Marquette University
e-Publications@Marquette

Civil and Environmental Engineering Faculty Research and Publications | Civil and Environmental Engineering, Department of

10-1-2011

Tertiary-Treated Municipal Wastewater is a Significant Point Source of Antibiotic Resistance Genes Into Duluth-Superior Harbor

Patrick J. McNamara
Marquette University, patrick.mcnamara@marquette.edu

Tucker R. Burch

Timothy M. LaPara

David T. Tan

Mi Yan

See next page for additional authors

Accepted version. *Environmental Science & Technology*, Vol. 45, No. 22 (2011): 9543-9549. DOI. © 2011 American Chemical Society. Used with permission.
Patrick J. McNamara was affiliated with The University of Minnesota at the time of publication.

Authors
Patrick J. McNamara, Tucker R. Burch, Timothy M. LaPara, David T. Tan, Mi Yan, and Jessica J. Eichmiller

This article is available at e-Publications@Marquette: https://epublications.marquette.edu/civengin_fac/1

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

Tertiary-Treated Municipal Wastewater is a Significant Point Source of Antibiotic Resistance Genes into Duluth-Superior Harbor

Timothy M. LaPara*
Department of Civil Engineering, University of Minnesota, Minneapolis, MN
Tucker R. Burch
Department of Civil Engineering, University of Minnesota, Minneapolis, MN
Patrick J. McNamara
Department of Civil Engineering, University of Minnesota, Minneapolis, MN
David T. Tan
Department of Civil Engineering, University of Minnesota, Minneapolis, MN
Mi Yan
Department of Bioproducts and Biosystems Engineering, University of Minnesota, St. Paul, MN

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

Jessica J. Eichmiller

Department of Soil, Water, and Climate, University of Minnesota, St. Paul, MN

Abstract

In this study, the impact of tertiary-treated municipal wastewater on the quantity of several antibiotic resistance determinants in Duluth-Superior Harbor was investigated by collecting surface water and sediment samples from 13 locations in Duluth-Superior Harbor, the St. Louis River, and Lake Superior. Quantitative PCR (qPCR) was used to target three different genes encoding resistance to tetracycline (*tet*(A), *tet*(X), and *tet*(W)), the gene encoding the integrase of class 1 integrons (*intI1*), and total bacterial abundance (16S rRNA genes) as well as total and human fecal contamination levels (16S rRNA genes specific to the genus *Bacteroides*). The quantities of *tet*(A), *tet*(X), *tet*(W), *intI1*, total *Bacteroides*, and human-specific *Bacteroides* were typically 20-fold higher in the tertiary-treated wastewater than in nearby surface water samples. In contrast, the quantities of these genes in the St. Louis River and Lake Superior were typically below detection. Analysis of sequences of *tet*(W) gene fragments from four different samples

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

collected throughout the study site supported the conclusion that tertiary-treated municipal wastewater is a point source of resistance genes into Duluth-Superior Harbor. This study demonstrates that the discharge of exceptionally treated municipal wastewater can have a statistically significant effect on the quantities of antibiotic resistance genes in otherwise pristine surface waters.

Introduction

Over the past several decades, antibiotic-resistant bacterial infections have become increasingly prevalent, increasing morbidity and mortality as well as the cost of treatment.[1-3] In response to these clinical concerns, there has been increasing focus on environmental reservoirs of antibiotic resistance over the past several years.[4-8] Antibiotic use in agriculture, for example, has been heavily scrutinized[9,10] and recently banned in the European Union. In contrast, the role of treated municipal wastewater has received relatively little attention as a reservoir of resistance, in spite of numerous reports suggesting that bacteria resistant to multiple antibiotics[11-13] and antibiotic resistance genes[14-21] are abundant in municipal wastewater.

Determining the relative importance of treated municipal wastewater as a reservoir of antibiotic resistance is a potentially difficult task. The first challenge is to enumerate "antibiotic resistance" in some meaningful way. Historically, antibiotic resistance would have been quantified by cultivating bacteria based on their phenotypic resistance to a specific antibiotic or set of antibiotics. This approach, however, is insufficient because cultivation-based methods are well-known to underestimate the quantities and diversity of bacteria.[22,23] The second challenge is to distinguish the impact of treated municipal wastewater from the background level of resistance because antibiotic resistant bacteria and antibiotic resistance genes are natural phenomena[5,24] and because other human activities (i.e., other than the release of municipal wastewater) have presumably perturbed the majority of surface waters to some extent.

In this study, we examined the impact of tertiary-treated municipal wastewater on the quantities of three tetracycline resistance genes (*tet*(A), *tet*(X), and *tet*(W)) and the integrase gene of class 1 integrons (*intI1*) in the St. Louis River, Duluth-Superior Harbor, and Lake Superior. This ecosystem represents an ideal locale for studying

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

the importance of treated municipal wastewater as a reservoir of antibiotic resistance because the St. Louis River and Lake Superior are surprisingly pristine surface waters with very low background levels of bacteria,[25] which suggests that the levels of antibiotic resistant bacteria also should be very low. Furthermore, the quality of treatment at the Western Lake Superior Sanitary District (WLSSD), which operates the municipal wastewater treatment facility in Duluth, MN, is exemplary. The WLSSD facility treats approximately 40 million gallons of residential, commercial, and industrial wastewater each day via a conventional system consisting of bar screens, grit removal, and a state-of-the-art, high-purity oxygen activated sludge process. The WLSSD wastewater treatment facility, however, is unique in that it further treats the wastewater by passing it through a mixed media filter (consisting of anthracite coal, silica sand, and garnet) before disinfecting (using sodium hypochlorite) the wastewater and discharging it to Duluth-Superior Harbor.

Materials and Methods

Sample Collection

Surface water (sample volume = 250 mL) and sediment (sample mass = ~0.75 g wet sediment) samples were collected on October 1, 2010 from the St. Louis River, Duluth-Superior Harbor, and Lake Superior while aboard the R/V Blue Heron (Figure 1). Most of the surface water samples were collected manually at a distance of 0.5 m below the water surface using sterile polystyrene bottles. A small fraction of the samples (those from Lake Superior) were collected using an SBE 32 Carousel Water Sampler (Sea-Bird Electronics, Inc., Bellevue, WA) at a depth of 5 m below the water surface. Sediment samples were collected using either a multicorer (Ocean Instruments, San Diego, CA) or a gravity-corer (HTH Teknik; Luleå, Sweden). Sediment samples represent a composite sample of the top 2.5 cm of sediment.

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

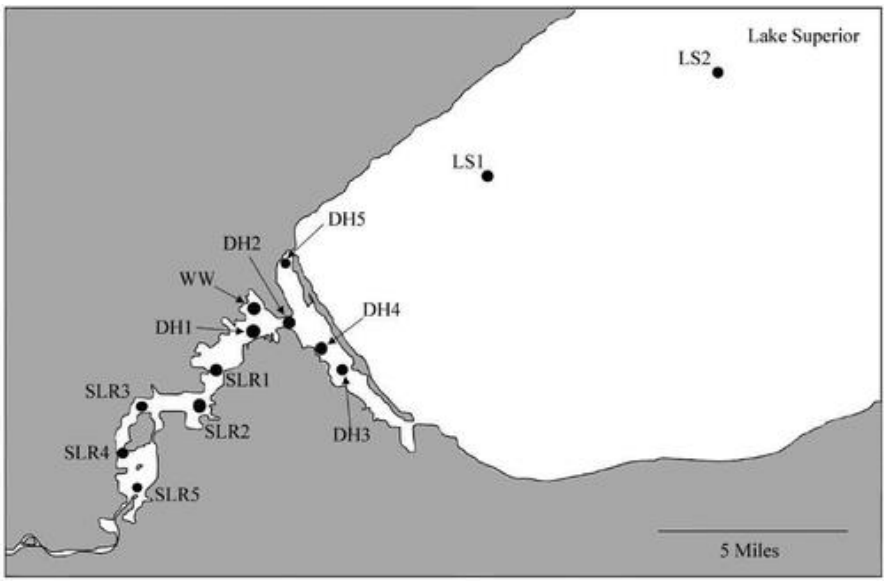

Figure 1. Map of the St. Louis River, Duluth-Superior Harbor, and Lake Superior, identifying the locations from which samples were collected.

As soon as possible after collection (typically less than 30 min; always less than 2 h), surface water samples were passed through a 47 mm diameter nitrocellulose filter (pore size = 0.22 μm) to concentrate microbial biomass. Filters were then immersed in 0.5 mL of lysis buffer (120 mM phosphate buffer, pH = 8.0, 5% sodium dodecyl sulfate) to preserve the sample until genomic DNA could be extracted and purified. All samples were stored on ice while they were transported to the University of Minnesota (within 12 h), after which they were stored at −20 °C until processed further.

Genomic DNA Extraction

Water samples (preserved in lysis buffer) underwent three consecutive freeze–thaw cycles and an incubation of 90 min at 70 °C to lyse cells. Genomic DNA was then extracted and purified from these samples using the FastDNA Spin Kit (MP Biomedicals, Solon, OH) according to manufacturer's instructions. Genomic DNA was also extracted from sediment samples (~500 mg of wet weight per sample) using a bead beater to lyse cells. Genomic DNA was then extracted and purified from sediment samples using a FastDNA Spin Kit for soil (MP Biomedicals, Solon, OH). All genomic DNA extractions were performed in triplicate and stored at −20 °C until needed.

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

Community Analysis

The composition of the bacterial communities in the aquatic samples was compared by automated ribosomal intergenic spacer analysis (ARISA). The ribosomal intergenic spacer (ITS) regions of *Bacteria* were amplified using primers ITSF (5′-GTC GTA ACA AGG TAG CCG TA-3′) and ITSReub (5′-GCC AAG GCA TCC ACC-3′)[26] as described previously.[27] Fragment analysis was performed by denaturing capillary electrophoresis at the Biomedical Genomics Center at the University of Minnesota using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The length of the fragments was estimated using the Map Marker 1000 size standard.

Quantitative PCR

Quantitative real-time PCR (qPCR) was used to quantify the presence of three genes encoding tetracycline resistance (*tet*(A), *tet*(W), and *tet*(X)) and the integrase gene of class 1 integrons (*intI1*) as described previously.[15] These genes were targeted in this study because our prior work demonstrated that these genes were easily detectable in untreated wastewater solids[15,16] and because these genes encode proteins that confer tetracycline resistance via each of the three known mechanisms of resistance.[28] qPCR was also used to quantify the 16S rRNA genes of all members of the domain *Bacteria* as well as total and human-specific *Bacteroides* spp. as described previously.[29-31]

The qPCR analysis was conducted using an Eppendorf Mastercycler ep *realplex* thermal cycler (Eppendorf, Westbury, NY) or an ABI Prism7000 Sequence Detection System (Applied Biosystems). Each qPCR run consisted of initial denaturation for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, and anneal and extension at 60 °C (most targets) or at 56 °C (human-specific *Bacteroides*) for 1 min. A 25 µL reaction mixture contained 12.5 µL of iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA), 25 µg bovine serum albumin (Roche Applied Science, Indianapolis, IN), optimized quantities of forward and reverse primers, and a specified volume of template DNA (usually 0.5 µL). The precise volume and concentration of template DNA were empirically optimized for each

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

sample to generate the lowest detection limit while minimizing inhibition of PCR. Additional information on the qPCR primers, their quantification limits, and their associated products are provided in the Supporting Information.

The quantity of target DNA in unknown samples was calculated based on a standard curve generated using known quantities of template DNA. Standards for qPCR were prepared by PCR amplification of genes from positive controls, followed by ligation into a cloning vector (either the StrataClone PCR kit (Stratagene, Santa Clara, CA) or pGEM-T Easy (Promega, Madison, WI)), and transformation into *E. coli* JM109. Plasmids were purified using the alkaline lysis procedure.[32] Plasmid DNA was quantified by staining with Hoechst 33258 dye and measured on a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) using calf thymus as a DNA standard. Tenfold serial dilutions of plasmid DNA were prepared and run on the thermal cycler to generate standard curves ($r^2 > 0.99$). Following qPCR, melting curves were generated and analyzed to verify that nonspecific amplification did not occur.

Clone Libraries

Fragments of *tet*(W) genes from four different surface water samples (samples SLR5, DH2, WW, and LS2) were amplified by PCR, purified, ligated into the pGEM-T Easy cloning vector, transformed into *Escherichia coli* JM109, and plated onto LB agar plates supplemented with 40 μg/mL of ampicillin. This resulted in libraries of *tet*(W) gene fragments from each of these samples, allowing their nucleotide sequences to be determined. Approximately 30 colonies from each library were randomly picked so that plasmids could be extracted and purified using the alkaline lysis method. Extracted plasmids were then used as template for nucleotide sequence analysis using M13F and M13R as sequencing primers. Bidirectional sequence information was then used to produce a consensus sequence. Approximately 20% of the plasmids contained primer–dimer rather than a genuine *tet*(W) gene fragment; these sequences were excluded from further analysis.

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

Data Analysis

Nonmetric multidimensional scaling (nMDS) was used on triplicate ARISA profiles to evaluate differences in bacterial community composition based on the presence and intensity of peaks in the electropherograms. The relative intensity of peaks, obtained by dividing the individual intensities by total intensity of all the peaks, was used in the analysis. Peaks falling below 1% of the total intensity were excluded from the analysis. nMDS was performed using the ade4 package in R, version 2.4.1.[33]

Prior to statistical analysis, samples with gene concentrations below the method detection limit were assigned a value equal to half the detection limit. All gene concentrations were then log-transformed, and this log-transformed data set was used for all subsequent statistical analysis. One-way analysis of variance (ANOVA) was performed with R version 2.12.0 for all gene targets. An F-test was conducted to determine if results from a specific surface water sample location exhibited gene concentrations that were significantly different from results at the other sample locations. Tukey's honestly significant difference (HSD) test was conducted for each gene target to determine the difference in mean gene concentrations between each possible pair of surface water samples sites. Pearson correlation coefficients of gene concentrations were also calculated using R version 2.12.0 for all possible pairs of gene targets. The detailed results of all statistical analyses (i.e., *P* values and/or Pearson correlation coefficients) are provided in the Supporting Information.

All nucleotide sequences were initially compared with sequences in the GenBank database[34] to verify that the cloned fragments were genuine *tet*(W) gene fragments. Sequences were then aligned using the ClustalW algorithm[35] using DNAMan version 7.0 software (Lynnon Biosoft, Vaudreuil-Dorion, Quebec, Canada). To avoid artifacts stemming from misamplification during PCR and nucleotide sequencing error,[36] all sequences for which there was not a replicate were excluded from further analysis.

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

Results

Bacterial Community Composition

The composition of the bacterial communities in surface water samples collected along a length of the St. Louis River, Duluth-Superior Harbor, and Lake Superior was assayed by automated ribosomal intergenic spacer analysis (ARISA) (Figure 2). The bacterial community composition gradually transitioned along the length of the St. Louis River, into Duluth-Superior Harbor, and out into Lake Superior. In contrast, the composition of bacteria in the treated municipal wastewater from the Western Lake Superior Sanitary District (WLSSD) was significantly different than all of the surface water samples.

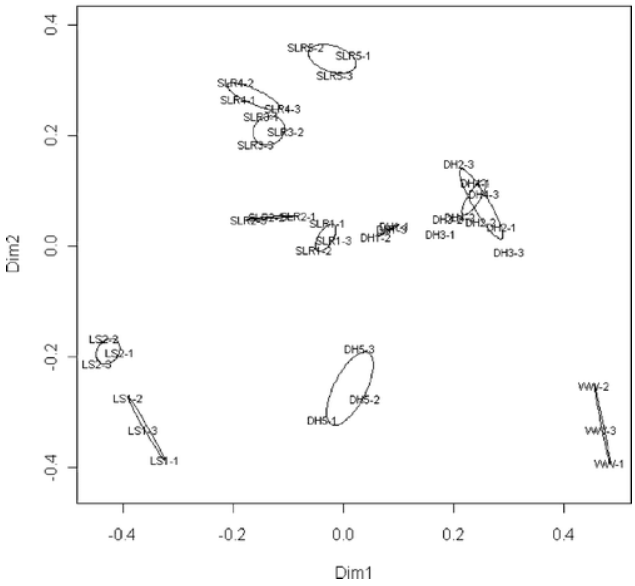

Figure 2. Results of nonmetric multidimensional scaling (nMDS) analysis of bacterial community composition as determined by automated ribosomal intergenic spacer analysis. Ellipses show the 95% confidence limit of triplicate water samples. Samples were collected from the St. Louis River (identified as "SLR"), Duluth-Superior Harbor (identified as "DH"), and Lake Superior (identified as "LS"); the precise locations from which samples were collected are shown in Figure 1.

Quantitative PCR

The amount of bacterial biomass was quantified in the surface water samples by real-time PCR of 16S rRNA gene fragments (Figure

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

3). Bacterial biomass in the different surface water samples varied substantially from as high as 3.6×10^6 gene copies per mL (sample location = SLR2) to as low as 2.1×10^5 gene copies per mL (sample location = LS2). These quantifications of 16S rRNA gene copies are substantially lower than that previously reported from the Haihe River in China (10^8–10^9 copies per mL)[37] and from a drinking water source in Michigan (3.4×10^9 copies per mL),[38] but are consistent with previously reported direct cell counts from Lake Superior (1×10^5 cells/mL).[25] The quantity of bacterial biomass in the treated WLSSD effluent was 5.4×10^6 gene copies per mL, which was higher than any surface water sample.

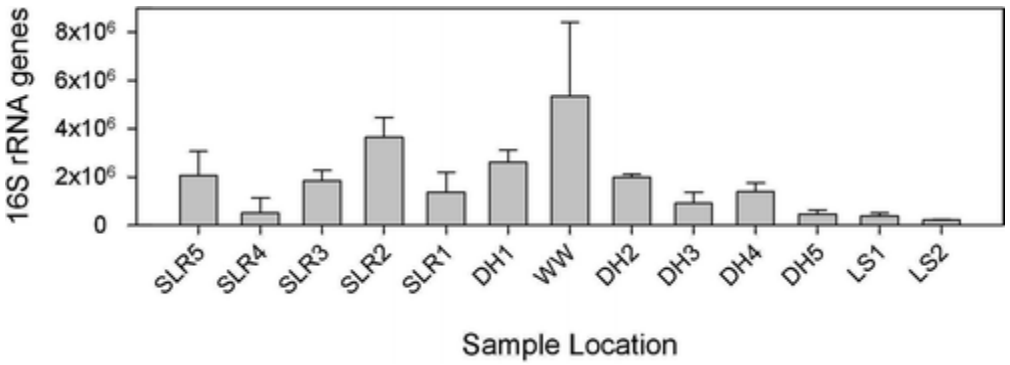

Figure 3. Quantities (gene copies per mL) of 16S rRNA genes in water samples collected from the St. Louis River, Duluth-Superior Harbor, the outfall from the Western Lake Superior Sanitary District, and Lake Superior. Values shown are the arithmetic means; error bars show the standard deviation of the mean. The locations from which samples were collected are shown in Figure 1.

The quantities of three different genes that encode resistance to tetracycline (*tet*(A), *tet*(X), and *tet*(W)) as well as the quantity of the integrase gene (*intI1*) of class 1 integrons were also determined along the St. Louis River, in Duluth-Superior Harbor, and in Lake Superior (Figure 4; for the same data normalized to 16S rRNA genes, see Supporting Information). The quantities of *tet*(A) and *tet*(X) followed similar patterns in the aquatic samples; both of these genes were at relatively high concentrations in the WLSSD effluent (*tet*(A): 6.3×10^2 copies per mL; *tet*(X): 1.2×10^3 copies per mL), slightly above the detection limit at several locations within Duluth-Superior Harbor, and below the detection limit in the St. Louis River and in Lake Superior. The pattern of *intI1* genes was somewhat similar to that observed with *tet*(A) and *tet*(X), except that a more distinct hump-shaped profile, albeit slightly skewed into Duluth-Superior Harbor, was observed; this

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

hump-shaped profile began in the St. Louis River and encompassed all but one sample collected from Duluth-Superior Harbor. An entirely different profile was observed with respect to the quantity of *tet*(W) genes, which were quantifiable in every aquatic sample with only the WLSSD effluent (1.8×10^4 gene copies per mL) and one sample from Duluth-Superior Harbor (sample DH4: 5.3×10^3 gene copies per mL) being statistically greater ($P < 0.05$) than the other samples. Because the quantities of 16S rRNA genes were relatively constant among the different water samples (i.e., within an order of magnitude), the quantities of *tet*(A), *tet*(X), *tet*(W), and *intI1* normalized to 16S rRNA genes follow similar patterns to those described above (see Supporting Information for more details).

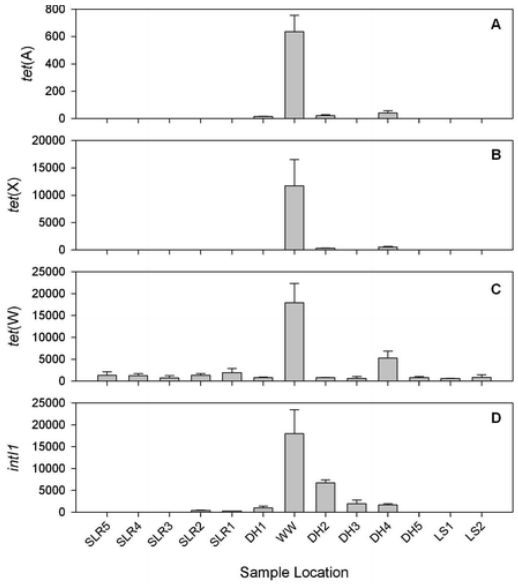

Figure 4. Quantities (gene copies per mL of water) of *tet*(A), *tet*(W), *tet*(X), and the integrase gene of class 1 integrons (*intI1*) in samples collected from the St. Louis River, Duluth-Superior Harbor, the outfall from the Western Lake Superior Sanitary District, and Lake Superior. Values shown are the arithmetic means; error bars show the standard deviation of the mean. The locations from which samples were collected are shown in Figure 1.

The quantities of 16S rRNA genes from all *Bacteroides* spp. in the aquatic samples followed a trend similar to that observed with the *tet*(W) quantities (Figure 5A). The highest quantity of *Bacteroides* spp. was found in the WLSSD effluent (6.8×10^3 gene copies per mL), but otherwise most of the samples had relatively low concentrations that were similar. In contrast, the quantities of human-specific *Bacteriodes* spp. followed a trend like that of *tet*(A) and *tet*(X), in which a

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

relatively high concentration was detected in the WLSSD effluent (1.0×10^2 gene copies per mL); two samples from Duluth-Superior Harbor had quantities slightly higher than the detection limit, but then all other samples were below the detection limit.

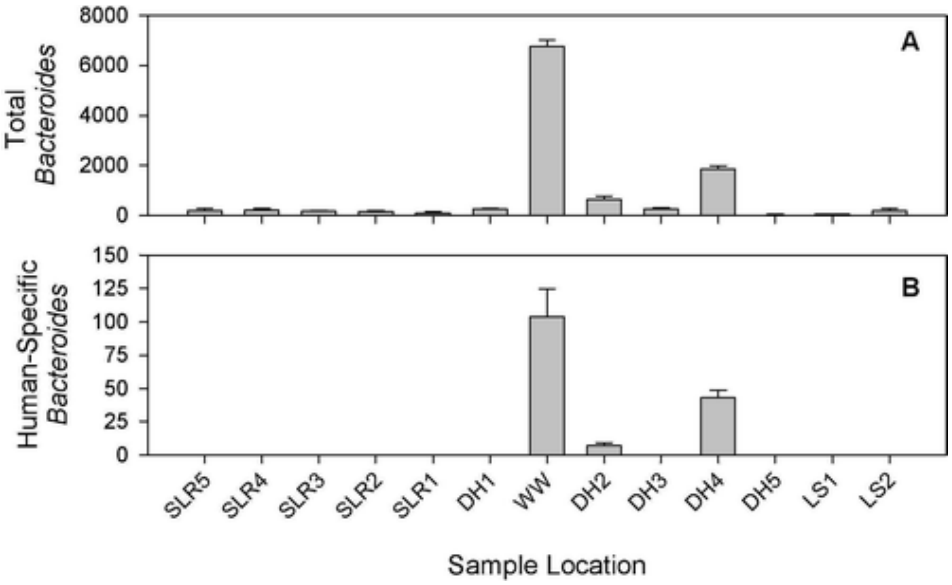

Figure 5. Quantities (gene copies per mL of water) of 16S rRNA genes from all *Bacteriodes* spp. and from human-specific *Bacteroides* spp. in water samples collected from the St. Louis River, Duluth-Superior Harbor, the outfall from the Western Lake Superior Sanitary District, and Lake Superior. Values shown are the arithmetic means; error bars show the standard deviation of the mean. The locations from which samples were collected are shown in Figure 1.

Four sediment samples were also collected from Duluth-Superior Harbor and Lake Superior (Table 1). Each of these samples had similar concentrations of total bacteria, as measured by qPCR of 16S rRNA genes. The quantities of the other genetic markers tracked in this study, however, varied significantly depending on sample location (except for human-specific *Bacteroides* spp., which were not detected in any of the sediment samples). The highest concentrations of these genetic markers were detected in sediment samples collected from near the WLSSD outfall (samples WW and DH1) compared to the samples collected from the Duluth-Superior Harbor channel (sample DH3) and from Lake Superior (sample LS2).

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

Table 1. Arithmetic Means (Units = gene copies per wet gram of sediment; *n* = 3) of Various Genes Detected in Sediment Samples Collected near the WLSSD Outfall, from Duluth-Superior Harbor, and from Lake Superior (See Figure 1 for Actual Locations)[a]

sample location	all 16S rRNA	all *Bacteroides* spp.	*tet*(A)	*tet*(X)	*tet*(W)	*intI1*
WW	1.9×10^{10}(6.5×10^{9})	6.9×10^{4}(3.9×10^{4})	2.1×10^{6}(6.8×10^{5})	2.9×10^{4}(5.4×10^{3})	1.9×10^{4}(9.3×10^{3})	2.4×10^{6}(6.6×10^{5})
DH1	2.1×10^{10}(5.1×10^{9})	2.3×10^{5}(1.4×10^{5})	7.4×10^{5}(1.9×10^{5})	1.2×10^{4}(2.3×10^{3})	6.5×10^{4}(3.7×10^{4})	2.5×10^{6}(5.5×10^{5})
DH3	1.7×10^{10}(5.1×10^{8})	4.9×10^{4}(2.8×10^{4})	3.7×10^{5}(6.8×10^{4})	b.d.[b]	2.6×10^{4}(1.4×10^{4})	7.7×10^{5}(4.3×10^{4})
LS2	1.9×10^{10}(5.7×10^{9})	2.3×10^{4}(3.2×10^{3})	1.2×10^{5}(1.2×10^{4})	b.d.	1.1×10^{4}(4.1×10^{3})	4.9×10^{5}(8.7×10^{4})

[a]Human-specific *Bacteroides* spp. were also targeted by real-time PCR, but were below the quantification limit in all four sample locations. The numbers in parentheses represent the standard deviation of the mean.
[b]b.d., below detection.

PCR Cloning of tet(W) Gene Fragments

In a previous study, *tet*(W) gene sequences corresponded to the location from which they originated (i.e., from agriculture, from municipal wastewater, etc.).[39] Nucleotide sequences, therefore, were determined from four different clone libraries (from samples SLR5, WW, DH2, and LS2) of *tet*(W) gene fragments to determine whether or not the type of *tet*(W) genes varied in the St. Louis River, Duluth-Superior Harbor, and Lake Superior. Comparing only nucleotide sequences for which a matching nucleotide sequence was detected (i.e., singletons were excluded from consideration), only two distinct clones were detected. The first of these clone types (100% sequence identity to GenBank accession no. GU116971) comprised 100% of the clone library from the St. Louis River sample (sample = SLR5; *n* = 17), slightly less than half of the clone library from the Duluth-Superior Harbor sample (sample = DH2; 8 out of 17 clones), and the majority of the clones from the Lake Superior sample (sample = LS2; 17 out of 20 clones). In contrast, the second clone type (100% sequence identity to GenBank accession no. AP012212) represented 100% of the library from the sample collected from the tertiary-treated wastewater (sample = WW; *n* = 14), slightly more than half of

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

the Duluth-Superior Harbor clone library (9 out of 17 clones), and a small fraction of the LS2 library (3 out of 20 clones).

Discussion

The importance of municipal wastewater treatment as a necessary component of modern society is without question.[40,41] The primary goal of municipal wastewater treatment is to protect surface water quality from the adverse effects of the relatively high concentration of nutrients (biodegradable carbon, nitrogen, and phosphorus) in the sewage; the secondary goal of municipal wastewater treatment is to protect public health from direct exposure to pathogens (usually via accidental ingestion of surface water).[41] An unintended consequence of municipal wastewater treatment, however, is the creation of a centralized location where bacteria from the microflora of healthy and unhealthy humans coalesce. Municipal wastewater and municipal wastewater treatment, therefore, simultaneously represent a pertinent reservoir of resistance and a potential opportunity to ameliorate this reservoir of resistance, respectively.

The present study demonstrates that treated municipal wastewater is a statistically significant point source of three tetracycline resistance determinants as well as the integrase gene of class 1 integrons into Duluth-Superior Harbor. The tertiary-treated wastewater had approximately 20-fold higher concentrations of various antibiotic resistance determinants than the local background levels in the St. Louis River and Lake Superior. Furthermore, the concentrations of antibiotic resistance genes generally correlated to either all *Bacteroides* spp. (a measure of total fecal material) or human-specific *Bacteroides* spp. (a measure of human-generated fecal material) (see Supporting Information for more details). Finally, the sequence of *tet*(W) gene fragments in tertiary-treated wastewater was unique compared to that compared to that found in the St. Louis River and Lake Superior, again suggesting that the tertiary-treated municipal wastewater was a significant source of antibiotic resistant determinants into Duluth-Superior Harbor – where approximately equal amounts of these two gene sequences were detected.

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

The present study is unique and novel because of its ability to clearly identify tertiary-treated municipal wastewater as a point source of antibiotic resistance genes, which have been identified as an emerging pollutant of concern.[42] Previous studies in which treated municipal wastewater was implicated as a source of antibiotic resistance determinants were substantially more convoluted because multiple sources of antibiotic resistance genes existed, such as agricultural activity and industrial wastewater discharges.[39,43] In contrast, the current study is considerably more straightforward to interpret because of the general transition from pristine (St. Louis River) to relatively perturbed (Duluth-Superior Harbor) back to pristine (Lake Superior), with virtually no known anthropogenic sources of antibiotic resistance genes other than a large input of tertiary-treated municipal wastewater from WLSSD (flow rate = 40 million gallons per day) and a small input of secondary-treated municipal wastewater from Superior, Wisconsin (flow rate = 5 million gallons per day; near sample location DH4).

In conclusion, municipal wastewater treatment operations need to be more carefully considered as an important factor in the global ecology of antibiotic resistance. Municipal wastewater contains numerous types of waste, of which human fecal material is known to have substantial concentrations of both antibiotic resistant bacteria and antibiotic resistance genes.[44] Municipal wastewater treatment operations undoubtedly remove a very large fraction of the antibiotic resistance genes in untreated sewage prior to discharging the treated effluent. This study demonstrates that even tertiary-treated municipal wastewater is a statistically significant source of antibiotic resistance genes in otherwise pristine surface waters; additional research is needed to determine the importance of treated municipal wastewater in the overall proliferation of antibiotic resistance.

Acknowledgment

This work was supported by the National Science Foundation (CBET-0967176) and by the Minnesota Environment and Natural Resources Trust Fund (for ship time on the R/V Blue Heron). The majority of this work was performed during the laboratory portion of CE 8551: *Environmental Microbiology—Molecular Theory and Methods* during the Fall 2010 semester at the University of Minnesota.

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

The assistance of the crew of the R/V Blue Heron is gratefully acknowledged. Dan Engstrom, Bill Arnold, Cale Anger, Noah Hensley, and Hao Pang are also gratefully acknowledged for their assistance with the collection and processing of sediment samples.

Supporting Information

Information regarding the qPCR primers and conditions (Table S1) and statistical analyses (Tables S2–S10). Additional results are also included regarding tetracycline resistance and *intI1* genes normalized to 16S rRNA genes (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

References

[1]Andersson, D. I.; Hughes, D.Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* 2010, 8, 260– 271

[2]Levy, S. B. *The antibiotic paradox: How miracle drugs are destroying the miracle*; Plenum Press: New York, 1992.

[3]Levy, S. B.Antibiotic resistance: Consequences of inaction *Clin. Infect. Dis.* 2001, 33, S124– S129

[4]Alonso, A.; Sánchez, P.; Martínez, J. L.Environmental selection of antibiotic resistance genes *Environ. Microbiol*. 2001, 3, 1– 9

[5]Allen, H. K.; Donato, J.; Wuimi Wang, H.; Cloud-Hansen, K. A.; Davies, J.; Handelsman, J.Call of the wild: Antibiotic resistance genes in natural environments *Nat. Rev. Microbiol*. 2010, 8, 251– 259

[6]Canton, R.Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting *Clin. Microbiol. Infect*. 2009, 15, 20– 25

[7]D'Costa, V. M.; Griffiths, E.; Wright, G. D.Expanding the soil antibiotic resistome: exploring environmental diversity *Curr. Opin. Microbiol.* 2007, 10, 481– 489

[8]Martínez, J.Antibiotics and antibiotic resistance genes in natural environments *Science* 2008, 321, 365– 367

[9]Lipsitch, M.; Singer, R. S.; Levin, B. R.Antibiotics in agriculture: when is it time to close the barn door? *Proc. Natl. Acad. Sci. U.S.A.* 2002, 99, 5752– 5754

[10]Smith, D. L.; Dushoff, J.; Morris, J. G., Jr.Agricultural antibiotics and human health *PLoS Med.* 2005, 2, 731– 735

[11]Merlin, C.; Bonot, S.; Courtois, S.; Block, J.-C.Persistence and dissemination of the multiple-antibiotic-resistance plasmid pB10 in the microbial communities of wastewater sludge microcosms *Water Res.* 2011, 45, 2897– 2905

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

[12]Novo, A.; Manaia, C. M.Factors influencing antibiotic resistance burden in municipal wastewater treatment plants *Appl. Microbiol. Biotechnol.* 2010, 87, 1157– 1166

[13]Ramsden, S. J.; Ghosh, S.; Bohl, L. J.; LaPara, T. M.Phenotypic and genotypic analysis of bacteria isolated from three municipal wastewater treatment plants on tetracycline-amended and ciprofloxacin-amended growth media *J. Appl. Microbiol.* 2010, 109, 1609– 1618

[14]Auerbach, E. A.; Seyfried, E. E.; McMahon, K. D.Tetracycline resistance genes in activated sludge wastewater treatment plants *Water Res.* 2007, 41, 1143– 1151

[15]Diehl, D. L.; LaPara, T. M.Effect of temperature on the fate of genes encoding tetracycline resistance and the integrase of class 1 integrons within anaerobic and aerobic digesters treating municipal wastewater solids *Environ. Sci. Technol*. 2010, 44, 9128– 9133

[16]Ghosh, S.; Ramsden, S. J.; LaPara, T. M.The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants *Appl. Microbiol. Biotechnol.* 2009, 84, 791– 796

[17]Moura, A.; Henriques, I.; Smalla, K.; Correia, A.Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes *Res. Microbiol.* 2010, 161, 58– 66

[18]Munir, M.; Wong, K.; Xagoraraki, I.Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan *Water Res.* 2011, 45, 681– 693

[19]Zhang, T.; Zhang, M.; Zhang, X.; Fang, H. H. P.Tetracycline resistance genes and tetracycline resistant lactose-fermenting *Enterobacteriaceae* in activated sludge of sewage treatment plants *Environ. Sci. Technol.* 2009, 43, 3455– 3460

[20]Zhang, X.; Zhang, T.; Zhang, M.; Fang, H. H. P; Cheng, S.-P.Characterization and quantification of class 1 integrons and associated gene cassettes in sewage treatment plants *Appl. Microbiol. Biotechnol.* 2009, 82, 1169– 1177

[21]Zhang, X.-X.; Zhang, T.Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations *Environ. Sci. Technol.* 2011, 45, 2598– 2604

[22]Amann, R. I.; Ludwig, W.; Schleifer, K. H.Phylogenetic identification and in situ detection of individual microbial cells without cultivation *Microbiol. Rev.* 1995, 59, 143– 169

[23]Staley, J. T.; Konopka, A.Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats *Annu. Rev. Microbiol.* 1985, 39, 321– 346

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

[24]Davies, J.; Davies, D.Origins and evolution of antibiotic resistance *Microbiol. Mol. Biol. Rev.* 2010, 74, 417– 433

[25]Cotner, J. B.; Ogdahl, M. L.; Biddanda, B. A.Double-stranded DNA measurement in lakes with the fluorescent stain PicoGreen and the application to bacterial bioassays *Aquat. Microb. Ecol.* 2001, 25, 65– 74

[26]Cardinale, M.; Brusetti, L.; Quatrinia, P.; Borin, S.; Puglia, A.; Rizzi, A.; Zanardini, E.; Sorlini, C.; Corselli, C.; Daffonchio, D.Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities *Appl. Environ. Microbiol.* 2004, 70, 6147– 6156

[27]Nelson, D. K.; LaPara, T. M.; Novak, P. J.Effects of ethanol-based fuel contamination: Microbial community changes, production of regulated compounds, and methane generation *Environ. Sci. Technol.* 2010, 44, 4525– 4530

[28]Chopra, I.; Roberts, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 2001, 65, 232– 260.

[29]Berhardt, A. E.; Field, K. G. 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. *Appl. Environ. Microbiol.* 2000, 66, 1587– 1594.

[30]Berhardt, A. E.; Field, K. G.A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA *Appl. Environ. Microbiol.* 2000, 66, 4571– 4574

[31]Seurinck, S.; Defiordt, T.; Verstraete, W.; Siciliano, S. D.Detection and quantification of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater *Environ. Microbiol.* 2005, 7, 249– 259

[32]Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A laboratory manual,* 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.

[33]Thioulouse, J.; Chessel, D.; Dolédec, S.; Olivier, J. M.Ade-4: a multivariate analysis and graphical display software *Stat. Comput.* 1997, 7, 75– 83

[34]Benson, D. A.; Karsch-Mizrachi, I.; Lipman, D. J.; Ostell, J.; Rapp, B. A.; Wheeler, D. L.GenBank *Nucleic Acids Res.* 2002, 30, 17– 20

[35]Thompson, J. D.; Higgins, D. G.; Gibson, T. J.CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice *Nucleic Acids Res.* 1994, 22, 4673– 4680

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.

***NOT THE PUBLISHED VERSION;* this is the author's final, peer-reviewed manuscript.** The published version may be accessed by following the link in the citation at the bottom of the page.

[36]Ling, L. L.; Keohavong, P.; Dias, C.; Thilly, W. G.Optimization of the polymerase chain reaction with regard to fidelity: modified T7, Taq, and vent DNA polymerases *PCR Methods Appl*. 1991, 1, 63– 69

[37]Yi, L.; Daqing, M.; Rysz, M.; Qixing, Z.; Hongjie, Z.; Lin, X.; Alvarez, P. J. J.Trends in antibiotic resistance genes occurrence in the Haihe River, China *Environ. Sci. Technol*. 2010, 44, 7220– 7225

[38]Xi, C.; Zhang, Y.; Marrs, C. F.; Ye, W.; Simon, C.; Foxman, B.; Nriagu, J.Prevalence of antibiotic resistance in drinking water treatment and distribution systems *Appl. Environ. Microbiol*. 2009, 75, 5714– 5718

[39]Storteboom, H.; Arabi, M.; Davis, J. G; Crimi, B.; Pruden, A.Tracking antibiotic resistance genes in the South Platte River basin using molecular signatures of urban, agricultural, and pristine sources *Environ. Sci. Technol*. 2010, 44, 7397– 7404

[40]Arthurson, V.Proper sanitization of sewage sludge: a critical issue for a sustainable society *Appl. Environ. Microbiol*. 2008, 74, 5267– 5275

[41]Tchobanoglous, G.; Burton, F. L.; Stensel, H. D. *Wastewater engineering: Treatment and Reuse,* 4th ed.; Metcalf and Eddy, Inc., McGraw-Hill: Boston, MA, 2003.

[42]Pruden, A.; Pei, R.; Storteboom, H.; Carlson, K. H.Antibiotic resistance genes as emerging contaminants: studies in northern Colorado *Environ. Sci. Technol.* 2006, 40, 7445– 7450

[43]Graham, D. W.; Olivares-Rieumont, S.; Knapp, C. W.; Lima, L.; Werner, D.; Bowen, E.Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, *Cuba Environ. Sci. Technol*. 2011, 45, 418– 424

[44]Sommer, M. O. A.; Dantas, G.; Church, G. M.Functional characterization of the antibiotic resistance reservoir in the human microflora *Science* 2009, 325, 1128– 1131

*Tel.: (612) 624-6028. Fax: (612) 626-7750. E-mail: lapar001@umn.edu.

Environmental Science & Technology, Vol 45, No. 22 (2011): pg. 9543-9549. DOI. This article is © American Chemical Society and permission has been granted for this version to appear in e-Publications@Marquette. American Chemical Society does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from American Chemical Society.