A PCR-DGGE approach to evaluate the impact of wastewater source on the antibiotic resistance diversity in treated wastewater effluent

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ABSTRACT

Increased incidence of antibiotics in human-affected environments is raising concerns about increase in acquired antibiotic resistance by environmental bacteria. Wastewater collection and treatment systems are likely significant anthropogenic sinks and vectors for antibiotics and associated antibiotic resistance. Typical municipal treatment plants collect wastewaters of various sources, including well-established antibiotic resistance reservoirs such as hospitals, intensive care units and nursing homes, and integrate them with sources not commonly identified as major sources of antibiotic resistance, such as residential or industrial sources. A comprehensive PCR-DGGE diversity analysis of wastewater antibiotic-resistant bacteria was performed to evaluate the role of various wastewater sources in the discharge of antibiotic resistance by a municipal treatment plant. Wastewater sources are clearly inducing resistance in the final effluent but the role of each source type is highly variable, likely as a function of variable environmental conditions or water use patterns. Comparisons between primary treatment and secondary treatment stages indicate a strong role of the intensity of the wastewater treatment in the diversity profiles of antibiotic-resistant bacteria. While pervasiveness of antibiotic resistance in the system impedes clear discrimination between sources in the tested system, there are indications of specific source type related impacts.

Key words | antibiotic resistance, PCR-DGGE, raw wastewater, wastewater treatment

INTRODUCTION

Treatment of wastewater is employed worldwide to decrease biological oxygen demand of released effluent and to prevent proliferation of disease agents into environmental sinks. Microbiological studies have focused on the overall quality of treatment (Blatchley et al. 2007; Nagulapally et al. 2009), microbial diversity in activated sludge samples (Boon et al. 2002), throughout the treatment process (Wéry et al. 2008; Börjesson et al. 2009) or on the impact of treated effluent on the quality of the receiving water bodies (Mispagel & Gray 2005; Li et al. 2010). Wastewater treatment efficiency is constantly reconsidered as our understanding of associated risks improves or as new challenges emerge, such as risks associated with the environmental dissemination of antibiotic-resistant bacteria. It is becoming more obvious that microbial quality must not be determined solely by the counts of indicator bacteria present in treated effluents, but also by the presence of genetic determinants of resistance harboired by certain bacteria or in the sample matrix (Pauwels & Verstraete 2006; Kim & Aga 2007).

While the proportion of antibiotic-resistant bacteria that survive wastewater treatment is rather small, it is possible that genes that confer resistance are released into the environment, where they may transfer horizontally to microorganisms native to water, soils or sediments (Reinthaler et al. 2003; Ferreira da Silva et al. 2006; Szczepanowski et al. 2009). Moreover, pathogenic bacteria have been detected in insufficiently treated wastewater (Wéry et al. 2008). Understanding of how antibiotic resistance is affected by wastewater treatment and the significance of wastewater sources is limited. Sources for the emergence of antibiotic resistance and dissemination of resistance to the environment have typically been assumed to be hospitals (Pauwels & Verstraete 2006), intensive care units, long-term nursing homes, or animal farms using antibiotics for growth
promotion (Randall et al. 2007). Nevertheless, typical household activities such as use of intensive cleaning agents may induce enhanced antibiotic resistance (SCENIHR 2009). Use of non-prescription antibiotics also contributes to increasing prevalence of antibiotic resistance in domestic wastewater (Hoiby 2000).

Thus, wastewater systems act as integrators of antibiotic and associated resistances from a variety of sources and possibly drive the spread or emergence of antibiotic-resistant bacterial populations in the environment (Baquero et al. 2008). Therefore, it is critical to understand the influence of source on the quality of treated effluent. Domestic sources most commonly dominate by volume. If any source is identified as the origin of large loads of antibiotic resistance, then targeted alternative treatment approaches could be considered.

In any environment, bacterial numbers and diversity are large, and this may challenge any assessment approach. Denaturing gradient gel electrophoresis (DGGE) is a relatively common method that can be easily employed to study microbial diversity (Muyzer et al. 1993). Its general ease, adaptability, and ability to examine a large number of samples are its strengths and have made it a standard method for analysing complex environmental samples. DGGE captures microbial diversity as electrophoresis fingerprints that allow comparison between samples in large datasets. Boon et al. (2002) used DGGE extensively to study diversity of microbial groups and entire microbial diversity in activated sludge samples from 15 treatment plants.

Our goal was to determine the extent to which wastewater source types and wastewater treatment stages affect presence of antibiotic resistance in the treated effluent. We used a targeted PCR-DGGE approach to distinguish among bacterial consortia capable of growth on substrates amended with antibiotics at concentrations generally used to determine presence of acquired antibiotic resistance. The antibiotics used were cefaclor, ciprofloxacin, doxycycline, and erythromycin. They represent various antibiotic classes and modes of action and thus various resistance mechanisms. Cefaclor is a clinically recent second generation β-lactam antibiotic that inhibits peptidoglycan synthesis in bacterial cell walls. It is mostly prescribed for respiratory tract infections and it is selective for Gram-positive and Gram-negative bacteria (Meyers 2000). Ciprofloxacin is a fluoroquinolone that binds to DNA gyrase and inhibits bacterial DNA replication. It is a broad-spectrum antibiotic, effective against Gram-positive and Gram-negative bacteria (Bryskier 2005), commonly used to treat urinary tract infections. Doxycycline belongs to the class of tetracycline and it inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. Tetracyclines are used for clinical and non-clinical uses, such as in livestock, and are broad-spectrum (Li et al. 2010). Erythromycin is a macrolide antibiotic that binds to bacterial 50S rRNA, inhibiting protein synthesis by inhibiting translocation of tRNA between the aminoacyl and peptidyl sites along the rRNA during translation (Bryskier & Bergogne-Bérézin 2005). The latter two are bacteriostatic. Literature on erythromycin is ample, reflecting its wide use to treat and prevent infections caused by various Gram-positive and -negative bacteria.

**MATERIALS AND METHODS**

**Sampling**

Sampling of wastewater was carried out at system-scale for a medium sized municipality (<100,000 people) in the arid US Southwest (Las Cruces, NM; 32°19’11” N 106°45’55” W). This included both collection system and in plant treatment stages. Raw, untreated wastewater samples were collected from wastewater lift stations (pumping stations) located throughout the municipality. Each lift station collects and integrates wastewater from known sources identified as residential, university (integrating residential and industrial type sources), mixed residential/hospital, or mixed residential/industrial. Wastewater treatment plant samples were collected at: influent, mixing, effluent of primary clarifier, effluent of trickling filter, aeration basin, effluent of the secondary clarifier, and final effluent after the chlorination/de-chlorination tank prior to discharge to surface waters (Figure 1).

Wastewater was sampled on the 9th and 10th of February 2010, and on the 3rd and 4th of August 2010. In February (winter), each location was sampled once a day in the morning. In August (summer), samples were collected in the morning and afternoon of the first day and the morning of the second day. Samples were composited separately for each season. Sample volumes varied from 200 to 3,800 mL according to the expected bacterial concentration. Sterile glass or HDPE containers were used and samples were stored on ice for <3 h until delivered to the laboratory.

**Sample culturing**

Composite samples were cultured within 24 h of last sample collection. Heterotrophic bacteria counts were obtained on nutrient rich Mueller-Hinton agar (MHA, Oxoid) targeting
copiotrophs or nutrient poor R2A (Difco) targeting oligotrophs. Agars were amended with antibiotics at concentrations indicative of acquired resistance.

Epidemiological cutoff values (ECOFF, http://www.eucast.org/mic_distributions) or the minimal inhibitory concentration (MIC) (CLSI 2010) were used to determine antibiotic concentrations for the two agars. Concentrations used were above the highest ECOFF for all bacteria listed in EUCAST database. Tests were carried out according to CLSI protocols (CLSI 2010). Escherichia coli ATTC 25922, resistant to the four antibiotics, was used as a positive control. Antibiotics used were cefaclor (16 μg mL⁻¹), ciprofloxacin (8 μg mL⁻¹), doxycycline (16 μg mL⁻¹), or erythromycin (64 μg mL⁻¹). Fungal growth was prevented by adding 75 μg L⁻¹ cycloheximide to all plates including control (Szczepanowski et al. 2009). Cycloheximide and erythromycin were dissolved in 35% ethyl alcohol solution (v/v). Ciprofloxacin and doxycycline were dissolved in water. Cefaclor was dissolved in 0.004 M HCl. When required, pH of stock solution was corrected to 7.2 with NaH₂PO₄.

For most samples plating was done by agar plate spread method. The more diluted secondary clarifier and effluent samples were plated by membrane filtration. Tests were carried out in triplicate. Plates were incubated for 36 h at 37 °C. Colony counts were collected for doxycycline and ciprofloxacin plates for winter and for all antibiotics for summer.

**DNA extraction, PCR and DGGE analysis**

All colonies from a plate (target range 30–300 cfu per plate) were collected in a plate sweep and stored at −80 °C in 1 mL of 20% glycerol, 1% NaCl solution before DNA extraction (Edenborn & Sexstone 2007). Stored cells were washed and DNA extracted with MoBio Ultra-Clean Microbial DNA isolation kits. DNA concentration and purity were assessed by spectrophotometry (NanoDrop 1000).

Primers for the V3 hypervariable region of the 16S rDNA gene, with a GC clamp attached to the forward primer, produced 204 bp PCR amplicons (Yang et al. 2009). Each PCR reaction contained 2.5 μL Dreamtaq 10x buffer, 0.2 mM of each dNTP, 0.3 μM of each primer, 0.5 μL BSA (20 mg/mL stock), 0.25 μL de-ionized formamide, 0.125 μL Dreamtaq DNA polymerase, 1–10 μL template DNA to extracted DNA concentration, and DNase-/RNase-free water up to 25 μL.
Thermocycling was run at 96 °C for 5 min, 31 cycles of 94 °C for 30 s, 59 °C for 30 s, 72 °C for 15 s, and a final 5 min elongation step at 72 °C. Post-PCR cleanup with ExoSAP-IT (Affymetrix USB, Santa Clara, CA) was used to determine presence of bands in smeared regions.

DGGE was run on a Bio-Rad DCode system on a 40–60% denaturing gradient, at 50 V and 60 °C for 14 h. Ethidium bromide stained polyacrylamide gels were visualized on a Kodak ImageStation 2000R transilluminator and images were processed with GelComparII (Applied Maths NV, Sint-Martens-Latem, Belgium). Cluster analysis was performed using the Dice similarity index and Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

RESULTS AND DISCUSSION

Physico-chemical parameters

Temperatures in the system vary seasonally and between sampling locations. In the collection network, upstream from the treatment plant entry point, water temperatures at sampling ranged from 15.6 to 20.8 °C in February and from 24.7 to 32.5 °C in August. Warmer wastewater was usually associated with the larger integrative stations (Figure 1). Within the treatment plant temperatures were more uniform, ranging from 16.9 to 18.5 °C in February and 28.2 to 29.3 °C in August. In the collection network pH ranged from 7.0 to 8.5 in February and 7.3 to 8.5 in August. Within the treatment plant the pH ranged from 6.5 to 7.5 in February and 6.8 to 8.0 in August. The lower pHs were measured in the aeration basin and the secondary clarifier effluents. Wastewater fluxes varied from 12 to 1,060 m³ d⁻¹ at the residential lift stations and from 486 to 4,736 m³ d⁻¹ in the lift stations that integrate large upstream area. The August volumes were either similar to or larger than the February volumes. This was also true for the wastewater plant effluent volumes (33,312 m³ d⁻¹ vs 31,041 m³ d⁻¹). For the larger integrative lift stations, flow rates were always larger in August. It is estimated that about 3,000 m³ d⁻¹ of water is lost in the wastewater treatment plant between the influent and effluent due to diversion into biosolids stream and evaporation.

Bacterial counts

Primary lift station nodes that integrate exclusively residential wastewaters had consistently higher plate counts. Plate counts ranging from 5.4 to 6.7 log cfu mL⁻¹ for raw wastewater were consistent with previous reports (Nagulapally et al. 2009; Manaia et al. 2010). As the collection system integrates more wastewater volumes, bacterial counts tended to decrease. At the wastewater treatment plant lower plate counts were obvious after the first biological treatment, the roughing (trickling) filters, with an accelerated drop in counts after the aeration basin (Figure 2). A two-way

![Figure 2](https://iwaponline.com/wst/article-pdf/65/7/1323/442708/1323.pdf)
factorial ANOVA with media as block design was used to analyse the plate counts for the summer event. Sampling location, antibiotic and their interaction were found to be significant ($\alpha < 0.001$, $r^2 = 0.93$).

Seasonal spikes in antibiotic use may induce differences in resistance profiles. Börjesson et al. (2009) reported highest counts of methicillin-resistant *Staphylococcus aureus* during winter. In arid environments such as in the sampled municipality, another factor is the increased wastewater flow during the summer, which increases dilution and consequently lowers bacterial load.

One point of discussion is whether the cultured bacteria are a true representation of resistant diversity. Because we used non-selective media it is possible that some of the cultured bacteria are inherently more resistant than other species. We took this into consideration, and used the EUCAST database to determine a concentration that would select for bacteria with acquired resistance as opposed to natural resistance. A similar approach that targets a diverse group of bacteria has been used to culture ciprofloxacin-resistant enterobacteria in wastewater treatment plants in Portugal (Manaia et al. 2010). For the other antibiotics, we used sufficiently large concentrations such that only bacteria with acquired resistance were likely cultured, and their DNA extracted and amplified. A potential bias is eliminating resistant species with lower ECOFF value than the ones used. On the other hand, DGGE fingerprints shown represent only bacteria with acquired resistance.

Of all antibiotics, doxycycline resulted in the lowest incidence of resistance, similarly to other reports (Li et al. 2010). The highest incidences of resistance were observed for cefaclor and erythromycin (Figure 2). Ciprofloxacin resistance occurred more often. Frequency of cefaclor and erythromycin resistance suggests the wide use of β-lactam and macrolide antibiotics and also the vector potential of wastewater (Figure 2). Foci of resistance to cefaclor, erythromycin, and occasionally ciprofloxacin could be observed in both residential areas (Res4, Res7, Res9) and the hospital source. Erythromycin resistance was highest at the sampling point that integrates hospital waste. Passage of the wastewater stream through the aeration basin led to an increase in cefaclor and erythromycin proportional resistance. Aeration basin and chlorination, both oxidative treatment stages, seemed to have allowed preferential survival of cefaclor-resistant bacteria.

**DGGE fingerprints analysis**

DGGE fingerprints dendrogram similarity analyses considered sampling season, culturing media and antibiotics. Dice similarity indices thus calculated are visualized in Figure 3.

Similarity analyses showed great diversity in antibiotic resistance profiles within the 11 residential lift stations (Figure 3(a)). Three mixed industrial/residential lift stations and a mixed hospital/residential lift station often produced similar DGGE fingerprints suggesting similar conditions. Given the occasionally non-normal distribution of the Dice similarities datasets, we opted for a nonparametric test to assess differences in the clustering patterns and similarity indices between the final effluent and each sampling location. Kruskal–Wallis (K–W) median tests showed no difference in the similarity indices between effluent and all sampling locations, suggesting that no single source overwhelmingly controls bacterial diversity in the treated effluent.

Another factor considered was method of cultivation. We used both MHA and R2A, targeting copiotrophs or oligotrophs. Similarity analysis (Figure 3) showed that for the most part MHA and R2A fingerprints followed comparable trends. K–W test showed the DGGE fingerprint of the effluent to be more similar for the R2A-DGGE fingerprints than for the MHA-DGGE fingerprints (54% versus 47%). Moreover, for the summer event the average similarity between collection points and effluent was greater than in winter (54% versus 46%).

For the summer event the effluent R2A-DGGE fingerprints from antibiotic free control plates (which may include both resistant and non-resistant bacteria) were >85% similar to three of the residential sources (Figure 3(a)). Generally control profiles were the most conserved throughout the treatment plant, from influent through effluent (57%).

Antibiotic treatments produced fingerprints of variable similarities throughout the treatment process. K–W tests showed that differences in antibiotic resistance were statistically significant. Erythromycin resistance fingerprints showed the largest degree of conservation between raw and treated samples at 55%; for the other antibiotics values varied from 45 to 49%. As previously reported (Reinthaler et al. 2003; Ferreira da Silva et al. 2006), we found that, although fewer, resistant bacteria were detected throughout the treatment. Our results indicate that consortia of resistant strains are carried throughout the treatment process. Nevertheless, large percentages of resistant bacteria at certain collection points did not guarantee that the respective consortia might be identified beyond the primary treatment stages. This again suggests a strong selective impact for the oxidative treatment stages.
Figure 3: Heatmap of statistically significant (p < 0.05) Dice similarity indices for the DGGE fingerprints. Similarity values range on a grey scale from 50 to 100%, the latter being represented as black. White indicates non-significant similarity indices. Numbers above columns refer to treatment locations, influent (1), mixing (2), primary clarifier (3), roughing filters (4), aeration basin (5), secondary clarifier (6), and effluent (7); for rows, ‘W’ and ‘S’ indicate winter and summer sampling events, respectively.
DGGE fingerprinting revealed relatively stable bacterial control, cefaclor- and erythromycin-resistant consortia in the early treatment stages, from influent to roughing filters (Figure 3). This was not as obvious for the consortia selected with the other two antibiotics, suggesting that shifts in bacterial diversity (Pholchan et al. 2010) occur at different rates for different bacteria types. This was true for all recovery options or seasons. These results support the conclusion that antibiotic-resistant bacteria compose a subpopulation consistently recoverable from locations imposing similar abiotic conditions.

The evidence points to the return activated sludge aeration basin as a likely significant modifier of wastewater bacterial diversity ahead of the final chlorination. The genetic fingerprints downstream of the aeration basin consistently diverged from the upstream sampling points across season, growth media, and antibiotics. Erythromycin resistance of the R2A recovered consortia was conserved throughout the last stages of the treatment. This was similar to the control consortia. On the contrary, the erythromycin resistance of consortia recovered on MHA was better conserved along the first stages of the treatment. For both MHA and R2A the similarities in the cefaclor-resistant consortia were disrupted after the aeration basin. It might be therefore assumed that fingerprints downstream of the aeration basin mirror the role of the return activated sludge process. Although stresses imposed on the microbial consortia at the secondary clarifier and final chlorination/dechlorination stages are unique, our test approach could not single out their effects on microbial diversity, both

![Figure 4](https://iwaponline.com/wst/article-pdf/65/7/1323/442708/1323.pdf)

**Figure 4** Principal component analysis (PCA) of Dice similarity indices to assess the correlation in bacterial diversities between the final effluent and wastewater source types (a) or treatment stages (b). MHA and R2A are the agar substrates. ‘W’ indicates winter and ‘S’ summer sampling. For the wastewater source samples (a) the PCs described 20% (PC1), 17% (PC2), 14% (PC3) and 12% (PC4) of the variance in the data set, while for the treatment stages (b) the PCs described 38% (PC1), 26% (PC2), 18% (PC3) and 12% (PC4) of the variance in the data set.
mirroring to some degree the impact of the aeration basin. It would be worthwhile to assess the return activated sludge process as a possible sink and vector for antimicrobial resistance and its role for mitigating antibiotic resistance risks associated with discharged effluents.

We attempted to determine the possibility of linking the bacterial diversity profiles between each of the tested location and the final effluent. The inherent complexity in such a large system led us to use exploratory methods such as principal component analysis (PCA). This way the variability and similarities in the location of bands in the DGGE fingerprints obtained from the different selective treatments were used to assess potential similarities between each sampling location and the effluent. Thus, locations that are grouped this way may be assumed to be similar in their relationship to the DGGE fingerprints of the effluent and, by extension, among themselves. Figure 4(a) shows this for Dice similarity indices between the effluent and all source sampling locations. DGGE diversity of bacterial consortia at each location varies as a function of the selective conditions, agar and antibiotic type, as well as seasonally.

The relatively low explanatory value of the eigenvectors associated with the PCA dimensions (Figure 4(a)) points at the complexity of the system. However, although likely not statistically significant, these observations are beneficial for developing more focused hypotheses. Firstly, residential sources seemed to associate into two distinct groups. One residential lift station (Res9) clustered with more complex industrial sources. The PCA biplot shows that of all wastewater sources, the hospital lift station was the most distinct. Nevertheless, its distinctiveness was induced by varying similarity matrices across the sampling season and recovery substrate. The analysis revealed that greater similarities between the doxycycline-resistant DGGE fingerprints of this sampling location and the effluent were partly responsible for setting this location apart from the rest. In addition, the same was true for erythromycin associated DGGE fingerprint similarities (i.e. see mostly positive PC1 associated eigenvalues), but with greater differences among sampling events or recovery substrates. Thus, specific sources induce specific resistance profiles in the effluent but these profiles are variable across space and time. The significance of this heterogeneity may be better evaluated in a more detailed analysis including intensive monitoring and more focused recovery protocols.

PCA of similarity indices across treatment sampling points revealed that diversity of antibiotic-resistant populations is relatively stable before the wastewater flow reaches the oxidative treatment stages (Figure 4(b)).

Influent, mixing, and primary clarifier samples cluster in the PCA biplot, reinforcing our previous analysis that these locations maintained consistency in antibiotic resistance profile. Roughing filters, aeration basin, and secondary clarifier are all separated in the PCA biplot, suggesting that treatment at these locations completely altered their microbial composition. This analysis also suggests that the conditions at the treatment plant can be reproducible, as we found that we can generally group antibiotic recovery method in the same direction of the eigenvector.

CONCLUSIONS

We evaluated antibiotic resistance related diversity at wastewater sources and throughout the treatment system. Antibiotic resistance profiles for residential wastewaters vary widely across sampling locations. Oxidative stresses in the aeration basin and at chlorination treatment have a strong selective impact on antibiotic resistance profiles, favouring resistance to cefaclor, a β-lactam. Our results agree with other reports in the number of bacteria recovered and resistant groups detected, specifically low incidence of doxycycline resistance. Further persistence of these resistant organisms and the relevant genetic determinants in the environment needs to be clearly evaluated. Similarity analyses using the PCR-DGGE fingerprints of cultured antibiotic-resistant bacterial consortia could be used in a complex analysis to group sources according to their impact on the quality of the treated effluent. Establishing a more direct link between sources and effluent, as well as among sources, will require more comprehensive sampling approaches.

ACKNOWLEDGEMENTS

Funding was provided by the New Mexico State University, New Mexico Water Resources Research Institute, and New Mexico Louis Stokes Alliance for Minority Participation Bridge to the Doctorate Program (HRD#0929343). We thank the City of Las Cruces for sampling support.

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First received 11 October 2011; accepted in revised form 2 December 2011