

**IMPACT OF WASTEWATER EFFLUENTS ON THE
STRUCTURE AND ANTIBIOTIC RESISTANCE OF
NATIVE MICROBIAL COMMUNITIES**

by

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ABSTRACT OF THE THESIS

Impact of Wastewater Effluent on the Structure and Antibiotic Resistance of Native Microbial Communities

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In this work, we investigated the impact of antibiotic resistant bacteria from final effluents of a sewage discharge on the selection of antibiotic resistance in natural bacterial communities in sites downstream the treatment plant. Samples were collected from the final effluents of the wastewater treatment plant and from two sites along the receiving stream. A fourth site, upstream of the discharge, was used as control. Phylogenetic analysis of the 16S rRNA gene sequences was performed to derive the composition and structure of the four microbial communities. Clone library data and TRFLP profiles showed that the four communities were all dominated by Betaproteobacteria, which constituted approximately two thirds of the final effluent community. The diversity and abundance of other bacterial phylotypes varied across sites. Bacterial diversity included 9 phylotypes in the final effluent, 15 and 13 phylotypes

respectively in the sites downstream the discharge. Furthermore, bacteria were isolated from the same samples using culture-dependent techniques, which allowed to link antibiotic resistance to particular bacterial species. While clone libraries were dominated by Betaproteobacteria, culturable isolates mainly belonged to the class of Gammaproteobacteria. Specifically, the analysis of the isolates from the final effluent showed the presence of *Bacillus*, *Enterobacter*, *Acinetobacter*, and *Staphylococcus* strains, while samples collected downstream from the plant were characterized by species belonging to the genera *Brevibacterium*, *Chryseobacterium*, *Aeromonas* and *Delftia*. All the isolates were resistant to amoxicillin, and most displayed resistance to multiple antibiotics. The distribution of β -lactamase genes across the four sites was also assessed. Phylogenetic analysis revealed that the distribution of the *bla* TEM4 gene is divided to two main clusters. The first cluster contains sequences that are exclusively detected at the plant and receiving water, but not at the upstream site, while sequences of the second cluster were present at all of the four sites.

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CHAPTER 1

Introduction

The spread of antibiotic resistant bacteria is a significant concern for public health, and it is well established that aquatic ecosystems constitute reservoirs of antibiotic-resistant bacteria (Allen et al., 2010; Batt et al., 2007; Hirsch et al., 1998). It is known that municipal wastewaters provide an optimal environment for the proliferation and exchange of genetic material between strains, and as such they constitute important reservoirs of antibiotic resistant bacteria. Although wastewaters are treated to reduce the bacterial load before their release in the environment, a small amount of resistant bacteria still reach the natural environment. The main objective of this work was to examine the impact of wastewater treatment plants (WWTPs) on the community structure and diversity of antibiotic resistance in receiving water bodies.

Genetic determinants of antibiotic resistance

Bacteria will show either positive or negative chemotaxis in response to chemical substances. A positive response occurs when they move toward the substrate and utilize it, whereas in negative chemotaxis they will escape from the chemical. Antibiotics as toxic compounds selectively inhibit bacteria by targeting a specific cell structure or function. While some bacteria have natural or intrinsic mechanisms of drug resistance to tolerate these toxic compounds, some others can develop resistance through the uptake of exogenous resistance genes. Therefore, the occurrence of both antibiotic resistant bacteria and resistance gene are not surprising (Hamamura et al., 2010). Primary or intrinsic antibiotic resistance is a trait of a microbial species that is inherited through

cell division and is defined as the natural sensitivity of organisms to the specific antibiotic. Various species, including opportunistic pathogens (e.g., *Pseudomonas* and *Acinetobacter*), are metabolically adaptable and encode a large number of genes for the degradation of toxic compounds. Some of these genes may be involved in the non-specific degradation of antibiotics. In addition to the primary resistance, some of the microorganisms are able to obtain the resistance genes under natural selection such as exposure to antibiotics or resistant microorganisms through horizontal gene transfer. Most resistance genes found in pathogens are acquired via horizontal gene transfer (HGT) mechanisms such as plasmid conjugation, bacteriophage transduction, and transformation. Through conjugation, DNA is transferred to compatible recipient bacteria by plasmids capable of being maintained in variety of bacteria (Xu et. al., 2007; Sorensen et. al., 2005). Transduction is the process during which microbial DNA is taken accidentally into the bacteriophage's capsid during phage assembly. When the transduced phage infects another bacterial cell, the fragment of bacterial DNA can be integrated into the new host DNA through recombination and become part of the genome. Transduction is a specific type of HGT process, because bacteriophage can only infect certain hosts. Finally, transformation is the uptake of free DNA by a bacterial cell followed by integration into the bacterial genome. Transformation is one of the common types of HGT within naturally transformable bacteria in the environment. Therefore, through the acquisition of new genes via horizontal gene transfer, bacteria can adapt to environmental changes (Osterloh, 2004; Sorensen et al., 2005).

Medical studies have shown that transfers of resistance genes and the persistence of already resistant bacteria are promoted by utilization of antibiotics and

long term exposure to sub-therapeutic concentrations (Schluter et al., 2007; Kummerer 2009). Therefore, the increased use of antibiotic for therapeutic applications on human and animals leads to their loss of effectiveness by selecting for resistant microbes and eventually becomes an environmental problem. Some of the environmental microbes that are either non-pathogenic, such as antibiotic producing bacteria, or opportunistic pathogens, such as *Pseudomonas aeruginosa*, are often highly resistant to antibiotics compared to pathogenic bacteria (Wright, 2010). The presence of antibiotic-producing bacteria and their resistance genes in nutrient-enriched environments could be considered as a factor contributing to the occurrence of antibiotic resistance genes in the environment. Naturally produced antibiotics, mostly by *Streptomyces*, can help the soil bacterial community to acquire resistance genes under a natural selection process (Munir and Xagorarakis, 2010; Zhang et al., 2009; Takano, 2006). The therapeutic use of antibiotics in human, veterinary and agriculture for more than 50 years is having a major impact on bacterial communities, resulting in various types of resistance to antibiotics. In general the resistance to antibiotics is determined genetically by antibiotic resistance genes, (ARGs), which are commonly detected in various aquatic environments (Zhang et al., 2009).

The β -lactamase family is one well characterized and widespread family of antibiotic resistance genes, and includes the TEM lactamase types that are mainly found in clinical isolates. TEM β -lactamases derive their name from a patient, Temoniera, who provided the *E.coli* strain where the enzyme representative of this class was originally identified (Bradford, 2001; Davies and Davies, 2010). It has been shown that some of the TEM β -lactamases are broad spectrum such as TEM-1 (hydrolyze penicillins and narrow

spectrum cephalosporins) and some are extended spectrum β -lactamases (ESBLs) such as TEM-3 which have even a broader substrate spectrum. In general, ESBLs types are mutant, plasmid-encoded β -lactamases originated from older, broad-spectrum β -lactamases such as TEM-1, TEM-2, SHV-1. Therefore, the ESBL type of β -lactamase is able to hydrolyse more β -lactam antibiotics compared to the broad spectrum type of enzymes, but they are sensitive to β -lactamase inhibitors such as clavulanic acid. TEM β -lactamases are commonly found to be ESBLs but recently some of them also were shown to be resistant to β -lactamase inhibitors. It has been shown that this phenotypic diversity within the TEM enzyme is determined by few select point mutations (Lachmayr et al., 2009).

Evolution and diversity of antibiotic resistance in the natural environment

To understand how environmental factors and conditions may contribute to the evolution and spread of the resistance genes in bacteria, we need to know what the origin and function of these genes in the environment are. There is disagreement regarding the evidence of resistance genes in plasmids predating the use of antibiotics. While it has been reported that plasmids found in bacterial collections of pathogens that predate the antibiotic era did not contain resistance genes (Wright 2010; Osterloh 2004), other evidence indicates that antibiotic resistance genes existed, even on plasmids, before the use of antibiotics. Phylogenetic analyses of serine β -lactamases have shown that this enzyme originated more than 2 billion years ago (Allen et al, 2010). However, some of these antibiotic resistance genes are likely to have other primary roles in the environment. For example, ability to pump various toxins, such as heavy metals and other toxic

molecules, out of the cell provides general mechanisms of resistance to the cell. The existence of these resistance genes is due to natural and anthropogenic selective pressures. Human activities such as massive production and consumption of antibiotics in both medicine, animal farming and agriculture is considered as selective pressure for antibiotic resistance genes in the environment (Baquero et al., 2008; Allen et al., 2010). In addition, there are other factors that contributed to the selection of antibiotic resistance gene. Constant exposure to different chemical compounds and conditions that occur in the environment may provide additional selection. It is known that antibiotic-producing strains are resistant to their own antibiotic and often resistance genes may be found in the same gene cluster as the antibiotic biosynthesis pathway genes. Antibiotics produced and released in the environment by some microorganisms may apply selective pressure on microbes sharing the same environment. Recent studies have shown the presence of the antibiotic-resistant bacteria, resulted from anthropogenic activity, in marine and fresh water ecosystems (Baquero et al., 2008). Antibiotic resistance genes confer resistance through different mechanisms, including target modification and modification of the action sites of antibiotics by mutational changes, loss of the enzyme coding gene, use of efflux pumps, by structural alterations of the cellular membrane that make it less permeable to the drugs, and antibiotic inactivation (Zhang et al., 2009; Allen et al., 2010).

Presence of antibiotics in the environment

There are not accurate data about the annual production of antibiotics, but it can be expected that many millions of metric tons of antibiotic have been introduced into the environment during the last 50 years (Davies and Davies, 2010). Analytical methods

such as GC-MS, MS/MS and radioimmunoassay (RIA) techniques allowed the detection of low levels of antibiotics in the water. The amount of antibiotics that are annually detected in the environment is associated with the antibiotic structure and the variation in annual utilization, which is typically higher in the winter. Unlike β -lactame antibiotics that are hardly observed in the environment due to their instability at ambient temperature, some classes of antibiotics such as sulfonamides, macrolides, trimethoprim, cephalosporins and fluoroquinolones can be detected at potentially active concentrations in wastewater (Baquero et al., 2008). The US Geological Survey measured several classes of antibiotics in water samples from a network of streams across 30 states during 1999 and 2000 (Fig. 1.1). Antibiotic concentrations as high as 1.9 $\mu\text{g/l}$ were detected, while other studies also indicated that different concentrations of antibiotics can be detected from sewage and water sample (Costanzo et al., 2005; Yang and Carlson, 2004; Hirsch et al. 1998). The considerable amount of antibiotics that are used in animal farming and agriculture leads to the contamination of manure, which is often used as fertilizer. What is currently known about the impact of antibiotics at very low concentration is that they might act as signaling agents (hormone-like) in microbial communities. They may have an impact on cell functions by induction or repression of bacterial genes, including those responsible for transferring antibiotic resistance (Kummerer, 2009; Goh et al., 2002).

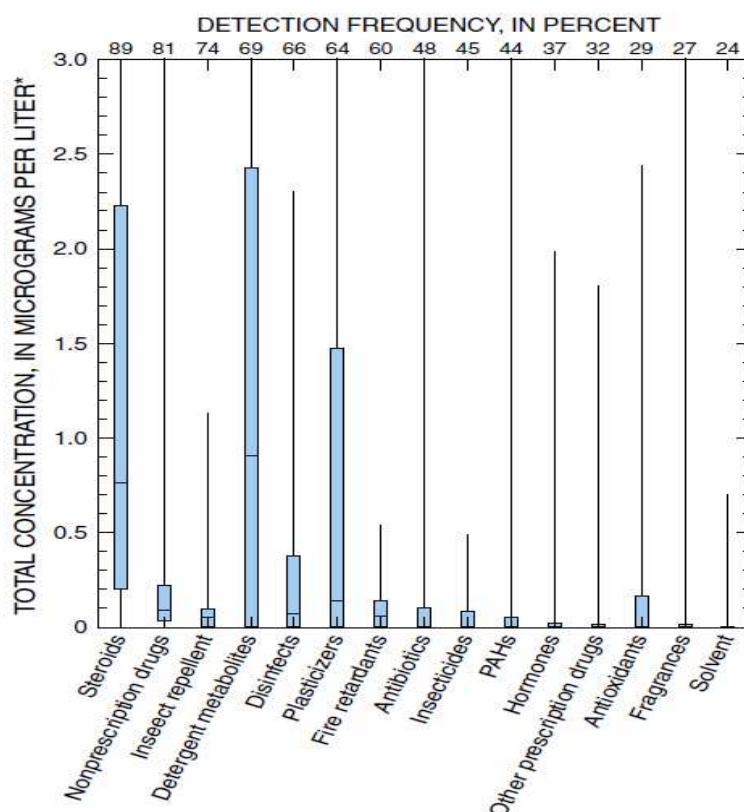


Figure 1.1 Detection of various categories of organic compounds downstream water treatment plants. (Courtesy of USGS, Fact Sheet FS-027-02)

Mechanism of action of antibiotics and development of structural variants

Antibiotics are mostly water soluble compounds, about 30- 90% of the amount consumed by humans and animals will be excreted in urine and then into the aquatic environment. Antibiotics are divided in two major groups of either naturally occurring or man-made chemicals that can be further divided in different classes, including β -lactams, quinolones, lincosamids, tetracyclines, macrolides and sulfonamides (Ding et al., 2011; Kummer, 2009). β - lactams such as penicillin G inhibit the synthesis of the peptidoglycan cell wall, which is necessary for bacteria to multiply and survive;

therefore they are very efficient against Gram-positive bacteria but inefficient against Gram-negative bacteria. However, some modifications of the structure result in compounds that show significant activity against Gram-negative bacteria and thus are broad-spectrum antibiotics. Amoxicillin is one of the moderate-spectrum β -lactam antibiotics. The fluoroquinolones are a relatively new group of antibiotics and derive from the modification of quinolones. This class of antibiotics kills bacteria by interfering with the DNA gyrase, the enzyme that rewind the DNA after replication, thereby stopping the synthesis of DNA. The fluoroquinolones are a family of synthetic, broad-spectrum antibacterial agents with bactericidal activity. As fluoroquinolones were the first antibiotics that could be used orally for the treatment of serious infections caused by Gram-negative organisms, such as *Pseudomonas* species, they have been extensively used. The new generations of fluoroquinolones have a broader spectrum and show effectiveness against Gram-positive and Gram-negative aerobic and anaerobic organisms. Ciprofloxacin is the second generation of quinolones, and it is widely administered for the treatment of *P. aeruginosa*. Lincosamides disrupt cell functions by binding to the 23S RNA portion of the 50S subunit of bacterial ribosomes and inhibit protein synthesis. Lincosamide antibiotics are known as the most useful antibiotic against the Gram-positive cocci. Comparatively, Clindamycin has a broader spectrum of antimicrobial activity among the lincosamids, and is also helpful against protozoans such as *Toxoplasma* and *Mycoplasma* as well as many anaerobic bacteria. Macrolides are inhibitors of prokaryotic protein synthesis. Because they are large and hydrophobic, their inhibitory action is mostly limited to Gram-positive bacteria. However some synthetic ones such as azithromycin show significant anti Gram-negative activity. Tetracyclines are

the inhibitors of eubacterial protein synthesis. They are relatively hydrophilic because of the presence of several hydroxyl groups, an amide moiety, and a tertiary amine substituent, so they can cross the outer membrane of Gram-negative bacteria efficiently. Within the Tetracyclines antibiotics that are active against both Gram-positive and Gram-negative bacteria, minocycline, has a broader spectrum than the other members of the group (Glazer and Nikado, 1995). Trimethoprim is a synthetic antibiotic which interfere with the synthesis of tetrahydrofolic acid by targeting the bacterial dihydrofolate reductase Trimethoprim is effective against a wide variety of bacteria.

Table 1.1 Characteristics of different classes of antibiotics.

	Group	MW	MW	MF	Range
Amoxicillin	β -lactam	Cell wall synthesis	365.40	$C_{16}H_{19}N_3O_5S$	Gr+, some Gr-
Azithromycin	Macrolide (MA)	Protein synthesis	748.98	$C_{38}H_{72}N_2O_{12}$	Gr-, Gr+, <i>Mycoplasma</i> , <i>Treponema</i>
Ciprofloxacin	Fluoroquinolone (FQ)	DNA synthesis	331.34	$C_{17}H_{18}FN_3O_3$	Aerobic Gr-, Gr+, <i>Mycobacterium</i>
Clindamycin hydrochloride	Lincosamid	Protein synthesis	461.44	$C_{18}H_{33}ClN_2O_5S.HCL$	Gr(+), cocci, protozoan, anaerobic bacteria
Minocycline hydrochloride	Tetracyclin (TC)	Protein synthesis	493.94	$C_{23}H_{27}N_3O_7.HCL$	Gr-, Gr+, <i>Mycoplasma</i>
Trimethoprim	Antifolants	DNA synthesis	290.32	$C_{14}H_{18}N_4O_3$	Gr-, Gr+

Antibiotic resistant bacteria in wastewater treatment plants

Before their release in the environment, municipal and industrial sewage undergo specific steps of treatment. In general, wastewater treatment consists of a primary, secondary, and sometimes tertiary or advanced treatment. The primary step of the treatment includes removal of solid substances from the wastewater, and it is common to all wastewater treatment plants (WWTP). However, during the secondary treatment, biological processes can be applied. These processes can vary from plant to plant. The main purposes of the secondary treatment are the biological reduction of biochemical oxygen demand (BOD), suspended solids (SS), and, in the case of industrial wastewater, the reduction in the toxic components. Moreover, the biological step also reduces the amount of nutrients providing an effluent that is less conducive to the growth of microorganisms. All the activities during the secondary treatment process are carried out by the microbial community. However, deficiency in some steps of the wastewater treatment such as bulking, foaming, and settling may lead to the prolific growth of certain microorganisms in wastewater effluent that might be health threatening (Arroyo et al., 2010; Gilbride et al., 2006). As a result, the efficiency and robustness of a WWTP essentially rely on the composition and activity of its microbial community. Even though biological treatment of the wastewater has been applied for many years, our knowledge of the microbiology of this process is limited to the past decade due to methodological limitations. The study of the composition and diversity of the microbial community was possible after the introduction of molecular techniques in the study of these systems (Gilbride et al., 2006).

In some WWTPs the tertiary treatment, which typically includes chlorination and/or ultraviolet radiation, is applied to the effluent. Although the bacteria load is decreased during the water treatment, the water released with the effluents still contains bacteria of human origin and is likely to carry antibiotic resistance genes that may be laterally transferred to the natural population (Lachmayr et al., 2009; Kim et al., 2010; Batt et al., 2006).

In general, compounds such as heavy metals, anti-microbial agents and detergents, which have been associated with antibiotic resistance, are commonly found in the influent of wastewater treatment plants (Batt et al., 2006). Because micropollutants such as antibiotics are not completely eliminated during the wastewater treatment, they are released into the environment through sewage effluents in much higher concentrations than they occur naturally. In fact, antibiotic residues have been detected in the final effluents of wastewater treatment plants in Canada, Europe, and the United States. The presence of antibiotics in the aquatic ecosystem has the potential to adversely affect the quality of drinking water, promote the persistence of antibiotic resistance bacteria, and finally it may have negative effects on microorganisms responsible for important processes such as denitrification (Costanzo et al., 2005). A study comparing wastewaters that included hospital effluents to a control, without hospital effluents, showed that the numbers of resistant bacteria were in the same range in both cases. Considering that hospital effluents may contribute to less than 1% of the entire amount of municipal wastewater, it is likely that hospitals are not the only source for resistant bacteria in wastewater. Frequently resistant bacteria are also detected in municipal wastewater not receiving hospital effluents. This can be related to the consumption of the

antibiotic at home (Allen et al., 2010; Kummerer, 2009). Wastewater treatment plants are important ecosystems since they link different environmental condition, such as hospitals and surface waters, and consequently may promote gene exchange between these habitats (Schuluter et al., 2007). Recent molecular analysis of wastewater treatment plants indicated that they represent ecosystems rich in antibiotic resistant microorganisms and resistance genes. Furthermore, these resistance genes are often encoded in genomic islands on transmissible plasmids (Davies and Davies, 2010). Some environmental conditions such as high bacterial densities and metabolic activities, biofilm and flock formation during the wastewater treatment process, are expected to facilitate genetic exchange by conjugation in these environments. Therefore, wastewater treatment plants seem to promote recombination and distribution of genes responsible for adaptive traits such as antimicrobial resistance. Furthermore, residual amounts of pollutants such as drugs, xenobiotics, surfactants, heavy metals in wastewater, select for genes responsible for degradation or resistance of those compounds (Schluter et al., 2007).

Techniques for the microbiological analysis of wastewaters

The aquatic environment of a waste water treatment plant is known to be an important reservoir for antibiotic resistance genes and microbial pathogens that can cause a threat to the environment and human health (Zhang et al., 2009; Gilbride et al., 2006). Thus, it is essential to understand composition and diversity of the microbial communities present in water and wastewater treatment systems (Martin, 2002).

Before applying currently available molecular and culture independent techniques, the detection of pathogens in environmental samples had been limited to

culture dependent techniques. However, since the majority of bacteria cannot be easily grown on general purpose media, culturable strains do not precisely reflect the composition and diversity of natural microbial communities. Therefore there is a considerable advantage in using molecular techniques over culture dependent ones in the study of environmental organisms. 16S rRNA gene amplification by PCR using universal primers, followed by sequencing, has been widely used for monitoring microbial communities. Further analysis of the composition and diversity of the community is achieved by using other methods including denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (TRFLP) (Cho and Kim, 2000; Kapley et al. 2007). All the mentioned techniques have been applied to different wastewater treatment systems such as anaerobic digesters, activated sludge, or membrane bioreactors (Miura et al. 2007; Kapley et al., 2007; Arroyo et al., 2010).

Objectives of this Study

In this project I wanted to investigate the extent of antibiotic resistance in microbial communities of a tributary of the Raritan Canal and to determine the impact of the effluents of a wastewater treatment plant on antibiotic resistance in the same communities.

The rationale for this investigation is that the antibiotics in wastewater treatment plant effluents released in the environment, although at low level, may lead to the selection of a resistant microbial population that has the ability to transfer resistant genes to other species, including pathogens (Kummerer, 2004; Dantas et al., 2008).

My objectives were: i) to investigate the structure, composition and diversity of microbial communities from the effluent of the WWTP of the Raritan and two locations downstream from the plant discharge; ii) to look for the antibiotic resistant pattern within the isolates from each site of sampling; iii) to investigate the distribution and diversity of the antibiotic resistance genes in the whole community. These might later on serve to study the transfer of antibiotic resistances between sewage bacteria.

CHAPTER 2

Community composition and antibiotic profiling of the wastewater treatment plant and receiving stream

Introduction

Environments contaminated with antibiotics draw attention because of the possible increase and spread of antibiotic-resistant genes to the microbial communities. The treatment of the wastewater generates an environment that contains higher concentrations of antibiotics than open aquatic environments. Therefore, wastewater treatment plants may play an important role in the selection and spread of antibiotic resistance inside natural communities of both pathogenic and non-pathogenic strains (Li et al., 2009).

The goal of this work was to investigate the impact of wastewater effluents on the composition and the antibiotic resistance of the native microbial communities. My hypothesis is that while the diversity of the microbial communities exposed to the wastewater effluents may not change, the antibiotic resistance of the communities downstream the effluent discharge will be affected and result in higher diversity and level of resistance.

This study has been approached using different strategies that will be described in this chapter. The composition and diversity of the microbial communities at the sites of interest was analyzed using a culture-independent approach, including genetic fingerprinting techniques such as terminal restriction fragment length polymorphism (TRFLP) and sequencing of 16S rRNA clone libraries. These molecular techniques have been widely applied to study the microbial communities, since they do not depend on isolation or growth of the microorganisms based on the culture dependant approaches. To examine the distribution of antibiotic resistance within individual genera in the four communities, bacterial isolates were obtained from the final effluent of the wastewater treatment plants and receiving water using non-selective culture media. Susceptibility to antibiotics belonging to different classes was tested by disc diffusion method and minimal inhibitory concentration techniques. In addition, given that resistance to high concentrations of amoxicillin (a β -lactam antibiotic) was found in all the isolates, I focused on this type of resistance. To explore the distribution of β -lactamase genes at different sites, community DNA libraries were employed to isolate and identify variants of these genes.

Materials and Methods

Study area and sample collection

The Somerset Raritan Valley Sewerage Authority, established in 1958, is a regional wastewater treatment plant located in Somerset County, NJ, that mostly treats

domestic wastewater, with only limited amounts of industrial waste. The Authority operates a 21.3 mgd (Millions of Gallons per Day) secondary advanced wastewater treatment system. Sludge is managed with a fluidized bed incinerator. After treatment, the treated wastewater from the secondary settling tank is discharged without any further treatment into a channel, which eventually merges in the Raritan river. Samples were collected from the final effluents of the plant, indicated with P in Figure 2.1, and from two locations downstream the sewage discharge, at coordinates N 40° 33.287' W 74° 34.069' and at N 40°33.160' W 74° 33.797', indicated with RI and RII, respectively.

There was no other discharge to the river between the two sampling sites. The distance between the RI and RII sites is approximately 500 m. As a control for the whole experiment, a water sample from a site upstream of the plant was collected, indicated as US. Water was collected at the end of September 2009. Each composite sample consisted of a total of 2000 ml of water collected in a sterile bottle, which was kept in an ice bath, in the dark, during transportation to the laboratory.

Strain isolation and identification

Approximately 100 ml of each collected water sample was filtered through a 0.45 µm pore membrane (Pall, USA). The membrane was then transferred to a Falcon tube and rinsed with 1 ml of the same collected water sample, and 100 µl of the resulting cell suspension was spread on Muller Hinton Agar and incubated overnight at 37 °C. To obtain pure cultures of the isolates, individual colonies were isolated by repeated streak plating on Muller Hinton Agar. Colonies were collected based on their different

morphology and grown overnight at 37 °C on Muller Hinton broth. All strains were stored at -80 °C as glycerol stocks.

Genomic DNA was extracted following the modified protocol of Kerkhof et al. (Kerkhof et al., 2000). Cultures were grown overnight in 2 ml of LB, at 37 °C. The cells were harvested by centrifugation at 13000 rpm for 1 minute. The supernatant was removed and the pellet was lysed by freeze-thawing with liquid nitrogen for 4-5 times. The sample was resuspended in 467 µl TE buffer, 30 µl of SDS 10% W/V and lysozyme to a final concentration of 2 mg/ml and incubated for the 15 min at 37 °C. After cells lysis, the DNA was extracted twice with equal volumes of Tris saturated phenol (pH 8), and twice with equal volume of chloroform /isoamyl alcohol (24:1). The DNA was then precipitated with 1/10 volumes of 3.0 M sodium acetate and 2 volumes of cold 100% ethanol. The quantity and quality of the DNA obtained was evaluated both using a Nanodrop ND-1000 spectrophotometer (NanoDrop, USA) and by agarose gel electrophoresis. Gel images were acquired using a Gel Logic 440 Imaging System (Eastman Kodak, USA).

The identity of culturable strains was assessed by PCR amplification of their 16S rRNA gene and sequencing. The pair of universal primers Bact-8F 5'-AGA GTT TGA TCC TGG CTC AG-3' and Univ-1517R 5'- ACG GCT ACC TTG TTA CGA CTT- 3' was used in a PCR reaction containing 2.5 µl of 10x buffer, 0.5 µl of 10 mM dNTPs, 0.25 µl of 0.05 nM of each of the primers, 250 ng of genomic DNA and 0.05 U JumpStart AccuTaq LA DNA polymerase (Sigma-Aldrich, USA) in a 25 µl reaction. PCR conditions were as follows: initial denaturing at 94 °C for 1 min, followed by 30 cycles of the 0.5 min at 94°C, 0.5 min at 55 °C, 1:30 min at 72 °C and final extension step

at 72 °C for 7 min. Aliquots of 5 µl of each reaction solution were analyzed by electrophoresis on 0.8% agarose gel and the gel images were acquired using a Gel Logic 440 Imaging System (Eastman Kodak, USA). PCR products corresponding to the 16S rRNA gene were purified using the Qiaex II Gel Purification Kit (Qiagen, USA) and submitted for sequencing (GeneWiz, USA). Sequences were compared to databases of 16S rRNA gene sequences at NCBI and the Ribosomal Database Project (RDP) (Cole et al., 2008).

Disc diffusion susceptibility test

The sensitivity to different antibiotics was assessed using the disc-diffusion method on Muller Hinton Agar. Disk diffusion is considered as one of the traditional techniques to test antimicrobial susceptibility of microorganisms, and yet it is one of the most commonly used tests in routine clinical application. It is convenient for testing the majority of the microorganisms' including bacterial pathogens. Muller Hinton Agar has been accepted of the international recognition for antimicrobial susceptibility testing. This medium is recommended in the document M44-P for disc diffusion susceptibility testing for yeast and other microorganisms. Some characteristics of the Muller Hinton Agar such as batch-to-batch reproducibility for susceptibility testing, low in sulfonamide, trimethoprim, and tetracycline inhibitors and its support for the growth of most nonfastidious pathogens makes it suitable for antibiotic testing (Serrano et al., 2004). Six antibiotics were selected as representative of commonly used antibiotic classes. This method consists of placing discs of absorbent paper containing the antibiotic of interest (all purchased from Sigma, USA) (i) amoxicillin (50 mg/ml), (ii) azithromycin (25

mg/ml), (iii) clindamycin (50 mg/ml), (iv) ciprofloxacin (25 mg/ml), (v) minocycline (7 mg/ml) and (vi) trimethoprim (50 mg/ml), on a plate inoculated with the bacteria collected from all four sites of sampling. Plates were incubated 20 h at 37 °C to allow growth of the bacteria and time for the antibiotics to diffuse into the agar. Strains were classified as resistant to the antibiotic tested when no inhibition zone was observed around the disk, or as sensitive, when a clear zone of inhibition was seen.

Determination of antibiotic minimal inhibitory concentrations (MIC)

To quantify the antibiotic sensitivity of the isolates, the MIC for each antibiotic was determined by utilizing serial dilutions of the initial concentration of the selected antibiotics (Table 1.1) in 200 µl of the Muller Hinton Broth, in microwell plates. Isolates were grown overnight on LB broth. The test was performed in triplicate in 96 well flat bottom plates (Costar, USA) with each column, comprised of 8 wells, corresponding to one susceptibility test. The first three columns were used as a drug free growth control. Serial dilutions, from 10000 to 0.008 µg/ml, of each of the six antibiotics, were tested. Cultures of each strain were incubated for 18 h at 37 °C and growth was assessed using a TECAN spectrophotometer and OD₆₀₀ measurement.

Molecular detection of the β -lactamase gene in individual isolates

Molecular screening for the presence of β -lactamase genes was performed on both culturable strains and community DNA from each sites of sampling with primers targeting conserved regions of the β -lactamase genes (Table 2.3). All the isolates were grown in LB overnight, at 37 °C. In preliminary experiments to detect the β -lactamase

gene in the isolates, a mixed culture of isolates from each sampling sites was prepared by taking 100-200 µl of an overnight culture of each isolate. Plasmid extraction from the mixed culture of each site was performed with a Qiagen Miniprep kit according to the manufacturer's instructions (Qiagen, USA). The mixed-culture plasmids were targeted for PCR amplification with 7 different sets of the available primers to amplify the internal region of the *bla*TEM and *bla*SHV (Table 2.3). PCR reaction containing 2.5 µl of 10x buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of 0.05 nM of each of the primers, 250 ng of genomic DNA and 0.05 U JumpStart AccuTaq LA DNA polymerase (Sigma-Aldrich, USA) in a 25 µl reaction were conducted. PCR conditions were as follows: initial denaturing at 94 °C for 1 min, followed by 30 cycles of the 0.5 min at 94 °C, 0.5 min at 55 °C, 2 min at 72 °C and final extension step at 72 °C for 10 min. Aliquots of 5 µl of each reaction solution were analyzed by electrophoresis on 0.8% agarose gel and the gel images. Next, to detect the presence of the β-lactamase in individual isolates, plasmid extraction from overnight growth cultures of single isolates by Qiagen Miniprep were performed. Isolated plasmids were targeted for PCR amplification with TEM4 primer pairs as previously mentioned.

Community DNA isolation

Total nucleic acids were extracted directly from whole filters according to the modified methods of Kerkhof et al. (Kerkhof et al., 2000). To concentrate the sample and to prevent any change in the number of the species present, 1000 ml of each site of sampling was immediately filtered using 0.2 µm membrane. The membrane was kept at -80 °C for subsequent DNA extraction. All the steps are carried out in micro-centrifuge

tubes as quickly as possible. The frozen membrane was resuspended in 100 µl of buffer (50 mM glucose/10 mM EDTA/25 mM Tris, pH 8.0) and underwent five freeze/thaw cycles at -80 °C/55 °C. To this bacterial suspension, 100 µl of buffer, 100 µl of lysozyme, and 75 µl of 500 mM EDTA were added. The mixture was incubated at room temperature for 5-10 min before lysing the cells with 50 µl of 10% sodium dodecyl sulfate (SDS). The resulting cell lysate was extracted twice with 800 µl of Tris (pH 8.2)-saturated phenol:chloroform:isoamyl alcohol (25:24:1) with 0.1% 8-hydroxyquinoline. The mixture was vortexed vigorously to form an emulsion and centrifuged at 14000Xg for 3 min. 1/10 volumes of 3.0 M sodium acetate and along with 2 µl of 20 mg/ml glycogen was added to the aqueous phase and the DNA was precipitated with 2 volumes of cold 100% ethanol. DNA was pelleted at 16000Xg for 15 min at 4 °C, and the pellet was resuspended in 250 µl nuclease free water. DNA concentration were measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop, USA) and by agarose gel electrophoresis. The DNA was purified in a cesium chloride density gradient with ethidium bromide: 0.53 g cesium chloride was added to a DNA solution to a final volume of 500 µl and 2 µl of 1% solution of ethidium bromide and centrifuged for at least 16 h at 10,000 g and 18 °C in a TFT 6513 Kontron rotor. The band corresponding to community DNA (indicated by the arrow) was extracted (Fig. 2.5). The DNA was then dialyzed against 10 mM Tris, pH 8, on Millipore 0.025 µm membrane for 45 min to eliminate the cesium chloride.

Construction of libraries of 16s rRNA and β -lactamase genes

Libraries were constructed using the community DNA from each of the four sites of sampling. For 16S rRNA gene libraries, the 16S rRNA gene was amplified using the pair of universal bacteria primers 27F (5'-AGA GTT TGA TCM TGG CTC AG -3') and 1492R (5'-GGY TAC CTT GTT ACG ACT T -3'). PCR amplification was carried out with a thermal cycler (Eppendorf AG 22331, USA) under the following conditions: initial denaturation at 94 °C for 1 min, followed by 30 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C, 1:30 min at 72 °C and final extension step at 72 °C for 7 min. For β -lactamase genes libraries, the bacterial *bla* gene was amplified with the two primers TEM4-F (5'-ATC AGC AAT AAA CCA GC-3P -3') and TEM4-R (5'-CCC CGA AGA ACG TTT TC -3'). PCR amplification was carried out with a thermal cycler (Eppendorf AG 22331, USA) under the following conditions: initial denaturation at 94 °C for 1 min, followed by 30 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C, 2 min at 72 °C and final extension step at 72 °C for 10 min. All PCR reactions contained 2.5 μ l of 10x buffer, 0.5 μ l of 10 mM dNTPs, 0.5 μ l of 0.05 nM of each of the primers, 250 ng of genomic DNA and 0.05 U JumpStart AccuTaq LA DNA polymerase (Sigma-Aldrich, USA). Aliquots of 5 μ l of each reaction solution were analyzed by electrophoresis on 0.8% agarose gel and the gel images were acquired using a Gel Logic 440 Imaging System (Eastman Kodak, USA). Amplified community DNA inserts were ligated into the pCR 2.1-TOPO plasmid using the TOPO-TA expression kit (Invitrogen, USA). This approach takes advantage of the ligase activity of topoisomerase I, which is bound to the linearized vector. Ligation mix was used to transform *E. coli* TOP10 (Invitrogen, USA) as recommended by the manufacturer. Transformed cells were plated on LB agar supplemented with 50 μ g/ml

Kanamycin and 40 mg/ml X-gal. Following overnight growth at 37 °C, the colonies were transferred to 96 microwell plates containing LB with 50 µg/ml Kanamycin and glycerol and stored at -80 °C.

To confirm the presence of the insert, a PCR screening was performed directly on overnight cultures of the single colonies under the following conditions: initial denaturation at 94 °C for 1 min, followed by 30 cycles of the 0.5 min at 94 °C, 3 min at 55 °C, 1:30 min at 72 °C and final extension step at 72 °C for 7 min. The PCR reaction contained 2.5 µl of 10x buffer, 0.5 µl of 10 mM dNTPs, 0.25 µl of 0.05 nM of each primer, 2 µl of the liquid culture and 0.05 U JumpStart AccuTaq LA DNA polymerase (Sigma-Aldrich, USA). Plasmids for DNA sequencing were isolated using the Qiagen Miniprep kit according to the manufacturer's instructions (Qiagen, USA). A total of 20 clones from each one of the four sites were sequenced by using sequencing primer M13 for the distribution of the β -lactamase gene.

To determine RFLP patterns of cloned 16S rRNA sequences, inserts were amplified by PCR directly from the overnight cultures of the single colonies under the following conditions: initial denaturing at 94 °C for 1 min, followed by 30 cycles of the 0.5 min at 94 °C, 3 min at 55 °C, 1:30 min at 72 °C and final extension step at 72 °C for 7 min. PCR reaction contained 2.5 µl of 10x buffer, 0.5 µl of 10 mM dNTPs, 0.25 µl of 0.05 nM of each of the primers, 2 µl of the liquid culture and 0.05 U JumpStart AccuTaq LA DNA polymerase (Sigma-Aldrich, USA). 10 µl of PCR product were digested for 3 h at 37 °C with 10 U/λ of *HaeIII* and *MnII* (Biolab, USA). The resulting fragments were visualized by 2.5% methaphore gel electrophoresis. RFLP patterns for each library were

identified and representative clones were selected for sequencing by Genewiz, Inc. (USA). Plasmids for DNA sequencing were isolated using the Qiagen Miniprep kit according to the manufacturer's instructions (Qiagen, USA). A total of 92, 136, 200 and 124 16S rRNA clones sequenced using one end of the inserted DNA fragment by using sequencing primer M13, from the upstream (US), plant (P), RI and RII sites, respectively. As for the β -lactamase gene library, a total of 80 clones were sequenced using the M13 primer.

TRFLP analysis of 16S rRNA genes

Terminal restriction fragment length polymorphism (TRFLP) analysis, a direct DNA fingerprinting method, was used to assess the wastewater bacterial community at the four sites of sampling. The method is based on amplification of 16S rRNA genes (~1.5 kb) using the universal bacterial primers 27-Forward (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1100-Reverse (5'-AGG GTT GCG CTC GTT G -3'). The 27-Forward primer is fluorescently labeled with 6-FAM (5(6)-carboxy-fluorescein) on the 5' end (Sigma, USA) (Lane, 1991). The following amplification parameters were used to amplify and label the 16S rRNA genes from the community DNA: 10 ng of template DNA and 20 pmol of each of the primers in a 10 μ l reaction, initial denaturation at 94 °C for 5 min, then 25 cycles at 94 °C for 0.5 min, 57 °C for 0.5 min and 72 °C for 1.5 min, and a final extension at 72 °C for 10 min in a GeneAmp PCR system 2700 thermal cycler (Applied Biosystem, USA). This fluorescently labeled PCR product was then digested for 6 h at 37 °C with the restriction endonuclease *MnII* (New England Biolabs, USA) to produce a mixture of variable length, end-labeled 16S rRNA fragments.

After digestion, the DNA was precipitated using 75 mM sodium acetate and 37 μ l of 95% ethanol. The pellet was rinsed with 70% ethanol and vacuumed dried for 30 min. Fragment analysis was performed by using 20 ng of the labeled PCR, and the sizes of the 5' terminal restriction fragments (TRFs) and the intensities of their fluorescence emission signals (peak area) were calculated using ABI 310 genetic analyzer (Perkin-Elmer) with GeneScan Analysis software and ROX size standard. The term operational taxonomic unit (OTU) was used to refer to individual restriction fragments in TRFLP patterns.

To further characterize the TRFLP peaks, selected 16S rRNA clones from the gene libraries described above (see Construction of libraries section) were used to assess and interpret the TRFLP profiles. Using this method the TRF size of each 16S rRNA was predicted *in silico* using the NEB cutter V2.0 program (Vincze et al., 2003) to find *MnII* restriction sites, and furthermore fragment sizes were experimentally verified by digesting and directly running a TRFLP analysis of individual fragments, obtained from the digestion of known 16S rRNA genes amplified from the clone libraries. Then, we used this dataset of known fragments to validate each of the peaks of our TRFLP profiles.

Phylogenetic analysis of community 16S rRNA and bla gene sequences

16S rRNA gene sequences obtained from the communities libraries were used to search databases of sequences at the National Center for Biotechnology Information (NCBI) using Blastn, and at the Ribosomal Database Project (RDP classifier) (Cole et al., 2008). Similarity to top matches was used to establish the phylogenetic affiliation of the 16S rRNA gene sequences at the level of genus, class, or phylum, depending on the level of identity (blast.ncbi.nlm.nih.gov). The sequences determined in this study and selected

reference sequences retrieved from the database were aligned using ClustalW (Thompson et al., 1994), and the alignments were trimmed manually to exclude vector sequences and uncertain positions. Neighbor-Joining trees were constructed using MEGA 5.0 software package (Tamura et al., 2011) and performing one hundred bootstrap replicates.

In addition, sequences obtained from community libraries of *bla* genes were compared to databases of sequences at NCBI using Blastx to confirm their identity. All the sequences were aligned using ClustalW followed by construction of neighbor-joining tree using MEGA 5.0 as described before.

Result and Discussion

The main objective of this work was to examine the impact of wastewater treatment plants (WWTPs) on the community structure and diversity of antibiotic resistance in receiving water bodies. This work is structured as follows:

- (1) Using a culture-dependent approach, type and level of antibiotic resistance was assessed and linked to specific strains isolated from the environment.
- (2) The diversity and composition of communities at sites surrounding the wastewater discharge was assessed applying culture-independent techniques.

Antibiotic resistance in environmental bacterial isolates

To determine the diversity of antibiotic resistance and quantify the minimal inhibitory concentrations (MIC) for a range of antibiotics, we isolated individual species

from environmental water samples from the final effluents of the Somerset Raritan Valley Sewage Authority in New Jersey (samples were indicated with “P”), from two locations downstream the sewage discharge (indicated with “RI” and “RII”), and upstream of the plant (indicated with “US”), Fig. 2.1. Culturable bacteria were identified using culture-dependent techniques and 16S rRNA gene sequencing, as described in the Methods section. Sequence data was then compared with currently available sequences in GenBank by using BlastN, or in RDP (Cole et al., 2008). Bacteria belonging to 6, 7, 8 and 7 different bacterial genera were isolated from the upstream site, the effluent, and the two sites downstream the plant discharge, respectively. The majority of the isolates from the effluent belonged to the class of Gammaproteobacteria, and they included members of the genera *Aeromonas*, *Acinetobacter*, *Pantoea* and *Enterobacter*. According to Schmidt et al., 2003, different members of these genera have been found to dominate different wastewater treatment plants effluents and receiving water. The most frequent culturable strains isolated from all of the four sites belonged to *Bacillus*. In addition, Betaproteobacteria, Actinobacteria, Bacilli and Flavobacteria, including strains belonging to the genera *Delftia*, *Brevibacterium*, *Corynebacterium*, *Bacillus*, *Staphylococcus*, and *Chryseobacterium* from different sites could be found. Among the isolates, some appeared to be unique to the site of sampling, for example *Corynebacterium sp.* was only isolated from the upstream site, while *Pantoea sp.*, *Enterobacter sp.* and *Staphylococcus* were only present in the plant effluent, and *Brevibacterium sp.* and *Delftia sp.* were detected only at the sites RI and RII, respectively. This is perhaps due to the fact that the small size of the sample did not represent the actual population of culturable species.

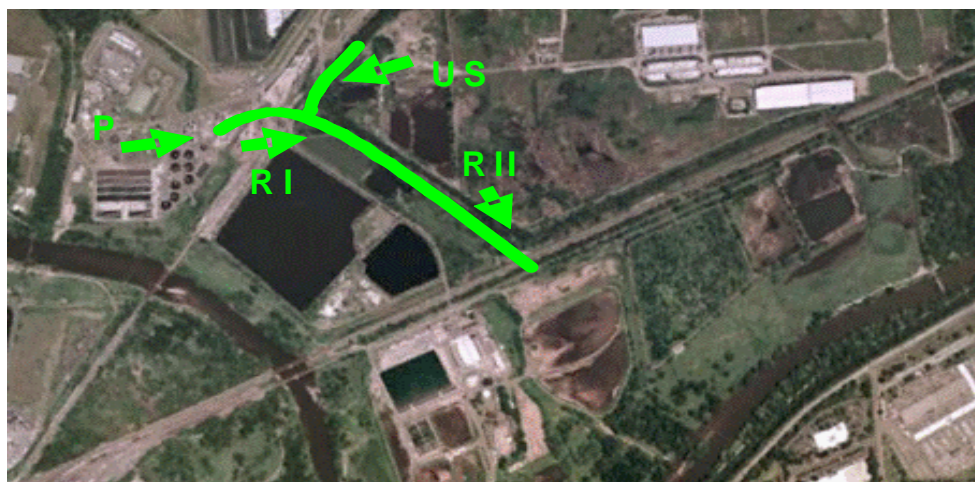


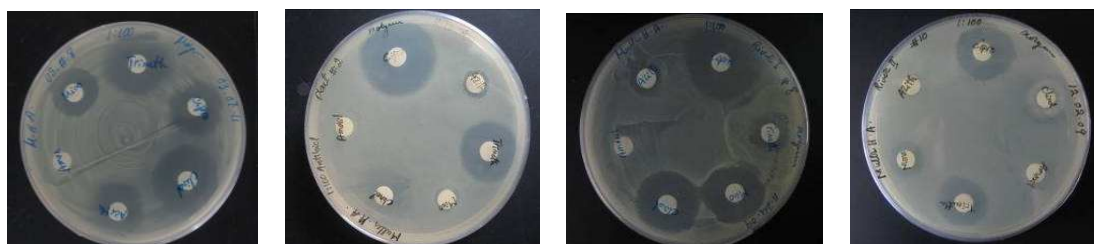
Fig. 2.1. Aerial view of the Raritan Valley wastewater treatment plant (Google Earth map, 2009). Sites of sampling are indicated as follows: plant “P”, the two locations downstream of the plant are “RI” and “RII”, and the one upstream is the site “US”. Arrows indicate direction of water flow.

In order to determine the resistance pattern of the isolates, their antibiotic susceptibility phenotypes were determined using the disc-diffusion method. Antibiotics belonging to six different classes were selected among the most frequently prescribed, and they included amoxicillin, azithromycin, clindamycin, ciprofloxacin, minocycline, and trimethoprim (Table 1.1). Most isolates resulted resistant to high concentrations of amoxicillin (50 mg/ml), while some of the bacteria, regardless of the site of sampling, displayed resistance to low concentrations of all the other antibiotics (Fig. 2.2). Previous studies have shown that while the production of lactamases is particularly common among Gram-negative bacteria, these enzymes have been identified in virtually all bacterial species, with notable exceptions being most *Enterococci* and *Salmonellae* (Lachmayr et al., 2009). The resistance to minocycline was consistently the lowest and all the isolates were sensitive to it. In fact, all the bacteria were resistant to at least two of the

six antibiotics tested. In addition to amoxicillin, resistance to azithromycin, trimethoprim, clindamycin, and ciprofloxacin was observed in some isolates. These results are summarized in Table 2.1.

To accurately quantify the level of antibiotic resistance of the isolates, the MIC of each antibiotic was determined by microbroth dilution (see Methods section). Consistent with the disc-diffusion tests, most of the isolates were resistant to amoxicillin (96%), and specifically about 35% were resistant to high amoxicillin levels (10,000 $\mu\text{g/ml}$). The next highest MIC was observed for trimethoprim, where 35% of the isolates were resistant to greater than 400 $\mu\text{g/ml}$ of the antibiotic. The most effective antibiotic was minocycline, since all of the isolates were sensitive to low concentrations of it, as shown in Fig. 2.3A. The distribution of antibiotic resistance within isolates did not appear to be correlated with the sites of sampling, with the exception of the azithromycin and minocycline. In fact, only the isolates from site US showed sensitivity to low concentrations of the azithromycin (Fig. 2.3B) and minocycline (Fig. 2.3C).

The MIC test confirmed the results obtained with the disc diffusion test and provided a qualitative assessment of the sensitivity to each antibiotic for the isolates. Most isolates were resistant to elevated concentrations of amoxicillin. In addition, resistance to multiple classes of antibiotics was detected among the isolates.

*Bacillus sp.**Aeromonas sp.**Brevibacterium sp.**Delftia sp.*

US #8

P#2

RI #8

RII #10

Fig. 2.2. Disc diffusion susceptibility test. The disc diffusion method, on Muller Hinton Agar, was used for a qualitative assessment of the sensitivity to various antibiotics in isolates from plant effluents and up- and downstream the wastewater treatment plant. Each paper disc contains a different antibiotic. The figure shows representative plates, each spread with one isolate. Resistance to multiple antibiotics (absence of a clear zone of inhibition) was observed for all the isolates.

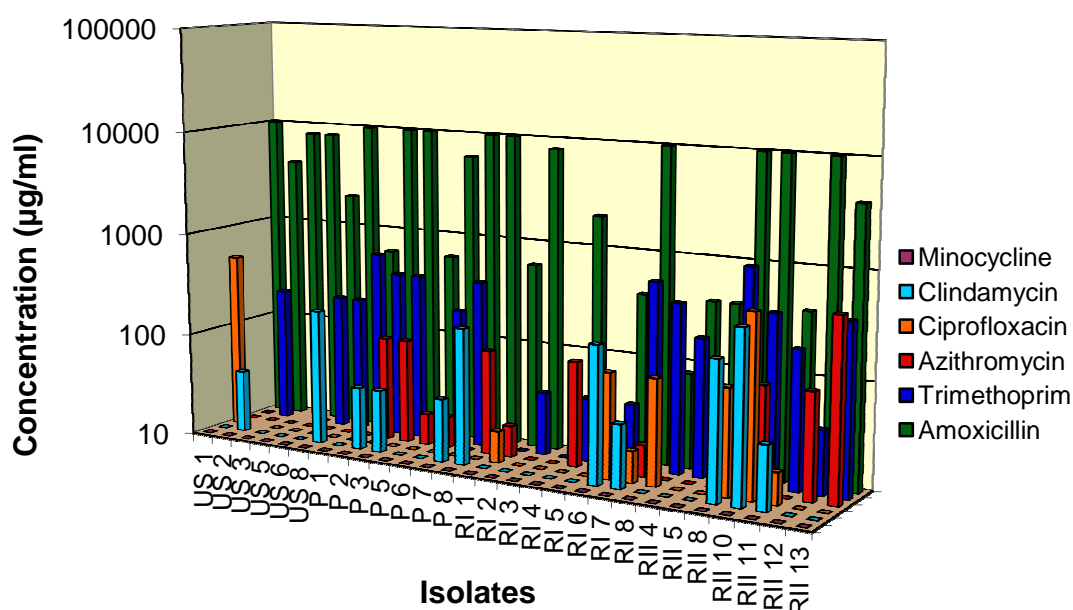
Table 2.1. Antibiotic resistance in isolates from the four sites, based on the disc diffusion test.

	Sites ^a				Total ^a	% ^b
	US	P	RI	RII		
Amoxicillin	6	7	7	7	27	96
Trimethoprim	1	4	2	2	9	32
Azithromycin	3	2	5	2	12	42
Minocycline	2	1	0	1	4	14
Clindamycin	2	4	1	1	10	28

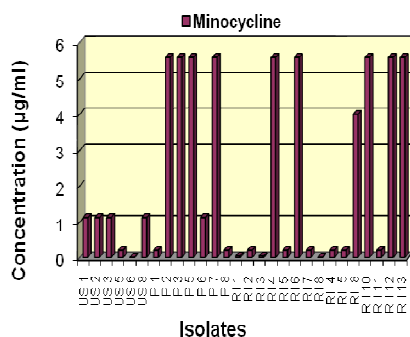
^a Total number of isolates that showed resistance to each antibiotic

^b A total of 28 isolates were tested using the disc diffusion method. The percentage was calculated from the number of the isolates resistant to a specific antibiotic divided to the total number of the isolates

A



B



C

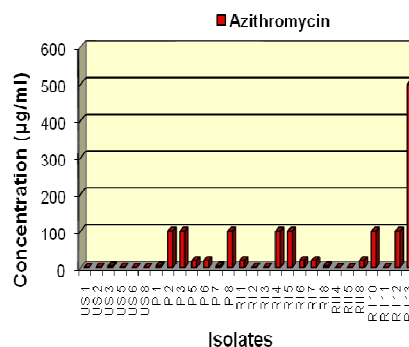


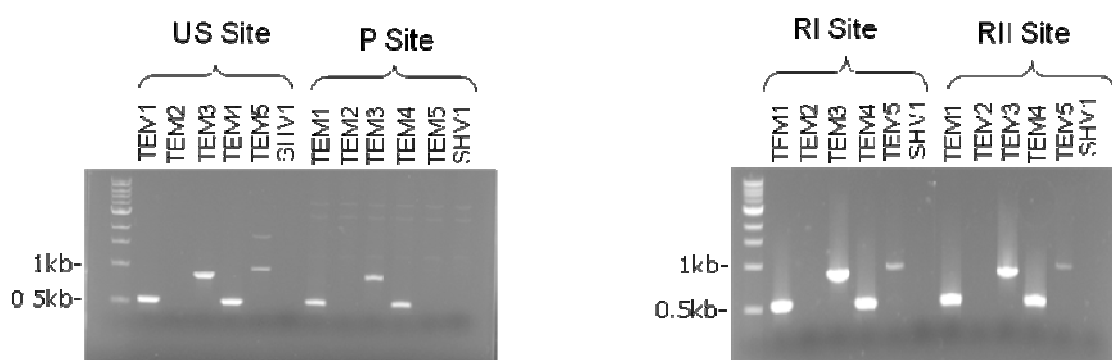
Fig. 2. 3. Antibiotic minimal inhibitory concentrations. To quantify the antibiotic sensitivity of the isolates, the MIC for each antibiotic was determined on Muller Hinton Broth, in microwell plates. Isolates were exposed to serial dilutions, from 10,000 to 0.008 µg/ml, of each of the six antibiotics. In this experiment, MIC was defined as the lowest antibiotic concentration that prevented cell growth (A). Sensitivity of the isolates to the antibiotics minocycline (B) and azithromycin (C).

Given that amoxicillin resistance was the most frequent type of antibiotic resistance observed in all the sites of sampling, we wanted to find out how many different variants of the β -lactamase gene responsible for resistance were present and whether these variants were prevalent at specific sites or equally distributed in the aquatic environment.

Plasmids were extracted from combined cultures of isolates for each of the four sites. Each plasmid mixture was tested using all of the 7 pairs of primers. Only 4 of the 7 pairs of primers produced a PCR product. All of the isolates resistant to amoxicillin carried the TEM 4 gene on their plasmid, as demonstrated by the presence of an amplification band (Fig. 2.4). Furthermore, the comparison of the *bla* gene sequence from the isolated plasmids showed that all the plasmids considered shared highly similar β -lactamase gene. The fact that isolates from different sites, belonging to different taxonomic groups, carried highly similar β -lactamase genes on their plasmids, strongly support the hypothesis that the same resistance gene has spread to species sharing the same environment.

Table 2.3. Oligonucleotide primers used for the *bla* gene detection.

<i>bla</i> _{TEM/SHV}	Primer name	Sequence 5'-3'	PCR size (bp)	Reference
<i>bla</i> _{TEM}	TEM1	F (TGG GTG CAC GAG TGG GTT AC) R (TTA TCC GCC TCC ATC CAG TC)	526	Tenover et al., 1994
<i>bla</i> _{TEM}	TEM2	F (CACTATTCTCAGAATGACTTGGT) R (CATGACAGTAAGAGAATTATGCA)	65-90	Lachmayr et al., 2009
<i>bla</i> _{TEM}	TEM3	F (GAGTATTCAACATTTCCGTGTC) R (TAATCAGTGAGGCACCTATCTC)	850	Messai et al., 2006
<i>bla</i> _{TEM}	TEM4	F (ATCAGCAATAAACCAGC) R (CCCCGAAGAACGTTTTC)	516	Colom et al., 2003
<i>bla</i> _{TEM}	TEM5	F (TCGGGGAAATGTGCGCG) R (TGCTTAATCAGTGAGGCACC)	971	Pitout et al., 1998
<i>bla</i> _{SHV}	SHV1	F (AGGATTGACTGCCTTTTTTG) R (ATTTGCTGATTTCGCTCG)	392	Colom et al., 2003
<i>bla</i> _{SHV}	SHV2	F (CACTCAAGGATGTATTGTG) R (TTAGCGTTGCCAGTGCTCG)	885	Pitout et al., 1998

**Fig. 2.4. Presence of the *bla* gene in the combined plasmids from each site.** Variants of the *bla* gene were detected by PCR assays, using the primers indicated at the top of the gel. Isolates from each site were grown in the presence of amoxicillin and combined before plasmid extraction. The amplification products from the sites US, P, RI and RII were visualized on 0.8% agarose gel.

Communities' structure and composition

The molecular diversity of the bacterial communities in the Raritan Valley wastewater plant and surrounding areas was analyzed to investigate the presence and estimate the diversity of bacterial species, and to assess the impact of the wastewater treatment plant effluent on the bacterial communities of the receiving waters. The comparison of the bacterial communities at the four sites was carried out using two approaches, TRFLP, and sequencing of cloned 16S rRNA gene libraries. Water samples were collected at the four study sites (Fig. 2.1), and total community bacterial DNA from each site of sampling was extracted as described in Materials and Methods.

The preliminary composition of bacterial communities were investigated and analyzed by TRFLP. TRFLP peaks were generated by digesting PCR amplicons obtained with a 6-FAM (5(6)-carboxy-fluorescein)-labeled 27F primer paired with a 1100R primer and digesting the product with *MnII* as described in the methods section. The presence of different TRFLP peaks, defined as operational taxonomic units (OTUs), and their relative abundances, was determined in each sample (Fig. 2.6). Comparing the 16S rRNA-based TRFLP community fingerprint patterns of the different sites, it was observed that the overall community composition of the plant effluent and the RI and RII sites are much closer to each other than the upstream site. The TRFLP fingerprint of the upstream site was quite different from the other three sites of sampling. Specifically, the TRFLP profiles show that the relative abundance of bacterial OTUs slightly increased from the plant effluent to the RI site (see peaks corresponding to fragment size at 58, 125, 130, 210, 250 and 275 bp in Fig. 2.6), indicating that the abundance of specific bacterial taxa was higher in the RI site compared to the plant effluent. The increase presumably

corresponds to the change in the environmental conditions at the two sites (transition from the plant effluent to the environment).

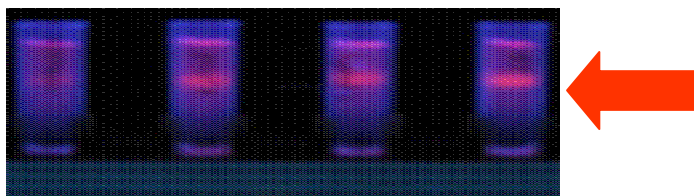


Fig. 2.5. Preparation of community DNA. Water samples were collected at the sites in Fig. 2.1. The DNA was extracted immediately after sample collection, and purified through a cesium chloride gradient. The band corresponding to the microbial community DNA (indicated by the arrow) was extracted and dialyzed before further analysis. The unlabeled band at the top is the meniscus.

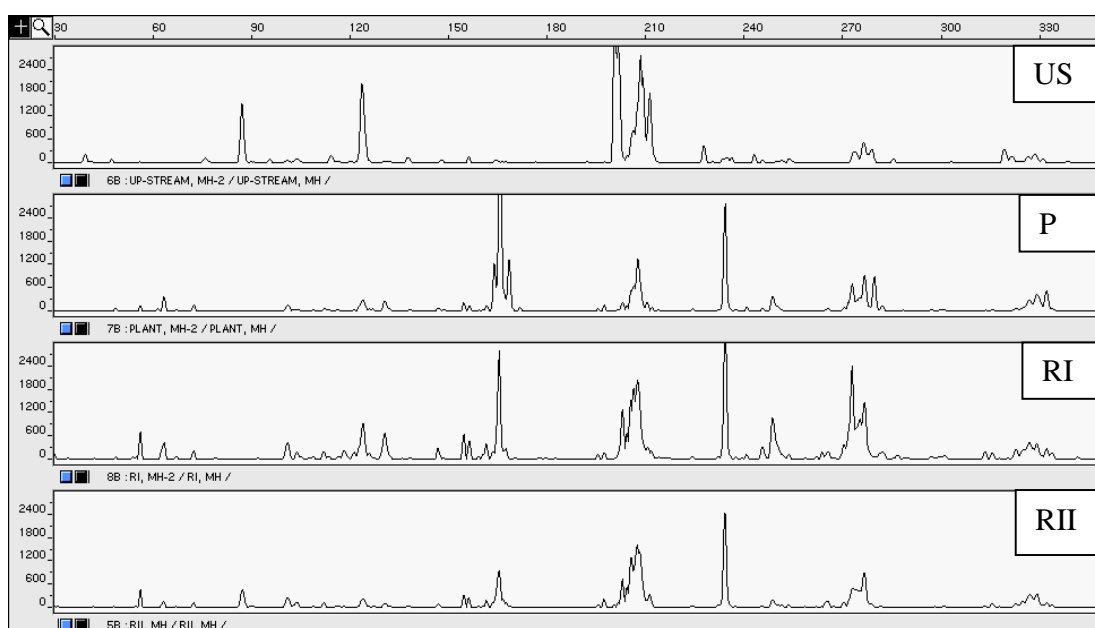


Fig. 2.6. Biodiversity pattern. TRFLP (terminal restriction fragment length polymorphism) of 16S rRNA genes amplified from the community DNA. Peaks correspond to 16S rRNA genes having different digestion patterns, and each TRFLP profile provides a fingerprint of the microbial community under analysis. The comparison of the four samples indicates that some peaks are common to all samples, although their intensity may vary, and some are unique. In particular, the composition of the “Upstream” community appears most dissimilar from the sites P, RI and RII.

The TRFLP analysis yielded a comprehensive survey of the four communities. To identify the bacterial groups that correspond to each peak, the TRFs of individual known 16S rRNA genes were analyzed and compared to the community TRFLP profiles. Both the *in silico* analysis and direct TRFLP analysis on the 16S rRNA clone library was performed as described in Materials and Methods. The results of both analyses were in agreements, with some exceptions that will be mentioned later. For all four sites of microbial communities, a total of 24 different 16S rRNA TRFs was observed across all fingerprints. By comparing the results (Table 2.4) of the computer simulation and direct TRFLP analysis of the clone libraries we attempted to assign bacterial groups to some OTUs. The 168-bp and 208-bp OTUs seemed to mainly represent Betaproteobacteria, a class which has been detected in all four sites in large relative quantity. Gammaproteobacteria and Alphaproteobacteria were predicted to be at 197, 230-bp and 249-bp, respectively. Epsilonproteobacteria at 123 bp and Bacteroidetes (Flavobacteria and Sphingobacteria) were detected at 113 bp, 101 bp and 334 bp, respectively. Some of the clone numbers such as US #38, P #89, RI #67, RI #98 and RII #76 (Table 2.4) indicated that the observed peaks from both analysis are not in agreement; therefore they were not assigned to any bacterial classes.

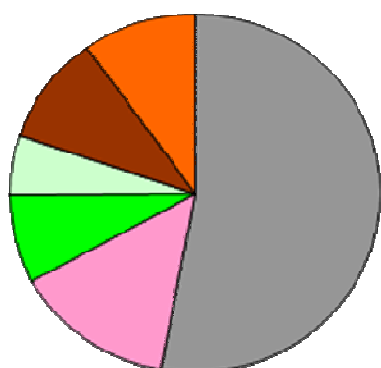
Table 2.4. Correspondence of TRFLP peaks to bacteria

Clone number	Size <i>in silico</i> (bp)	TRFLP size (bp)	Clone identity
US #6	211	208	β - proteobacteria
US #16	134	123	ϵ -proteobacteria
US #38	90	124	α -proteobacteria
US #30	91	101	Flavobacteria
US #73	174	172	δ -proteobacteria
US #26	232	230	γ -proteobacteria
P #155	211	208	β - proteobacteria
P #123	168	168	β - proteobacteria
P #127	335	334	Sphingobacteria
P #173	175	173	Bacterioidetes
P #174	228	225	δ -proteobacteria
P # 89	253	271	α -proteobacteria
RI #4	211	205	β - proteobacteria (Comamonadacea)
RI #2	168	165	β - proteobacteria (Burkholderiaceae)
RI #202	199	197	γ -proteobacteria
RI #67	121	101	Flavobacteria
RI #98	131	120	δ -proteobacteria
RI #99	253	249	α -proteobacteria
RII #80	211	207	β - proteobacteria (Oxalobacteriaceae)
RII #132	169	167	β - proteobacteria (Burkholderiales)
RII #148	199	197	γ -proteobacteria
RII #100	121	113	Flavobacteria
RII #76	86	56	Unclassified bacteria
RII #108	204	201	α -proteobacteria

Further support for the TRFLP analysis came from the sequencing of a total of 92, 136, 200 and 124 clones which were recovered and identified from US, P RI and RII sites respectively. Sequences obtained from individual clones were used to query databases of 16S rRNA gene sequences available in GenBank and in the Ribosomal Database Project (RDP) (Cole et al., 2008). Sequences were assigned to different taxonomic categories according to their level of identity. The distribution of Phyla and classes for each site is illustrated by pie charts in Fig. 2.7. The majority of the bacterial genera were common residents of wastewater treatment plants, such as Alpha-, Beta- and Gammaproteobacteria (Fig. 2.8). Bacterial belonging to those classes were proposed to be responsible for removal of various organic matters from municipal wastewater (Miura et al., 2007; Ding et al., 2011). The abundance and distribution of the 16S rRNA sequences from the P, RI and RII sites were highly similar with slightly differences with respect to the US site. Betaproteobacteria were the most abundant bacterial group in wastewater, and they were represented in more than half of the clone libraries. Other studies have also shown that the dominant members of aerobic reactors treating municipal wastewater belong to the beta subdivision of the Proteobacteria (LaPara et al., 2000; Del Casale et al., 2011). The majority of the Betaproteobacteria sequences were members of the genera Comamonadaceae and Rhodocyclaceae with variation in abundance related to the site of sampling. Betaproteobacteria belonging to Burkholderiales and Oxalobacteraceae were found only in RI and RII. The next most common group at all sites was represented by the Gammaproteobacteria and Bacteroidetes including Sphingobacteria and Flavobacteria. Both Flavobacteria and Bacteroidetes were shown to dominate the community of an industrial treatment facility (LaPara et al., 2000). Interestingly, the

Epsilonproteobacteria division was only found in the upstream site, while Sphingobacteria are present in all sites except the upstream one. In addition, few Clostridia 16S rRNA clones were detected at the RI sites. Cyanobacteria were uniquely found at the RII site. Cyanobacteria are common residents of the eutrophic natural waters. In general, Cyanobacteria are favored by warm, stable and nutrient-enriched waters and they may constitute an important part of the phytoplankton community in WWTPs. The presence of the Cyanobacteria have been reported in other WWTP and have been used to treat wastewater in other parts of the world. Cyanobacteria are usually detected in complex communities in association with nonphotosynthetic bacterial communities composed of different phyla such as Proteobacteria, Actinobacteria and Bacteroidetes (Pope and Patel, 2008; Hoshino et al. 2005; Vasconcelos and Pereira, 2001).

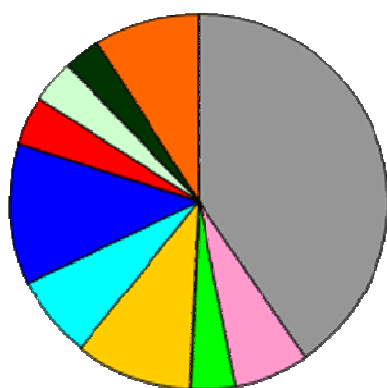
US Community Composition



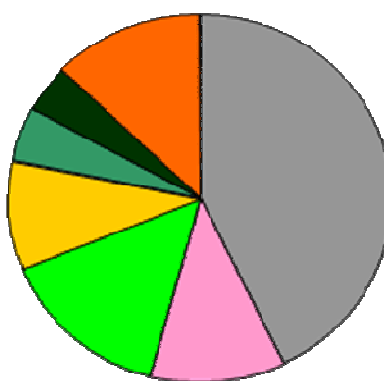
Plant Community Composition



RI Community Composition

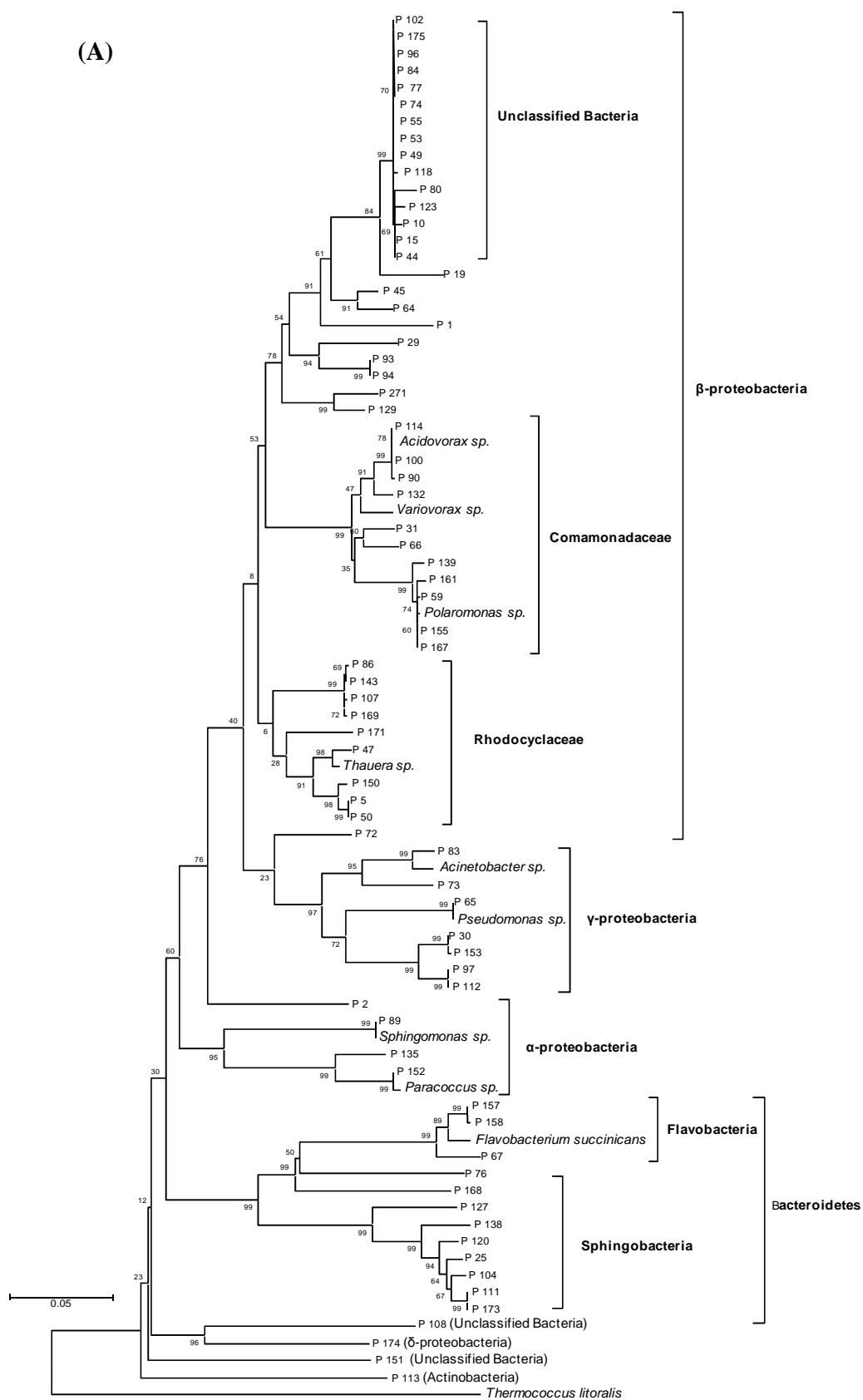


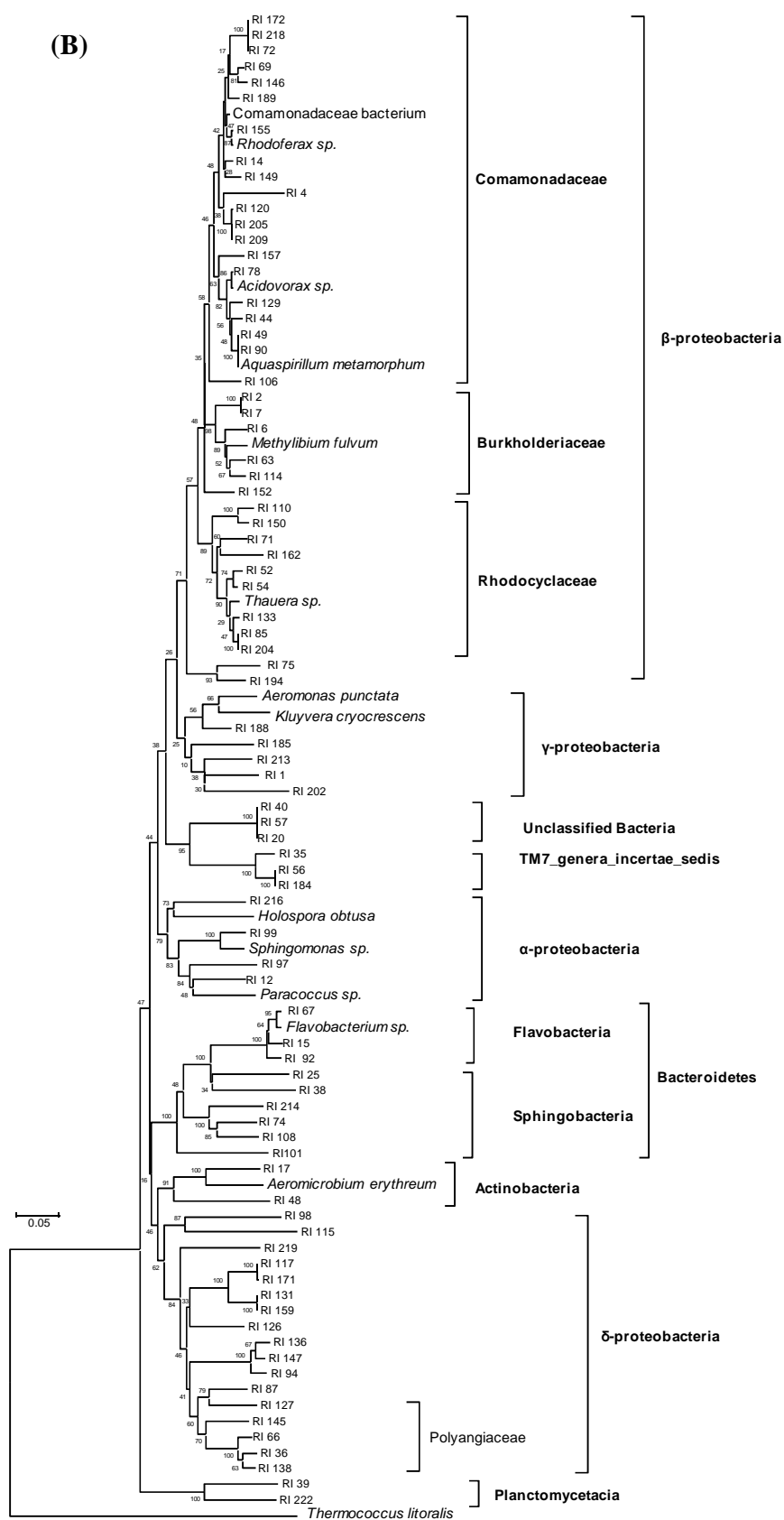
RII Community Composition

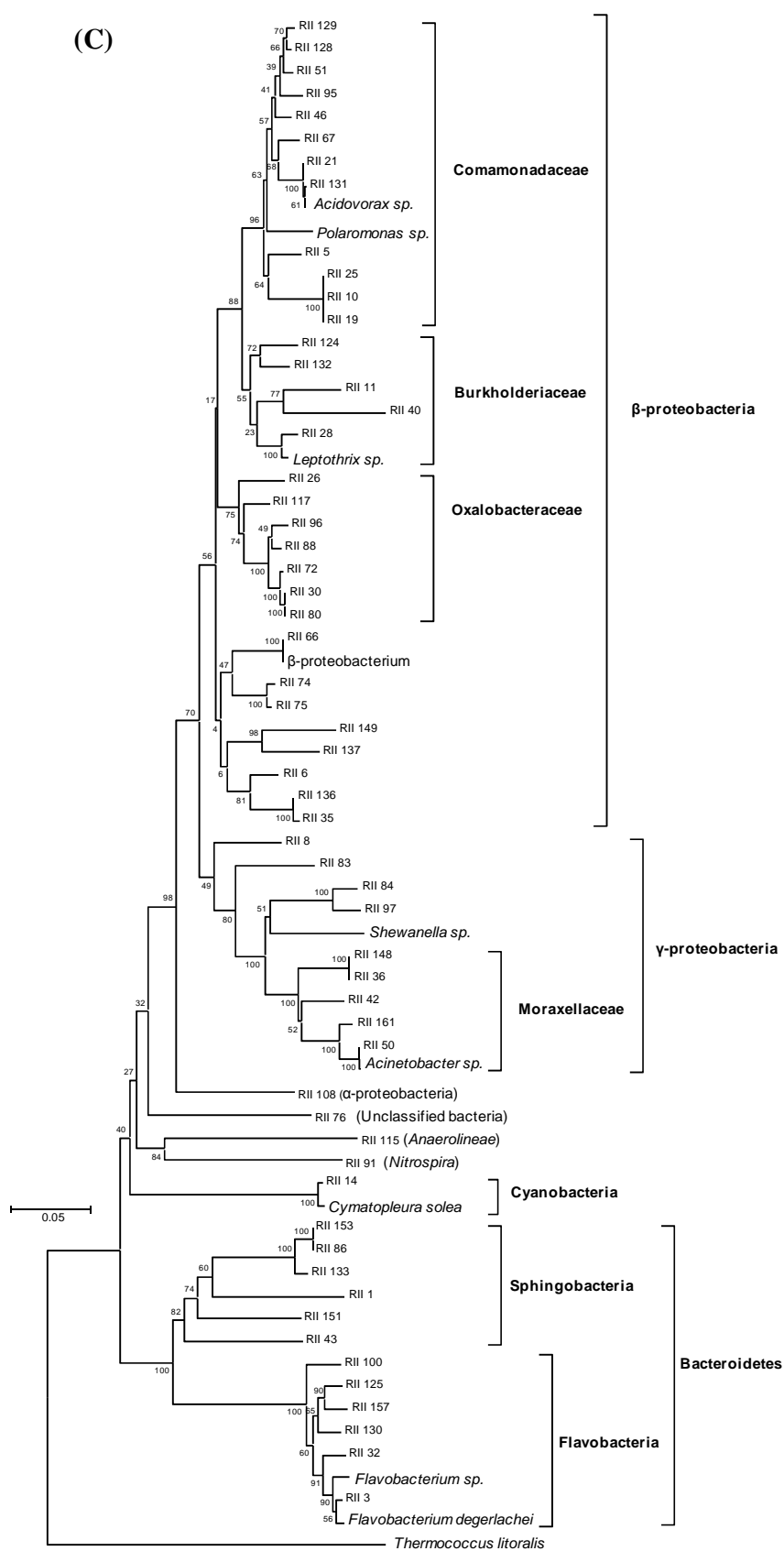


- Betaproteobacteria
- Deltaproteobacteria
- Sphingobacteria
- Alphaproteobacteria
- Gammaproteobacteria
- Epsilonproteobacteria
- Un_Proteobacteria
- Un_ Classified bacteria
- Flavobacteria
- Cyanobacteria
- Clostridia
- Others

Fig. 2.7. Frequency of bacterial phyla at each site of sampling. Slices with a values lower than 4 were combined together within the “Other” slice.







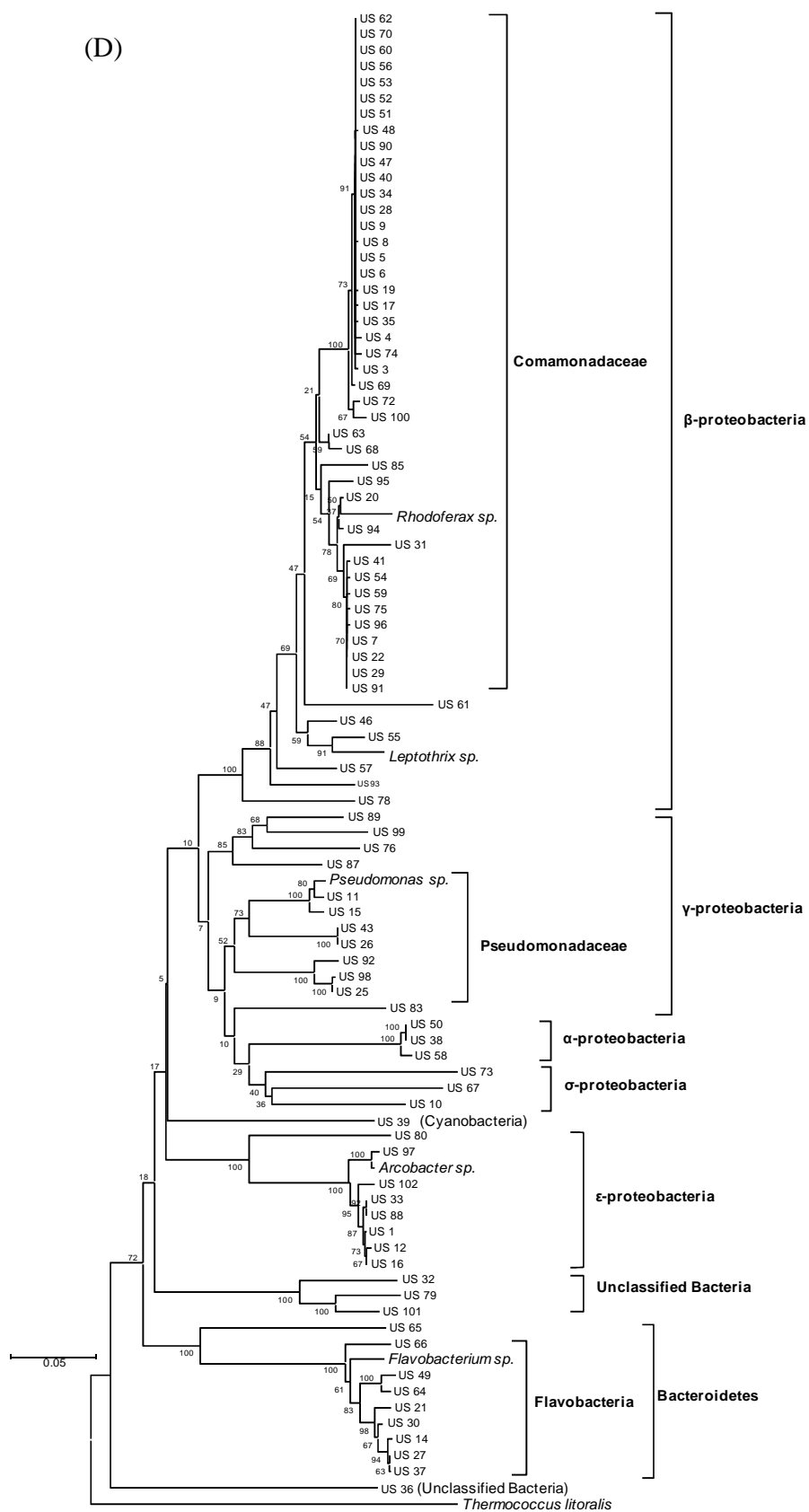


Fig. 2.8. Phylogenetic tree. Neighbor-joining phylogenetic trees of 16S rRNA gene sequences amplified from the wastewater treatment plant (A), from two location downstream RI (B) and RII (C), and upstream (D) of the plant. Trees were constructed using Mega 5 as described in Material and Methods. Major groupings (e.g. Betaproteobacteria) are indicated in parenthesis. Trees were bootstrapped 100 times. *Thermococcus litoralis* was used as outgroup. Scale bars represent a 5% estimate sequence.

Distribution of the β -lactamase resistance genes at the four sites

In order to evaluate the distribution of *bla* TEM4 gene variants within our isolates and the whole community, a library of the *bla* TEM4 was constructed for each of the four sites of sampling. The gene was also amplified from plasmids extracted from individual isolates, which allowed us to link the gene to specific genera and antibiotic resistance. The TEM4 primer pair amplifies about 500-bp of the β -lactamase gene, and the fragments obtained provides sufficient information to look at the distribution of variants of the β -lactamase gene at the four sites of sampling.

The nucleotide sequence of a total of 80 clones was determined for all four sites. To find a possible correlation between the clone library of the β -lactamase gene sequences and their phylogenetic affiliation, we used as reference the TEM4 sequences from known strains that were isolated from the four sites. The phylogenetic relationships among the *bla* TEM4 sequences from the gene libraries and environmental isolates are depicted in Fig. 2.9. The tree revealed two major clusters of the *bla* TEM4 gene. The first cluster is constituted of sequences that are exclusively found at the sites P, RI and RII and were not detected at the upstream site US, whereas the second cluster contains sequences found at all sites (Fig. 2.9). The nucleotide sequence alignment of the second cluster resulted highly similar among the sequences except for one nucleotide position.

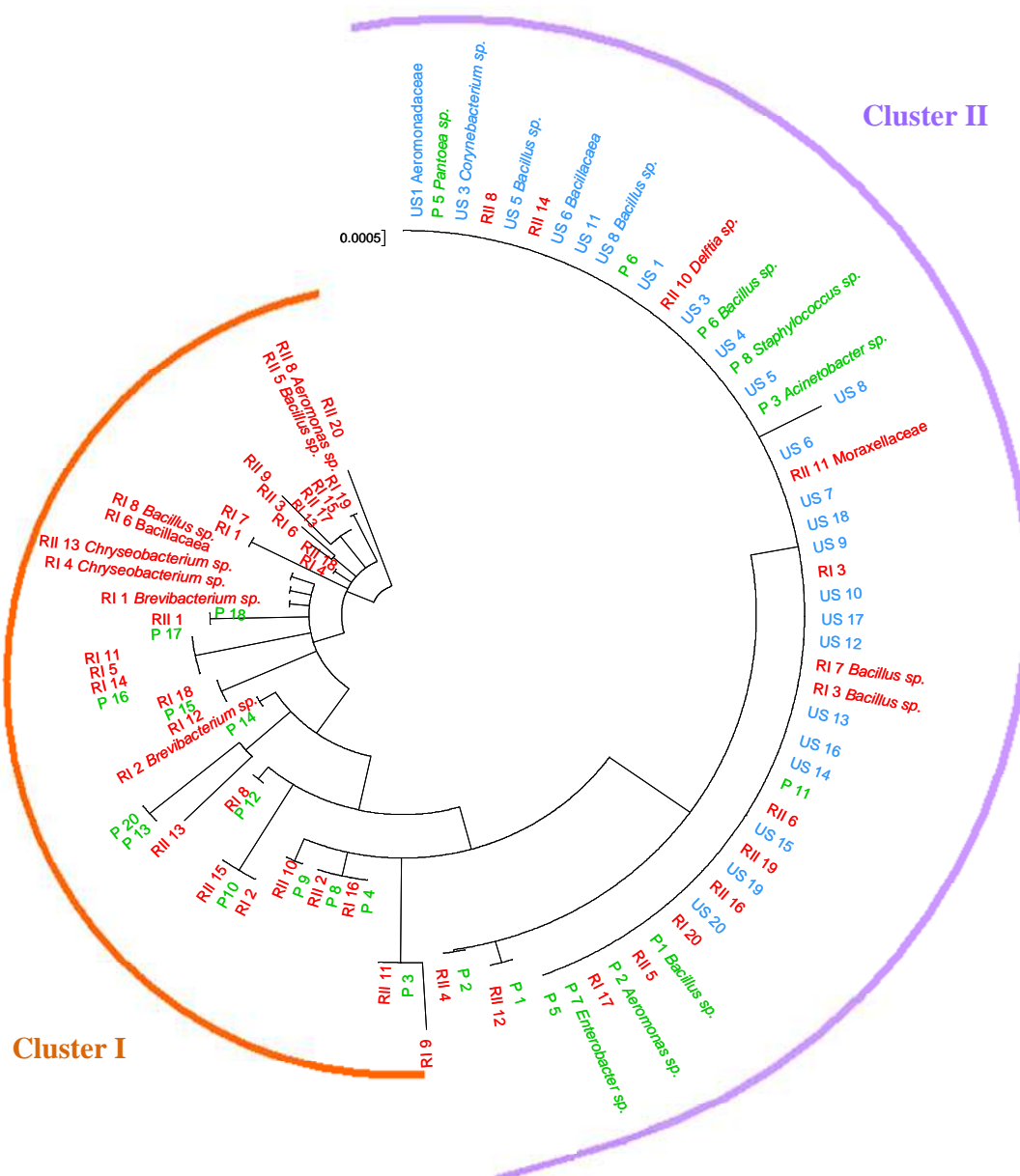


Fig. 2.9. Distribution of the TEM4 genes. Phylogenetic analysis of TEM4 fragments of the β -lactamase genes amplified from community DNA and plasmids from environmental isolates. The tree was obtained with Mega 5, using the neighbor-joining method.

Conclusion

Previous studies have addressed the distribution of antibiotic resistance in WWTPs, their effluents and receiving body of waters. However, most of the reports are restricted to specific taxonomic groups, which are regarded as good indicators for monitoring antibiotic resistance in the environment, for example *Acinetobacter sp.* (Zhang et al., 2009), or Enterobacteria (Huang et al., 2011). This is a reasonable assumption based on our knowledge of the antibiotic resistance properties of these culturable species. As a matter of fact, unculturable species account for the vast majority of the species present, and it is not known what these species are and what their prevalence in these environments is. Throughout this study, we assessed the global microbial biodiversity in wastewater final effluents and in the area downstream from one of wastewater treatment plants in the Somerset Raritan Valley. As a result of the phylogenetic analysis, the microbial community of the Somerset Raritan Valley Sewage Authority and two locations downstream of the plant consisted mainly of Proteobacteria and specifically Betaproteobacteria (Fig. 2.8). However, small differences were observed between sites of sampling, such as presence or absence of specific class or genera (see Results section for details). Our observations are consistent with previously reported studies, as Proteobacteria are commonly detected as the dominant phylotype in WWTPs (LaPara et al., 2000; Del Casale et al., 2011). When the 16S rRNA sequences of the environmental isolates are compared with the results obtained from the clone libraries, which encompass the entire bacterial community of each site, the limits of culture dependent techniques become evident. Only a fraction of the bacteria present in the clone libraries, in our case dominated by the Gammaproteobacteria were actually

culturable, whereas in whole community composition it was determined that Betaproteobacteria are the dominant class.

The evidence supports our proposition that wastewater effluents and receiving environments are reservoir of resistant microorganisms and their associated genes, in agreement with previous reports (Lachmayr et al., 2009; Batt et al., 2006). The data obtained from the antibiotic profiling (Fig. 2.3) of the isolates showed resistance to high concentration of β -lactam antibiotics, including amoxicillin, and multi-drug resistance, which are regarded as common features of isolates from wastewater (Costanzo et al., 2005). However, the analysis of the distribution of plasmid-encoded *bla* TEM genes (Fig. 2.4) of the isolates suggests that the spread of resistance genes might also occur through HGT among different genera, and that isolates not previously reported in the literature may indeed contribute to the spread of resistance.

By looking at the distribution of the *bla* TEM gene within our communities, it was found that genes grouping within cluster 1 (Fig. 2.9) are remarkable in that they are exclusively found in the plant, RI and RII sites, but they were not detected in the US site. We can speculate that *bla* TEM genes specific to the community of the plant effluent moved to the community of receiving body of water, which would explain the particular distribution of the *bla* TEM genes belonging to cluster 1. In contrast, *bla* TEM genes belonging to cluster 2 are ubiquitous, and do not seem to be prevalent at any specific site. Previous study to investigate the impact of the wastewater treatment plant on the antibiotic resistance in *Actinobacter* sp. indicated the possible spread on antibiotic resistance from the WWTPs to the receiving water bodies (Zhang et al., 2009). This

further support the hypothesis that the discharge of wastewater effluents may contribute to the dissemination of antibiotic resistance in the aquatic environment.

Future work will aim to the isolation of novel antibiotic resistance genes by constructing metagenomic expression libraries. An alternative approach will consist of “capturing” natural plasmids, potential carriers of antibiotic resistance genes, for amplification in *E. coli* and sequencing.

Appendix A.

Comparisons of Microbial Community Memberships and Structures

To further evaluate the diversity pattern of our four communities (US, P, RI and RII), some computational approaches were applied. Sequences were initially aligned using CLUSTAL X2 (Thompson et al., 1997), and the alignments were visually inspected and manually edited using BioEdit (Cho and Kim, 2000). Maximum likelihood analyses were conducted using the program PHYML 3.0 (Guindon et al., 2005) using the generalized time reversible (GTR) substitution model with 100 bootstrap resampling (Dorador et al., 2008, Hugler, 2011). Topology of the tree was visualized with the program TreeView (Page, 1996). Simulated phylogenies were rescaled with the APE function *chronoPL* to calibrate the root node. Phylodiversity values were determined using Phylocom v4.0.1 to assess the degree of differentiation between microbial communities by calculating the diversity index (Webb et al., 2008). Phylocom was used to construct a community phylogenetic distance matrix using Rao phylogenetic distances. We performed an unrooted hierarchical clustering based on 1000 bootstraps of the clustering using the *pvclust* package in R (<http://www.is.titech.ac.jp/~shimo/prog/pvclust/>). Hierarchical clustering was based on Ward's agglomerative correlation method (Hamilton et al, 2011). *Pvclust* results include two types of probability values: AU (Approximately Unbiased; represented in red in Fig. A.1) P-value, and BP (Bootstrap Probability; represented in green in Figure A.1) value. Clusters (edges) with high AU probability values (e.g. 95%) are strongly supported.

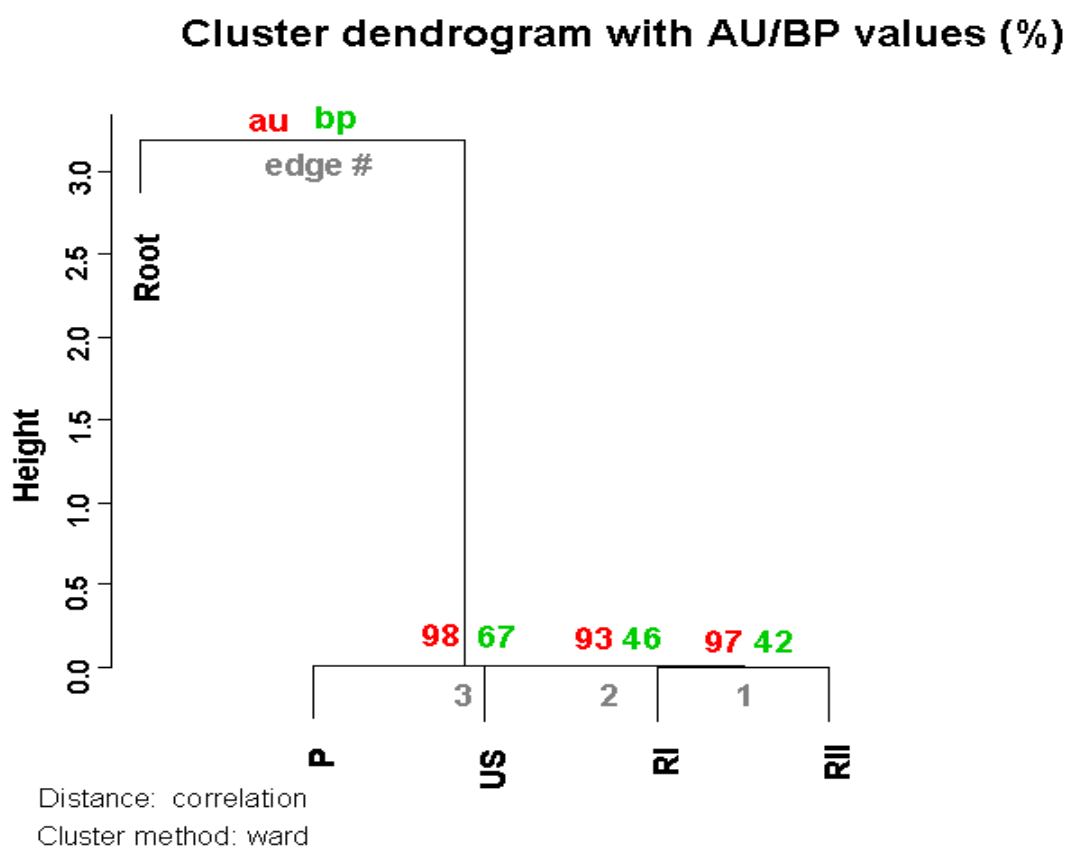


Fig. A.1. A dendrogram based on 16S rRNA gene sequence. Hierarchical clustering analysis was obtained with pvclust, by applying the R project. The approximately unbiased P values (numbers on nodes, in percent) were determined with a multiscale bootstrap (B= 1,000)

Maximum-likelihood tree constructed from the alignment of partial 16S rRNA gene sequences using CLUSTAL X2, and a PhyML tree was constructed from the alignment with the bootstrap value of the 100 (Data not shown).

Taking into consideration that it is unlikely that the habitat evolve according to the same rate-variation patterns as the molecular data, chronogram analysis was performed on the maximum likelihood tree. These chronograms were calculated using the

chronopl function in APE version 2.2 (Paradis et al., 2004), with lambda of 0.50 Rao's phylogenetic diversity index (Dp).

Clustering analysis was computed on the principal component analysis with the R package pvclust (<http://cran.rproject.org/web/packages/pvclust>) using Ward method with correlation distances between the different microbiota profiles at the phylotype level and bootstrap resampling. For each hierarchical cluster, quantities called Probability values are calculated. Probability value of a cluster is calculated between 0 and 1, indicating how strong the cluster is supported by data. Pvclust provides two types of probability values: AU (Approximately Unbiased) P-value and BP (Bootstrap Probability) value. The BP value of a cluster (edge) is the frequency that it appears in the bootstrap replicates. The AU Probability value is calculated by multiscale bootstrap resampling for each cluster in the results of hierarchical clustering. As BP value is described as less accurate than AU value as probability value, we decided to base our clustering approach on the AU probability value. One can consider that clusters (edges) with high AU values (e.g. 95%) are strongly supported by the data. Surprisingly, Dp did not vary significantly show diversity among collected. Clustering tree indicated an unexpected compositional overlap among US, P, RI and RII.

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