Project Report

Assessment of a Novel Source-Tracking Protocol for Evaluating the Significance of Municipal Wastewater Sources on the Microbial Contaminant Levels of Discharged Wastewater

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Abstract

Municipal wastewater treatment plants treat wastewaters generated from residential, industrial, hospital, and other sources. A growing concern worldwide is the prevalence of antibiotic resistant microorganisms in wastewater treatment plants that can potentially survive treatment and be released to the environment through liquid effluent or biosolids. Our research goal was to identify potential protocols to determine the contribution of antibiotic resistant microorganisms from various sources within a municipality. Wastewater samples were collected from ten locations, four from distinct sources before entering wastewater treatment plant (representing hospital, residential, university, and mixed residential/industrial sources) and six samples within the treatment plant at the inflow (mixed), primary clarifiers, trickling filters, aeration basin, secondary clarifiers, and after the chlorination/de-chlorination tank prior to being released as treated effluent. All samples were cultured under antibiotic stress using four antibiotics (cefaclor, ciprofloxacin, doxycycline, and erythromycin) each at two to four concentrations. The lowest concentrations were at the average epidemiological breakpoint common for enterobacteria and the highest concentration at about 16 times above the respective average breakpoint as described by EUCAST (www.eucast.org). Two agars were used in order to favor either copiotrophes or oligotrophes in the sample. DNA extracted from the bacterial populations thus recovered was analyzed through PCR with rpoB primers and separated on DGGE (denaturing gradient gel electrophoresis) polyacrylamide gels. Resultant rpoB-DGGE fingerprints (profile) from each sample were silver-stained; polyacrylamide gels were scanned and processed using BioNumerics software. Principal component analyses, canonical analyses and cluster analyses were carried out in Minitab™ and Genstat™. Results show that our selective approach may distinguish among sources; typically, higher antibiotic concentrations were more useful in discriminating among different sample locations. Same DGGE fingerprints can also be used to infer the effectiveness of treatment options on the diversity of antibiotic resistant microorganisms.

Introduction

The wide use of antibiotics in clinical and residential settings has resulted in increasing incidences of antibiotic resistance in the environment. Additionally, the prolonged use of antibiotics allowed for resistance to develop in many clinically important microorganisms readily detected in the environment (Kim and Aga, 2007; Mispagel and Gray, 2005). Municipal wastewater treatment plants are a critical location for detection of antibiotic resistant bacteria from anthropogenic sources. Studies have shown that treatment is not effective at eliminating all bacteria and there are indications that some bacteria can become more stress resistant, including antibiotic resistance, following treatment (Ferreira da Silva et al., 2006; Ferreira da Silva et al., 2007). After treatment, waste is released into the environment as effluent or biosolid, thus emerging as a potential health and environmental health risks.

The objective of our research is to determine the effects that certain wastewater sources have on the parameters of treated effluents. There are plenty of data to support shortcoming in treatment options when treating antibiotic resistant bacteria (Kim and Aga, 2007; Pauwels and Verstraete, 2006). However, there are lack of data that determine the sources of antibiotic resistant bacteria, specifically the sources that are most responsible for resistant bacteria surviving treatment. Current approaches have usually tended to compare wastewater treatment plants with a single source influent. The procedure in Boon et al. (2001) comparing activated sludge communities in wastewater treatment plants treating municipal, hospital, and a variety of industrial sources is common. In order to compare integrated wastewater sources within the same treatment plant, we develop a source tracking method that can be used to source track antibiotic resistant bacteria in source-integrated wastewater samples. Similar source tracking methods were developed for water samples containing fecal matter from mixed sources including human and non-human (Griffith et al., 2003). We sampled several distinct wastewater sources and treatment plant stages. Molecular fingerprints using primers specific for bacteria allow for the assessment of antibiotic resistant bacterial diversity present in the wastewater samples (Dahllöf et al., 2000; Peixoto et al., 2002). Our method has the potential to support discriminant comparison among sources and treatment stages to assess transport of antibiotic resistance along wastewater collection and treatment stages and the effectiveness of treatment options.

Denaturing gradient gel electrophoresis (DGGE) was used to study the microbial diversity of all wastewater samples. DGGE has been used by many researchers in different microbial diversity applications, including wastewater microbial diversity (Boon et al., 2002). Similar to other electrophoretic method, DGGE separates nucleic acid fragments that have been amplified by polymerase chain reaction (PCR) using primers specific for group of interest. These fragments are separated differentially in DGGE polyacrylamide gels by base pairing sequence of amplified fragment. DNA denaturants, urea and formamide, are present in a gradient in the gels, with lowest concentration at the loading top, and highest concentration of denaturants at the bottom of the gel. The gel assembly is submersed in a buffered solution, and an electric current is applied so that DNA fragments can migrate through the charge gradient; in the case of DGGE the charged current passes directly through the gel. Increasing denaturant concentration causes disruption in the DNA strands, which causes strand separation in some regions and hinders migration of DNA fragments (Okubo and Sugiyama, 2009). Because of different base pairing in all bacterial species, fragment migration will be offset for each unique species or subspecies, dependent on the base-pairing, thus resulting in differential migration and separation of heterogeneous mixture of DNA fragments as is the case in mixed samples from wastewater samples. Alternative molecular based approaches to analyze bacterial diversity are discussed in Okubo and Sugiyama (2009) and include terminal-restriction fragment length polymorphisms (T-RFLP) and automated ribosomal intergenic spacer analysis (ARISA). While there are advantageous and disadvantages to each method, DGGE is the most suitable for our application because DGGE coupled with proper statistical analyses method will discriminate microbial diversity among groups in applications where there is high heterogeneity and unknown species such as in wastewater samples (Okubo and Sugiyama, 2009). In addition, DGGE gels can be

further analyzed by excising individual bands (which are DNA fragments amplified by PCR) and cloned and purified for sequencing (Boon et al., 2002) or directly sequenced.

We tested for antibiotic resistant bacteria based on the use of four antibiotics widely used. These antibiotics were selected based on their distinct mode of action against bacteria; all the major groups of antibiotics are represented in our study. Cefaclor is a member of the β-lactam antibiotics, more specifically an α-amino cephalosporin. Similar to many β-lactam antibiotics, cefaclor is bactericidal by inhibiting peptidoglycan synthesis (Bryskier and Lebel, 2005). Ciprofloxacin is a fluoroquinolone that inhibits bacterial DNA replication by inhibiting the activity of DNA gyrase (Bryskier, 2005B). Doxycycline is classified as a tetracycline antibiotic and its mode of action is through inhibition of protein synthesis of the 30S RNA in bacteria (Bryskier, 2005A). Lastly, erythromycin is a macrolide antibiotic and its mode of action is through inhibition of protein synthesis by binding to the 50S subunit of bacterial ribosomes (Bryskier and Bergogne-Bérézin, 2005).

Materials and methods

Sampling of wastewater

Wastewater samples were collected over a period of two days in the fall of 2008 from a municipal wastewater collection system and secondary stage treatment plant in Las Cruces, New Mexico, USA. A total of ten sampling locations were included in this study. Four pre-treatment collection system locations throughout the city were sampled (lift stations). Lift stations collect raw wastewater from discrete locations that can be described as a unique wastewater source. The four sources sampled include hospital, university, residential, and mixed residential-industrial sources. Six sampling locations within the wastewater treatment plant were included from the inflow (mixed sources), primary clarifiers, trickling filters, aeration basin, secondary clarifiers, and after the chlorination/de-chlorination tank prior to effluent release to surface waters. One liter samples were transported on ice to the laboratory and stored at 4°C until analyzed. Samples were analyzed within 24h from sampling.

Sample culturing

Sample extracts were cultured on Mueller-Hinton Agar (MHA, Oxoid) and R2A (Remel) with incubation temperatures of 35°C for a period of 36 hours. The MHA agar allows for growth of fast growing, copiotrophic, organisms while R2A agar, a low nutrient agar, allows selective growth of slow growing, oligotrophic organisms. We followed the agar dilution protocol by Wiegand et al. (2008) to prepare antibiotics and test for susceptibility to antibiotic stress. Two to four concentrations of each antibiotic were prepared, with a low concentration of antibiotic below the "epidemiological cut-off value" (ECOFF) (Wiegand, 2008) and a high concentration of antibiotic up to 16 times the average ECOFF value as listed in EUCAST (www.eucast.org). For cefaclor, a relatively new antibiotic not currently reported by EUCAST, the concentrations were based on published data by Bryskier and Lebel (2005).

DNA extraction, PCR and DGGE methods

Following culturing, we performed DNA extraction of whole plate cell growth using a standard microbial culture DNA extraction kit (MoBio, Carlsbad, CA). The PCR amplification program using rpoB primers was carried out as described by Peixoto et al. (2002) with the addition of four cycles of denaturing, annealing, and extension prior to the final extension step, for a total of 29 steps. The rpoB primers used were 1698F (with a GC clamp) and 2041R; we used Taq polymerase from Fermentas (Glen Burnie, MD, USA). PCR amplification was carried out on a Bio-Rad (Hercules, CA) thermal cycler.

Amplified genomic DNA was then separated by DGGE using 6% polyacrylamide gels and a 40 to 60% denaturing gradient. DGGE was performed for 14 hr at 85 V in 1X TAE buffer using a Bio-Rad DCode Universal Mutation Detection System. Following DGGE, gels were either silver stained using the protocol by Han et al. (2008) or stained by using the standard ethidium bromide method. Silver-stained gels were preserved in 4% glycerol solution for long-term storage at 4°C.

Gel analysis and statistical analysis

Silver stained gels were scanned while the image of ethidium bromide gels were collected using a Kodak UV transilluminator. Gel fingerprint analyses were carried out using BioNumerics software and we compared the rpoB profiles from each sample. Principal component analyses, canonical variant analyses and cluster analyses were carried out in Minitab™ and Genstat™. Dendrograms were produced using Jaccard similarity and nearest neighbor linking method.

Results and discussion

DGGE band analysis revealed observable differences between samples, antibiotic, antibiotic concentration, and growth media.

Initial analyses estimated the capability of the variable concentrations of antibiotics to select for distinct bacterial populations, with distinct rpoB-DGGE fingerprints (Figure 1). Results show that to be mostly true although this was not necessarily statistically significant as indicated by the overlap of the 95% confidence intervals in Fig. 1; this may possibly be more an indication of the limited number of fingerprints used in the analysis. Dissimilarities in population diversities were also introduced by the type of agar used, thus suggesting differential recovery of antibiotic resistant oligotrophes or copiotrophes. The most obvious selective action of antibiotics was observed on the doxycycline amended R2A agar, for all tested antibiotic concentrations (Figure 1). A likely explanation for this occurrence is the wide use of doxycycline or other tetracycline antibiotics in different sources, which allow resistance to be widespread, and resistance profiles to be similar.

In a second step the capability of the different tested antibiotics to select for different bacterial population was evaluated. The results, presented in Figure 2, were obtained with the 16x ECOFF concentration for each antibiotic. Thus it is expected that the organisms selected here do exhibit acquired resistance. The rpoB-DGGE fingerprints obtained from

the different antibiotics on the MHA agar have shown erythromycin, doxycycline and ciprofloxacin to select for bacterial populations distinct from control; cefaclor resistant population, on the other hand, was similar to the control population. These results suggest that 1) copiotrophes resistant to cefaclor may have a competitive advantage and thus outcompete all other bacteria on the control plates and 2) that populations resistant (putative acquired resistance) to the other three antibiotics are present in the sample but may be eliminated from a non-selective growth substrate. The antibiotic amended R2A agar, that allows oligotrophic organisms to compete against faster growing copiotrophes, has shown that all antibiotics do allow the recovery of distinct bacterial populations. This suggests that oligotrophic bacteria, while present, may not compete well against non-resistant bacteria in non-selective environments. Overlap of several fingerprints may also suggest organisms exhibiting multiple antibiotic resistances.

Source discrimination analyses were attempted by Principal Component and Hierarchical Clustering analyses. Here we present the latter for a high concentration of antibiotics (Figures 3 and 4). For copiotrophes the discriminant power of the doxycycline resulted in little similarity among most samples; the trickling filters, aeration basin and the University input had 40% to 60% similarity suggesting that same doxycyline resistant bacteria are found at all these locations. On the other hand cefaclor resistance, a relatively newer but widely used antibiotic, was found in all sources except the mixed source and more importantly also has shown some similarity with the bacteria surviving chlorination. The low similarity index simply indicates that not all organisms that exhibited cefaclor resistance passed through the system but only a few. The fingerprints of ciprofloxacin resistant bacteria were largely separated in what may be called an input cluster and a treatment cluster. This suggests that the resistant population is parsed as it travels through the treatment stages and that the ones dominating in the sources are not the same as the ones recovered in the treatment plant. Given that our testing did not cover all input locations (only four lift stations out of eleven) it is possible that the ciprofloxacin resistant population recovered in the treatment plant is more representative of the other sources. Erythromycin dendrogram suggests that certain dominant bacteria from sources including the hospital may be recovered after chlorination (Reinthaler, 2003; Baquero, 2008). A comparison of antibiotic resistance profiles from hospital wastewater compared to other end stage wastewater treatment samples is noteworthy; our results suggest that this similarity supports the idea that of the municipal sources, hospital wastewater contributes greatly to the incidence of antibiotic resistant bacteria recovered after wastewater treatment. Further sampling coupled with sequencing of similar resistant isolates could reinforce this hypothesis.

For the oligotrophes (Figure 4) the erythromycin dendrogram suggests that the population resisting chlorination is not correlated with any of the other tested locations, which may mean that the dominant organism(s) resistant to erythromycin recovered after chlorination is likely a minor member of the pre-chlorination population. Doxycycline has proven to be a likely candidate for source tracking for oligotrophes.

A common issue in our DGGE gels is often few observable bands from several samples, some of which produced no clear bands, which we eliminated from our analysis. A possible explanation is most likely low DNA extraction yields and not PCR amplification

biases (Calábria de Araújo et al., 2008). Although our method results in some simplifications, the nature of microbial diversity in wastewater samples is complex and problems with low band resolution has been described before in DGGE analysis of wastewater samples (Boon et al., 2002).

Conclusions

We have developed a PCR-DGGE approach using rpoB gene bacterial diversity method to source track antibiotic resistant bacteria in wastewater treatment plants. By using antibiotic resistance as a selective factor we can simplify part of the complex diversity in wastewater samples. Through statistical and dendrogram analyses of the DGGE sample profile, our results demonstrate that our method can discriminate waste sources. We typically found that a high concentration of antibiotic was more effective than a low concentration at discriminating between sources. We have shown that within the group of antibiotic resistant bacteria, many differences in diversity are present, such as variable selection based on antibiotic presence and the concentration of antibiotic in the amended agar. Of the tested antibiotics, doxycycline is most suitable to discriminate resistant groups from the control cultures. Erythromycin and cefaclor at high concentrations are usually found to discriminate hospital source from other sources. Erythromycin resistance diversity was found to be most similar at the hospital source and the end stage treatment including effluent sample based on the dendrogram clustering result, thus the possibility of a potential source-tracking antibiotic for hospital resistance loads.

We have analyzed wastewater samples collected in one temporal period, to determine the reproducibility of results it is necessary to extend sampling periods throughout the year as well as cover all lift stations in the municipality that contribute to the influent (total of eleven). Future work should also identify individual antibiotic resistant strains directly from DGGE gels.

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Figures

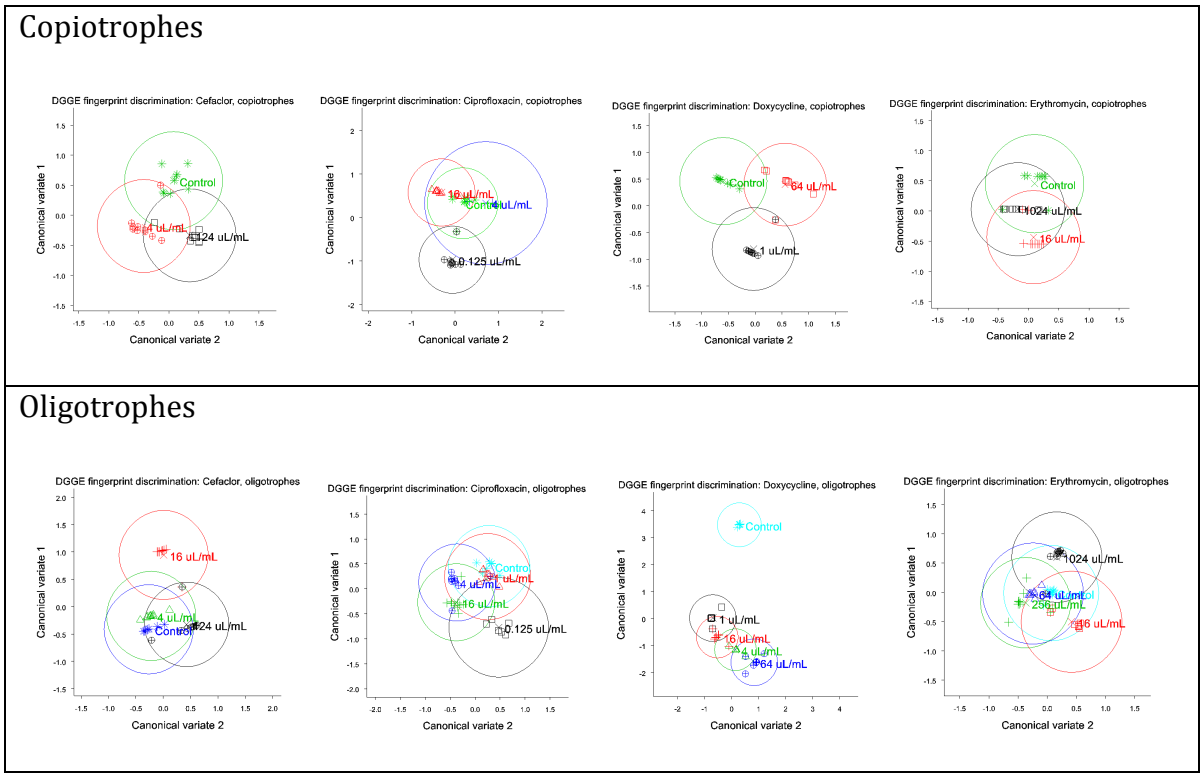

Figure 1. Discriminant influence of variable antibiotic concentrations as inferred from rpoB-DGGE fingerprints.

Note: Each data point represents one rpoB-DGGE fingerprint of one sample; the circles indicate a 95% CI; copiotrophic organisms are the ones recovered on MHA while oligotrophic organisms are the ones recovered on R2A agar.

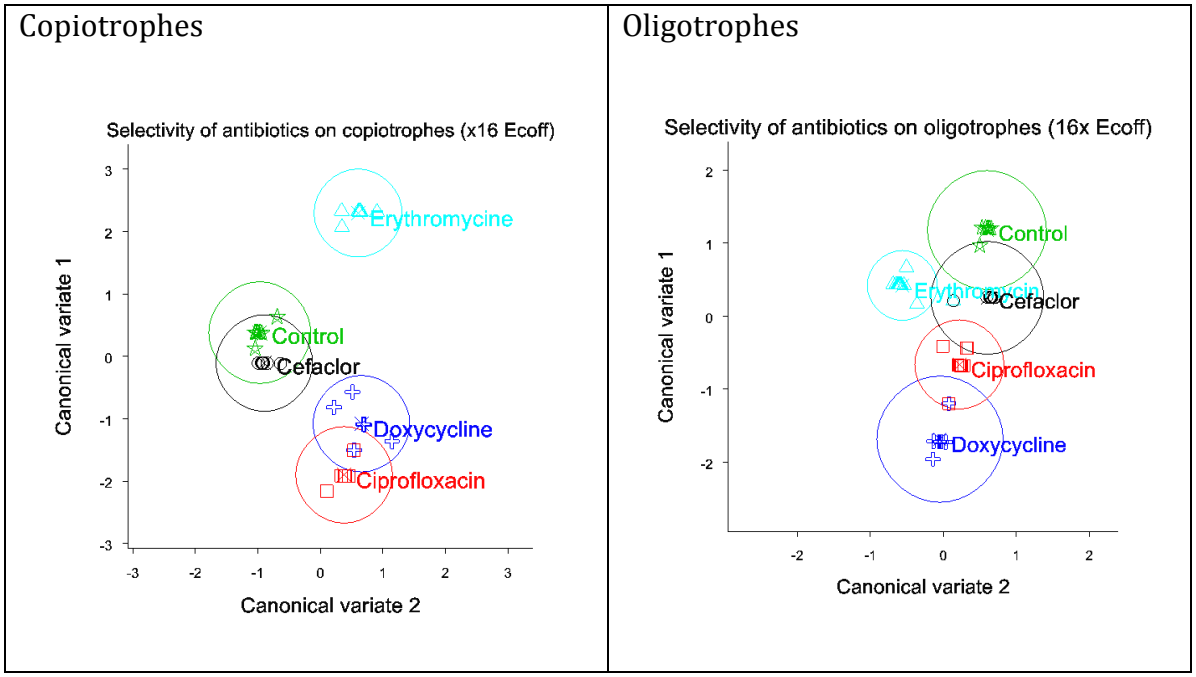

Figure 2. Discriminant influence of the high concentration of antibiotics 16x the ECOFF

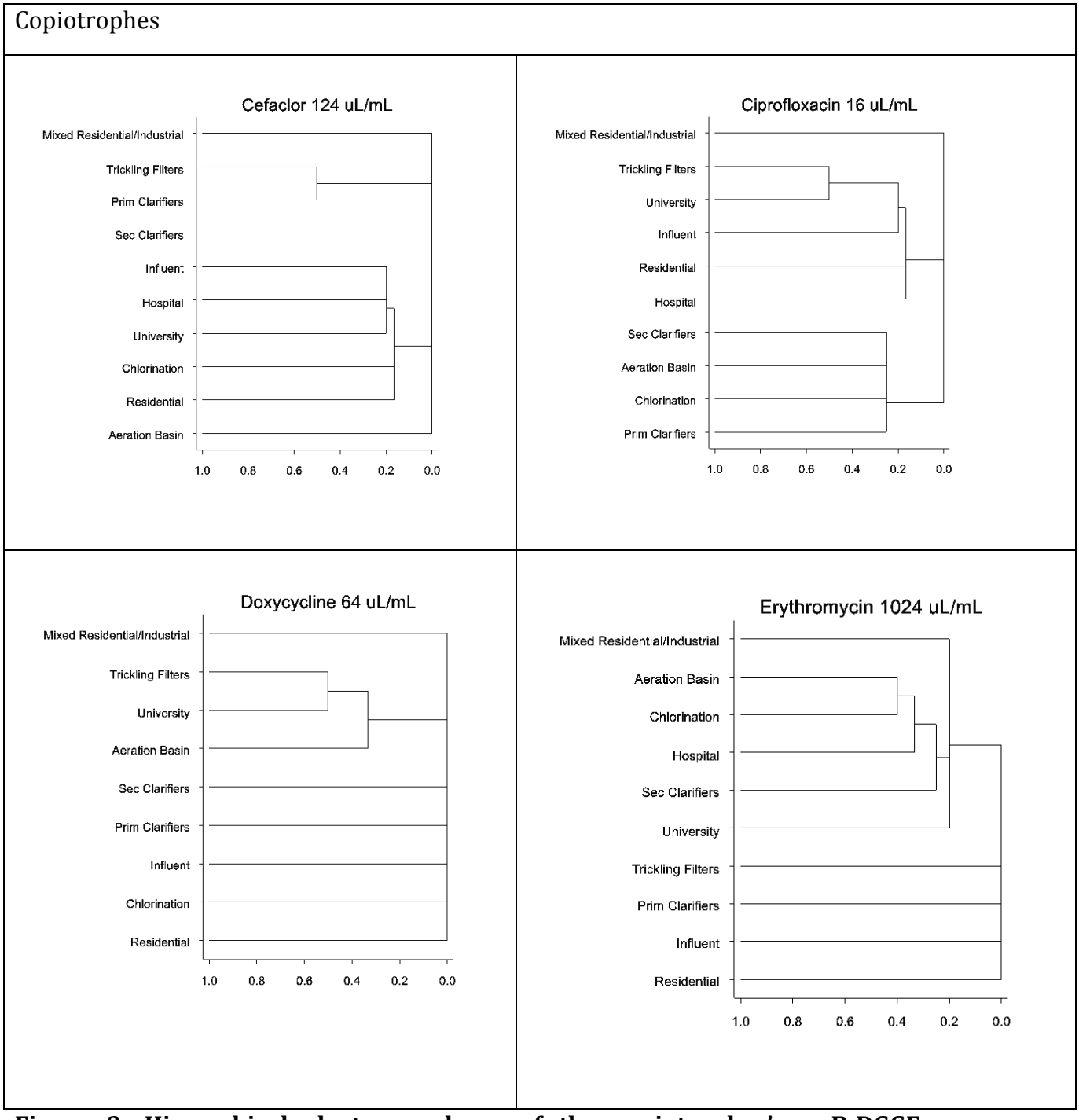

Figure 3. Hierarchical cluster analyses of the copiotrophes' rpoB-DGGE fingerprints obtained for the four antibiotics at 16x ECOFF

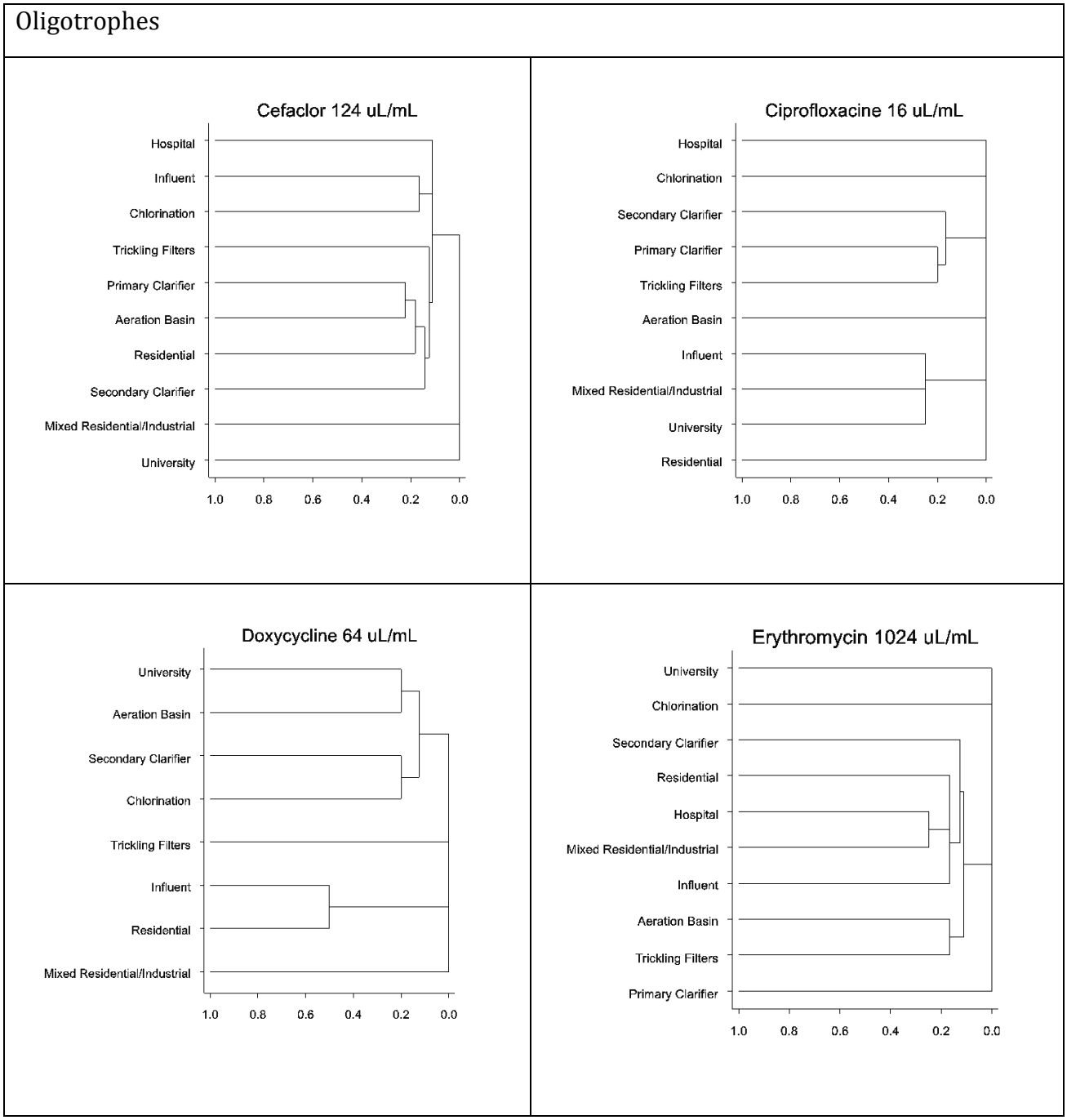

Figure 4. Hierarchical cluster analyses of the oligotrophes' rpoB-DGGE fingerprints obtained for the four antibiotics at 16x ECOFF

Deliverables

Publications:

1. Sigala, J, Unc, A. 2009. Assessment of the contribution of wastewater sources to the diversity of antibiotic resistance in wastewater treatment plants. Proceedings of the 14th European Biosolids and Organic Resources Conference, November 2007, Lowe P. (ed), Aqua Enviro, Manchester, UK, ISBN # 978-1-903958-35-3

Public presentations:

1. Sigala, J, Unc, A. 2009. Assessment of the contribution of wastewater sources to the diversity of antibiotic resistance in wastewater treatment plants. 14th European Biosolids and Organic Resources Conference, 9-11th November 2009, The Royal Armouries, Leeds, UK
2. Sigala, J, Unc, A. 2009. Assessment of a novel source-tracking protocol for evaluating the significance of municipal wastewater sources on the microbial contaminant profiles of discharged wastewaters. New Mexico Water Research Symposium, Aug. 11, New Mexico Tech Macey Center, Socorro, NM

Other:

The student (Jesus Sigala) has used the project activity to support his application, in collaboration with Dr, Adrian Unc, to the Bridge to Doctorate graduate support (NSF program). He was accepted and is currently a graduate student under the supervision of Dr. Adrian Unc.