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Indirect Evidence of Transposon-Mediated Selection of Antibiotic Resistance Genes in Aquatic Systems at Low-Level Oxytetracycline Exposures

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Subinhibitory levels of antibiotics can promote the development of antibiotic resistance in bacteria. However, it is unclear whether antibiotic concentrations released into aquatic systems exert adequate pressure to select populations with resistance traits. To examine this issue, 15 mesocosms containing pristine surface water were treated with oxytetracycline (OTC) for 56 days at five levels (0, 5, 20, 50, and 250 μg L^{-1}), and six tetracycline-resistance genes (*tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), and *tet*(W)), the sum of those genes (tet^R), "total" 16S-rRNA genes, and transposons (Tn*916* and Tn*1545*) were monitored using real-time PCR. Absolute water-column resistance-gene abundances did not change at any OTC exposure. However, an increase was observed in the ratio of tet^R to 16S-rRNA genes in the 250 μg L^{-1} OTC units, and an increase in the selection rate of Tc^r genes (relative to 16S-rRNA genes) was seen when OTC levels were at 20 μg L^{-1}. Furthermore, *tet*(M) and Tn*916*/*1545* gene abundances correlated among all treatments ($r^2 = 0.701$, $p = 0.05$), and there were similar selection patterns of tet^R and Tn*916*/*1545* genes relative to the OTC level, suggesting a possible mechanism for retention of specific resistance genes within the systems.

Introduction

Tetracyclines have been used extensively as antibiotics in North America and Europe for over 50 years in both public health and agriculture (*1–3*). Considerable evidence now exists that exposure to subinhibitory doses results in the development and spread of antibiotic resistant bacteria in both clinical and food-supply scenarios (*4, 7*), and higher levels of antibiotics, including tetracyclines, have been correlated with elevated resistance gene abundances (*8–10*). However, less is known about how chronic low-level antibiotic exposures might affect resistance development (*11*), especially at low concentrations detected in aquatic systems (*3, 12–16*). It is possible that low antibiotic levels might provide adequate pressure to select for resistance in bacteria, especially over prolonged exposure times, although this has not been proven because it is difficult to separate intrinsic selection of resistance based on in situ antibiotic exposure from extrinsic contamination by resistance genes resulting from previous anthropogenic activity (e.g., antibiotic use in animal operations, medicine, etc).

This study examines intrinsic resistance development resulting from exposure to low levels of oxytetracycline (OTC), a naturally occurring antibiotic produced by *Streptomyces rimosus*. Although tetracycline- and oxytetracycline-resistance genes are naturally present in the environment (note: there are very few inherent differences in function or range of action between the tetracycline and the oxytetracycline class of genes (*1*)), such resistance genes are now being viewed as emerging contaminants (*10*). The practical question is how much the intrinsic selection of resistance genes resulting from OTC exposure (itself) contributes to "net resistance" in exposed environmental organisms. Although this question is obvious, providing an answer is not trivial because of the inherent problem of segregating intrinsic versus extrinsic genes in real systems.

To address this problem, replicate aquatic field mesocosms that contained water with no previous direct exposure to antibiotics were semicontinuously loaded with subinhibitory levels of OTC to simulate the effect of chronic antibiotic loadings in the systems. Further, these mesocosms had no extrinsic input of resistance genes, were at a field scale with extended exposures, and had replication among treatments, which permitted the statistical segregation of small differences among different OTC exposures (*17, 18*). Gene selection was quantified using real-time polymerase chain reaction (PCR) detection of selected tetracycline resistance genes (*tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), and *tet*(W)), total 16S-rRNA genes, and a regulatory gene in Tn*916* and Tn*1545* to determine possible transposon-related effects on selection. The working hypothesis was that low OTC loadings would, in fact, exert selective pressure on susceptible populations to gain or retain resistance traits.

Materials and Methods

Study Design. This study was performed at the Nelson Environmental Study Area (NESA) in Lawrence, KS, using procedures similar to those used in previous mesocosm studies (*17–20*). Three sets of five 11.3 m^3 cylindrical tank mesocosms were placed in parallel water-containing shallow "host" ponds to minimize temperature fluctuations. Mesocosms were filled with water to a depth of 1.2 m from an isolated surface pond located on site. Three trays (39 cm × 53 cm) containing sediment and macrophytes were then placed on the bottom of each unit as described previously (*21*).

The experiment commenced on September 14, 2004, with OTC (Sigma Aldrich, St. Louis, MO) being added on the first night to nominal OTC levels of 0, 5, 20, 50, and 250 μg L^{-1} (each in triplicate), respectively. Sampling for most parameters was performed between 9:00 a.m. and 10:00 a.m. on sample days (including the first morning); however, collection

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of samples for OTC analysis was more frequent, especially early in the experiment when OTC disappearance patterns were needed to establish OTC replenishment patterns for the experiment (see Supporting Information). The goal was to maintain pseudosteady OTC levels near nominal values for the study. Unfortunately, low OTC concentrations are typically not persistant in clear, surface waters due to photodegradation (*14*), which is experimentally problematic. As such, early OTC disappearance data indicated that OTC addition was required every 2 days (added at night, approximately 6:00 p.m.). OTC replenishment rates were reduced to every third day later in the experiment as autumn progressed and OTC disappearance rates declined.

Sample Collection and Processing. Field water quality measurements were performed weekly and included dissolved oxygen (DO), pH, conductivity, turbidity, water temperature, and light intensity. All measurements, except light intensity, were performed using a water checker field monitor (Horiba Instruments, Inc., Kyoto, Japan). Specifically, measurements were made at 0.3, 0.7, and 1.0 m depths, and average values from the three estimates were reported for each unit. Light levels (PAR; photosynthetically active radiation) were determined at 0.0, 0.5, and 1.0 m depths for each tank using a LI-COR spherical quantum sensor (LI185A, Lincoln, NE). Light extinction coefficients were estimated by curve-fitting light intensity values using the equation

$$I_z = I_0\, e^{-\varepsilon z} \quad (1)$$

where I_z is the light intensity (μmol-photons m^{-2} s^{-1}) at depth z (m), ε is the light extinction coefficient (m^{-1}), and I_0 is the light intensity just below the water surface.

Samples for wet chemistry and OTC analysis were collected at the time of field measurements using treatment-dedicated, depth-integrated samplers made of rigid PVC tubes with a check valve attached at one end (*17*). Typically, the tube sampler was prerinsed with tank water, and samples were withdrawn by gently passing the tube down the water column. The resulting sample was immediately transferred to acid-washed 500 mL amber glass bottles and stored on ice before returning to the laboratory. Samples for molecular analysis were collected identically to the chemistry samples except the tube sampler was sequentially rinsed with ethanol and three volumes of tank water before sampling. Sample bottles were presterilized prior to use.

OTC and Water Chemistry Analyses. Regular OTC analysis was performed using ELISA (RIDASCREEN Tetracycline kit, R-Biopharm, Darmstadt, Germany) according to manufacturer instructions. Samples were prefiltered with glass-fiber Whatman GF/F filters to remove particulates, and they were diluted with buffer as required to obtain readings in the middle of the standard curve. Additional standards were prepared using diluted OTC to determine analytical efficiencies. A subset of samples ($n = 20$) were also analyzed via LC ESI MS/MS (Agilent Technologies 1100 Series HPLC, Santa Clara, CA; Micromass Quatro Ultima, Waters Corporation, Milford, MA) to verify accuracy of the ELISA readings. Parallel samples for LC ESI MS/MS analysis were extracted (30% methanol, 0.05 M EDTA, and 0.1 M citric acid), evaporated to <1 mL, and resuspended in 2 mL of 30% acetonitrile prior to injection. OTC levels determined by ELISA and LC ESI MS/MS significantly correlated ($r^2 = 0.94$, $p < 0.01$).

Samples for regular water chemistry were processed the day of sampling and analyzed within two days. Total phosphorus (TP) and total nitrogen (TN) were both analyzed spectrophotometrically (Shimadzu UV-160) on unfiltered samples with TP being determined by the ascorbic acid reaction following digestion with potassium persulfate and TN being determined following alkaline persulfate digestion (*22*). Samples for chlorophyll *a* were filtered with Whatman GF/F filters, extracted using hot ethanol, and quantified spectrophotometrically (*23*). Filtrate from the chlorophyll *a* preparation was used for dissolved organic carbon (DOC) analysis. The samples were acidified (pH 2) with concentrated phosphoric acid and injected into a precalibrated Dohrmann organic carbon analyzer.

DNA Extraction. To harvest cells for DNA extraction, 500 mL subsamples were separated and filtered through 0.2 μm Nalgene presterilized funnel filters (Rochester, NY) in duplicate and frozen immediately at −80 °C. The filters were stored frozen until the end of the experiment when DNA was extracted from all samples at the same time. Extraction was performed directly from the filters using the MoBio UltraClean Soil DNA kit (Solona Beach, CA), with some modifications to the manufacturer's protocol (*24*). Filters, beads, and initial extraction buffer were agitated using a FastPrep (Qbiogene, Irvine, CA) cell disruptor for 45 s at 6.0-speed to breakdown the filters. The pulped filters were then incubated at 70 °C for 10 min to enhance lysis of Gram-positive bacteria, and then they were reagitated for 30 s at 5.0-speed. Extracts were retained at −20 °C until real-time PCR analysis was performed.

Real-Time PCR Assays. Real-time PCR analysis was used to quantify six common tetracycline resistance genes, including *tet*(B), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), and *tet*(W); 16S-rRNA genes; and a regulatory gene for the Tn*916*/Tn*1545* transposon family. Primers, probes, and plasmid standards have been reported previously for the six resistance genes (*16, 25*) and the 16S-rRNA genes (*26*). Primers, probe, and standards were newly designed and constructed using Beacon Designer (v.5.10 Premier Biosoft International, Palo Alto, CA) from existing GenBank data (#U09422) to target the *ORF5* gene region (*27*) of Tn*916*, which has similar sequences to Tn*1545*, another *tet*(M)-related transposon (*28*). The new sequences for probe/primers were tested for specificity using *BLASTn* and were as follows: sense 5′-ATACTCCCATACAGTCAATAGTCC, antisense 5′-AGTTCCACCCCTGCATGG, and TaqMan probe FAM-AGCCATACTCATTGCCTGCGACGG-TAMRA. All primers and probes were synthesized by Sigma-Genosys (Woodlands, TX).

Duplicate real-time PCR assays were performed using a BioRad iCycler with the iCycler iQ fluorescence detector and iCycler software version 2.3 (BioRad, Hercules, CA). Plate well factors were determined prior to each PCR run to normalize background fluorescence intensities from each single well, and diluted plasmid standards calibrated each run. The 25 μL reactions for the tetracycline resistance genes typically contained iQ Supermix (BioRad), 900 nM primers, 100 nM Taqman probe, and 2−3 μL of DNA template, and they were run under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles at 95 °C for 15 s and 60 °C for 30 s. 16S-rRNA gene quantification was performed according to Harms et al. (*26*) as described previously (*16*). The Tn*916*/Tn*1545* assay was performed at 50 °C for 2 min, 95 °C for 10 min, and 45 cycles at 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s.

The presence of inhibitory substances in the sample matrix was checked by spiking the samples with known amounts of DNA template and comparing differences in concentration threshold values (C_T). PCR efficiencies were further examined by comparing serial dilutions of selected samples (using 16S-rRNA assay) and diluted plasmid controls in a clean matrix. Very little inhibition was found (data not provided).

Data Analysis. PCR sensitivity for the tetracycline resistance genes was 0.02 genes mL^{-1}, whereas the limit was 10 genes mL^{-1} for the 16S rRNA gene; values below these limits were reported as zero. Rates of OTC disappearance and rates of normalized resistance-gene appearance were best represented as first-order decay functions and were calculated accordingly. However, an exceedingly high 16S-rRNA signal

TABLE 1. Mean Physical and Chemical Conditions among the Five OTC Treatments[a]

targeted OTC level (μg L^{-1})	measured OTC level (μg L^{-1})[b]	pH	dissolved oxygen (mg L^{-1})	water temperature (°C)	total phosphorus (mg-P L^{-1})	total nitrogen (mg-N L^{-1})	dissolved organic carbon (mg-C L^{-1})	chlorophyll *a* (mg L^{-1})	PAR attenuation (m^{-1})
0	1.9 (1.8)	8.0 (0.2)	11.0 (0.4)	16.0 (0.2)	0.018 (0.005)	0.53 (0.04)	5.8 (0.1)	0.006 (0.002)	0.39 (0.02)
5	8.4 (1.8)	8.0 (0.0)	11.2 (0.3)	16.0 (0.2)	0.010 (0.002)	0.49 (0.05)	6.2 (0.2)	0.003 (0.001)	0.45 (0.04)
20	22.6 (4.2)	8.1 (0.0)	11.4 (0.4)	15.9 (0.2)	0.011 (0.002)	0.53 (0.04)	6.3 (0.2)	0.006 (0.001)	0.48 (0.02)
50	51.0 (12.0)	7.9 (0.1)	10.9 (0.4)	15.9 (0.2)	0.012 (0.003)	0.51 (0.03)	6.2 (0.2)	0.002 (0.001)	0.55 (0.03)
250	279 (71.8)	7.8 (0.0)	9.7 (0.2)	15.9 (0.2)	0.014 (0.008)	0.63 (0.07)	8.1 (0.5)	0.002 (0.002)	0.79 (0.02)

[a] 95% confidence intervals are denoted in parentheses. [b] Note: Samples were collected at 9:00 a.m., +15 h following the addition of OTC the previous evening.

TABLE 2. Mean Absolute Gene Abundances (genes mL^{-1}) of Detected Individual Tetracycline Resistance and Total 16S-rRNA Genes over the Duration of the Experiment among Treatments[a]

nominal OTC treatment (μg L^{-1})	*tet*(M)	*tet*(O)	*tet*(Q)	*tet*(W)	16S-rRNA genes
0	0.31 (0.19)[b]	0.090 (0.100)	0.011 (0.049)	0.24 (0.13)	11240 (4660)
5	0.49 (0.08)	0.029 (0.023)	0.009 (0.006)	0.12 (0.07)	10790 (2510)
20	0.46 (0.21)	0.032 (0.025)	0.051 (0.082)	0.19 (0.18)	12380 (6440)
50	0.34 (0.07)	0.038 (0.010)	0.015 (0.010)	0.15 (0.01)	11890 (3720)
250	0.32 (0.06)	0.033 (0.026)	0.006 (0.006)	0.11 (0.08)	3180 (1190)

[a] Note: No significant levels of *tet*(B) or *tet*(L) were detected (<0.02 genes mL^{-1}). [b] 95% confidence intervals around grand means based on $n = 5$ or 6 treatment averages over time.

was seen in one outlier (Day 42, 20 μg/L treatment), which was subsequently not used in regression analysis.

All statistical analyses were performed using SPSS (v. 11.01; Chicago, IL). Data normality was tested using the Kolmogorov−Smirnov test for population distributions and, in most cases, gene-abundance values required log-transformation prior to statistical analysis. Arithmetic means and associated 95% confidence intervals were used as statistical descriptors. A mixed-order nested *ANOVA* on water chemistry and gene abundances in tanks within each treatment (as the nested factor) was used to make treatment comparisons. Fortunately, no significant differences among tanks within any treatment were found. Levels of significance for all tests were assigned as $p < 0.05$. Tukey poststatistical analysis was conducted to identify treatment differences in the *ANOVA*.

Results

Water Chemistry in Mesocosms. Mean OTC levels and other water chemistry conditions in the five mesocosm treatments are presented in Table 1. In summary, detected OTC levels were very close to nominal values for all treatments, typically within 10% of the targeted value. Further, water chemistry conditions were statistically identical for temperature, TP, and TN among treatments (*ANOVA*, $p > 0.05$). However, pH ($F_{4,10} = 4.31$; $p = 0.03$), DO ($F_{4,10} = 15.2$; $p < 0.01$), PAR ($F_{4,10} = 140$; $p < 0.01$), chlorophyll *a* ($F_{4,10} = 4.10$; $p = 0.03$), and DOC ($F_{4,10} = 42.9$; $p < 0.01$) differed in the 250 μg L^{-1} units relative to the other four treatments. DO, pH, and chlorophyll *a* were significantly lower in the 250 μg L^{-1} units, whereas DOC and PAR attenuation were significantly higher, suggesting that this level of OTC likely impaired photosynthesis in these units. Such effects have been seen in previous studies (*29, 30*), and they are attributed to either antibiotic effects of the OTC or by reduced light levels from color development due to OTC and breakdown-product accumulation in the water column (*21, 29*). Both factors likely affected water conditions in the 250 μg L^{-1} units here.

Measures of Resistance and Abundance of 16S-rRNA Genes. Of the six resistance genes quantified, only *tet*(M), *tet*(O), *tet*(Q), and *tet*(W) were detectable in mesocosm waters (*tet*(B) and *tet*(L) were always below detection limits; 0.02 genes mL^{-1}), and *tet*(W) and *tet*(M) were dominant among detected genes (Table 2). However, the absolute abundance of each detected resistance gene did not significantly differ among the five treatments (*ANOVA*; all $p > 0.34$). In contrast, 16S-rRNA gene levels, which provided a surrogate measure of bacterial abundances, were significantly lower in the 250 μg L^{-1} units relative to other treatments (4-fold; *ANOVA*, $F_{4,10} = 7.04$, $p = 0.01$), indicating that reduced light supply or antibacterial effects reduced the bacterial community size in this treatment.

To assess possible treatment effects on relative resistance-gene selection, resistance-gene abundances were normalized to ambient 16S-rRNA gene levels for each treatment (Figure 1). As OTC exposure proceeded, the ratio of resistance genes to 16S-rRNA genes consistently increased, which was most apparent in the 250 μg-OTC L^{-1} units; i.e., the relative selection of populations containing resistance genes appear to be occurring as time proceeded. In most cases, this effect appeared subtle; however, when first-order "selection rates" were plotted versus OTC level, selection rate differences among treatments became more apparent. Figure 2A shows that as the OTC level increases, the rate of increase in normalized resistance-gene abundances also increases, although the relationship between selection rate and OTC level is mixed-order. Further, surprisingly low OTC levels appeared to influence the rate of resistance-gene selection relative to the 16S-rRNA gene pool, although differences at the lowest concentrations were not statistically significant. Regardless, quasi-steady OTC exposure at low levels may contribute to subtle selection of resistance genes in the mesocosms.

Figure 1 shows that *tet*(W), and most notably *tet*(M), were the main selected resistance genes in all treatments. Selection of these genes in aquatic systems has been noted previously (*31, 32*); therefore, it was speculated that their selection might result from specific gene transfer or retention mechanisms. As such, Tn*916*/Tn*1545* gene abundances were also quantified among samples as a possible explanation for elevated *tet*(M). Tn*916* and Tn*1545* are two closely related transposons that carry the *tet*(M) gene (*28, 33, 34*). Figure 3 shows a strong

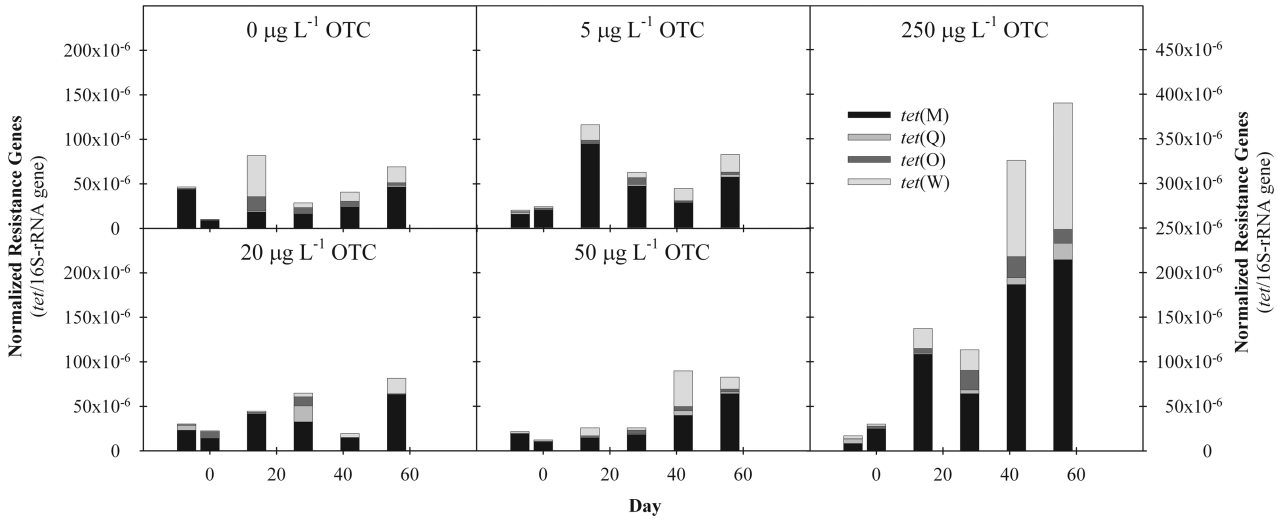

FIGURE 1. Abundances of *tet*(M), *tet*(Q), *tet*(O), and *tet*(W) over time normalized to 16S-rRNA gene levels in mesocosms amended with (A) 0, (B) 5, (C) 20, (D) 50, and (E) 250 μg L^{-1}oxytetracycline.

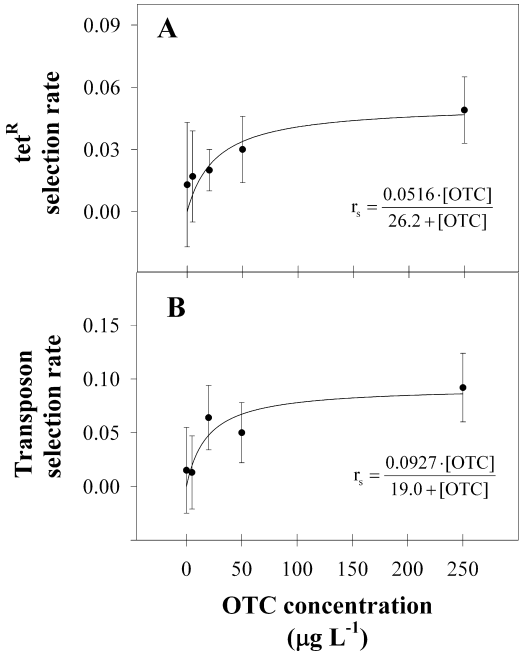

FIGURE 2. First-order rates of increase (r_s) of (A) the sum of six resistance genes (tet^R) normalized to 16S-rRNA genes and (B) Tn*916*/Tn*1545* genes normalized to 16S-rRNA genes over oxytetracycline concentrations.

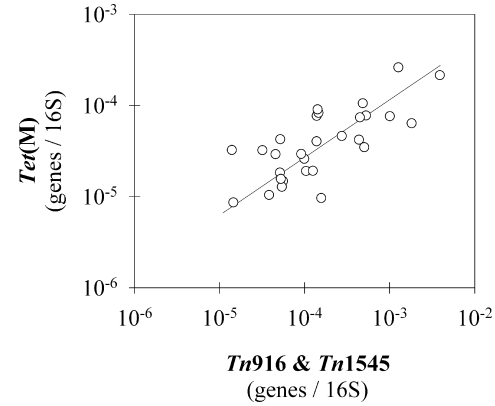

FIGURE 3. Log−log relationship between *tet*(M) and Tn*916*/Tn*1545* genes normalized to 16S-rRNA genes for water-column samples collected from all treatments. Pearson correlation coefficient: $r^2 = 0.701$.

Pearson correlation between Tn*916*+Tn*1545* and *tet*(M) abundances ($r^2 = 0.701$, $p = 0.05$), implying that Tn*916*/Tn*1545* (or analogous transposons) might be playing a direct or indirect role in the retention of these genes in tank waters. To qualitatively test this speculation, the rate of selection of relative Tn*916*/Tn*1545* abundances versus OTC level is presented in Figure 2B, and a very similar pattern is seen between overall resistance-gene-selection rate and the selection rate of related transposons.

Discussion

There is growing concern that the use of antibiotics, including tetracyclines, is increasing resistance in pathogenic, commensal, and environmental bacteria (*7*), which is supported by increased resistance in resident organisms in the gastrointestinal tract, feedlot wastewater lagoons, animal manure and compost, hospital wastewater, and aquatic sediments (*4, 5, 7*). Due to the broadening prevalence of resistant organisms, resistance-conferring genes themselves are now considered emerging contaminants, especially genes selected in one location (e.g., the gut of an animal) and spread to the environment via surface and groundwater flow (*10, 35*). Unfortunately, because strong evidence exists for such extrinsic release of resistance genes to the environment, the possibility of resistance selection based on antibiotic exposures themselves (i.e., intrinsic selection) is being less considered. Early evidence implied that low antibiotic levels typical of the environment do not appear to significantly increase the resistance gene pool. However, such experiments were of short durations that may not have been adequate to detect the effects of chronic low antibiotic exposure levels (*36*).

Increased resistance in the environment can result from bacteria capable of growing in the presence of the antibiotic, which can be natural or anthropogenic in origin. However, the transfer of resistance traits can occur from either new mutations or through the acquisition of genes coding for resistance in bacteria (*7*), which is often via transferable genetic elements that allow selection based upon antibiotic exposure (*37*). In the case of tetracycline resistance, genes encoding efflux pumps (e.g., *tet*(B)) are generally associated with large plasmids that are spread via conjugation (*38, 39*), which is why their presence is detected among diverse species (*40*). Alternately, genes for ribosomal protection proteins (RPP), another mechanism of tetracycline resistance, have variable means of

spreading. *tet*(O) is commonly associated with conjugative plasmids, whereas *tet*(M), *tet*(Q), and *tet*(W) are usually associated with mobile chromosomal elements that can code for their own transfer (e.g., conjugative transposons (*33, 34, 41*). As such, mobile genes, like those carried by transposons, by their increased relative presence in the environment might more readily move among exposed species facilitating their possible selection even in the presence of comparatively low antibiotic levels.

Figures 1 and 2 show a clear relationship between OTC exposures and increased rate of selection of resistance genes within the gene pool. Figure 1 demonstrates this most dramatically in the highest exposure systems (i.e., 250 μg L^{-1} OTC); however, Figure 2 hints that increased selection was also apparent at exposures as low as 20 μg L^{-1} OTC. Further, slightly elevated selection rates, although not statistically significant, were noted at even lower OTC levels. It is not clear whether this is due to weak selection resulting from OTC exposure or the natural accumulation of resistance genes as general defense responses within certain organisms.

A possible mechanism for environmental selection of populations containing resistance determinants is suggested here. *tet*(M), which was (and has been in past studies) the most common resistance gene observed in aquatic systems, is known to be conjugative transposon-mediated (*28, 33, 34*). Therefore, our statistically significant correlation between *tet*(M) and Tn*916*+Tn*1545* gene abundances provides a possible link between horizontal gene transfer and its selective effect on resistance to antibiotics in the environment. This is partially confirmed by Figure 2, which shows that the rate of resistance-gene selection and the rate of transposon selection follow similar patterns and are also antibiotic concentration dependent. This further hints that low sub-inhibitory antibiotic levels can selectively influence resistance genes in the environment.

In summary, these data are the first field-scale demonstration of increased in situ antibiotic resistance selection at low antibiotic exposures. It is not clear yet as to how broadly significant intrinsic rates of selection are relative to extrinsic gene sources; however, these data imply that the antibiotics themselves must also be considered in locations where extrinsic gene sources are less substantial. Overall, new strategies are clearly needed that consider both intrinsic and extrinsic impacts on resistance-gene levels in the environment because both sources might be conditionally important depending on local conditions.

Acknowledgments

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Supporting Information Available

Additional information about OTC fate (Figure S1) and gene abundances, both tetracycline-resistance (Figure S2) and 16S-rRNA (Figure S3), are provided in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Chopra, I.; Roberts, M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **2001**, *65* (2), 232–260.
(2) Levy, S. B. *The Antibiotic Paradox: How Miracle Drugs are Destroying the Miracle*; Plenum Press: New York, 1992.
(3) Sarmah, A. K.; Meyer, M. T.; Boxall, A. B. A. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* **2006**, *65* (5), 725–759.
(4) Anderson, A. D.; Nelson, J. M.; Rossiter, S.; Angulo, F. J. Public health consequences of use of antimicrobial agents in food animals in the United States. *Microb. Drug Resist. (Larchmont, N.Y.)* **2003**, *9* (4), 373–379.
(5) O'Brien, T. F. Emergence, spread, and environmental effect of antimicrobial resistance: How use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. *Clin. Infect. Dis.* **2002**, *34*, S78−S84.
(6) Summers, A. O. Generally overlooked fundamentals of bacterial genetics and ecology. *Clin. Infect. Dis.* **2002**, *34*, S85−S92.
(7) Witte, W. Medical consequences of antibiotic use in agriculture. *Science* **1998**, *279* (5353), 996–997.
(8) Auerbach, E. A.; Seyfried, E. E.; McMahon, K. D. Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* **2007**, *41*, 1143–1151.
(9) Pei, R. T.; Kim, S. C.; Carlson, K. H.; Pruden, A. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* **2006**, *40* (12), 2427–2435.
(10) Pruden, A.; Pei, R. T.; Storteboom, H.; Carlson, K. H. Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environ. Sci. Technol.* **2006**, *40* (23), 7445–7450.
(11) Kummerer, K. Significance of antibiotics in the environment. *J. Antimicrob. Chemother.* **2003**, *52* (1), 5–7.
(12) Yang, S. W.; Carlson, K. Evolution of antibiotic occurrence in a river through pristine, urban and agricultural landscapes. *Water Res.* **2003**, *37* (19), 4645–4656.
(13) Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton, H. T. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999−2000: a national reconnaissance. *Environ. Sci. Technol.* **2002**, *36* (6), 1202–1211.
(14) Hirsch, R.; Ternes, T.; Haberer, K.; Kratz, K. L. Occurrence of antibiotics in the aquatic environment. *Sci. Total Environ.* **1999**, *225* (1−2), 109–118.
(15) Aga, D. S.; O'Conner, S.; Ensley, S.; Payero, J. O.; Snow, D.; Tarkalson, D. Determination of the persistence of tetracycline antibiotics and their degradates in manure-amended soil using enzyme-linked immunosorbent assay and liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 7165–7171.
(16) Peak, N.; Knapp, C. W.; Yang, R. K.; Hanfelt, M. M.; Smith, M. S.; Aga, D. S.; Graham, D. W. Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. *Environ. Microbiol.* **2007**, *9* (1), 143–151.
(17) Graham, W. H.; Graham, D. W.; Denoyelles, F.; Smith, V. H.; Larive, C. K.; Thurman, E. M. Metolachlor and alachlor breakdown product formation patterns in aquatic field mesocosms. *Environ. Sci. Technol.* **1999**, *33* (24), 4471–4476.
(18) Knapp, C. W.; Graham, D. W.; Berardesco, G.; deNoyelles, F.; Cutak, B. J.; Larive, C. K. Nutrient level, microbial activity, and alachlor transformation in aerobic aquatic systems. *Water Res.* **2003**, *37* (19), 4761–4769.
(19) Ensz, A. P.; Knapp, C. W.; Graham, D. W. Influence of autochthonous dissolved organic carbon and nutrient limitation on alachlor biotransformation in aerobic aquatic systems. *Environ. Sci. Technol.* **2003**, *37* (18), 4157–4162.
(20) Knapp, C. W.; Cardoza, L. A.; Hawes, J. N.; Wellington, E. M. H.; Larive, C. K.; Graham, D. W. Fate and effects of enrofloxacin in aquatic systems under different light conditions. *Environ. Sci. Technol.* **2005**, *39* (23), 9140–9146.
(21) Hanson, M. L.; Knapp, C. W.; Graham, D. W. Field assessment of oxytetracycline exposure to the freshwater macrophytes *Egeria densa* Planch. and *Ceratophyllum demersum* L. *Environ. Pollut.* **2006**, *141* (3), 434–442.
(22) American Public Heath Association (APHA); American Water Works Association; World Environment Federation. *Standard Methods for the Examination of Water and Wastewater*, 20th ed.; APHA: Washington, DC, 1998.
(23) Nusch, E. A. Comparison of different methods for chlorophyll and phaeopigment determination. *Arch. Hydrobiol. Beih. Ergebn. Limnol.* **1980**, *14*, 14–36.
(24) Mo-Bio-Laboratories. Alternative protocol (for maximum yields). In *UltraClean(TM) Soil DNA Isolation Kit Instruction Manual*; Mo-Bio-Laboratories: Solona, CA, 2004.
(25) Smith, M. S.; Yang, R. K.; Knapp, C. W.; Niu, Y. F.; Peak, N.; Hanfelt, M. M.; Galland, J. C.; Graham, D. W. Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. *Appl. Environ. Microbiol.* **2004**, *70* (12), 7372–7377.
(26) Harms, G.; Layton, A. C.; Dionisi, H. M.; Gregory, I. R.; Garrett, V. M.; Hawkins, S. A.; Robinson, K. G.; Sayler, G. S. Real-time

PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. *Environ. Sci. Technol.* **2003**, *37* (2), 343–351.

(27) Su, Y. A.; Clewell, D. B. Characterization of the left 4 kb of conjugative transposon Tn916: determinants involved in excision. *Plasmid* **1993**, *30*, 234–250.

(28) Flannagan, S. E.; Zitzow, L. A.; Su, Y. A.; Clewell, D. B. Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from from *Enterococcus faecalis*. *Plasmid* **1994**, *32*, 350–354.

(29) Brain, R. A.; Johnson, D. J.; Richards, S. M.; Hanson, M. L.; Sanderson, H.; Lam, M. W.; Young, C.; Mabury, S. A.; Sibley, P. K.; Solomon, K. R. Microcosm evaluation of the effects of an eight pharmaceutical mixture to the aquatic macrophytes *Lemna gibba* and *Myriophyllum sibiricum*. *Aquat. Toxicol.* **2004**, *70* (1), 23–40.

(30) Brain, R. A.; Wilson, C. J.; Johnson, D. J.; Sanderson, H.; Bestari, K.; Hanson, M. L.; Sibley, P. K.; Solomon, K. R. Effects of a mixture of tetracyclines to *Lemna gibba* and *Myriophyllum sibiricum* evaluated in aquatic microcosms. *Environ. Pollut.* **2005**, *138* (3), 425–442.

(31) Engemann, C. A.; Adams, L.; Knapp, C. W.; Graham, D. W. Disappearance of oxytetracycline resistance genes in aquatic systems. *FEMS Microbiol. Lett.* **2006**, *263* (2), 176–182.

(32) Engemann, C. A.; Keen, P. L.; Knapp, C. W.; Hall, K. J.; Graham, D. W. Fate of oxytetracycline resistance genes in aquatic systems: migration from water column to benthic biofilms. *Environ. Sci. Technol.* **2008**, *42*, 5131−5136.

(33) Agerso, Y.; Pedersen, A. G.; Aarestrup, F. M. Identification of Tn5397-like and Tn916-like transposons and diversity of the tetracycline resistance gene tet(M) in enterococci from humans, pigs and poultry. *J. Antimicrob. Chemother.* **2006**, *57* (5), 832–839.

(34) Franke, A. E.; Clewell, D. B. Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Stretococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. *J. Bacteriol.* **1981**, *183*, 2947–2951.

(35) Koike, S.; Krapac, I. G.; Oliver, H. D.; Yannarell, A. C.; Chee-Sanford, J. C.; Aminov, R. I.; Mackie, R. I. Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period. *Appl. Environ. Microbiol.* **2007**, *73* (15), 4813–4823.

(36) Munoz-Aguayo, J.; Lang, K. S.; LaPara, T. M.; Gonzalez, G.; Singer, R. S. Evaluating the effects of chlortetracycline on the proliferation of antibiotic-resistant bacteria in a simulated river water ecosystem. *Appl. Environ. Microbiol.* **2007**, *73* (17), 5421–5425.

(37) Levy, S. B. Resistance to the tetracyclines. In *Antimicrobial Drug Resistance*; Bryan, L. E., Ed.; Academic Press: Orlando, FL, 1984.

(38) Jones, C. S.; Osborne, D. J.; Stanley, J. Enterobacterial tetracycline resistance in relation to plasmid incompatibility. *Mol. Cell. Probes* **1992**, *6* (4), 313–317.

(39) Mendez, B.; Tachibana, C.; Levy, S. B. Heterogeneity of tetracycline resistance determinants. *Plasmid* **1980**, *3* (2), 99–108.

(40) Roberts, M. C. Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.* **2005**, *245* (2), 195–203.

(41) Roberts, M. C. Tetracycline resistance determinants: Mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol. Rev.* **1996**, *19* (1), 1–24.

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