source was used for the second heat exchanger. A switch valve was used to control water returning to the recatchment tank if the water was not properly disinfested. McPherson et al. (1995) used commercially available heat exchangers for milk heat treatment. Poncet et al. (2001) used the "L'ien thermodisinfection" co heat-exchange system. Their disinfestation system was similar to that described by Runia et al. (1988) as the drainage water was treated successively through two heat exchangers.

Re-circulated irrigation water in commercial greenhouse circulation systems can be heat treated similarly to commercial heat treatment of other fluids, such as milk and drinking water (Evans 1994). The basic principles are the same as in the disinfestation system built by Runia et al. (1988). Two heat exchangers with one being linked to an external heat source are used for heat treatment of the irrigation water with an initial filtering of water through a rapid sand filter to remove plant parts and other debris (Ehret et al. 2001). It is important that the quality of the water to be treated is high to prevent elevated levels of sodium and chloride that would result in phytotoxicity. The holding tanks used to store disinfested water must be in an area where the water can be protected from new contamination prior to use. Corrosion-free materials, such as stainless steel and synthetic materials should be used to build the equipment, also copper- and

zinc- based materials should be avoided due to their phytotoxicity (Runia et al. 1988). Because high temperatures cause calcium salts to precipitate inside the equipment thus leading to a lower efficiency of the disinfestation system over time, washing the equipment with an acid solution (pH 2 to 3) for a few minutes before each disinfestation cycle is advised to remove accumulated salts (van Os et al. 1988). The use of heat exchangers increased the efficiency of energy use and reduced the energy input to a relatively low level (Runia et al. 1988; Runia 1995). Overall, heat treatment systems are not difficult to install and they are easy to monitor with a thermostat (Ehret et al. 2001).

Heat treatment involves no chemicals and thus presents no health hazard to workers (Gurol 2005). It also presents minimal environmental hazards compared to chemical treatments. Some chemical disinfestants such as copper will not degrade in water and will easily accumulate. Also, excessive or accidental applications associated with recirculation of water treated with these disinfestants can easily lead to damaging chemical concentrations in water, which can be taken up by the plants and soil, causing phytotoxicity (Gurol 2005; Blom 2008). Heat treatment on the other hand will not have secondary effects on the environment.

The major limitations of current heat treatment protocols for re-circulated irrigation water are energy inefficiency, the cost of required equipment, the volume of the storage tank needed to hold treated water, and the killing of beneficial microorganisms (Runia et al. 1988; van Os et al. 1988; Ehret et al. 2001; Hong and Moorman 2005).

The cost of heat treatment is relatively high due to the initial investment in the equipment and continuous consumption of energy to produce heat. The investment for construction of the heat disinfestation system is substantial (Poncet et al. 2001), so it is not quite affordable for smaller floriculture farms. Runia et al. (1988) indicated that their disinfestation system needed

about 1.5 m^3 natural gas per 1 m^3 drainwater, resulting in usage of about 2700 m^3 gas per hectare. Blom (2008) also reported that as high as 20.2 to 39.6 m^3 of fuel was needed to heat every 1 m^3 of water, and resulted in an estimated cost of 7.7 to 15.2 US dollar per 1 m^3 water heated according to the current price average 10.87 dollar per 1000 ft^3 of natural gas in 2012 (EIA 2012). Also the burning of fossil fuels emits a large amount of greenhouse gases, especially carbon dioxide (CO_2). These greenhouse gases are major contributors to global warming, which has become a worldwide concern (De Oliveira et al. 2005). In addition to the continuously increasing cost of energy, the natural gas consumption for heating the contaminated water can be energy inefficient and cost prohibitive when there are no other sustainable energy sources. Application of the described heat-exchangers to recover some energy for preheating water will reduce the cost and it may also be feasible to use heat-treated water for only the critical stages of crop production such as during the propagation of highly susceptible plants (Bewley and Buddin 1921).

The worsening energy crisis is further hurting the economics of this highly effective water treatment until a more efficient heat treatment system is developed. The possibility of substituting heat with treatment time and alternative energy sources have been studied.

The efficacy of heat treatment depends on both temperature and treatment time. Sublethal temperatures, usually below 50 ℃, can be lethal to soilborne plant pathogens if maintained for long periods (Smith 1923; Munnecke et al. 1976; Vanuden and Vidalleiria 1976; Gallo et al. 2007). Sublethal heating of infected roots in soil reduced survival of *Armillaria mellea*. The fungus was killed in the roots of citrus seedlings at 40 ℃ for 2 to 3 h (Munnecke et al. 1976). Runia et al. (1988) also reported that the viability of spores of *Fusarium oxysporum* f. sp. *melongenae* was reduced with increasing exposure time at the same heat treatment temperature.

It is thus likely that reducing treatment temperature while increasing exposure time will still have the same efficacy as the current heat treatment protocol. By using a heating system developed by a commercial company and laboratory setups, Runia and Amsing (2001) indicated that heating at 85 ℃ for 3 min had the same efficacy as heating at 95 ℃ for 30 s for both Tomato mosaic virus and *F. oxysporum* f. sp. *lycopersici*. The burrowing nematode *Radopholus similis* was eliminated at 48, 50, and 52.5 ℃ for 5, 2 min, and 30 s, respectively. Also temperature-exposure times lethal to pathogens were well established for soil steam sterilization where exposure time ranges from 0.5 to 12 h (Bollen 1985; Evans 1994), so inactivation of plant pathogens in irrigation water might be achieved at a lower temperature when extending treatment time from seconds to hours.

The energy consumption and cost of water disinfestation will be substantially reduced if new heat practices can be developed for horticulture. Also, new sustainable energy sources such as solar irradiation and geothermal energy have great potential to be applied in heat treatment of re-circulated irrigation water, once the appropriate disinfestation systems have been developed.

Solar water pasteurization has been used to disinfest drinking water, but solar radiation is seldom used in treating re-circulated irrigation water (Jorgensen et al. 1998; Duff and Hodgson 2005; Kang et al. 2006). As early as 1984, solar cookers were used to decontaminate relatively small amounts of drinking water (Ciochetti and Metcalf 1984). Conroy et al. (1996) employed drinking water contained in transparent plastic bottles exposed to the sun for a few hours, which reduced the rate of childhood diarrhea by 10%. There are two main types of solar water pasteurization systems: batch and continuous flow. In batch systems, refillable vessels are used, and it usually takes a full day of sun for a batch system to disinfest water (Ciochetti and Metcalf 1984; Andreatta et al. 1994; Conroy et al. 1996). In a continuous flow-through system, water from a supply reservoir flows through a heat exchanger at first, then through a solar collector,

such as copper pipes, which heats the water up to a desired temperature. Heat exchangers here are also used to preheat untreated water and a thermostatic valve is used to control temperature and flow (Jorgensen et al. 1998; Duff and Hodgson 2005). Jorgensen et al. (1998) used a water decontaminator device (HS 231545; Roerslev Smedie, Roerslev, Denmark) for heating drinking water by solar radiation in a flow-through system of copper pipes. The water temperature could be raised up to 85 ℃. When the required temperature was reached and detected by a sensor, the water flows out through an open valve. The heating temperature was set at 65 ℃. Daily production was about 50 L of decontaminated water per m^2 of solar panel, and it was possible to double the amount by using a heat exchanger to recycle the heat. Since the water decontamination device can be used for years and the running cost is low, the cost of pasteurized water can be estimated at less than US dollar 0.008 per liter.

Water disinfestation systems using solar panels are energy-efficient, cost-effective, and easy to install and transport (Duff and Hodgson 2005; Kang et al. 2006). However, these systems may not be able to hold water at a high temperature for a relatively long period, and can only treat small amounts of water. Therefore, current solar water disinfestation systems which have been developed probably are not very useful for commercial soilless culturing greenhouses, where as much as 175 to 275 m^3 per hectare per day of runoff water needs to be disinfested (Blom 2008). New advanced solar water heating systems with a much larger water-treating capacity need to be developed for common usage in agriculture.

Geothermal energy, which is the energy contained as heat within the earth's interior, could be used to replace fossil fuels to some extent (Lund et al. 2005; Ozgener et al. 2005). Geothermal energy has been commercially utilized for over 80 years and was used in more than 72 countries in 2005 (Lund et al. 2005; Ozgener et al. 2005). In the United States, geothermal

energy has been directly used in heating of pools and spas, greenhouse and aquaculture facilities, space and district heating, snow melting, agricultural drying, ground-source heat pumps; and generating electricity (Ozgener et al. 2005). Most of the applications have experienced continual increase over the years; however, there is no record of geothermal energy being directly used to disinfest water. Because using geothermal energy instead of fuels could significantly reduce the emission of pollutants, such as greenhouse gases, and since only a small fraction of the geothermal potential has been developed so far, there is plenty of space for an increased use of geothermal energy (Fridleifsson 2001; Lund et al. 2005). Geothermal energy thus has great potential in heat treatment of contaminated water.

Characterization of microbial diversity in irrigation water

To evaluate and choose the most appropriate and efficient disinfestation treatments in irrigation water, knowledge of the microbial diversity within the system is an important precondition (Hong and Moorman 2005). Knowledge of the microbial diversity and how it changes would facilitate understanding of the dynamics of plant pathogens in irrigation water. Specifically, these data would help identify naturally occurring biological control agents against plant pathogenic species. It could also support the physicochemical assessment of water quality and health in irrigation systems (Liu et al. 2011).

Microbial communities can be characterized using different methods. Traditional morphological and culture-dependent technologies, such as plating, and culture-independent molecular technologies, such as polymerase chain reaction (PCR) and molecular fingerprinting, give a picture of the structural composition of the microbial community in water (Alsanius and Jung 2003). However, morphological and culture-dependent approaches, especially for bacterial

species, can only detect a very small proportion of the total microbial community due to the difficulties of discriminating morphologically similar organisms, and the limitations of a majority of microbes being unculturable in field samples (Amann et al. 1995; Vartoukian et al. 2010). Molecular techniques have been shown to be very powerful to investigate the composition and structure of environmental microbial communities besides the traditional culture-dependent approaches (Case et al. 2007).

Microbial communities can be regarded as a mixture of microbial genomes. Genomic DNA sequences and their numbers indicate the community structure, which has been defined as the amount and distribution of (genomic) information in a particular habitat (Xie et al. 2009). Phylogenetically meaningful sequences, such as randomly amplified genome fragments, small subunit ribosomal RNA (rRNA) genes and conserved functional genes, have been used as molecular markers to analyze microbial communities by different technical strategies, including molecular hybridization, clone library profiling, and genetic fingerprinting by denaturing gradient gel electrophoresis (DGGE) (Wikstroim et al. 1999; Xie et al. 2009). Since the 1980s, rRNA-PCR allows microorganisms in a sample to be phylogenetically typed and quantified based on the sequence of their rRNA genes (Eisen 2007).

Since 1990, the 16S rRNA gene has been used as a molecular tool to investigate environmental microbial communities (Giovannoni et al. 1990). The 16S rRNA gene is highly conserved between different bacteria and archaea species, but the nine hypervariable regions (V1 to V9) with different sequence diversity among different species can be used as phylogenetic markers to detect bacterial community composition (Van de Peer et al. 1996). In addition, Chakravorty et al. (2007) demonstrated that none of the single regions can distinguish all bacteria species, but the V6 region can be used to identify most bacterial species except

enterobacteriaceae. Genomic DNAs are extracted directly from an environmental sample, such as water, soil or biofilm, and the 16s rRNA genes of the microbial community are amplified from the mixture by PCR. Molecular tools utilizing the16S rRNA gene to study microbial communities include cloning libraries, in-situ hybridization, and fingerprinting methods, such as DGGE (Tringe and Rubin 2005; Case et al. 2007).

PCR-DGGE has been used as a rapid and efficient approach to characterize microbial communities of many environmental samples, such as soil, different water sources, eg, groundwater, river, lake, sea water, drinking water; biofilms, and even the symbiotic microbial community of other organisms, eg. sponges (Kozdroj and van Elsas 2001; Kawai et al. 2002; Cho et al. 2003; Feris et al. 2003; Thoms et al. 2003; Brakstad and Lodeng 2005; Lyautey et al. 2005; Yan et al. 2007). Muyzer et al. (1993) was the first to report on the use of DGGE to separate PCR amplified fragments of genes coding for 16S rRNA, in order to analyze the genetic diversity of complex microbial populations. In DGGE, PCR products of similar size but different base-pair sequence are separated based on the different electrophoretic mobility of partially melted double-stranded fragments in polyacrylamide gels (Fischer and Lerman 1979). Theoretically, numbers and intensity of bands represent phylotype number and abundance in a microbial community (Muyzer and Smalla 1998; Nubel et al. 1999; Sigler et al. 2004). The separation of PCR products with optimum resolution is very critical to a good DGGE-based community structure analysis (Sheffield et al. 1989). This technique was particularly useful in the detection and identification of unculturable microbial populations and those present in low abundance (Holben et al. 2004). Cherif et al. (2008) demonstrated that PCR-DGGE was more accurate for bacterial identification in soil samples than other molecular methods. Lyautey et al.

(2005) also indicated that PCR-DGGE is useful for assessing the bacterial diversity of epilithic biofilms in rivers.

However, for irrigation water samples, the reliability and stability of PCR-DGGE to investigate the microbial community diversity compared with traditional culture-dependent approaches have not been determined. Brakstad and Lodeng (2005) revealed more than thirty different phylotypes in seawater from the North Sea by using only PCR-DGGE fingerprinting of 16S rRNA. Yan et al. (2007) investigated the microbial diversity of a plankton community in lakes by PCR-DGGE fingerprinting and concluded that PCR-DGGE fingerprinting might be more sensitive and effective to characterize the microbial diversity within an aquatic ecosystem than traditional methods. Liu et al. (2011) studied the microbial planktonic community diversity in a subtropical river by using PCR-DGGE fingerprinting. On the other hand, some researchers demonstrated that traditional culture-dependent methods were needed along with the molecular fingerprinting techniques to investigate the microbial communities (Kawai et al. 2002; Cho et al. 2003). Both cultured colonies growing on nutrient agar media after plating water samples and direct analysis of genomic bacterial DNAs from these samples by PCR-DGGE were utilized to study the bacterial communities in groundwater (Cho et al. 2003). Kawai et al. (2002) also indicated the importance of culture-independent methods to investigate the bacterial diversity in partially purified water, and conventional plating methods on media complemented the study.

Previous PCR-DGGE based microbial diversity studies have used different conditions, including the denaturant concentration ranges, electrophoresis voltage and time. Sigler et al. (2004) demonstrated that the DGGE profile of a bacterial community changed as the electrophoresis running time increased, and they recommended minimizing running time for optimum band resolution. In practice, various DGGE denaturant concentrations running under

different electrophoretic conditions have been used to study different environmental samples. DGGE with the denaturant concentration of 30 to 55% (a 100% denaturant concentration is defined as 40% [v/v] formamide and 7 M urea) running at 200 V for 3 h was used to study the microbial diversity of groundwater samples (Cho et al. 2003). Bacterial communities in partially purified water were analyzed by PCR-DGGE with a 40 to 60% denaturant concentration running for 12 h at 100 V (Kawai et al. 2002). The diversity of planktonic communities in lakes was determined by DGGE having denaturant concentrations of 40 to 60% running at 120 V for 16 h (Yan et al. 2007). The bacterial diversity of the epilithic biofilm in a river was assessed by PCR-DGGE with denaturant concentrations of 35 to 70% running at 100 V for 18 h (Lyautey et al. 2005). Thoms et al. (2003) investigated the bacterial community of the sponge *Aplysina cavernicola* by DGGE with 30 to 70% denaturant concentrations at 150 V for 6 h. Kozdroj and van Elsas (2001) characterized different bacterial richness in soil by using PCR-DGGE with a 45 to 65% denaturant concentration running at 100 V for 16 h. Cherif et al. (2008) also investigated microbial communities in soils by PCR-DGGE, but they used 40 to 60% denaturant concentration and electrophoretic conditions of 99 V for 17 h. But very few studies have been conducted to characterize the bacterial community diversity in irrigation water systems.

A better knowledge of dynamics of microorganisms in water will enable a more rapid and detailed assessment of the naturally occurring biological control agents against plant pathogenic species (Mazzola 2004; Liu et al. 2011). By manipulating and enhancing the beneficial microorganisms that exist in water, a pathogen-suppressing biological control system in irrigation water with minimum negative impacts may be obtained in greenhouses (Weller et al. 2002; Welbaum et al. 2004).

Numerous studies have described and characterized the diversity of soil microorganisms, and tested them as biocontrol agents of diseases caused by soilborne plant pathogens. The most extensively studied bacterial organisms, including *Pseudomonas* spp., *Bacillus subtilis*, and *Enterobacter cloacae*, have been reported to control many seedling diseases and root rots on several crops (Punja 1997). One of the major mechanisms is that bacteria produce substances that have a direct and deleterious effect on plant pathogens (Whipps 2001; Kobayashi and Crouch 2009). Other interactions between bacterial species and fungal pathogens range from parasitism and competition, to antibiosis, all of which have been exploited in the area of biological control of plant pathogenic (Whipps 2001; Duffy et al. 2003).

Parasitic interaction is characterized by the intimate association of two dissimilar organisms where one partner increases its fitness at the expense of the second partner, and it may be considered as pathogenic if a parasitic association with a bacterium leads to disease in the plant pathogen (Kobayashi and Crouch 2009). Bacteria can parasitize and degrade fungal plant pathogens, thus take off nutrients to inhibit the fungal growth. Parasitism could range from simple attachment of cells to hyphae, such as *Enterobacter cloacae* inhibiting *Pythium ultimum* (Nelson et al. 1986), to complete lysis and degradation of hyphae, such as *Pseudomonas putida* strain 06909 suppressing *Phytophthora nicotianae* (Yang et al. 1994).

Competition between bacterial and fungal plant pathogens for space, nutrients, or iron is another biocontrol mechanism (Whipps 2001). Scher and Baker (1982) indicated that *Pseudomonas putida* competing for iron in the infection court induced the soil suppressiveness to Fusarium wilt pathogens. Duijff et al. (1993) also demonstrated that the mechanism of *Pseudomonas putida* WCS358r suppressing Fusarium wilt of carnation caused by *Fusarium oxysporum* f. sp. *dianthi* depended on siderophore-mediated competition for iron.

Antibiosis is the antagonistic relationship between the bacterium and plant pathogenic species by the metabolic substances produced by bacteria. It may be nonspecific and conducted at a distance (Whipps 2001). For example, bacteria produce antifungal compounds to cause host cell leakage or lysis, and then use the released cellular contents as a nutrient source (Kobayashi and Crouch 2009). *Pseudomonas fluorescens* strain Pf-5 provides highly effective biocontrol activity across a broad range of fungal plant pathogens through the production of several secondary metabolites, including antifungal compounds such as pyoluteorin, pyrrolnitrin, and 2,4- diacylphloroglucinol, as well as several rhizoxin derivatives (Loper et al. 2007; Loper et al. 2008).

Bacillus subtilis Strain QST713 have a spectrum of antagonistic activity against over 40 plant diseases including, gray mold, damping-off, and powdery mildews through a mixed mechanisms of competition, parasitism, antibiosis, and induction of systemic acquired resistance (SAR) (Paulitz and Belanger 2001). And *Lysobacter enzymogenes* is antagonistic to a broad range of plant pathogens by infecting the pathogens intracellularly (Christensen and Cook 1978; Kobayashi and Crouch 2009).

Most biocontrol interactions are provided by single agents, but using mixtures or a combination of biocontrol agents would improve the biocontrol effect. That's because the mixed agents may have different or complementary mechanisms against plant pathogens. These multiple interactions are very common in the rhizosphere, for example, in suppressive soil or compost (Whipps 2001). Raupach and Kloepper (1998) proved that seed treatments using mixture of *Bacillus pumilus* strains INR7, *Bacillus subtilis* strain GB03, and *Curtobacterium flaccumfaciens* strain ME1 showed a greater control on the cucumber pathogens *Colletotrichum*

orbiculare, *Pseudomonas syringae* pv. *lachrymans*, and *Erwinia tracheiphila* than any single application.

A number of studies have investigated the microbial diversity in water; however, there are only very few attempts that effectively link these descriptive studies to the function of microbial communities and their influence on controlling plant pathogens in water. *Brevibacillus laterosporus* BPM3 isolated from a natural hot water spring strongly inhibited growth of plant pathogenic fungi, including *Fusarium oxysporum* f. sp. *ciceri*, *F. semitectum*, *Magnaporthe grisea* and *Rhizoctonia oryzae*) (Saikia et al. 2011). Biocontrol agents have been used to control plant pathogens in a hydroponic system for decades in Canada. *Pseudomonas fluorescens* and *P. corrugata* strains are used in a greenhouse in Canada to control Pythium diseases by reducing zoospore germination and chemotaxis directly or by producing an antifungal compound (Paulitz and Belanger 2001).

Research objectives:

The ultimate goal of this dissertation was to reinvigorate heat treatment for irrigation water by improving its energy efficiency. Specific objectives are described below.

Objective 1: To test the hypothesis that water temperature required to inactivate major pathogens in water may be lowered substantially from 95 ℃ with extended exposure time to improve energy efficiency and reduce environmental footprint.

- To evaluate effect of water temperature on survival of zoospores of *P. nicotianae* in the laboratory and on annual vinca (*Catharanthus roseus* cv. Little Bright Eye) in the greenhouse;

- To determine how life stages of *Phytophthora* species other than zoospores respond to water temperatures;
- To test how bacterial species respond to water temperatures.

Objective 2: To develop a scheme for collection, detection, and identification of bacterial species present in irrigation water.

- To compare the culture-dependent and –independent strategies;
- To determine the most efficient method for bacteria concentration in water samples;
- To identify the most appropriate conditions for PCR-DGGE profiling the 16S rRNA sequences of bacterial diversity.

Objective 3. To test the hypothesis that the bacterial community structure shifts under heat treatments at 42 and 48 ℃ in irrigation water which enhances antagonistic activities against plant pathogens.

- To use both culture-dependent and -independent strategies to characterize the bacterial community structures in irrigation water samples treated at 42 and 48 ℃ and compare with those at 25 ℃ (control);
- To identify potential pathogen-suppressing bacteria in the irrigation reservoir of study.

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Chapter 2

Inactivation of *Phytophthora* and bacterial species in water by a potential energy-saving heat treatment

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Abstract

Plant pathogens in re-circulated irrigation water, especially *Phytophthora* and bacterial species, present a significant health risk to nursery and greenhouse crops. Heat treatment at 95 ℃ for 30 s is one of the most reliable technologies for irrigation water decontamination. The primary objective here was to examine whether the water temperature required to inactivate major pathogens in re-circulated irrigation water can be lowered from 95 ℃ to conserve energy and improve horticultural profitability while reducing environmental footprint. Specifically, we investigated the effect of water temperature on *Phytophthora nicotianae* zoospore survival in the laboratory and on annual vinca under greenhouse conditions. We also assessed the effect of water temperature on survival of chlamydospores of *P. nicotianae*, oospores of *P. pini*, six plant pathogenic bacterial species and *Escherichia coli*. The zoospores of *P. nicotianae* did not survive or cause any disease on annual vinca when exposed to 42 ℃ for 12 h or 48 ℃ for 6 h. No chlamydospores of *P. nicotianae* survived at 42 ℃ for 24 h or 48 ℃ for 6 h, nor did the oospores of *P. pini* at 42 ℃ for 12 h or 48 ℃ for 6 h. In addition, none of the seven bacterial species survived 48 ℃ for 24 h. These results indicate that the water temperature required to eliminate *Phytophthora* and bacterial species may be lowered to 48 ℃ from 95 ℃ by longer exposure time, improving the economics and reducing environmental footprint.

Introduction

Many horticultural enterprises have implemented collection, recovery, and reuse of irrigation water to reduce water consumption and the release of pollutants, such as fertilizers and pesticides, into the environment (Bush et al. 2003; Hong et al. 2003; Hong and Moorman 2005; Cayanan et al. 2009). However, plant pathogens may be recycled and disseminated onto crops through the irrigation system. A large number of studies have revealed that contaminated irrigation water is one of the principal sources of disease inoculum in numerous crops (Thomson and Allen 1974; van Kuik 1992; McPherson et al. 1995; Pettitt et al. 1998; Bush et al. 2003; Hong and Moorman 2005; Cayanan et al. 2009). As reviewed by Hong and Moorman (2005), 17 *Phytophthora* species, 26 *Pythium* species, 27 genera of fungi, 8 species of bacteria, 10 viruses, and 13 species of plant parasitic nematodes have been found in irrigation water sources all over the world, including ponds, rivers, canals, streams, lakes, watersheds, reservoirs, wells, holding tanks, and runoff water.

Zoosporic pathogens are the most common and destructive plant pathogens carried in water, particularly those belonging to the genera *Pythium* Pringsh. and *Phytophthora* de Bary (Baker and Matkin 1978; Stanghellini et al. 1996a; Stanghellini et al. 1996b; Bush et al. 2003; Hong and Moorman 2005). *Phytophthora* species release zoospores as the primary dispersal and infective propagules. They also produce oospores as adaptive spores, and along with chlamydospores as survival structures (Erwin and Ribeiro 1996). Bacterial species including *Erwinia* species, *Ralstonia solanacearum,* and *Xanthomonas campestris,* are also destructive plant pathogens detected in water which attack a wide range of host plants (Hong and Moorman 2005). Water decontamination has become a priority for the floriculture industry to reduce the spread of plant pathogens in re-circulated irrigation systems (Hong and Moorman 2005).

Heat is one of the most reliable water treatments (Hong and Moorman 2005). It also poses minimal human health and environmental hazards. Current heat treatment protocols recommend raising and maintaining water temperature at 95 ℃ for 10 to 30 s, based primarily on the studies performed in the Netherlands (Runia et al. 1988) and the United Kingdom (McPherson et al. 1995). This water treatment is widely used in Europe. However, it is used to a lesser extent in the United States due to concerns in regard to low energy efficiency and high cost (Hong and Moorman 2005).

Previous studies have shown that the efficacy of heat treatment against plant pathogens depends on both temperature and exposure time. Exposing plant pathogens to sublethal temperatures, usually below 50 ℃, can inactivate them if maintained for long periods (Smith 1923; Munnecke et al. 1976; Runia et al. 1988; Gallo et al. 2007). Temperature-time exposures lethal to plant pathogens have been well established for soil steam sterilization where exposure time ranges from 0.5 to 12 h (Bollen 1985; Evans 1994). Heat treatment of infected roots with a continuous flow of steam-air mix passing over the infected root pieces of *Citrus* species in sand reduced survival of *Armillaria mellea* (Munnecke et al. 1976). The fungus was not viable on agar after treatment at 49 ℃ for 0.5 h, 41 ℃ for 4 to 7 h, and 38 ℃ for 24 to 38 h. Heat treatment in a water bath at 40 ℃ for 1 to 2 h inhibited the growth of mycelium and chlamydospores of *Phytophthora cinnamomi* on cornmeal agar (Gallo et al. 2007). Runia et al. (1988) reported that the viability of macroconidia of *Fusarium oxysporum* f. sp. *melongenae* in sterilized drain water was reduced with increasing exposure time at the same heat treatment temperature. Runia and Amsing (2001) indicated that heating of drain water to 85 ℃ for 3 min had the same efficacy as heating to 95 ℃ for 30 s for both tomato mosaic virus and *F. oxysporum* f. sp. *lycopersici*.

Similarly, the burrowing nematode (*Radopholus similis*) was eliminated at 48, 50, and 52.5 ℃ for 5, 2 min, and 30 s, respectively.

Based on the above studies and our own preliminary observations we hypothesized that water temperature required to inactivate major pathogens in recycled irrigation water may be lowered substantially from 95 ℃. The present study was to test this hypothesis and substantiate the findings for energy-saving heat treatment. The specific objectives of this study were to (1) evaluate effect of water temperature on survival of zoospores of *P. nicotianae* in the laboratory and on annual vinca (*Catharanthus roseus* cv. Little Bright Eye) in the greenhouse; (2) determine how life stages of *Phytophthora* species other than zoospores respond to water temperatures; and (3) test how bacterial species respond to water temperatures.

Materials and methods

Temperature treatments of *Phytophthora* zoospores

P. nicotianae isolate 31A3, recovered from *Petunia* species in 2004 in Virginia, and isolate 38E5, recovered from annual vinca (*Catharanthus roseus* cv. Pacific Red) in 2006 in Virginia, were used in the zoospore survival tests. The "Wet-plate" method (Ahonsi et al. 2007) with minor modifications was used to produce zoospores. In brief, three to four 3 mm diameter V8-agar culture mycelial plugs (three to four weeks old) were incubated in 10 mL 20% V8 broth in a 10 cm diameter Petri dish at 25 ℃ in dark. The broth was washed off after one week with growing mycelia attached to the bottom of the Petri dish. The Petri dishes with washed mycelia were further incubated in the dark at 25 ℃ for two to three weeks. Two hours before heat treatment, a volume of 20 mL of pre-chilled (4 ℃) sterile de-ionized water (SDW) was added to each Petri dish, which was then incubated at room temperature under fluorescent light (about

10000 lux) for zoospore release. The zoospore suspension was then collected in glass flasks for later use.

Zoospore survival was first tested in the laboratory. An estimated 150 motile zoospores or an estimated 200 forcefully encysted (by vortexing in 50 ml sterile Falcon tube) zoospores were added to 8 mL of PARP-V8 broth (Erwin and Ribeiro 1996) in a 10 cm diameter Petri dish. Three replicate plates were incubated at the respective combinations of treatment temperatures and exposure times described below in temperature-controlled incubators (Intellus Environmental Controllers, Percival Scientific, Inc.). The incubators were calibrated and monitored before and during the temperature experiment using a HOBO Pendant® temp/light data logger (Onset Computer Corporation). Temperature treatments included 25 (as control), 34, 36, 38 and 40 ℃ combined with an exposure time of 4, 6, 8, 10, 12, 14, and 16 h for motile zoospores, and 25 (as control), 34, 36, 38, 40, and 42 ℃ treated for 3, 6, 12, 24, 30, 36, and 48 h for encysted zoospores. Immediately after temperature treatments, the plates were moved to room temperature (25 ℃) for further incubation. Emerging colonies were counted 48 h after the start of the experiments.

Additional tests were performed in the greenhouse. Annual vinca (*Catharanthus roseus* cv. Little Bright Eye) were seeded in Metro Mix 360 (Sun Gro Horticulture) in multi-cell flats. After germination, each seedling plug was potted into a 10 cm square plastic pot with pine bark medium. Two hundred plants were maintained in a greenhouse (day hour/night hour = 12/12, 35 ± 3 ℃ at day time, 26 ± 3 ℃ at night). Slow release fertilizer Osmocote PLUS (N-P-K = 15-9-12) was applied when the seedlings were transplanted from flats into pots. Plants were watered as needed. Plants which were fifty to sixty days old were used for inoculation experiments.

Zoospore suspensions at 5000 spores/mL were heat-treated at different temperatures for different durations before inoculation. Eight treatments were included in the experiment: four different temperatures (25 as control, 40, 42, and 48 ℃) combined with two different time intervals (6 and 12 h). Temperature-controlled incubators were used for the temperature treatments and one hour was needed to raise this volume of zoospore suspension from room temperature to 48 ℃ in the incubator in the preliminary experiments. Glass flasks (250 mL) were used to hold the zoospore suspensions in incubators. The temperature treatments were started early in the morning so that inoculations could be done in the evening. Plants were watered manually before inoculation. Sixteen plants were used for each treatment. Once the temperature treatments were completed, a volume of 7.5 mL zoospore suspension was sprayed onto the foliage of each plant using a hand sprayer. Sixteen plants were sprayed with SDW as a negative control. Inoculated plants for each treatment were placed under a plastic tent at room temperature in the dark overnight to maintain moisture and facilitate infection. Plants were then moved to the greenhouse for further symptom development. Numbers of total and diseased plants and shoots in each treatment were counted. Subsequently, the percentage of diseased plants and shoots were calculated as estimates of zoospore survival for each heat treatment.

The experiments were conducted three times. Because the results from the two isolates were similar, only data from isolate 38E5 are presented here.

Production of chlamydospores and oospores by *Phytophthora* species

In preliminary experiments with four isolates of *P. nicotianae*, the isolate 26E7 produced the most chlamydospores. Subsequently, this isolate was used in the chlamydospore tests. This isolate was recovered from tobacco by Dr. D. Shew, North Carolina State University.

Chlamydospores were produced using the protocol described by Tsao (1971) with minor modifications. In brief, three 7 mm diameter one-week-old V8 agar culture plugs were cut and incubated in 36 mL 10 % V8 broth in a 500 mL glass bottle in the dark at 25 ℃ for a week for the mycelium mat to develop. Then 100 mL SDW was added to the bottle to deeply submerge the mycelium mat. Bottles with mycelium mats were further incubated at 18 ℃ in the dark for another two to three weeks to produce chlamydospores. For chlamydospore separation, the mycelium mat was blended at low speed using a variable speed laboratory blender (Waring Laboratory) for 3 min in 100 mL SDW. Resultant chlamydospores and mycelium fragments in the suspension were separated by centrifugation at 1500 g twice for 1 min. Chlamydospores in the pellet were re-suspended in 10 mL SDW and diluted to a concentration of 250 spores/mL for temperature treatments.

The homothallic species *P. pini* isolate 43J6, which always produced a large number of oospores, was used for the oospore heat treatment study. This isolate was recovered from *Rhododendron catawbiense* cv. Boursault in 2007 in Virginia. Oospores were produced using a protocol adapted from Ann and Ko (1988). One-month-old V8-agar culture (in a 10 cm diameter Petri dish), which had a large amount of oospores already, was blended using a variable speed laboratory blender at high speed for 2 min in 100 mL SDW. The resultant oospore and mycelium fragment mixtures were sieved successively through 125, 38, and 20 μm sieves. The 125 and 38 μm sieves were used to remove agar particles and the 20 μm sieve retained the oospores with an average diameter of 28 μm. The oospores were collected in SDW and mixed with an equal volume of 0.5% $KMnO_4$ for 20 min to stimulate germination by breaking dormancy (Ann and Ko 1988). Then, the oospores were washed and re-suspended in 10 mL SDW and diluted to 250 spores/mL for temperature treatments.

Temperature treatments of *Phytophthora* chlamydospores and oospores

Chlamydospore and oospore suspensions were incubated at different temperatures for different durations as designated. Specifically, chlamydospore suspensions were treated at five temperatures: 25 (as control), 38, 40, 42, or 48 ℃ each for five durations (6, 12, 24, 48, and 72 h). Oospore suspensions were treated at the same temperatures for three durations (6, 12, and 24 h). Temperature-controlled incubators were used for the temperature treatments. One milliliter of spore suspension in a 1.5 ml microtube was used as a replicate. Three replicates were included for each treatment. Microtubes were placed into a tube rack in the incubator for temperature treatments.

After heat treatment, an aliquot of 200 µl of chlamydospore suspension (approximately 50 spores) was plated onto 10% V8 agar medium (10 cm diameter Petri dish) for each replicate and incubated at 25 ℃ in the dark for 1 to 3 d. The germinated spores in each plate were counted under a dissecting microscope (Model SMZ800, Nikon Instruments Inc.) and the germination rate was calculated. Similarly, a 200 µl aliquot of heat-treated oospore suspension was plated on S+L salt medium plate (Ann and Ko 1988). Germinated oospores were counted under a dissecting microscope after incubating at 25 ℃ under light for 2 to 5 d.

The water temperature treatment experiments for chlamydospores and oospores were repeated three times.

Temperature treatments of bacterial species

Six common bacterial plant pathogens *Agrobacterium tumefaciens*, *Erwinia carotovora*, *E. amylovora*, *Pseudomonas syringae* pv. tomato, *Ralstonia solanacearum*, *Xanthomonas*

campestris, and a laboratory strain of the human commensal *Escherichia coli* DH52 were tested in this experiment. Three bacteria strains, *A. tumefaciens, E. carotovora,* and *R. solanacearum* were kindly provided by Dr. A. Baudoin, and *E. amylovora* was provided by Plant Disease Clinic, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech. The other three bacterial strains were from the lab collection. The six plant bacteria strains were isolated from plant tissues of different hosts. *A. tumefaciens, E. amylovora, E. carotovora, R. solanacearum, X. campestris* and *E. coli* were grown on Luria-Bertani agar (LB) medium (10 cm diameter Petri dish), and *P. syringae* on King's B (KB) medium. Bacteria were streaked out on media plates from stocks, incubated at 28 ℃ (37 ℃ for *E. coli*) overnight in the dark, then transferred to fresh media plates and grown for one more day. Each bacterial strain was transferred to a sterile tube containing 4 ml SDW using a transfer loop. Bacterial clusters were re-suspended and diluted to an optical density at 600 nm (OD600) of 0.01 with SDW.

Bacterial suspensions were heat-treated at different temperatures for 24 h. Six temperature treatments were included: 28 ℃ as control (with exception of 37 ℃ for *E. coli*), 40, 42, 44, 46, and 48 ℃. Four replicates of 2 mL bacterial suspensions were used for each treatment. After temperature treatments, bacterial suspensions were diluted to 1:1000, and the diluted suspensions were streaked out onto media plates. Emerged colonies were counted for 1 to 3 d. The experiment was performed three times.

Because *X. campestris* and *E. coli* did not grow well in the initial assays including the controls (few colonies emerged at 28 ℃ for *X. campestris* and 37 ℃ for *E. coli*), an additional experiment was conducted for these species. In brief, bacterial suspensions at OD600 of 0.01 were treated at 28/37 ℃ (as control) and 48 ℃ for 24 h. Three concentrations of the bacterial

suspension: undiluted, 1:10, and 1:100 dilutions, were streaked out onto LB media plates to check for survival. The experiment was performed three times.

Statistical analysis

A completely randomized design was used in the greenhouse experiments. Data obtained from repeated experiments were evaluated for homogeneity using R statistical software (R Development Core Team, R Foundation for Statistical Computing. 2011. ISBN 3-900051-07-0), and then pooled together. Student's *t* test and analysis of variance (ANOVA) were conducted using SAS (version 9.2, SAS Institute) to determine the effect of treatment temperatures and exposure time on the survival of zoospores, on the germination rate of chlamydospores and oospores, and on colony forming units of bacterial species.

Results

Water temperature effect on *Phytophthora* zoospores survival

Plating motile zoospore suspensions of *P. nicotianae* incubated at 36 ℃ or above for 10 h or longer did not result in any colonies whereas those at 25 ℃ (control) produced 18 to 20 colonies per Petri dish (Fig. 2.1a). Similarly, plating encysted zoospore suspensions treated at 36 ℃ or above for 12 h or longer resulted in zero colony development whereas those held at 25 ℃ generated over a hundred colonies per Petri dish (Fig. 2.1b).

For the greenhouse zoospore survival tests, no disease developed on plants sprayed with unheated SDW (negative control) while all plants inoculated with zoospore suspension held at 25 ℃ for 6 and 12 h (positive controls) developed severe foliage blight (Fig. 2.2). The percentage of diseased plants and blighted shoots declined significantly as the temperature increased. For 6-

h heat treatments, the percentage of diseased plants declined to 16% at 42 ℃, and to 0% at 48 ℃. Likewise, the percentage of blighted shoots decreased from 83% in the control (25 ℃) to 2% at 42 ℃ and to 0% at 48 ℃. After 12 h of heat treatment, the percentage of diseased plants dropped to 34% at 40 ℃ and 0% at 42 ℃. Similarly, the percentage of diseased shoots declined to less than 10% at 40 ℃ and 0% at 42 ℃ (Fig. 2.2).

Water temperature effect on *Phytophthora* chlamydospore and oospore survival

Chlamydospore germination of *P. nicotianae* declined as the temperature and treatment time increased (Fig. 2.3). Nearly 99% of chlamydospores did not germinate after heat treatment at 40 ℃ for 48 h, and 100% inhibition of germination occurred at 40 ℃ for 72 h, at 42 ℃ for 24 h, or at 48 ℃ for 6 h. Chlamydospore germination also decreased with time even at 25 ℃.

Oospore germination of *P. pini* also declined with increasing treatment temperature and time (Fig. 2.4). Heat treatments at 40 ℃ for 24 h, at 42 ℃ for 12 h, or at 48 ℃ for 6 h stopped the germination of more than 98% oospores. At 25 ℃ (control), oospores of *P. pini* had a relatively high germination rate no matter how long they were exposed (Fig. 2.4).

Water temperature effect on bacterial species

Among the seven bacterial species, *P. syringae* was the most heat-sensitive and did not survive at a water temperature of 40 ℃. *A. tumefaciens* and *E. amylovora* were slightly more tolerant but did not survive at temperatures higher than 42 ℃. *E. carotovora* survived at 44 ℃ at a very low rate, but did not survive at 46 ℃. *R. solanacearum* survived at 46 ℃ but was completely killed when the water temperature was raised to 48 ℃ (Table 2.1). Both *X. campestris*

and *E. coli* survived at 46 ℃, but neither survived at 48 ℃ (Tables 2.1 and 2.2). Overall, none of the seven species survived at 48 ℃ for 24 h.

Discussion

This study demonstrated that heat treatments at 42 ℃ for 24 h or at 48 ℃ for 6 h inactivated the major life stages of *Phytophthora* species, and heat treatments at 48 ℃ for 24 h inactivated all seven tested bacterial species. The results indicate that raising and maintaining water temperature at 48 ℃ for 24 h may be an alternative to the current heat treatment protocol which uses 95 ℃ for 30 s. This new temperature and exposure time combination could reduce energy consumption and thus will have practical implications.

Phytophthora nicotianae represents high-temperature tolerant oomycetes, which are known as water molds and the most frequently isolated plant pathogens in irrigation water (Hong and Moorman 2005; Gallegly and Hong 2008). Therefore, it is expected that the heat and exposure time combination that worked for *P. nicotianae* would work for a vast majority, if not all, oomycete pathogens in irrigation water. Specifically, zoospores are the primary dispersal propagules of *Phytophthora* and *Pythium* species in water (Thomson and Allen 1974; Stanghellini et al. 1996a; Stanghellini et al. 1996b). Heating the infested water at 48 ℃ for 6 h provided 100% control of the aerial blight on annual vinca caused by zoospores of *P. nicotianae* in the greenhouse. The required temperature to kill motile and encysted zoospores in the laboratory was even lower due to the smaller volume of zoospore suspensions. Heat treatment at 48 ℃ for 6 h also completely inhibited the viability of chlamydospores of *P. nicotianae* and oospores of *P. pini*. The inactivation of the main life stages of *P. nicotianae* and *P. pini* were all achieved at 48 ℃ within 6 h instead of 24 h. Considering the size and wall thickness of

chlamydospores of *P. nicotianae* and oospores of *P. pini*, 48 ℃ and 6 hour may suffice to kill most oomycete pathogens and extending treatment time certainly will further improve the heat treatment efficacy.

Heat treatment at 48 ℃ for 24 h eliminated the growth of all seven bacterial species, each of which showed different water temperature sensitivities. Hong and Moorman (2005) pointed out that *Erwinia* species, *P. syringae, R. solanacearum,* and *X. campestris* are the most common bacterial plant pathogens found in irrigation water. Our results suggest that heat treatment of water at 48 ℃ for 24 h could inactivate these bacterial species and *Phytophthora* species, both of which make up the two of the major plant pathogen groups in water. More remarkably, this study has shown that there is no risk of sacrificing the efficacy of heat treatment by lowering treatment temperature significantly from 95 ℃ in the current protocol.

Compared to current heat treatment (95 ℃ for 30 s), the new recommendation (48 ℃ for 24 h) could save energy consumption by 50% or more depending on the temperature of returning water and the insulation of the holding tank. Assuming that the temperature of returning water is 20 ℃, only 55,000 Btu is needed to heat 500 L water to 48 ℃ whereas 148,500 Btu is required to heat the same quantity of water to 95 ℃. At the same time, modern insulation technology has reduced heat loss to a minimum. According to the minimum energy performance standard (MEPS and Labelling Requirements 2005) levels, the maximum allowable standing heat loss from 500 L hot water (~50 ℃) of mains pressure electric water heaters is 3.15 kWh or 10,748 Btu within 24 h. The heat loss within 30 s for the current protocol is negligible. Therefore, the energy required to raise and maintain the water temperature at 48 ℃ for 24 h is approximately 65,748 Btu for the new heat treatment protocol, which is less than one half of what is needed for the current protocol (148,500 Btu). Additional investigations are warranted to assess these

potential energy savings at production facilities, identify alternative energy generation and retention sources and develop strategies to further improve the economics and environmental footprint of this new heat treatment protocol.

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Table 2.1 Growth of six bacterial plant pathogens and a human commensal bacterium after heat treatment at different temperatures for 24 h.

Bacteria species	Colony-forming unit per 10 µL (1:1000 dilution)					
	Control[a]	40 ℃	42 ℃	44 ℃	46 ℃	48 ℃
Agrobacterium tumefaciens	208 A[b]	105 B	0 C	0 C	0 C	0 C
Erwinia amylovora	207 A	50 B	5 C	0 C	0 C	0 C
E. carotovora	548 A	492 B	64 C	1 D	0 D	0 D
Pseudomonas syringae	253 A	0 B	0 B	0 B	0 B	0 B
Ralstonia solanacearum	300 A	263 B	159 C	25 D	14 D	0 E
Xanthomonas campestris	19 A	5 B	2 B	2 B	1 B	1 B
Escherichia coli	18 A	9 B	7 BC	2 CD	1 D	0 D

[a] Controls were incubated at 28 ℃ except *E. coli* which was incubated at 37 ℃.

[b] Values followed by a different capitalized letter in each row differed significantly at a *P* value of < 0.05 using the Student's *t* test.

Table 2.2 Growth of *Xanthomonas campestris* and *Escherichia coli* after heat treatment at 48 ℃ for 24 h.

Bacteria species	Temperature treatment	Colony-forming unit per 10 μL		
		Undiluted suspension	1:10 dilution	1:100 dilution
X. campestris	Control[a]	Countless	583 A[b]	150 A
	48 ℃	0	0 B	0 B
E. coli	Control[a]	Countless	622 A	127 A
	48 ℃	0	0 B	0 B

[a] Controls were incubated at 28 ℃ for *X. campestris* and 37 ℃ for *E. coli*.

[b] Values followed by a different capitalized letter in each row differed significantly at a *P* value of < 0.05 using the Student's *t* test.

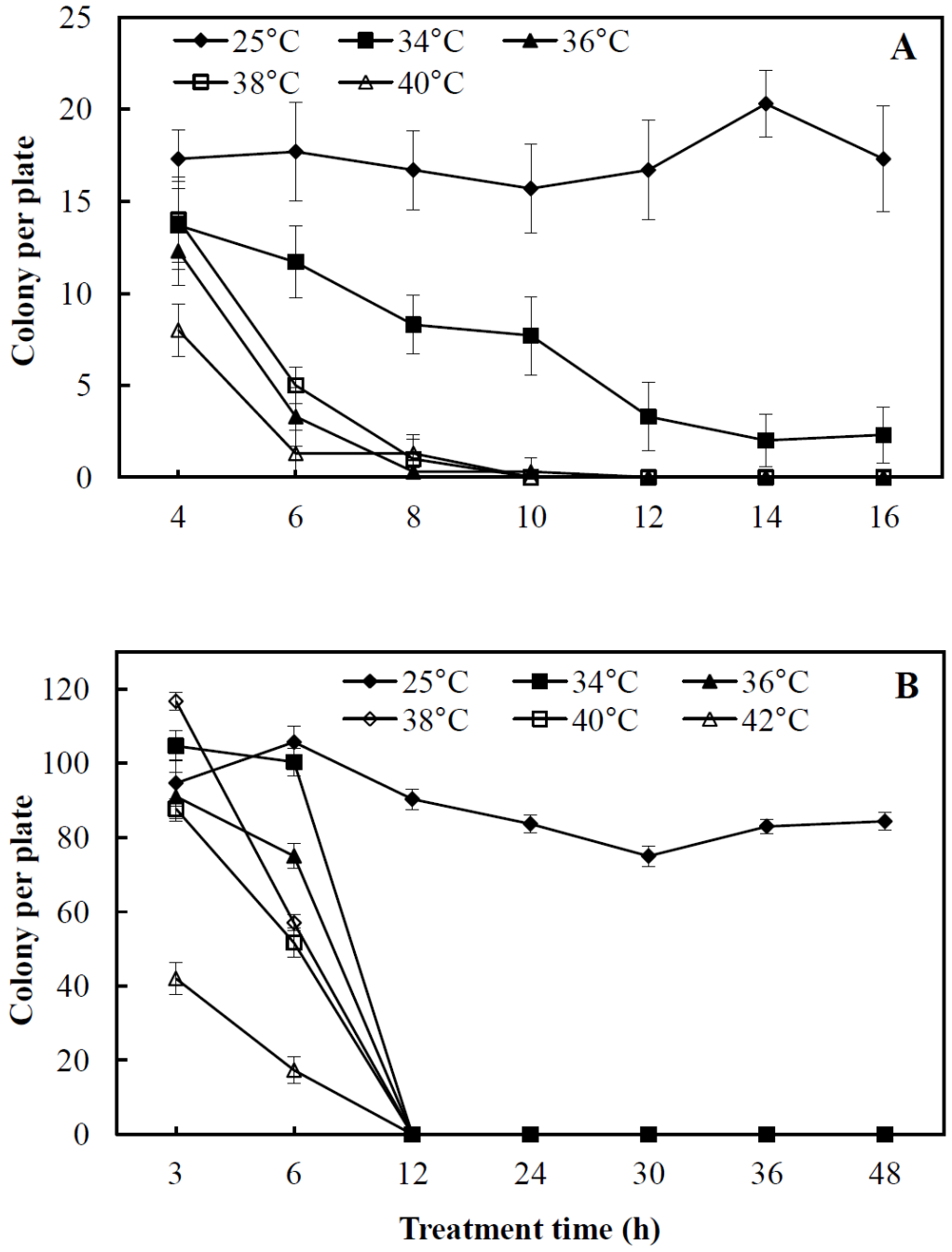

Figure 2.1 Effect of heat treatments on survival of (a) motile zoospores and (b) encysted zoospores of *Phytophthora nicotianae* in laboratory. The vertical bars represent standard errors.

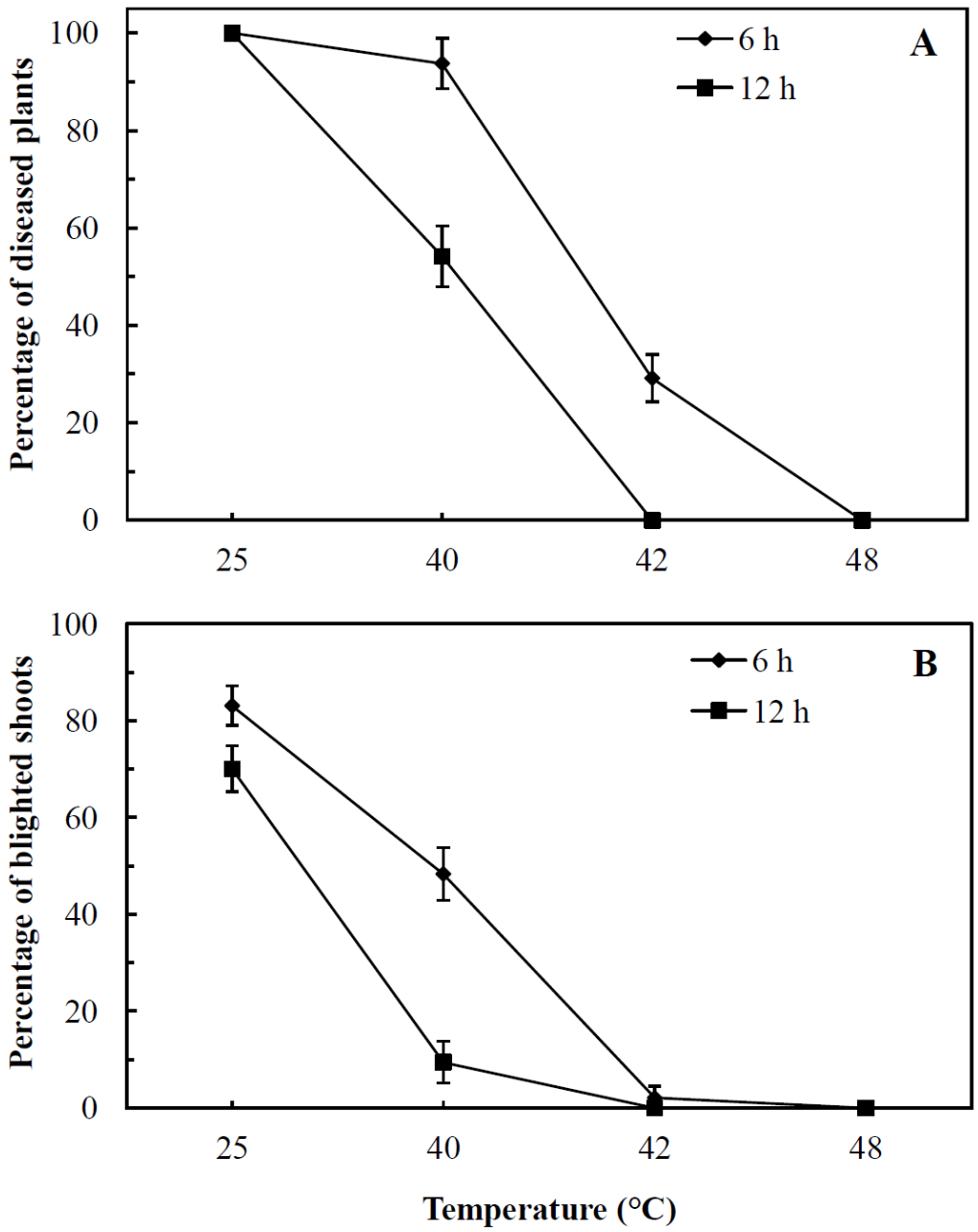

Figure 2.2 Effect of heat treatments on survival of zoospores of *Phytophthora nicotianae* as indicated in the (a) percentage diseased plants and (b) percentage blighted shoots of annual vinca 7 d after inoculation in the greenhouse. The vertical bars represent standard errors.

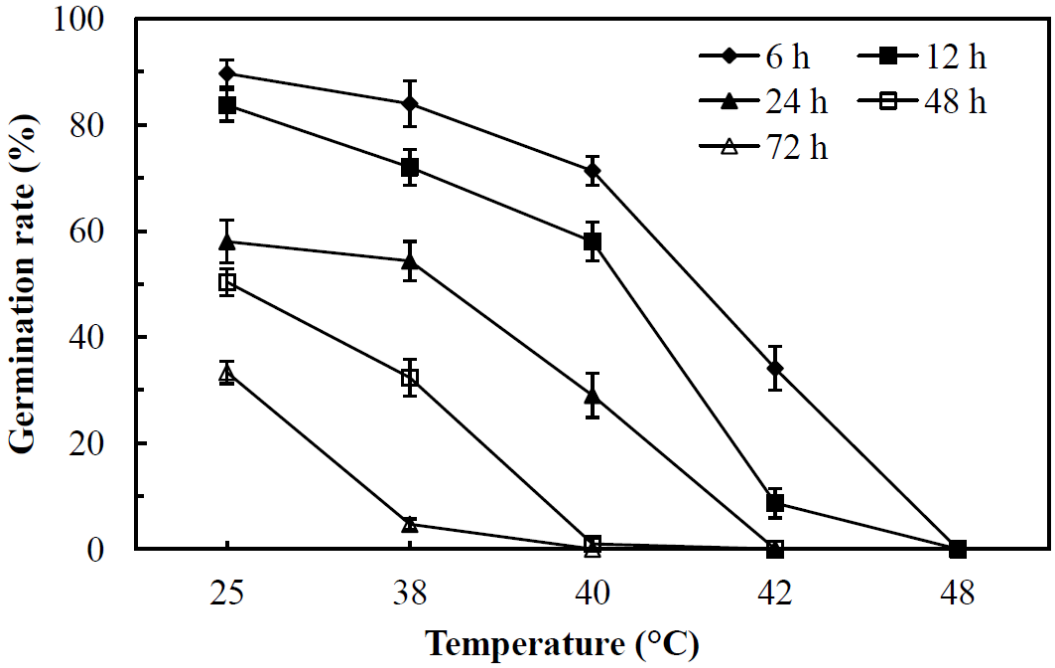

Figure 2.3 Germination of chlamydospores of *Phytophthora nicotianae* as affected by heat treatments. The vertical bars represent standard errors.

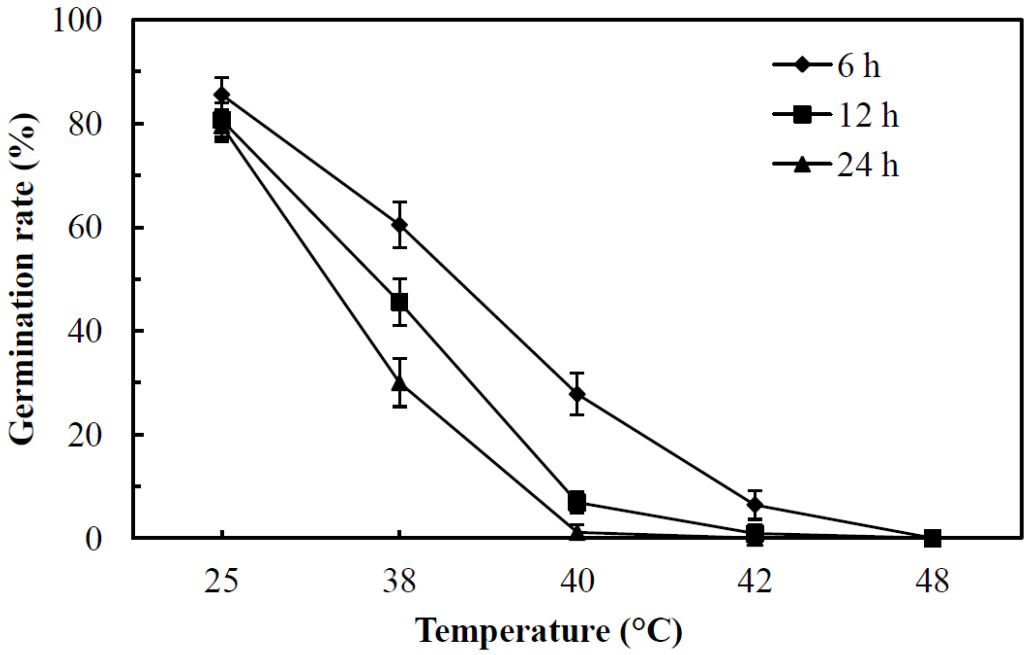

Figure 2.4 Germination of oospores of *Phytophthora pini* as affected by heat treatments. The vertical bars represent standard errors.

Chapter 3

Temperature effect on *Phytophthora* zoospore survival in irrigation water

Our previous study in Chapter 2 (Hao et al. 2012) revealed the required temperature of water heat treatment to inactivate major groups of plant pathogens can be lowered to 48 ℃ for 24 h from the currently recommendation of 95 ℃ for 30 s without efficacy sacrifice. Sterile deionized water was consistently used in these experiments. However, what is going on in irrigation water during the heat treatments at 42 and 48 ℃ have not been studied. The objective of this study was to compare the zoospore survival of *Phytophthora nicotianae* in irrigation water (IW) and sterile deionized water (SDW) at heat treatment temperatures 42 and 48 ℃.

Materials and Methods

Water samples were collected from an irrigation reservoir at a local nursery in eastern Virginia in June 2012. All samples were collected from the surface water column in the center of the reservoir in sterile bottles. Three replicates of 1 L each were collected. Water samples were then transported to the lab and immediately subjected to following experiments.

P. nicotianae isolate 38E5, recovered from annual vinca (*Catharanthus roseus* cv. Pacific Red) in Virginia in 2006, were used in the zoospore survival tests. The "Wet-plate" method (Ahonsi et al. 2007) with minor modifications was used to produce zoospores. In brief, three to four 3 mm-diameter V8-agar culture mycelial plugs (three to four weeks old) were incubated in 10 mL 20% V8 broth in a 10-cm diameter Petri dish at 25 ℃ in dark for a week. The broth was washed off with growing mycelia attached to the bottom of the Petri dish. The Petri dishes with

washed mycelia were further incubated in dark at 25 ℃ for 2 to 3 week. Two hours before heat treatment, a volume of 20 mL of pre-chilled (4 ℃) SDW was added to each Petri dish, which was then incubated at room temperature under fluorescent light (about 10,000 lux) for zoospore release. The zoospore suspension was then collected in glass flasks.

Zoospore suspensions were diluted to 5,000 spore/mL with IW or SDW and heat-treated at three different temperatures (25 as control, 42, and 48 ℃) for four different durations (6, 12, 24, and 48 h). A volume of 10 mL zoospore suspension was used as a replicate, and three replicates were included each treatment. Temperature-controlled incubators (Intellus Environmental Controllers, Percival Scientific, Inc.) were used for the temperature treatments. The incubators were calibrated and monitored before and during the temperature experiment using a HOBO® temp/light pendant data logger (Onset Computer Corporation). Immediately after temperature treatments, an aliquot of 100 µl of zoospore suspension (approximately 500 spores) was plated onto a 10-cm diameter Petri dish with 10% V8 agar medium plate. Three plates each replicate were incubated at 25 ℃ in the dark. Emerging colonies were counted after 48 h. The experiment was performed three times.

Data obtained from repeated experiments were evaluated for homogeneity and pooled together. Student's *t* test with a P value of 0.05 and analysis of variance (ANOVA) were conducted to determine the significance of differences among treatment means. R statistical software (R Development Core Team, R Foundation for Statistical Computing. 2011. ISBN 3-900051-07-0) was used for all statistical analyses.

Results and discussion

Zoospores of *P. nicotianae* survived longer and at greater rates in SDW than IW (Fig. 3.1). This was consistently at 25 ℃ across all the exposure times from 6 to 48 h, as indicated by the number of cfu per plate. A large portion of zoospores in SDW survived but none of those in IW did after a 6-h heat treatment at 42 ℃. No colony was observed in any dishes plated with zoospores treated at the same temperature (42 ℃) for longer periods of time or at higher temperature (48 ℃) in this study.

These results implicate that there were agents in irrigation water that worked against the *Phytophthora* zoospore when compared to SDW. These agents may be microbes including bacterial species and/or their metabolites. Additional studies are warranted to investigate what microorganisms produce these substances, whether and how they can be integrated into the heat treatment to further lower the treatment temperature, improving its economics and reducing environmental footprint which was a central themes of Chapter 4 and 5.

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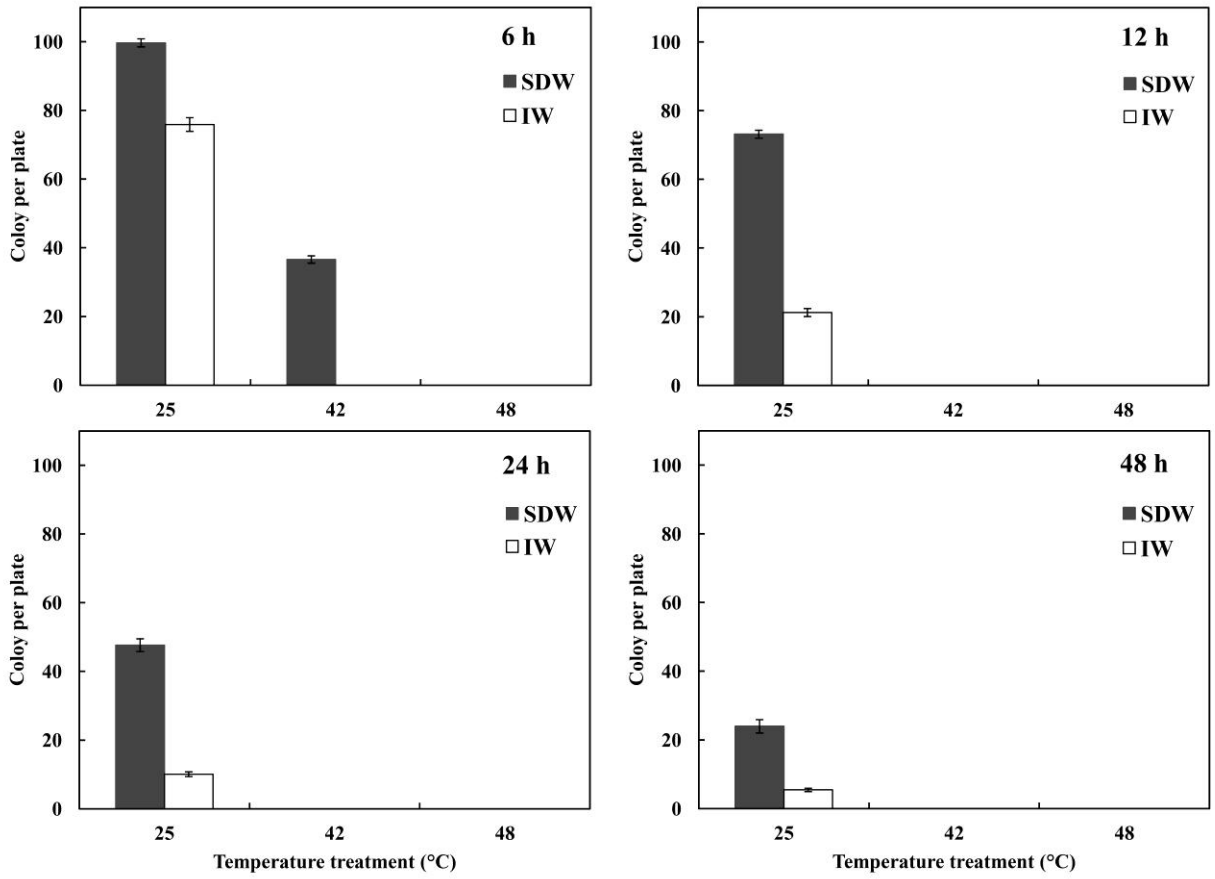

Figure 3.1 Effect of heat treatments (25 ℃ as control, 42 and 48 ℃) on survival of zoospore of *Phytophthora nicotianae* in sterile de-ionized water (SDW) and irrigation water (IW) at (a) 6 h, (b) 12 h, (c) 24 h, and (d) 48 h. The vertical bars represent standard errors.

Chapter 4

Characterization of microbial diversity in irrigation water: sample concentration and processing strategies

Abstract

Understanding the microbial diversity in irrigation reservoirs is essential to manage pathogens originating and spreading through water and to identify naturally occurring biological control agents against plant pathogens. The objective of this study was to develop an optimized methodology for collection, detection, and identification of bacterial species present in irrigation water. Specifically, (1) culture-dependent and -independent methods, (2) three water sample concentration methods, and (3) different DGGE denaturant concentrations and different electrophoresis conditions were compared for recovery of bacterial diversity from irrigation water. Species composition determined by culture-dependent and -independent strategies were substantially different. In the culture-independent strategy, more bacteria were detected in water samples via a combination of centrifugation and filtration than when using each alone. PCR-DGGE with a 40 to 60% denaturant concentration running at 70 V for 16 h revealed the highest bacterial diversity. In conclusion, a step-wise methodology to characterize bacterial diversity in irrigation water has been developed. In this scheme, both culture-dependent and -independent strategies are employed. Centrifugation is used along with filtration to concentrate water samples, and DGGE with 40 to 60% concentration at 70 V for 16 h is performed in the culture-independent strategy.

Introduction

Understanding the microbial diversity in irrigation reservoirs is essential to assess and manage crop health risk incited by plant pathogens originating and spreading through irrigation systems (Hong & Moorman, 2005). Such studies help identify naturally occurring biological control agents against plant pathogenic species, and also improve the physicochemical assessment of water quality and health in irrigation systems (Liu et al. 2011). Of particular interest to this project is whether there is bacterial community structure shift in irrigation water after heat treatments and whether and how such shifts may have contributed to the pathogen suppression at reduced heat treatment temperatures of 42 and 48 ℃.

Both culture-dependent and -independent approaches have been utilized for investigation into microbial diversity in the environment. The culture-dependent approach consists of growing microbes on general or selective media followed by morphological, biochemical, or molecular identification. The main limitation of this approach is that the majority of environmental microbes are unculturable (Amann et al. 1995; Vartoukian et al. 2010). The culture-independent approach employs molecular techniques including clone libraries, in-situ hybridization, molecular fingerprinting methods, and metagenomics (Case et al. 2007; Shokralla et al. 2012).

Denaturing gradient gel electrophoresis (DGGE) is a rapid and efficient DNA fingerprinting technique for characterizing microbial communities (Kozdroj and van Elsas 2001; Kawai et al. 2002; Cho et al. 2003; Feris et al. 2003; Thoms et al. 2003; Brakstad and Lodeng 2005; Lyautey et al. 2005; Yan et al. 2007). Muyzer et al. (1993) first used DGGE to separate polymerase chain reaction (PCR) amplified 16S rRNA gene fragments in order to analyze the genetic diversity of complex microbial populations. In DGGE, PCR products of similar size but different base-pair sequences are separated based on their electrophoretic mobility (Fischer and

Lerman 1979). The DNA fragments encounter increasing concentrations of chemical denaturant as they migrate through a polyacrylamide gel. Upon reaching a threshold denaturant concentration, the weaker melting domains, which are the stretches of base pairs in the PCR amplicon with an identical melting temperature, begin to denature. The DNA molecules transition from helical to partially melted, at which time migration slows dramatically. The different concentrations of denaturant and sequence variation within the DNA fragments result in a pattern of bands as the DNA migrates through the gel. The separation of PCR products with optimum resolution is very critical for a good DGGE-based community structure analysis (Sheffield et al. 1989). Theoretically, the number and intensity of bands represents phylotype number and abundance within a sample (Muyzer and Smalla 1998; Nubel et al. 1999; Sigler et al. 2004). Cherif et al. (2008) demonstrated that PCR-DGGE was more accurate for bacterial identification in soil samples than other molecular methods. Lyautey et al. (2005) also indicated that PCR-DGGE was useful for assessing bacterial diversity of epilithic biofilms in rivers.

The efficiency of PCR-DGGE as opposed to traditional culture-dependent approaches for characterization of microbial community diversity in aquatic environments has not been determined. Some researchers used only PCR-DGGE to investigate microbial diversity in the North Sea (Brakstad and Lodeng 2005) and planktonic community diversity in rivers and lakes (Yan et al. 2007; Liu et al. 2011). Yan et al. (2007) concluded that PCR-DGGE fingerprinting might be sensitive and effective to characterize the microbial diversity within an aquatic ecosystem. On the other hand, other studies used traditional culture-dependent approaches along with culture-independent molecular fingerprinting techniques to investigate microbial communities, for example, in groundwater and partially purified water (Kawai et al. 2002; Cho et al. 2003).

Various DGGE conditions, including different ranges of denaturant concentration, electrophoresis voltage, and time have been used in previous PCR-DGGE based microbial diversity studies of environmental samples (Kozdroj and van Elsas 2001; Kawai et al. 2002; Cho et al. 2003; Thoms et al. 2003; Lyautey et al. 2005; Yan et al. 2007; Cherif et al. 2008). However, how different DGGE conditions influence the results of a DGGE analysis has not been investigated.

The aim of this study was to develop an optimized and standardized methodology to collect, detect, and identify bacterial species present in greenhouse and nursery irrigation water. The specific objectives were to (1) compare the culture-dependent strategy with -independent strategy for investigation of bacterial diversity, (2) determine the most efficient method to concentrate water samples, and (3) identify the most appropriate conditions for DGGE to analyze bacterial species in irrigation water.

Materials and methods

Water samples

Water samples were collected from an irrigation reservoir at a local nursery in eastern Virginia in September 2011 and May 2012. All samples were collected in sterile bottles from the surface of a water column in the center of the reservoir. Three replicates of 1 L each were collected. Water temperature and other water quality parameters were recorded on-site using a Hydrolab DS5X multiparameter water quality meter (Hach®, Loveland, CO, USA). Water samples were then transported to the lab and immediately passed through nylon membrane filters (pore size 10 μm) to remove larger debris and algae. The filtrate was then used for further processing.

Culture-dependent strategy

Plating

One hundred microliters of the filtrate was evenly spread in a 10-cm diameter Petri dish with Nutrient Agar (NA) (Difco™, Detroit, MI) using a sterile glass spreader. Three replicate plates were used for each sample and incubated at 28 ℃ for 2 d.

Colony PCR & SSCP analysis

Colonies on NA plates were grouped based on color and size. The total numbers of colonies in each group were recorded. About 20 to 30% of the colonies from each group were sub-cultured into 48 well plates containing NA medium, and incubated at 28 ℃ for 2 d. Colony PCRs were performed to amplify the hypervariable regions V6 to V8 of 16S rDNA with primers 16S f968 and r1401 (Nubel et al. 1996; Peixoto et al. 2002). Reagents supplied with Takara *Taq* (Takara Bio Inc., Japan) were used in the reactions. The PCR mixture (25 μl) contained 0.1 μl Takara *Taq* (5 units/ μl), 2.5 μl 10× PCR buffer (containing 1.5 mM $MgCl_2$), 2 μl dNTP mixture (2.5 mM each), 1 μl of each primer (10 μM), and 18.4 μl DNA-free nanopure water. Bacterial colonies were picked directly from culture plates and added to the mixture. Cycling conditions consisted of initial denaturation at 96 ℃ for 2 min, followed by 40 cycles of denaturation at 94 ℃ for 0.5 min, annealing at 55 ℃ for 0.5 min, and an extension at 72 ℃ for 1 min. For the last cycle, the extension time was increased to 10 min.

Single-strand conformation polymorphism (SSCP) analyses were performed on the PCR products as described by Kong et al. (2003) with minor modifications. In brief, the amplicons were mixed with loading dye (1:4, v:v) and denatured by heating at 96 ℃ for 10 min then chilled

on ice. Two microliters were loaded into each well on an 8% polyacrylamide gel (acrylamide/bisacrylamide = 29:1) running at 220 V for 2.5 h in chilled 1 × TBE buffer. After electrophoresis, the gels were stained with silver nitrate (Beidler et al. 1982). SSCP banding patterns of individual colonies were determined with the aid of a 100 bp DNA ladder (Promega Corp., Madison, WI). PCR products with the same SSCP fingerprint were grouped and stored for sequence analysis.

Culture-independent strategy

Irrigation water sample concentration

Three methods were evaluated for concentrating irrigation water samples after filtration through a 10-μm membrane. In the first method (P), the filtrate was centrifuged at 12,200 g for 50 min, and the supernatant was discarded. The pellet was re-suspended in 1.5 mL SDW and centrifuged at 11,750 g for 5 min. Genomic DNA was extracted from the final pellet. For the second method (F), the filtrate was passed through nylon membrane filters (pore size 0.2 μm), and the filters trapping bacterial cells were used for genomic DNA extraction. The third method (C), involved centrifugation of the filtrate at 12,200 g for 50 min, the pellet was saved, and the supernatant was then filtered through 0.2 μm filters. Total genomic DNA was extracted from the pellet and the 0.2 μm filters and combined for analysis.

Genomic DNA extraction

The UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA) was used to extract bacterial genomic DNAs from the pellets following the instruction manual.

The PowerWater DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA) was used to extract bacterial genomic DNA from the 0.2-μm filters.

PCR amplification and evaluation of DGGE conditions

For DGGE analysis, 16S rRNA fragments were amplified with primers GC-clamp-EUB f933 and EUB r1387 covering the three hypervariable regions V6 to V8 (Kawai et al. 2002). PCR mixture (50 μl) contained 0.2 μl Takara *Taq* (5 units/ μl), 5 μl 10× PCR buffer (containing $MgCl_2$), 7.5 μl BSA (0.3 mg/ml), 4 μl dNTP mixture (2.5 mM each), 2 μl of each primer (10 μM), 19.3 μl DNA-free nanopure water, and 10 μl DNA extract. Hot-start PCR was performed at 94 ℃ for 4 min, followed by touchdown PCR with the following parameters: denaturation at 94 ℃ for 1 min, annealing at 65 ℃ decreasing by 1 ℃ every cycle (1.5 min) to 55 ℃. Twenty additional cycles were carried out at 55 ℃. Primer extension was carried out at 72 ℃ for 2.5 min. The final extension step was 7 min at 72 ℃.

DGGE was performed using a Bio-Rad Dcode^{TM} Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. PCR products were electrophoresed in 1 mm thick vertical 8% polyacrylamide gels (acrylamide/bisacrylamide = 37.5:1) in 1 × TAE buffer. Gels were prepared with three denaturant concentrations: 30 to 60%, 40 to 60%, and 40 to 70%. A 100% denaturant concentration is defined as 40% [v/v] formamide and 7 M urea. Electrophoresis at 60 ℃ was performed under three conditions: at 70 V for 16 h, at 90 V for 16 h, and at 120 V for 12 h. After electrophoresis, the gels were stained with silver nitrate (Beidler et al. 1982).

Statistical analysis

DGGE gels were digitally documented using Epi Chemi II darkroom and then analyzed using LabWorks software (UVP Laboratory, Upland, CA, USA). Each lane was detected automatically with the width and length adjusted manually. Bands in each lane were detected automatically and corrected manually. The Shannon index of diversity (H) was used to determine the diversity of the bacterial community. It was calculated as $H = - \Sigma P_i \ln P_i$, the relative intensity P_i of each band in the lanes was calculated as $P_i = n_i/N$, n_i is the maximum optical density (MOD) of the band i, and N is the sum of the MOD for all the bands within the lane (Shannon and Weaver 1963; Eichner et al. 1999). Bands with MOD greater than 3% of the sum of MODs were considered. H values and band numbers of each replicate were analyzed by analysis of variance (ANOVA) using R statistical software (R Development Core Team, R Foundation for Statistical Computing. 2011. ISBN 3-900051-07-0) to determine the effects of the employed water concentration method and DGGE conditions on recovery of bacterial diversity.

Re-isolation of DNA from gel and Cloning

Selected bands were excised from the DGGE gel using a "crush and soak" method (Sambrook and Russell 2001) with minor modifications. In brief, bands were excised from the gel using sterile gel cutting tips (BioExpress, Kaysville, UT, USA), transferred to 1.7 ml tubes, and washed briefly with 0.5 mL TE buffer. The gel slice was then crushed using a sterile pestle in 400 µl elution buffer (0.5 M $NH_4O(C_2H_3O)$, 10 mM EDTA), incubated at 37 ℃ overnight in an incubator shaker to allow DNA diffusion from the gel, then centrifuged for 5 min at 12,000 rpm to pellet acrylamide gel fragments. The supernatant was transferred and DNA precipitated in 1 mL 100% ethanol with 16 µl 5M NaCl at -20 ℃ overnight, then centrifuged at 12,000 rpm for 20 min. Pellets were vacuum dried and re-suspended in 20 µl TE buffer. The extracted DNA was

re-amplified with 16S rRNA primers EUB f933 and EUB r1387 (Kawai et al. 2002) using the same cycling conditions as colony PCR. These PCR amplicons were cloned using the pGEM-T Easy Vector System (Promega Biosciences, LLC. San Luis Obispo, CA, USA). Randomly picked white colonies were PCR amplified with the plasmid primers T7f (5' - TAA TAC GAC TCA CTA TAG GG - 3') and SP6r (5' - ATT TAG GTG ACA CTA TAG AA - 3') under the same cycling conditions used for 16S rRNA colony PCR. The PCR products were screened on SSCP gels and stored for sequencing analysis.

Sequence analysis

About 20 to 30% of the colony PCR products of each SSCP pattern and all representative PCR products from the culture-independent strategy were sequenced by Advanced Genetic Technologies Center, University of Kentucky. Sequences were edited using the BioEdit Sequence Alignment Editor (version 7.1.3.0) (Hall 1999), and subjected to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the closest matches in GenBank. The phylogenetic trees for the partial bacterial 16S rDNA sequences cut into the same size were built using the maximum likelihood method based on the Tamura-Nei model (Felsenstein 1981) in MEGA 5.05 (Build#: 5110426) (Tamura and Nei 1993; Tamura et al. 2011) and iTOL (Interactive tree of life, http://itol.embl.de/index.shtml) (Letunic and Bork 2007, 2011).

A total of 155 partial 16S rRNA sequences have been deposited in the GenBank sequence database under accession numbers JX628632 to JX628749 and JX657295 to JX657331.

Results

Water quality

The turbidity values were relatively low on both water sampling dates (Table 4.1). The water temperatures were also similar between two sampling dates, ranging from about 23 to 25 ℃, and both pH values were slightly alkaline.

Culture-dependent strategy

About 350 to 450 colonies were counted on each NA plate. By colony size and color, these colonies were grouped into eight different types: white large, white medium, white small, white dry, yellow, brown, orange, and purple. Eleven and sixteen different SSCP banding patterns were observed among the subcultures from September 2011and May 2012, respectively (Fig. 4.1).

All 16S rRNA gene sequences were aligned to their closest matches in GenBank using BLAST. A phylogenetic tree was built based on the partial 16S rRNA gene sequences and their closest matching references (Fig. 4.2). Fifty-two distinct bacterial sequences were identified. About half of the bacteria belonged to Firmicutes, and a quarter belonged to β-proteobacteria. Some were γ-proteobacteria, and only a few were α-proteobacteria or Actinobacteria.

Culture-independent strategy

Comparison of the sample concentration methods

Combining centrifugation with filtration to concentrate irrigation water samples resulted in the identification of the most diverse bacterial population (Fig. 4.3). Specifically, centrifugation- and filtration-based concentration methods alone resulted in only 23 and 26 bands while the combination of both resulted in 32 bands from water sample collected in May 2012

(Table 4.2). Similar differences among the three concentration methods were observed in the Shannon index, a common diversity measurement (Table 4.2).

Evaluation of DGGE gel denaturant concentrations and running conditions

Regardless of denaturant concentration, the largest number of bands and greatest Shannon index were observed on the gel running at 70 V for 16 h (Table 4.2). Overall a 40 to 60% gel running at 70 V for 16 h resulted in the largest number of bands, with a mean of 32 bands from sample collected in May 2012, and the greatest Shannon index (Table 4.2, Fig. 4.4).

Bacterial identities via PCR-DGGE

Cloning and sequencing analysis of the 16S rRNA fragments excised from distinct bands on the 40 to 60% DGGE gels running at 70 V for 16 h revealed 32 distinct bacterial identities using the culture-independent strategy. A phylogenetic tree was constructed and is shown in Fig. 4.5. Identified bacteria belonged to five different subdivisions: α-proteobacteria, β-proteobacteria, γ-proteobacteria, Actinobacteria, and Bacteroidetes, and sixteen unknown bacterial species were also recovered.

Comparison between culture-dependent and -independent strategies

The closest matches in GenBank of the 16S rRNA gene sequences from both sampling dates and both culture-dependent and -independent strategies are summarized in Table 4.3. The culture-independent PCR-DGGE analysis revealed very different bacterial diversities compared to those from the culture-dependent strategy. There was no overlap at the species level between the two strategies. There were four overlapping subdivisions: α-proteobacteria, β-proteobacteria,

γ-proteobacteria, and Actinobacteria, but their relative abundance and assignment to subdivisions was different between two strategies. In addition, the largest bacterial subdivisions detected were different between the two strategies. Most bacterial isolates found via the culture-dependent strategy were γ-proteobacteria and Firmicutes, and most bacteria identified via culture-independent PCR-DGGE belonged to Actinobacteria and Bacteroidetes.

Discussion

This study produced a sample concentration and processing scheme for typing the greatest bacterial diversity in irrigation water (Fig. 4.6). Of the essence in this scheme are to (1) employ both culture-dependent and -independent strategies as each detects a subset of bacteria; (2) use both centrifugation and filtration to concentrate water samples and (3) perform DGGE with 40 to 60% denaturant at 70 V for 16 h in the culture-independent strategies. This scheme has already facilitated the detection of diverse bacteria in heat-treated and control water in the present study and will be a useful tool for future investigations into bacterial diversity in agricultural water systems.

The fact that culture-dependent and -independent strategies detected substantially different bacterial groups demonstrated that both strategies are needed to detect the most bacterial diversity in water. Culture-dependent strategy here included, culturable bacteria recovered account for a very small portion of the total microorganisms in irrigation water (Amann et al., 1995; Vartoukian et al., 2010). In the present study, only one type of general medium (nutrient agar) was used to culture bacteria which may have further limited the recovery of some groups of bacteria. The water sample collected was 1 L each replicate, which was a very

small size comparing to total size of the irrigation reservoir, so some bacteria groups may not just be present in the water samples we collected.

Two critical steps in the culture-dependent strategy to ensure detection of the greatest diversity of bacteria in irrigation water samples at the minimum cost are to (1) select and subculture colonies based on color and size and (2) group the subcultures by colony PCR-SSCP banding pattern. As encountered in the present study, hundreds of colonies grew in each plate, it would be cost prohibitive to sequence all bacterial colonies from multiple samples with at least three replicates with three plates each. Randomly selecting colonies for DNA sequencing may miss some important bacterial groups and consequently compromise the data quality. Selection of colonies by color and size followed by grouping them based on the SSCP pattern substantially reduce such risk. Multiple SSCP patterns were detected from the subcultures of the same colony size and color and two or more species were identified from the PCR product with the same SSCP pattern. Thus it is advisable that at least 20% colonies are subcultured from each color and size group and multiple PCR products of the same SSCP pattern are sequenced when using the culture-dependent strategy.

In the culture-independent strategy, concentrating irrigation water sample by combining centrifugation with filtration, and subsequent PCR-DGGE analysis using 40 to 60% denaturant concentrations at 70 V for 16 h ensure the quickening water sample processing and detection of the highest level of bacterial diversity based on partial bacterial 16S rRNA sequences. Different DGGE denaturant concentrations were used for different samples in previous studies, but in general in the range was from 30 to 70% (Kozdroj and van Elsas 2001; Kawai et al. 2002; Lyautey et al. 2005; Bouskill et al. 2010; Essahale et al. 2010; Liu et al. 2011). In this study, to determine the most appropriate range of denaturant concentrations in a DGGE gel for irrigation

water samples, a wider range of denaturant concentrations were initially tested (30 to 60% and 40 to 70%) then narrowed down to 40 to 60%, under three different electrophoretic conditions (voltage × time). The optimum condition in terms of denaturant concentration, running voltage and time for PCR-DGGE analysis of irrigation water was determined by comparing the bacterial diversity among treatments.

The recovery of *Bacillus, Brevibacillus, Enterobacter, Peanibacillus* and *Pseudomonas* species in the present study underscores the practical significance of investigations into the microbial diversity in irrigation reservoirs. Strains of these species are known to have antagonistic activities against plant pathogens. For example, Saikia et al. (2011) found that *Brevibacillus laterosporus* BPM3 isolated from a natural hot spring strongly inhibited the growth of *Fusarium oxysporum* f. sp. *ciceri, F. semitectum, Magnaporthe grisea* and *Rhizoctonia oryzae*. Strains from *Pseudomonas* and *Bacillus* species have been reported to control many seedling diseases and root rots on several crops (Yang et al. 1994; Punja 1997; Paulitz and Belanger 2001; Loper et al. 2007). Other bacterial species belonging to genera of *Enterobacter* and *Peanibacillus* also could have biocontrol activities as reported previously (Singh et al. 1999; Saikia et al. 2011). Further studies are warranted to determine the dominant bacterial groups, species and strains with the greatest biocontrol potential across and how these groups may be utilized along with other pathogen avoidance and mitigation methods including heat treatment to realize their full potential.

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Table 4.1 Water quality measurements in September 2011 and May 2012.

	September 2011[a]	May 2012[a]
Temperature (℃)	24.78 ±0.01	23.45 ±0.01
pH	8.93 ±0.01	9.81 ±0.01
ORP (mV)[b]	315.67 ±0.88	412.50 ±1.15
Sp Conductivity (µS/cm)[c]	181.20 ±0.15	266.30 ±0.21
LDO[d] (mg/l)	13.78 ±0.01	14.43 ±0.15
CHLV[e] (Volts)	0.10 ±0.01	0.77 ±0.01
Turbidity (NTU)	28.83 ±0.25	53.30 ±0.05

[a] Values represented by mean ± SE of three replicates

[b] ORP - the oxidation reduction potential

[c] Sp Conductivity - specific conductivity

[d] LDO - luminescent dissolved oxygen

[e] CHLV - the chlorophyll voltage

Table 4.2 Effects of sample concentration method, DGGE denaturant concentration and running conditions on the number of resultant bands and Shannon index (H) of bacterial diversity in irrigation water sampled in September 2011 and May 2012.

Treatment		September 2011		May 2012	
		Band #[b]	H[b]	Band #	H
Sample concentration method [a]					
Centrifugation alone		15.00 ±0.58	2.53 ±0.05	23.33 ±2.40	2.88 ±0.10
Filtration alone		16.00 ±1.15	2.61 ±0.08	26 ±2.52	3.00 ±0.08
Centrifugation and filtration		22.33 ±0	2.99 ±0.01	32.33 ±3.29	3.25 ±0.13
DGGE denaturant concentrations & running conditions [c]					
40 - 70%	70 V for 16 h	12.00 ±0.58	2.26 ±0.05	20.33 ±0.67	2.89 ±0.02
	90 V for 16 h	8.33 ±0.33	2.12 ±0.03	15 ±0.58	2.53 ±0.07
	120 V for 12 h	8.33 ±0.33	2.01 ±0.06	15.33 ±1.45	2.61 ±0.10
30 - 60%	70 V for 16 h	10.00 ±0.58	2.25 ±0.05	22 ±0.58	2.88 ±0.05
	90 V for 16 h	9.00 ±0	2.02 ±0.02	14.3 ±0.67	2.55 ±0.07
	120 V for 12 h	7.00 ±0	1.85 ±0.01	15.67 ±0.33	2.56 ±0.01
40 - 60%	70 V for 16 h	22.33 ±0.33	2.99 ±0.01	32.33 ±3.28	3.25 ±0.13
	90 V for 16 h	10.67 ±0.33	2.14 ±0.02	25 ±0.58	3.13 ±0.02
	120 V for 12 h	11.00 ±0	2.26 ±0.01	15.67 ±0.33	2.69 ±0.02

[a] PCR-DGGE with a 40 to 60% denaturant concentration running at 70 V for 16 h

[b] Values represented by mean ± SE of three replicates

[c] PCR-DGGE using bacterial cells collected via a combination of centrifugation and filtration method

Table 4.3 Alignment of 16S rRNA gene sequences obtained by colony PCR and excised DGGE bands from irrigation water sampled in September 2011 and May 2012.

Subdivision	Closest match (Accession number)[a,b]	Source[c]			
		September 2011		May 2012	
		Colony PCR	DGGE	Colony PCR	DGGE
γ-proteobacteria	*Aeromonas* sp. NM-21 (JQ894500.1)			+	
	Aeromonas sp. PBB3 (JQ681230.1)	+			
	Enterobacter sp. X40 (HE662680)			+	
	Pseudomonas sp. Cf0-5 (JN836275.1)	+			
	Pseudomonas sp. HM05 (JQ396178.1)			+	
	Pseudomonas sp. NCCP-279 (AB641888.1)			+	
	Pseudomonas sp. MLB28 (JQ765424.1)			+	
	Providencia sp. SRS82 (JN381552.1)	+			
	Rheinheimera soli B29 (JN377675.1)			+	
	Rheinheimera sp. CF12-15 (FJ170029.1)			+	
	Rheinheimera sp. E49 (JQ922424.1)			+	
	Gamma proteobacterium F8 (AY077611.1)			+	
	Uncultured *Rheinheimera* sp. 48PP (JF278072.1)				+
β-proteobacteria	*Acidovorax delafieldii* A3RO7 (JQ689177.1)			+	
	Acidovorax sp. DPS2 (HQ704415.1)			+	
	Acidovorax sp. NF1078 (JQ782387.2)			+	
	Chitinibacter sp. SK16 (JN981166.1)			+	
	Chromobacterium haemolyticum strain MDA0585 (NR_043957.1)	+			
	Chromobacterium violaceum strain NBRC 12614 (AB680302.1)	+			
	Chromobacterium violaceum strain 968 (HM449690.1)	+			
	Chromobacterium sp. IITR-71 (JN210566.1)	+			
	Chromobacterium sp. PMR-A (HM047300.1)	+			
	Hydrogenophaga sp. BAC55P (EU130964.1)			+	
	Hydrogenophaga sp. WLSH-50 (JF682017.1)			+	
	Hydrogenophaga sp. p3 (HQ652595.1)			+	
	Limnohabitans sp. Rim8 (HE600682.1)		+		
	Mitsuaria sp. Vi30 (JF501194.1)			+	
	Pelomonas sp. R7W-1-1 (JQ928692.1)			+	
	Polynucleobacter sp. NBRC 101963 (AB681633.1)		+		
	Uncultured *Herbaspirillum* sp. FL 52 (HQ008597.1)		+		
	Uncultured *Polynucleobacter* sp. YL205 (HM856566.1)				+
α-proteobacteria	*Azospirillum* sp. DJM2D4 (JF753513.1)			+	
	Mesorhizobium sp. RITF 741 (JQ697665.1)			+	
	Candidatus Azospirillum massiliensis URAM1 (EF394925.1)			+	
	Uncultured *Rhodobacter* sp. rm15b3c02 (HM003636.1)				+
Bacteroidetes	Uncultured Bacteroidetes bacterium 30LAKE43F10 (HQ531074.1)		+		

	Uncultured Bacteroidetes bacterium ME002C1 (FJ827913.1)				+
	Uncultured *Sphingobacterium* sp. w21 (HE654931.1)		+		
Actinobacteria	*Microbacterium* sp. E56 (JF501120.1)			+	
	Microbacterium sp. FI_1010 (JQ691553.1)			+	
	Microbacterium sp. I_29-J6NFA10A (JQ917793.1)			+	
	Uncultured *actinobacterium* clone 30LAKE01B08 (HQ530574.1)		+		
	Uncultured *actinobacterium* clone 30LAKE01E01 (HQ530592.1)				+
	Uncultured *actinobacterium* clone KWK1S.54 (JN656761.1)		+		
	Uncultured *actinobacterium* clone KWK6F.62 (JN656787.1)		+		
	Uncultured *actinobacterium* clone w60 (HE654966.1)		+		
	Uncultured *actinobacterium* clone w70 (HE654972.1)		+		
	Uncultured *actinobacterium* clone w98 (HE654991.1)		+		
Firmicutes	*Bacillus cereus* strain CT25 (EU111736.1)	+			
	Bacillus cereus strain IARI-S-9 (JN411483.1)	+			
	Bacillus cereus strain LZ027 (JN210566.1)	+			
	Bacillus marisflavi p53 B09 (JQ834634.1)			+	
	Bacillus mycoides TN12 (JQ415984.1)			+	
	Bacillus pumilus strain MSII-6 (JN993718.1)	+			
	Bacillus pumilus strain TAD174 (FJ225317.1)	+			
	Bacillus thuringiensis strain GTG-40 (JQ004436.1)	+			
	Bacillus thuringiensis strain IARI-A-7 (JN411428.1)	+			
	Bacillus sp. ARA1-1 (AB677952.1)	+			
	Bacillus sp. BWDY-6 (DQ314534.1)	+			
	Bacillus sp. cmc28 (JQ917994.1)			+	
	Bacillus sp. MGB13 (JN000926.1)	+			
	Bacillus sp. PZ 35 (JQ808528.1)			+	
	Brevibacillus brevis strain NBRC 100599 (AB681205.1)	+			
	Lysinibacillus sphaericus strain SP19_LP11 (JQ289050.1)	+			
	Lysinibacillus sp. C250R (GQ342695.1)	+			
	Lysinibacillus sp. ZSG2-3 (JN990432.1)	+			
	Paenibacillus sp.FSL H3-469 (EF203107.1)			+	
	Paenibacillus sp. XP8 (GU080335.1)			+	
Unknown	Uncultured bacterium clone 060127_T6S1_W_T_SDP_144 (FJ350708.1)				+
	Uncultured bacterium clone BST15-17 (HQ436607.1)		+		
	Uncultured bacterium clone CRD99-65 (AF428643.1)		+		
	Uncultured bacterium clone CRP99-73 (AF428727.1)		+		
	Uncultured bacterium clone Filt WallC02 (GQ247163.1)				+

	Uncultured bacterium clone JC43 (JN868893.1)		+
	Uncultured bacterium clone Lc2z ML 055 (FJ355132.1)		+
	Uncultured bacterium clone LRE22B42 (HQ420131.1)	+	
	Uncultured bacterium clone Lc2z ML 058 (FJ355128.1)	+	
	Uncultured bacterium clone ncd2310d03c1 (JF198212.1)	+	
	Uncultured bacterium clone ncd2331g09c1 (JF204216.1)	+	
	Uncultured bacterium clone SING575 (HM129208.1)	+	
	Uncultured bacterium clone SINN1113 (HM128496.1)		+
	Uncultured bacterium clone SINN828 (HM128813.1)		+
	Uncultured bacterium clone S0052 (FJ820417.1)		+
	Uncultured bacterium clone XX0076 (FJ820462.1)	+	

[a] Sequences aligned to their closest matches in GenBank with BLAST

[b] All the closest relatives of sequences with a similarity ≥ 97%.

[c] + indicating the presence of the bacterial identity.

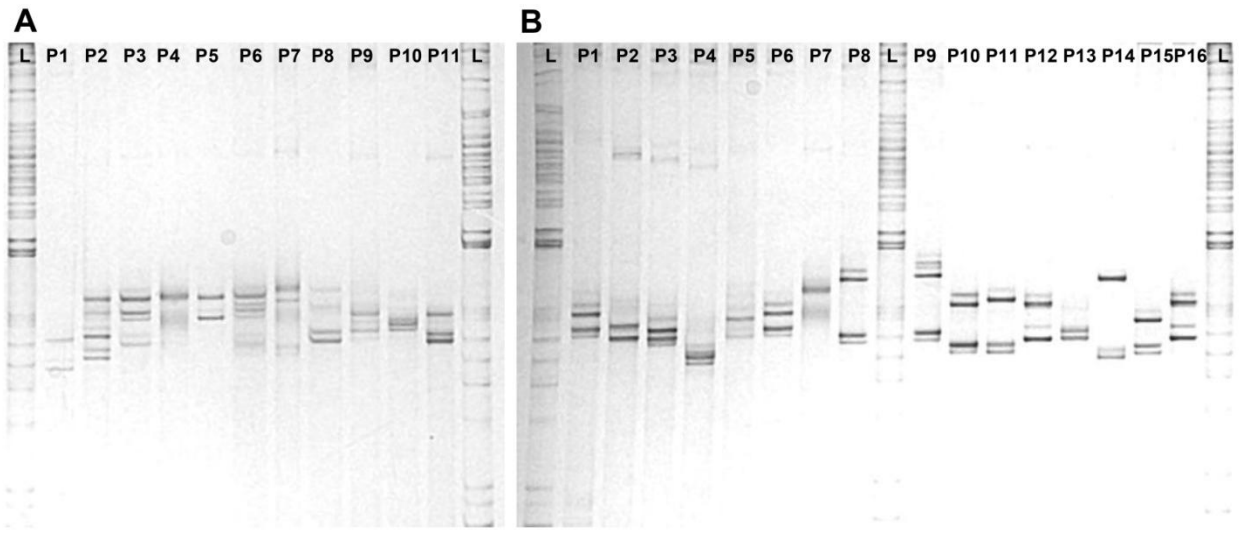

Figure 4.1 SSCP banding patterns of bacterial colonies recovered from irrigation water sampled in (a) September 2011 and (b) May 2012. The bacterial colonies were subjected to direct colony PCR with 16S rRNA primers, and the PCR products were denatured and electrophoresed in 8% native acrylamide gels. Specific banding patterns were indicated as P(#) on the top of each lane; L indicates the 100 DNA ladder.

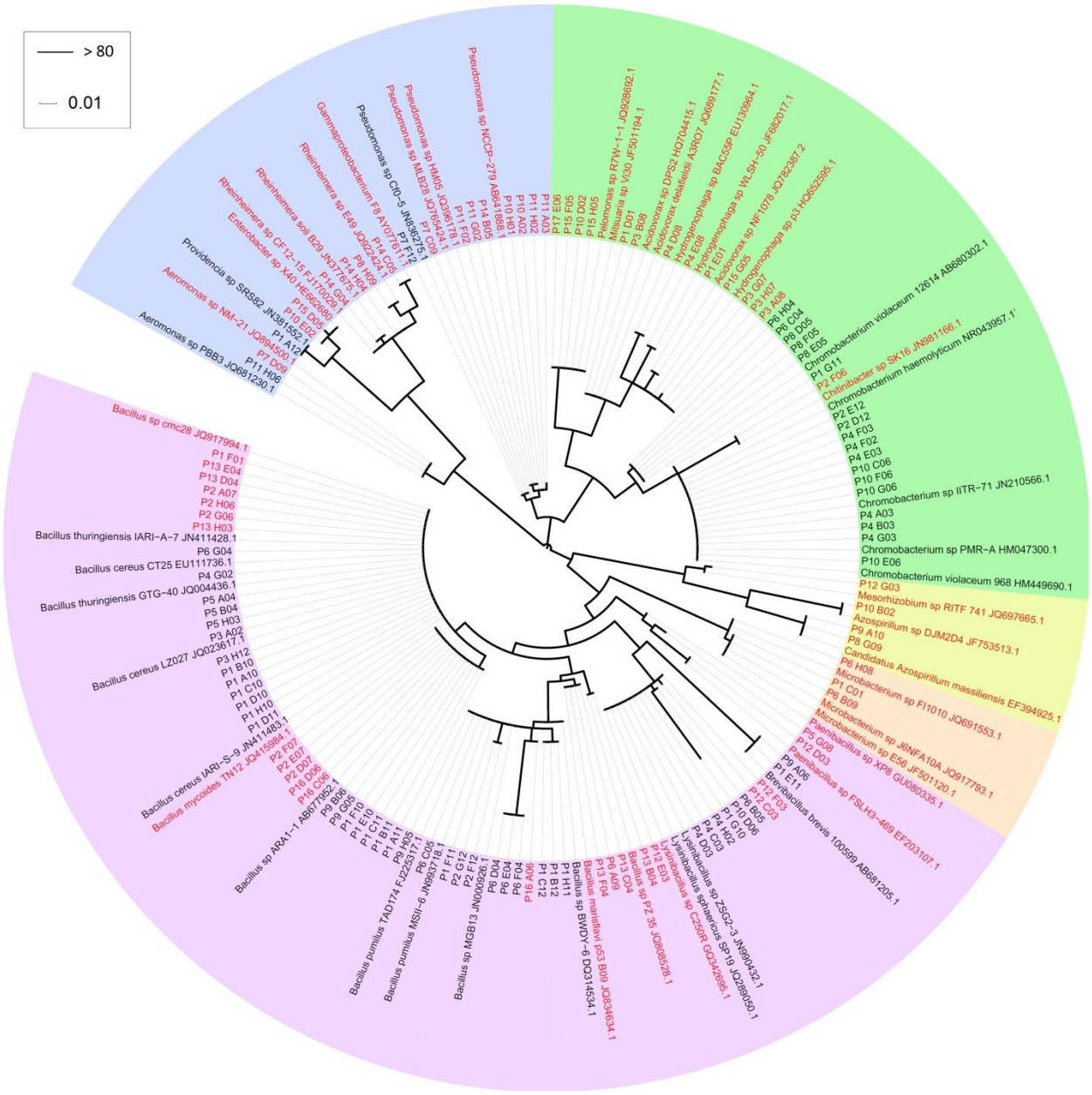

Figure 4.2 Bacterial diversity in irrigation water samples as revealed through culture dependent strategy based on partial sequences of 16S rDNA genes. The Maximum Likelihood method based on the Tamura-Nei model was used. Bootstrap analyses were based on 1000 replicates. Different color sections indicate various subdivisions of bacteria: blue, γ-proteobacteria; green, β-proteobacteria; yellow, α-proteobacteria; orange, Actinobacteria; purple, Firmicutes. Red

labels denote sequences from May 2012, and black labels denote sequences from September 2011.

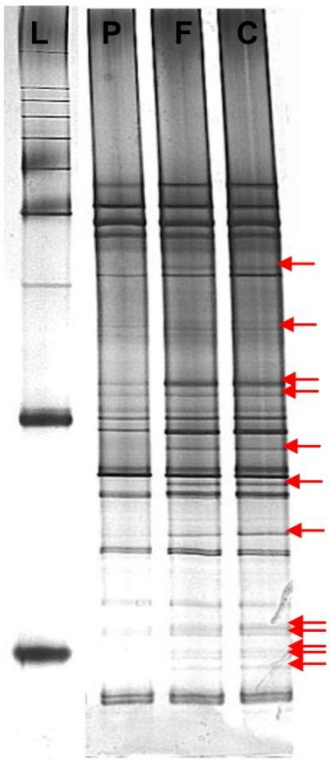

Figure 4.3 Comparison of DGGE fingerprints of bacterial 16S rRNA amplicons from irrigation water samples via three concentration methods: centrifugation (P), 0.2 μm filtration (F), and a combination of centrifugation and filtration (C), with a 40 to 60% DGGE gel denaturant concentration at 70 V for 16 h. L represents 100 bp DNA ladder. Red arrows represent changed bands among concentration methods.

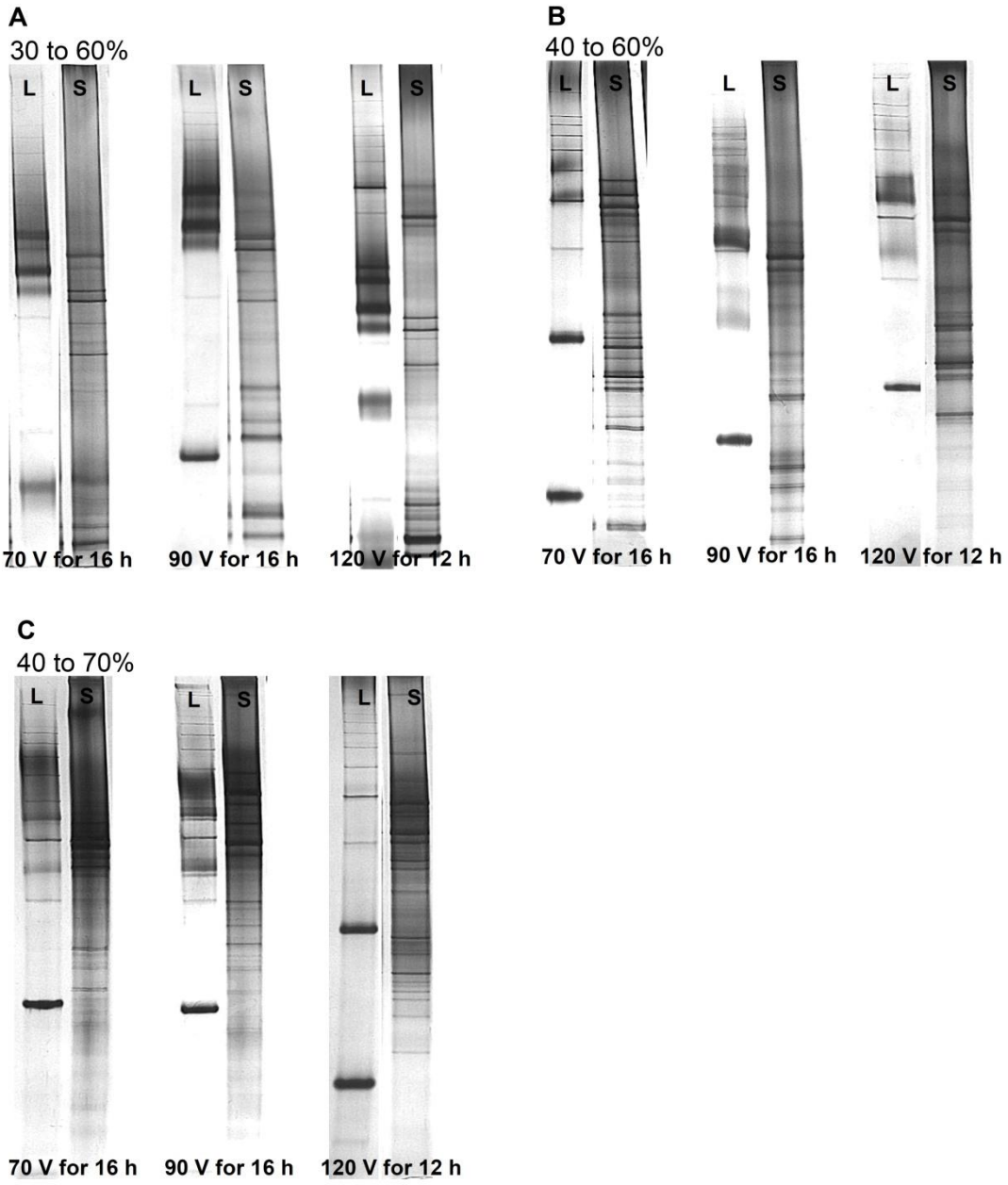

Figure 4.4 DGGE fingerprints of bacterial 16S rRNA fragments amplified from irrigation water as affected by gel denaturant concentrations (a) 30 to 60%, (b) 40 to 60%, and (c) 40 to 70% under three conditions (70 V for 16 h, 90 V for 16 h, and 120 V for 12 h). A combination of centrifugation and filtration was used to concentrate water sample. L represents 100 bp DNA ladder. S represents samples.

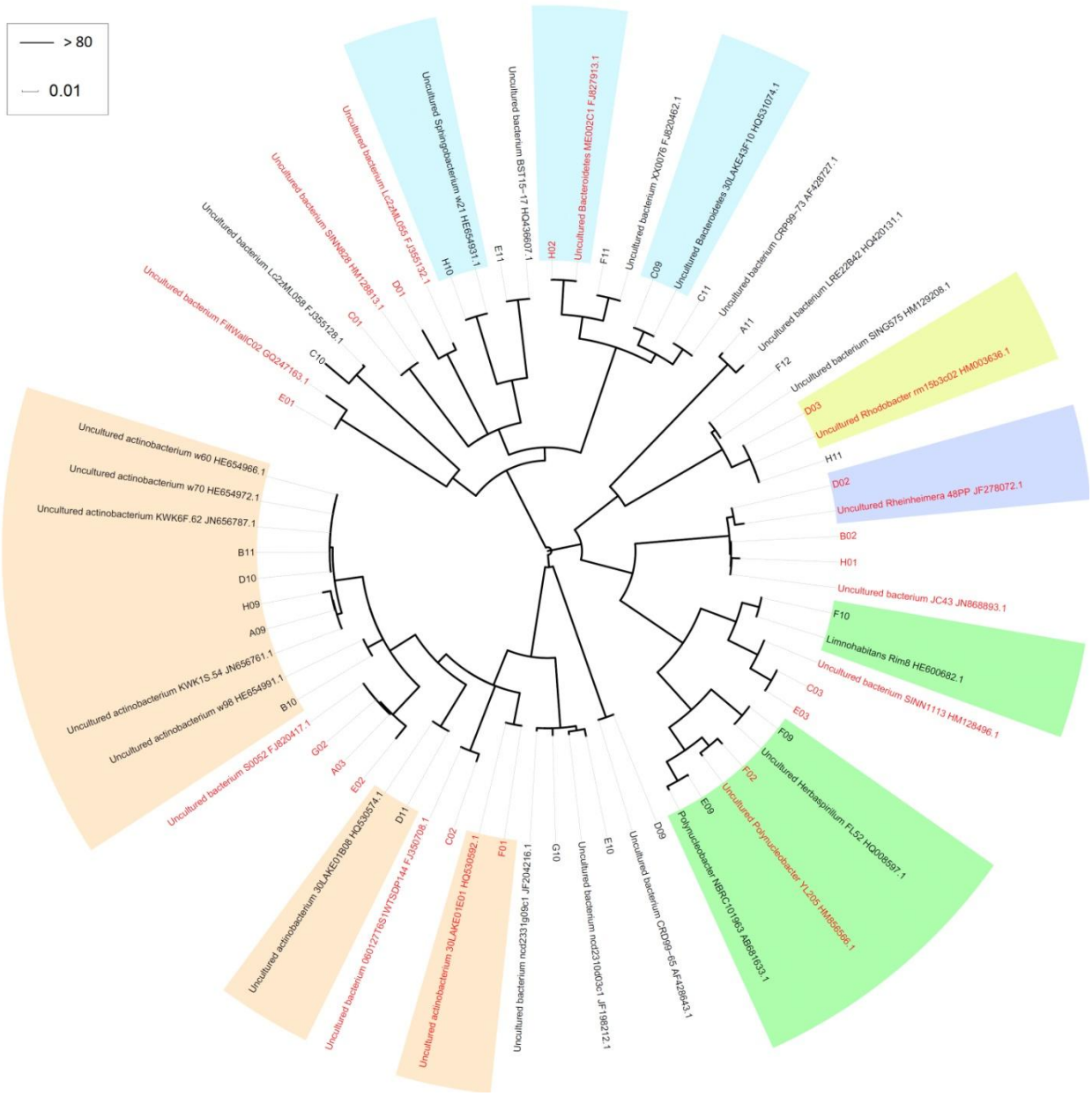

Figure 4.5 Bacterial diversity in irrigation water samples as revealed through culture-independent strategy (PCR-DGGE) from partial sequences of 16S rDNA amplicons. The Maximum Likelihood method based on the Tamura-Nei model was used. Bootstrap analyses were based on 1000 replicates. Different color sections indicate various subdivisions of bacteria: blue, γ-proteobacteria; green, β-proteobacteria; yellow, α-proteobacteria; orange, Actinobacteria;

light blue, Bacteroidetes; unshaded, unknown bacterial species. Red labels denote sequences from May 2012, and black labels denote sequences from September 2011.

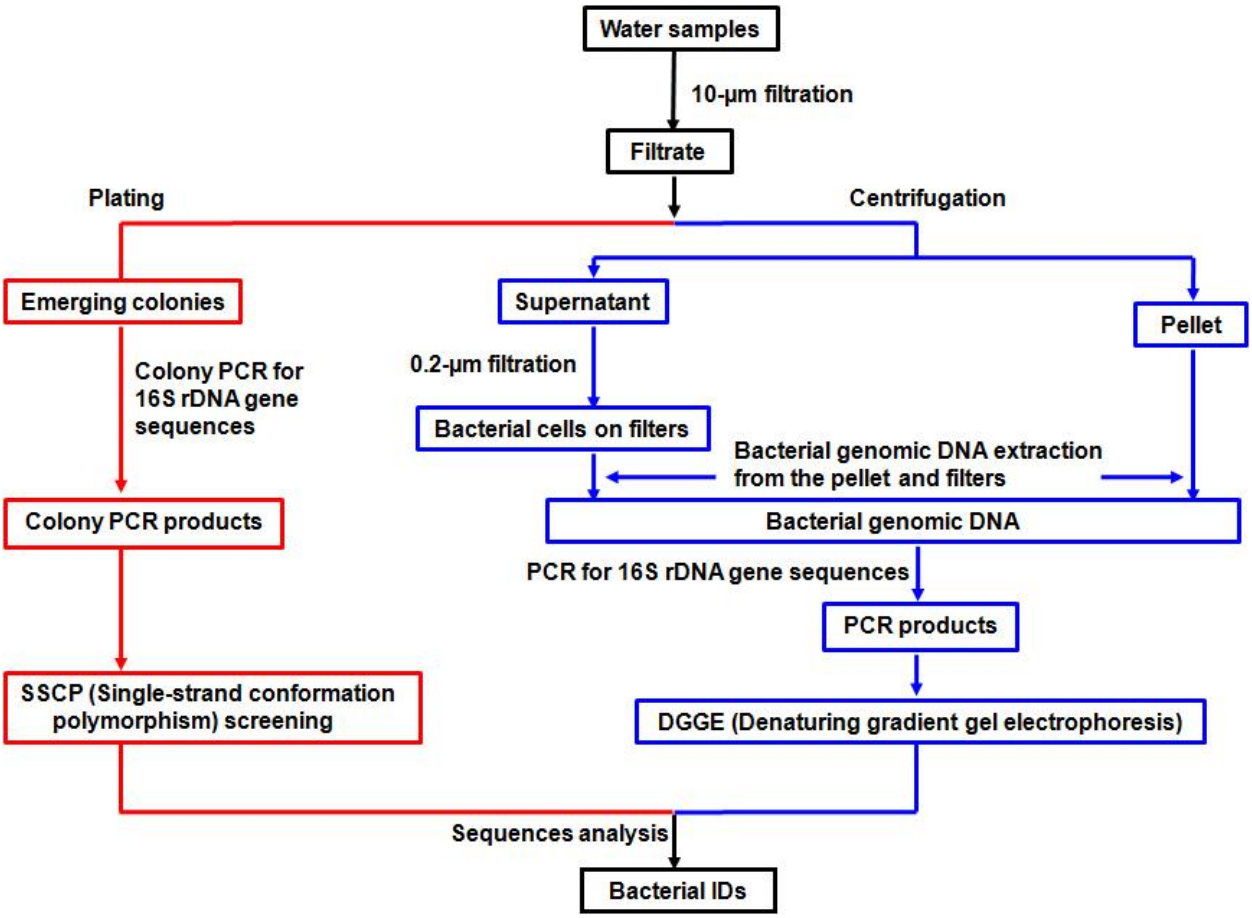

Figure 4.6 Flow chart illustrating steps in characterization of bacterial species in irrigation water. Red lines denote culture-dependent strategy, blue lines denote culture-independent strategy.

Chapter 5

Bacterial community structure shifts in irrigation water under heat treatment

Abstract

Our previous study revealed the required temperature of heat treatment can be lowered to 48 ℃ for 24 h from the currently recommendation of 95 ℃ for 30 s without sacrificing decontamination efficacy. However, the mechanisms by which plant pathogens in irrigation water are suppressed or killed at 42 and 48 ℃ are yet to be elucidated. The objective of this study was to determine whether such temperatures shift bacterial community structure in irrigation water, enhancing antagonistic activities against plant pathogens. Both culture-dependent and -independent strategies were employed. The structure of both culturable and uncultured bacterial community in irrigation water substantially shifted after the heat treatments. In contrast to that at 25 ℃, the two largest bacterial groups detected at 42 and 48 ℃ were γ-proteobacteria and Firmicutes. Specifically, these included genera of *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Brevibacillus*, and *Lysobacter*. These bacteria are known to have biocontrol potential; their increased abundance implicated bacterial contributions to heat treatment of irrigation water at 42 and 48 ℃. Further experiments are being conducted to evaluate those bacteria for biocontrol activities against Phytophthora species.

Introduction

Heat pasteurization is one of the safest and most reliable methods for water decontamination in horticulture (Runia et al. 1988; Runia 1995; van Os 1999). Our recent study

revealed that water treatment temperature can be lowered to 48 ℃ for 24 h from the current recommendation of 95 ℃ for 30 s without sacrificing decontamination efficacy (Hao et al. 2012). This is a major improvement in energy efficiency and economics, two major hurdles currently affecting the adoption of this water treatment (Hong and Moorman 2005). With the current protocol, all microorganisms in water are killed at 95 ℃, resulting in a biological vacuum allowing invasion by plant pathogens through other avenues such as contaminated plants and planting materials (Schumann and D'Arcy 2006).

The question asked in this part of the dissertation is whether and how the water temperatures of 48 and 42 ℃ may affect the bacteria especially those beneficial bacteria in irrigation water. We hypothesized that these water temperatures shift the bacterial community structure in irrigation water and enhance antagonistic activities against plant pathogens, thus contributes to the suppression or killing of plant pathogens. The present study was performed to test this hypothesis with irrigation water samples from a local nursery in eastern Virginia. Specific objectives were to (1) to characterize the bacterial community structures in water samples treated at 42 and 48 ℃ and compare with those at 25 ℃ (control) using the scheme described in the previous chapter, and (2) identify potential pathogen-suppressing bacteria in the irrigation reservoir of study.

Materials and Methods

Water samples

Water samples were collected from an irrigation reservoir at a local nursery in eastern **Virginia** in February and May 2012. All samples were collected in sterile bottles from the surface of water columns in the center of the reservoir. Three replicates of 10 L each were

collected. Water temperature and other water quality parameters were recorded on-site using a Hydrolab DS5X multiparameter water quality meter (Hach®, Loveland, CO, USA). Water samples were then transported to the lab and immediately used for following experiments.

Temperature treatments

Irrigation water samples were heat-treated at three temperatures of 25, 42, and 48 ℃ for 48 h. For each treatment three 1-L volume replicates were transferred to 1-L Erlenmeyer flasks and placed in temperature-controlled incubators (Intellus Environmental Controllers, Percival Scientific, Inc.). The incubators were calibrated and monitored before and during the temperature experiment using a HOBO Pendant® temp/light data logger (Onset Computer Corporation). Once the temperature treatments were completed, water was passed through nylon membrane filters (pore size 10 μm) to remove larger debris and algae.

Culture-dependent strategy

Plating

One hundred microliters of the filtrate was evenly spread on Nutrient Agar (NA) (Difco™, Detroit, MI) in a 10-cm diameter Petri dish using a sterile glass spreader. Three NA medium plates were used for each replicate per treatment and incubated at 28 ℃ for 2 d.

Colony PCR & SSCP analysis

The emerging colonies on NA plates were grouped based on color, texture and size. The total numbers of colonies in each group were recorded. About 20 to 30% of colonies from each group were subcultured into 48-well plates containing NA medium, and incubated at 28 ℃ for 2

d. Colony PCRs amplifying bacterial 16S rDNA were performed for each subculture with primers 16S f968 and r1401 containing hypervariable regions V6 to V8 (Nubel et al. 1996; Peixoto et al. 2002). Reagents supplied with Takara *Taq* (Takara Bio Inc., Japan) were used in 25 μl reactions containing 0.1 μl Takara *Taq* (5 units/μl), 2.5 μl 10× PCR buffer (containing 1.5 mM $MgCl_2$), 2 μl dNTP mixture (2.5 mM each), 1 μl of each primer (10 μM), and 18.4 μl DNA-free nanopure water. The bacterial colonies were picked directly from the culture plate and added to the mixture. Cycling conditions were an initial denaturation at 96 ℃ for 2 min, followed by 40 cycles of denaturation at 94 ℃ for 0.5 min, annealing at 55 ℃ for 0.5 min, and extension at 72 ℃ for 1 min. For the last cycle, the extension time was increased to 10 min.

Single-strand conformation polymorphism (SSCP) analyses were performed for the colony PCR products as described by Kong et al. (2003) with minor modifications. In brief, the amplicons were mixed with loading dye (1:4, v:v) and denatured by heating at 96 ℃ for 10 min then chilled on ice. Two microliters of each sample was loaded on 8% polyacrylamide gels (acrylamide/bisacrylamide = 29:1) running at 220 V for 2.5 h in chilled 1 × TBE buffer. After electrophoresis, the gels were stained with silver nitrate (Beidler et al. 1982). SSCP banding patterns of individual colonies were categorized with the aid of a 100 bp DNA ladder (Promega Corp., Madison, WI). Colony PCR products were grouped by SSCP fingerprint subsequently selected for DNA sequencing.

Culture-independent strategy

Genomic DNA extraction

The filtrate passing through 10-μm filters was concentrated through a combination of centrifugation and 0.2-μm filtration. In brief, the filtrate was centrifuged at 12,200 g for 50 min,

the pellet was saved, and the supernatant was filtered through 0.2-μm filters. The UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA) was used to extract genomic DNA from the pellets following the instruction manual. The PowerWater DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA) was used to extract genomic DNA from the 0.2-μm filters. Total genomic DNA from both pellets and filters were combined for DGGE analyses.

PCR amplification and DGGE

For DGGE analysis, 16S rRNA fragments containing three hypervariable regions V6 to V8 were amplified with primers GC-clamp-EUB f933 and EUB r1387 (Kawai et al. 2002). The PCR mixture (50 μl) was adapted to contain 0.2 μl Takara *Taq* (5 units/μl), 5 μl 10× PCR buffer (containing 1.5 mM $MgCl_2$), 7.5 μl BSA (0.3 mg/ml), 4 μl dNTP mixture (2.5 mM each), 2 μl of each primer (10 μM), 19.3 μl DNA-free nanopure water and 10 μl DNA extract. A hot-start PCR was performed at 94 ℃ for 4 min, followed by touchdown PCR with the following parameters: the denaturation was set at 94 ℃ for 1 min, the annealing temperature was initially set at 65 ℃ and then decreased by 1 ℃ every cycle (1.5 min) until it reached 55 ℃. Twenty additional cycles were carried out at 55 ℃. Primer extension was carried out at 72 ℃ for 2.5 min. with a final extension step for 7 min at 72 ℃.

DGGE was performed using a Bio-Rad Dcode^{TM} Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. PCR products were electrophoresed in 1 mm thick denaturing 8% polyacrylamide gel (acrylamide:bisacrylamide = 37.5:1) in 1 × TAE buffer. Gels were prepared with a denaturant concentration of 40 to 60%.

Electrophoresis at 60 ℃ was run at 70 V for 16. After electrophoresis, the gels were stained with silver nitrate (Beidler et al. 1982).

DGGE gels were digitally documented using the Epi Chemi II darkroom and analyzed using LabWorks software (UVP Laboratory, Upland, CA). Each lane was detected automatically with the width and length adjusted manually. Bands in each lane were detected automatically and corrected manually.

DNA reisolation and cloning

Unique bands among temperature treatments were selected from the DGGE gels. DNAs were re-isolated from the acrylamide gel using a "crush and soak" method with minor modifications (Sambrook and Russell 2001). In brief, bands were excised using sterile gel cutting tips (BioExpress, Kaysville, UT), transferred to 1.7 ml tubes and washed briefly with 0.5 mL TE buffer. The gel fragment was then crushed using a sterile pestle in 400 µl elution buffer (0.5 M NH_4OAc, 10 mM EDTA), incubated at 37 ℃ overnight in an incubator shaker to allow DNA diffusion from the gel, then centrifuged for 5 minutes at 12,000 rpm to pellet the acrylamide. The supernatant was transferred and DNA precipitated in 1 mL 100% ethanol with 16 µl 5M NaCl at -20 ℃ overnight, then centrifuged at 12,000 rpm for 20 min. The pellets were vacuum dried and re-suspended in 20 µl TE buffer. The extracted DNAs were re-amplified with 16S rRNA primers EUB f933 and EUB r1387 (Kawai et al. 2002) using the same cycling conditions as colony PCR. These PCR amplicons were cloned using the pGEM-T Easy Vector System (Promega). Randomly picked white (positive) colonies were PCR amplified with the plasmid primers T7 (5' - TAA TAC GAC TCA CTA TAG GG - 3') and SP6 (5' - ATT TAG

GTG ACA CTA TAG AA - 3') under the same cycling conditions as 16S rRNA colony PCR. The PCR products were screened on SSCP gels and stored for sequencing analysis.

Sequence analysis

For each temperature treatment, about 20 to 30% of the colony PCR products of each SSCP fingerprint from the culture-dependent strategy and all representative PCR products from the culture-independent strategy were sequenced at the Advanced Genetic Technologies Center, University of Kentucky. Sequences were edited using the BioEdit Sequence Alignment Editor v. 7.1.3.0 (Hall 1999), and subjected to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the closest matches in GenBank. Phylogenetic trees for the partial bacterial 16S rDNA sequences were built using the maximum likelihood based on the Tamura-Nei model (Felsenstein 1981) in MEGA 5.05 (Build#: 5110426) (Tamura and Nei 1993; Tamura et al. 2011) and iTOL (Interactive tree of life, http://itol.embl.de/index.shtml) (Letunic and Bork 2007, 2011).

A total of 394 partial 16S rRNA sequences have been deposited in the GenBank sequence database under accession numbers JX870915 to JX871216, JX628692 to JX628749, and JX657293 to JX657330.

Data analysis

CFU data of cultured bacterial populations from all three temperature treatments were used in an analysis of variance. The significance of differences among treatment means was determined by using Student *t* test with a P value of 0.05 using R statistical software (R Development Core Team, R Foundation for Statistical Computing. 2011. ISBN 3-900051-07-0).

For PCR-DGGE, the Shannon index of diversity (H) was used to determine the bacterial diversity within each treatment. It was calculated as $H = - \Sigma P_i \ln P_i$, where, the relative intensity of each band in the lanes (P_i) was calculated as $P_i = n_i/N$, where n_i is the maximum optimal density (MOD) of the band i, and N is the sum of the MOD for all the bands within the lane (Shannon and Weaver 1963; Eichner et al. 1999). Bands with MOD greater than 3% of the sum of MODs were considered. H values and band numbers in each replicate were analyzed by analysis of variance (ANOVA) using R statistical software. The cluster analysis was used to investigate similarity of bacterial communities between treatments. A binary matrix of the DGGE band pattern was made based on the presence (1) or absence (0) of the bands. The binary data representing the band patterns were used to generate a distance matrix by using the Euclidean distances. The distance matrix was used to construct a dendrogram by cluster analysis with Ward's Hierarchical Clustering Method (Ward 1963). The cluster analysis and dendrogram generation were carried out using the R statistical software.

Results

Water quality

As expected, water temperature was low in February 2012 and mild in May 2012 (Table 5.1). Both water samples were only slightly turbid, their oxidation-reduction potential, electrical conductivity and dissolved oxygen readings were similar but chlorophyll *a* and pH were higher in May than February.

Comparing culturable bacteria among temperature treatments

Overall bacterial population in water was the highest at 42 ℃ and the lowest population was observed at 48 ℃ (Table 5.2). Ten different colony types were observed: white big, white medium, white small, white dry, cream, yellow, light yellow, thread, orange, and pink. The most diverse colony types were observed at 25 ℃, fewer colony types were at 42 and 48 ℃.

Bacterial groups and species identified by analysis of the partial 16S rRNA gene sequences are illustrated in Fig. 5.1. Bacteria belonging to the γ-proteobacteria, β-proteobacteria, and Firmicute subdivisions were detected on both water sampling dates. Additional bacterial subdivisions including α-proteobacteria and Actinobacteria were detected in the water samples collected in May 2012 (Fig. 5.1b). Overall, γ-proteobacteria and Firmicutes accounted for most of the culturable bacteria in samples collected in February and May 2012.

There was a stark contrast in bacterial community structure between water at 25 and 42/48 ℃. The majority of Firmicutes detected were from water treated at 42 and 48 ℃. They included species of *Bacillus, Paenibacillus, Brevibacillus*. Among them, *Bacillus* species were most abundant. Approximately half of γ-proteobacteria identified were from water treated at 42 and 48 ℃ and they all belong to the genus *Pseudomonas*. On the other hand, most β-proteobacteria identified were from water at 25 ℃. Similarly, the majority of α-proteobacteria and Actinobacteria detected in May were from water at 25 ℃.

Comparing non-culturable bacteria among temperature treatments

There was a clear shift in bacterial community structure among water at three different temperatures. The DGGE banding pattern differed among the control and two heat treatments (Fig. 5.2). The total number of bands observed on DGGE and Shannon diversity index did not change significantly between the control and the 42 ℃ treatment (Table 5.3). However, there was

a significant decrease in both the number of bands and Shannon index values at 48 ℃. The similarity analysis of bacterial community structure among temperature treatments based on Ward's Hierarchical clustering of DGGE banding patterns illustrates the differences among temperature treatments (Fig. 5.3). In the dendrograms, the distance between the points reflects the similarity of the DGGE profiles. In samples collected in February 2012, banding patterns from temperature treatments 42 and 48 ℃ grouped together, showing higher similarity. But in samples collected in May 2012, the banding pattern from the 42 ℃ treatment grouped with the 25 ℃ control, indicating their high degree of similarity.

Bacterial community structure shifts after heat treatments also were observed through cloning and sequence analysis of the 16S rRNA fragments from the selected DGGE bands. In total, twenty-three and twenty-one bands were analyzed for samples collected in February and May 2012, respectively (Fig. 5.2). Phylogenetic trees of partial sequences of the 16S rDNA gene were constructed (Fig. 5.4). In samples collected in February 2012, five subdivisions of bacteria were detected, including β-proteobacteria, α-proteobacteria, γ-proteobacteria, Actinobacteria, and Bacteroidetes. These five subdivisions as well as the subdivision Firmicutes were detected in samples collected in May 2012. In February 2012, the subdivision γ-proteobacteria was exclusively detected in the samples treated at 42 and 48 ℃. In addition, the majority of α-proteobacteria, and about half of the β-proteobacteria and Bacteroidetes were detected at 42 and 48 ℃. All of the Actinobacteria, and a few α-proteobacteria and Bacteroidetes were detected in the 25 ℃ control. In May 2012, the population structure was similar with the exception of the unique subdivision Firmicutes detected in the 48 ℃ treatment. All Firmicutes detected in May 2012 were identified as uncultured *Bacillus* species.

Bacterial community structure shift with heat treatment

Culturable bacteria in the subdivisions Firmicutes and γ-proteobacteria increased in abundance in the 42 and 48 ℃ treated irrigation water (Table 5.4). The three major bacterial groups identified were the genera *Bacillus* and *Paenibacillus* in Firmicutes, and *Pseudomonas* in γ-proteobacteria. And other some minor groups found were *Brevibacillus* and *Lysobacter* species. Bacteria identified via PCR-DGGE belonging to the subdivisions α-proteobacteria, γ-proteobacteria, and Bacteroidetes were found only at 42 and 48 ℃ treatments. Bacteria similar/identical to *Pseudomonas aeruginosa* strain NTS6 were the most common detected on two sampling dates by both culturing and PCR-DGGE at heat-treated irrigation water. The majority of bacteria identified in the subdivisions β-proteobacteria and Actinobacteria were from control water.

Discussion

This study demonstrated that the dominant bacterial populations in the water samples shifted after heat treatments at 42 and 48 ℃ when compared with those at the 25 ℃ control. The two largest groups of bacteria detected in heat-treated irrigation water were γ-proteobacteria and Firmicutes. These findings may help understand the underlying biological mechanism by which *Phytophthora* and bacterial pathogens are killed or suppressed under the new heat treatment.

Our previous study (Chapter 2) showed that water temperature required to inactivate major groups of plant pathogens can be lowered to 48 ℃ for 24 h in sterile deionized water (SDW). In addition, the survival rate of *Phytophthora* zoospores at 42 ℃ was lower in irrigation water than SDW (Chapter 3). The enhanced inactivation of *Phytophthora* zoospores in irrigation water as well as the killing of major groups of plant pathogens at heat temperatures of 42 and

48 ℃ may be attribute partially to the bacterial community shift at such temperatures. Current heat treatment protocol recommending a high temperature of 95 ℃ destroys most microorganisms in water. Thus, the aquatic ecosystem is off biological balance, because microorganisms constitute the base of food webs and changes in composition and structure can profoundly affect the stability and sustainability of the ecosystem (Fuhrman and Ouverney 1998; Pomeroy et al. 2007). The proposed heat treatment temperature of 48 ℃ would not break the entire microbial community in irrigation water since it is much lower than the lethal temperature of most microorganisms (Baker 1962; Schumann 1991). In fact, the heat temperatures of 42 and 48 ℃ changed the composition and structure of bacterial communities in irrigation water, leading to an increased abundance of several bacterial groups compared to the 25 ℃ control. These bacterial groups may be antagonistic and thus enhance the control of plant pathogens present in irrigation water.

Bacteria in the subdivisions Firmicutes and γ-proteobacteria, including *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Brevibacillus*, and *Lysobacter* species, may contribute to inactivation of plant pathogens in irrigation water after heat treatment by biocontrol activities. Firmicutes and γ-proteobacteria were the two largest bacteria groups identified in heat-treated irrigation water, and *Bacillus*, *Paenibacillus*, and *Pseudomonas* were the three major bacterial genera. *Pseudomonas* species are the most extensively studied bacterial organisms utilized for biocontrol of many seedling diseases and root rots on several crops (Punja 1997). *Pseudomonas putida* strain 06909 suppresses *Phytophthora nicotianae* by complete lysis and degradation of hyphae of *P. nicotianae* (Yang et al. 1994). *Pseudomonas putida* WCS358r suppresses fusarium wilt of carnation caused by *Fusarium oxysporum* f. sp. *dianthi* via siderophore-mediated competition for iron (Duijff et al. 1993). *Pseudomonas fluorescens* strain Pf-5 provides highly

effective biocontrol activity against a broad range of fungal plant pathogens through the production of several secondary metabolites, including antifungal compounds such as pyoluteorin, pyrrolnitrin, and 2,4- diacylphloroglucinol, as well as several rhizoxin derivatives (Loper et al. 2007; Loper et al. 2008). This strain has been used in a Canadian greenhouse to control pythium diseases by reducing zoospore germination and chemotaxis (Paulitz and Belanger 2001). *Bacillus* species have also been investigated frequently as biocontrol agents. *Bacillus pumilus* strains INR7 and *Bacillus subtilis* strain GB03 control the cucumber pathogens *Colletotrichum orbiculare*, *Pseudomonas syringae* pv. *1achrymans*, and *Erwinia tracheiphila* (Raupach and Kloepper 1998). *Bacillus subtilis* Strain QST713 has a spectrum of antagonistic activity against over 40 plant diseases, including gray mold, damping-off, and powdery mildews, through a mixture of mechanisms consisted of competition, parasitism, antibiosis, and induction of systemic acquired resistance (SAR) (Paulitz and Belanger 2001). *Paenibacillus* species are not commonly tested as biocontrol agents against plant pathogens, but *Paenibacillus* sp. 300 did suppress fusarium wilt of cucumber (*Cucumis sativus*) caused by *Fusarium oxysporum* f. sp. *cucumerinum* in nonsterile, soilless potting medium (Singh et al. 1999). In addition, several other bacterial species in the genera *Brevibacillus* and *Lysobacter*, which only detected in heat-treated irrigation water, have been reported to biologically control a number of plant pathogens. *Brevibacillus laterosporus* BPM3 isolated from a natural hot water spring strongly inhibited growth of plant pathogenic fungi, including *Fusarium oxysporum* f. sp. *ciceri*, *F. semitectum*, *Magnaporthe grisea* and *Rhizoctonia oryzae*) (Saikia et al. 2011). *Lysobacter enzymogenes* is antagonistic to a broad range of plant pathogens by infecting the pathogens intracellularly (Christensen and Cook 1978; Kobayashi and Crouch 2009). Therefore, the increase of these

bacterial groups after heat treatment at 42 and 48 ℃ may result in more antagonistic activities against plant pathogens in irrigation water, contributing to the efficacy of the new heat treatment.

There are probably more bacteria than those detected in this study with biocontrol effect contributing to control of plant pathogens in irrigation water at 42 and 48 ℃. The culturable bacteria recovered from irrigation water here just compose a very small portion of the total microorganisms because the majority of environmental microbes are unculturable (Amann et al. 1995; Vartoukian et al. 2010). Moreover, most bacteria detected through culture-independent PCR-DGGE were unknown species and could not be identified to the genus level. In addition, the microbial community population in irrigation water varies with environmental conditions.

To further investigate the potential biological mechanisms of pathogen controlling at 42 and 48 ℃, *in vitro* and *in vivo* experiments are being conducted to test the biocontrol activities of selected bacterial isolates. Once the nature-occurring bacteria with biological activity are confirmed existing in the irrigation system, by manipulating and enhancing those beneficial microorganisms would build a pathogen-suppressing biological control system with minimal negative impact (Weller et al. 2002; Welbaum et al. 2004).

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Table 5.1 Water quality measurements in February and May 2012

	February 2012[a]	May 2012[a]
Temperature (℃)	8.04 ± 0.01	23.45 ± 0.01
pH	8.20 ± 0.01	9.81 ± 0.01
ORP (mV)[b]	549.00 ± 1.15	412.50 ± 1.15
Sp Conductivity (μS/cm)[c]	247.90 ± 0.21	266.30 ± 0.21
LDO[d] (mg/l)	13.38 ± 0.15	14.43 ± 0.15
CHLV[e] (Volts)	0.05 ± 0.01	0.77 ± 0.01
Turbidity (NTU)	52.43 ± 0.26	53.30 ± 0.05

[a] Values represented by mean ± SE of three replicates

[b] ORP - the oxidation reduction potential

[c] Sp Conductivity - specific conductivity

[d] LDO - luminescent dissolved oxygen

[e] CHLV - the chlorophyll sensor voltage

Table 5.2 Total colony-forming units per plate on nutrient agar as affected by temperature [a, b]

Treatment (℃)	February 2012	May 2012
25 (control)	349.33 ± 26.72	347.67 ± 18.32
42	401 ± 5.57	471.33 ± 45.62
48	155 ± 2	127 ± 16.46

[a] Values represented by mean ± SE of three replicates

[b] A volume of 100 µl water plated on each nutrient agar plate

Table 5.3 The number of resultant bands and Shannon diversity index (H) of bacterial diversity at temperature treatments of irrigation water sampled in February and May 2012.

Temperature treatment (℃)	February 2012		May 2012	
	Band #[a]	H[a]	Band #	H
25 (control)	22.67 ± 0.33	2.90 ± 0.01	20.67 ± 0.88	2.87 ± 0.05
42	23.67 ± 2.19	2.90 ± 0.10	22.67 ± 0.88	2.93 ± 0.02
48	11.67 ± 1.33	2.16 ± 0.11	14.67 ± 0.88	2.44 ± 0.06

[a] Values represented by mean ± SE of three replicates

Table 5.4 Bacteria in heat-treated water at 42 and 48 ℃ and control (25 ℃) as identified by partial sequences of 16S rDNA genes via culture-dependent and -independent strategies.

Subdivision	Closest match (Accession number)[a,b,c]	Temperature treatment[d] (℃)					
		February 2012			May 2012		
		25[e]	42	48	25[e]	42	48
α-proteobacteria	*Azospirillum* sp. DJM2D4 (JF753513.1) **		+				
	Candidatus Azospirillum massiliensis strain URAM1 (EF394925.1) *				+		
	Mesorhizobium sp. RITF 741 (JQ697665.1) *				+		
	Microvirga flocculans strain TFB (JF438969.1) **		+				
	Uncultured *Azospirillum* sp. clone GE7GXPU01AVTUJ (HQ044242.1) **					+	
	Uncultured *Balneimonas* sp. clone UHAS4.3 (JN037960.1) **			+			
	Uncultured *Rhodobacter* sp. clone rm15b3c02 (HM003636.1) **	+			+		
	Uncultured Alphaproteobacteria bacterium clone QEDV2BC02 (JQ696175.1) **		+	+		+	
	Uncultured Alphaproteobacterium clone DC5-50-1 (AY145582.1) **		+			+	
β-proteobacteria	*Acidovorax delafieldii strain* A3R07 (JQ689177.1) *	+			+		
	Acidovorax sp. DP52 (HQ704415.1) *				+		
	Acidovorax sp. NF1078 (JQ782387.2) *				+		
	Chitinibacter sp. SK16 (JN981166.1) *				+		
	Hydrogenophaga sp. WLSH-321 (JF682011.1) **		+				
	Hydrogenophaga sp. p3(2011) (HQ652595.1) *				+		
	Mitsuaria sp. Vi30 (JF501194.1) *				+		
	Pelomonas sp. UKW24 (JQ687102.1) *	+			+		
	Vogesella sp. TPS6 (FJ821602.1) *					+	
	Xenophilus aerolatus strain NML 100527 (JN585330.1) *	+					
	Uncultured *Herbaspirillum* sp. clone FL_52 (HQ008597.1) **	+					
	Uncultured *Polynucleobacter* sp. clone YL205 (HM856566.1) **	+			+		
γ-proteobacteria	*Aeromonas sp.* NM-21 (JQ894500.1) *		+				
	Enterobacter sp. X40 *(*HE662680.1) *				+		
	Pseudomonas aeruginosa strain NTS6 (HE858286.1) ***		+			+	
	Pseudomonas sp. Cf0-5 (JF683673.1) **		+				
	Pseudomonas sp. DCA-9 (FJ493141.1) *					+	
	Pseudomonas sp. HM05 (JQ396178.1) *	+	+		+	+	
	Pseudomonas sp. J61 (JQ670671.1) *					+	
	Pseudomonas sp. K7 (FR874238.1) *					+	
	Pseudomonas sp. MLB28 (JQ765424.1) *	+	+		+	+	
	Pseudomonas sp. NBRC 101713 (AB681538.1) *		+				
	Pseudomonas sp. NCCP-279 (AB641888.1) *	+			+		
	Pseudomonas sp. R3S4-1 (JQ928687.1) *			+			
	Lysobacter sp. 2-1 (JN942143.1) **		+				
	Lysobacter sp. YIM 77875 (JQ746036.1) **					+	+
	Rheinheimera texasensis strain JN-9 (JF496548.1) *	+					
	Rheinheimera sp. E49 (JQ922424.1) *				+		
	Uncultured *Pseudomonas* sp. clone GE7GXPU01AGHHE (HM973327.1) **						

	Uncultured *Rheinheimera* sp. clone 48PP (JF278072.1) **				+	+	
	Uncultured Gammaproteobacterium clone 30LAKE02G02 (HQ530658.1) **					+	
Actinobacteria	*Microbacterium* sp. F1 1010 (JQ691553.1) ***	+		+			
	Microbacterium sp. E56 (JF501120.1) *			+			
	Uncultured *Mycobacterium* sp. clone AMPE9 (AM935142.1) **	+					
	Uncultured actinobacterium clone 30LAKE01B08 (HQ530574.1) **	+			+		
	Uncultured actinobacterium clone KWK1S.54 (JN656761.1) **	+					
	Uncultured actinobacterium clone KWK6F.62 (JN656787.1) **	+					
	Uncultured actinobacterium clone w60 (HE654966.1) **	+					
	Uncultured actinobacterium clone w70 (HE654972.1) **	+					
	Uncultured actinobacterium clone w98 (HE654991.1) **	+					
Bacteroidetes	*Chitinophaga* sp. BS27 (JF806525.1) **			+			+
	Uncultured *Fluviicola* sp. clone JXS1-92 (JN873202.1) **					+	
	Uncultured Sphinogobacteriales bacterium clone W-LLR195 (HE654931.1) **		++			+	+
	Uncultured *Sphinogobacterium* sp. clone w21 (HE654931.1) **	+					
	Uncultured Bacteroidetes bacterium clone 30LAKE43F10 (HQ531074.1) **	+				+	+
Firmicutes	*Bacillus cereus* strain CT25 (EU111736.1) *	+	+	+			
	Bacillus cibi strain IARI-S-22 (JN411495.1) *			+			
	Bacillus ginsengihumi strain ANA15 (HQ219846.1) *						+
	Bacillus koreensis strain WIF49 (HM480320.1) *						+
	Bacillus marisflavi strain BVC64 (JQ407799.1) *	+		+			
	Bacillus marisflavi strain p53_B09 (JQ834634.1) *				+		+
	Bacillus mycoides strain TN12 (JQ415984.1) *						
	Bacillus pumilus strain CE4 (JQ435701.1) *				+		+
	Bacillus pumilus strain JR5-4 (JQ229695.1) *		+				
	Bacillus pumilus strain SRS83 (JQ965500.1) *		+	+			
	Bacillus pumilus strain TAD088 (FJ225317.1) *	+	+	+			
	Bacillus vietnamensis KL2-3 (AB697709.1) *	+					
	Bacillus sp. cmc28 (JQ917994.1) *			+	+	+	+
	Bacillus sp. DHC04 (JQ904715.1) *	+					+
	Bacillus sp. Pt1 (JF900600.1) *						+
	Bacillus sp. PZ_7 (JQ808525.1) *				+		+
	Bacillus sp. PZ_27 (JQ808526.1) *		+	+			
	Bacillus sp. PZ_35 (JQ808528.1) *			+	+		+
	Bacillus sp. s-bf-PMW-5.1.1 (FR774585.1) *			+			
	Brevibacillus parabrevis strain IFO 12334 (NR_040981.1) *					+	
	Brevibacillus sp. SE12 (JQ714095.1) *						+
	Cohnella sp. CC-Alfalfa-35 (JN806384.1) *						+
	Exiguobacterium aurantiacum strain TSL7 (JQ885975.1) *				+		
	Exiguobacterium profundum strain CIBA BW17 (JQ073758.1) *		+				
	Paenibacillus sp. 140SI (JF825471.1) *			+			+
	Paenibacillus sp. BL16-1 (EU912455.1) *			+			+
	Paenibacillus sp. DHC15 (JQ904726.1) *				+		+

Paenibacillus sp. Dv34 (HQ728085.1) *			+
Paenibacillus sp. KMC003 (EU219972.1) *			+
Paenibacillus sp. MN2-8 (JQ396530.1) *			+
Paenibacillus sp. WYT011 (JQ807858.1) *			+
Paenibacillus terrigena strain B30 (JN252080.1) *		+	+
Paenibacillus timonensis strain 3584BRRJ (JF309265.1) *			+

[a] Sequences aligned to their closest matches in GenBank using BLAST.

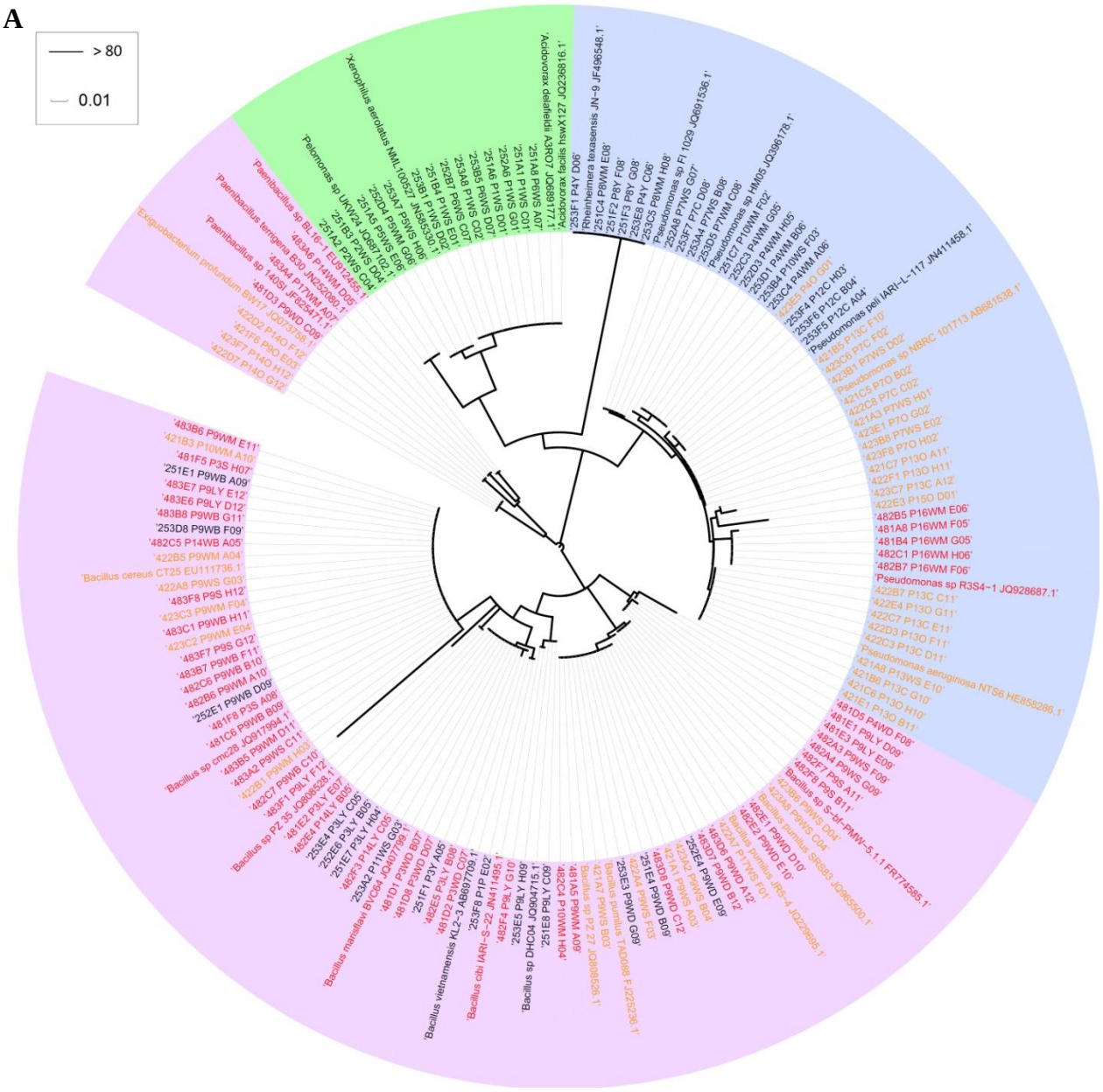
[b] All the closest relatives of sequences with a similarity ≥ 96%.

[c] * indicating detection via culture-dependent strategy, ** indicating detection via culture-independent strategy, *** indicating detection via both strategies

[d] + indicating the presence of the bacterial identity

[e] 25 ℃ as control

A



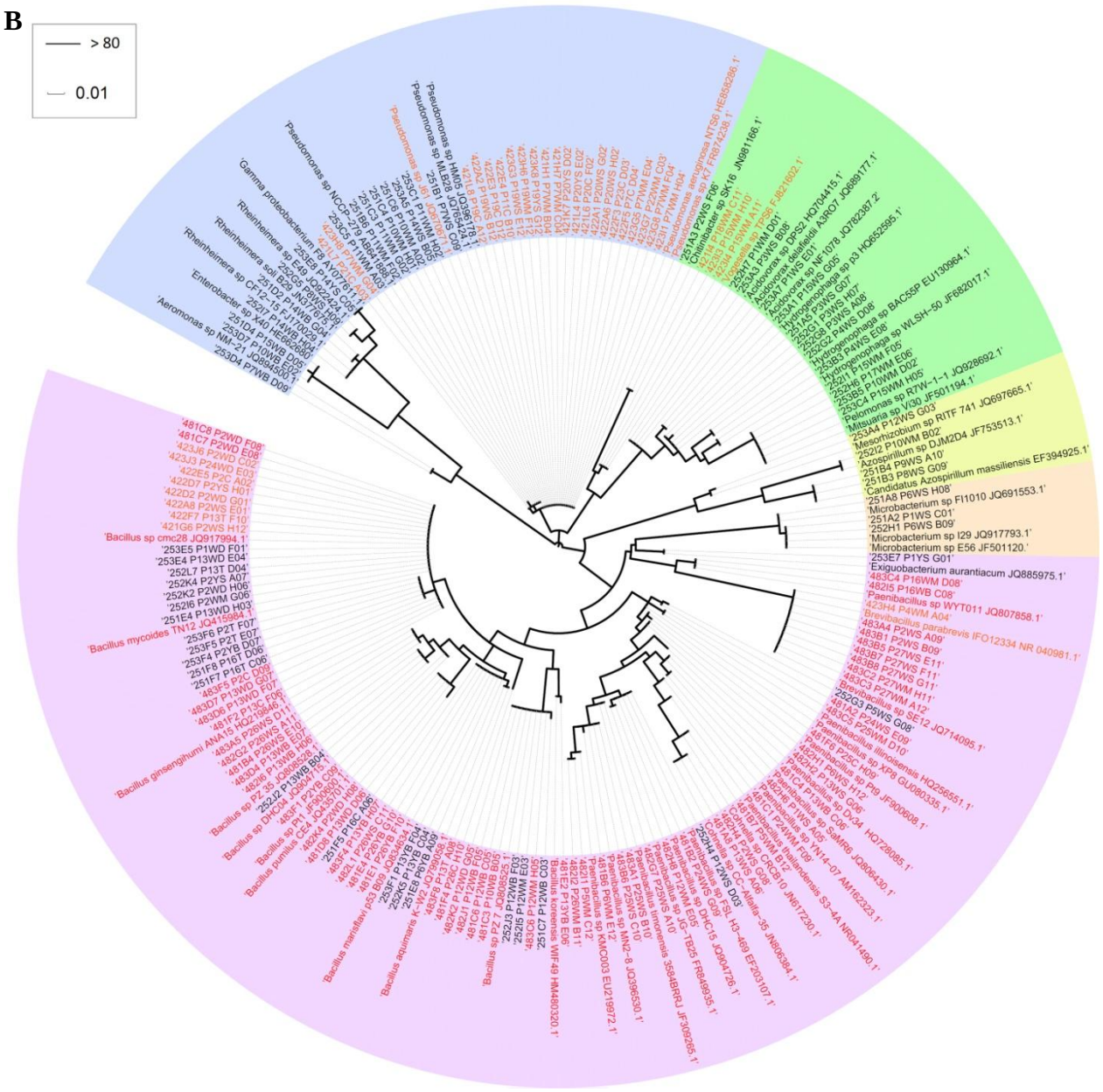


Figure 5.1 Phylogenetic analysis of partial 16S rDNA sequences of representative bacterial isolates from irrigation water samples collected in (A) February and (B) May 2012 after heat treatments at 25 (as control, black), 42 (orange), and 48 ℃ (red). The Maximum Likelihood method based on the Tamura-Nei model was used. Bootstrap analyses were based on 1000 replicates. Different color shadings indicate various subdivisions of bacteria: blue, γ-

proteobacteria; green, β-proteobacteria; yellow, α-proteobacteria, purple, Firmicutes; and orange, Actinobacteria.

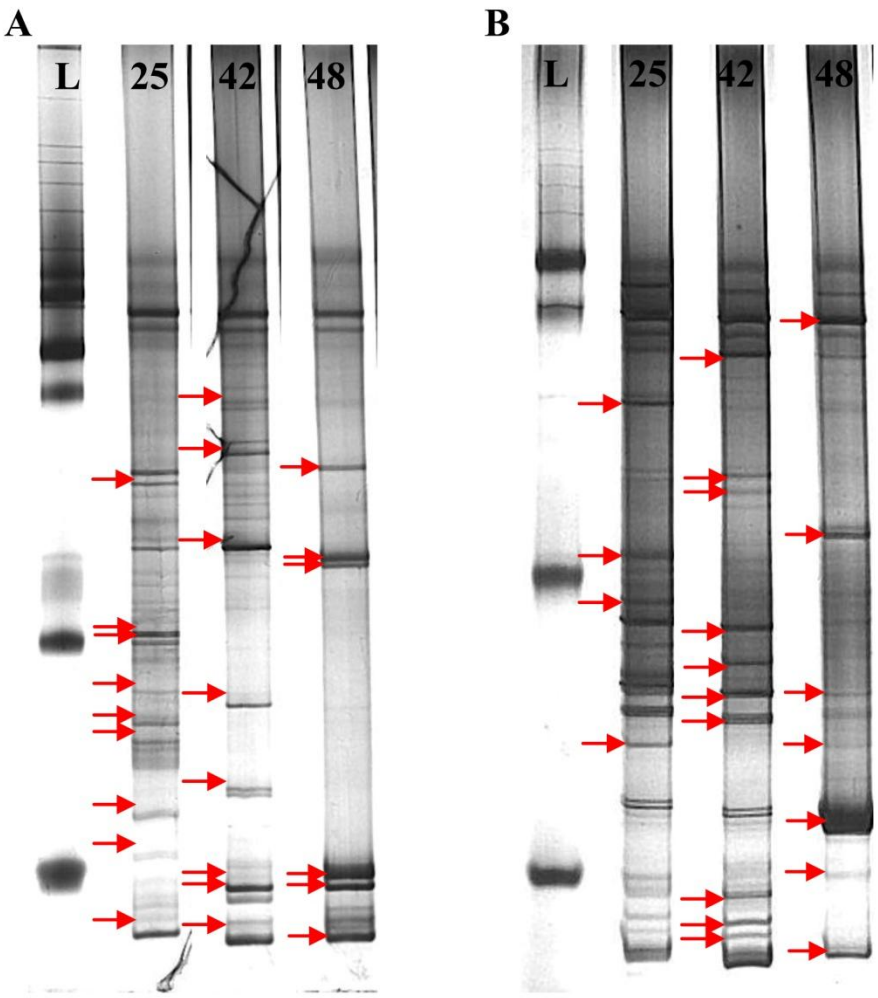


Figure 5.2 DGGE fingerprinting of bacterial 16S rRNA fragments in irrigation water sampled in A) February and B) May 2012 after heat treatments at 25 (control), 42, and 48 ℃. The DGGE was run at70 V for 16 h with a gel denaturant concentration of 40 to 60%. Red arrows represent selected bands. L represents 100 bp DNA ladder.

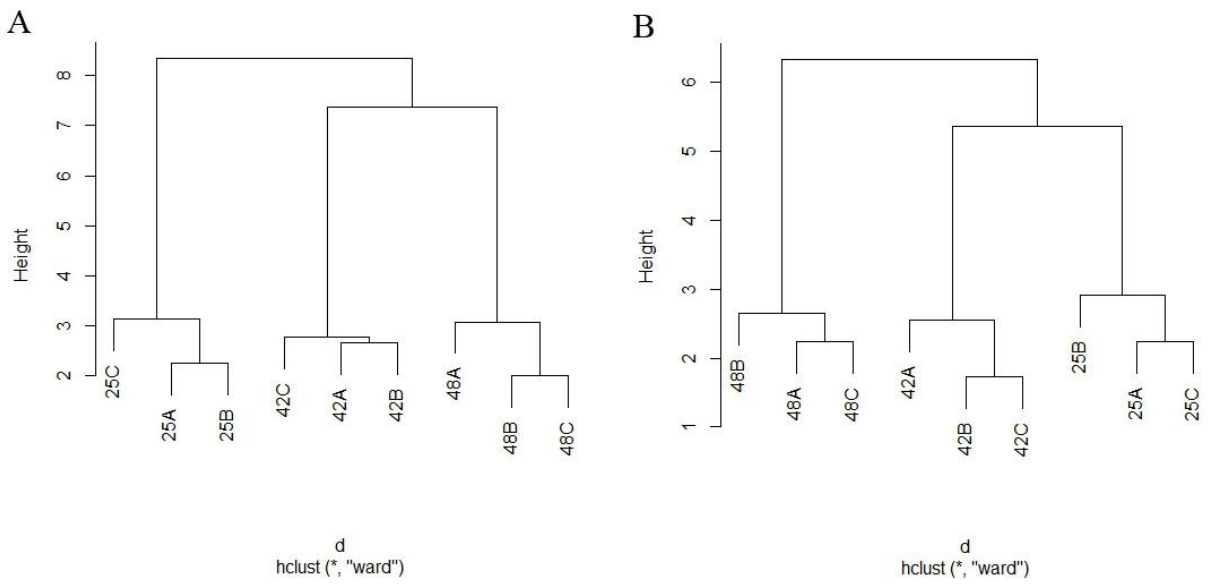
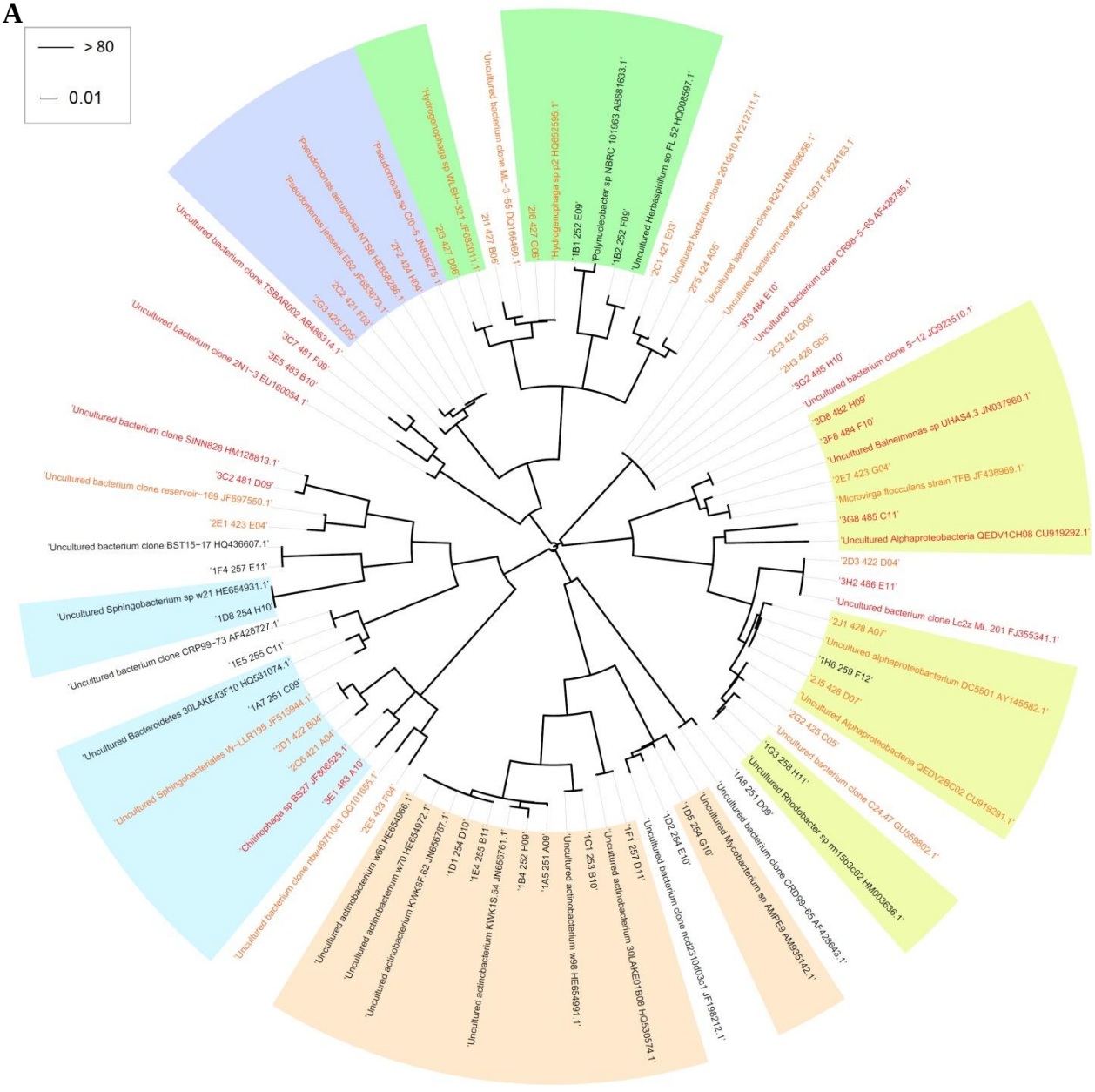


Figure 5.3 Dendrogram derived from a distance analysis of DGGE bands and the Ward's Hierarchical Method of clustering to show relationships between DGGE patterns for different temperature treatments (25 as control, 42, and 48 ℃) in irrigation water sampled in A) February and B) May 2012. R statistical software (R Development Core Team, R Foundation for Statistical Computing. 2011. ISBN 3-900051-07-0) was used for the analyses.

A


> 80
0.01
'Uncultured bacterium clone ML−3−55 DQ166480.1'
'2I1 427 B06'
'Hydrogenophaga sp WLSH−321 JF682011.1'
'2I3 427 D06'
'Pseudomonas sp Ch0−5 JN636275.1'
'Pseudomonas aeruginosa NTS8 HE858036.1'
'Pseudomonas jessenii E62 JF683973.1'
'2F2 424 H04'
'2C2 421 F03'
'2G3 425 D05'
'Uncultured bacterium clone TSBAR002 AB486314.1'
'Uncultured bacterium clone 2N1−3 EU160054.1'
'3C7 481 F09'
'3E5 483 B10'
'Uncultured bacterium clone SINN828 HM128813.1'
'3C2 481 D09'
'Uncultured bacterium clone reservoir−169 JF697550.1'
'2E1 423 E04'
'Uncultured bacterium clone BST15−17 HQ436607.1'
'1F4 257 E11'
'Uncultured Sphingobacterium sp w21 HE654931.1'
'1D8 254 H10'
'Uncultured bacterium clone CRP99−73 AF428727.1'
'1E5 255 C11'
'Uncultured Bacteroidetes 30LAKE43F10 HQ531074.1'
'1A7 251 C09'
'Uncultured Sphingobacteriales W−LLR195 JF515944.1'
'2D1 422 B04'
'2C6 421 A04'
'3E1 483 A10'
'Chitinophaga sp BS27 JF806525.1'
'Uncultured bacterium clone nbw497f10c1 GQ101655.1'
'2E5 423 F04'
'Hydrogenophaga sp cd2 HQ652565.1'
'2I6 427 G06'
'1B1 252 E09'
'Polynucleobacter sp NBRC 101963 AB681633.1'
'1B2 252 F09'
'Uncultured Herbaspirillum sp FL 52 HQ008597.1'
'2C1 421 E03'
'Uncultured bacterium clone 2610ds10 AY212711.1'
'2F5 424 A05'
'Uncultured bacterium clone RC42 HM666056.1'
'Uncultured bacterium clone MFC 19D7 FJ624163.1'
'3F5 484 E10'
'Uncultured bacterium clone CR98−5−65 AF428795.1'
'2C3 421 G03'
'2H3 426 G05'
'3G2 485 H10'
'Uncultured bacterium clone 5−12 JQ923510.1'
'3D8 482 H09'
'3F8 484 F10'
'Uncultured Balneimonas sp UHAS4.3 JN037960.1'
'2E7 423 G04'
'Microvirga flocculans strain TFB JF438969.1'
'3G8 485 C11'
'Uncultured Alphaproteobacteria QEDV1CH08 CU919292.1'
'2D3 422 D04'
'3H2 486 E11'
'Uncultured bacterium clone Lc2z ML 201 FJ355341.1'
'2J1 426 A07'
'Uncultured alphaproteobacterium DC5501 AY145582.1'
'1H6 259 F12'
'2J5 428 D07'
'Uncultured Alphaproteobacteria QEDV2BC02 CU919291.1'
'2G2 425 C05'
'Uncultured bacterium clone C24.47 GU358602.1'
'1G3 258 H11'
'Uncultured Rhodobacter sp rm15b3c02 HM003636.1'
'1A8 251 D09'
'Uncultured bacterium clone CRD99−65 AF428843.1'
'Uncultured Mycobacterium sp AMPEB AM935142.1'
'1D5 254 G10'
'1D2 254 E10'
'Uncultured bacterium clone nov23100f3c1 JF198212.1'
'1F1 257 D11'
'Uncultured actinobacterium 30LAKE01B08 HQ530574.1'
'1C1 253 B10'
'Uncultured actinobacterium w98 HE654991.1'
'1A5 251 A09'
'1B4 252 H09'
'Uncultured actinobacterium KWK1S.54 JN656761.1'
'1E4 255 B11'
'1D1 254 D10'
'Uncultured actinobacterium KWK6F.62 JN656787.1'
'Uncultured actinobacterium w70 HE654972.1'
'Uncultured actinobacterium w60 HE654966.1'

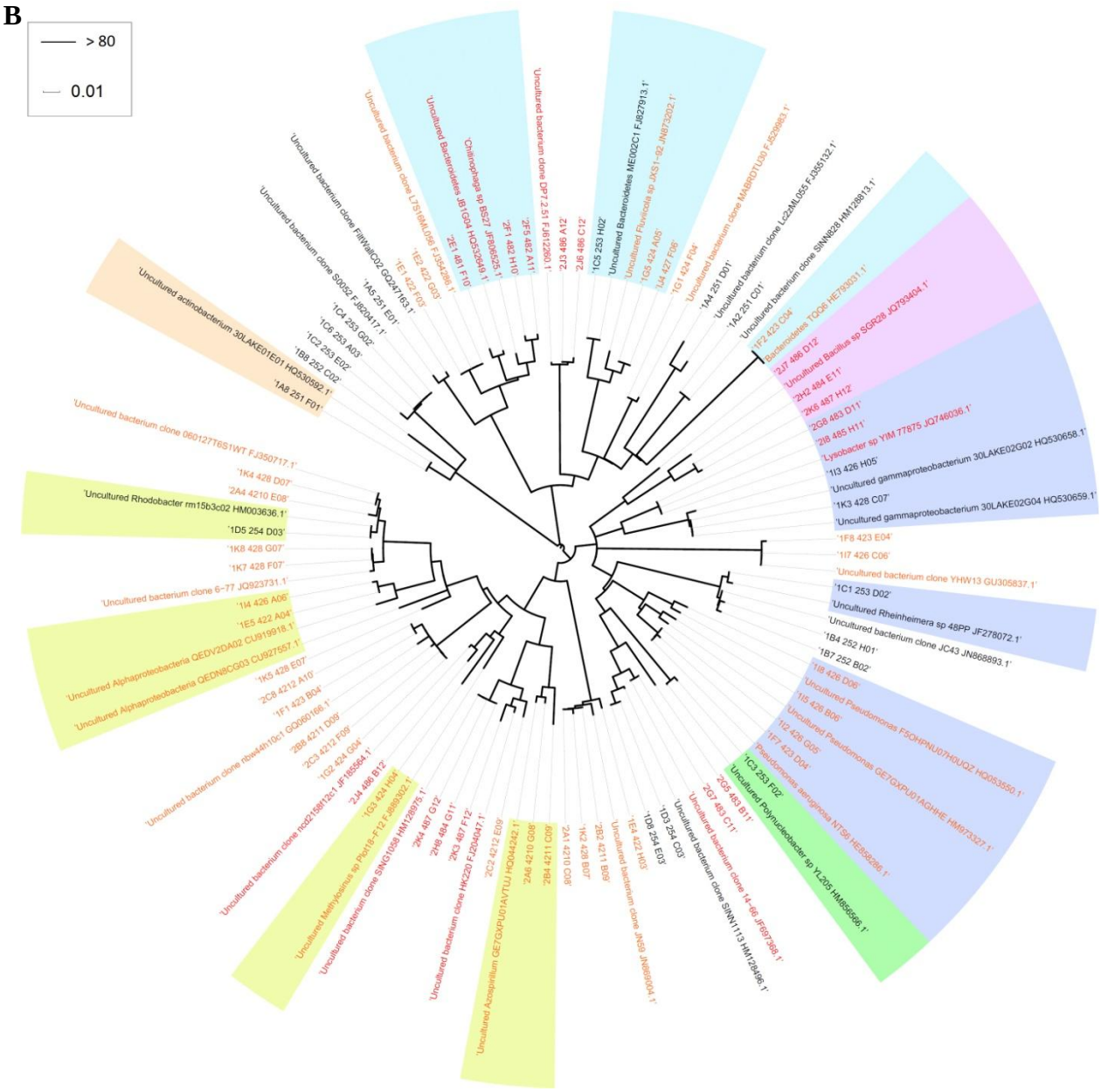


Figure 5.4 Phylogenetic tree of partial sequences of 16S rDNA from representative bacteria in irrigation water samples collected in (A) February and (B) May 2012 after heat treatments at 25 (as control, black), 42 (orange), and 48 ℃ (red) via culture-independent strategy (PCR-DGGE). The Maximum Likelihood method based on the Tamura-Nei model was used. Bootstrap analyses were based on 1000 replicates. Different color shadings indicate various subdivisions of bacteria:

blue, γ-proteobacteria; green, β-proteobacteria; yellow, α-proteobacteria; purple, Firmicutes; orange, Actinobacteria; light blue, Bacteroidetes; unshaded, unknown bacterial species.

Chapter 6

Summary and future directions

Heat treatment is a reliable and effective decontamination technology for re-circulated irrigation water in the ornamental horticulture industry (van Os et al. 1988; Hong and Moorman 2005; Hao et al. 2012). The current protocol recommends raising water temperature to 95 ℃ and maintaining it at that temperature for 30 s (Runia et al. 1988; McPherson et al. 1995). The major limitations associated with this water treatment are its energy inefficiency, high costs, and environmental footprint (Poncet et al. 2001; Hong and Moorman 2005). The overall goal of this dissertation was to make this technology economically more attractive while reducing its environmental impact. We hypothesized that the temperature required to inactivate major pathogens in re-circulated water may be substantially lowered from 95 ℃. The specific objectives of this dissertation were to test the above hypothesis, including investigation into biological mechanisms by which plant pathogens are killed at the substantially lowered water temperature.

The effect of water temperature on the survival of *Phytophthora* and bacterial species, two major groups of plant pathogens in re-circulating water systems, was examined. *Phytophthora* species, known as water molds, are the most frequently isolated plant pathogens in irrigation water (Hong and Moorman 2005; Gallegly and Hong 2008). *P. nicotianae,* a high-temperature tolerant species, was used as a major model system in this study. Heat treatments at 42 ℃ for 24 h or at 48 ℃ for 6 h inactivated the major life stages of *Phytophthora* species in the laboratory and greenhouse tests. It is expected that the heat and exposure time combination that

worked for *P. nicotianae* would work for a vast majority, if not all, oomycete pathogens in irrigation water. Heat treatment at 48 ℃ for 24 h inactivated all seven tested bacterial species, some of which are the most common bacterial plant pathogens found in irrigation water (Hong and Moorman 2005). These results indicated that raising water temperature to 48 ℃ and maintaining it for 24 h could be an alternative to the current heat treatment protocol which uses 95 ℃ for 30 s. Use of a lower temperature with a longer exposure time could substantially reduce energy consumption, consequently improve the economics of heat treatment, and hence the profitability of the ornamental horticulture industry while reducing its environmental footprint.

Two steps were taken to elucidate the underlying biological mechanisms of pathogen removal at the substantially lowered water temperature. Firstly, a water sample concentration and processing scheme was developed for typing bacterial species in irrigation water samples. The key component of this scheme to detect the greatest diversity of bacteria in irrigation water samples were to (1) employ both culture-dependent and -independent technologies as each detects a subset of bacteria, (2) use both centrifugation and filtration to concentrate water samples and (3) perform PCR-DGGE with 40 to 60% denaturant at 70 V for 16 h. Secondly, water samples were taken from an irrigation reservoir in a local nursery and analyzed for bacterial diversity following heat treatments at 42 and 48 ℃. A substantial shift in the dominant bacterial populations were found at 42 and 48 ℃ when compared with the control at 25 ℃. Of the most significance were γ-proteobacteria and Firmicutes becoming dominant in heat-treated water. These included *Bacillus*, *Pseudomonas, Paenibacillus, Brevibacillus,* and *Lysobacter* species, which are known to have a great potential as biological control agents. For example, *Bacillus* and *Pseudomonas* species have been investigated extensively as biocontrol agents (Punja 1997). Some species and isolates in the genera *Paenibacillus*, *Brevibacillus*, and

Lysobacter have also been reported to biologically control a number of plant pathogens (Christensen and Cook 1978; Singh et al. 1999; Kobayashi and Crouch 2009; Saikia et al. 2011). These results indicated that bacterial community shifts may have contributed to the pathogen removal under heat treatments at 42 and 48 ℃.

To substantiate the findings from the above studies, additional investigations are needed to evaluate the new heat treatment temperatures at production facilities, identify alternative energy generation and retention sources. To further elucidate its underlying biological mechanisms additional experiments will need to determine the biological control activities of selected bacterial species and isolates. Moreover, investigation into the abundance and frequency of these bacterial species in irrigation water at multiple locations also are warranted.

In summary, this dissertation provided the scientific basis for developing a more energy-efficient and environmentally sound heat treatment for re-circulated irrigation water.

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Appendix A

Bacterial isolates recovered from irrigation water in Chapter 4

Table A.1 Bacterial isolates obtained via culture-dependent strategy from irrigation water sample in Chapter 4.

Sampling date	Sequence #	Colony #	Colony type	SSCP banding pattern	Accession #	Closest match (Accession #)[a]	Similarity (%)
Sep 2011	1a13_2AP1_A10	1A4	WS	P1	JX628632	*Bacillus cereus* strain IARI-S-9 (JN411483.1)	99
	1a13_2AP1_B10	1B1	WS	P1	JX628633	*Bacillus cereus* strain IARI-S-9 (JN411483.1)	99
	1a13_2AP1_C10	1B4	WS	P1	JX628634	*Bacillus cereus* strain IARI-S-9 (JN411483.1)	99
	1a13_2AP1_D11	1H5	WD	P1	JX628637	*Bacillus cereus* strain IARI-S-9 (JN411483.1)	99
	1a13_2AP1_H10	1G4	WD	P1	JX628636	*Bacillus cereus* strain IARI-S-9 (JN411483.1)	99
	1a13_2AP1_D10	1C3	WM	P1	JX628635	*Bacillus cereus* strain IARI-S-9 (JN411483.1)	99
	1a13_2AP1_G10	1F1	WM	P1	JX628638	*Lysinibacillus* sp. ZSG2-3 (JN990432.1)	99
	1a13_2AP1_A11	1H1	WB	P1	JX628681	*Bacillus* sp. ARA1-1 (AB677952.1)	100
	1a13_2AP1_C11	1H4	WS	P1	JX628683	*Bacillus* sp. ARA1-1 (AB677952.1)	100
	1a13_2AP1_E10	1C4	WS	P1	JX628684	*Bacillus* sp. ARA1-1 (AB677952.1)	100
	1a13_2AP1_B11	1H2	WB	P1	JX628682	*Bacillus* sp. ARA1-1 (AB677952.1)	100
	1a13_2AP1_F10	1D5	WM	P1	JX628685	*Bacillus* sp. ARA1-1 (AB677952.1)	100
	1a13_2AP1_E11	2B2	WM	P1	JX628642	*Brevibacillus brevis* strain NBRC 100599 (AB681205.1)	100
	1a13_2AP1_F11	4B4	WD	P1	JX628644	*Bacillus pumilus* strain MSII-6 (JN993718.1)	99
	1a13_2AP1_G11	4B8	P	P1	JX628645	*Chromobacterium haemolyticum* strain MDA0585 (NR_043957.1)	99
	1a13_2AP1_H11	4D4	Y	P1	JX628646	*Bacillus* sp. BWDY-6 (DQ314534.1)	98
	1a13_2AP1_B12	5B2	Y	P1	JX628647	*Bacillus* sp. BWDY-6 (DQ314534.1)	98
	1a13_2AP1_C12	5E5	Y	P1	JX628648	*Bacillus* sp. BWDY-6 (DQ314534.1)	98
	1a13_2AP1_A12	4D8	Y	P1	JX628649	*Providencia* sp. SRS82 (JN381552.1)	100
	1a13_2AP2_E12	4B5	B	P2	JX628650	*Chromobacterium* sp. IITR-71 (JN210566.1)	98
	1a13_2AP2_D12	1A6	B	P2	JX628651	*Chromobacterium* sp. IITR-71 (JN210566.1)	98

1a13_2AP2_F12	4B10	Y	P2	JX628658	*Bacillus pumilus* strain TAD174 (FJ225317.1)	98
1a13_2AP2_G12	5C7	WB	P2	JX628659	*Bacillus pumilus* strain TAD174 (FJ225317.1)	98
1a13_2AP3_H12	1B7	WS	P3	JX628664	*Bacillus cereus* strain LZ027 (JQ023617.1)	99
1a15_2AP3_A02	1B8	WD	P3	JX628665	*Bacillus thuringiensis* strain GTG-40 (JQ004436.1)	100
1a15_2AP4_G02	1C4	Y	P4	JX628669	*Bacillus cereus* strain CT25 (EU111736.1)	100
1a15_2AP4_H02	1F1	O	P4	JX628639	*Lysinibacillus* sp. ZSG2-3 (JN990432.1)	99
1a15_2AP4_C03	4B9	Y	P4	JX628640	*Lysinibacillus* sp. ZSG2-3 (JN990432.1)	99
1a15_2AP4_D03	4D1	Y	P4	JX628641	*Lysinibacillus* sp. ZSG2-3 (JN990432.1)	99
1a15_2AP4_A03	3B6	P	P4	JX628670	*Chromobacterium* sp. PMR-A (HM047300.1)	99
1a15_2AP4_B03	4B8	B	P4	JX628671	*Chromobacterium* sp. PMR-A (HM047300.1)	99
1a15_2AP4_G03	5F3	Y	P4	JX628672	*Chromobacterium* sp. PMR-A (HM047300.1)	99
1a15_2AP4_F03	5E2	B	P4	JX628652	*Chromobacterium* sp. IITR-71 (JN210566.1)	99
1a15_2AP4_F02	1C3	Y	P4	JX628653	*Chromobacterium* sp. IITR-71 (JN210566.1)	99
1a15_2AP4_E03	4D3	Y	P4	JX628654	*Chromobacterium* sp. IITR-71 (JN210566.1)	99
1a15_2AP5_H03	1F11	WS	P5	JX628666	*Bacillus thuringiensis* strain GTG-40 (JQ004436.1)	100
1a15_2AP5_B04	2B12	WS	P5	JX628667	*Bacillus thuringiensis* strain GTG-40 (JQ004436.1)	100
1a15_2AP5_A04	2A6	WS	P5	JX628668	*Bacillus thuringiensis* strain GTG-40 (JQ004436.1)	99
1a15_2AP6_C04	1G6	P	P6	JX628673	*Chromobacterium violaceum* strain NBRC 12614 (AB680302.1)	99
1a15_2AP6_H04	2D6	WS	P6	JX628674	*Chromobacterium violaceum* strain NBRC 12614 (AB680302.1)	99
1a15_2AP6_F04	2D1	WS	P6	JX628661	*Bacillus pumilus* strain RRLJ SMAD (DQ299945.1)	97
1a15_2AP6_E04	2c7	WS	P6	JX628662	*Bacillus pumilus* strain RRLJ SMAD (DQ299945.1)	97
1a15_2AP6_D04	2A3	WD	P6	JX628663	*Bacillus pumilus* strain RRLJ SMAD (DQ299945.1)	97
1a15_2AP6_G04	2D3	WS	P6	JX628678	*Bacillus thuringiensis* strain IARI-A-7 (JN411428.1)	99
1a15_2AP6_B05	3A8	O	P6	JX628679	*Lysinibacillus sphaericus* strain SP19_LP11 (JQ289050.1)	99
1a15_2AP6_C05	3B7	WM	P6	JX628660	*Bacillus pumilus* strain TAD174 (FJ225317.1)	100
1a15_2AP7_F12	1G12	WB	P7	JX628680	*Pseudomonas* sp. Cf0-5 (JN836275.1)	99
1a15_2AP8_D05	2D5	P	P8	JX628675	*Chromobacterium violaceum* strain NBRC 12614 (AB680302.1)	100
1a15_2AP8_F05	2D7	P	P8	JX628676	*Chromobacterium violaceum* strain NBRC 12614 (AB680302.1)	100
1a15_2AP8_E05	2D9	P	P8	JX628677	*Chromobacterium violaceum* strain NBRC 12614 (AB680302.1)	100
1a15_2AP9_G05	3B8	WS	P9	JX628686	*Bacillus* sp. ARA1-1 (AB677952.1)	99
1a15_2AP9_B06	4A6	WS	P9	JX628687	*Bacillus* sp. ARA1-1 (AB677952.1)	99

	1a15_2AP9_H05	3C9	WS	P9	JX628688	*Bacillus* sp. MGB13 (JN000926.1)	99
	1a15_2AP9_A06	4A2	WM	P9	JX628643	*Brevibacillus brevis* strain NBRC 100599 (AB681205.1)	99
	1a15_2AP10_C06	5B2	B	P10	JX628655	*Chromobacterium* sp. IITR-71 (JN210566.1)	99
	1a15_2AP10_F06	5E7	P	P10	JX628656	*Chromobacterium* sp. IITR-71 (JN210566.1)	99
	1a15_2AP10_G06	5E8	P	P10	JX628657	*Chromobacterium* sp. IITR-71 (JN210566.1)	99
	1a15_2AP10_D06	5B8	O	P10	JX628689	*Lysinibacillus* sp. C250R (GQ342695.1)	97
	1a15_2AP10_E06	5E5	P	P10	JX628690	*Chromobacterium violaceum* strain 968 (HM449690.1)	100
	1a15_2AP11_H06	5B3	Y	P11	JX628691	*Aeromonas* sp. PBB3 (JQ681230.1)	99
May 2012	1a24_253A3_P3WS_B08	253A3	WS	P3	JX628732	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
	1a24_252H7_P1WM_D01	252H7	WM	P1	JX628719	*Acidovorax* sp. DPS2 (HQ704415.1)	100
	1a24_253A7_P1WS_E01	253A7	WS	P1	JX628735	*Acidovorax* sp. NF1078 (JQ782387.2)	100
	1a24_253D4_P7WB_D09	253D4	WB	P7	JX628741	*Aeromonas* sp. NM-21 (JQ894500.1)	100
	1a24_252I2_P10WM_B02	252I2	WM	P10	JX628721	*Azospirillum* sp. DJM2D4 (JF753513.1)	100
	1a24_251F5_P16C_A06	251F5	WB	P16	JX628708	*Bacillus altitudinis* strain SGb146 (HQ224625.1)	100
	1a24_252K5_P13YB_C04	252K5	YB	P13	JX628729	*Bacillus marisflavi* strain p53_B09 (JQ834634.1)	100
	1a24_253F1_P13YB_F04	253F1	YB	P13	JX628746	*Bacillus marisflavi* strain p53_B09 (JQ834634.1)	100
	1a24_251E8_P6YB_A09	251E8	YB	P6	JX628707	*Bacillus marisflavi* strain p53_B09 (JQ834634.1)	100
	1a24_251F7_P16T_C06	251F7	WB	P16	JX628709	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
	1a24_251F8_P16T_D06	251F8	WB	P16	JX628710	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
	1a24_253F5_P2T_E07	253F5	WB	P2	JX628748	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
	1a24_253F6_P2T_F07	253F6	WB	P2	JX628749	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
	1a24_253F4_P2YB_D07	253F4	Y	P2	JX628747	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
	1a24_253E4_P13WD_E04	253E4	WD	P13	JX628743	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_252L7_P13T_D04	252L7	WB	P13	JX628730	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_251E4_P13WD_H03	251E4	WD	P13	JX628706	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_252I6_P2WM_G06	252I6	WM	P2	JX628723	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1A24_252K2_P2WD_H06	252K2	WD	P2	JX628727	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_252K4_P2YS_A07	252K4	B	P2	JX628728	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_253E5_P1WD_F01	253E5	WD	P1	JX628744	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_252J2_P13WB_B04	252J2	WB	P13	JX628725	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_251C7_P12WB_C03	251C7	WB	P12	JX628703	*Bacillus* sp. PZ_7 (JQ808525.1)	99
	1a24_252J3_P12WB_F03	252J3	WB	P12	JX628726	*Bacillus* sp. PZ_7 (JQ808525.1)	100
	1a24_252I5_P12WM_E03	252I5	WM	P12	JX628722	*Bacillus* sp. PZ_7 (JQ808525.1)	99
	1a24_251B3_P8WS_G09	251B3	WS	P8	JX628697	*Candidatus Azospirillum massiliensis* strain URAM1 (EF394925.1)	99

1a24_1_251B4_P9WS_A10	251B4	WS	P9	JX628698	*Candidatus Azospirillum massiliensis* strain URAM1 (EF394925.1)	99
1a24_251A3_P2WS_F06	251A3	WS	P2	JX628693	*Chitinibacter* sp. SK16 (JN981166.1)	100
1a24_253D7_P10WB_E02	253D7	WB	P10	JX628742	*Enterobacter* sp. X40 (HE662680.1)	100
1a24_251D4_P15WB_D05	251D4	WB	P15	JX628705	*Enterobacter* sp. X40 (HE662680.1)	100
1a24_253E8_P14YS_C05	253E8	B	P14	JX628745	Gamma proteobacterium F8 (AY077611.1)	100
1a24_252G2_P4WS_D08	252G2	WS	P4	JX628712	*Hydrogenophaga* sp. BAC55P (EU130964.1)	100
1a24_253A1_P15WS_G05	253A1	WS	P15	JX628731	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
1a24_251A5_P3WS_G07	251A5	WS	P3	JX628694	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
1a24_252G1_P3WS_H07	252G1	WS	P3	JX628711	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
1a24_252G8_P3WS_A08	252G8	WS	P3	JX628715	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
1a24_253B3_P4WS_E08	253B3	WS	P4	JX628736	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
1a24_253A4__P12WS_G03	253A4	WS	P12	JX628733	*Mesorhizobium* sp. RITF 741 (JQ697665.1)	99
1a24_252H1_P6WS_B09	252H1	WS	P6	JX628716	*Microbacterium* sp. E56 (JF501120.1)	100
1a24_251A8_P6WS_H08	251A8	WS	P6	JX628695	*Microbacterium* sp. FI 1010 (JQ691553.1)	100
1a24_251A2_P1WS_C01	251A2	WS	P1	JX628692	*Microbacterium* sp. I_29-J6NFA10A (JQ917793.1)	100
1a24_253B5_P10WM_D02	253B5	WM	P10	JX628737	*Mitsuaria* sp. Vi30 (JF501194.1)	99
1a24_252I1_P15WM_F05	252I1	WM	P15	JX628720	*Mitsuaria* sp. Vi30 (JF501194.1)	100
1a24_253C4_P15WM_H05	253C4	WM	P15	JX628739	*Mitsuaria* sp. Vi30 (JF501194.1)	100
1a24_252H4__P12WS_D03	252H4	WS	P12	JX628717	*Paenibacillus* sp. FSL_H3-469 (EF203107.1)	100
1a24_252G3_P5WS_G08	252G3	WS	P5	JX628713	*Paenibacillus* sp. XP8 (GU080335.1)	100
1a24_252H6_P17WM_E06	252H6	WM	P16	JX628718	*Pelomonas* sp. R7W-1-1 (JQ928692.1)	100
1a24_251C4_P10WM_H01	251C4	WM	P10	JX628701	*Pseudomonas* sp. HM05 (JQ396178.1)	99
1a24_251C6_P10WM_A02	251C6	WM	P10	JX628702	*Pseudomonas* sp. HM05 (JQ396178.1)	99
1a24_253C1_P11WM_H02	253C1	WM	P11	JX628738	*Pseudomonas* sp. HM05 (JQ396178.1)	99
1a24_253C5_P11WM_A03	253C5	WM	P11	JX628740	*Pseudomonas* sp. HM05 (JQ396178.1)	99
1a24_251B1_P7WS_C09	251B1	WS	P7	JX628696	*Pseudomonas* sp. MLB28 (JQ765424.1)	100
1a24_251B6_P11WM_F02	251B6	WM	P11	JX628699	*Pseudomonas* sp. NCCP-279 (AB641888.1)	100
1a24_251C3_P11WM_G02	251C3	WM	P11	JX628700	*Pseudomonas* sp. NCCP-279 (AB641888.1)	100
1a24_253A5_P14WS_B05	253A5	WS	P14	JX628734	*Pseudomonas* sp. NCCP-279 (AB641888.1)	100
1a24_251D2_P14WB_G04	251D2	WB	P14	JX628704	*Rheinheimera soli* strain B29 (JN377675.1)	100
1a24_252I7_P14WB_H04	252I7	WB	P14	JX628724	*Rheinheimera* sp. CF12-15 (FJ170029.1)	100
1a24_252G5_P8WS_H09	252G5	WS	P8	JX628714	*Rheinheimera* sp. E49 (JQ922424.1)	100

[a] Sequences aligned to their closest matches in GenBank with BLAST

Table A.2 Bacterial isolates obtained via culture-independent strategy (PCR-DGGE) from irrigation water sample in Chapter 4.

Sampling date	Sequence #	DGGE band #	Accession #	Closest match (Accession #)[a]	Similarity (%)
Sep 2012	1a20_1A5_251_A09	1	JX657295	Uncultured actinobacterium clone KWK1S.54 (JN656761.1)	99
	1a20_1A7_251_C09	1	JX657296	Uncultured Bacteroidetes bacterium clone 30LAKE43F10 (HQ531074.1)	99
	1a20_1A8_251_D09	1	JX657297	Uncultured bacterium clone CRD99-65 (AF428643.1)	99
	1a20_1B1_252_E09	2	JX657298	*Polynucleobacter* sp. NBRC 101963 (AB681633.1)	99
	1a20_1B2_252_F09	2	JX657299	Uncultured *Herbaspirillum* sp. clone FL_52 (HQ008597.1)	99
	1a20_1B4_252_H09	2	JX657300	Uncultured actinobacterium clone KWK6F.62 (JN656787.1)	99
	1a20_1C1_253_B10	3	JX657301	Uncultured actinobacterium clone w98 (HE654991.1)	100
	1a20_1C8_253_C10	3	JX657302	Uncultured bacterium clone Lc2z_ML_058 (FJ355128.1)	98
	1a20_1D1_254_D10	4	JX657303	Uncultured actinobacterium clone w70 (HE654972.1)	100
	1a20_1D2_254_E10	4	JX657304	Uncultured bacterium clone ncd2310d03c1 (JF198212.1)	99
	1a20_1D3_254_F10	4	JX657305	*Limnohabitans* sp. Rim8 (HE600682.1)	100
	1a20_1D5_254_G10	4	JX657306	Uncultured *Mycobacterium* sp. clone AMPE9 (AM935142.1)	99
	1a20_1D8_254_H10	4	JX657307	Uncultured *Sphingobacterium* sp. clone w21 (HE654931.1)	100
	1a20_1E1_255_A11	5	JX657308	Uncultured alpha proteobacterium clone SW06-paRBL.05-46 (JF917200.1)	99
	1a20_1E4_255_B11	5	JX657309	Uncultured actinobacterium clone w60 (HE654966.1)	100
	1a20_1E5_255_C11	5	JX657310	Uncultured bacterium clone CRP99-73 (AF428727.1)	99
	1a20_1F1_257_D11	7	JX657311	Uncultured actinobacterium clone 30LAKE01B08 (HQ530574.1)	100
	1a20_1F4_257_E11	7	JX657312	Uncultured bacterium clone BST15-17 (HQ436607.1)	100
	1a20_1F8_257_F11	7	JX657313	Uncultured bacterium clone XX0076 (FJ820462.1)	99
	1a20_1G3_258_H11	8	JX657314	Uncultured *Rhodobacter* sp. clone rm15b3c02 (HM003636.1)	98
	1a20_1H6_259_F12	9	JX657315	Uncultured alpha proteobacterium clone DC5-50-1 (AY145582.1)	99
May 2012	1a26_1A2_251_C01	1	JX657316	Uncultured bacterium clone SINN828 (HM128813.1)	100
	1a26_1A4_251_D01	1	JX657317	Uncultured bacterium clone Lc2z_ML_055 (FJ355132.1)	96
	1a26_1A5_251_E01	1	JX657318	Uncultured bacterium clone Filt_WallC02 (GQ247163.1)	98
	1a26_1A8_251_F01	1	JX657319	Uncultured actinobacterium clone 30LAKE01E01 (HQ530592.1)	99
	1a26_1B4_252_H01	2	JX657320	Uncultured bacterium clone JC43 (JN868893.1)	99
	1a26_1B7_252_B02	2	JX657321	Uncultured bacterium clone JC43 (JN868893.1)	99
	1a26_1B8_252_C02	2	JX657322	Uncultured bacterium clone 060127_T6S1_W_T_SDP_144 (FJ350708.1)	99
	1a26_1C1_253_D02	3	JX657323	Uncultured *Rheinheimera* sp. clone 48PP (JF278072.1)	99
	1a26_1C2_253_E02	3	JX657324	Uncultured bacterium clone S0052 (FJ820417.1)	99
	1a26_1C3_253_F02	3	JX657325	Uncultured *Polynucleobacter* sp. clone YL205 (HM856566.1)	99
	1a26_1C4_253_G02	3	JX657326	Uncultured bacterium clone S0052 (FJ820417.1)	99

1a26_1C5_253_H02	3	JX657327	Uncultured Bacteroidetes bacterium clone ME002C1 (FJ827913.1)	100
1a26_1C6_253_A03	3	JX657328	Uncultured bacterium clone S0052 (FJ820417.1)	99
1a26_1D3_254_C03	4	JX657329	Uncultured bacterium clone SINN1113 (HM128496.1)	99
1a26_1D5_254_D03	4	JX657330	Uncultured *Rhodobacter* sp. clone rm15b3c02 (HM003636.1)	100
1a26_1D8_254_E03	4	JX657331	Uncultured bacterium clone SINN1113 (HM128496.1)	99

[a] Sequences aligned to their closest matches in GenBank with BLAST

Appendix B

Bacterial isolates recovered from irrigation water in Chapter 5

Table B.1 Bacterial isolates recovered from irrigation water sample at control (25 ℃), 42 and 48 ℃ via culture-dependent strategy in Chapter 5.

Sampling date	Treatment (℃)	Sequence #	Colony #	Colony type	SSCP banding pattern	Accession #	Closest match (Accession #)[a]	Similarity (%)
Feb 2012	25	1a22_251A1_P1WS_C01	251A1	WS	P1	JX870915	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
		1a22_251A2_P2WS_C04	251A2	WS	P2	JX870916	*Pelomonas* sp. UKW24 (JQ687102.1)	100
		1a22_251A5_P5WS_E06	251A5	WS	P5	JX870917	*Xenophilus aerolatus* strain NML100527 (JN585330.1)	99
		1a22_251A6_P1WS_D01	251A6	WS	P1	JX870918	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
		1a22_251A8_P6WS_A07	251A8	WS	P6	JX870919	*Acidovorax facilis* strain hswX127 (JQ236816.1)	100
		1a22_251B3_P2WS_D04	251B3	WS	P2	JX870920	*Pelomonas* sp. UKW24 (JQ687102.1)	100
		1a22_251B4_P1WS_E01	251B4	WS	P1	JX870921	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
		1a22_251C4_P8WM_E08	251C4	WM	P8	JX870922	*Rheinheimera texasensis* strain JN-9 (JF496548.1)	100
		1a22_251C7_P10WM_F02	251C7	WM	P10	JX870923	*Pseudomonas* sp. HM05 (JQ396178.1)	99
		1a22_251E1_P9WB_A09	251E1	WB	P9	JX870924	*Bacillus cereus* strain CT25 (EU111736.1)	100
		1a22_251E4_P9WD_B09	251E4	WD	P9	JX870925	*Bacillus pumilus* strain TAD088 (FJ225236.1)	100
		1a22_251E7_P3LY_H04	251E7	Y	P3	JX870926	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	100
		1a22_251E8_P9LY_C09	251E8	Y	P9	JX870927	*Bacillus* sp. DHC04 (JQ904715.1)	100

1a22_251F1_P3Y_A05	251F1	Y	P3	JX870928	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	100
1a22_251F2_P8Y_F08	251F2	Y	P8	JX870929	*Rheinheimera texasensis* strain JN-9 (JF496548.1)	100
1a22_251F3_P8Y_G08	251F3	Y	P8	JX870930	*Rheinheimera texasensis* strain JN-9 (JF496548.1)	99
1a22_252A6_P1WS_G01	252A6	WS	P1	JX870931	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
1a22_252A8_P7WS_G07	252A8	WS	P7	JX870932	*Pseudomonas* sp. FI 1029 (JQ691536.1)	100
1a22_252B7_P6WS_C07	252B7	WS	P6	JX870933	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
1a22_252C3_P4WM_G05	252C3	WM	P4	JX870934	*Pseudomonas* sp. HM05 (JQ396178.1)	99
1a22_252D3_P4WM_H05	252D3	WM	P4	JX870935	*Pseudomonas* sp. HM05 (JQ396178.1)	99
1a22_252D4_P5WM_G06	252D4	WM	P5	JX870936	*Xenophilus aerolatus* strain NML100527 (JN585330.1)	99
1a22_252E1_P9WB_D09	252E1	WB	P9	JX870937	*Bacillus pumilus* strain TAD088 (FJ225236.1)	100
1a22_252E4_P9WD_E09	252E4	WD	P9	JX870938	*Bacillus pumilus* strain TAD088 (FJ225236.1)	100
1a22_252E6_P3LY_B05	252E6	Y	P3	JX870939	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	100
1a22_253A2_P11WS_G03	253A2	WS	P11	JX870940	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	99
1a22_253A4_P7WS_B08	253A4	WS	P7	JX870941	*Pseudomonas* sp. FI 1029 (JQ691536.1)	100
1a22_253A7_P5WS_H06	253A7	WS	P5	JX870942	*Xenophilus aerolatus* strain NML100527 (JN585330.1)	99
1a22_253A8_P1WS_C02	253A8	WS	P1	JX870943	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
1a22_253B1_P1WS_D02	253B1	WS	P1	JX870944	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
1a22_253B4_P10WS_F03	253B4	WS	P10	JX870945	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	99
1a22_253B5_P6WS_D07	253B5	WS	P6	JX870946	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
1a22_253C4_P4WM_A06	253C4	WM	P4	JX870947	*Pseudomonas* sp. HM05 (JQ396178.1)	99
1a22_253C5_P8WM_H08	253C5	WM	P8	JX870948	*Rheinheimera texasensis* strain JN-9 (JF496548.1)	100
1a22_253D1_P4WM_B06	253D1	WM	P4	JX870949	*Pseudomonas* sp. HM05 (JQ396178.1)	99
1a22_253D5_P7WM_C08	253D5	WM	P7	JX870950	*Pseudomonas* sp. FI 1029 (JQ691536.1)	100

1a22_253D8_P9WB_F09	253D8	WB	P9	JX870951	*Bacillus cereus* strain CT25 (EU111736.1)	100
1a22_253E3_P9WD_G09	253E3	WD	P9	JX870952	*Bacillus* sp. DHC04 (JQ904715.1)	100
1a22_253E4_P3LY_C05	253E4	WM	P3	JX870953	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	100
1a22_253E5_P9LY_H09	253E5	Y	P9	JX870954	*Bacillus* sp. DHC04 (JQ904715.1)	100
1a22_253E8_P4Y_C06	253E8	Y	P4	JX870955	*Rheinheimera texasensis* strain JN-9 (JF496548.1)	99
1a22_253F1_P4Y_D06	253F1	Y	P4	JX870956	*Rheinheimera texasensis* strain JN-9 (JF496548.1)	99
1a22_253F4_P12C_H03	253F4	C	P12	JX870957	*Pseudomonas peli* strain IARI-L-117 (JN411458.1)	100
1a22_253F5_P12C_A04	253F5	C	P12	JX870958	*Pseudomonas peli* strain IARI-L-117 (JN411458.1)	100
1a22_253F6_P12C_B04	253F6	C	P12	JX870959	*Pseudomonas peli* strain IARI-L-117 (JN411458.1)	100
1a22_253F7_P7C_D08	253F7	C	P7	JX870960	*Pseudomonas* sp. FI 1029 (JQ691536.1)	100
1a22_253F8_P1P_E02	253F8	P	P1	JX870961	*Bacillus vietnamensis* strain KL2-3 (AB697709.1)	100
1a22_421A8_P13WS_E10	421A8	WS	P13	JX870962	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
1a22_421B3_P10WM_A10	421B3	WM	P10	JX870963	*Bacillus* cereus strain CT25 (EU111736.1)	100
1a22_421B5_P13C_F10	421B5	C	P13	JX870964	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
1a22_421B6_P13C_G10	421B6	C	P13	JX870965	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
1a22_421C6_P13O_H10	421C6	O	P13	JX870966	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
1a22_421C7_P13O_A11	421C7	O	P13	JX870967	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
1a22_421E1_P13O_B11	421E1	O	P13	JX870968	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
1a22_422B7_P13C_C11	422B7	C	P13	JX870969	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
1a22_422C3_P13C_D11	422C3	C	P13	JX870970	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
1a22_422C7_P13C_E11	422C7	C	P13	JX870971	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
1a22_422D2_P14O_F12	422D2	O	P14	JX870972	*Exiguobacterium profundum* strain CIBA	100

						BW17 (JQ073758.1)	
	1a22_422D3_P13O_F11	422D3	O	P13	JX870973	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
	1a22_422D7_P14O_G12	422D7	O	P14	JX870974	*Exiguobacterium profundum* strain CIBA BW17 (JQ073758.1)	100
	1a22_422E4_P13O_G11	422E4	O	P13	JX870975	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
	1a22_422F1_P13O_H11	422F1	O	P13	JX870976	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
	1a22_423C7_P13C_A12	423C7	C	P13	JX870977	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
	1a22_423F7_P14O_H12	423F7	O	P14	JX870978	*Exiguobacterium profundum* strain CIBA BW17 (JQ073758.1)	100
42	1a23_421A1_P9WS_A03	421A1	WS	P9	JX870979	*Bacillus pumilus* strain TAD088 (FJ225236.1)	100
	1a23_421A3_P7WS_H01	421A3	WS	P7	JX870980	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
	1a23_421A7_P9WS_B03	421A7	WS	P9	JX870981	*Bacillus pumilus* strain TAD088 (FJ225236.1)	100
	1a23_421C5_P7O_B02	421C5	O	P7	JX870982	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
	1a23_421F6_P9O_E03	421F6	O	P9	JX870983	*Exiguobacterium profundum* strain CIBA BW17 (JQ073758.1)	100
	1a23_422A4_P9WS_F03	422A4	WS	P9	JX870984	*Bacillus pumilus* strain TAD088 (FJ225236.1)	100
	1a23_422A7_P17WS_F01	422A7	WS	P17	JX870985	*Bacillus pumilus* strain JR5-4 (JQ229695.1)	100
	1a23_422A8_P9WS_G03	422A8	WS	P9	JX870986	*Bacillus cereus* strain CT25 (EU111736.1)	100
	1a23_422B1_P9WM_H03	422B1	WM	P9	JX870987	*Bacillus cereus* strain CT25 (EU111736.1)	100
	1a23_422B5_P9WM_A04	422B5	WM	P9	JX870988	*Bacillus cereus* strain CT25 (EU111736.1)	100
	1a23_422C8_P7C_C02	422C8	C	P7	JX870989	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
	1a23_422E3_P15O_D01	422E3	O	P15	JX870990	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	97
	1a23_423A2_P9WS_B04	423A2	WS	P9	JX870991	*Bacillus* sp. PZ_27 (JQ808526.1)	100
	1a23_423A8_P9WS_C04	423A8	WS	P9	JX870992	*Bacillus pumilus* strain SRS83 (JQ965500.1)	100

	1a23_423B1_P7WS_D02	423B1	WS	P7	JX870993	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
	1a23_423B6_P9WS_D04	423B6	WS	P9	JX870994	*Bacillus pumilus* strain SRS83 (JQ965500.1)	100
	1a23_423B8_P7WS_E02	423B8	WS	P7	JX870995	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
	1a23_423C2_P9WM_E04	423C2	WM	P9	JX870996	*Bacillus cereus* strain CT25 (EU111736.1)	100
	1a23_423C3_P9WM_F04	423C3	WM	P9	JX870997	*Bacillus cereus* strain CT25 (EU111736.1)	100
	1a23_423C6_P7C_F02	423C6	C	P7	JX870998	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
	1a23_423E1_P7O_G02	423E1	O	P7	JX870999	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
	1a23_423E5_P4O_G01	423E5	O	P4	JX871000	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	99
	1a23_423F8_P7O_H02	423F8	O	P7	JX871001	*Pseudomonas* sp. NBRC 101713 (AB681538.1)	100
48	1a23_481A5_P9WM_A09	481A5	WM	P9	JX871002	*Bacillus* sp. PZ_27 (JQ808526.1)	100
	1a23_481A8_P16WM_F05	481A8	WM	P16	JX871003	*Pseudomonas* sp. R3S4-1 (JQ928687.1)	100
	1a23_481B4_P16WM_G05	481B4	WM	P16	JX871004	*Pseudomonas* sp. R3S4-1 (JQ928687.1)	100
	1a23_481C6_P9WB_B09	481C6	WB	P9	JX871005	*Bacillus cereus* strain CT25 (EU111736.1)	100
	1a23_481D1_P3WD_B07	481D1	WD	P3	JX871006	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	99
	1a23_481D2_P3WD_C07	481D2	WD	P3	JX871007	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	99
	1a23_481D3_P9WD_C09	481D3	WD	P9	JX871008	*Paenibacillus* sp. 140SI (JF825471.1)	100
	1a23_481D5_P4WD_F08	481D5	WD	P4	JX871009	*Bacillus* sp. S-bf-PMW-5.1.1 (FR774585.1)	97
	1a23_481D8_P3WD_D07	481D8	WD	P3	JX871010	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	99
	1a23_481E1_P9LY_D09	481E1	Y	P9	JX871011	*Bacillus* sp. S-bf-PMW-5.1.1 (FR774585.1)	99
	1a23_481E2_P3LY_E07	481E2	Y	P3	JX871012	*Bacillus* sp. PZ_35 (JQ808528.1)	100
	1a23_481E3_P9LY_E09	481E3	Y	P9	JX871013	*Bacillus* sp. S-bf-PMW-5.1.1 (FR774585.1)	99
	1a23_481F5_P3S_H07	481F5	T	P3	JX871014	*Bacillus cereus* strain CT25 (EU111736.1)	100

1a23_481F8_P3S_A08	481F8	T	P3	JX871015	*Bacillus cereus* strain CT25 (EU111736.1)	100
1a23_482A3_P9WS_F09	482A3	WS	P9	JX871016	*Bacillus* sp. S-bf-PMW-5.1.1 (FR774585.1)	99
1a23_482A4_P9WS_G09	482A4	WS	P9	JX871017	*Bacillus* sp. S-bf-PMW-5.1.1 (FR774585.1)	99
1a23_482B5_P16WM_E06	482B5	WM	P16	JX871018	*Pseudomonas* sp. R3S4-1 (JQ928687.1)	99
1a23_482B6_P9WM_A10	482B6	WM	P9	JX871019	*Paenibacillus* sp. 140SI (JF825471.1)	100
1a23_482B7_P16WM_F06	482B7	WM	P16	JX871020	*Pseudomonas* sp. R3S4-1 (JQ928687.1)	100
1a23_482C1_P16WM_H06	482C1	WM	P16	JX871021	*Pseudomonas* sp. R3S4-1 (JQ928687.1)	100
1a23_482C4_P10WM_H04	482C4	WM	P10	JX871022	*Bacillus* sp. PZ_27 (JQ808526.1)	100
1a23_482C5_P14WB_A05	482C5	WB	P14	JX871023	*Bacillus cereus* strain CT25 (EU111736.1)	100
1a23_482C6_P9WB_B10	482C6	WB	P9	JX871024	*Bacillus cereus* strain CT25 (EU111736.1)	100
1a23_482C7_P9WB_C10	482C7	WB	P9	JX871025	*Bacillus cereus* strain CT25 (EU111736.1)	100
1a23_482E1_P9WD_D10	482E1	WD	P9	JX871026	*Bacillus* sp. PZ_27 (JQ808526.1)	100
1a23_482E2_P9WD_E10	482E2	WD	P9	JX871027	*Bacillus pumilus* strain SRS83 (JQ965500.1)	100
1a23_482E4_P14LY_B05	482E4	Y	P14	JX871028	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	99
1a23_482E5_P3LY_B08	482E5	Y	P3	JX871029	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	99
1a23_482F3_P14LY_C05	482F3	Y	P14	JX871030	*Bacillus marisflavi* strain BVC64 (JQ407799.1)	99
1a23_482F4_P9LY_G10	482F4	Y	P9	JX871031	*Bacillus cibi* strain IARI-S-22 (JN411495.1)	100
1a23_482F7_P9S_A11	482F7	T	P9	JX871032	*Bacillus* sp. S-bf-PMW-5.1.1 (FR774585.1)	99
1a23_482F8_P9S_B11	482F8	T	P9	JX871033	*Bacillus* sp. S-bf-PMW-5.1.1 (FR774585.1)	99
1a23_483A2_P9WS_C11	483A2	WS	P9	JX871034	*Bacillus cereus* strain CT25 (EU111736.1)	100
1a23_483A4_P17WM_A07	483A4	WM	P17	JX871035	*Paenibacillus terrigena* strain B30 (JN252080.1)	100
1a23_483A6_P14WM_D05	483A6	WM	P14	JX871036	*Paenibacillus* sp. BL16-1 (EU912455.1)	100
1a23_483B5_P9WM_D11	483B5	WM	P9	JX871037	*Bacillus cereus* strain CT25 (EU111736.1)	100

		1a23_483B6_P9WM_E11	483B6	WM	P9	JX871038	*Bacillus cereus* strain CT25 (EU111736.1)	100
		1a23_483B7_P9WB_F11	483B7	WB	P9	JX871039	*Bacillus cereus* strain CT25 (EU111736.1)	100
		1a23_483B8_P9WB_G11	483B8	WB	P9	JX871040	*Bacillus cereus* strain CT25 (EU111736.1)	100
		1a23_483C1_P9WB_H11	483C1	WB	P9	JX871041	*Bacillus cereus* strain CT25 (EU111736.1)	100
		1a23_483D6_P9WD_A12	483D6	WD	P9	JX871042	*Bacillus pumilus* strain TAD088 (FJ225236.1)	100
		1a23_483D7_P9WD_B12	483D7	WD	P9	JX871043	*Bacillus pumilus* strain TAD088 (FJ225236.1)	100
		1a23_483D8_P9WD_C12	483D8	WD	P9	JX871044	*Bacillus pumilus* strain TAD088 (FJ225236.1)	100
		1a23_483E6_P9LY_D12	483E6	Y	P9	JX871045	*Bacillus* sp. cmc28 (JQ917994.1)	100
		1a23_483E7_P9LY_E12	483E7	Y	P9	JX871046	*Bacillus* sp. cmc28 (JQ917994.1)	100
		1a23_483F1_P9LY_F12	483F1	Y	P9	JX871047	*Bacillus* sp. cmc28 (JQ917994.1)	100
		1a23_483F7_P9S_G12	483F7	T	P9	JX871048	*Bacillus cereus* strain CT25 (EU111736.1)	100
		1a23_483F8_P9S_H12	483F8	T	P9	JX871049	*Bacillus cereus* strain CT25 (EU111736.1)	100
May 2012	25	1a24_1_251B4_P9WS_A10	251B4	WS	P9	JX628698	*Candidatus Azospirillum massiliensis* strain URAM1 (EF394925.1)	99
		1a24_251A2_P1WS_C01	251A2	WS	P1	JX628692	*Microbacterium* sp. I_29-J6NFA10A (JQ917793.1)	100
		1a24_251A3_P2WS_F06	251A3	WS	P2	JX628693	*Chitinibacter* sp. SK16 (JN981166.1)	100
		1a24_251A5_P3WS_G07	251A5	WS	P3	JX628694	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
		1a24_251A8_P6WS_H08	251A8	WS	P6	JX628695	*Microbacterium* sp. FI 1010 (JQ691553.1)	100
		1a24_251B1_P7WS_C09	251B1	WS	P7	JX628696	*Pseudomonas* sp. MLB28 (JQ765424.1)	100
		1a24_251B3_P8WS_G09	251B3	WS	P8	JX628697	*Candidatus Azospirillum massiliensis* strain URAM1 (EF394925.1)	99
		1a24_251B6_P11WM_F02	251B6	WM	P11	JX628699	*Pseudomonas* sp. NCCP-279 (AB641888.1)	100
		1a24_251C3_P11WM_G02	251C3	WM	P11	JX628700	*Pseudomonas* sp. NCCP-279 (AB641888.1)	100
		1a24_251C4_P10WM_H01	251C4	WM	P10	JX628701	*Pseudomonas* sp. HM05 (JQ396178.1)	99
		1a24_251C6_P10WM_A02	251C6	WM	P10	JX628702	*Pseudomonas* sp. HM05 (JQ396178.1)	99

1a24_251C7_P12WB_C03	251C7	WB	P12	JX628703	*Bacillus* sp. PZ_7 (JQ808525.1)	99
1a24_251D2_P14WB_G04	251D2	WB	P14	JX628704	*Rheinheimera soli* strain B29 (JN377675.1)	100
1a24_251D4_P15WB_D05	251D4	WB	P15	JX628705	*Enterobacter* sp. X40 (HE662680.1)	100
1a24_251E4_P13WD_H03	251E4	WD	P13	JX628706	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a24_251E8_P6YB_A09	251E8	YB	P6	JX628707	*Bacillus marisflavi* strain p53_B09 (JQ834634.1)	100
1a24_251F5_P16C_A06	251F5	C	P16	JX628708	*Bacillus altitudinis* strain SGb146 (HQ224625.1)	100
1a24_251F7_P16T_C06	251F7	T	P16	JX628709	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
1a24_251F8_P16T_D06	251F8	T	P16	JX628710	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
1a24_252G1_P3WS_H07	252G1	WS	P3	JX628711	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
1a24_252G2_P4WS_D08	252G2	WS	P4	JX628712	*Hydrogenophaga* sp. BAC55P (EU130964.1)	100
1a24_252G3_P5WS_G08	252G3	WS	P5	JX628713	*Paenibacillus* sp. XP8 (GU080335.1)	100
1a24_252G5_P8WS_H09	252G5	WS	P8	JX628714	*Rheinheimera* sp. E49 (JQ922424.1)	100
1a24_252G8_P3WS_A08	252G8	WS	P3	JX628715	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
1a24_252H1_P6WS_B09	252H1	WS	P6	JX628716	*Microbacterium* sp. E56 (JF501120.1)	100
1a24_252H4__P12WS_D03	252H4	WS	P12	JX628717	*Paenibacillus* sp. FSL_H3-469 (EF203107.1)	100
1a24_252H6_P17WM_E06	252H6	WM	P17	JX628718	*Pelomonas* sp. R7W-1-1 (JQ928692.1)	100
1a24_252H7_P1WM_D01	252H7	WM	P1	JX628719	*Acidovorax* sp. DPS2 (HQ704415.1)	100
1a24_252I1_P15WM_F05	252I1	WM	P15	JX628720	*Mitsuaria* sp. Vi30 (JF501194.1)	100
1a24_252I2_P10WM_B02	252I2	WM	P10	JX628721	*Azospirillum* sp. DJM2D4 (JF753513.1)	100
1a24_252I5_P12WM_E03	252I5	WM	P12	JX628722	*Bacillus* sp. PZ_7 (JQ808525.1)	99
1a24_252I6_P2WM_G06	252I6	WM	P2	JX628723	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a24_252I7_P14WB_H04	252I7	WB	P14	JX628724	*Rheinheimera* sp. CF12-15 (FJ170029.1)	100
1a24_252J2_P13WB_B04	252J2	WB	P13	JX628725	*Bacillus* sp. PZ_35 (JQ808528.1)	100
1a24_252J3_P12WB_F03	252J3	WB	P12	JX628726	*Bacillus* sp. PZ_7 (JQ808525.1)	100
1A24_252K2_P2WD_H06	252K2	WD	P2	JX628727	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a24_252K4_P2YS_A07	252K4	YS	P2	JX628728	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a24_252K5_P13YB_C04	252K5	YB	P13	JX628729	*Bacillus marisflavi* strain p53_B09 (JQ834634.1)	100
1a24_252L7_P13T_D04	252L7	T	P13	JX628730	*Bacillus* sp. cmc28 (JQ917994.1)	100

	1a24_253A1_P15WS_G05	253A1	WS	P15	JX628731	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
	1a24_253A3_P3WS_B08	253A3	WS	P3	JX628732	*Acidovorax delafieldii* strain A3RO7 (JQ689177.1)	100
	1a24_253A4__P12WS_G03	253A4	WS	P12	JX628733	*Mesorhizobium* sp. RITF 741 (JQ697665.1)	99
	1a24_253A5_P14WS_B05	253A5	WS	P14	JX628734	*Pseudomonas* sp. NCCP-279 (AB641888.1)	100
	1a24_253A7_P1WS_E01	253A7	WS	P1	JX628735	*Acidovorax* sp. NF1078 (JQ782387.2)	100
	1a24_253B3_P4WS_E08	253B3	WS	P4	JX628736	*Hydrogenophaga* sp. WLSH-50 (JF682017.1)	100
	1a24_253B5_P10WM_D02	253B5	WM	P10	JX628737	*Mitsuaria* sp. Vi30 (JF501194.1)	99
	1a24_253C1_P11WM_H02	253C1	WM	P11	JX628738	*Pseudomonas* sp. HM05 (JQ396178.1)	99
	1a24_253C4_P15WM_H05	253C4	WM	P15	JX628739	*Mitsuaria* sp. Vi30 (JF501194.1)	100
	1a24_253C5_P11WM_A03	253C5	WM	P11	JX628740	*Pseudomonas* sp. HM05 (JQ396178.1)	99
	1a24_253D4_P7WB_D09	253D4	WB	P7	JX628741	*Aeromonas* sp. NM-21 (JQ894500.1)	100
	1a24_253D7_P10WB_E02	253D7	WB	P10	JX628742	*Enterobacter* sp. X40 (HE662680.1)	100
	1a24_253E4_P13WD_E04	253E4	WD	P13	JX628743	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_253E5_P1WD_F01	253E5	WD	P1	JX628744	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_253E8_P14YS_C05	253E8	YS	P14	JX628745	Gamma proteobacterium F8 (AY077611.1)	100
	1a24_253F1_P13YB_F04	253F1	YB	P13	JX628746	*Bacillus marisflavi* strain p53_B09 (JQ834634.1)	100
	1a24_253F4_P2YB_D07	253F4	YB	P2	JX628747	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
	1a24_253F5_P2T_E07	253F5	T	P2	JX628748	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
	1a24_253F6_P2T_F07	253F6	T	P2	JX628749	*Bacillus mycoides* strain TN12 (JQ415984.1)	100
42	1a24_421G6_P2WS_H12	421G6	WS	P2	JX871050	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_421I4_P18WM_C11	421I4	WM	P18	JX871051	*Vogesella* sp. TPS6 (FJ821602.1)	100
	1a24_421L8_P19C_A12	421L8	C	P19	JX871052	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	100
	1a24_422A2_P19WS_B12	422A2	WS	P19	JX871053	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	100
	1a24_422E3_P19C_D12	422E3	C	P19	JX871054	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	100
	1a24_422E4_P11C_B10	422E4	C	P11	JX871055	*Pseudomonas* sp. K7 (FR874238.1)	100

	1a24_422F7_P13T_F10	422F7	T	P13	JX871056	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a24_423G3_P19WS_E12	423G3	WS	P19	JX871057	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	100
	1a24_423H6_P19WM_F12	423H6	WM	P19	JX871058	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	100
	1a24_423I3_P15WM_H10	423I3	WM	P15	JX871059	*Vogesella* sp. TPS6 (FJ821602.1)	100
	1a24_423I4_P15WM_A11	423I4	WM	P15	JX871060	*Vogesella* sp. TPS6 (FJ821602.1)	100
	1a24_423K8_P19YS_G12	423K8	YS	P19	JX871061	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	100
	1a25_421H1_P7WM_B04	421H1	WM	P7	JX871062	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_421H7_P7WM_C04	421H7	WM	P7	JX871063	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_421K7_P20YS_D02	421K7	YS	P20	JX871064	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_421L4_P20YS_E02	421L4	YS	P20	JX871065	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_421L6_P20C_F02	421L6	C	P20	JX871066	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_421L7_P21C_A03	421L7	C	P21	JX871067	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	100
	1a25_422A1_P20WS_G02	422A1	WS	P20	JX871068	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_422A6_P20WS_H02	422A6	WS	P20	JX871069	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_422A8_P2WS_E01	422A8	WS	P2	JX871070	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a25_422D2_P2WD_G01	422D2	WD	P2	JX871071	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a25_422D7_P2YS_H01	422D7	YS	P2	JX871072	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a25_422E5_P2C_A02	422E5	C	P2	JX871073	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a25_422F3_P23C_D03	422F3	C	P23	JX871074	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_422F5_P7C_D04	422F5	C	P7	JX871075	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_423G5_P7WM_E04	423G5	WM	P7	JX871076	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_423G7_P22WM_C03	422D5	YS	P22	JX87107	*Pseudomonas* sp. DCA-9 (FJ493141.1)	100
	1a25_423G8_P7WM_F04	423G8	WM	P7	JX871078	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_423H4_P4WM_A04	423H4	WM	P4	JX871079	*Brevibacillus parabrevis* strain IFO 12334 (NR_040981.1)	100
	1a25_423H8_P7WM_G04	423H8	WM	P7	JX871080	*Pseudomonas* sp. J61 (JQ670671.1)	99
	1a25_423I1_P7WM_H04	423I1	WM	P7	JX871081	*Pseudomonas* sp. J61 (JQ670671.1)	100
	1a25_423J3_P24WD_E03	423J3	WD	P24	JX871082	*Bacillus* sp. cmc28 (JQ917994.1)	100
	1a25_423J6_P2WD_C02	423J6	WD	P2	JX871083	*Bacillus* sp. cmc28 (JQ917994.1)	100
48	1a25_481A2_P24WS_E09	481A2	WS	P24	JX871084	*Paenibacillus* sp. XP8 (GU080335.1)	100
	1a25_481A8_P13WS_A06	481A8	WS	P13	JX871085	*Cohnella* sp. CC-Alfalfa-35 (JN806384.1)	99
	1a25_481B2_P24WS_G09	481B2	WS	P25	JX871086	*Paenibacillus* sp. DHC15 (JQ904726.1)	100
	1a25_481B4_P26WS_E10	481B4	WS	P26	JX871087	*Bacillus ginsengihumi* strain ANA15	100

					(HQ219846.1)	
1a25_481B6_P6WM_E12	481B6	WM	P6	JX871088	*Paenibacillus* sp. MN2-8 (JQ396530.1)	99
1a25_481B7_P5WM_B12	481B7	WM	P5	JX871089	*Cohnella* sp. CRCB10 (JN617230.1)	99
1a25_481C1_P24WM_F09	481C1	WM	P24	JX871090	*Paenibacillus thailandensis* strain S3-4A (NR_041490.1)	98
1a25_481C3_P10WB_B05	481C3	WB	P10	JX871091	*Bacillus* sp. PZ_7 (JQ808525.1)	100
1a25_481C4_P13WB_C06	481C4	WB	P13	JX871092	*Paenibacillus* sp. SaMR6 (JQ806430.1)	100
1a25_481C6_P12WB_C05	481C6	WB	P12	JX871093	*Bacillus* sp. PZ_7 (JQ808525.1)	100
1a25_481C7_P2WD_E08	481C7	WD	P2	JX871094	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a25_481C8_P2WD_F08	481C8	WD	P2	JX871095	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a25_481D8_P13WD_D06	481D8	WD	P13	JX871096	*Bacillus* sp. Pt1 (JF900600.1)	100
1a25_481E1_P26YB_F10	481E1	YB	P26	JX871097	*Bacillus marisflavi* strain p53_B09 (JQ834634.1)	100
1a25_481E2_P13YB_E06	481E2	YB	P13	JX871098	*Bacillus koreensis* strain WIF49 (HM480320.1)	99
1a25_481E4_P26YB_G10	481E4	YB	P26	JX871099	*Bacillus marisflavi* strain p53_B09 (JQ834634.1)	100
1a25_481F2_P13C_F06	481F2	C	P13	JX871100	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a25_481F6_P25C_H09	481F6	C	P25	JX871102	*Paenibacillus* sp. Pt9 (JF900608.1)	99
1a25_482G2_P26WS_A11	482G2	WS	P26	JX871103	*Bacillus ginsengihumi* strain ANA15 (HQ219846.1)	100
1a25_482G7_P25WS_A10	482G7	WS	P25	JX871104	*Paenibacillus timonensis* strain 3584BRRJ (JF309265.1)	100
1a25_482H1_P6WS_H12	482H1	WS	P6	JX871105	*Paenibacillus* sp. Dv34 (HQ728085.1)	98
1a25_482H2_P13WS_G06	482H2	WS	P13	JX871106	*Paenibacillus* sp. Dv34 (HQ728085.1)	98
1a25_482H4_P2WS_G08	482H4	WS	P2	JX871107	*Cohnella* sp. CC-Alfalfa-35 (JN806384.1)	100
1a25_482H6_P1WS_A05	482H6	WS	P1	JX871108	*Paenibacillus* sp. YN14-07 (AM162323.1)	99
1a25_482H8_P12WM_E05	482H8	WM	P12	JX871109	*Paenibacillus* sp. JG-TB25 (FR849935.1)	100
1a25_482I1_P5WM_C12	482I1	WM	P5	JX871110	*Paenibacillus* sp. KMC003 (EU219972.1)	99
1a25_482I2_P26WM_B11	482I2	WM	P26	JX871111	*Paenibacillus* sp. KMC003 (EU219972.1)	99
1a25_482I5_P16WB_C08	482I5	WB	P16	JX871112	*Paenibacillus* sp. WYT011 (JQ807858.1)	100
1a25_482I6_P13WB_H06	482I6	WB	P13	JX871113	*Bacillus* sp. PZ_35 (JQ808528.1)	100
1a25_482J1_P12WB_F05	482J1	WB	P12	JX871114	*Bacillus* sp. PZ_7 (JQ808525.1)	100
1a25_482K2_P12WD_G05	482K2	WD	P12	JX871115	*Bacillus* sp. PZ_7 (JQ808525.1)	100
1a25_482K4_P2WD_H08	482K4	WD	P2	JX871116	*Bacillus pumilus* strain CE4 (JQ435701.1)	100
1a25_482L1_P26WS_C11	482L1	YB	P26	JX871117	*Bacillus marisflavi* strain p53_B09	100

					(JQ834634.1)	
1a25_483A1_P25WS_B10	483A1	WS	P25	JX871118	*Paenibacillus* sp. MN2-8 (JQ396530.1)	100
1a25_483A4_P2WS_A09	483A4	WS	P2	JX871119	*Brevibacillus* sp. SE12 (JQ714095.1)	100
1a25_483A5_P26WS_D11	483A5	WS	P26	JX871120	*Bacillus ginsengihumi* strain ANA15 (HQ219846.1)	100
1a25_483B1_P2WS_B09	483B1	WS	P2	JX871121	*Brevibacillus* sp. SE12 (JQ714095.1)	100
1a25_483B5_P27WS_E11	483B5	WS	P27	JX871122	*Brevibacillus* sp. SE12 (JQ714095.1)	100
1a25_483B6_P25WS_C10	483B6	WS	P25	JX871123	*Paenibacillus* sp. MN2-8 (JQ396530.1)	100
1a25_483B7_P27WS_F11	483B7	WS	P27	JX871124	*Brevibacillus* sp. SE12 (JQ714095.1)	100
1a25_483B8_P27WS_G11	483B8	WS	P27	JX871125	*Brevibacillus* sp. SE12 (JQ714095.1)	100
1a25_483C2_P27WM_H11	483C2	WM	P27	JX871126	*Brevibacillus* sp. SE12 (JQ714095.1)	100
1a25_483C3_P27WM_A12	483C3	WM	P27	JX871127	*Brevibacillus* sp. SE12 (JQ714095.1)	100
1a25_483C4_P16WM_D08	483C4	WM	P16	JX871128	*Paenibacillus* sp. WYT011 (JQ807858.1)	100
1a25_483C5_P25WM_D10	483C5	WM	P25	JX871129	*Paenibacillus illinoisensis* strain NBB11 (HQ256551.1)	100
1a25_483C6_P12WM_H05	483C6	WM	P12	JX871130	*Bacillus* sp. PZ_7 (JQ808525.1)	100
1a25_483D4_P13WB_E07	483D4	WB	P13	JX871131	*Bacillus* sp. PZ_35 (JQ808528.1)	100
1a25_483D6_P13WD_F07	483D6	WD	P13	JX871132	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a25_483D7_P13WD_G07	483D7	WD	P13	JX871133	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a25_483F1_P2YB_C09	483F1	YB	P2	JX871134	*Bacillus* sp. DHC04 (JQ904715.1)	100
1a25_483F4_P13YB_H07	483F4	YB	P13	JX871135	*Bacillus marisflavi* strain p53_B09 (JQ834634.1)	100
1a25_483F5_P2C_D09	483F5	C	P2	JX871136	*Bacillus* sp. cmc28 (JQ917994.1)	100
1a25_483F8_P13T_A08	483F8	T	P13	JX871137	*Bacillus aquimaris* strain K-W2 (JQ799058.1)	100

[a] Sequences aligned to their closest matches in GenBank with BLAST

Table B.2 Bacterial isolates recovered from irrigation water sample at control (25 ℃), 42 and 48 ℃ via culture-independent strategy (PCR-DGGE) in Chapter 5.

Sampling date	Treatment (℃)	Sequence #	DGGE band #	Accession #	Closest match (Accession #)[a]	Similarity (%)
Feb 2012	25	1a20_1A5_251_A09	1	JX657295	Uncultured actinobacterium clone KWK1S.54 (JN656761.1)	99
		1a20_1A7_251_C09	1	JX657296	Uncultured Bacteroidetes bacterium clone 30LAKE43F10 (HQ531074.1)	99
		1a20_1A8_251_D09	1	JX657297	Uncultured bacterium clone CRD99-65 (AF428643.1)	99
		1a20_1B1_252_E09	2	JX657298	*Polynucleobacter* sp. NBRC 101963 (AB681633.1)	99
		1a20_1B2_252_F09	2	JX657299	Uncultured *Herbaspirillum* sp. clone FL_52 (HQ008597.1)	99
		1a20_1B4_252_H09	2	JX657300	Uncultured actinobacterium clone KWK6F.62 (JN656787.1)	99
		1a20_1C1_253_B10	3	JX657301	Uncultured actinobacterium clone w98 (HE654991.1)	100
		1a20_1C8_253_C10	3	JX657302	Uncultured bacterium clone Lc2z_ML_058 (FJ355128.1)	98
		1a20_1D1_254_D10	4	JX657303	Uncultured actinobacterium clone w70 (HE654972.1)	100
		1a20_1D2_254_E10	4	JX657304	Uncultured bacterium clone ncd2310d03c1 (JF198212.1)	99
		1a20_1D3_254_F10	4	JX657305	*Limnohabitans* sp. Rim8 (HE600682.1)	100
		1a20_1D5_254_G10	4	JX657306	Uncultured *Mycobacterium* sp. clone AMPE9 (AM935142.1)	99
		1a20_1D8_254_H10	4	JX657307	Uncultured *Sphingobacterium* sp. clone w21 (HE654931.1)	100
		1a20_1E1_255_A11	5	JX657308	Uncultured alpha proteobacterium clone SW06-paRBL.05-46 (JF917200.1)	99
		1a20_1E4_255_B11	5	JX657309	Uncultured actinobacterium clone w60 (HE654966.1)	100
		1a20_1E5_255_C11	5	JX657310	Uncultured bacterium clone CRP99-73 (AF428727.1)	99
		1a20_1F1_257_D11	7	JX657311	Uncultured actinobacterium clone 30LAKE01B08 (HQ530574.1)	100
		1a20_1F4_257_E11	7	JX657312	Uncultured bacterium clone BST15-17 (HQ436607.1)	100
		1a20_1F8_257_F11	7	JX657313	Uncultured bacterium clone XX0076 (FJ820462.1)	99
		1a20_1G3_258_H11	8	JX657314	Uncultured *Rhodobacter* sp. clone rm15b3c02 (HM003636.1)	98
		1a20_1H6_259_F12	9	JX657315	Uncultured alpha proteobacterium clone DC5-50-1 (AY145582.1)	99
	42	1a21_2C1_421_E03	1	JX871138	Uncultured bacterium clone 261ds10 (AY212711.1)	99
		1a21_2C2_421_F03	1	JX871139	*Pseudomonas jessenii* strain E62 (JF683673.1)	99
		1a21_2C3_421_G03	1	JX871140	Uncultured bacterium clone CR98-5-65 (AF428795.1)	99
		1a21_2C6_421_A04	1	JX871141	Uncultured *Sphingobacteriales* bacterium clone W-LLR195 (JF515944.1)	99
		1a21_2D1_422_B04	2	JX871142	Uncultured *Sphingobacteriales* bacterium clone W-LLR195 (JF515944.1)	100

		1a21_2D3_422_D04	2	JX871143	Uncultured bacterium clone Lc2z_ML_201 (FJ355341.1)	99
		1a21_2E1_423_E04	3	JX871144	Uncultured bacterium clone reservoir-169 (JF697550.1)	99
		1a21_2E5_423_F04	3	JX871145	Uncultured bacterium clone nbw497f10c1 (GQ101655.1)	98
		1a21_2E7_423_G04	3	JX871146	*Microvirga flocculans* strain TFB (JF438969.1)	100
		1a21_2F2_424_H04	4	JX871147	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	100
		1a21_2F5_424_A05	4	JX871148	Uncultured bacterium clone R242 (HM069056.1)	100
		1a21_2G2_425_C05	5	JX871149	Uncultured bacterium clone C24.47 (GU559802.1)	99
		1a21_2G3_425_D05	5	JX871150	*Pseudomonas* sp. Cf0-5 (JN836275.1)	98
		1a21_2H3_426_G05	6	JX871151	Uncultured bacterium clone CR98-5-65 (AF428795.1)	100
		1a21_2I1_427_B06	7	JX871152	Uncultured bacterium clone ML-3-55 (DQ166460.1)	99
		1a21_2I3_427_D06	7	JX871153	*Hydrogenophaga* sp. WLSH-321 (JF682011.1)	99
		1a21_2I6_427_G06	7	JX871154	*Hydrogenophaga* sp. p3(2011) (HQ652595.1)	100
		1a21_2J1_428_A07	8	JX871155	Uncultured Alphaproteobacteria bacterium clone QEDV2BC02 (JQ696175.1)	99
		1a21_2J5_428_D07	8	JX871156	Uncultured Alphaproteobacterium clone DC5-50-1 (AY145582.1)	100
	48	1a21_3C2_481_D09	1	JX871157	Uncultured bacterium clone SINN828 (HM128813.1)	99
		1a21_3C7_481_F09	1	JX871158	Uncultured bacterium clone TSBAR002 (AB486314.1)	99
		1a21_3D8_482_H09	2	JX871159	Uncultured *Balneimonas* sp. clone UHAS4.3 (JN037960.1)	98
		1a21_3E1_483_A10	3	JX871160	*Chitinophaga* sp. BS27 (JF806525.1)	97
		1a21_3E5_483_B10	3	JX871161	Uncultured bacterium clone 2N1-3 (EU160054.1)	97
		1a21_3F5_484_E10	4	JX871162	Uncultured bacterium clone 5-12 (JQ923510.1)	98
		1a21_3F8_484_F10	4	JX871163	Uncultured *Balneimonas* sp. clone UHAS4.3 (JN037960.1)	98
		1a21_3G2_485_H10	5	JX871164	Uncultured bacterium clone MFC_19D7 (JF204132.1)	99
		1a21_3G8_485_C11	5	JX871165	Uncultured Alphaproteobacteria bacterium clone QEDV1CH08 (CU919292.1)	96
May 2012	25	1a26_1A2_251_C01	1	JX657316	Uncultured bacterium clone SINN828 (HM128813.1)	100
		1a26_1A4_251_D01	1	JX657317	Uncultured bacterium clone Lc2z_ML_055 (FJ355132.1)	96
		1a26_1A5_251_E01	1	JX657318	Uncultured bacterium clone Filt_WallC02 (GQ247163.1)	98
		1a26_1A8_251_F01	1	JX657319	Uncultured actinobacterium clone 30LAKE01E01 (HQ530592.1)	99
		1a26_1B4_252_H01	2	JX657320	Uncultured bacterium clone JC43 (JN868893.1)	99
		1a26_1B7_252_B02	2	JX657321	Uncultured bacterium clone JC43 (JN868893.1)	99
		1a26_1B8_252_C02	2	JX657322	Uncultured bacterium clone 060127_T6S1_W_T_SDP_144 (FJ350708.1)	99
		1a26_1C1_253_D02	3	JX657323	Uncultured *Rheinheimera* sp. clone 48PP (JF278072.1)	99
		1a26_1C2_253_E02	3	JX657324	Uncultured bacterium clone S0052 (FJ820417.1)	99
		1a26_1C3_253_F02	3	JX657325	Uncultured *Polynucleobacter* sp. clone YL205 (HM856566.1)	99

	1a26_1C4_253_G02	3	JX657326	Uncultured bacterium clone S0052 (FJ820417.1)	99
	1a26_1C5_253_H02	3	JX657327	Uncultured Bacteroidetes bacterium clone ME002C1 (FJ827913.1)	100
	1a26_1C6_253_A03	3	JX657328	Uncultured bacterium clone S0052 (FJ820417.1)	99
	1a26_1D3_254_C03	4	JX657329	Uncultured bacterium clone SINN1113 (HM128496.1)	99
	1a26_1D5_254_D03	4	JX657330	Uncultured *Rhodobacter* sp. clone rm15b3c02 (HM003636.1)	100
	1a26_1D8_254_E03	4	JX657331	Uncultured bacterium clone SINN1113 (HM128496.1)	99
42	1a26_1E1_422_F03	2	JX871167	Uncultured bacterium clone L7S16_ML_056 (FJ354286.1)	100
	1a26_1E2_422_G03	2	JX871168	Uncultured bacterium clone L7S16_ML_056 (FJ354286.1)	100
	1a26_1E4_422_H03	2	JX871169	Uncultured bacterium clone JN59 (JN869004.1)	99
	1a26_1E5_422_A04	2	JX871170	Uncultured Alphaproteobacteria bacterium QEDN8CG03 (CU927557.1)	97
	1a26_1F1_423_B04	3	JX871171	Uncultured bacterium clone nbw44h10c1 (GQ060166.1)	99
	1a26_1F2_423_C04	3	JX871172	Bacteroidetes bacterium TQQ6 (HE793031.1)	99
	1a26_1F7_423_D04	3	JX871173	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	99
	1a26_1F8_423_E04	3	JX871174	Uncultured bacterium clone YHW13 (GU305837.1)	99
	1a26_1G1_424_F04	4	JX871175	Uncultured bacterium clone MABRDTU30 (FJ529983.1)	99
	1a26_1G2_424_G04	4	JX871176	Uncultured bacterium clone nbw44h10c1 (GQ060166.1)	99
	1a26_1G3_424_H04	4	JX871177	Uncultured *Methylosinus* sp. clone Plot18-F12 (FJ889302.1)	96
	1a26_1G5_424_A05	4	JX871178	Uncultured *Fluviicola* sp. clone JXS1-92 (JN873202.1)	97
	1a26_1I2_426_G05	6	JX871179	*Pseudomonas aeruginosa* strain NTS6 (HE858286.1)	100
	1a26_1I3_426_H05	6	JX871180	Uncultured gamma proteobacterium clone 30LAKE02G04 (HQ530659.1)	99
	1a26_1I4_426_A06	6	JX871181	Uncultured Alphaproteobacteria bacterium QEDN8CG03 (CU927557.1)	97
	1a26_1I5_426_B06	6	JX871182	Uncultured *Pseudomonas* sp. clone GE7GXPU01AGHHE (HM973327.1)	96
	1a26_1I7_426_C06	6	JX871183	Uncultured bacterium clone YHW13 (GU305837.1)	99
	1a26_1I8_426_D06	6	JX871184	Uncultured *Pseudomonas* sp. clone F5OHPNU07H0UQZ (HQ053550.1)	96
	1a26_IJ4_427_F06	7	JX871185	Uncultured *Fluviicola* sp. clone JXS1-92 (JN873202.1)	97
	1a26_1K2_428_B07	8	JX871186	Uncultured bacterium clone JN59 (JN869004.1)	99
	1a26_1K3_428_C07	8	JX871187	Uncultured gamma proteobacterium clone 30LAKE02G02 (HQ530658.1)	99
	1a26_1K4_428_D07	8	JX871188	Uncultured bacterium clone 060127_T6S1_W_T_SDP_186 (FJ350717.1)	100
	1a26_1K5_428_E07	8	JX871189	Uncultured Alphaproteobacteria bacterium QEDN8CG03 (CU927557.1)	96

	1a26_1K7_428_F07	8	JX871190	Uncultured bacterium clone 6-77 (JQ923731.1)	99
	1a26_1K8_428_G07	8	JX871191	Uncultured bacterium clone 6-77 (JQ923731.1)	99
	1a26_2A1_4210_C08	10	JX871192	Uncultured bacterium clone JN59 (JN869004.1)	99
	1a26_2A4_4210_E08	10	JX871193	Uncultured Alphaproteobacteria bacterium QEDN8CG03 (CU927557.1)	99
	1a26_2A6_4210_G08	10	JX871194	Uncultured *Azospirillum* sp. clone GE7GXPU01AVTUJ (HQ044242.1)	99
	1a26_2B2_4211_B09	11	JX871195	Uncultured bacterium clone JN59 (JN869004.1)	99
	1a26_2B4_4211_C09	11	JX871196	Uncultured *Azospirillum* sp. clone GE7GXPU01AVTUJ (HQ044242.1)	96
	1a26_2B8_4211_D09	11	JX871197	Uncultured bacterium clone nbw44h10c1 (GQ060166.1)	99
	1a26_2C2_4212_E09	12	JX871198	Uncultured bacterium clone HK220 (FJ204047.1)	97
	1a26_2C3_4212_F09	12	JX871199	Uncultured bacterium clone nbw44h10c1 (GQ060166.1)	99
	1a26_2C8_4212_A10	12	JX871200	Uncultured bacterium clone nbw44h10c1 (GQ060166.1)	99
48	1a26_2E1_481_F10	1	JX871201	Uncultured Bacteroidetes bacterium clone JB1G04 (HQ532649.1)	99
	1a26_2F1_482_H10	2	JX871202	*Chitinophaga* sp. BS27 (JF806525.1)	97
	1a26_2F5_482_A11	2	JX871203	*Chitinophaga* sp. BS27 (JF806525.1)	97
	1a26_2G5_483_B11	3	JX871204	Uncultured *Sphingomonadales* bacterium clone SSF018 (JF733360.1)	97
	1a26_2G7_483_C11	3	JX871205	Uncultured bacterium clone 14-66 (JF697368.1)	100
	1a26_2G8_483_D11	3	JX871206	*Lysobacter* sp. YIM 77875 (JQ746036.1)	99
	1a26_2H2_484_E11	4	JX871207	Uncultured *Bacillus* sp. clone SGR28 (JQ793404.1)	98
	1a26_2H8_484_G11	4	JX871208	Uncultured bacterium clone SING1058 (HM128975.1)	99
	1a26_2I8_485_H11	5	JX871209	*Lysobacter* sp. YIM 77875 (JQ746036.1)	99
	1a26_2J3_486_A12	6	JX871210	Uncultured bacterium clone DP7.2.51 (FJ612260.1)	98
	1a26_2J4_486_B12	6	JX871211	Uncultured bacterium clone ncd2158f12c1 (JF185564.1)	98
	1a26_2J6_486_C12	6	JX871212	Uncultured bacterium clone DP7.2.51 (FJ612260.1)	98
	1a26_2J7_486_D12	6	JX871213	Uncultured bacterium clone Fec185 (FJ911492.1)	96
	1a26_2K3_487_F12	7	JX871214	Uncultured bacterium clone HK220 (FJ204047.1)	96
	1a26_2K4_487_G12	7	JX871215	Uncultured bacterium clone SING1058 (HM128975.1)	99
	1a26_2K6_487_H12	7	JX871216	Uncultured *Bacillus* sp. clone SGR28 (JQ793404.1)	97

[a] Sequences aligned to their closest matches in GenBank with BLAST