Pyrosequencing estimates of the diversity of antibiotic resistant bacteria in a wastewater system

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ABSTRACT

Standard protocols for monitoring wastewater treatment efficacy target Escherichia coli and fecal coliforms. This might not accurately describe risks associated with antibiotic resistance in the bacterial population of treated wastewaters. We modified a standard agar recovery method by amending it with various antibiotics. The resulting bacterial colonies were submitted to 454 pyrosequencing; thus we identified the diversity of culturable antibiotic resistant bacteria from treated and raw wastewaters. This approach produced 209,706 high quality reads of >300 bp. Operational taxonomic units (OTUs) within Proteobacteria dominated the system. The Shannon–Wiener H’ index showed bacterial populations recovered on ciprofloxacin amended agars to be the least diverse. Principal component analysis of OTU distribution at phylum level showed that Proteobacteria accounted for most of the variability. The same analysis revealed most of the samples to have similar diversities at phylum level being dominated by Proteobacteria, though a few samples, typically recovered from ciprofloxacin or doxycycline amended agars were often dissimilar. Arcobacter spp. or E. coli were dominant in the bacterial communities recovered on agars amended with ciprofloxacin or doxycycline, respectively. Genera containing putative pathogens were mostly representatives of Gamma and Epsilon proteobacteria. Bacterial populations containing multiple antibiotic resistance (MAR) in the final treated effluent was a possibility.

Key words | 454 pyrosequencing, antibiotic resistance, Proteobacteria, wastewater collection and treatment system

INTRODUCTION

Standard measures of assessing the quality of treated wastewater focus on Escherichia coli and fecal coliform counts. Technological advances (Aw & Rose 2012) have made it possible to go beyond the current set of indicators for a more extensive assessment of total microbial population diversity in the estimates of wastewater treatment (WWT) efficacy. Although not regulated, antibiotic resistance in bacteria is a critical parameter that may be used in an integrated assessment of the risks associated with receiving surface waters (Unc et al. 2012) or with wastewater effluent, and thus in relevant decision support systems.

Pyrosequencing is an essential tool in assessing complex microbial diversity in diverse habitats. It has been used to detect pathogenic bacteria in wastewater and biosolids treatment (Bibby et al. 2010; Ye & Zhang 2011) and to reveal total bacterial diversity in fixed-film reactors (Kwon et al. 2010). Pyrosequencing of the plasmid metagenome has shown antibiotic resistance genes to be prominent in activated sludge samples (Sanapareddy et al. 2009; Zhang et al. 2011). These studies show wastewater bacteria to harbor antibiotic resistance genes and to also be carriers of all accessory genes and mobile genetic elements necessary for dissemination of resistance genes, thus establishing the potential for resistance genes to be carried forth and disseminate via wastewater.

What is less understood is the proximate source of these resistance genes and what factors favor their resilience in the microbial population exposed to WWT stresses. Hence the importance of understanding wastewater source types as potential differential sources of resistance genes, and the role of biotic and abiotic stresses on the persistence or possibly selection of antibiotic resistance. To understand the significance of variable sources of wastewater and the extent of treatment on microbial populations, we assessed
by pyrosequencing the diversity of antibiotic resistant bacterial consortia associated with a wastewater collection and treatment system. Specifically, we compared various raw wastewater sources and treatment plant samples, including the final treated effluent. Thus we assessed the possible role of wastewater source type and a return activated sludge treatment on the diversity of the bacterial population capable of growth on antibiotic amended recovery substrates.

MATERIALS AND METHODS

Sampling and recovery methods

A municipal wastewater collection and treatment plant was sampled as described previously (Sigala & Unc 2012). The treatment plant employs a return activated sludge system followed by a final treatment step of chlorination/dechlorination. Wastewater samples were collected in winter and summer 2010. We analyzed five locations, three collection points representing diverse raw wastewater sources (residential [Res], hospital [Hosp], and a mixed residential/industrial [Ind]) and two WWT plant (WWTP) stages (influent [Inf] and final treated effluent [Eff]). Note that for the tested municipality, industrial type effluent is mainly associated with service-type establishments. Wastewater samples were cultured in triplicate on Mueller–Hinton agar (MHA). Final effluent was also cultured on R2 agar (R2A). Substrates were amended with cefaclor (CEF, 16 µg mL⁻¹), ciprofloxacin (CIP, 8 µg mL⁻¹), doxycycline (DOX, 16 µg mL⁻¹), or erythromycin (ERY, 64 µgL⁻¹). A control (CON) with no antibiotic was also included. All plates were also amended with cycloheximide (75 µg mL⁻¹) to prevent fungal growth. Antibiotics were added at concentrations above the epidemiological cut-off values (ECOFF) for all bacteria listed in the EUCAST database (http://mic.eucast.org/).

Mixed cultures from whole plates were collected, washed in saline solution (1X PBS), and genomic DNA was extracted using a MoBio (Carlsbad, CA) microbial DNA isolation kit. DNA extracts for the same location, antibiotic, and agar type were composited for the two seasons. In total, 30 samples were analyzed by pyrosequencing.

Sequencing and analysis

Pyrosequencing was performed at the Research and Testing Laboratory (Lubbock, TX). Tagged Gray28F primer (Ishak et al. 2011) was used to initiate pyrosequencing. This sequenced a 16S rDNA sequence spanning the V1 through V3 hypervariable region, numbered in relation to E. coli 16S rDNA. The diversity of bacterial populations recovered on the antibiotic amended agar plates was evaluated from the 16S rDNA amplicon mixtures using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) as described previously (Ishak et al. 2011) using the titanium sequencing platform rather than FLX (Roche Applied Science). Finally, rather than the double PCR utilized in the previous methods, only a single step reaction (35 cycles) was utilized and a 1U of HotStar HiFidelity Polymerase (Qiagen) added to each reaction. Raw data from bTEFAP were screened and trimmed based upon quality scores and binned into individual sample collections. Sequence collections were then depleted of short reads (<300 bp) using the QC flow in CAMERA (Sun et al. 2011). Data obtained (fasta files) were used for metagenomic analyses using the following analysis flow: 454 fasta files were uploaded into CAMERA for consensus sequence clustering (>96% similarity) (CD-HIT) (Niu et al. 2010); consensus, non-redundant sequences fasta files were used for BLASTn (Altschul et al. 1997) against the (CAMERA_REF)NCBI RefSeq Microbial Genomes database using standard NCBI settings. Consensus sequences were considered to represent distinct operational taxonomic units (OTUs). Taxonomic summarization and identification of the most similar diagnostic rank was carried out on MEGAN 4 (Huson et al. 2011). Most standard parameters for the least common ancestor algorithm were maintained; however since duplicate reads were consolidated when obtaining consensus sequence clustering we set the minimum taxonomic support to 1.

MEGAN 4 was used to perform comparisons of multiple samples. Identified taxa structures, matched to assigned OTUs (distinct OTUs), were exported for further analysis. In addition, desired taxa could be selected and a cross-sample comparison carried out using the MEGAN 4 network comparison method (Mitra et al. 2010). Normalized Euclidean distances were exported as nexus files for use in SplitsTree4 (Huson & Bryant 2006) to produce cladograms. Two of these were included in the results, one for the top Proteobacterial orders and another for selected genera containing putative pathogens.

Statistics were carried out using GenStat11. Diversity indices Shannon–Wiener H' and J' (Hill et al. 2003) were calculated for sequencing reads as

\[
H' = - \sum \left( \frac{n_i}{N} \right) \ln \left( \frac{n_i}{N} \right)
\]

\[
J' = H' / \ln S
\]
where \( n_i \) is the number of sequence reads, ‘individuals,’ in the \( i \)th OTU and \( N \) is the total number of sequence reads in the sample. When calculating \( f \) the species richness, \( S \), equals the number of OTUs in each sample.

OTU datasets exported from the MEGAN 4 sample comparison were used for principal component analysis (PCA) and related biplots. Two PCA and biplots are presented; the first analyzes OTU distribution across phyla for all MHA samples. Two preliminary steps were necessary to prepare the data for PCA. First, rare phyla were removed; these were phyla with OTU abundance <0.3% of the total. Next, OTU counts were normalized to percentage of total sample OTUs; normalizing OTU counts was necessary and applicable since samples had variable OTUs and sequence reads, and the main research interest was to compare the relative abundance of taxa throughout samples. PCA was performed using the covariance matrix, with taxa as the response variate. The heatmap shown was generated using R 2.13.1 (www.R-project.org), using relative OTU abundance of putative genera.

**RESULTS AND DISCUSSION**

**Sequence results**

Sequence reads were submitted to NCBI Sequence Read Archive (SRA) under accession number SRA053374. Pyrosequencing produced a total sum of 225,452 high quality reads. Of these, 209,706 were longer than 300 bp (292 bp after removing tag sequence) and thus retained for further analysis. The average read length was 464 bp, while the maximum read length was 630 bp. For the 30 samples analyzed, a cumulative total of 6,573 consensus, non-redundant reads were obtained, for an average of 219 OTUs per sample.

**OTU diversity and identification**

Microbial communities recovered across combinations of sampling location and antibiotic treatment were examined by OTU-based diversity indices. The results are shown in Table 1 and include Shannon–Wiener \( H' \) and \( f \). The \( H' \) index varied from 2.15 to 4.40. Ciprofloxacin amended plates produced the least diverse populations with a 2.70 group average \( H' \) index. Evenness (\( f' \) index) was also the lowest for ciprofloxacin plates, reflecting the high number of reads from these samples yet distributed across fewer OTUs compared to other antibiotics. Richness (OTUs) ranged from 80 to 418. It was quite obvious that richness was higher in raw samples, 262 (±76) OTUs, than in treatment plant samples, 176 (±61) OTUs.

The combination of non-specific bacterial recovery on antibiotic amended media and pyrosequencing of recovered cultures allowed for taxa-based identification of composite populations, putatively resistant to antibiotics. All reads were classified in the bacteria domain. Proteobacterial reads represented 59–93% of assigned OTUs per sample with obvious differences across sampling gradients and antibiotic treatments. The top five phyla, to which reads were assigned according to BLASTn, were, in decreasing order, Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, and Actinobacteria; 4% of reads could not be classified to any phylum (these constituted the fourth most common ‘operational phylum’). Only Proteobacteria, Bacteroidetes, and Cyanobacteria were found in all samples; Firmicutes were found in all but two samples.

A correlation was found between occurrence of OTUs within specific phyla and the number of assigned reads represented (Supplementary Figure S1, available online at http://www.iwaponline.com/wst/067/026.pdf). As shown, Proteobacteria were always represented by a large number of OTUs and sequence reads. Though phyla identification for reads was possible, more specific identification was often hindered beyond bacterial class due to sequence length (average read length was 464 bp) and incompleteness of the GenBank database for non-cultured organisms (Supplementary Figure S2 – available online at http://www.iwaponline.com/wst/067/026.pdf – summarizes identification of sequence reads from phyla to genera). At the extreme classification of genera, most sequences (66%) could not be classified.

Since the Proteobacteria phylum dominated, a large part of the analyses focused on examining this important group in closer detail. Most reads were assigned to Gammaproteobacteria (Supplementary Figure S2). Characteristically, Gamma and Alphaproteobacteria were found in all samples (Supplementary Figure S3, available online at http://www.iwaponline.com/wst/067/026.pdf) while Betaproteobacteria were found in all but one sample (Inf DOX). Deltaproteobacteria were found sparingly throughout all samples, while Epsilon proteobacteria (which include the important genera *Campylobacter*, *Helicobacter* and *Arcobacter*) were detected more commonly in raw rather than treated wastewater. Pseudomonadales and Enterobacteriales were the most commonly recovered orders of Proteobacteria in wastewater. Burkholderiales were found in all but one sample. For some samples, Burkholderiales were more abundant than Enterobacteriales. Previously, we found the hospital source could be discriminated from other sources based on...
antibiotic resistant diversity profiles (Sigala & Unc 2012). Results herein show that for all antibiotics except cefaclor, the relative OTU abundance of Gammaproteobacteria was larger in the hospital source compared to other sources (Supplementary Figure S3). In addition, the proportions of OTUs belonging to Alpha and Betaproteobacteria were smaller in the hospital source than in the other sources.

The abundance of Bacteroidetes OTUs increased substantially in the WWTP, especially in the treated effluent. Bacteroidetes, which are mostly anaerobic, are often reported in wastewater by culture-independent methods in ranges from 0 to 30% (Sanapareddy et al. 2009; Bibby et al. 2010; Kwon et al. 2010). Our results yielded an overall abundance of Bacteroidetes of 3.5%. Likely, this was an

### Table 1

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Note: Diversity indices calculated for OTUs (see Materials and methods section).

aAb, antibiotic.

bCD-HIT used to define OTUs as described in methods section.

cIn text 17 phyla were stated, here we included ‘unclassified bacteria’ as a group.
underestimate due to the aerobic recovery protocol we employed. Nevertheless, while very few reads from raw wastewater were assigned to Bacteroidetes, more were found in the treatment plant influent and effluent. While typically most Bacteroidetes are obligate anaerobes, some members have been detected using aerobic conditions and aerobic Bacteroidetes have been described in WWT including novel species of flavobacteria (Ryu et al. 2007).

While Bacteroidetes OTU richness increased in the treatment plant, Firmicutes decreased from raw sewage to WWTP samples. Bacteroidetes and Firmicutes are known to contain novel resistances genes (Sommer et al. 2009) and indeed these two phyla merit more research to determine what role they can play in dissemination of antibiotic resistant determinants in wastewater. In two samples (ciprofloxacin and cefaclor based recovery of effluent bacteria) Firmicutes were not detected by our method. Cyanobacteria were found in all samples; although Cyanobacteria are assumed not to be cultured under aerobic conditions, the use of antibiotic amended media together with growth of heterotrophic bacteria possibly allowed for at least a few colonies to grow (Morris et al. 2008). Most likely, Cyanobacteria were introduced to wastewater through soil and dust where they are ubiquitous. These were found in low abundance and typically very low identity at phylogenetic levels, below phyla, probably due to poor taxonomic resolution among cyanobacterial 16S rDNA sequences. Actinobacteria were found in 25 out of 30 samples, both in raw and treated wastewater, with slightly greater numbers in raw wastewater. Among antibiotics, Actinobacteria were present in greater numbers on ciprofloxacin amended media.

Overall, 17 phyla were classified according to the NCBI database alignment results summarized in the MEGAN 4 software (Huson et al. 2011). Included in these 17 was one candidate phylum, candidate division TM7, though only three OTUs were assigned to it. Although not tested, it is reasonable to hypothesize that the detection of several taxa which are not known to be cultured by standard methods can be attributed to co-culturing, due to suitable growth conditions near heterotrophic colonies resulting from use of antibiotics, crowding, or to limited die-off of plated but not growing bacteria which were not removed under our recovery conditions. These taxa were always in a low number of reads.

Thirty samples taken from cultured wastewater communities were analyzed. Results are presented for several taxonomic levels, from phyla to species. Firstly, we may conclude that the resistant population in wastewater was dominated by Proteobacteria. More in-depth examination has shown that most Proteobacteria were Gammaproteobacteria of the Enterobacteriales and Pseudomonadales orders. Sequencing of aeration basin samples by clone library sequencing and 454 pyrosequencing also found Proteobacteria to dominate followed to a lesser extent by Bacteroidetes and Firmicutes (Sanapareddy et al. 2009). Previous 454 pyrosequencing has revealed Chloroflexi to be the most dominant phylum in anaerobic treatment of biosolids, followed by Proteobacteria, Bacteroidetes, and Firmicutes (Bibby et al. 2010).

Comparison of MHA and R2A as growth media

For all samples we used MHA recovery media. R2A was only used for the final effluent; this was decided based on our previous results, which indicated no major differential diversity, as estimated by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), due to the recovery substrate, with the exception of the last stages of treatment (Sigala & Unc 2012). MHA and R2A are selective for copiotrophes and oligotrophes, respectively. We found similar phyla level diversity recovered for most samples. Proteobacteria showed the most variability. Notably, control as well as ciprofloxacin amended plates allowed recovery of more OTUs on R2A, while doxycycline and erythromycin allowed recovery of more OTUs on MHA.

While most of the same phyla were recovered for the effluent on both the MHA and the R2A recovery media, their relative abundance varied. A more detailed examination of lower level taxonomy can offer a hint of possible mechanisms but is beyond the scope of the paper. Interestingly, the only two reads which were not assigned by MEGAN 4 software in the first workflow were found separately in MHA or R2A cultures of treated effluent and were possibly the same organism. A megablast analysis indicated that these reads were 99% similar to a species of Microbacterium (Genbank accession DQ985071) which was described as Microbacterium sp. JL1116 sequence deposited from cultured marine samples.

PCA discriminant

A PCA of phyla distribution of OTUs revealed that four components were significant (eigenvalues sum ~ 1), and the first two components explained 82% of the variability (Figure 1(a)). Proteobacteria dominated the first principal component, which explained 61% of the variability. PCA biplots allow for scanning of major data trends, and
general statements can be made. As seen in the biplot (Figure 1(a)), most of the experimental combinations fall within one large cluster, indicating that most samples were similarly distributed with the major identities Proteobacteria, Bacteroidetes and Firmicutes. A few samples fall outside this major cluster; these were typically recovered from ciprofloxacin and doxycycline amended agars and associated with decrease in the resistance among Proteobacteria. Possibly, this was due to differential selection of resistant populations within these antibiotic treatments. Furthermore, Bacteroidetes had an important presence in the effluent sample; this was shown by most effluent samples running parallel to the Bacteroidetes eigenvector.

The distribution and abundance of the top 10 (Pseudomonadales excluded since these were not anticipated to

**Figure 1**  (a) PCA biplot of phyla distribution recovered from MHA (25 samples). MEGAN 4 exported OTU data were normalized in Excel to percent OTU per phylum per sample. Covariate matrix with phyla as variates. Although 17 phyla were recovered, only those with overall OTU abundance >0.3% were included. (b) PCA biplot of top 10 Proteobacteria orders (not including Pseudomonadales). Less dominant orders not shown to reduce cluttering but included in the analysis: Xanthomonadales and Caulobacterales.
be of fecal origin) Proteobacteria orders was assessed. A cladogram was produced in SplitsTree4 (Huson & Bryant 2006) to compare location and antibiotic datasets. An obvious cluster associated effluent diversities from both resistant and non-resistant groups (Figure 2). The PCA biplot (Figure 1(b)) confirmed the similarities in diversity of the resistant bacteria in effluent; these were characterized by more Alteromonadales (and other minor players) and fewer Enterobacteriales. The similarities in Proteobacteria diversities in the effluent, most obvious for the populations resistant to erythromycin, cefaclor and ciprofloxacin, might suggest multiple antibiotic resistances (MARs) within effluent bacterial communities.

Decrease in Proteobacterial abundance in effluent coincided with increased diversity of other phyla. Throughout the wastewater collection system and up to the influent, resistance was associated with Pseudomonadales and Enterobacteriales; in the treated effluent wastewater, resistance was spread widely among other taxa. The bacterial community in the effluent contained a population which could be recovered on several antibiotics, as shown in Figure 2. The cladogram’s structure is mostly a representation of the top Proteobacteria bacterial orders. These results show at least part of the bacterial community probably features MAR at end-stage WWT. These results would not be uncommon, WWT has been previously reported to increase the proportion of MAR in bacteria recovered (Zhang et al. 2009), likely being influenced by advantageous genetic exchange.

High Proteobacteria diversity and abundance in wastewater is likely a reflection of both their ubiquity in human feces and ability to survive in the wastewater environment. The added capability of coping with antibiotics at concentrations above the ECOFF indicates acquired resistance. Wastewater bacteria have been shown to exhibit various antibiotic resistances and gene transfer mechanisms (Zhang et al. 2011) and Proteobacteria abundance, in particular, is very often promoted after antibiotic exposure (Antonopoulos et al. 2009). A firm conclusion establishing a link to multiple stresses encountered by wastewater bacteria cannot be drawn here to explain the dominance of Proteobacteria.

One of the major challenges in wastewater microbiology is understanding which parameters drive bacterial diversity and how composition affects effectiveness of treatment (Lee et al. 2004). Although it is fair to assume that variability in the source types for raw wastewater would be reflected in distinct variability in bacterial diversity, the results offered only marginal support for this hypothesis, with the possible exception of the hospital source. Variability of physical and chemical conditions may change dramatically over small distances and over time. In wastewater, chemical stresses are common, including antibiotics detected at subclinical concentrations. Probably the most important in typical treatment plants is oxidative stress, which is known to induce specific responses. Other chemical stresses were definitely present, though it is difficult to regard these in isolation to determine patterns of resistance for each.

Putative pathogen diversities

A total of 23 genera known to include pathogens (Ye & Zhang 2011) were detected. Of these, Pseudomonas and Escherichia were the most commonly detected. Because Pseudomonas spp. are ubiquitous in the environment and with many likely saprophytes, we further limited our analysis to include only one species known to be pathogenic, P. aeruginosa. Also, because the majority of Escherichia identified were E. coli, we further limited our analysis to this species, which resulted in very little difference, numerically.

Figure 3 shows both a cladogram that separates treatments based on target pathogenic genera and a heatmap of the relative abundance in each treatment. Most diversity
in antibiotic resistance was identified in Gamma proteobacteria (E. coli, P. aeruginosa, Vibrio spp., and Yersinia spp.) and in the Epsilon proteobacteria (Arcobacter spp.). One striking observation is the separation of two clusters, a doxycycline and ciprofloxacin resistant groups. These clusters follow closely with the dominance of E. coli or Arcobacter spp. It is noteworthy to indicate that these clusters are driven by antibiotic recovery type and are independent of location sampled, suggesting distinct antibiotic resistance profiles for different species. It has been noted previously that the most important species of Arcobacter, A. butzleri, is resistant to several antibiotics, including ciprofloxacin (Collado & Figueras 2011).

It would be worthwhile to determine which resistance mechanisms are favored in wastewater, given that doxycycline and ciprofloxacin have different targets in the bacterial cell, the first inhibiting protein synthesis and the latter targeting DNA replication. Identifying the conduit of resistance dissemination would carry important implications to research in environmental antibiotic resistance and health risks associated with wastewater. Though quinolone (which includes ciprofloxacin) resistance determinants are mostly found on chromosomes, the latest research shows a growing number to be plasmid-mediated (Robicsek et al. 2006). A large number of tetracycline (which include doxycycline) resistance genes are known, which are located mostly on plasmids, integrons, transposons, and conjugative transposons (Chopra & Roberts 2001).

CONCLUSIONS

Wastewater diversity was analyzed in relation to the antibiotic resistant population. This study was carried out to resolve source-associated resistant populations in wastewater systems. Proteobacteria were found most commonly
throughout the treatment process, final stage treatment, which featured chlorination, led to a relative proportional reduction of Proteobacteria. Hospital source contained a larger abundance of Gamma proteobacteria compared to other sources for all antibiotics except cefaclor. Effluent bacterial populations cultured on different antibiotics have similar diversity structures offering support for a MAR hypothesis following WWT. This study also shows ciprofloxacin resistance in an important genus, *Arcobacter*, to persist throughout WWT as well as doxycycline resistant *E. coli*.

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**REFERENCES**


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