Effect of Treated Wastewater Irrigation on the Proliferation of Antibiotic Resistance in Agricultural Soils

Thesis

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By

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Abstract

In Israel with its semi-arid climate, water is a limited resource and yet, in the last two decades about 60% of all of Israel's water resources are directed for agricultural use. This is feasible due to the capacity to recycle water. Treated wastewater (TWW) has become an important source of irrigation water in Israel and other parts of the world due to the pressures of increasing population and the limited amount of high quality water. Today, approximately 50% of agricultural irrigation in Israel is carried out with TWW.

Irrigation with TWW has significant immediate benefits as it mitigates water shortages, and enables continuation of agricultural development and expansion, in response to the growing needs of the population. However, there are some disadvantages and potential risks associated with its long-term use that are not fully understood.

TWW may contain pathogenic microorganisms, effluent-borne anthropogenic chemicals such as pesticides, and a large group of compounds collectively referred to as Pharmaceuticals and Personal Care Products (PPCPs) as pollutants. A major concern is that compounds such as hormones and antibiotics can detrimentally affect the environment and public health due to biological interactions when released into soil and water environments through TWW-irrigation.

In this work, I evaluated the environmental impact of one group of TWW-associated PCPPs – antibiotics, by assessing levels of antibiotic resistance and antibiotic resistant genes (ARG's) of the soil bacteria, in TWW and TWW-irrigated soils compared to fresh water (FW) irrigated soils.

Indigenous soil-dwelling bacteria both produce and encounter a variety of antibiotics, resulting in evolution of diverse and novel resistance mechanisms. These bacteria may serve as reservoirs of resistance determinants that can be mobilized into the human-associated microbial community. On a molecular level, antibiotic resistance may evolve from either spontaneous mutation of genes, or by *inter*- or *intra*-species horizontal gene transfer (HGT) of existing antibiotic resistance genes.

Both of these mechanisms are believed to occur spontaneously at relatively low frequencies. However, exposure to antibiotics can result in the proliferation of these mechanisms due to natural selection.

Proliferation of resistant bacteria due to natural selection in areas with anthropogenic effects (such as manure-fertilized soils, rivers receiving treated wastewater effluent, etc) has been well documented. Nevertheless, there is no current research, which focuses on the direct effect of irrigation with TWW on the resistance of the soil bacteria to antibiotics.

In this study, I assessed the resistance level of four different antibiotics (that differ significantly in chemical composition, source and modes of action): tetracycline, erythromycin, sulfonamide and ciprofloxacin in four agricultural soils irrigated with fresh water and TWW. Traditional microbiological isolation methods and culture-independent molecular analysis using quantitative real time PCR targeting the antibiotic resistant genes: tet*O*, erm*B*, erm*F*, *sul*1 and *sul*2 were implemented.

The working hypothesis of this thesis was that TWW irrigation increases antibiotic resistance levels in irrigated soils due to selective pressure generated by residual antibiotic compounds and the presence of antibiotic resistance bacteria. We compared the levels of antibiotic resistant bacteria in soils irrigated with TWW to identical soils irrigated with freshwater. Overall, the main findings of this work did not support the hypothesis and revealed interesting points:

i. Based on conventional isolation methods and a cultivation–independent approach, irrigation with TWW does not cause an increase in the resistance level of the soil bacteria. This is despite of the high levels of AR bacteria and ARG's that are present in the TWW.
ii. Soil moisture induces bacterial resistance to tetracycline and ciprofloxacin, in both TWW and FW irrigated soils.
iii. The significant levels of antibiotic resistance detected in the agricultural soils analyzed in this study, are mainly attributed to native bacteria and not to wastewater-derived bacteria.

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List of abbreviations:

AR- Antibiotic resistance

ARG's- Antibiotic resistance genes

CFU- Colony forming unit

DGGE- Denaturing gradient gel-electrophoresis

FW- Fresh water

TWW- Treated wastewater

LB- Luria Broth

MDR- Multi drug resistance

PCR- Polymerase-chain reaction

PPCPs- Pharmaceuticals and Personal Care Products

WWTP- Wastewater treatment plant

1. Introduction

1.1 Treated wastewater

In Israel with its semi-arid climate, water is a limited resource, and yet in the last two decades about 60% of all of Israel's water resources are directed for agricultural use (Blank, 2000). This is feasible due to the capacity to recycle water. Treated wastewater (TWW) has become an important source of irrigation water in Israel and other parts of the world due to the pressures of increasing population and the limited amount of high quality water. Today, approximately 50% of agricultural irrigation in Israel is carried out with TWW (Navon et al., 2011).

Most wastewater is treated in industrial-scale wastewater treatment plants (WWTPs) which may include physical, chemical and biological (aerobic and anaerobic) treatment processes. The primary aerobic treatment used in large-scale wastewater treatment systems is the activated sludge process. This process is based on maintenance, and recirculation of complex biomass composed of micro-organisms able to absorb organic matter from the wastewater. Anaerobic processes are also widely applied in the treatment of industrial wastewaters and in additional purification of biological sludge from the activated sludge process in municipal wastewater treatment. According to the Israeli Ministry of Environmental Protection, the amount of sewage generated in Israel stands at 510 million cubic meters (MCM), of which approximately 480 MCM are recycled for irrigation. In recent years, new upgraded intensive WWTPs have been set up in municipalities throughout the country.

Irrigation with TWW has significant immediate benefits as it mitigates water shortages, and enables continuation of agricultural development and expansion, in response to the growing needs of the population. However, there are some disadvantages and potential risks associated with its long-term use that are not fully understood. These include:

A) Evidence indicating that TWW irrigation of arable lands can have detrimental impact on the environment (Wallach and Graber, 2007). This includes Israel's aquifer contamination by nitrates, heavy metals, and pesticide residues (Amiel A et al., 1990; Avisar et al., 2009; Muszkat et al., 1993).

B) Irrigation with TWW has sometimes been reported to adversely affect soil physical and hydraulic properties such as decreased hydraulic conductivity and infiltration rates, clay dispersion by organic acids and electrolytes, and development of soil water repellency (Graber et al., 2006; Wallach et al., 2005).

C) TWW may contain pathogenic microorganisms and effluent-borne anthropogenic chemicals such as pesticides, and a large group of compounds collectively referred to as Pharmaceuticals and Personal Care Products (PPCPs) as pollutants - discussed below. A major concern is that compounds such as hormones and antibiotics can detrimentally affect the environment and public health due to biological interactions when released into soil and water environments through TWW-irrigation. For example, hormones can affect the sex of aquatic organisms (Larsson et al., 2000) and accumulation of antibiotic compounds in soil may exert selective pressure on the bacterial populations, resulting in proliferation of antibiotic resistance in indigenous bacteria. In addition, there is the possible uptake of these anthropogenic compounds by plants and crops (Graber and Gerstl, 2011; Shenker et al., 2011).

1.2 Pharmaceuticals and Personal Care Products

PPCPs as pollutants, refers to any product used by individuals for personal health or cosmetic reasons, or used by agribusiness to enhance growth or health of livestock (Graber and Gerstl, 2011). PPCPs consist of thousands of chemical substances, including prescription and over-the-counter therapeutic drugs, veterinary drugs, fragrances, and cosmetics. PPCPs have been around for decades but only in the past few years, they have been recognized and named. Their effect on the environment is now an important area of research.

PPCPs have probably been present in wastewater and the environment since humans have been using them. The drugs taken by humans and animals are not entirely absorbed in the body, and are excreted and passed into wastewater and potentially to surface water. Recently, the appearance of more sensitive tests for monitoring environmental contamination has revealed the presence of these types of contaminants at nanograms per liter levels. With advances in research, and in analytical instrumentation, it is now possible to detect and

quantify these chemicals, and identify what effects, if any, these chemicals have on human's ecosystem and environmental health.

The environmental fate of PCPPs depends mainly on the physicochemical properties, such as water solubility, adsorption behavior, and volatility on their degradability, which is affected by microorganisms (biological degradation) present in sewage treatment plants, surface waters, and soils. These fate-relevant properties control the distribution of a chemical in the various environmental compartments (water, soil, air) and its final removal by degradation processes (Graber and Gerstl, 2011).

In this work, I evaluated the environmental impact of one group of TWW-associated PCPPs – antibiotics, by assessing levels of antibiotic resistance and antibiotic resistant genes (ARG's) in TWW and TWW-irrigated soils.

1.3 Antibiotic resistance

The discovery of antibiotics in the 1930's was one of the most important medical revolutions in the history of the world. Antibiotic compounds play a crucial role in controlling infectious diseases. Many antibiotics used in the clinical setting originate from bacteria and fungi that were isolated from natural environments, such that resistance to antibiotics among bacteria predates their modern clinical use (Ghosh and LaPara, 2007).

During the past decade, an increase in antimicrobial resistance has been recognized worldwide and increased frequencies of multidrug-resistant (MDR) bacteria in the clinical setting have been observed (Canton, 2009). The pervasive use of antibiotics for both therapeutic and non-therapeutic purposes has been blamed for the widespread occurrence of antibiotic resistant (AR) bacteria, because the use of antibiotics selects for resistant bacteria.

Although overuse and misuse of antibiotics in the community (hospitals, health care institutions, etc) is undoubtedly a major contributor to the spread of antibiotic resistance among bacteria in the community, it is becoming increasingly clear that certain anthropogenic activities, including: animal husbandry, agronomic practices and municipal wastewater

treatment plants (WWTPs), may also be key generators of AR propagation. This is due to the release of residual antibiotic compounds that can cause a selective pressure and proliferation of resistance bacteria, as well as the release of AR bacteria, and AR-associated genetic elements, into natural environments.

Agricultural use accounts for at least half of the antibiotics produced in the United States (Lipsitch et al., 2002). Antibiotics are used in agriculture for the treatment of sick animals, disease prophylaxis, growth promotion and crop dusting. Application of stabilized (composted) or non-stabilized animal waste to agricultural soils is a widespread process worldwide, due to the high nitrogen and phosphorus concentrations in the manure. Nonetheless, these wastes frequently contain residual concentrations of antibiotic compounds and high loads of resistant bacteria, which can cause proliferation of resistance among the microbial community in the soil. Today, there is large debate regarding the necessity of administering sub-therapeutic doses of antibiotics as animal growth promoting agents, given the threat this practice poses to human health.

Several reports showed the link between subtherapeutic antibiotic concentrations and AR resistance in the environment (Aarestrup et al., 2001; Ghosh and LaPara, 2007; Khachatourians, 1998; Liu et al., 2011). Gullberg et al., (2011) recently confirmed that selection of resistant bacteria can occur at extremely low antibiotic concentrations that are present in many natural environments. Such data led Denmark to ban subtherapeutic dosing of antibiotics in livestock.

One potential hot-spot for horizontal transfer of genetic material and proliferation of AR is WWTPs. WWTPs are connected to private households and hospitals where antibiotics are used and resistances in bacteria might arise. Once AR bacteria reach WWTPs, they can potentially disseminate resistance genes to members of the endogenous microbial community. Evidence for horizontal transfer of resistance elements in sewage habitats has been obtained for model systems (Geisenberger et al., 1999; Marcinek et al., 1998; Nüsslein et al., 1992). A recent study detected 123 resistance genes obtained from total plasmid DNA originating from bacteria of the final-effluent compartment (Szczepanowski et al., 2009).

Another phenomenon of antibiotic resistance refers to the native resistance found in natural soil bacterial communities. D'Costa et al., (2006) introduced the term 'antibiotic resistome' for the collection of all ARG's in a specific environment. These include antibiotic resistance genes associated with both pathogenic and nonpathogenic bacteria. The authors screened a collection of 480 soil-derived *Streptomyces* sp. strains against 21 antibiotics. Within this library, they identified resistance genes in all strains and resistance mechanisms against both natural and synthetic antimicrobials showing that non-clinical bacteria from natural environments have a wide array of antibiotic resistance mechanisms. Some of these resistance mechanisms, e.g. inactivation mechanisms affecting daptomycin or telithromycin (two of the newest antimicrobials introduced in therapeutics), have not yet been characterized in clinical isolates, while other mechanisms were similar to those found in clinical isolates suggesting that the reservoir of resistance determinants in soil can be mobilized into the human-associated microbial community by gene transfer.

Anthropogenic and native antibiotic resistances are interlinked; however understanding how these phenomena interact in the environment, especially in the context of agricultural soils is still vague.

Antibiotics are commonly classified based on their mechanism of action, chemical structure, or spectrum of activity. Most antibiotics target bacterial functions or growth processes such as bacterial cell wall synthesis (such as penicillins and cephalosporins), cell membranes, interfere with essential bacterial enzymes (such as quinolones and sulfonamides) or have bactericidal activities. Those that target protein synthesis, such as the aminoglycosides, macrolides, and tetracyclines, are usually bacteriostatic (Chee-Sanford et al., 2009).

Once antibiotic resistant bacteria and their corresponding suite of resistance genes enter the soil and water, the persistence and fate of the introduced determinant is dependent on several factors. First, on the nature and viability of the host bacteria harboring the determinant(s), and second, on the partitioning of free genetic material following cell lysis that may be subjected to degradation, sorption, or uptake by new cells. As long as a resistance gene is present in the environment, the possibility for its transfer exists. Genetic mechanisms involved in lateral

exchange of antibiotic resistance genes may include: (i) conjugative transfer via plasmids, transposons, integrons; (ii) transduction by bacteriophage; (iii) transformation, which is dependent on the native competent state of bacteria as well as cells acquiring induced competency (e.g., the presence of calcium, lightning event) (Chee-Sanford et al., 2009).

Four different antibiotic families that differ significantly in chemical composition, source (synthetic *vs.* natural products) and modes of action were chosen in this work, in order to assess the resistance level of the soil bacteria in agricultural soils irrigated with TWW and FW. A brief overview of their characteristics is detailed below:

i. The fluoroquinolones are a broad-spectrum family of antibiotics extensively used in the treatment of a great variety of human infections. Fluoroquinolones act by inhibiting the action of DNA gyrase and topoisomerase IV, enzymes involved in DNA synthesis. There are two mechanisms of resistance to fluoroquinolones in bacteria: the first, alterations in the targets of fluoroquinolones which is widespread in many bacteria, due to amino acid substitutions in the quinolone-resistance determining region (QRDR) of the target enzymes (Piddock, 1999). The second, a decrease in accumulation inside the bacteria due to impermeability of the membrane and/or an over expression of efflux pump systems (Spigaglia et al., 2009). Additionally, there is fluoroquinolone resistance that is mediated by plasmids. This was first reported in a *Klebsiella pneumoniae* clinical isolate from the USA in 1998 (Martinez-Martinez et al., 1998). The gene *qnrA*, which is responsible for this resistance, codes for a 218 amino acid protein belonging to the pentapeptide family that protects DNA from quinolone binding to gyrase and topoisomerase IV (Robicsek et al., 2006). The QnrA determinant has been reported worldwide from unrelated enterobacterial species and six variants of QnrA are known (QnrA1 to QnrA6) (Robicsek et al., 2006).

Recently, two other plasmid-mediated quinolone resistance genes, namely, *qnr*B and *qnr*S, have been identified that code for QnrB (six variants) and QnrS (two variants) belonging also to the pentapeptide repeat family (Cattoir et al., 2007).

Ciprofloxacin is a synthetic second-generation fluoroquinolone antibacterial compound. It is effective against a large number of bacteria, and is particularly useful against gram-negative bacteria. It is used to treat a wide range of infections, including infections of the chest, urinary tract and of the gastrointestinal system. Fluoroquinolone concentrations in natural environments can vary extensively depending on the particular environment. For example, in pollution from pharmaceutical industries or at sewage outlets from hospitals the concentrations can reach very high levels (mg/ml) (Gullberg et al., 2011), whereas in aquatic environments or in soil levels are typically much lower (Klaus, 2009). Ciprofloxacin in vitro minimum inhibitory concentrations (MICs) are $\geq$4 μg/ml against strains of the following clinical microorganisms; Enterobacteriacae, Enterococcus faecalis, Methicillin susceptible Staphlyococcus species, Pseudomonas aerogenosa, Haemophilus influenza, Haemophilus parainfluenzae, Penicillin susceptible Streptococcus pneumonia, Streptococcus pyogenes and Neisseria gonorrhoeae (from-Ciprofloxacin official FDA information).

ii. Since their introduction in the 1950s, tetracyclines have been widely used in human and veterinary medicine, as growth promoters in animal industry, and for prophylaxis in plant agriculture and aquaculture. At present, resistance to tetracyclines has spread to almost all bacterial genera, and this situation perhaps is the consequence of previous overuse (Aminov et al., 2001). Tetracyclines belong to a family of broad-spectrum antibiotics that includes tetracycline, chlortetracycline, doxycycline, and minocycline. These antibiotics inhibit protein synthesis in gram-positive and gram-negative bacteria by preventing the binding of aminoacyl-tRNA molecules to the 30S ribosomal subunit (Schnappinger and Hillen, 1996). Bacterial resistance to tetracycline is mediated mainly by two mechanisms, protection of ribosomes by large cytoplasmic proteins and energy dependent efflux pumps of tetracycline (Aminov et al., 2001). A third mechanism, enzymatic inactivation of tetracycline, is relatively uncommon (Aminov et al., 2001). The tetracycline resistant genes associated with efflux mechanisms are *tet* A, B, C, D, E, G, I, M and K. The tetracycline resistance genes associated with a ribosomal protection mechanism and/or efflux mechanism are *tet* K, L, M, O, S, P, Q, B, D, H and C. The only example of a tetracycline resistance gene causing the enzymatic alteration of tetracycline is *tet* X (Ng et al., 2001). Tet O-related sequences were determined first in plasmids from

campylobacteria (Sougakoff et al., 1987; Taylor et al., 1987), then in streptococci (LeBlanc et al., 1998; Widdowson et al., 1996), and in a rumen bacterium, *Butyrivibrio fibrisolvens* (Barbosa et al., 1999).

iii. Sulfonamides are among the most widely used veterinary antibiotics in the European Union (Kools et al., 2008), of which sulfadiazine (SDZ) is most extensively used and therefore has high potential to enter the environment (Byrne-Bailey et al., 2009). They act as a structural analogue of *p*-amino-benzoic acid and bind dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway, resulting in the inhibition of dihydrofolic acid formation (Skold, 2000). Resistance is conferred by mutations in the DHPS gene (*folP*) (Swedberg et al., 1993) or from the acquisition of an alternative DHPS gene (*sul*) (Heuer et al., 2011; Perreten and Boerlin, 2003). The genes occur in a wide range of bacterial species, because they are often located on transposable elements of self-transferable or mobilizable broad-host-range plasmids (Byrne-Bailey et al., 2009). The first of the known alternative DHPS genes, *sul1*, is usually located on the 3' conserved region of a class 1 integron (Skold, 2000) and is frequently identified with this potentially mobile element in the slurry and soil environment (Guerra et al., 2003; Rosser and Young, 1999). The *sul2* gene was first identified on RSF1010 in *Escherichia coli* and has been found on small nonconjugative resistance plasmids (Radstrom and Swedberg, 1988). The prevalence of the sulfonamide resistance genes varies among published studies, depending on environments and bacterial species sampled.

iv. Erythromycin, the last type of antibiotic that was used in our analyses, belongs to the macrolide-lincosamide-streptogramin B (MLS_B) superfamily of antibiotics. In structure, this macrocyclic compound contains a 14-membered lactone ring with ten asymmetric centers and two sugars (L-cladinose and D-desosamine), making it very difficult to produce via synthetic methods. It is produced by a strain of the actinomycete *Saccharopolyspora erythraea*. Erythromycin, like all macrolide antibiotics, prevents bacterial cells from growing and multiplying by interfering with their ability to make proteins while not affecting human cells. It is used to treat several types of infections: upper/lower respiratory tract infections, skin infections, acute pelvic inflammatory disease, erythrasma, etc. caused by bacteria such as

Streptococcus pyogenes, Streptococcus pneumoniae, Mycoplasma pneumoniae, Staphylococcus aureus, Neisseria gonorrhoeae, and many others.

Erythromycin is used for treatment of both humans and livestock (Chen et al., 2007). Resistance to erythromycin is mediated by the *erm* genes which encode the 23S rRNA adenine-specific N_6-methyltransferases, that methylate the 23S rRNA of bacteria (Roberts, 2003). Such methylation results in decreased binding of all MLS_B drugs to their target (bacterial ribosomes) and thus resistance to all MLS_B antibiotics (Chen et al., 2007). The *erm* genes are among the most common AR genes of MLSB, and 32 classes of *erm* genes ($\geq$80% amino acid sequence identity within each class) have been identified and sequenced to date among many different genera of bacteria (Roberts, 2004). Furthermore, *erm* genes are among the most common acquired resistance genes in bacteria and the only genes conferring resistance to MLS_B currently found in anaerobes (Roberts, 2003).

1.4 The family *Flavobacteriaceae*

Flavobacteria are gram-negative bacteria belonging to the phylum *Bacteroidetes*, which comprises the classes *Bacteroides*, *Flavobacteria*, and *Sphingobacteria* (Garrity and Holt, 2001). The *Flavobacteriaceae* family is widely distributed in nature. They are ubiquitous and are common members of the microbial community in bulk soil and in the rhizosphere of plant roots (Johansen et al., 2009). In addition, they are known to be highly resistant to several antibiotic compounds. *Flavobacteriaceae* also plays a role in mineralizing various types of poorly degradable organic matter including chitin and cellulose. They often possess an arsenal of extracellular enzymes, which enable them to degrade bacteria, fungi, insects and nematodes. In order to acquire insight into native soil antibiotic resistant bacteria, several flavobacterial strains (isolate name: E21, D43, K4, J26,M1,M34 and M52) isolated by me and others in my lab were characterized in this work.

1.5 Anthropogenic impact on antibiotic resistance in natural environments

As discussed above, community-associated antibiotic resistance is a growing threat to public health in both Israel and worldwide. This is largely due to overuse and misuse of antibiotics in both clinical and community settings, but may also stem from proliferation of antibiotic resistance in natural environments potentially stimulated by additional factors that are not directly linked to human consumption of antibiotics. Certain anthropogenic processes such as application of animal manure and municipal biosolids to agricultural soils, discharge of wastewater effluents to natural water reservoirs and TWW irrigation, have been linked to elevated levels of antibiotic resistance levels in the environment. Proliferation of resistant bacteria due to natural selection in areas exposed to anthropogenic effects has been well documented (Byrne-Bailey et al., 2009; Ghosh and LaPara, 2007; Lipsitch et al., 2002; Pei et al., 2006; Robicsek et al., 2006). Nevertheless, there is no current research, which focuses on the direct effect of irrigation with TWW on the soil resistome.

1.6. Research hypothesis

The working hypothesis of this thesis was that TWW irrigation increases antibiotic resistance levels in irrigated soils due to selective pressure generated by residual antibiotic compounds and the presence of antibiotic resistance bacteria. We assessed the abundance of antibiotic resistant bacteria and genes in soils irrigated with TWW relative to identical soils irrigated with freshwater

1.7. Research objectives

The objectives of this study were:

1. To determine whether irrigation with TWW causes an increase in the relative abundance of antibiotic resistant soil bacteria and in the prevalence of clinically-associated antibiotic resistance genes.

2. To determine how selective pressure in the form of different antibiotic concentrations, affects antibiotic resistance levels and bacterial community composition of the soil bacteria.

3. To assess the physiological characteristics of multidrug resistant bacteria isolated from TWW-irrigated soils, specifically focusing on isolates from the flavobacterial genera.

Objective one focused on soils from field experiments and objective two was accomplished in bench-scale incubation experiments.

The resistance level was determined by both cultivation-based approaches and by cultivation-independent analyses of antibiotic resistance genes.

2. Materials and Methods

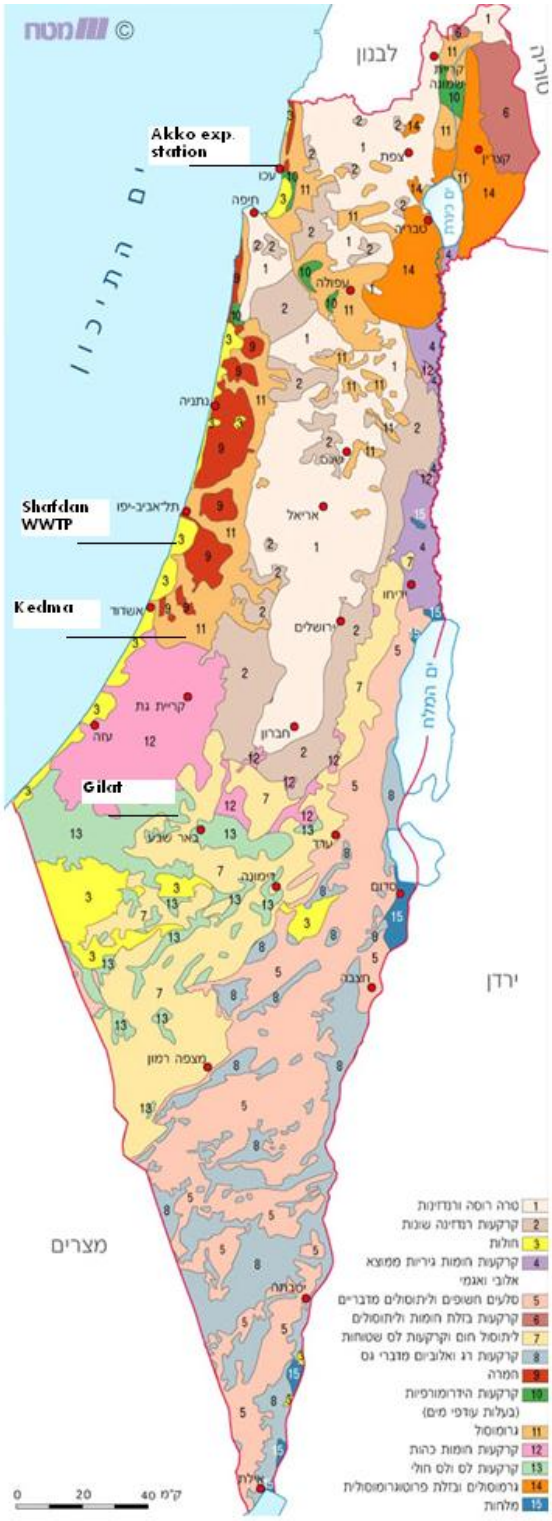

Figure 1: Different types of soil in Israel and their location (Fein et al., 2007). Text boxes indicate the study site locations.

2.1 Study sites and sample collection

Four agricultural soils with distinct physicochemical properties from diverse geographic locations in Israel were used in this study (Fig. 1, Table1). Two of the soils (Akko and Rishon LeZion) were used for both cultivation and direct molecular analyses of antibiotic resistance genes, while the other two (Gilat and Kedma) were only used for molecular analyses.

- Akko:

Soil samples were taken from an experimental Avocado orchard supervised by agricultural extension advisor Anat Lowengart-Aycicegi in the Akko Agricultural Experimental Station. Soil at this site is a Vertisol, with 60% clay, 21% silt, and 19% sand. The orchard has been irrigated with secondary TWW and with FW for the past 12 years. The secondary TWW used in the study are from domestic sewage from the towns Shomrat, Bustan, Kfar Masser, and Gadida, which was treated at the Shomrat-Agamit Wastewater Treatment Facility. TWW-irrigation water did not have fertilizers added. FW was supplemented with KCl, phosphoric acid, and NH_3 or Urea. No manure-based fertilizers were used. Irrigation of the orchard was carried out during the irrigation season between March and October (start after the last rain).

Soil samples were collected directly under irrigation drippers (WET) from the top 5 cm of soil (after removing residual debris). Biological replicates (~50g soil each) were sampled from three different locations along the FW and TWW irrigated tree rows. In the 2010 profile, samples were also taken from less hydrated soil at a distance of 50 cm from the dripping holes (DRY). Water samples (TWW and FW) were taken from the irrigation tap at the site.

- Rishon LeZion:

Soil samples were taken from an experiment conducted by Dr. Pinhas Fine in the Dan Region wastewater treatment plant, Shafdan in Rishon LeZion. The experiments were conducted in 200 liter steel barrel lysimeters containing Oroblanco Citrus planted in dune quartz sand. The lysimeters were irrigated throughout the whole year with either secondary wastewater effluent from the activated sludge mechanical-biological treatment plant, or with tertiary infiltrated water (IW)*- secondary effluent from the Shafdan plant that is recharged through the soil and the vadose zone into groundwater reservoirs (aquifers) where it undergoes natural physical, biological and chemical processes. (Arye et al., 2011). Water samples were taken from the irrigation tap at the site.

Between 30-40 ml of TWW samples from Akko and Rishon LeZion, were filtered through 0.2 µm mixed cellulose ester filters (Whatman®, Brentford, UK) and stored at -80°C for DNA extraction at a later point. In addition, raw sludge and activated sludge samples from the Shafdan WWTP, whose effluent feeds the TWW used to irrigate the Rishon LeZion lysimeters were taken in triplicates from different areas of the sewage inlet and activated sludge reactors, respectively, in 50 ml sterile tubes and stored on ice until arrival at the laboratory. Fifty millimeters of activated sludge samples were also filtered and stored at -80°C for DNA extraction at later point. 50 ml of raw sludge samples were centrifuged at 1000 rpm for 10 min to obtain the pellet. About 0.15g from the pellet was taken for DNA extraction at a later point.

All soil samples were transferred from field sites to the laboratory on ice.

Bacterial platting from soil subsets was performed on the day of sampling and the remaining soil samples were stored at -80°C for DNA extraction at a later point.

- Gilat:

Soil samples were taken from a plot located near the Agricultural Experiment Station in Gilat. The soil type is Loam with 20% sand, 35% silt and 45% clay. The plot has been irrigated with secondary TWW from the Sde-Timan WTTP and with fresh water, for the last 15 years. The main crops at the plot were wheat and cotton. Irrigation of the field was held during the irrigation season between March and October.

- Kedma:

Soil samples were taken from an Olive orchard located in the coastal plain of central Israel (31º42’50”N, 34º46’32”E), represented by a Mediterranean climate. The soil texture was clayey (24% sand, 25% silt and 51 % clay). Two water sources were utilized throughout the experiment. Secondary-treated domestic wastewater from Jerusalem and well water originating from the local coastal aquifer (fresh) were used for irrigation. Irrigation of the orchard was conducted during the irrigation season between March and October (start after the last rain).

Table 1 Description and soil characterization of the four experimental sites that were examined in this work.

Site	Soil type	TWW quality	FW quality	Irrigation period	Crop	Plot type	Duration of experiment
Akko	**Vertisol (60% clay)**	**Secondary TWW from Shomrat-Agamit treatment facility**	**Fresh water**	**Irrigation season**	**Avocado**	**Orchard**	**12 years**
Rishon LeZion	**Dune quartz sand**	**wastewater effluent from the Shafdan mechanical-biological treatment plant**	**Injected TWW**	**All the time**	**Oroblanco Citrus**	**200 liters Lysimeters**	**12 years**
Gilat	**Loam (20% clay)**	**Secondary TWW from Sde-Timan treatment facility**	**Fresh water**	**Irrigation season**	**Cotton and wheat**	**Field**	**15 years**
Kedma	**Vertisol (52% clay)**	**Secondary-TWW from the city of Jerusalem**	**Well water from the local coastal aquifer**	**Irrigation season**	**Olive tree**	**Orchard**	**6 years**

2.2 Enumeration of resistant soil bacteria

Bacteria were dislodged from soil matrices by suspending 1 g (wet weight) of soil in 9 ml of saline (0.85% NaCl) and vortexing at maximal speed for 30 s followed by shaking on a reciprocating shaker (Edmund Bühler GmbH, Hechingen, Germany) for 30 min at 130 rpm. Samples were then serially diluted 10-fold in saline and applied to agar plates using standard spread plating techniques. Water samples were also serially diluted and applied to agar plates. Agar plates (dilute (25%) Luria–Bertani growth medium) were amended with either 20 mg/L of tetracycline, 4 mg/L of ciprofloxacin or 10 mg/L of erythromycin to enumerate resistant bacteria. Antibiotic resistance levels were calculated as the ratio of CFUs growing on plates supplemented with antibiotics compared to the number of CFUs growing on plates without antibiotics. All initial plate counts were done in triplicate and averaged before calculating resistance levels for each soil sample. The average resistance levels for each of three soil samples collected at a site was subsequently used to calculate mean and standard deviations of resistance levels at a site. Plates were incubated for 1–4 days at 30°C. All growth media were sterilized by autoclaving (20 min; 121°C; 15 psi).

2.3 DNA extraction

DNA was extracted from soil, water, raw sludge and activated sludge samples using a modified bead-beating method as described previously (Inbar et al., 2005) . Briefly, DNA was recovered from 0.5 g of soil, from 0.2 µm nitrocellulose filters (containing 20-30 ml of TWW) and from the raw and activated sludge samples (described above), via bead-beating (Fast Prep FP 120, Bio101, Savant Instruments Inc., Holbrook, NY) in an extraction buffer [100 mM Tris HCl, pH 8.0; 100 mM potassium phosphate buffer pH 8.0; 1% cetyltrimethylammonium bromide (CTAB); and 2% sodium dodecyl sulphate (SDS)]. The crude extracts were mixed with KCl to a final concentration of 0.5 M, incubated for 5 min, followed by ethanol precipitation. Pellets were resuspended in Tris-EDTA (TE). DNA present in the supernatant was bound to glassmilk (0.5–10 µm silica particles, Sigma Chemical Co., St. Louis, MO) with NaI as described elsewhere (Boyle and Lew, 1995). The silica was then resuspended in an ethanol-based wash

buffer solution (Boyle and Lew, 1995) and this solution, containing the glassmilk, was transferred to a centrifuge tube filter (0.22 µm cut-off nylon filter, Costar, Corning Inc., Corning, NY). After the glassmilk was dried via centrifugation, DNA bound to the silica and retained on the filter was eluted with TE into a sterile tube and stored at -20°C prior to use.

2.4 Polymerase chain-reaction (PCR)

All primers used in this study were synthesized by Integrated DNA technologies, Inc. (Iowa, USA). All PCR and Multiplex PCR reactions were conducted on a T-Gradient thermocycler (Biometra, Goettingen, Gremany) in sterile 0.2 mL polypropylene tubes. The different primer pairs and reaction conditions used are specified in Table 2.

2.5 Phylogenetic identification of resistant bacteria isolates

Several antibiotic-resistant colonies were randomly picked from plates containing 20–200 colonies and directly resuspended in the PCR mixture. The PCR reaction was done in a final volume of 25 µl containing 12.5 µl Taq DNA Polymerase Master Mix RED kit (Ampliqon, Denmark), distilled water and 25 pmol of universal Eubacterial primers 11F and 1392R, that target the 16S rRNA gene, as previously described (Green et al., 2004) . PCR products were sequenced by Macrogen Inc. (Seoul, Korea), and the phylogenetic affiliation of the colonies was determined using BLASTn (http://blast.ncbi.nlm.nih.gov).

2.6 Real time quantitative PCR (QPCR)

QPCR was applied to quantify selected ARG in the soil, water samples from the four agricultural sites, and in raw sewage and activated sludge from the Shafdan WWTP. All QPCR conditions and primers are listed in Table2. Plasmids comprising the target ARG were used as standard DNA templates for each of the QPCR reactions. The plasmids were decimally diluted to range from 10^9 to 10^0 copy number. All the DNA amplifications and quantifications were performed using a MX 3000 Real Time PCR system (Stratagene, La Jolla, CA). Each 25µL reaction

contained 12.5µL Absolute Blue SYBR Green ROX Mix (Thermo Fisher Scientific, Surrey, UK), 1µL of each primer (0.2mM), 1µL of DNA template and 9.5µL ultra pure PCR grade water. The PCR program began with an initial hot start step of 15 min at 95°C required for the activation of the DNA polymerase, followed by 40 cycles each consisting of the following steps: 95°C for 30s, the respective annealing temp (Table 1) for 30s, 72°C for 30s, and a final melt curve stage with temperature ramping from 55 to 95°C. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal originated from specific PCR products. Quantification of total bacteria was also performed using the general bacterial primer set Eub519F and Univ907.

Baseline and threshold calculations were performed with the MxPro™ QPCR software analysis tools (Stratagene, La Jolla, Ca). Following real-time PCR, the products were confirmed by agarose gel electrophoresis. PCR products of each targeted gene were cloned (Fermentas, CloneJET™ PCR Cloning Kit) and sequenced to confirm specificity. All QPCR reactions were done in triplicate for both the standards and the microbial community DNA samples.

2.7 Incubation experiment

Plastic containers (0.5 L) were packed with Vertisol soil taken from the Akko experiment (soil irrigated with FW) and were irrigated with four different treatments, each supplemented with a different concentration of ciprofloxacin: 0 (control), 0.4 µg/L, 4 µg/L and 40 µg/L. All treatments were done in triplicate. Antibiotic resistance levels were determined by plating on 25% Luria–Bertani agar supplemented with 0.4 µg/ml or 4 µg/ml ciprofloxacin, one day, one week, two months and three months after experiment initiation. DNA was extracted at each time point as previously described and stored at -80°C.

Multiplex PCR for detection of plasmid-borne quinolone resistance (qnr) genes (*qnr*A *qnr*B and *qnr*S) was performed on the extracted soil DNA and on the ciprofloxacin-resistant colonies from each of the incubation experiment treatments. DNA (2 µL) was subjected to multiplex PCR in a 50 µL reaction mixture containing 1XPCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl], 1.5 mM MgCl2, 200 µM each deoxynucleotide triphosphate, 20 pmol of each of the six primers

(Table 1) and 2.5 U of Taq DNA polymerase (Red Taq; Sigma, St. Louis, MO). Amplification was carried out with the following thermal cycling profile: 10 min at 95°C and 35 cycles of amplification consisting of 1 min at 95°C,1 min at 54°C and 1 min at 72°C and 10 min at 72°C for the final extension. DNA fragments were analyzed by electrophoresis in a 2% agarose gel at 100 V for 1 h in 1X TAE [40 mM Tris–HCl (pH8.3), 2 mM acetate and 1 mM EDTA] containing 0.05 mg/L ethidium bromide (Cattoir et al., 2007).

2.8 Denaturing gradient gel-electrophoresis (DGGE)

DGGE was performed in order to assess the impact of antibiotic amendment on compositions of the bacterial community in the incubation experiment.

PCR for the DGGE analysis was performed as described previously (Green et al., 2004) using the general bacterial primer set 341F-GC containing a 40-bp GC-clamp to enhance DGGE separation (clamp sequence in italics), *CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G*CC TAC GGG AGG CAG CAG , and 907R, CCG TCA ATT CMT TTG AGT TT (Muyzer et al., 1998). DGGE analysis was performed using a 20-70% denaturant gradient. The gel was photographed using a UV transillumination table (302 nm) with a Kodak digital camera (Rochester, NY).

2.9 Physiological characteristics of flavobacteria isolates

Some of the phylogenetically-characterized antibiotic-resistant soil isolates were associated with the genus *Flavobacterium* from the *Bacteriodetes* phylum. This group is common in the soil and rhizosphere (Johansen et al., 2009), and many of its strains are known to have multiple antibiotic resistance. Therefore, I chose to study the antibiotic resistance characteristics of these isolates more carefully. Isolates from TWW- and FW-irrigated soils described above, as well as isolates from pepper roots from previously described studies conducted in the lab were used (Kolton and Cytryn, 2011). Physiological analyses of the flavobacterial isolates were performed as described elsewhere (Berić. T., 2009; Johansen et al., 2009; Yu et al., 2009).

Several assays were used to characterize the flabobacterial isolates: PCR analysis was used to identify specific antibiotic resistance genes (tetracycline, ciprofloxacin, erythromycin and sulfonamide resistance genes) (Ng et al., 2001; (Cattoir et al., 2007; Pei et al., 2006). The disk-

diffusion method (see section 2.10), was used to test resistance of isolates to a spectrum of antibiotics (tetracycline, ciprofloxacin, erythromycin, sulfonamide, kanamycin, streptomycin, and ampicilin). A variety of enzymatic activities was tested including chitinase, protease, catalase and -β-galactosidase, and NH_3 production was measured. In addition, gliding motility and the production of flexirubin pigment was tested.

2.10 Antibiotic disc diffusion method

Bacterial isolates were analyzed for antibiotic resistance by the disk-diffusion method (Kirby-Bauer). Briefly, agar plates (Luria–Bertani growth medium) were uniformly inoculated with the isolate liquid culture (O.D 0.1 nm) and a paper disk (HiMedia Laboratories, Mumbai, India) impregnated with a fixed concentration of an antibiotic was placed on the agar surface. Plates were incubated overnight at 30ºC and the diameter of the inhibition zone was measured using a standard table of antibiotic susceptibilities. Laboratory *E.coli* DHα-5 was used for a positive control to the antibiotics, as a sensitive strain.

All growth media and antibiotics were purchased from Sigma-Aldrich Israel.

2.11 Statistical analysis

Processing and analyzing the data was done using Microsoft Office Excel 2007 and SPSS program for statistics (one way ANOVA and Non-parametric Kruskal-Wallis tests).

Table 2: List of primers used for multiplex PCR and quantitative real-time PCR in this study

Primer	Class target	Sequences	Traditional PCR annealing temp	Q-PCR annealing temp	Amplicon size (bp)	References
multiplex PCR for tet genes						
tet(A)F	tet(A)	GCT ACA TCC TGC TTG CCT TC	55°C		210	(Ng et al., 2001)
tet(A)R		CAT AGA TCG CCG TGA AGA GG				
tet(B)F	tet(B)	TTG GTT AGG GGC AAG TTT TG	55°C		659	
tet(B)R		GTA ATG GGC CAA TAA CAC CG				
tet(C)F	tet(C)	CTT GAG AGC CTT CAA CCC AG	55°C		418	
tet(C)R		ATG GTC GTC ATC TAC CTG CC				
tet(D)F	tet(D)	AAA CCA TTA CGG CAT TCT GC	55°C		787	
tet(D)R		GAC CGG ATA CAC CAT CCA TC				
tet(E)F	tet(E)	AAA CCA CAT CCT CCA TAC GC	55°C		278	
tet(E)R		AAA TAG GCC ACA ACC GTC AG				
tet(K)F	tet(K)	TCG ATA GGA ACA GCA GTA	55°C		169	
tet(K)R		CAG CAG ATC CTA CTC CTT				
tet(L)F	tet(L)	TCG TTA GCG TGC TGT CAT TC	55°C		267	(Ng et al., 2001)
tet(L)R		GTA TCC CAC CAA TGT AGC CG				
tet(M)F	tet(M)	GTG GAC AAA GGT ACA ACG AG	55°C		406	
tet(M)R		CGG TAA AGT TCG TCA CAC AC				
tet(O)F	tet(O)	AAC TTA GGC ATT CTG GCT CAC	55°C		515	
tet(O)R		TCC CAC TGT TCC ATA TCG TCA				
tet(Q)F	tet(Q)	TTA TAC TTC CTC CGG CAT CG	55°C		904	
tet(Q)R		ATC GGT TCG AGA ATG TCC AC				
tet(X)F	tet(X)	CAA TAA TTG GTG GTG GAC CC	55°C		468	
tet(X)R		TTC TTA CCT TGG ACA TCC CG				
11F	16S rRNA	GTTTGATCMTGGCTCAG	58°C			(Green et al., 2004)
1392R		ACGGGCGGTGTGTAC				(D.J, 1991)

multiplex PCR for qnr genes						
QnrAm-F	qnrA1-A6	AGAGGATTTCTCACGCCAGG	54°C		580	(Cattoir et al., 2007)
QnrAm-R		TGCCAGGCACAGATCTTGAC				
QnrBm-F	qnrB1-B6	GGMATHGAAATTCGCCACTG	54°C		264	
QnrBm-R		TTTGCYGYYCGCCAGTCGAA				
QnrSm-F	qnrS1-S2	GCAAGTTCATTGAACAGGGT	54°C		428	
QnrSm-R		TCTAAACCGTCGAGTTCGGCG				
Real Time QPCR Primers						
sul1-FW	sul(1)	CGCACCGGAAACATCGCTGCAC	55.9°C	55.9°C	163	(Pei et al., 2006)
sul1-RV		TGAAGTTCCGCCGCAAGGCTCG				
sul2-FW	sul(2)	TCCGGTGGAGGCCGGTATCTGG	60.8°C	60.8°C	191	
sul2-RV		CGGGAATGCCATCTGCCTTGAG				
TetO-FW	tet(O)	ACGGARAGTTTATTGTATACC	60°C	60°C	171	(Aminov et al., 2001)
TetO-RV		TGGCGTATCTATAATGTTGAC				
erm(F)-189f	erm(F)	CGA CAC AGC TTT GGT TGA AC	56°C	56°C	309	(Chen et al., 2007)
erm(F)-497r		GGA CCT ACC TCA TAG ACA AG				
erm(B)-91fc	erm(B)	GAT ACC GTT TAC GAA ATT GG	58°C	58°C	364	
erm(B)-454rc		GAA TCG AGA CTT GAG TGT GC				
qnrA32F	qnr(A)	AGGATTTCTCACGCCAGGATT	57°C	57°C	124	(Cummings et al., 2010)
qnrA155R		CCGCTTTCAATGAAACTGCA				
Univ 907R	16S rRNA	CCGTCAATTCMTTTGAGTTT	58°C	58°C	388	(Muyzer et al., 1998)
Eub 519F		CAGCMGCCGCGGTAANWC				(D.J, 1991)

3. Results

3.1 Heterotrophic bacterial plate counts on antibiotic-selective media

The relative abundances of antibiotic resistant bacteria was assessed by serial-dilution plating on dilute (25%) nutrient-rich Luria broth (LB) medium in a clay/organic-rich soil from the western Galilee (Akko), and in dune quartz sand from the coastal plain of Israel (Rishon LeZion). The characterization of soil quality and the description of the experimental sites are listed in the Materials and Method section (Table 1).

Akko

Two profiles from the end (September 2010) and middle (July 2011) of the irrigation season were sampled from the Akko soils (Table 1). In the September 2010 profile, approximately 2-6% and 8-16% of the isolates were resistant to tetracycline and ciprofloxacin, respectively (Fig. 2). Irrigation with treated wastewater (TWW) did not increase the percentage of tetracycline- and ciprofloxacin-resistant soil bacteria when compared to soils irrigated with freshwater (FW). On the contrary, levels of tetracycline resistance were higher ($p<0.05$) in FW- irrigated soils than in TWW-irrigated soils, in the wet samples (Fig. 2 Top).

Soil moisture had a significant positive effect on the bacterial resistance levels to both tetracycline and ciprofloxacin. For ciprofloxacin, the percentage of resistant bacteria in FW-irrigated soils was higher ($p<0.05$) in the wet samples than in the dry samples (Fig. 2 Bottom). Soil samples taken directly under the irrigation drippers (*i.e* outlet holes- WET) showed higher antibiotic resistance levels than samples taken from soils, 50 cm from the drippers (DRY). This however, was significant only for FW-irrigated soils samples ($p<0.05$).

Tetracycline- and ciprofloxacin-resistant levels were also examined in water samples that were used for irrigation. Bacterial abundance (CFUs ml^{-1}) in TWW samples, measured directly from water pipes feeding the irrigation tubes, was up to six orders of magnitude higher than in freshwater samples (Fig. 3). The relative abundance of resistant bacteria found in the TWW samples was up to 0.1% for ciprofloxacin and 0.005% for tetracycline. In freshwater samples, resistant bacteria were not detected. Fig. 4 shows the visual differences between TWW and FW samples.

Analyses of the July 2011 profile was conducted only on wet soil samples (taken directly under the drippers). In addition, the relative abundance of erythromycin-resistance was also evaluated. The results showed a similar trend to the September 2010 profile. There were no significant differences in antibiotic-resistant bacterial abundance between the TWW- and FW-irrigated soils (Fig. 5).

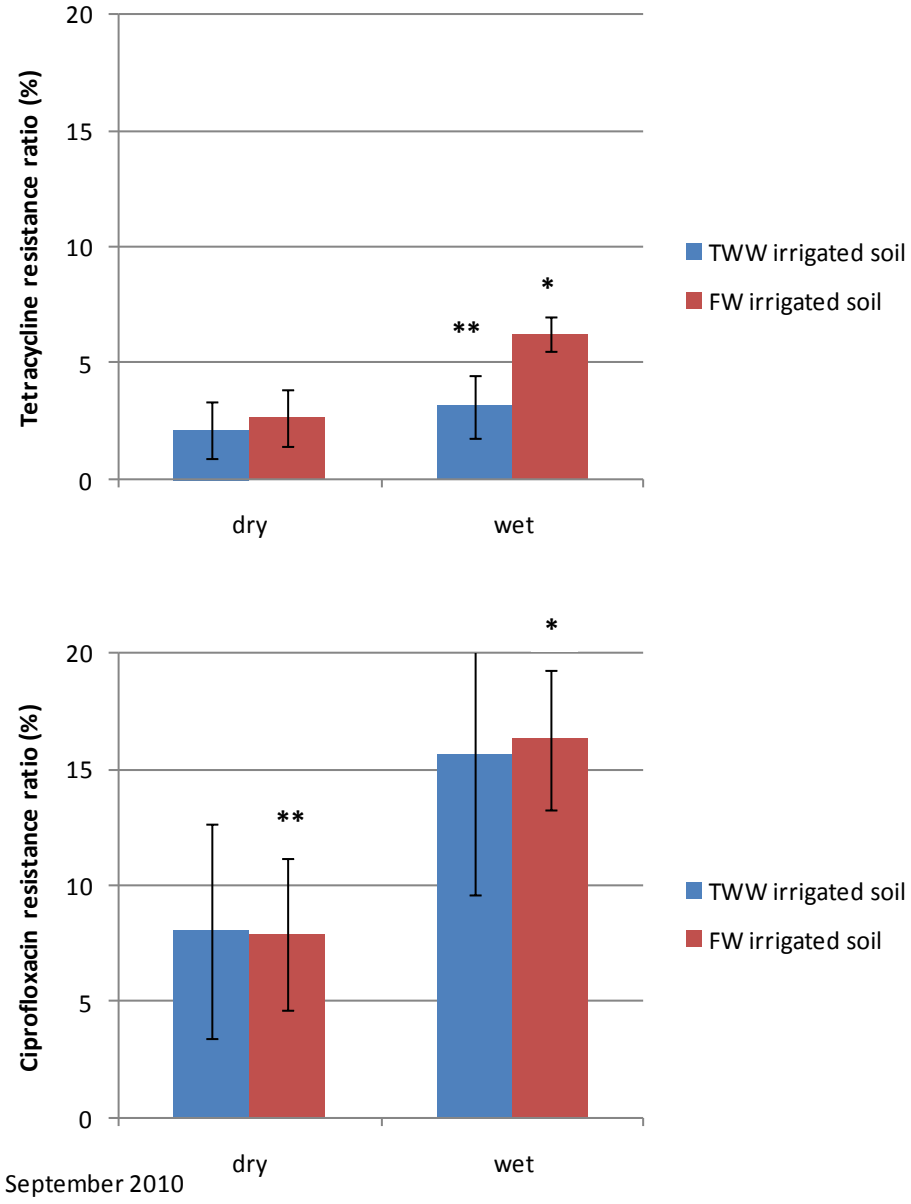

Figure 2 Relative abundance of antibiotic-resistant bacteria (CFUs on antibiotic media /CFUs on media with no antibiotic) in FW- and TWW-irrigated soils from the Akko experiement. Samples were taken under irrigation drippers (wet) and 50 cm from the drippers (dry).Top: Tetracycline resistance; Bottom: Ciprofloxacin resistnce. Error bars indicate the standard deviations of three bilogical replicates.

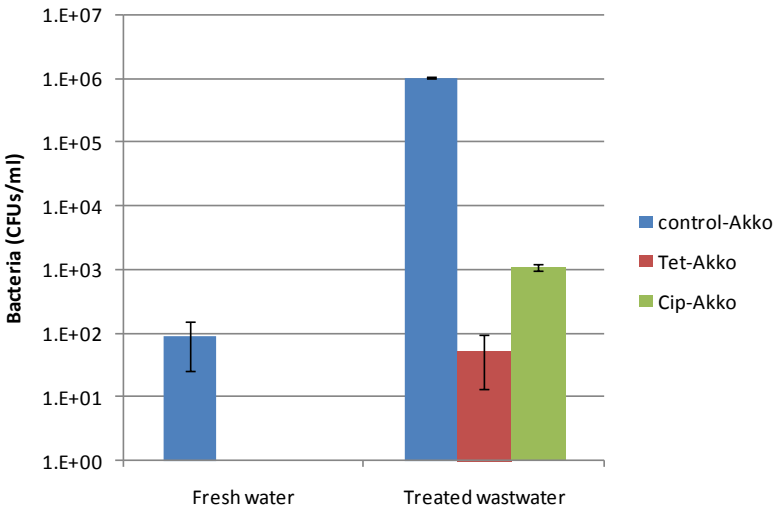

Figure 3 Tetracycline and ciprofloxacin resistant bacteria in water samples from Akko- FW and TWW. Resistant bacteria were detected only in the TWW samples.

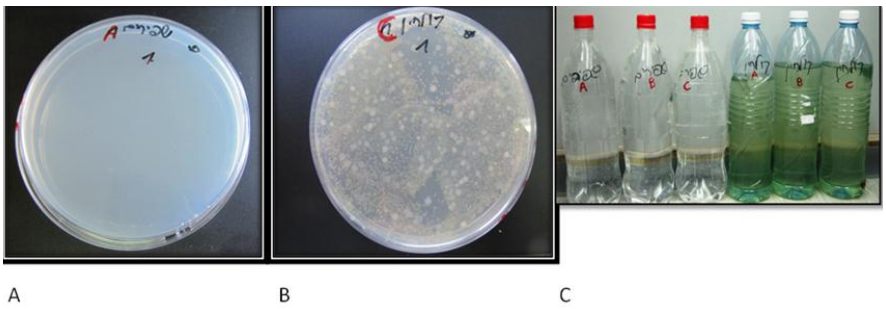

Figure 4 Bacterial growth on dillute LB plates isolated from fresh water (A) and TWW (B) used for irrigation at the Akko site. Illustration of samples from fresh water (C:left) and TWW (C:right).

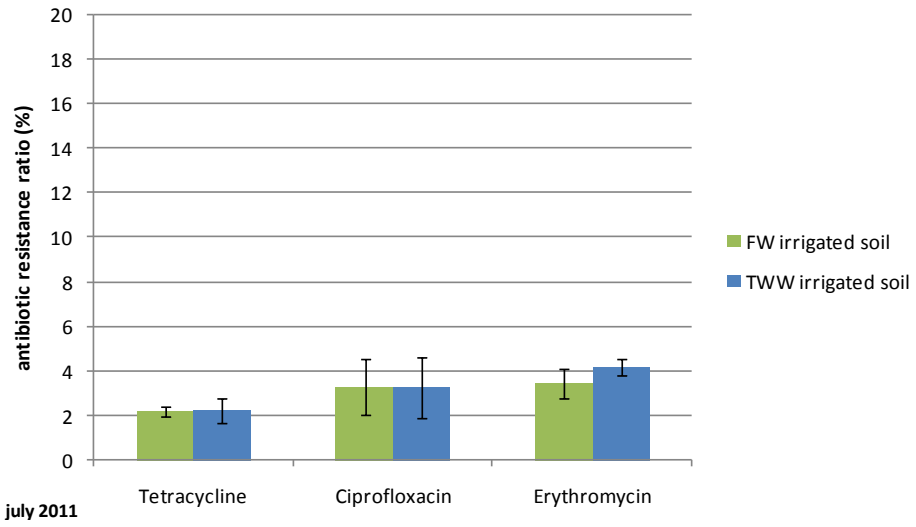

Figure 5 Relative abundance of antibiotic-resistant bacteria (CFUs on antibiotic media /CFUs on media with no antibiotic) in FW- and TWW-irrigated soil in Akko. Three antibiotics were tested: tetracycline, ciprofloxacin and erythromycin. Error bars indicate the standard deviations of three replicates

Rishon LeZion

The Rishon LeZion sand is characterized by very different physiochemical parameters relative to the soil from the Akko experiment described above (Table 1). Nonetheless, the impact of TWW-irrigation on soil AR was very similar to Akko (Fig. 6). There were no significant differences in tetracycline or erythromycin resistance levels of the bacterial isolates in the TWW-*vs.* the FW-irrigated soils. Moreover, the relative abundance of ciprofloxacin-resistant bacteria was higher ($p<0.05$) in the FW-irrigated soils than in the TWW-irrigated soils.

The relative abundance of AR bacterial isolates in the water samples, taken from the irrigation tap at the site was also similar to the irrigation water from the Akko plot (Fig. 7). Bacterial levels (CFUs/ml) in the TWW samples were up to five orders of magnitude higher than those measured in FW samples (Fig. 7). The relative abundances of AR bacteria found in the TWW samples were up to 0.21% for ciprofloxacin and 0.13% for tetracycline. In freshwater samples, resistant bacteria were not detected.

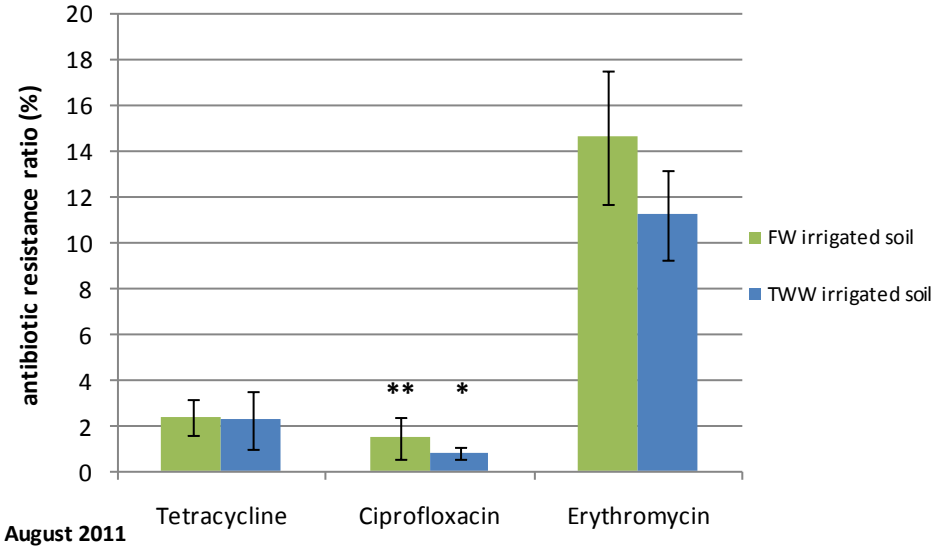

Figure 6 Relative abundance of antibiotic-resistant bacteria (CFUs on antibiotic media /CFUs on media with no antibiotic) in FW- and TWW-irrigated soil in Rishon LeZion. Three antibiotics were tested: tetracycline, ciprofloxacin and erythromycin. Error bars indicate the standard deviations of three replicates.

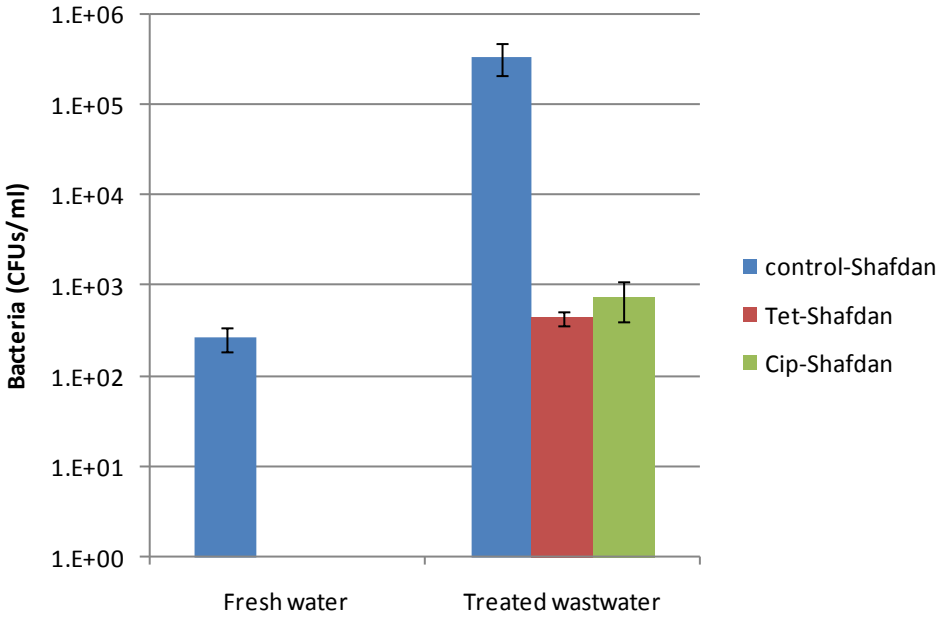

Figure 7 Tetracycline and ciprofloxacin resistant bacteria in water samples from Shafdan FW and TWW. Resistant bacteria were found only in the TWW samples.

3.2 Quantification of antibiotic resistance genes

In order to examine if irrigation with TWW has an impact on the abundance of ARG's, we applied cultivation-independent Q-PCR analyses targeting five different ARG's: *tet*O (tetracycline resistance), *sul1* and *sul*2 (sulfofnamide resistance), and *erm*B and *erm*F (macrolide resistance) (Fig. 8, Fig. 9). We also tested the abundance of *qnr*A, associated with fluoroquinolone resistance. However, *qnr*A gene amplicons were below detection limits in all of the sites sampled (data not showed).

In addition to the Akko and Rishon LeZion sites, we also tested ARG levels in two additional TWW-irrigated agricultural soils in southern Israel: (A) a loamy soil in the northern Negev Desert (Gilat) that receives TWW from the Sde-Timan WTTP in Beer Sheva; and (B) in a vertisol soil in the southern lowlands of Israel near Kiriat Malachi (Kedma) that is irrigated with effluent from the Nachal Soreq WWTP in Jerusalem (Table 1).

ARG relative abundance was first normalized to 16S rRNA gene abundance to minimize variance caused by differential extraction and analytical efficiencies and difference in background bacterial abundance. The total bacterial abundance in each sample was quantified and expressed as the number of 16S rRNA gene copies per gram or ml of sample (Table 3). As indicated in Table 3, raw sludge samples had the highest value of total bacterial abundance. In the soil samples this value was two orders of magnitude smaller, and the TWW effluent sample had the lowest number of bacteria. We used Q-PCR to assess the relative abundance of ARGs in biosolid samples from various stages of the Shafdan WWTP and in TWW effluents samples in addition to the soil samples.

Table 3 Summary of total bacterial abundance in each sample as the number of 16S rRNA gene copies per gram or ml of sample

Site	Sample	Copy number of 16S rRNA per gram or ml
Rishon LeZion	Shafdan raw sludge	**1.0E+11** ±9.0E+09 g^{-1}
	Shafdan activated sludge	**4.8E+07**±1.2E+07 ml^{-1}
	TWW effluent	**3.6E+07**±3.3E+07 ml^{-1}
	TWW irrigated soil	**1.1E+09**±6.2E+08 g^{-1}
	FW irrigated soil	**1.5E+09**±3.3E+08 g^{-1}
Akko	TWW effluent	**2.7E+06**±1.1E+06 ml^{-1}
	TWW irrigated soil	**2.2E+09**±3.3E+08 g^{-1}
	FW irrigated soil	**1.4E+09**± 1.0E+09g^{-1}
Gilat	TWW effluent	**5.4E+06**±2.5E+06 ml^{-1}
	TWW irrigated soil	**6.5E+08**±3.3E+08 g^{-1}
	FW irrigated soil	**2.9E+08**±1.0E+08 g^{-1}
Kedma	TWW effluent	**7.0E+07**±1.3E+07 ml^{-1}
	TWW irrigated soil	**1.8E+09**±1.3E+09 g^{-1}
	FW irrigated soil	**1.3E+09**± 1.0E+09g^{-1}

*tet*O was only detected in the raw sludge, activated sludge and TWW of Shafdan samples. Its relative abundance was significantly lower ($p<0.05$) in the water sample than in the raw and activated sludge (Fig. 8).

Overall, on the Rishon LeZion site, the relative abundance of all the ARG were significantly higher in the different water samples (raw sludge, activated sludge and TWW) than in the soil samples ($p<0.05$) (Fig. 8).
The FW irrigated soil samples had higher levels off *sul*(1) *sul*(2) and *erm*(F) than in the TWW soil samples ($p<0.05$) (fig8).

In Akko and Gilat, we witnessed a similar trend. The water samples had higher relative abundance of ARG than the soil samples ($p<0.05$). Once more, the FW- irrigated soil samples from Gilat had higher levels off ARG than the TWW soil samples ($p<0.05$). In the TWW irrigated soil from Akko, none of the ARG's were above the detection limit (Fig. 9).

On the other hand, on the Kedma site we saw that the TWW irrigated samples had significantly higher levels of ARG than in the FW irrigated samples ($p<0.05$, Fig. 9).

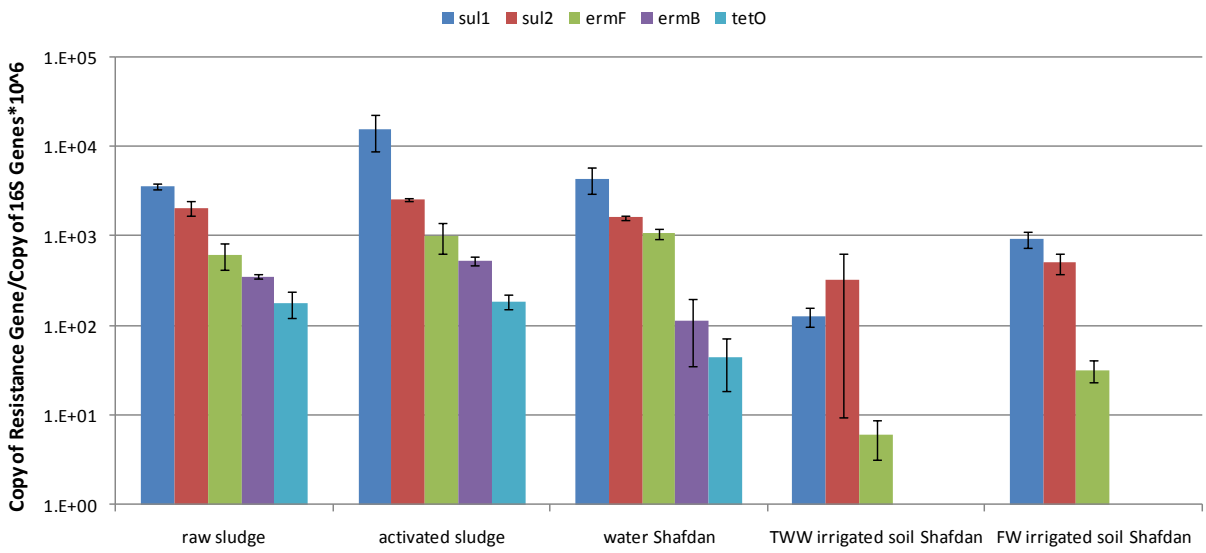

Figure 8 Copies of resistance genes normalized to the number of bacterial 16S rRNA genes *10^^{6} at different sites from Shafdan. Error bars indicate the standard deviation of three replicates in three independent Q-PCR runs.

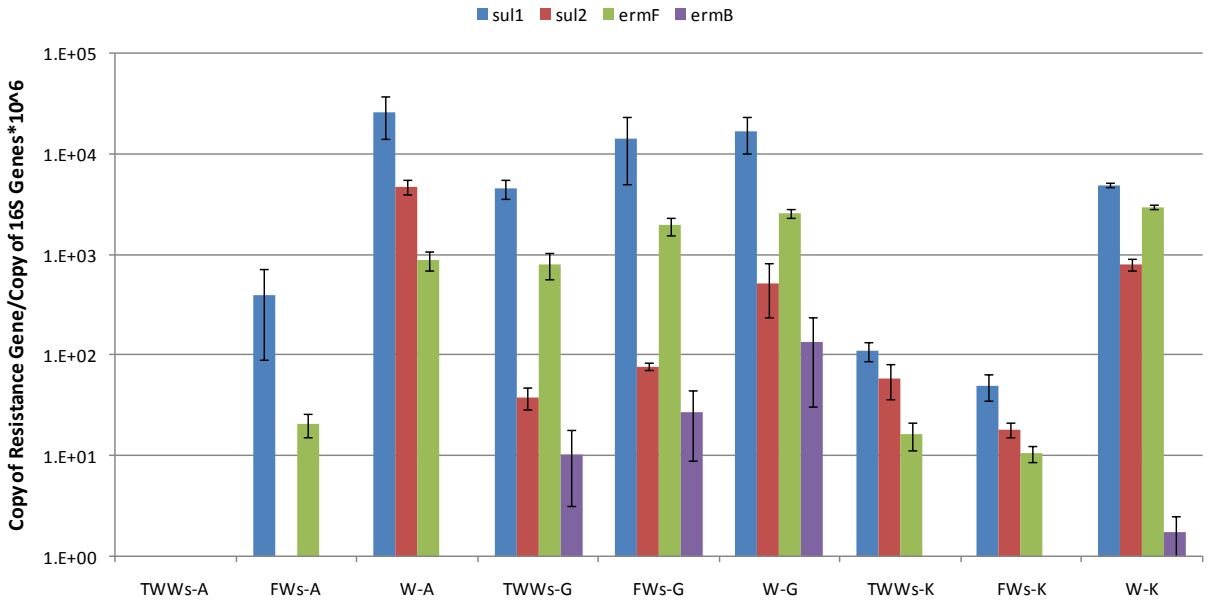

Figure 9 Copies of resistance genes normalized to the number of Bacterial 16S rRNA genes *10^{6} from Akko, Gilat and Kedma. Error bars indicate the standard deviation of three replicates in three independent Q-PCR runs. **TWWs**: TWW irrigated soil, **FWs**: FW irrigated soil, **W**: TWW effluent. **A-Akko, G-Gilat, K-Kedma**

3.3 Incubation experiment

In this experiment, soils were incubated for a period of three months during which they were periodically irrigated with water containing various antibiotic concentrations. Resistance of the soil bacteria to ciprofloxacin was measured as CFUs on antibiotic media relative to CFUs on media without antibiotic. Two concentrations of antibiotic were used in the growth media for the plate counts: low concentration- 0.4 µg/ml (Fig. 10)- generally used to detect plasmid-associated fluoroquinolone resistance, and high concentration-4 µg/ml (Fig. 11)- the characteristic minimal inhibitory concentration (MIC) for many bacteria harboring chromosomal associated fluoroquinolone resistance. The measured resistance levels ranged between 45.8%-80% and 7.5%-31% for the low and high concentration, respectively (Fig. 10, Fig. 11).

In the first month of the incubation experiment there was an increase in the relative levels of resistant bacteria for all three antibiotic treatments (0.4 μg/L, 4 μg/L and 40 μg/L) compared to the control (no antibiotic). This trend appeared on both low and high concentration of antibiotic on plates (Fig. 10, Fig. 11). Nevertheless, a statistically significant increase was only seen between the control soils (no ciprofloxacin) and the soils irrigated with 40 μg/L ciprofloxacin ($p<0.05$). Following the first 30 days of the experiment, there was a decline in the resistance levels of all of the samples and the difference between all the four treatments became negligible. Furthermore, 90 days from the beginning of the experiment the resistance levels returned to the same level as they were at the beginning of the experiment.

The bacterial community composition of the ciprofloxacin-irrigated and control soils were determined by PCR-DGGE analysis of 16S rRNA gene fragments, using general bacterial PCR primers. DNA was extracted from the control treatment (no ciprofloxacin) and the high ciprofloxacin-irrigated soils (40 μg/L) from three time points – directly following-, one month after- and three months after experimental initiation. Fig. 12 shows the community ribotypes obtained from the analysis.

Bacterial community ribotypes of the ciprofloxacin-irrigated soils did not differ from the control soils (Fig. 12). Furthermore, the patterns obtained after three months were similar to one month and time zero.

Multiplex PCR analysis was done to detect plasmid-mediated quinolone resistance genes-*qnr*A, *qnr*B and *qnr*S in the experimental soil samples. Libraries of 50 colonies that grew on ciprofloxacin-amended media from each of the four treatments were tested (Fig. 13). Overall, there were between 6-10 colonies in each library, which were suspected to possess the resistant genes (12-20%). There was no influence of the type of treatment (no Cip, Cip0.4μg/L, Cip4 μg/L and Cip40 μg/L) on the amount of the resistance genes that was found. Furthermore, the ciprofloxacin concentration on the plates (0.4 μg/ml or 4 μg/ml) also did not have an effect on the amount of resistance genes that were found.

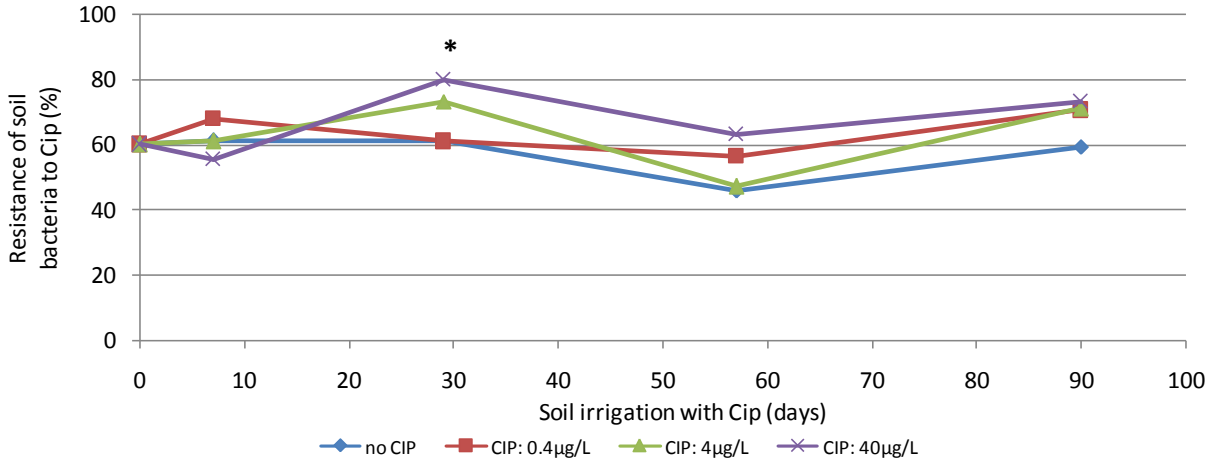

Figure 10 Resistance levels to Ciprofloxacin at low concentration on plates (0.4 µg/ml). Four treatments: irrigation with ciprofloxacin at 0.4 µg/L, 4 µg/L, 40 µg/L and control- no ciprofloxacin. * indicates statistically significant differences between the 40 µg/L treatment and the control treatment.

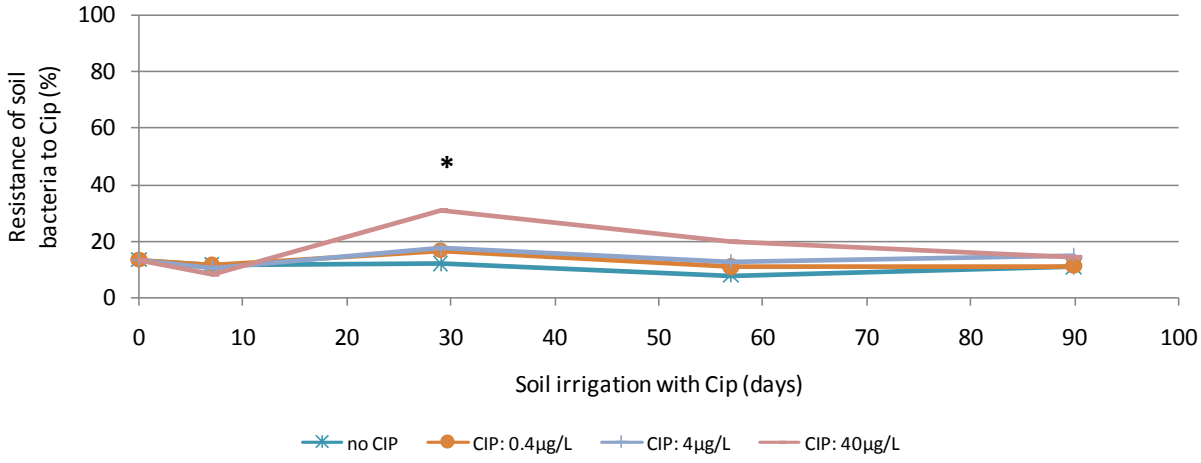

Figure 11 Resistance levels to ciprofloxacin at high concentration on plates (4 µg/ml). Four treatments: irrigation with ciprofloxacin at 0.4 µg/L, 4 µg/L, 40 µg/L and control-no ciprofloxacin. * indicates statistically significant differences between the 40 µg/L treatment and the control treatment.

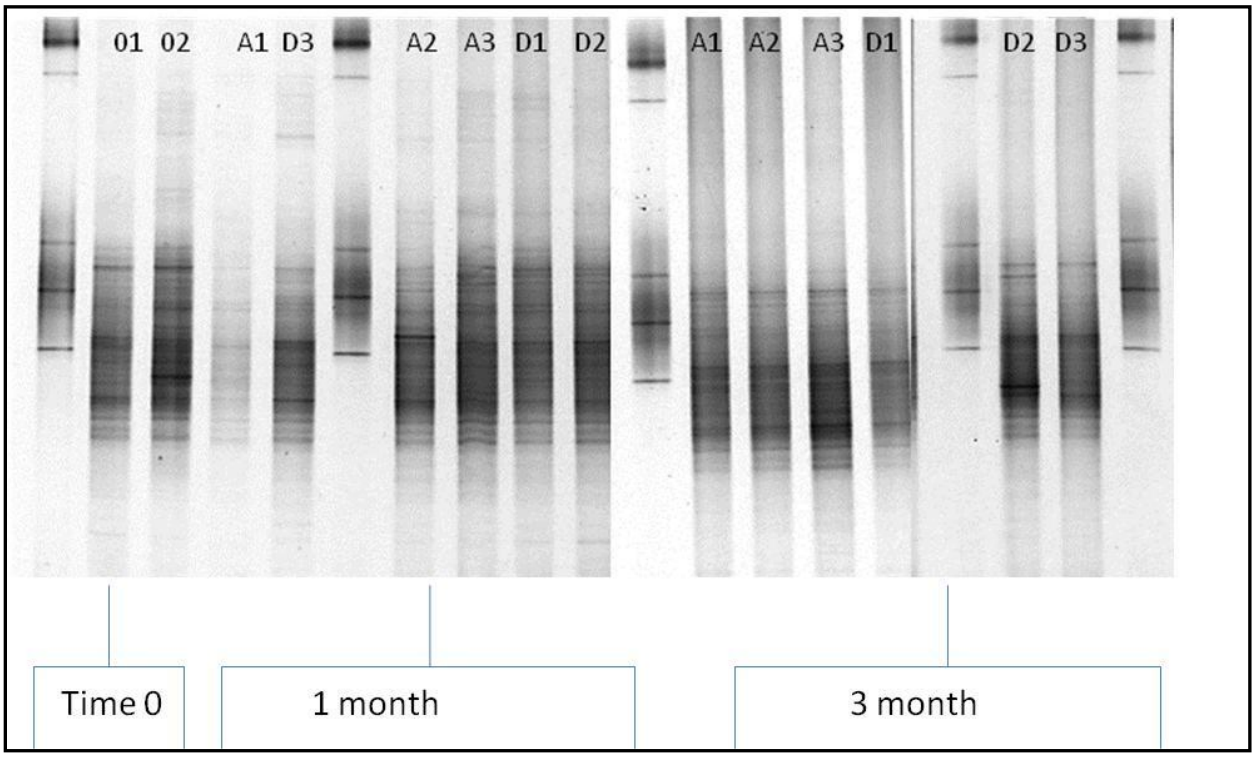

Figure 12 Effects of irrigation with ciprofloxacin for a period of 3 months on bacterial community composition. Bacterial community composition was compared based on PCR-DGGE patterns of 16S rRNA gene fragments. **0(**1-2): time0, **A(**1-3): control (no Cip), **D**(1-3): irrigation with ciprofloxacin at 40 µg/L. Non labeled lanes: markers.

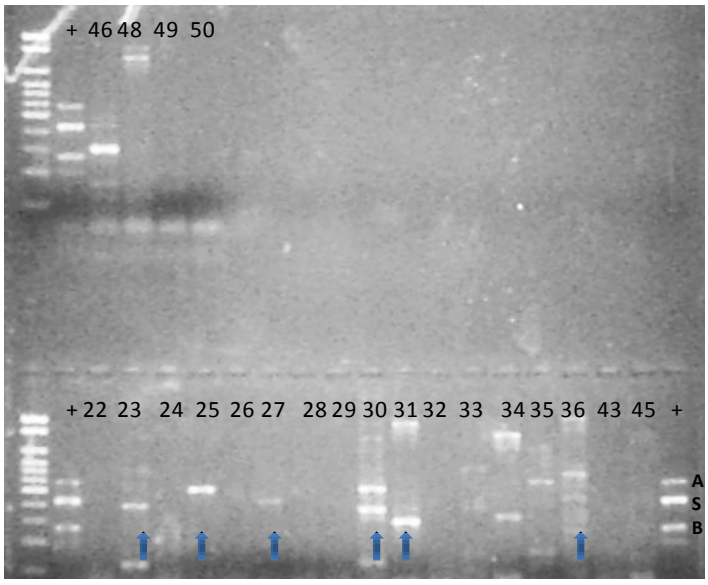

Figure 13 Multiplex PCR analyses of ciprofloxacin resistance genes: qnr A, S and B in resistant isolates (numbered in black). Lanes with (+): positive control. Blue arrows indicated colonies with suspected resistance gene

3 .4 Identification of resistant bacteria from Akko

Bacterial isolates from TWW- and FW- irrigated soil samples from Akko that were resistance to both antibiotics ciprofloxacin and tetracycline were phylogenetically analyzed by 16S rRNA gene PCR amplification and sequencing analyses (Fig. 14). Four of these isolates , which were detected only in the TWW-- irrigated soil, belonged to the *Flavobacterium* genus from the *Bacteroidetes* phylum. Furthermore, we identified the presence of both gram- positive and gram-negative bacteria associated with a wide array of bacterial genera.

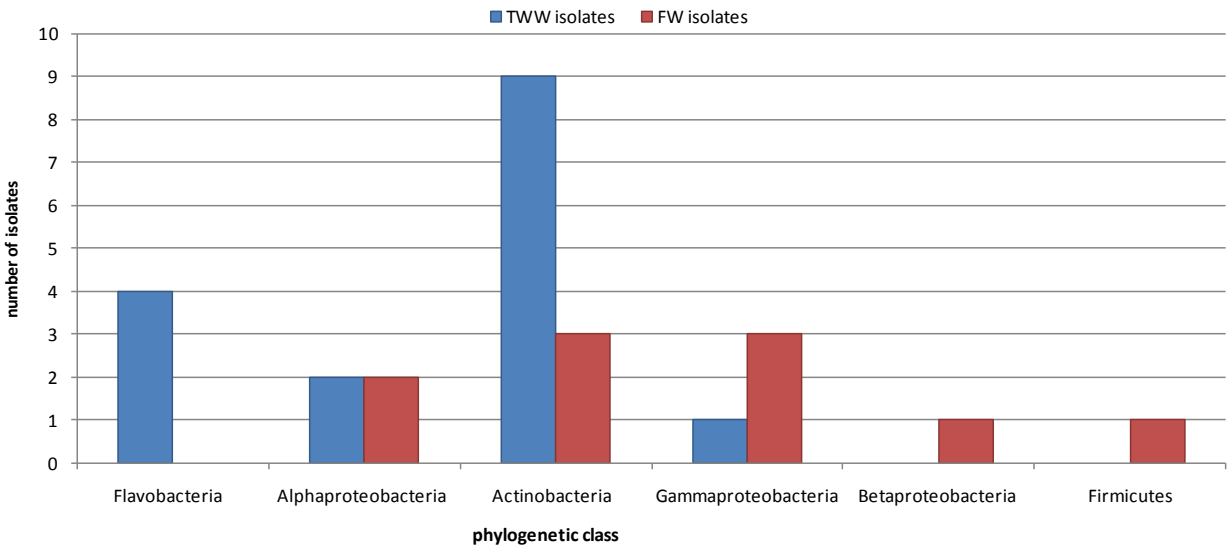

Figure 14 Phylogenetic distribution of multi-resistant (ciprofloxacin and tetracycline) isolates from TWW- and FW-irrigated soils from Akko

3.5 Physiological characterization of flavobacteria isolates

Further analyses were made on flavobacteria resistant isolates from the soils in Akko (isolates: E21, D43 and K4) and they were compared to root- associated strains (isolates: M52, M34 and M1) isolated from pepper roots in a greenhouse experiment conducted in the lab.
Physiological analysis of these isolates has revealed a variety of enzymatic activities (Table 4). In addition, all of them could grow at a wide range of temperatures, had rapid gliding motility and produce carotenoid-like flexirubin pigments (Fig. 15, Fig. 16). PCR analyses using specific primers showed that they possess a number of antibiotic resistance genes (Table 4, Fig. 17).
All isolates from Akko were resistant to all of the antibiotics (except for erythromycin in isolates D43 and K4) and had b-Galactosidase activity differing from the root-associated isolates, which were sensitive to tetracycline and ciprofloxacin. Furthermore, they did not have b-Galactosidase activity (Fig. 18, Table 4).

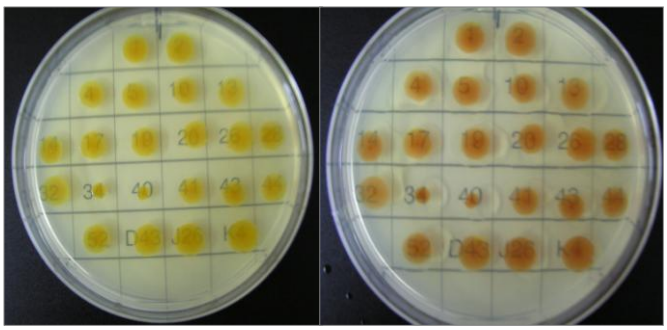

Figure 15 Analysis of flavobacteria isolates for flexirubin pigment. Flexirubin-positive cells were yellow at neutral pH and red under alkaline conditions (10N KOH).

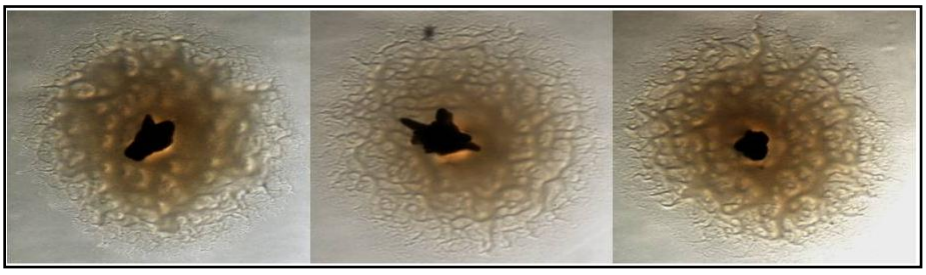

Figure 16: Gliding motility of flavobacteria isolates: K4, M52, D43 (left to right)

Table 4 Physiological and antibiotic resistance characterization of flavobacteria isolates (ND-not detected)

	Isolate name	E21	D43	K4	M1	M34	M52
	closest relative (%16S RNA gene identity)	*Chryseobacterium ginsengisoli* (99%)	*Flavobacterium anhuiense* (100%)	*Flavobacterium johnsoniae* UW101 (96%)	*Flavobacterium anhuiense* (99%)	*Flavobacterium johnsoniae* (99%)	*Flavobacterium anhuiense* (99%)
Antibiotics resistance	Tetracycline	+++	++	++	-	-	-
	Ciprofloxacin	++	++	++	-	-	-
	Sulfamethoxazole	++	++	++	++	-	++
	Ampicilin	+++	+++	+++	+++	+++	+++
	Earythromycin	+++	-	-	++	++	++
	Kanamycin	+++	+++	+++	+++	+++	+++
	Streptomycine	+++	++	++	++	-	-
	Gliding PY2	+	+	+	+	+	+
	Flexirubin	+	+	+	+	+	+
Enzimatic activiyy	Protease	ND	-	-	+	week	+
	Catalase	+	+	+	+	+	+
	NH3 production	+	+	+	+	+	+
	b-Galactosidase	+	+	+	-	-	-
	Resistant genes	*tetC tetO*	*tetE,tetC*	*tetE, tetC,tetB,tetM*	*tetC,tetO,qnrA*	*tetC,tetE,sul2,qnrA,,ermF*	*tetC,tetE, sul2,qnrA*

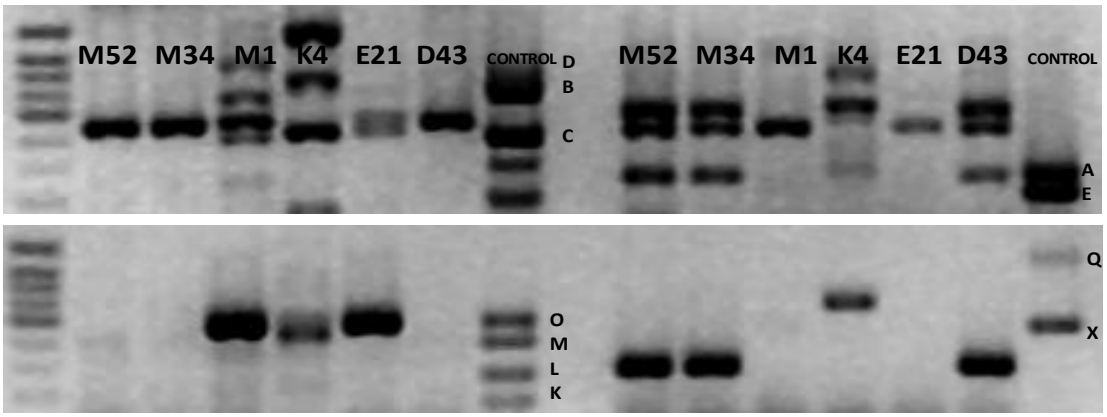

Figure 17 Multiplex PCR analysis of tetracycline resistance genes (tet: D,B,C,A,E,O,M,L,K Q and X)

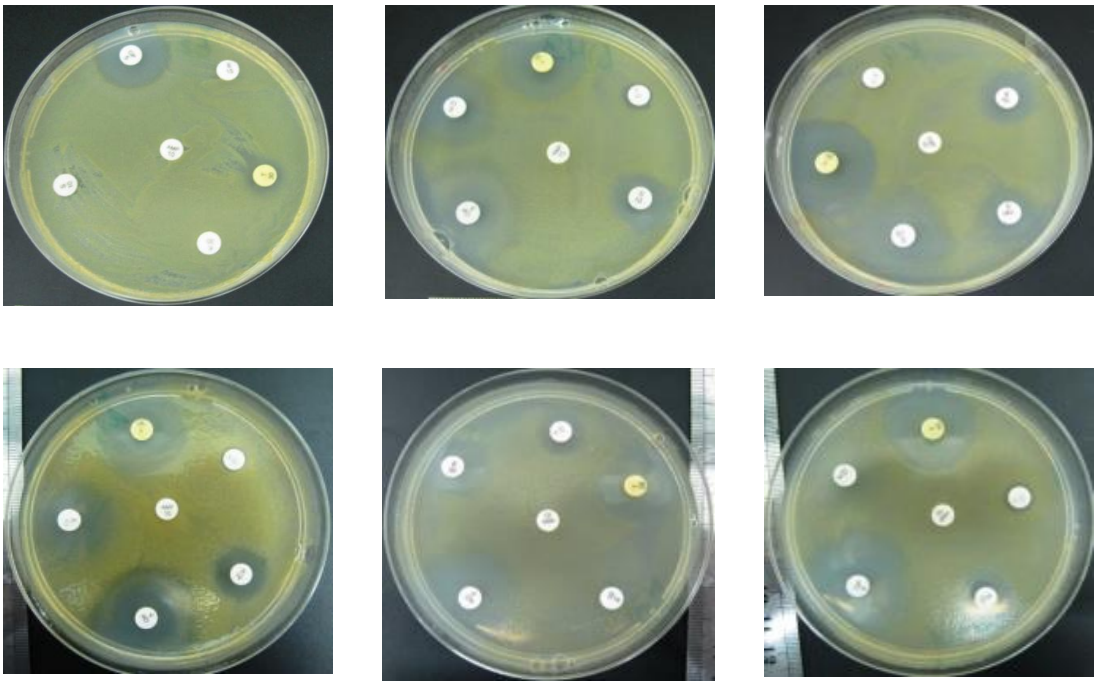

Figure 18 Determination of antibiotic resistance in flavobacteria isolates on LB plates using the antibiotic disk diffusion method. The antibiotic discs that were used: ciprofloxacin, erythromycin, kanamycin, streptomycin, ampicilin and tetracycline. Top: isolates: E21, D43 and K4. Bottom: isolates: M52, M34 and M1 (left to right).

4. Discussion and conclusion

4.1 Heterotrophic bacterial plate counts on antibiotic-selective media

Antibiotics play an important role in controlling infectious disease. The past few decades, however, have witnessed a steady increase in the number and diversity of antibiotic resistant bacteria- rendering some bacterial infections untreatable (Ghosh and LaPara, 2007). In this study, antibiotic-resistant bacteria were isolated, quantified and characterized in soils irrigated *in tandem* with TWW and FW. The goal of this study was to determine the impact of residual antibiotic compounds and antibiotic resistant bacteria in TWW on the occurrence and abundance of cultivable AR bacteria and ARG's in these soils.

Contrary to our hypothesis, there was no difference in levels of tetracycline, ciprofloxacin and erythromycin resistance between TWW irrigated soils, and FW irrigated soils. This phenomenon was observed in both the Akko and the Rishon LeZion sites. Moreover, levels of tetracycline resistance in Akko soils and ciprofloxacin resistance in Rishon soils were significantly higher in the FW irrigated soils than in the TWW irrigated soils (Fig. 2, Fig. 6).

The different Akko (September 2010 and July 20110) and Rishon LeZion (August 2011) profiles showed substantial variance in the resistance levels to all three antibiotics, which ranged between 2%-16%. This variation could be the result of various factors. For example, seasonal fluctuations in the microbial community composition, the type of soil and bias associated with the serial-dilution plating technique. This technique is often inaccurate because bacteria have the tendency to create aggregates and not fully separate in the dilutions. For instance, the higher level of resistance to erythromycin seen in the Rishon LeZion profile compared to the Akko profile could be a result of some resistant bacteria that did not separate during the serial-dilution.

A very interesting phenomenon was observed in the September profile from Akko, where, the soil moisture had a significant positive effect on bacterial resistance levels to both tetracycline and ciprofloxacin (Fig. 2). One possible reason could be that is that there is positive correlation

between the soil moisture and the microbial activity (Barros et al., 1995). Soil moisture directly affects the physiological status of bacteria (Harris, 1981). Water availability affects the osmotic status of bacterial cells and can indirectly regulate substrate availability, diffusion of gases, soil pH, and temperature (Griffiths et al., 2003). Further, moisture deficit will stress plants and, as a result, may affect bacterial communities through changes in rhizodeposition and nutrient allocation below ground (Lynch and Whipps, 1990).The population of the soil bacteria is more active in a wet environment (Barros et al., 1995) and therefore more resistant bacteria could be present. Furthermore, in hydrated environments lateral genetic exchange of resistant genes between the soil bacteria may occur more frequently (Paul, 1999), which can lead to, elevated levels of resistance.

The heterotrophic bacterial plate counts from the water samples showed a different trend. Both in Akko and Rishon LeZion, in the FW samples used for irrigation, total bacterial counts were very low and AR bacteria were not detected (Fig. 3, Fig. 4, Fig. 7). Relatively high levels of total bacteria were detected in the TWW samples used for irrigation ($3.5x10^5$ ml^{-1}-$1.07x10^6$ ml^{-1}), though, AR bacterial levels were low (0.05%-0.21%) relative to the soil samples. However, given the irrigation rates of the sample sites (approximately 130 and 97 L m^{-2} $month^{-1}$ in the irrigation season), it can be roughly calculated that the number of total bacteria that enters the soils each month in the irrigation season, throughout irrigation with TWW is 9.7*10^^{10} and 4.5*10^^{10} (for Akko and Rishon LeZion, respectively). Furthermore, approximately 9.710^^{6}-9.7*10^^{7} and 1.3*10^^{7} -1.3*10^^{8} of AR bacteria are transmitted to each m^2 of soil each month in the irrigation season (Akko and Rishon LeZion respectively). This indicates that although a large number of water-associated AR bacteria enter the soil through the irrigation, they probably do not survive in a viable state in the soil and do not affect the soil resistance levels.

Based on analysis of the results of resistance levels in the soil and water samples we suggest that the antibiotic resistance observed in soils is almost entirely due to native soil bacteria, and is not influenced by irrigation with TWW. High levels of AR bacteria and ARG's in soil are supported in studies by D'costa et al. (2006) and others (Allen et al., 2008; Riesenfeld et al., 2004; Torres-Cortés et al., 2011). These results also support the work of Gosh and La Para

(2007), which showed that application of animal manure onto agricultural land in agronomically acceptable rates, did not lead to an increase in the quantity of resistant bacteria.

It can be said that soil bacteria form a strong and stable community that is not easily affected by the influx of foreign bacteria. Regarding selective pressure due to presence of antibiotic compounds or other compounds in the TWW, it can be assumed that either: (a) the concentrations that enter the soil from the TWW are too low to select for AR bacteria; (b) antibiotic compounds bind to clay particles or organic matter in the soil (Navon et al., 2011) and therefore do not exert selective pressure on the soil bacteria; or (c) those compounds are rapidly metabolized by native bacteria.

Soil microbial communities can be characterized by having a relatively short generation time and a high metabolic rate and thus can rapidly respond to environmental changes (Prosser et al., 2007). A healthy bacterial community in soil can increase soil organic matter content, help prevent plant disease and decompose contaminants .The microorganisms in the soil are responsible for filtering, buffering, degrading, immobilizing, and detoxifying organic and inorganic materials, including industrial and municipal by-products and atmospheric deposits. Furthermore, soil can absorb contaminants from both water and air.

4.2 Quantification of ARG's in agricultural soils

The limitation of the cultivation- based approach described above is that it is known to detect only a small fraction (about 1%) of the total soil bacterial community (Amann et al., 1995). Therefore, it can only give us a partial understanding of the bacterial dynamics in soil and water environments. In order to try to expand our understanding of how TWW-irrigation affects AR dynamics in soil, we used a cultivation–independent approach, applying real time PCR to assess the relative abundance of specific ARG's in the different environments. However, this cultivation-independent approach is complicated because it generally targets a specific antibiotic resistance gene, whereas resistance to many antibiotics can be attributed to

numerous ARG's. For instance, more than 40 different genes are known to encode for tetracycline resistance (Roberts, 2005). Furthermore, PCR analysis of resistance genes detects the DNA of both live and dead cells.

In the current study, the real-time PCR approach was used in order to assess the prevalence of ARG's in: (a) different stages of the wastewater treatment process; (b) in TWW used for irrigation; and (c) among the population of the soil bacteria in agricultural soils irrigated with TWW and FW in the four targeted experimental plots. Normalizing the number of copies of each resistance gene to the number of copies of bacterial 16SrRNA genes provides a means to assess the level of resistance proportional to the size of the overall population. The total number of copies of 16SrRNA genes in the different soil samples was found to be relatively consistent between sites (except for Gilat, which was one order of magnitude lower).

Other researchers have recently investigated the abundance of ARG's in anthropogenically impacted environments. A recent study (Knapp et al., 2009), examined the changes in antibiotic resistance in soil bacteria in samples taken and archived in the Netherlands between 1940 and 2008. According to the study, there is growing evidence that resistance to antibiotics in the sampled soils increased both in native and pathogenic bacteria, posing an emerging threat to public and environmental health in the future. A few studies (Chee-Sanford et al., 2009; Pei et al., 2006; Storteboom et al., 2010) demonstrated the occurrence of ARG's in animal feedlot lagoons (from farms that administer sub-therapeutic doses of antibiotics to livestock as growth promoters) and in areas immediately adjacent to them. These ARG's can spread through soil and water matrixes, where they can potentially reach food webs through drinking water and irrigation. For instance Chee-Sanford et al., show a high occurrence of tetracycline resistance genes in the groundwater underlying swine production facilities. More recently, Pei et al. reported an increase in ARG's in river sediments corresponding to increases in human and agricultural activity.

Surprisingly, the overall trend revealed in this study, contrary to our initial hypothesis and to other studies published, was that soils irrigated with TWW did not show higher relative abundance of ARG's compared to FW irrigated soils (Fig. 8, Fig. 9). This phenomenon was

consistent in all of the study sites except for Kedma. This finding further reinforced what we observed in the cultivation- based approach.

At the different stages of water treatment (raw sludge, activated sludge and the TWW) in the Rishon LeZion site, the relative abundance of all of the ARG's analyzed showed similar trends. The most prevalent gene in all of the sites was *sul*1. In some cases, its relative abundance was up to four orders of magnitude higher than the other ARG's. This finding correlates with other publications that assessed the prevalence of sulfonamide resistance genes (Byrne-Bailey et al., 2009; Pei et al., 2006; Pruden et al., 2006). High prevalence of sulfonamide resistance has been observed in gram-negative bacteria from animals and humans all over the world (Bean et al., 2009). *Sul1* has almost exclusively been found on large conjugative plasmids and on class 1 integrons (Hammerum et al., 2006). Genetic localization of *sul* genes on efficient mobile genetic structures probably contribute to the wide spread of sulfonamides resistance. Plasmids unavoidably play an important role in carrying and mobilizing *sul* genes (Radstrom et al., 1991).

The abundance of these genes could be due to natural selection triggered by the presence of residual concentrations of sulfonamides in the environment. Sulfonamides exhibit weak sorption to soil and, likely are the most mobile of the antibiotics (Tolls, 2001). This is also evident from the work of Avisar who detected sulfamethoxazole (SMX) in an aquifer under a field irrigated with TWW (Avisar et al., 2009).

Soil characteristics could explain some of the differences in the prevalence of the ARG's in all of the four sites. Silt and loamy sand soils are known to have lower adsorption properties for antibiotics (especially for tetracyclines and sulfonamides) than clay soils (Boxall et al., 2002; De Liguoro et al., 2003; Pruden et al., 2006). Therefore, we would expect to find more ARG's in Akko and Kedma soils than in Rishon LeZion and Gilat. However, this was not revealed in the results. It may be that the antibiotics that are bound to soil particles are not active and those in the water phase are, therefore this could explain the lower AR levels in the clay soils. Another possible reason for this is the variation in the efficiency of extracting the DNA. DNA extraction from clay soils is especially difficult (Andersen et al., 1998; Braid et al., 2003), given the tight binding of DNA strands to clay soil particles (Cai et al., 2006; Khanna and Stotzky., 1992).

Additionally, extracellular DNA binds to and is co-purified with soil humic substances (Crecchio and Stotzky, 1998), which inhibit the activity of enzymes such as restriction endonucleases and DNA polymerase (Burgmann et al., 2001; Dong et al., 2006).

The persistence and transport of pathogenic microorganisms in the environment continues to be a concern for environmental quality, food safety, as well as human and animal health. Gavalchin and Katz (1994) concluded that the longer an antibiotic persists in the soil in an active form, the greater the potential for native soil bacterial populations to be affected. Biologically active antibiotics (or antibiotic breakdown products) introduced to the soil may confer a selective advantage for commensal soil bacteria carrying resistance genes, or exert selective pressure for acquisition of resistance genes in commensal soil populations (Chee-Sanford et al., 2009). On the other hand, the length of time that introduced organisms can persist in the soil varies with temperature, moisture, pH, and the indigenous community present. A recent study examining the survival of *E. coli* and *Salmonella typhimurium* applied to a clay soil with swine effluent, found considerably short persistence times (21 d for *E.coli* and 7 d for *Salmonella typhimurium*) (Boes et al., 2005). Sengelov et al., (2003) studied the persistence of cultureable aerobic, heterotrophic, tetracycline resistant bacteria in four Danish farm soils following variable rates of pig slurry application. Five months following application, the proportion of tetracycline resistant bacteria in all of the treated soils had returned to levels within the range of the non-manured control samples. An additional study found that enterococci levels decreased approximately five orders of magnitude in soil microcosms over a 5-wk period(Andrews Jr et al., 2004). These studies suggest a certain rebound effect of the native bacterial community in the soil following an initial spike, although there may be sufficient time and opportunity for mechanisms of resistance selection and gene transfer to occur.

Accordingly, this rebound effect is also seen in this study. The relative abundance of ARG's in the TWW is high compared to their abundance in the soils. It seems that when the resistance genes (within the resistant bacteria) reach the soils through the irrigation with TWW they do not proliferate. This may be due to the fact that their concentration is not high enough to make a significant change. Furthermore, the concentration of the antibiotics in the TWW may not be

high enough to create selective pressure that can affect the population of the soil bacteria. The soil acts as some kind of buffer to the influx of bacteria and antibiotic compounds that comes with the irrigation. Another reason for why there is not selective pressure in the soils irrigated with TWW is that antibiotic compounds can attach to the organic matter in the TWW and to the clay particle in the soil (Navon et al., 2011). Consequently, the antibiotic is not available to soil bacteria. In addition, TWW-derived AR bacteria may not survive in the soil due to: (a) different environmental conditions (nutrient deficiency, lower temperatures, radiation, lower levels of hydration, etc), (b) lack of capacity to compete for resources with the native soil bacteria, and (c) the presence of antibiotics and other bactericidal chemicals in the soils.

The answer of why the relative abundance of the ARG's in many of the soils was significantly higher in the FW irrigated soils than the TWW irrigated soils is still not certain. One explanation is that the TWW contains different kinds of organic matter, which can affect the composition of the soil bacteria. Bacteria without plasmids containing resistant genes (which are energetically expensive to maintain) may outcompete resistant strains. The AR sensitive strains may be more capable of handling TWW-associated xenobiotic compounds, and therefore they are more abundant than the resistant strains. Consequently, when the prevalence of the ARG's was examined by the real time PCR, soils irrigated with TWW showed lower levels than FW irrigated soils.

There are two limitations to this study. First, we did not have data on the concentration of the antibiotics in the different water and soil samples, due to technical reasons. However, there are several reports on residual concentrations of antibiotics in the effluents of WWTP's and in the soils themselves (Avisar et al., 2009; Chefetz et al., 2008; Navon et al., 2011). Second, we did not test the abundance of the ARG's at different time period as we did in the cultivation- based approach (in the irrigation season and in the wet season), which could have given us a better understanding on the processes in the soil microbiome that are influenced by irrigation with TWW.

4.3 Incubation experiment

In a separate part of my research, I incubated agricultural soils irrigated with water containing different concentrations of the antibiotic ciprofloxacin (0.4, 4 and 40 μg/L) for a time of three month to assess the impact of selective pressure on the diversity, distribution and relative abundance of AR bacteria. In these incubation experiments, we expected to see an increase in the relative abundance of heterotrophic AR bacteria [growing on media with ciprofloxacin at the minimal inhibitory concentration (MIC): 0.4 and 4 μg/ml] in the treatments irrigated with antibiotics compared to the control, due to the selective pressure caused by the antibiotic. Nevertheless, induced AR was only witnessed in the first month of incubation, and was significant only in soil samples irrigated with high concentrations of antibiotic. DGGE (microbial community fingerprinting) analysis also did not show any significant differences in the bacterial community composition between the soils irrigated with antibiotic-amended water relative to the control, throughout the three month of the experiment (Fig. 12). After the first month, the resistance levels of soil bacteria returned to the initial point in all of the treatments (Fig. 10, Fig. 11).

This phenomenon is consistent with the heterotrophic plate counts on antibiotic-selective media in the Akko and Rishon Lezion sites and with the direct quantification of ARG's in the soil samples using real time PCR. Apparently, the concentration of ciprofloxacin that was used in the irrigation of this experiment was not high enough to exert selective pressure. Even the highest concentration- 40 μg/L which is one order of magnitude higher than the concentration found in effluents of WWTP (Bhandari, 2008; Karthikeyan and Meyer, 2006), was not enough to make a change. Again, it is likely that there was a rebound effect of the soil, which started with initial spike of the resistance level and then returned to the natural level at the beginning.

There could be a number of reasons why we did not see the selective pressure effect we expected. During the experiment there was no addition of organic matter to the soil, therefore it is likely that the bacteria were not active and possibly went into a dormant or stationary growth stage. Many antibiotics including ciprofloxacin are much more toxic to bacteria in the growth phase than in the stationary phase and therefore the sensitive soil bacteria may not

have been affected by the antibiotics. In addition, mechanisms that can affect the resistance of soil bacteria (HGT, mutation) are more common in the growth stage rather than the stationary stage that many bacteria probably went into. Lack of induction of AR bacteria may also be the result of the strong interactions of antimicrobials with natural organic matter and humic substances, which are believed to reduce their bioavailability, retard their abiotic and biotic degradation, and facilitate their persistence in soils and aquatic sediments (Aristilde and Sposito, 2010). Therefore, it is possible that ciprofloxacin bound to the humic substance or other organic matter and was not available to the bacteria to exert selective pressure.

4.4 Identification of resistant bacteria and characterization of flavobacteria isolates

Phylogenetic identification of resistant bacteria isolated from TWW and FW irrigated soil samples from Akko revealed a variety of bacteria associated with six different phyla, which represent a part of the soil microbiome. This included several root and bulk soil isolates from the flavobacterial family, which showed multiple antibiotic resistance and a wide range of extracellular enzymatic activity. Furthermore, there were some difference between the root-associated isolates and bulk soil isolates in the resistance profile and some of the enzymatic activity. Although the soil isolate library defined above represents only a small fraction of the natural soil microbial population, it demonstrates the high taxonomic diversity of antibiotic resistance in native soil bacteria. In order to achieve a better understanding of the composition of the soil microbial populations in TWW and FW irrigated soils we need to apply different approaches like the Next generation (Illumina, 454, etc) sequencing. The seasonal effect of TWW irrigation on microbial communities in agricultural soil was recently tested (Frenk et al., 2011). They showed a shift of the bacterial community composition due to TWW irrigation, compared to fresh water irrigation, and consequently a return to a winter "steady state". These results indicate a temporal seasonal variation, in the microbial community composition that is strongly enhanced by TWW irrigation. Although TWW-irrigation appears to affect the overall microbial community composition of the soil, it does not seem to affect the relative abundance of antibiotic resistance in this community.

More and more studies are demonstrating that soils represent a vast natural reservoir of antibiotic resistant bacteria and ARG's and that these genes have existed long before human use of antibiotics (Allen et al., 2008; D'Costa et al., 2011). For example, a recent study by D'Costa reported on a targeted metagenomic analyses of rigorously authenticated ancient DNA from 30,000-year-old Beringian permafrost sediments and the identification of a highly diverse collection of genes encoding resistance to b-lactam, tetracycline and glycopeptide antibiotics. These results show conclusively that antibiotic resistance is a natural phenomenon that predates the modern selective pressure of clinical antibiotic use. In addition, it is believed that ARG's evolved in the natural environment and subsequently moved into clinically relevant bacteria through horizontal gene transfer (Martinez, 2009). It has been suggested that soils may continue to be a reservoir of new ARG's. Future research should focus on identifying unclassified ARG's from native soil bacteria, and determining the potential of these genes to be transferred to clinically relevant bacteria.

To conclude, the purpose of this study was to estimate the affect of irrigation with TWW on the antibiotic resistance among the soil bacterial populations. The three major conclusions of this study were:

i. Based on conventional isolation methods and a cultivation–independent molecular approach, it appears that irrigation with TWW does not cause an increase in antibiotic resistance levels of the soil bacteria. This is despite of the high levels of AR bacteria and ARG's that are present in the TWW.

ii. Soil moisture induces bacterial resistance to tetracycline and ciprofloxacin, in both TWW and FW irrigated soils.

iii. The significant levels of antibiotic resistance detected in the agricultural soils analyzed in this study, are mainly attributed to native bacteria and not to wastewater-derived bacteria.

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תודה:

לאדי, על העזרה והתמיכה לאורך כל הדרך, שהדלת הייתה תמיד פתוחה לכל בעיה וקושי ושהכול נעשה תמיד עם חיוך.

למקס, על העזרה, ההתעניינות התמידית והאווירה.

לכל שאר חברי המעבדה, על העזרה ועל זה שהפכתם את מכון וולקני למקום כייפי להיות בו.

לאדוארד וזוהר, על העזרה והרעיונות.

וכמובן לניב.....

תודה לכולכם

בארבע קרקעות חקלאיות המושקות במי קולחין ובמים שפירים. יושמו שיטות בידוד מיקרוביאליות

קלאסיות ואנליזות מולקולאריות לא תלויות-בידוד, כגון quantitative real time PCR המגבירות את הגנים: tet*O*, erm*B*, erm*F*, *sul*1, *sul*2 .

היפותזת העבודה הייתה שהשקיה במי קולחין מגבירה את רמת העמידות בקרקעות אלו, עקב לחץ סלקטיבי הנוצר משאריות של תרכובות אנטיביוטיות ומנוכחות חיידקים עמידים לאנטיביוטיקה במי הקולחין. ציפינו לגלות רמות גבוהות יותר של חיידקים עמידים לאנטיביוטיקה בקרקעות המושקות במי קולחין יחסית לאותן קרקעות המושקות במים שפירים. אף על פי כן, הממצאים העיקריים של עבודה זו לא תמכו בהיפותזה וחשפו נקודות מעניינות:

i. בהתבסס על שיטות בידוד קונבנציונאליות ואנליזות מולקולאריות לא תלויות-בידוד, השקיה עם מי קולחין אינה גורמת לעלייה ברמת העמידות בקרב חיידקי הקרקע. זאת למרות רמות גבוהות של חיידקים עמידים לאנטיביוטיקה ושל גנים לעמידות הקיימות במי הקולחין.

ii. לחות הקרקע מעודדת עמידות חיידקים לטטראציקלין ולציפרופלוקססין, הן בקרקעות המושקות במי קולחין והן במים שפירים.

iii. הרמות המשמעותיות של עמידות לאנטיביוטיקה שנתגלו בקרקעות החקלאיות במחקר זה, משויכות בעיקר לחיידקים האנדמיים בקרקע ולא לחיידקים ממקור מי קולחין.

תקציר

במדינה בעלת אקלים סמי-ארידי כמו ישראל, מים הם משאב הנמצא בחסר ולמרות זאת, בעשור האחרון כ-60% מכלל כמות המים בארץ מופנית לחקלאות. דבר זה אפשרי בזכות היכולת למחזר מים. מי קולחין הפכו למקור השקיה חשוב בישראל ובשאר חלקי העולם עקב הלחץ של גדילת האוכלוסייה ומחסור במים באיכות גבוהה. היום, כ-50% מההשקיה בחקלאות בארץ היא באמצעות מי קולחין.

להשקיה במי קולחין ישנם יתרונות משמעותיים בכך שהם מקלים על המחסור במים שפירים, ועל ידי כך מאפשרים התפתחות חקלאית כתגובה לצרכים הגדלים של האוכלוסייה. אולם, ישנם מספר חסרונות וסיכונים פוטנציאלים עם השקיה במי בקולחין לטווח ארוך, שאינם ידועים עד היסוד.

מי קולחין עלולים להכיל מיקרואורגניזמים פתוגניים וכימיקלים ממקור אנתרופוגני כגון קוטלי מזיקים וקבוצה גדולה של תרכובות מזהמות הנקראת Pharmaceuticals and Personal Care Products (PPCPs). הדאגה העיקרית היא שתרכובות כגון הורמונים ואנטיביוטיקות אשר מגיעות לקרקעות ולמקורות מים דרך ההשקיה במי קולחין, יכולות להשפיע בצורה מזיקה על הסביבה ובריאות הציבור כתוצאה מאינטראקציות ביולוגיות.

בעבודה זו, הערכתי את ההשפעה הסביבתית של קבוצה אחת הקשורה לPCPPs- אנטיביוטיקות, על ידי אומדן רמת העמידות לאנטיביוטיקה ורמת הגנים לעמידות של חיידקי הקרקע, בקרקעות המושקות במי קולחין ובמים שפירים.

חיידקי קרקע אנדמיים מייצרים ונתקלים בספקטרום רחב של אנטיביוטיקות, דבר המוביל להתפתחות מנגנונים לעמידות חדשים ומגוונים. חיידקים אלו יכולים לשמש כמאגר לדטרמיננטות של עמידות היכולות לעבור לאוכלוסייה מיקרוביאלית הקשורה לאדם. מבחינה מולקולארית, עמידות לאנטיביוטיקה יכולה להתפתח ממוטציה ספונטנית של גנים, או על ידי העברת גנים לעמידות קיימים, באמצעות העברת גנים אופקית (HGT) בין ובתוך מינים. שני מנגנונים אלו מתרחשים ספונטנית, בשכיחות נמוכה יחסית. אולם, חשיפה לאנטיביוטיקה יכולה לגרום לשגשוג מהיר של מנגנונים אלו, כתוצאה מסלקציה טבעית.

התרבות חיידקים עמידים לאנטיביוטיקה עקב סלקציה טבעית באזורים בעלי השפעה אנתרופוגנית (כגון אדמות מדושנות בזבל בע"ח, נהרות המקבלים זרימה של ביוב מטופל וכדומה) תועדה היטב. אף על פי כן, אין מחקרים אשר מתמקדים בהשפעה הישירה של השקיה בקולחין על רמת העמידות לאנטיביוטיקה, בקרב חיידקי הקרקע.

במחקר זה, הערכתי את רמת העמידות כנגד ארבע אנטיביוטיקות שונות, אשר נבדלות אחת מהשנייה בהרכב הכימי, במקור ובמנגנון הפעולה: טטראציקלין, אריתרומיצין, סולפאנאמיד וציפרופלוקסצין,

השפעת השקיה במי קולחין על התפתחות עמידות לאנטיביוטיקה בקרקעות חקלאיות

"עבודת-גמר מוגשת לפקולטה לחקלאות, מזון וסביבה על שם רוברט ה.סמית של האוניברסיטה העברית בירושלים לשם קבלת 'תואר מוסמך למדעי החקלאות'"

יעל נגראנו

רחובות, דצמבר 2011