IN VITRO INDUCTION OF MULTI-ANTIBIOTIC RESISTANT PHENOTYPES IN *STAPHYLOCCOCUS AUREUS* BY EXPOSURE TO ENVIRONMENTAL WATERS

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ABSTRACT OF THE DISSERTATION

IN VITRO INDUCTION OF MULTI-ANTIBIOTIC RESISTANT PHENOTYPES

IN _STAPHYLOCCOCUS AUREUS_ BY EXPOSURE

TO ENVIRONMENTAL WATERS _IN VITRO_

WATERS

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In vitro exposure of *Staphylococcus aureus* strain ATTC 9144 for 10-days to environmental water samples including ambient fresh water and wastewater treatment plant influents and effluents increased the Minimum Inhibitory Concentration (MIC) values versus the controls in 11of the 51 (22%) samples assayed. Increases in the MIC values for all of the assayed antibiotics were observed in more than one sample. Antibiotic resistance, as measured by an increase in the MIC values greater than or equal to 4 times the control sample MIC value, was observed most frequently for tetracycline (22%) and the beta lactam ampicillin, (20%), and the beta lactam methicillin (18%). The aminoglycoside, kanamycin, increased the MIC values least frequently (9%). Methicillin was co-resistant with ampicillin and tetracycline in all of the methicillin resistant samples. For other antibiotics assayed for their MIC values in *Staphylococcus aureus* ATCC 9144 after exposure to environmental waters, all displayed multi-antibiotic resistance with ampicillin and tetracycline resistance, suggesting a common origin or assembly of resistance traits. Vancomycin resistance was present in 10% of the 40 samples assayed for its MIC, and coexisted with methicillin resistance in 75% of the vancomycin resistant samples.

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Introduction

The prevalence of antibiotic resistant pathogen strains is of widespread concern due to its decreasing the arsenal of antimicrobial agents available to treat human and animal infectious bacterial diseases (World Health Organization, 2000). The recent human morbidity and mortality in the United States resulting from infection caused by both nosocomial and community-associated methicillin resistant *Staphylococcus aureus* (MRSA) has further highlighted the high prevalence and commensurate public health implications of antibiotic resistant bacteria (Centers for Disease Control, 2007). In 2005, the U.S. Center for Disease Control (CDC) estimated the occurrence in the U.S. of 94,360 invasive MRSA infections. Approximately 18,650 persons died during hospital stays related to these infections (Klevens, et al., 2007). The more recent emergence of vancomycin resistant *S. aureus* indicates that further urgency is warranted to address the presence of antibiotic resistant bacteria, since vancomycin is one of the few remaining antibiotics effective for treatment of MRSA infections (Schaff, et al. 2002). The costs of treating human incidences of disease associated with antibiotic resistant pathogens alone are high, with likely conservative estimates of $3 - $5 billion per year in the U.S. alone (United States General Accounting Office, 2004; McGowan, 2001).

Literature Review

Pharmaceuticals and Antibiotics in the Environment

Both human and veterinary pharmaceuticals are employed in increasing quantities worldwide. I recently available information, market growth in pharmaceuticals was forecast to increase 6 - 7% in 2006, with a value of $640 to $650 billion (IMS Health,

Inc., 2006), but the actual values achieved are not currently publicly available. In the U.S., estimates run between about 30 and 50 million pounds of antibiotics are produced each year (Chandler, et al., 2007). Estimates suggest that about 70% of these antibiotics are used in agriculture (Mellon, et. al, 2001). Many therapeutics, including most if not all antibiotics, are largely unmetabolized and consequently excreted in urine and feces that can then enter the environment directly (e.g. from animal husbandry and livestock production) or after treatment (e.g. sanitary sewage) (Lienert, et al., 2007). Disposal of unused pharmaceuticals down sink drains or toilets including those at hospital and other health care facilities is yet another pathway for their introduction into the environment (Brown, et al., 2006; Bound and Voulvoulis, 2005; Daughton, 2002, 2003 (b); Emmanuel, et al. 2005).

Recent investigations have demonstrated the widespread presence of pharmacologically active compounds in ambient waters, wastewaters, drinking water, sewage sludge, and soil. (Abuin, et al., 2006; Batt et al., 2006; Brown, et al., 2006; Ellis, J. B., 2006; Hochereau, et al., 2005; Kolpin, et al., 2000; Managaki, et al., 2007). Detected compounds include both human and veterinary therapeutics, including vasoactive compounds, anti inflamatories, analgesics, steroids, and antibiotics. The presence of these compounds is generally associated with either locations receiving input from livestock production activities, treated sewage effluent discharges from wastewater treatment, or urban runoff.

The presence of pharmaceuticals and personal care products (PPCPs) in the environment has received increasing attention by investigators in Europe, Asia, and North America (Bound and Voulvoulis. 2005; Boyd, et al., 2003; Brown, et al. 2006; Bruchet, et

al., 2005; Kolpin, et al., 2002; Lishman, et al., 2006). While not intended to discount the importance of other compounds detected, the principal focus of this review is with respect to targeted antibiotics.

The first broad national survey of the occurrence of pharmacologically active pharmaceuticals and metabolites in U.S. waters identified 95 pharmaceuticals in 80% of 139 sampled fresh water bodies (Kolpin, et al., 2002). Only eight of the 31 antibiotic/antimicrobial or antibiotic/antimicrobial metabolites included for analysis were not detected. Trimethoprim, detected in 27.4% of samples, and erythromycin, detected in 21.5% of samples, were the most frequently detected antibiotic/antimicrobial. Concentrations of detected antibiotics/antimicrobials ranged from 0.02 ug/L (ciprofloxacin) to 1.9 ug/L (sulfamethoxazole). Although the sampled waters were selected to be representative of those with a higher potential for pharmaceutical detection (i.e., downstream of highly urbanized areas and treated sewage discharge or livestock production) the Kolpin, et al. study provided impetus for additional investigation into the occurrence, prevalence, sources, and transport and fate of pharmaceuticals including antibiotic/antimicrobial in U.S. waters (Batt, et al., 2006; Glassmeyer, et al., 2005; Karthikeyan and Meyer, 2006; Stackleberg, et al., 2007). Commensurately, spatially focused investigations in other countries have increased knowledge of the occurrence of PPCPs and antibiotic/antimicrobial in various environmental waters (Abuin, 2006; Andreozzi, et al., 2004; Bruchet, et al., 2005; Castiglioni, et al., 2006; Ellis, 2006; Giger, et al., 2003; Hirsch, et al., 1999; Jacobsen and Berglind, 1988; Lindberg, et al., 2005; McCardle, et al., 2003).

A study of 18 antibiotic/antimicrobial in wastewater treatment plant (WWTP)

effluent, surface waters, and groundwaters in Germany detected a variety of antibiotic/antimicrobial (Hirsch, et al., 1999). Five antibiotic/antimicrobial were detected in WWTP effluent. Erythromycin (as its principal hydrolysis product erythromycin-H_2O) was detected at the highest concentration of 6 ug/L, and was detected in every effluent sample at concentrations greater than 1 ug/L. Sulfamethoxazole and roxithromycin were also detected in every WWTP effluent sample with maximum concentrations greater than or equal to1 ug/L, and were detected in all samples at concentrations greater than 0.1 ug/L. Other antibiotics/antimicrobials examined were detected at lower frequencies, but when detected concentrations exceeded 0.1 ug/L. Antibiotics were detected less frequently and at lower concentrations in surface water samples. In surface water, erythromycin was both the most frequently detected antibiotic, and was also detected at the highest concentration, with detection in all but 3 of 52 samples greater than 100 ug/L, and in three samples greater than 1 ug/L. The 5 other targeted antibiotic/antimicrobial were detected in surface water in the sub ug/L range. Only 2 antibiotic/antimicrobial, sulfamethoxazole and sulfamethazone, were detected in groundwater. Sufamethoxazole was detected in 3 out of 59 groundwater samples at concentrations up to 0.49 ug/L. Sulfamethazone was detected in 3 out of 59 groundwater samples at concentrations up to 0.16 ug/L.

In England, a study of PPCPs in urban surface and groundwaters from London evaluated 6 antibiotic/antimicrobial as part of a larger PPCP suite (Ellis, 2006). No antibiotic/antimicrobial were detected in samples collected during dry weather flows either upstream or downstream of a WWTP, or in groundwater samples collected from the trunk line into the treatment plant. However, analytical sensitivity was reported to be variable

and relatively low compared with other studies.

A study of 5 macrolide antibiotics in water from three rivers in Spain, one in an agricultural area, one in a mixed agricultural – urban area, and one in a highly developed urban area, detected erythromycin, clarithromycin, and azithromycin at concentrations $\leq$ 0.2 ug/L (Abuin, et al., 2006). Erythromycin was detected in all 3 river samples, whereas clarithromycin and azithromycin were only detected in the sample collected from the highly urbanized river. Kitasamycin and roxithromycin were targeted in the analysis but were not detected.

An evaluation of 110 PPCPs in samples from 10 U.S. WWTP effluents and waters from upstream and downstream of the WWTP discharges included 10 veterinary, 5 human, and 6 mixed use (human and veterinary) antibiotic/antimicrobial (Glassmeyer, et al., 2005). Only 2 antibiotic/antimicrobial were detected. Sulfamethoxazole was detected in almost three quarters of samples with a median concentration of 0.835 ug/L and a maximum concentration of 2.9 ug/L. Trimethoprim was detected at the lower frequency of 60%, and at a lower median (0.011 ug/L) and maximum concentration (0.414 ug/L).

Other investigations have also detected antibiotic/antimicrobial in the vicinity of WWTP discharges. Five of 6 targeted antibiotic/antimicrobial were detected in effluents, and upstream and downstream samples from three WWTPs in New York State (Batt, et al., 2006). In WWTP effluents, ciprofloxacin and sulfamethoxazole were both the most frequently detected antibiotic/antimicrobial (89% each), and were detected at the highest concentrations in all samples. The maximum detected concentration of sulfamethoxazole was 6.0 ug/L; the maximum detected concentration of ciprofloxacin was 5.6 ug/L. Trimethoprim was detected in every sample, and clindamycin and tetracycline were

detected in some but not all effluents. No target antibiotics/antimicrobials were detected in upstream samples. Ciprofloxacin, sulfamethoxazole, and clindamycin were all detected in downstream samples where they were also detected in upstream WWTP effluent. Concentrations detected in samples collected 10 meters downstream from WWTP discharge, presumably within the mixing zone of the discharge, were generally similar to those detected in effluent. Concentrations diminished by an order of magnitude or more in samples collected 100 meters downstream of the WWTP discharge.

Six antibiotic/antimicrobial of 21 analyzed were detected in influents and effluents from 7 WWTPs in Wisconsin that discharge to either surface waters or groundwaters (Karthikeyan and Meyer, 2006). Sulfamethoxazole, tetracycline, trimethoprim, and erythromycin were the most frequently detected antibiotic/antimicrobial, and were variously detected in samples of both influent and effluent at concentrations ranging from 1.2 ug/L for sulfamethoxazole, to 0.04 ug/L for ciprofloxacin. Tetracycline and trimethoprim were detected in all effluent samples when they were detected in influent samples from the same WWTP, though at reduced levels, but generally in the same order of magnitude concentrations. Sulfamethoxazole was detected with nearly as high a frequency as tetracycline and trimethoprim, and was also detected in two groundwater monitoring wells at WWTPs that employ subsurface discharge. No other targeted antibiotics/antimicrobials were detected in groundwater. Seasonal variations corresponding to the use of antibiotic/antimicrobial, for example during cold and flu season, is suggested by the increased detected occurrences during winter months. However, the lack of consistent seasonal samples for all of the detected antibiotic/antimicrobial makes this association speculative.

The transport and fate of the antibiotic amoxicillin was investigated in 8 WWTPs in Italy (Andreozzi, et al., 2004). Amoxicillin was detected in 5 of the 8 sampled effluents with concentrations ranging from 0.0018 ug/L to 0.12 ug/L. Results of associated batch experiments showed a temperature dependent rate of amoxicillin degradation by direct photolysis, with rates increasing with increasing temperature up to 50^{o}C. Hydrolysis was the principal mechanism of amoxicillin removal, with a half life of less than 5 hours at 25^{o}C, whereas adsorption to activated sludge was observed to occur at a slower rate.

Two antibiotics/antimicrobials were detected in samples of water collected four months apart at 3 locations upstream and downstream of WWTP discharges to the Seine River near Paris, France (Bruchet, et al., 2005). Sulfamethoxazole was detected at a concentration of 202 ug/L in 1 of the 6 samples collected from the 3 sampling locations. The macrolide antibiotic roxithromycin was detected in 3 of the 6 samples, with a maximum detected concentration of 395 ug/L. Maximum concentrations of both were detected at the nearest downstream sampling point from a WWTP discharge. Additional analysis of a sample collected from the nearest downstream location to the WWTP also showed the presence of 4 additional antibiotics including erythromycin at 0.075 ug/L.

WWTP influent, effluent, and sludge collected during 2 sampling events from five WWTPs in Sweden were analyzed for 12 human use antibiotic/antimicrobial (Lindberg, et al., 2005). Doxycycline was detected in all 3 matrices, and at the highest concentration of any antibiotic detected (2.480 ug/L in an influent sample). The highest concentration of doxycycline detected in effluent (0.915 ug/L) was greater than the concentration detected in influent (0.333ug/L) from the same sampling event. Concentrations of doxycycline in sludge were below the quantitation level in all but 2 samples. Trimethoprim was detected

at the second highest concentration in influent (1300 ug/L), and was also detected in effluent and sludge. Similar to doxycycline, the effluent sample exhibiting the highest concentration (1.34 ug/L) for trimethoprim was greater than the influent sample from the same sampling event. The maximum detected concentrations of other antibiotic/antimicrobial surveyed (sulfamethoxazole, ciprofloxacin, ofloxacin, norfloxacin) were also detected in influent samples. All compounds were detected in every influent sample and most effluent and sludge samples, though in some cases (especially ofloxacin) below the quantitation limit. Detected concentrations of targeted antibiotic/antimicrobial in sludge samples ranged from 4.8 mg/kg (ciprofloxacin) to 0.1 mg/kg (norfloxacin and ofloxacin). Mass analysis of detected antibiotic/antimicrobial generally followed the order of sludge < influent < effluent with the exception of doxycycline which was not detected in 7 of 10 sludge samples. Sulfamethoxazole and trimethoprim were not detected in any sludge samples.

A similar investigation evaluated 10 human and veterinary antibiotics/antimicrobials among 26 pharmaceuticals, in samples of influent and effluent from 6 WWTPs in Italy (Castiglioni, et al., 2006). Observed concentrations in discrete samples were not provided; however population normalized WWTP influent and effluent loads were calculated. Only oxytetracycline and the veterinary antibiotic tilmicosin were not detected in any reported WWTP influent or effluent. Influent and effluent loads of ofloxacin were the highest of any detected antibiotic, and overall median removals were less than half of influent concentrations (57%). Loadings of ciprofloxacin and sulfamethoxazole were the second and third highest determined, respectively, with median removals for both of greater than 50%. However, median removals of all detected

antibiotic/antimicrobial were less than 40%. Loads in winter were greater than those measured in summer consistent with reported prescription patterns for the detected antibiotic/antimicrobial. Attenuation of the concentrations for detected antibiotic/antimicrobial in surface waters less than 1 km downstream from WWTP discharges varied from about 50% for spiramycin to less than 5% for sulfamethoxazole.

Evaluations of concentrations and loadings of macrolide and fluorquinolone antibiotics/antimicrobials in the Glatt River, 3 WWTPs, and in groundwater near Zurich, Switzerland indicated the presence of the macrolides erythromycin, clarithromycin, roxithromycin, tylosin, and spiramycin and the fluoroquinolones ciprofloxacin and norfloxacin (McCardle, et al., 2003; Giger, et al. 2003). About 90% of the quantified fluoroquinolones, ciprofloxacin and norfloxacin, were removed in the WWTP. Removals were largely associated with sorption to sludge, with residuals in the range of 1.4 to 2.4 mg/kg. However, no significant removal of antibiotic/antimicrobial in methanogenic sludge digesters was observed. Effluent concentrations of the fluoroquinolones detected ranged from 0.036 to 0.106 ug/L. In water samples collected from the Glatt River water downstream from the WWTP discharge, fluoroquinolone concentrations were less than 0.0019 ug/L with additional attenuation or dilution observed further downstream. Detected macrolide antibiotic concentrations showed a distribution similar to those predicted by reported therapeutic consumption, with clarithromycin being detected at up to 10 times the concentration of erythromycin and roxithromycin. Concentrations of macrolide antibiotics detected in samples collected during summer months were about double those detected in samples collected in winter. Macrolide antibiotics exhibited lower removals (about 20% on average) from treatment in WWTPs than

fluoroquinolones. However, no removal of erythromycin was observed. Residual concentrations of clarithromycin detected in water samples collected from the Glatt River downstream of WWTP discharges were detected in the range of 0.007 - 0.075 ug/L. No other macrolide antibiotic was detected in the downstream samples.

An analysis of 12 drinking-source water and finished drinking water samples from a facility in New Jersey detected 6 of 37 antibiotic/antimicrobial targeted (Stackleberg, et al., 2007). In source water samples, sulfamethazone was detected at the highest concentration (0.08 ug/L). Sulfamethazone was also the only targeted antibiotic detected in finished drinking water (0.01 ug/L), and was detected at equal frequencies (8%) in both source water and finished drinking water. Sulfamethoxazole was detected in source water at the second highest concentration (0.06 ug/L), and was the most frequently detected antibiotic (83%). Erythromycin and its common hydrolysis product erythromycin-H_2O were both detected in source water: the highest concentration of erythromycin and erythromycin-H_2O detected were 0.04 ug/L and 0.01 ug/L, respectively. However, erythromycin-H_2O was detected at a higher frequency (58%) than erythromycin (17%). Sulfadimethoxine was detected at a maximum concentration of 0.01 ug/L and was detected at the same frequency (17%) as erythromycin.

The presence of antibiotics/antimicrobials have also been investigated in samples of soils (Davis, et al., 2006), and aquaculture facilities and sediments (Jacobsen and Berglind, 1988; Kim and Carlson, 2007). Detections of antibiotics/antimicrobials in associated water samples were in the low to sub ug/L range, and were consistent with results for previously discussed investigations.

Evaluation of the runoff potential for antibiotics/antimicrobials from agricultural soils observed variable loss rates during a simulated rainfall from soils to which tetracycline, chlorotetracycline, sufathiazole, sulfamethazine, erythromycin, tylosin, and monensin had been applied (Davis, et al., 2006). The use of antibiotics/antimicrobials in aquaculture in Norway was shown to introduce oxytetracycline into sediments at active concentrations that persisted with a half life of about two and a half months (Jacobsen and Berglind, 1988).

Notwithstanding the low concentrations detected and the wide variability in analytical targets, the presence of antibiotics/antimicrobials in a wide array of environmental compartments is well established. The data suggest that the potential exists for ecological pressure favoring the selection of antibiotic resistant bacterial phenotypes resulting from chronic exposure in the environment. Moreover, the detection of several antibiotic/antimicrobial in most instances implies further pressure favoring multi-antibiotic resistant bacterial phenotypes that could be of concern for public health.

Antibiotic Resistant Bacteria and Genes in the Environment

The presence of antibiotic resistant bacteria in the environment including soils, manures, human and agricultural wastewater, freshwater, marine water, and in aquaculutural waters and fish have been the subject of widespread investigation over the past 3 decades. Groundbreaking work performed in the U.S. established the presence and transmissibility of antibiotic resistance in lactose fermenting bacteria isolated from sewage influent and effluent collected from five urban area WWTPs in Alabama (Sturtevant, et al., 1969). Low numbers of lactose fermenting bacteria (0.01% to 1% of total lactose

fermenting colonies, 2x 10^3 - $>10^6$ colonies/ml) isolated were resistant or multi resistant to streptomycin or tetracycline, with 10 – 100 fold less resistance observed for chloramphenicol. Multi-antibiotic resistances to various combinations of other antibiotic/antimicrobial including ampicillin, chloramphenicol, cephalothin, dihydrostreptomycin, tetracycline, kanamycin, and nalidixic acid were also reported; no isolate showed resistance to gentimicin or colistin. Antibiotic resistance patterns observed were very similar in influent and effluent derived colonies. Although the frequency of observed antibiotic resistance in isolated bacteria was low, *in vitro* transmittal of multiple antibiotic resistance from sewage isolated host lactose fermenters to an antibiotic sensitive *E. coli* strain was observed in about 50% of the isolated lactose fermenting bacteria.

A later investigation in the same laboratory supported the previous work (Sturtevant, et al., 1971). Generally low numbers ($\leq$6% of total colonies) of total coliforms and fecal coliforms isolated from duplicate samples of WWTP influent from 5 facilities in Alabama were resistant to both streptomycin and tetracycline. Two of the 10 samples assayed, however, exhibited higher frequencies of resistance to streptomycin and tetracycline (10% and 70% of total colonies, respectively). Colonies exhibiting resistance to streptomycin, tetracycline and ampicillin were also observed, but at a lower frequency ($\leq$ 3% of total colonies). About half of the antibiotic resistant colonies were capable of transmission of the observed multiple resistances to an antibiotic sensitive receptor strain of *E. coli*.

Research in Great Britain demonstrated the presence and transmissibility of antibiotic resistance in coliforms isolated from rivers and coastal marine waters (Smith, H. W., 1970; Smith, H. W., 1971). Fresh water samples were collected from 98 locations on

54 rivers (Smith, H.W., 1970). Variability was observed between sampling locations in the incidence of coliforms resistant to the antibiotic/antimicrobial chloramphenicol, ampicillin, tetracycline, neomycin, and streptomycin, but consistency was generally observed for multiple samples collected at different times from the same location. A higher incidence of antibiotic resistant coliforms was observed in samples collected in urban, sewage impacted, areas than in samples collected in rural areas, and sewage treatment plant influents contained higher concentrations of antibiotic resistant coliforms than effluents. Most of the resistant strains were further characterized as *E. coli*. Antibiotic resistance was shown to be transmissible to a pathogenic *E. coli* strain and to *Salmonella typhimurium.* Subsequently, coastal marine water samples were collected from 10 locations each along stretches of 15 randomly selected beaches used for recreational bathing (Smith, H.W., 1971). Isolated *E. coli* demonstrated resistance to one or more of the antibiotic/antimicrobial chloramphenicol, ampicillin, tetracycline, neomycin, and streptomycin. Transmissibility of resistance to a pathogenic *E. coli* strain and to *Salmonella typhimurium* was observed in the majority of samples that were isolated based on chloramphenicol resistance. Antibiotic/antimicrobial resistant bacteria dissipated at room temperature to non detectable levels after 5-days. Comparison of results from the 2 Smith studies reveals that the incidence and transmissibility of ampicillin resistance was higher in polluted fresh water than in sewage impacted coastal marine water. Additionally, a study performed in Ireland demonstrated that transmissible ampicillin resistance factors were not influenced by dilution of raw sewage in marine water (Smith, P. R., et al., 1974).

A study of wastewaters and sewage from two hospitals in Pietermaritzburg, South Africa demonstrated the presence of antibiotic resistance in total coliforms to ampicillin,

cephaloridine, chloramphenicol, kanamycin, neomycin, oxytetracycline, streptomycin, sulfonamide, and tetracycline (Grabow and Prozesky, 1973). Total coliform concentrations in sewage were about double those found in hospital wastewater, but antibiotic resistant colonies were more numerous in hospital wastewater isolates, with the exception of colonies resistant to cephaloridine. *In vitro* transmittal of antibiotic resistance, denoted as resistance (R+) factors, from isolated antibiotic resistant total coliform colonies to a clinical nalidixic acid resistant strain of *E. coli* and *S. typhi*, showed that the R+ factor could be transferred. The most frequently observed transmittals of the R+ factor were for ampicillin, chloramphenicol, and tetracycline, with higher frequency of transmission observed for *E. coli* than *S. typhi* as recipient. Transfer of multiple antibiotic resistance was also observed. Co-resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline were the most abundant observed in both hospital and sewage isolates, as well as the pattern of co-resistance to the greatest number of tested antibiotic/antimicrobial. While non-transferable antibiotic resistance was observed, it was lower than the observed transferable resistance for each antibiotic studied.

A study examining the occurrence of multiple-antibiotic resistant bacteria among 2,653 standard plate count (SPC) bacteria isolates from 7 finished drinking waters in Oregon revealed that 33.9 % of samples contained bacteria resistant to at least 2 of the 5 antibiotics (sulfonamide, streptomycin, kanamycin, chloramphenicol, tetracycline) screened (Armstrong, et al., 1981). Analysis of 535 SPC isolates from 12 corresponding source waters indicated a reduced frequency of the multiple-antibiotic resistance phenotypes, suggesting that elements of treatment and residence in the distribution systems such as in biofilms, may enhance expression of multiple-antibiotic resistant

phenotypes. Additional characterization of the multiple-antibiotic resistant strains identified 60% to be Gram-negative rods, and 40% to be Gram-positive cocci and rods, including *Staphylococcus aureus.*

The ability of genetic elements conferring resistance to transfer from WWTP influent and effluent derived coliforms (78% identified as *E. coli)* resistant to chloramphenicol, streptomycin, and tetracycline to an isolated antibiotic sensitive *E. coli* strain *in situ* in membrane diffusion chambers was demonstrated in Florida (Altherr and Kasweck, 1982). In the isolated antibiotic resistant *E. coli*, resistance to streptomycin was most abundant (8.3 – 13.2%), followed by tetracycline resistance (5.0 – 9.3%), and chloramphenicol resistance (0.6 – 1.7%). Resistance of some isolates to sulfathiazole was also observed. *In situ*, resistance was transferred in a temperature dependent manner with optimal transfer at 25^{o}C. Transfer of antibiotic resistance was associated with identification of a 60 mega Dalton plasmid in transconjugants.

A survey of antibiotic resistant bacteria in 22 urban U.S. rivers reported that between 3.9% and 73% of isolated bacteria were ampicillin resistant (Ash, et al., 2002). The magnitude of ampicillin resistance, as determined by the Minimum Inhibitory Concentration (MIC) assay was >256 ug/ml for 98% of the resistant isolates, most of that were identified as Gram-negative strains. Multiple antibiotic resistance to other beta lactam antibiotic/antimicrobial was also observed, with resistance to cefalothin being the most prevalent, followed by cefotaxime, cefazidime, imipenim, and amoxicillin-clavalanic acid. About 20-30% of the cefotaxime resistant bacteria were determined to be Gram-positive organisms. Ampicillin resistance genes were carried on 70% of plasmids isolated from Gram-negative strains; 97% of these also carried another resistance trait.

Some similarities and differences were observed in results from samples collected upstream and downstream of a Pamplona, Spain WWTP discharge to the Arga River. Multiple antibiotic resistances were detected in isolated Gram-negative bacteria, which were non-transferable and therefore were suggested to be chromosomally mediated (Goni-Urriza, et al., 2000). More than half (58%) of the enterobacteria isolated did not demonstrate antibiotic resistance, but three quarters of the *Aeromonas* stains showed resistance to one or more antibiotic/antimicrobial. Antibiotic resistance increased downstream from the WWTP discharge for both strains, with the incidence of resistances observed, except for nalidixic acid, being about equal. Beta lactam and tetracycline resistances showed the greatest magnitude of increase in frequency for both enterobacteria and *Aeromonas* downstream of the WWTP discharge, from 5% or less upstream to over 20% each downstream of the WWTP discharge. The peak levels of resistance to nalidixic acid were observed in upstream samples and the furthest downstream samples, suggesting a source(s) other than the WWTP discharge.

The resistance of fecal coliforms to ampicillin, chloramphenicol, unidentified sulfonamides, tetracycline, and streptomycin were investigated in fecal coliform isolates from 14 water samples in Finland representing WWTP influent and effluent, livestock production effluent, polluted rivers and lakes, and the Baltic Sea (Niemi, et al., 1983). Resistance to one or more antibiotic/antimicrobial was observed in 31% of the fecal coliform isolates, and multiple resistance was observed in 11% of the isolates. Resistance to ampicillin was most frequently observed (20%), followed by tetracycline and sulfonamides (12% each), cloramphenicol (4%), and streptomycin (3%).

Analysis of the antibiotic susceptibility of coliform bacteria isolated from the

Sumjin River in Korea showed antibiotic resistance in over half (53.6%) of the 1,400 isolates analyzed (Park, et al., 2003). Antibiotics evaluated represented the beta lactams (ampicillin, carbenicillin, cefoxitin, cefotaxim), aminoglycosides (gentamycin, kanamycin, streptomycin, tobramycin), quinolones (nalidixic acid, ciprofloxacin), sulfonamides (sulfamethoxazole), and chloramphenicol, tetracycline, and trimethoprim. The decreasing order and magnitude of resistances observed in isolated colonies were sulfamethoxazole (61.3% of resistant colonies), aminoglycosides (60% of resistant colonies), beta lactams (57.9% of resistant colonies), tetracycline (33.3% of resistant colonies), trimethoprim (28% of resistant colonies), quinolones (21.3% of resistant colonies), and chloramphenicol (16% of resistant colonies). Analysis of class I integrons in 150 of the antibiotic resistant isolates revealed the presence of the *int-1* gene in 36 of the isolates, with 30 of these identified as residing in *E. coli*. Seven distinct antibiotic resistance-associated genes were identified in the integrons including two coding for aminoglycoside and trimethoprim resistance (*dfrA* and *aaa)*. However, over 60% of the integrons were determined to be incomplete or non-functional.

Variable multi-antibiotic resistance was observed in *E. coli* isolated from 5 locations along the Ganga River in India, which receives inputs of untreated sewage and other wastes despite its use as a water supply for drinking and irrigation water (Ram, et al., 2007). Of the 15 isolates investigated from each location almost all showed reduced susceptibility or clinically defined resistance to multiple antibiotic/antimicrobial. Resistance of the isolated *E. coli* to the cephalosporin cephalothin was most prevalent, with 29 isolates exhibiting resistance, followed by piperazine (19), amoxicillin (16), and tetracycline (15). All isolates from one site showed resistance to ampicillin, amoxicillin,

and piperacillin. Fourteen of the isolates from this location also showed resistance to cephalothin, and a lesser number to tetracycline (8), and cipropfloxacin (4). Moreover, shiga toxin or enterotoxin producing genes were determined to be present in all of the isolates assayed.

Listeria species were observed to be reduced by 69% to 99% in a WWTP discharging to the River Sar in Spain (Combarro, et al., 1997). However, *Listeria* species increased in receiving waters downstream of the WWTP, with the pathogen *L. monocytogenes* being most prevalent in the downstream samples. Moreover, *Listeria* were not reduced from numbers observed in the WWTP effluent over 25 Km downstream. All *Listeria* strains isolated from the river were resistant to nalidixic acid, with resistance also observed for aztreonam (94.4%), moxalactam (56.9%), cefotaxime (46.3%), nitrofurantoin (38.1%), and norfloxacin (6.3%). Resistances of less than 5% to tetracycline, streptomycin, kanamycin, tobramycin, mezlocillin, erythromycin and chloramphenicol were also observed. Over half of *Listeria* strains isolated from all sources exhibited multi-resistance to five or more antibiotic/antimicrobial (influent 61.8%; effluent 76.6%; Sar River water 52.9%).

The presence of antibiotic resistant bacterial strains, most notably pathogens or pathogen indicators, has led to evaluation of antibiotic resistant genes and the potential for transfer to non-resistant bacteria in the environment (Pruden, et al., 2006; Muela, et al., 1994; Pei, et al., 2007; Roe, et al., 2003; Szczepanowski, et al., 2007).

Results obtained from an evaluation of antibiotic resistance containing plasmid transfer efficiency between *E. coli* species from the River Burtron in Spain showed that the growth phase and cell length but not the observed physiologic state of donors affected

plasmid transfer (Muela, et al., 1994). The efficiency of transfer remained relatively stable during the same period for recipients. Exponential growth of donors disfavored transfer, which was maximal during the initial hours of growth and in the stationary growth phase. The number of transconjugants formed, however, steadily declined over time to non detectable levels after 30 hours of incubation in dark systems, and declined at an enhanced rate to non detectable levels after 24 hours in illuminated systems.

Twelve class I integrons encoding 19 different antibiotic resistance cassette arrays containing 21 different resistance gene cassettes were identified in 97 different multi-antibiotic resistance plasmids isolated from WWTP influent, activated sludge, and effluent in Germany (Tennstedt, et al., 2003). Multi-resistance profiles included various combinations of up to 4 antibiotic/antimicrobial including ampicillin, cefaclor, chloramphenicol, gentamicin, kanamycin, streptomycin, tobramycin, and trimethoprim. Among the cassette arrays identified, several had been described in human and veterinary isolates, and several were new. Cassettes coding for antibiotic resistance mechanisms included aminoglycoside modifiers, beta lactamase inhibitors, dihydrofolate reductases, chloramphenicol resistance proteins, and exporters.

In water samples collected from 16 coastal marine locations in Oahu, Hawaii, both methicillin resistance and toxin producing genes were identified in *S. aureus* isolates (Stotts, et al., 2007). Two genes associated with methicillin resistance (mecA and femA) were associated with both methicillin sensitive strains (5 isolates), and resistant strains (8 isolates), suggesting an unidentified determinant for methicillin resistance in some strains of *S. aureus.*

The presence of antibiotic/antimicrobial in the environment may favor selectivity

for antibiotic resistant bacteria. The observed presence of antibiotic resistant bacteria in the environment suggests that vector derived or naked DNA harboring antibiotic resistance genes may be transferring resistance factors between bacteria. Recently, DNA conveying resistance determinants was reported as free material and in bacteriophage isolates cultured from environmental samples, (Pei, et al., 2007; Pruden, et al., 2006; Szczepanowski, et al., 2007), as well as in bacteriophages (Muniesa, et al., 2004).

Coliphages containing determinants for expression of two beta lactamases were isolated from influent samples collected from 5 WWTPs, and pig, cattle, and poultry abattoir wastewaters, which also contained high concentrations of fecal coliforms. (Muniesa, et al. 2004). The high likelihood of transduction was indicated; however, characterization of the mechanism of transduction and presence of determinants for resistance to other antibiotic/antimicrobial was not performed.

Water samples and sediment collected from the Poudre River in Colorado containing Compounds for tetracycline and sulfonamides indicated that genetic material conveying identified antibiotic resistance can exist outside of a host organism (Pruden, et al., 2007). Genes for tetracycline resistance were present in “pristine” upstream samples, but genes for sulfonamides were not. The highest concentrations of genes for both tetracycline and sulfonamide resistance were detected in samples collected from a dairy wastewater lagoon that discharged its effluent to the Poudre River. Genes for resistance to both compounds were also detected in samples collected downstream from the dairy lagoon discharge, downstream of a WWTP discharge, and in an agricultural drainage ditch. While the presence of the targeted resistance genes was spatially consistent with expected inputs of antibiotic resistant genes (i.e. in the dairy lagoon and downstream of

discharges), flow weighted and temporal associations were inconclusive. Genes coding for resistance to tetracycline and sulfonamides were also detected in wastewater recycling effluent and drinking water treatment plant product. The transmissibility of the isolated genes does not appear to have been investigated since no results were reported.

General Mechanisms of Resistance and Their Conveyance

Antibiotic resistance was likely first described in 1956 in clinical isolates from Japan, within a few years after various antibiotics were introduced into widespread use (Davies, 1995). Since that time, antibiotic resistance in Gram-positive and Gram-negative bacteria to essentially every antibiotic has been identified. Moreover, widespread resistance to multiple antibiotic/antimicrobial is of increasing clinical concern.

Three broad mechanisms convey post-expression antibiotic resistance at the molecular level: (1) activation of antibiotic inactivation enzymes; (2) activation of genes that confer resistance to the drug's target; and (3) activation of genes that restrict drug entry into the cell, or which actively transport drugs from the cell thus preventing their accumulation (Hawkey, 1998; Levy, 1992). Examples include transformation of the antibiotic subsequent to entering the cell (e.g., for penicillin: beta lactamases, penicillin binding proteins); reduced influx into the cell (e.g., for fluoroquinolones and aminoglycosides), or enhancing efflux from the cell (e.g., for tetracyclines); alterations in the primary site of action (e.g., for penicillins); production of an alternative intracellular target (e.g., for methicillin) (Hawkey, 1986). Resistance may also occur via mechanisms that effect elements of transcription or translation, and these may be coincident with other mechanisms such as enhanced efflux for the same antibiotic, as for fluoroquinolones

(Woodford, 2005).

Genetic elements that confer the resistant trait can be acquired via conjugatory plasmid transfer, infection by phages, and uptake from the environment, or result from chromosomal mutations. Transfer of genetic elements may proceed by at least three mechanisms: uptake of DNA from the environment and bacterial cell transformation; transduction by phages; and conjugation (George and Levy, 1983, Madigan, et al., 2003; McMurray, et al., 1980; Ochman, et al., 2000).

Uptake from the environment and transformation is mediated in nature by favorable ecological conditions and expression of specific proteins required for capture and cross membrane transport of naked DNA (Rudin, et al., 1974, Madigan, et al., 2003). Transformation conferring antibiotic resistance traits has been demonstrated in both Gram-negative and Gram-positive bacteria, though at low frequency (Cohen, et al., 1972; Rudin, et al.,1997).

Phage mediated transfer and subsequent transformation occurs when a bacteriophage injects DNA into a bacterial cell thereby introducing the resistance gene which is later expressed in the infected bacteria (Madigan, et al., 2003). Identification of phage-like particles with concurrent expression of resistance to beta lactam antibiotics and antimicrobials suggested phage mediated acquisition of resistance in Gram-positive *Rhodococcus equi* isolated from AIDS patients (Nordmann, et al., 1972). Phage mediated transfer of resistance for novobiocin was demonstrated *in vitro* utilizing a clinically isolated *Staphylococcus aureus* strain. High efficiency transfer of coliphage genes with 20% viability in receptor *E. coli* was shown to occur *in vitro* indicating that transduction could be a major mechanism for introducing diversity into bacterial populations (Kenzaka,

et al., 2007).

Plasmid gene transfer and subsequent transduction occurs via sexual conjugations between bacteria of like or different strains, or more rarely by uptake of DNA freed into the media during cell lysis (Madigan, et al., 2003). Transposons and integrons are relatively short transducable genetic sequences that may contain specific resistance genes. Integrons are self regulating units that have the capacity to capture other genes and integrate them into plasmids or the host genome. Multiple adjacent genes contained in integrons are known as "cassettes", and many of those identified convey resistance to multiple antibiotic/antimicrobial. Integron mediated transfer of antibiotic resistance is perhaps the principal mechanism of multi-antibiotic resistance spread among Gram-negative bacteria, and has also been recognized as important for Gram-positive bacteria (Nandi, et al., 2004; Shi, et al., 2006). Chromosomal gene cassettes conferring multi-antibiotic resistance phenotypes in Gram-positive bacteria have also been described (Shittu, et al., 2007, Shi, et al. 2006).

Among prevalent mechanisms of general antibiotic resistance, energy dependent efflux has been demonstrated for a wide array of structurally and functionally dissimilar antibiotic/antimicrobial including tetracyclines, fluoroquinolones, and macrolides (Levy, 1992). A multi-antibiotic resistance (MAR) regulon has been described in *E. coli* that conveys resistance to an array of antibiotic/antimicrobial including tetracyclines, fluoroquinolones, and chloramphenicol in energy dependent influx inhibition and enhanced efflux. Alternatively, elevated mutation rates were shown to influence vancomycin resistance in *S. aureus* without increasing resistance to a wide array of other antibiotic/antimicrobial (Schaff, et al., 2002). Furthermore, the resistance of *S. aureus* to

vancomycin was shown to be related to cell wall thickening and a subsequent "clogging" of the cell wall with vancomycin itself, thereby inhibiting entry of the antibiotic into the bacterial cell (Cui, et al., 2006).

The presence in bacteria of multiple antibiotic resistance determinants and the horizontal transfer of the multi-antibiotic resistance trait between bacteria is believed to be enhancing development of multi-antibiotic resistance within and across bacterial species (Tenover, 2001). Integrons carrying multi-antibiotic resistance cassettes can result in resistance to an array of similar or dissimilar antibiotic/antimicrobial with equally varied mechanisms of resistance.

Exposure of *E. coli* to sub-inhibitory levels of tetracycline was shown to result in expression of four different plasmid associated determinants that regulate active efflux (McMurray, et al., 1980). Similarly, sub-inhibitory levels (1 - 5 ng/L) of antibiotic/antimicrobial were demonstrated to convey selective pressure for expression of antibiotic resistance via activation of chromosomal genes regulating efflux of tetracycline, chloramphenicol, beta lactams, puromycin, and nalidixic acid in *E. coli*. (George and Levy, 1983). Resistance was achieved in 50 - 200 generations of growth, and was reversed within 100 generations of growth when antibiotics/antimicrobials were removed from the growth medium.

In *Staphylococcus aureus*, transduction and transformation appear to be predominant mechanisms conveying antibiotic resistance determinants (Lyon and Scurray, 1987). *In vitro*, calcium ions are required for both transduction and transformation, and transformation and transformational competence, is maximal prior to exponential growth due to absence of extracellular deoxyribonuclease during this interval of the growth cycle.

Transformational competence is also dependent on the presence of a superinfecting phage or induced prophage. Spontaneous mutation that conveys resistance phenotypes occurs at a relatively low rate. Transformation has been shown to transfer plasmid antibiotic resistance determinants at high frequency, and chromosomal antibiotic resistance determinants at a relatively lower frequency. A process of phage-mediated conjugation has been suggested in which mutant phage enhances cell-cell contact. Moreover, sub-inhibitory concentrations of beta lactam antibiotic/antimicrobial were observed to increase donor – recipient cell aggregation by 2 – 3 orders of magnitude, though other antibiotic/antimicrobial including vancomycin had no effect (Barr, et al. 1986).

Three principal mechanisms of resistance to penicillin and other beta lactam antibiotics have been characterized in *S. aureus*: beta lactamase hydrolysis of the beta lactam ring; reduced affinity of penicillin binding proteins for the antibiotic; and tolerance to the bactericidal effect (Lyon and Scurray, 1987). Characterized plasmids conveying resistance genes to beta lactamases in *S. aureus* ubiquitously convey resistance to metals, disinfectants, and/or other classes of antibiotic/antimicrobial. *Staphylococcus aureus* strains carrying both plasmid associated and independent chromosomally encoded transposons have also been isolated. Methicillin resistance results from reduced affinity of this and analogous beta lactam antibiotic/antimicrobial for penicillin binding proteins, characteristics coded by the *mec* family of related gene cassettes (Woodford, 2005).

Reduced affinity between ribosomes and macrolide antibiotics due to a single alteration of an adenine nucleotide residue in the 23S ribosome, which induces a conformational shift in the ribosome, results in the resistance phenotypes in *S. aureus* for this class of antibiotic/antimicrobial (Barr, et al. 1986; Woodford, 2005). Erythromycin

induces methylation of the target adenine, resulting in the expression of plasmid or chromosomally encoded resistance.

Tetracycline resistance in *S. aureus* is conveyed by plasmid and chromosomal genes encoding for decreased uptake into the cell or, more prevalently, increased efflux from the cell (Barr, et al., 1986; Woodford, 2005). Chromosomal, but not plasmid, coded tetracycline resistance is inducible, and can also convey resistance to minocylcine. Ribosomal protection systems also appear to be associated with tetracycline resistance, but the mechanism(s) is not as yet understood

Protocol and Rational

The research presented herein focused on the investigation of environmental water samples to induce antibiotic resistance in a model microbial system. Based on studies described earlier that demonstrated the widespread occurrence of antibiotic/antimicrobial compounds and antibiotic/antimicrobial resistant bacterial strains in environmental waters and wastewaters, it was hypothesized that selective pressure and/or genetic factors that may convey antibiotic/antimicrobial resistant traits in non-resistant bacteria may be present. The hypothesis was tested *in vitro* using filter sterilized environmental water/wastewater samples, and a sensitive Gram-positive bacterial strain. Specifically, the experimental element of this study employed *Staphylococcus aureus* ATCC 9144 (*SA*), a strain known to be sensitive to a wide spectrum of antibiotic classes including the beta lactams, macrolides, aminoglycosides, tetracyclines, glycopeptides, fluoroquinolones, and sulfonamides. This *SA* strain has been used in other investigations concerning the ability of various natural and synthetic compounds to induce antibiotic resistance (Bordas, et al.,

1997; Brady and Katz, 1992). The broad inherent sensitivity of *SA* 9144 therefore potentially renders it a useful tool to assess environmental waters for their ability to induce antibiotic resistance.

Evaluating the effect on the model system of exposure to ambient environmental waters, treated wastewater influents and treated effluents, and pharmaceutical manufacturing effluents would demonstrate the potential for these waters to induce antibiotic resistance in native bacterial populations. Moreover, the results could suggest that the model system may be applicable as an indicator of the potential for an environmental water to induce antibiotic resistance in native bacterial populations.

Materials and Methods

Sample Collection

Samples were collected directly into certified clean sample containers (amber borosilicate glass or HDPE cubitainers), and placed on ice for transport to the laboratory where they were stored at 4^{o}C until preparation. Samples collected by U.S. Environmental Protection Agency (U.S. EPA) sampling personnel at facilities undergoing regulatory compliance inspections were labeled with anonymous codes, which allowed identification of the facility type (sewage treatment plant, pharmaceutical plant), sample type (influent, effluent), and the date of collection. Split samples (i.e. co-located samples) collected in the field from the same location and within minutes of each other, were available from most sampling events. Surface water samples collected by U.S. EPA sampling personnel were given identifiers as to the water body, location in the waterbody (e.g., upstream, downstream) and the date of collection. All other samples were collected

by the author. Locations were noted and in some cases identified using a global positioning system (GPS). Samples were labeled with a coded sample location or station identifier, and the date of collection. The time of collection, general ambient conditions, and other relevant information regarding the location were recorded.

Sample Preparation

Environmental samples were filter sterilized under constant low pressure without any manual disturbance, so as not to disrupt bacteria potentially present in the sample, through a 0.2 um absolute pore size glass fiber filter (Millipore) with sterile reservoir. In some cases, aseptic transfer to disposable sterile 50 ml plastic tubes was performed. Appropriate volumes, usually 1 ml, were aseptically transferred from the 50 ml sample and were then used for exposures. Sewage influent samples that would not pass through the 0.2 um filter were pre-filtered, under low pressure with no manual disturbance, through a 0.45 um absolute pore size glass fiber filter prior to filtering under low pressure with no manual disturbance, through the 0.2 um filter. After filtration, samples were stored at 4^{o}C unless under study.

Media and Reagents

Trypticase Soy Agar (TSA), Difco Lot # 414774B, and Trypticase Soy Broth (TSB), Difco Lot # 4112355, were prepared according to the manufacturers directions in deionized water, except for TSB medium used for exposures that were prepared at half (50%) strength to minimize any potential interference. Portions of medium were added to borosilicate culture tubes, capped, and sterilized by autoclaving (20 min @ 15 psi, 121^{o}C).

The TSA culture plates and slants were prepared by directly pouring autoclaved (sterile) medium into sterile petri plates and tubes, respectively. Sterile TSA slants and petri plates were stored for relatively short periods to ensure that the media were not compromised. Tubes or petri plates were prepared and used as needed. Tubes exhibiting any discoloration or growth during storage were discarded.

The following antibiotic/antimicrobial were used in this study: ampicillin (Amp); erythromycin (Ery); kanamycin (Kan); norfloxacin (Nor); sulfamethoxazole (Sul); tetracycline (Tet); vancomycin (Van); and methicillin (Met). The abbreviations for these compounds will be used in all tables and throughout the text without further description.

Antibiotics were prepared as follows:

Ampicillin (Sigma Lot # 77H0408): 12.8 mg was dissolved in 100 ml distilled deionized water. The final concentration was 128 ug/ml.

Erythromycin (Sigma Lot # 107H0644, 966 ug active ingredient/mg): 13.24 mg was dissolved in a small volume of methanol then diluted to 100 ml in deionized water. The final concentration of active ingredient was 128 ug/ml.

Kanamycin monosulfate (Sigma Lot # 101H01295, 783 ug active ingredient/mg): 16.35 mg was dissolved in 100 ml deionized water. The final concentration of active ingredient was 128 ug/ml.

Methicillin (Sigma): 12.8 mg was dissolved in 100 ml distilled deionized water. The final concentration of active ingredient was 128 ug/ml.

Norfloxacin (Sigma Lot # 83H0921): 12.8 mg was dissolved in a small volume of acetone

and diluted to 100 ml with deionized water. The final concentration of active ingredient was 128 ug/ml.

Tetracycline (Sigma, 987 ug active ingredient/mg): 12.97 mg dissolved in a small volume of 0.1N HCl and diluted to 100 ml with deionized water. The final concentration of active ingredient was 128 ug/ml.

Sulfamethoxazole (Sigma): 12.8, 25.6, or 51.2 mg was dissolved in a small volume of acetone and diluted to 100 ml with deionized water. The final concentrations were 128 ug/ml, 256 ug/ml, 512 ug/ml, respectively.

Vancomycin: 12.8 mg was dissolved in 100 ml distilled deionized water. The final concentration was 128 ug/ml.

All antibiotic stock solutions were filter sterilized (0.2 um absolute pore size, Millipore) immediately following preparation. Stock solutions were either stored at 4^{o}C for up to 30 days, or frozen for later use. Freezing was found not to diminish activity during the course of the study.

Staphylococcus aureus

American Type Culture Collection (ATCC) *Staphylococcus aureus* strain 9144 Lot # 1158720 (*SA*) was prepared for use as follows. A fresh loop of lyophilized *SA* obtained from ATCC was inoculated into TSB, mixed using a vortex mixer for 30 seconds, and incubated at 37^{o}C with shaking at 250 rpm for 12 - 24 hours. A loop of the resulting culture was streaked onto TSA plates followed by incubation at 37^{o} C overnight. Individual colonies were picked from the TSA plates and inoculated into TSB, mixed well,

and incubated overnight at 37°C with shaking at 250 rpm. Resulting cultures were streaked onto TSA slants, incubated at 37°C for 12 – 24 hours, and stored at 4° C until use. The day prior to use, a small amount of bacteria was aseptically transferred with a sterile loop from the slant to 1 ml of TSB, incubated at 37°C with shaking at 250 rpm for 12 – 24 hours. *Staphylococcus aureus* was then inoculated (10 ul) directly to exposure tubes. Slant cultures were stored for up to 3 weeks at 4°C. Fresh cultures were prepared from slants by repeating the procedure used for the initial lyophilized *SA*.

Exposures

All procedures were performed aseptically in a biological laboratory hood. Exposures of *SA* to evaluate the ability of the introduced environmental sample to induce antibiotic resistance were performed as follows. Sterile sample jars containing the filter sterilized environmental samples were manually shaken for 30 seconds immediately prior to withdrawing a sample. A 10 ml sterile borosilicate culture tube containing 1 ml of 50% TSB received 1 ml of environmental sample for each exposure. A 10 ul loop of *SA* was inoculated into each tube. Tubes were mixed using a vortex mixer and incubated at 37°C with shaking at 250 rpm for 16 – 24 hrs. On subsequent days throughout the duration of exposures, the procedure was replicated, with transfer of a 10 ul loop of the exposure culture inoculated the previous day. The procedure was repeated for 10-days unless stated otherwise. Following the exposure, 10 ul of each sample was either inoculated to 100% TSB and incubated at 37°C with shaking at 250 rpm for the MIC assay the following day, or inoculated to a TSA slant, incubated at 37°C for 16 – 24 hours, and stored up to 3 weeks at 4° C. For use, a small amount (1 ul) was collected from the TSA

slant culture with a sterile loop, inoculated into 100% TSB, mixed using a vortex mixer, and incubated at 37^{o}C with shaking at 250 rpm for the MIC assay the following day.

Control samples consisting of filter sterilized 0.85% NaCl (sterile saline) were exposed to SA and with each batch of environmental water samples, and treated identically to environmental water samples. Briefly, sterile saline (1 ml) in 1ml 50% TSB were incubated for the 10-day period, unless otherwise indicated. Post exposure handling of the control sample was also identical to the batch of environmental water samples for which the control sample was used as a baseline.

Minimum Inhibitory Concentration (MIC) Assay

The MIC assay was performed in the manner reported previously (Bordas, et al., 1997; Kleiner, et al., 2007). Exposure cultures from environmental water samples and the associated control sample (sterile saline) were grown for 16 - 24 hours in 100% TSB, and were serially diluted in sterile 0.85% NaCl (physiological saline) from an estimated starting concentration of 10^9 CFU/ml to a final concentration of approximately 10^5 CFU/ml. The diluted cultures (0.2 ml) were inoculated into 19.8 ml of TSB in a 50 ml borosilicate screw top culture tube, mixed using a vortex mixer, and carefully poured into a sterile multi-channel pipetter reservoir, for an assay concentration of about 10^3 CFU/ml.

MIC's were determined in 96-well factory sterilized culture plates (Corning 3595, 12 rows, 8 columns) as follows. An aliquot (125 ul) of one of the suite of antibiotic/antimicrobial was added to the first well of the first column of the 96 well culture plate (MIC assay plate). The procedure was repeated in each column of the MIC assay plate until the first well of each column, with the exception of the plate control as

described below, was charged with antibiotic/antimicrobial. Each MIC assay plate also contained a plate control (no antibiotic/antimicrobial), consisting of 125 ul sterile saline, which was treated identically to columns containing antibiotic/antimicrobial, to ensure consistency of *SA* growth in each row of the assay. Using a multi channel pipetter, a portion of the *SA* culture (125 ul) which had been exposed to the environmental water sample of interest or the control saline, was taken from the 20 ml reservoir and transferred sequentially to each well of each row of the MIC assay plate, beginning from the wells furthest from the wells charged with antibiotics/antimicrobials and control saline, proceeding toward the culture plate row containing the highest concentration of antibiotics/antimicrobials and the control saline well. An equal volume and concentration of *SA* was consequently added to each well of the MIC assay plate. Immediately upon introduction of *SA* culture previously exposed to environmental or control saline to the first well (that containing antibiotic/antimicrobial) the contents of the wells were rapidly mixed by pipeting up and down four times with the multi channel pipetter. The mixed suspension of *SA* and antibiotic/antimicrobial was then transferred to the next row of the MIC assay plate, mixed as for the initial portions, and sequentially transferred again until the last row on the plate was reached. The excess 125 ul in the last row was discarded to maintain equal volumes in each well on the MIC assay plate. The procedure results in a 2-fold serial dilution in each well. MIC assay plates were immediately covered, and incubated at 37^{o}C for 16 to 24 hours.

For the MIC assay, one or more, as necessary to include the full suite of antibiotic/antimicrobial evaluated, 96 well assay plates were devoted to each exposure culture. Likewise, MIC assay plates containing each of the antibiotic/antimicrobial

assayed were devoted to the *SA* exposed sample exposed to sterile saline over the duration of the exposure element of each experiment.

Following incubation, absorbance at 620nm was determined in each well using a Sunrise$_{TM}$ automated plate reader. Results for each plate were printed, and the results analyzed as described below.

Split samples (i.e. co-located samples) collected in the field from the same location and within minutes of each other, were available from most sampling events, were also exposed to SA and assayed. Each exposure sample batch and MIC assay contained at least one sample that was exposed to sterile saline and was otherwise processed identically to and within the same batch as environmental water samples. At least one exposure sample was also assayed for its MIC for at least one of the panel of antibiotics/antimicrobials in duplicate to evaluate the precision of the MIC assay (assay duplicates). Each 96 well assay plate contained an MIC assay control sample (sterile saline) to assess the presence/absence and relative uniformity of *SA* growth in each row of the MIC assay plate when no antibiotic/antimicrobial was present. The absorbance of samples was randomly measured in duplicate by the plate reader to ensure precision of the instrument.

Data Analysis

Data were analyzed using a standard protocol for determining the MIC. Growth inhibition in each well of the 96-well culture plate was determined from the absorbance measurement. An increase in absorbance greater than 2 times that of the previous well was used as the marker for reduced inhibition of growth, and the concentration of

antibiotic in the well just previous was determined as the MIC concentration. Data were also evaluated qualitatively by scoring the quantitative results as either positive (+) indicating an increase in antibiotic resistance (MIC) of greater than two wells in antibiotic concentration versus the related exposure control sample (sterile saline), or negative (-) indicating no change or a reduction of greater than one well in antibiotic concentration (MIC) versus the exposure control sample. In the rare cases where the exposure control sample was assayed for its MIC in duplicate and MIC values for a given antibiotic/antimicrobial were not identical, the more conservative estimate (i.e. higher value for an increase in MIC, lower value for decrease in MIC) was used in determining the MIC for the environmental water exposed sample. In cases where control duplicates did not agree and sample duplicates also provided different MIC values, values were also scored using the more conservative estimate. Samples that when assayed for the MIC in duplicate (either split samples or MIC assay replicates) produced contradictory results (e.g. one + and one -) were ranked as + since the sample did exhibit an ability to increase MIC versus the associated control (sterile saline) sample.

Results and Discussion

The exposure of bacteria to many if not all antibiotic or antimicrobial compounds, especially when such exposure occurs at sub-therapeutic concentrations, whether *in vitro*, *in vivo*, or in the external environment, can result in the selection of resistant bacterial strains. The widespread use of antibiotics and antimicrobials in human and veterinary medicine and as supplements to enhance growth or prevent disease in animal husbandry results in discharges to surface waters. In the U.S., excretion of drugs - that typically is in the form of active ingredient - generally results in conveyance to wastewater treatment,

whether on-site (e.g. septic system) or off-site (e.g. to sanitary sewer and centralized wastewater treatment). The polar nature and consequent appreciable solubility of many oral dosage antibiotic/antimicrobial compounds enhances passage through the majority of wastewater treatment processes. Animal wastes, including those from free ranging livestock, concentrated animal feeding operations, and aquaculture, also contain unmetabolized antibiotics that can be released to surface waters in recharge and runoff, or by direct defecation to streams. Cleaning and personal care products that contain antimicrobial compounds provide yet another source of such compounds to environmental waters.

The question arises as to the biological importance of antibiotic/antimicrobial compounds when discharged to, and subsequently diluted within, environmental waters. In this case, the development of antibiotic/antimicrobial resistance in bacteria from exposure to sub-therapeutic concentrations of these compounds is hypothesized, and is tested in an indicator bacterium. Given the documented presence of various combinations antibiotic and antimicrobial compounds, and other anthropogenic constituents in environmental waters, establishing a biological significance to their occurrence completes a link regarding their environmental effects and potential relevance to human and animal health.

Wastewater Treatment Plant (WWTP) Influent and Effluent

Influent and effluent samples were collected by trained samplers from the U.S. EPA from 3 anonymous wastewater treatment plants (WWTPs), denoted as A, B, and C, during routine compliance inspections. The identity of the WWTPs were not disclosed, as

previously described. Exposures to *SA* were started within 48 hours from the time of sample collection. Results are described below, and are shown in Table 1, and are summarized in Table 1a.

WWTP Influent A/Effluent A

Influent A and Effluent A exhibited a very large increase the MIC value versus the control for Amp, (426 times the control for both), Ery (16 and 8 times the control, respectively), Sul (32 times control for both), Tet (4 and 8 times control, respectively), and Met (32 times control for both). Kan and Van exhibited no increase in MIC value versus control. Nor exhibited a slight decrease of MIC value versus control.

WWTP Influent B/Effluent B

Influent B and effluent B assayed for the MIC value on June 23, 2005 exhibited neither an increased nor decreased MIC (> 2 wells) value versus control for either sample. The results suggest that no factor was present in the Influent B or Effluent B that conveys resistance to any of the antibiotic/antimicrobial tested.

WWTP Influent C/Effluent C

Influent C and effluent C assayed for the MIC value on June 23, 2005 exhibited neither an increase nor a decrease MIC (> 2 wells) versus control for either sample, except for Met which exhibited a 2 well decrease in the MIC value for Effluent C. The results suggest that no factor was present in the Influent C or Effluent C that conveys resistance to any of the antibiotic/antimicrobial tested.

Table 1: The MIC value results for Sewage Treatment Plant Influent A, B, and C and Effluent A, B, and C Samples. The MIC values represent the concentration (ug/ml) of antibiotic/antimicrobial in each sample. Multiple values within a cell represent results from duplicate MIC assay determinations. Duplicate MIC assay results are shown as multiple values in the appropriate cell in the table.

Assay Date 06/23/05 / **Compound**	**Influent A**	**Effluent A**	**Influent B**	**Effluent B**	**Influent C**	**Effluent C**	**Control**
Amp	21.3	21.3	0.005	0.005	0.011	0.0025	0.005
Ery	2	1	0.125	0.125	0.125	0.125	0.125
Kan	64	64	64	64	64	64	64
Nor	0.125	0.25	0.25	0.25	0.25	0.25	0.5
Sul	256	256	4	8	16	8	8
Tet	0.125	0.25	0.016	0.033	0.033	0.033	0.033
Van	1	1	1	1	1	2	1
Met	16	16	0.25	0.5	0.25 0.25	0.125	0.5

Table 1a: The MIC value scoring for Wastewater Treatment Plant Influent and Effluent Samples A, B, C. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. Duplicate assay results were identical and are shown as a single result.

Assay Date 6/23/2005 Compound	Influent A	Effluent A	Influent B	Effluent B	Influent C	Effluent C
Amp	+	+	-	-	-	-
Ery	+	+	-	-	-	-
Kan	-	-	-	-	-	-
Nor	-	-	-	-	-	-
Sul	+	+	-	-	-	-
Tet	+	+	-	-	-	-
Van	-	-	-	-	-	-
Met	+	+	-	-	-	-

Additional Evaluation of Influent A and Effluent A

The 10-day exposures of *SA* using Influent A and Effluent A were re-assayed for the MIC values on July 8, 2005, to further assess the observed potency of these samples at increasing the MIC value. Post exposure MIC value results are shown in Table 2 and the significance summarized in Table 2a.

Influent A and Effluent A samples, respectively, exhibited increases in the MIC values versus the control for Amp, Ery, Tet, and Met. Increases in the MIC values for these compounds were consistent, and ranged from 50 – 100 times control for Amp, about 8 – 16 times the control for Ery, about 4 – 16 times the control for Tet, and 128 times the control for Met. Effluent A exhibited an increase in the MIC values versus the control for Sul, which was assayed in duplicate, of 4 – 16 times the control range. No change in the MIC values greater than one well versus the control MIC values were observed for Kan, Nor, or Van. Results confirm the antibiotic resistance, measured as an increase in MIC values, conferred on the *SA* by both Influent A and Effluent A. Resistance in *SA* to Sul for Influent A appears diminished when compared to MIC results obtained from the June 23 assay, possibly reflecting a real decrease in activity between the dates that the SA was assayed, or an example of the generally observed variable responses to Sul during the course of this work. MIC results for Amp and Tet are also somewhat lower than in the previous (June 23) MIC assay. However, MIC results for Met are greater than those from the previous assay. It was not determined whether this is the result of variability in the assay itself or truly diminished or enhanced activity when samples are stored at 4^{o}C for 2 weeks, but likely reflects variability in the MIC assay.

Table 2: Additional evaluation of Influent and Effluent Sample A potency. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Duplicate MIC assay results are shown as multiple values in the appropriate cell in the table.

Assay Date 7/8/2005 Compound	Influent A	Effluent A	Control
Amp	21.3 21.3	10.7 21.3	0.021 0.021
Ery	1 2	2 2	0.125 0.125
Kan	64 64	64 64	64 64
Nor	0.125 0.125	0.25 0.25	0.125 0.25
Sul	4 8	128 64	8 16
Tet	0.25 0.5	0.5 0.25	0.063 0.033
Van	1 2	1 1	1 1
Met	16 16	16 16	0.125 0.125

Table 2a: Comparison of results for the MIC value scoring for Influent A and Effluent A assayed on 6/23/06 and 7/8/06. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. Results were scored as positive where the MIC of one sample of a duplicate was > 2 wells versus the control exposure sample MIC.

Assay Date	6/23/2005	6/23/2005	7/8/2005	7/8/2005
Compound	Influent A	Effluent A	Influent A	Effluent A
Amp	+	+	+	+
Ery	+	+	+	+
Kan	-	-	-	-
Nor	-	-	-	-
Sul	+	+	-	+
Tet	+	+	+	+
Van	-	-	-	-
Met	+	+	+	+

Influent A/Effluent A Exposure Time Course

To further assess the observed activity of Influent A and Effluent A at increasing the MIC values, exposures to *SA* of 1-day and 3-days to each were performed. Exposures of *SA* to Influent A were performed in duplicate for both the 1-day and the 3-days exposures. The MIC assay was performed on July 18, 2005. Samples were maintained at 4^{o}C on the different dates.

No increase of more than one well in the MIC values versus the control were observed for either the 1-day or 3-day exposures, except for an approximately 10-fold increase in the MIC value versus the control observed for a single Met duplicate sample. No difference in the MIC value versus the control was observed in the other Met duplicate. Results are shown in Table 3, and are summarized in Table 3a.

To provide an additional evaluation of the contrast between the original 10-day exposure results and those obtained after 1-day and 3-day exposures, samples collected after 3-days exposure to Effluent A were re-assayed on August 7, 2005. Results of the re-assay are shown in Table 4 and are summarized in Table 4a. The results confirm the time course MIC value assay results obtained on July 18, 2005. No sample showed an increase in MIC value with respect to the control, with the exception of Tet, which exhibited a slight increase in the MIC value versus the control in one of the duplicate MIC re-assays.

Table 3: Influent A and Effluent A exposure time course MIC values. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Duplicate MIC assay results are shown as multiple values in the appropriate cell in the table.

Assay Date 7/18/2005 Compound	Influent A, 1-day exposure	Effluent A, 1-day exposure	Control, 1-day exposure	Influent A, 3-day exposure	Effluent A, 3-day exposure	Control, 3-day exposure
Amp	0.011 0.011	0.005	0.011	0.011 0.011	0.005	0.021
Ery	0.25 0.25	0.25	0.125	0.25 0.25	0.125	0.25
Kan	2 4	4	4	4 4	2	2
Nor	0.25 0.25	0.25	0.25	0.25 0.25	0.25	0.25
Sul	4 8	8	4	8 16	16	8
Tet	0.063 0.125	0.063	0.063	0.125 0.125	0.063	0.063
Van	1 1	1	1	1 1	2	2
Met	0.125 0.125	0.125	0.125	0.25 2	0.125	0.25

Table 3a: The MIC value scoring of Influent A and Effluent A exposure time course. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. No sample increased the MIC value more than one well above the control.

Assay Date 7/18/2005 Compound	Influent A, 1-day exposure	Effluent A, exposure 1-day	Influent A, 3-day exposure	Effluent A, 3-day exposure
Amp	-	-	-	-
Ery	-	-	-	-
Kan	-	-	-	-
Nor	-	-	-	-
Sul	-	-	-	-
Tet	-	-	-	-
Van	-	-	-	-
Met	-	-	+	-

Table 4: Re-assay of MIC values for 3-day exposure time course samples of SA exposed to Effluent A. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample.

Assay Date 8/7/2005 Compound	Effluent A 3-day	Control
Amp	0.043 0.021	0.043 0.043
Ery	0.25 0.25	0.25 0.25
Kan	0.25* 8	16 8
Nor	0.5 0.5	0.25 0.5
Sul	32 8	64 64
Tet	1 2	0.5 0.5
Van	4 2	1 2
Met	0.5 0.25	0.5 0.25

*Result considered anomalous since inconsistent with other results for this compound in this assay

Table 4a: Scoring of results of re-assay of 3-day exposure time course samples for Effluent A. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. NA: Not Assayed.

Assay Date 8/7/2005 Compound	Effluent A 3-day exposure	Duplicate Effluent A 3-day exposure
Amp	-	-
Ery	-	-
Kan	-	-
Nor	-	-
Sul	-	-
Tet	-	+
Van	-	-
Met	-	-

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Influent A/Effluent A Dose Response

A dose response (50 ul, 100 ul, 250 ul, 500 ul, 1000 ul) over a 5-day exposure of SA to Influent A and Effluent A exhibited no difference from control except for Influent A at 250 ul for Amp, Ery, Kan, Nor and Tet; and Van, and in a single sample of duplicates at 1000 ul for Amp, Ery, Sul, and Tet. These results are considered suspect since no other assay of Influent A exhibited an increase in MIC versus control sample of Nor or Van, and since replicates exhibited very different results at a1000 ul volume of *SA* exposed to *SA* for 10 days, which exhibited no increase in MIC versus control of $\geq$2 wells. Results are shown in Table 5, and are summarized in Table 5a.

Table 5: The MIC values for Influent A and Effluent A exposures of *Staphylococcus aureus* to 50 ul, 100 ul, 250 ul, 1 ml volumes for 5-days and 10-days. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Where more than a 2 well difference was observed in replicate controls, the range between control samples was used to determine the 2 well difference between the control range and the environmental sample was used to determine a positive result. Duplicate MIC assay results are shown as multiple results in the appropriate table cell.

Assay Date 7/19/2005	5-day	5-day	5-day	5-day	5-day	10-day	10-day	10-day	10-day	10-day	10-day
Exposure Duration											
Compound	Influent 50 ul	Influent 100 ul	Influent 250 ul	Influent 500 ul	Influent 1 ml	Effluent 50 ul	Effluent 100 ul	Effluent 250 ul	Effluent 500 ul	Effluent 1 ml	Control
Amp	0.011	0.011	21.3	0.005	10.65 0.011	0.011	0.011	0.011	0.011	0.011	0.011 0.011
Ery	0.125	0.125	64	0.125	1 0.125	0.125	0.125	0.125	0.125	0.125	0.125 0.0625
Kan	4	2	64	2	4 2	4	2	2	2	2	2 1
Nor	0.25	0.25	4	0.125	0.25 0.25	0.25	0.25	0.25	0.25	0.25	0.125 0.125
Sul	4	4	8	4	64 4	4	4	4	4	4	4 16
Tet	0.063	0.063	0.50	0.033	0.25 0.063	0.063	0.063	0.063	0.063	0.033	0.033 0.063
Van	0.25	1	32	1	1 1	1	1	1	1	1	1 1

Table 5a: MIC Scoring of Influent A and Effluent A dose response determined from 5-day and 10-day exposures. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control.

Assay Date 7/19/2005 Exposure Duration	5-day	5-day	5-day	5-day	5-day	10-day	10-day	10-day	10-day	10-day
Compound	Influent A 50 ul	Influent A 100 ul	Influent A 250 ul	Influent A 500 ul	Influent A 1 ml	Effluent A 50 ul	Effluent A 100 ul	Effluent A 250 ul	Effluent A 500 ul	Effluent A 1 ml
Amp	-	-	+	-	+	-	-	-	-	-
Ery	-	-	+	-	+	-	-	-	-	-
Kan	+	-	+	-	-	-	-	-	-	-
Nor	-	-	-	-	-	-	-	-	-	-
Sul	-	-	+	-	+	-	-	-	-	-
Tet	-	-	+	-	+	-	-	-	-	-
Van	-	-	+	-	-	-	-	-	-	-

Pharmaceutical Manufacturing Effluents

Pharmaceutical manufacturing plant effluent samples were obtained by trained personnel from the U.S EPA during two routine compliance inspections at different facilities (Pharm A and Pharm B, respectively). Each facility's identity, for obvious reasons, was kept anonymous. Exposures were started within 48 hours from the time of sample collection. Assays for the MIC values were determined after exposures of Pharmaceutical Effluent A for 10-days, and Pharmaceutical Effluent B for 3-days and 10-days.

The MIC value for the 3-day exposures of *SA* to pharmaceutical plant effluent sample Pharm B are shown in Table 6, and results are summarized in Table 6a. *Staphylococcus aureus* exposed to Pharmaceutical Effluent B for 3-days exhibited neither an increase nor decrease in the MIC versus the control for any of the antibiotics/antimicrobials assayed, except for Sul which showed a 2 – 3 well decrease versus the control. *Staphylococcus aureus* exposed for 10-days to pharmaceutical effluent samples Pharm A and Pharm B exhibited neither an increase nor decrease in MIC versus control for any sample. Results are shown in Table 7, and are summarized in Table 7a.

Table 6: Time course MICs of Pharmaceutical Manufacturing Sample Pharm B Effluent. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Duplicate control MIC assay results are shown as multiple results in the appropriate table cell. NA: Not Assayed.

Assay Date 8/7/2005 Compound	Pharm. B Effluent 3-day incubation	Duplicate Pharm. B Effluent 3-day incubation	Control
Amp	0.043	0.043	0.043 0.043
Ery	0.25	0.5	0.25 0.25
Kan	4	8	16 8
Nor	0.5	0.5	0.25 0.5
Sul	16	8	64 64
Tet	0.25	0.25	0.5 0.5
Van	1	1	1 2
Met	0.25	0.25	0.5 0.25
Duplicate Met	NA	NA	0.5

Table 6a: MIC scoring of Pharmaceutical (Pharm) Manufacturing Effluent Time Course. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. No sample increased the MIC value more than one well above the control.

Assay Date 8/7/2005 Compound	Pharm. Effluent 3-day	Duplicate Pharm. Effluent 3-day
Amp	-	-
Ery	-	-
Kan	-	-
Nor	-	-
Sul	-	-
Tet	-	-
Van	-	-
Met	-	-

Table 7: MICs of Pharmaceutical Manufacturing Effluents Pharm A and Pharm B after 10-day exposure. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. The results for each assay date are compared to associated control sample. Duplicate assay results are shown as multiple values in the appropriate cell in the table. Duplicate MIC assay results are shown as multiple values in the Pharm. Effl. A and Control table cells.

Assay Date	**6/23/2005**	**6/23/2005**	**8/17/2005**	**8/17/2005**	**8/17/2005**
Compound	**Pharm. Effl. A**	**Control**	**Pharm. Effluent B**	**Duplicate Pharm. Effluent B**	**Control**
Amp	0.005 0.005	0.005	0.021	0.021	0.021 0.021
Ery	0.125 0.125	0.125	0.125	0.125	0.125 0.125
Kan	64 64	64	4	2	2 4
Nor	0.25 0.25	0.5	0.25	0.25	0.25 0.25
Sul	8 4	8	8	16	16 16
Tet	0.016 0.016	0.033	0.125	0.125	0.125 0.125
Van	1 1	1	1	1	1 1
Met	0.50 0.50	0.50	0.25	0.125	0.25 0.25

Table 7a: MIC scoring of Pharmaceutical Manufacturing Effluent (samples Pharm. A and Pharm. B) after 10-day exposure. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. No sample increased the MIC value more than one well above the control.

Assay Date	6/23/2005		8/17/2005	
Compound	Pharm. A Effluent	Duplicate Pharm. A Effluent	Pharm. B Effluent	Duplicate Pharm. B Effluent
Amp	-	-	-	-
Ery	-	-	-	-
Kan	-	-	-	-
Nor	-	-	-	-
Sul	-	-	-	-
Tet	-	-	-	-
Van	-	-	-	-
Met	-	-	-	-

Ambient Fresh Waters

Catskill-Delaware Upstate Tributaries

Samples were collected by U.S. EPA personnel from Fir Brook and a tributary to the Beaverkill River, both of which ultimately discharge to the Neversink Reservoir of the New York City Catskill - Delaware Watershed that provides drinking water to New York City. *SA* exposed to the environmental waters samples were assayed for MIC values following 3-days, and 10-days exposures. Control samples for all sample assays were generally within the one well response criteria, except for Sul for which duplicate samples both exhibited a two well (4x) higher response from others in the set in the 1-day exposure assay.

Fir Brook

SA exposed to Fir Brook water in duplicate for 3-days exhibited no increase of more than one well in MIC values versus the control results for any antibiotic/antimicrobial marker assayed. Results of 1-day and 3-day exposures are shown in Table 8, and are summarized in Table 8a. Duplicate 10-day exposures, which was the standard exposure protocol used in these studies of environmental waters, are shown in Table 9, and are summarized in Table 9a. MIC assay of the duplicate exposures showed marked disagreement, with one sample of the duplicate showing an increase in the MIC values compared to the control sample for Amp (1014 x control), Ery (512 x control), Sul (16 x control), Tet (16 x control), Van (64 x control), and Met (64 x control), and the duplicate exposure sample showing no difference from control. To evaluate the discrepancy, the samples were re-assayed. WWTP sample Effluent A was also grown

from a stored culture and re-assayed as a potential positive control sample. Re-assay confirmed the original MIC result indicating that the observed MIC value discrepancy was related to the sample and therefore was not an experimental anomaly. Both duplicate exposure samples exhibited an increase in the MIC value of greater than 64 times the associated control in each of 2 determinations using Met from a pre-thawed (Tube 1 used in previous assay) and a freshly thawed tube of antibiotic (Tube 2). Re-assay results are shown in Table 10, and are summarized and compared for the initial and the re-assay in Table 10a.

Beaverkill Tributary

Staphylococcus aureus exposed to Beaverkill Tributary water for 3-days and 10-days exhibited no difference in MIC values versus control results for all but one antibiotic/antimicrobial compound assayed. Results of 3-day exposures are shown in Table 8, and are summarized in Table 8a. Results of 10-day exposures are shown in Table 9, and are summarized in Table 9a. Exposure of *SA* to Beaverkill Tributary water did not result in an increase in MIC values versus the controls for any sample. However, the control MIC values in the 3-day exposure duplicate assays for Sul were less 2 and 3 wells less than the control exposure duplicates. This may be related to the variability generally observed in the MIC values for Sul.

Table 8: MIC results for three-day exposures of *Staphylococcus aureus* to Fir Brook and Beaverkill Tributary water. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Results from duplicate exposures are shown as multiple values in the appropriate cell in the table. A duplicate of Fir Brook and the Control exposures for MET were also assayed for the MIC in duplicate resulted in the triplicate values shown.

Assay Date 8/7/2005 Compound	Fir Brook 3-day	Beaverkill Tributary 3-day	Control
Amp	0.043 0.043	0.043 0.043	0.043 0.043
Ery	0.25 0.25	0.25 0.5	0.25 0.25
Kan	8 8	4 8	16 8
Nor	0.25 0.5	0.5 0.25	0.25 0.5
Sul	16 8	32 16	64 64
Tet	0.25 1	0.5 0.25	0.5 0.5
Van	1 1	1 1	1 2
Met	0.50 0.25 0.25	0.25 0.5	0.50 0.25 0.50

Table 8a: MIC scoring for three-day exposures of *SA* to Fir Brook and Beaverkill Tributary water. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. No sample increased the MIC value more than one well above the control.

Assay Date 8/7/2005 **Compound**	**Fir Brook 3-day exposure**	**Beaverkill Tributary 3-day exposure**
Amp	-	-
Ery	-	-
Kan	-	-
Nor	-	-
Sul	-	-
Tet	-	-
Van	-	-
Met	-	-

Table 9: MIC results for ten-day exposures of *SA* to Fir Brook and Beaverkill Tributary water. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample.

Assay Date 8/17/2005			
Resistance Marker	**Beaverkill Tributary**	**Fir Brook**	**Control**
Amp	0.021 0.021	0.021 21.3	0.021 0.021
Ery	0.125 0.125	0.125 64	0.125 0.125
Kan	4 4	4 4	2 4
Nor	0.125 0.25	0.25 0.125	0.25 0.25
Sul	8 16	16 256	16 16
Tet	0.125 0.125	0.125 2	0.125 0.125
Van	1 1	1 64	1 1
Met	0.25 0.25	0.25 16	0.25 0.50

Table 9a: MIC scoring for ten-day exposures of *SA* to Fir Brook and Beaverkill Tributary water. A + indicates an increase in the MIC of at least two wells greater than control. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control.

Assay Date 8/17/2005 / **Compound**	**Beaverkill Tributary**	**Duplicate Beaverkill Tributary**	**Fir Brook**
Amp	-	-	+
Ery	-	-	+
Kan	-	-	-
Nor	-	-	-
Sul	-	-	+
Tet	-	-	+
Van	-	-	+
Met	-	-	+

Table 10: The MIC values for re-assay of Fir Brook 10-day exposure samples. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Met (tube 1) and Met (tube 2) are as described in the text. MIC assay results from duplicate exposures are shown as multiple entries in the appropriate table cell.

Assay Date 8/19/05 (Repeat of Assay Date 08/17/05)			
Compound	**Fir Brook**	**WWTP Effluent A (Positive Control)**	**Control**
Amp	0.021 21.3	21.3	0.021
Ery	0.25 64	64	0.25
Kan	4 2	2	4
Nor	0.25 0.031	0.031	0.25
Sul	16 256	256	16
Tet	0.063 2	2	0.25
Van	0.25 64	64	1
Met (tube 1)	1 1	16	0.25
Met (tube 2)	0.25 16	16	16

Table 10a: Comparison of MIC values scoring for initial (assay date 8/17/05) and re-assay (assay date 8/19/05) of Fir Brook 10-day exposure samples. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. NA: Not assayed.

Assay Date 08/19/07 (Repeat of 08/17/05) / Compound	08/19/07 Fir Brook	Duplicate 08/19/07 Fir Brook	08/19/07 Effluent A (Positive Control)	08/17/05 Fir Brook	08/17/05 Duplicate Fir Brook
Amp	-	+	+	-	+
Ery	-	+	+	-	+
Kan	-	-	-	-	-
Nor	-	-	-	-	-
Sul	-	+	+	-	+
Tet	-	+	+	-	+
Van	-	+	+	-	+
Met antibiotic tube 1)	+	+	+	NA	NA
Met (antibiotic tube 2)	-	-	-	-	+

Kensico Reservoir and Tributaries

Samples were collected from the Kensico Reservoir and several of its tributaries in Westchester County, NY on four different occasions (8/22/05, 9/26/05, 12/17/05, 1/08/06) by experienced U.S. EPA personnel. Samples were provided anonymously with respect to the precise sampling location, and were only labeled with the date of sample collection and a unique identifier (A thru N). However, the samples were documented as having been collected from locations at increasing distances from the intake to the New York City water supply intake shafts, with sample A being closest and N being furthest.

Control samples generally exhibited good agreement in MICs in assays where exposure controls were run in duplicate. Exceptions were observed for Amp and Sul in the assay performed on 10/02/07, shown in Table 11. For Amp, a duplicate MIC values were unexpectedly divergent (0.021 and 5.32 ug/ml). The MIC values for Amp in other control samples in this series exhibited values in the lower range (0.021 and 0.005 ug/ml respectively), suggesting that the higher value is an anomaly. The MICs values for Sul in the duplicate control samples (16 and 256 ug/ml) also exhibited discrepancy. As for Amp, however, the lower value is in general agreement with other control samples, also suggesting that the higher value is an anomaly.

Kan exhibited a high, stable MIC for all samples including control. The MIC value was achieved at the highest concentration, or above, the assayed range, suggesting that the Kan stock preparation might have lost potency during storage. Results for this antibiotic are consequently not considered usable.

Upstream samples (Kensico C thru N) exhibited no increases greater than one well in the MIC values versus the control for any antibiotic/antimicrobial.

Results for samples Kensico A and Kensico B are shown in Table 11, and are summarized in Table 11a. Exposures for the control were run in duplicate. As indicated above, poor agreement was exhibited in the control duplicate values for Amp and Sul. Since the values obtained in one of the duplicate exposure control samples for these two antibiotics/antimicrobials were markedly different than those obtained in other assays, results for Amp and Sul are discussed omitting consideration of the anomalous high values (5.32 and 256 ug/ml, respectively)

. Amp (1014 x control), Sul (8 and 2 x control), Van (64 x control), and Met (32 and 64 x control) exhibited increases in the MIC values of one or more wells from control for sample Kensico A, assayed in duplicate. Sample Kensico B generally exhibited poor agreement between duplicate exposure results. At least one sample duplicate for Amp, Ery, Nor, Tet, Van and Met showed a greater tan two well increase in the MIC value versus the control. The other duplicate sample showed either no increase in the MIC value - or for Sul, Van, and Met - a decrease in MIC value versus the control MIC value.

Results for samples Kensico C thru Kensico G are shown in Table 12, and are summarized in Table 12a. Results for samples Kensico H thru Kensico L are shown in Table 13 and are summarized in Table 13a. Results for samples Kensico M and Kensico N are shown in Table 14 and are summarized in Table 14a. Samples Kensico C thru N exhibited no increases in the MIC values of two wells or more versus the control except for sample Kensico M which showed an increase for tetracycline in one sample of duplicate exposures. A decrease in the MIC value versus the control was observed in some samples for Amp, Sul, and Tet. Decreases in the MIC values were generally 1 to 2 wells less than the control MIC value.

Table 11: The MICs values results for Samples Kensico A and Kensico B. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Duplicate MIC assay results are shown as multiple entries in the appropriate table cell.

Assay Date 10/2/2005 Compound	Kensico A	Kensico B	Control
Amp	21.3 21.3	21.3 0.043	0.021 5.32
Ery	64 64	64 0.25	0.25 0.25
Kan	64 64	64 64	64 64
Nor	8 8	8 0.5	0.25 0.5
Sul	64 256	16 8	32 256
Tet	8 8	8 0.5	0.5 1
Van	64 64	64 0.5	1 1
Met	16 16	16 0.063	0.5 0.25

Table 11a: MIC value scoring for Samples Kensico A and Kensico B. A + indicates an increase in the MIC of at least two wells greater than control. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. Kan MIC values were not considered usable for this assay.

Assay Date 10/2/2005 Compound	Kensico A	Kensico B
Amp	+	+
Ery	+	+
Nor	+	+
Sul	-	-
Tet	+	+
Van	+	+
Met	+	-

Table 12: The MICs value results for samples Kensico C thru Kensico G. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample

Assay Date 10/10/2005 Compound	Kensico C	Kensico D	Kensico E	Kensico F	Kensico G	Control
Amp	0.043	0.011	0.011	0.021	0.021 0.021	0.021
Ery	0.25	0.25	0.25	0.125	0.25 0.25	0.25
Kan	64	64	64	64	64 64	64
Nor	0.5	0.25	0.25	0.25	0.25 0.25	0.25
Sul	4	8	8	4	8 32	16
Tet	0.25	0.25	0.25	0.5	0.25 0.25	1
Van	1	1	1	1	1 1	1
Met	0.25	0.25	0.25	0.125	0.25 0.25	0.25

Table 12a: MIC scoring for samples Kensico C thru Kensico G. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. No sample increased the MIC value versus the control.

Assay Date 10/10/2005	Kensico C	Kensico D	Kensico E	Kensico F	Kensico G
Amp	-	-	-	-	-
Ery	-	-	-	-	-
Kan	-	-	-	-	-
Nor	-	-	-	-	-
Sul	-	-	-	-	-
Tet	-	-	-	-	-
Van	-	-	-	-	-
Met	-	-	-	-	-

Table 13: The MIC value for samples Kensico H thru Kensico L. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Duplicate assay results are shown as multiple values in the appropriate cell in the table.

Assay Date 12/31/2005 / **Compound**	**Kensico H**	**Kensico I**	**Kensico J**	**Kensico K**	**Kensico L**	**Control**
Amp	0.005	0.005	0.011	0.005	0.005	0.005
Ery	0.25	0.125	0.25	0.125	0.125	0.25
Kan	4	4	4	4	4	4
Nor	0.25	0.25	0.25	0.25	0.25	0.25
Sul	8	4	8	8	8	16
Tet	0.25	0.25	0.25	0.25	0.25	0.25
Van	1	1	1	1	1	1
Met	0.063	0.063	0.063	0.063	0.125	0.063 0.063

Table13a: MIC scoring for samples Kensico H thru Kensico L. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. No sample increased the MIC value more than one well above the control.

Assay Date 12/31/2005 Compound	Kensico H	Kensico I	Kensico J	Kensico K	Kensico L
Amp	-	-	-	-	-
Ery	-	-	-	-	-
Kan	-	-	-	-	-
Nor	-	-	-	-	-
Sul	-	-	-	-	-
Tet	-	-	-	-	-
Van	-	-	-	-	-
Met	-	-	-	-	-

Table 14: The MIC values for samples Kensico M and Kensico N. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. MIC assay results for duplicate exposures are shown as multiple values in the appropriate cell in the table.

Assay Date 1/24/2006 Compound	Kensico M	Kensico N	Control
Amp	0.005 0.011	0.005	0.005
Ery	0.25 0.125	0.25	0.25
Kan	4 4	4	4
Nor	0.25 0.25	0.25	0.25
Sul	16 32	32	32
Tet	4 0.5	0.5	0.5
Van	2 1	1	2
Met	2 4	2	2
Duplicate Met	2 2	2	2

Table 14a: MIC scoring for samples M and N. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control.

Assay Date 1/24/2006		
Compound	**Kensico M**	**Kensico N**
Amp	-	-
Ery	-	-
Kan	-	-
Nor	-	-
Sul	-	-
Tet	+	-
Van	-	-
Met	-	-

South Branch Raritan River

Samples were collected on two occasions from the South Branch of the Raritan River in Hunterdon County, New Jersey. The furthest upstream samples were collected approximately 100 yards upstream of the Long Valley (LV) Sewage Treatment Plant (STP), the furthest downstream south of Ken Lockwood Gorge (KLG) on Rt. 31 and 513 (on Durham House property) in Clinton, NJ. Locations, organized from most upstream to most downstream sample, are provided in Table 15.

Table 15: South Branch Raritan River sampling locations listed consecutively from upstream to downstream. (LV – Long Valley, KLG – Ken Lockwood Gorge).

Sample	Description
LV-3	Intersection of Bartley and Coleman Rds. Furthest upstream sample
LV-1	Adjacent to Long Valley WWTP
LV-2	Downstream of Long Valley WWTP at intersection of Rts. 24 and 517
KLG-1	Upstream entrance to Ken Lockwood Gorge
KLG-2	Downstream terminus of Ken Lockwood Gorge
KLG-3	Rt. 31 and 513 (on Durham House property). Furthest downstream sample.

The MIC assay results for the South Branch Raritan River samples are shown in Table 16, and are summarized in Table 16a. Sample LV-1 collected during the first sampling event adjacent to the Long Valley WWTP, was the only sample to show an increase in MIC values versus the control. However, results for the duplicate LV-1 sample exposures showed poor agreement, with one sample exhibiting an increase in the MIC value versus the control for Amp (62 x control) and Met (4 x control) and the duplicate sample exhibiting an increase in the MIC value versus the control for Amp (495 x control), Ery (128 x control), Kan (8 x control), Sul (2 x control), Tet (8 x control), Van (32 x control) and Met (64 x control). The discrepancy in results was further evaluated by re-assaying the samples for the MIC. Results of the re-assay are shown and compared with the initial sample assay in Table 17, and the results are summarized in Table 17a. The results from the re-assay showed excellent agreement with the results obtained from the LV-1 duplicate sample from the initial assay, strongly suggesting that the initial result was an anomaly and that the LV-1 sample induced marked increases in the MIC values versus the control for Amp, Ery, Kan. Nor, Tet, Van, and Met. Sample LV-2 collected approximately one half mile downstream of LV-1 did not exhibit any increase in the MIC value versus the control. The furthest upstream sample (LV-3) also did not exhibit any increase in the MIC value versus the control. Results from the duplicate MIC determinations for LV-1 were inconsistent, however, so this sample was re-assayed two days after the initial MIC determination. The re-assay result verified that the sample collected adjacent to the Long Valley WWTP discharge exhibited an increase in the MIC values versus the control for Amp, Ery, Nor, Tet, Van, and Met.

Table 16: The MIC value results for South Branch Raritan River samples from first sampling event. LV – Long Valley, KLG – Ken Lockwood Gorge. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Results from assay of duplicate exposures are shown as multiple values in the same cell of the table.

Assay Date 11/15/05 **Compound**	**LV 1**	**LV 2**	**LV 3**	**KLG 1**	**KLG 2**	**KLG 3**	**Control**
Amp	2.65 21.3	0.043	0.043	0.043	0.043	0.043	0.043
Ery	0.125 16	0.125	0.125	0.125	0.125	0.125	0.125
Kan	8 64	8	8	8	8	8	8
Nor	0.25 8	0.25	0.125	0.125	0.25	0.25	0.25
Sul	256 128	64	128	128	64	128	64
Tet	0.5 4	0.5	0.5	0.5	0.5	0.5	0.5
Van	2 64	2	2	2	2	2	2
Met	1 16	0.25	0.5	0.25	0.25	0.25	0.25

Table 16a: MIC scoring for South Branch Raritan River samples from first sampling event. LV – Long Valley, KLG – Ken Lockwood Gorge. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. Results from duplicate exposures where one sample scored + relative to control in the MIC assay are scored as +.

Assay Date 11/15/2005 Compound	LV 1	LV 2	LV 3	KLG 1	KLG 2	KLG 3
Amp	+	-	-	-	-	-
Ery	+	-	-	-	-	-
Kan	+	-	-	-	-	-
Nor	+	-	-	-	-	-
Sul	+	-	-	-	-	-
Tet	+	-	-	-	-	-
Van	+	-	-	-	-	-

Met	**+**	**-**	**-**	**-**	**-**	**-**

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Table 17: Comparison of MIC values for samples collected on the South Branch of the Raritan River at Long Valley, NJ (station LV-1), assayed on 11/15/05 and 11/17/05. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Duplicates, for the samples shown, were exposed to the same sample as their associated sample.

Assay Date	**11/15/05**	**11/17/05**	**11/15/05**	**11/17/05**	**11/15/05**	**11/17/05**
Compound	**LV 1**	**LV 1**	**Duplicate LV 1**	**Duplicate LV 1**	**Control**	**Control**
Amp	2.65	21.3	21.3	21.3	0.043	0.043
Ery	0.125	64	16	64	0.125	0.25
Kan	8	64	64	64	8	8
Nor	0.25	8	8	8	0.25	0.25
Sul	256	256	128	256	64	128
Tet	0.5	8	4	8	0.5	0.063
Van	2	64	64	64	2	0.25
Met	1	8 4	16	16 16	0.25	0.25 0.25

Table 17a: Comparison of MIC scoring for samples collected on the South Branch of the Raritan River at Long Valley, NJ (station LV-1), assayed on 11/15/05 and 11/17/05. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control.
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Assay Date	11/15/05	11/17/05	11/15/05	11/17/05
Compound	LV 1	LV 1	Duplicate LV 1	Duplicate LV 1
Amp	+	+	+	+
Ery	-	+	+	+
Kan	-	+	+	+
Nor	-	+	+	+
Sul	+	-	-	-
Tet	+	+	+	+
Van	+	+	+	+
Met	+	+	+	+

To further evaluate the increase in the MIC values for the LV-1 sample, two sub-colonies were selected after isolation on TSA. Two control (no exposure to environmental water) sub colonies were similarly selected. LV-1 sub-colony samples were assayed for their MIC values in duplicate. Results are shown in Table 18, and are summarized in Table 18a. The MICs for both LV-1 sub colonies show consistent agreement between duplicates; however, the two selected sub-colonies exhibited differences in the MIC profile. One possibility for this result is that a mixed culture of multi-antibiotic resistant *SA* might have emerged from exposure to the LV-1 water sample. One of the colonies (Colony 1) exposed to sample LV-1 in duplicate did not exhibit any increase in the MIC values versus the control for Ery, Kan, Nor, Tet and Van, whereas the other colony (Colony 2) exposed to sample LV-1 in duplicate showed increases in the MIC values versus the control for these antibiotics. One of the Colony 2 duplicates did not exhibit an increase in the MIC value versus the control for Sul.

Table 18: The MIC values for sub-colonies cultured from primary exposure culture from South Branch Raritan River samples from first sampling event. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Duplicate assay results are shown as multiple values in the appropriate cell in the table.

Assay Date 11/23/2005 / **Compound**	**Control Colony 1**	**Control Colony 2**	**LV 1 Colony 1**	**LV 1 Colony 2**
Amp	0.043	0.043	21.3 10.65	21.3 21.3
Ery	0.125	0.25	0.125 0.125	64 64
Kan	8	8	16 16	64 64
Nor	0.25	0.25	0.25 0.25	8 2
Sul	64	32	256 256	128 64
Tet	0.5	0.5	0.5 0.5	4 4
Van	2	2	2 2	64 64
Met	0.25 0.125	4 4	16 16	16

Table 18a: MIC scoring for sub-colonies cultured from primary exposure culture from South Branch Raritan River samples from first sampling event. LV – Long Valley. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. Duplicate determinations where one sample was + relative to control are scored as +.

Assay Date 11/23/2005	LV 1Colony 1	LV 1 Colony 2
Amp	+	+
Ery	+	+
Kan	+	+
Nor	-	+
Sul	+	+
Tet	-	+
Van	-	+
Met	+	+

Samples were collected from the same locations at the South Branch Raritan River on a second sampling event approximately two months after the initial sample collection, and MICs were determined after exposure of *SA* to the samples for 10-days. Results are shown in Table 19, and are summarized in Table 19a. MICs were not greater than control for any sample collected during the second sampling event.

Table 19: The MIC values for South Branch Raritan River samples from second sampling event. LV – Long Valley, KLG – Ken Lockwood Gorge. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. MIC results from duplicate exposures are shown as multiple values in a sample cell. For MET, duplicate MIC assays were run for sample LV 1b, KLG 3 b, and the control with results shown in italics.

Assay Date 1/15/2006 **Compound**	**LV 1 b**	**LV 2b**	**LV 3b**	**KLG 1b**	**KLG 2b**	**KLG 3b**	**Control**
Amp	0.005 0.011	0.011	0.005	0.011 0.005	0.011	0.011	0.005
Ery	0.25 0.25	0.25	0.25	0.25 0.25	0.25	0.25	0.25
Kan	4 4	4	4	8 8	8	8	4
Nor	0.25 0.5	0.25	0.25	0.25 0.25	0.25	0.25	0.25
Sul	256 256	256	256	256 256	256	256	256
Tet	0.5 0.5	0.5	0.5	0.5 0.5	0.5	0.5	0.5
Van	1 1	1	1	1 1	1	1	1
Met	0.063 0.125 *0.063* *0.125*	0.125	0.125	0.125 0.125	0.125	*0.25* *0.25*	*0.063* *0.125*

Table 19a: MIC scoring for South Branch Raritan River samples from second sampling event. LV – Long Valley, KLG – Ken Lockwood Gorge. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. No sample increased the MIC value more than one well above the control. Duplicate assay results were of the same magnitude and are shown as single scores.

Assay Date 1/15/2006 Compound	LV 1 b	LV 2b	LV 3b	KLG 1b	KLG 2b	KLG 3b
Amp	-	-	-	-	-	-
Ery	-	-	-	-	-	-
Kan	-	-	-	-	-	-
Nor	-	-	-	-	-	-
Sul	-	-	-	-	-	-
Tet	-	-	-	-	-	-
Van	-	-	-	-	-	-
Met	-	-	-	-	-	-

Raritan River, City of New Brunswick Source Water and Tap Water

Samples were collected at three locations on two consecutive days from the Raritan River at Bound Brook, NJ, and the Delaware-Raritan Canal which serves as the source water for drinking water for the City of New Brunswick, NJ. Sampling on consecutive days (May 1 and 2, 2007) took advantage of potential runoff or sewer overflows resulting from the storm event that occurred on the previous night through the morning of the following day (0.85 inches at Middlesex, NJ, http://nj.usgs.gov). Previous rainfall occurred on April 27 when 4.23 inches of rainfall were recorded at Middlesex, NJ. Sampling locations were as follows: Sample RR 10-1and RR 10-2 were collected from the Raritan River in Bound Brook, NJ adjacent to the City of Bound Brook New Jersey - American Water Works drinking water treatment facility intake which is located just downstream of Lock 10 which will be further described. Sample DRC 10-1 and DRC 10-2 were collected at Lock 10 (a.k.a. 10 Mile Lock) just prior to overflow at the spillway; Sample DRC NB-1 and DRC NB-2 were collected approximately 50 yards upstream of the New Brunswick Water Company intake at the downstream terminus of the Canal at the Raritan River in New Brunswick, NJ. New Brunswick tap water was collected from the tap after running for three minutes in the laboratory at Lipman Hall, Cook College Campus, New Brunswick, NJ. The cold tap water sample was immediately treated with sodium thiosulfate to eliminate any chlorine residual.

Staphylococcus aureus exposed to the environmental water samples were assayed for their MIC values on 05/21/07. Exposure samples and the control sample, including duplicate exposures for some samples, were re-assayed on 06/01/07 to confirm results for samples that showed a discrepancy in their MIC values between duplicate exposures.

Results for the MIC value determinations in the samples are shown in Table 20, and data are summarized in Table 20a. The Van standard was apparently consumed by others working in the laboratory and consequently was not assayed for its MIC value.

The MIC values for the samples collected at Lock 10 on 05/01/07 (DRC 10-1) and 05/02/07 (DRC 10-2) were within the range of the duplicate control samples except for Amp (532 times control) and Met (32 times control) in one of the duplicate samples collected on 05/01/07. The MIC values in the samples collected at the New Brunswick water intake on 05/01/07 (DRC-NB-1) were identical to the controls. The MIC values for the samples collected on 05/02/07 (DRC NB-2) exhibited increases in the MIC values versus the control for Amp (213 times control), Kan (4 times control) and Met (32 times control). The MIC values for New Brunswick tap water (NB Water) samples were within the range of controls except for Amp in one duplicate sample (213 times control). The relatively high MIC value observed for Sul was consistent with the variability observed for this antimicrobial marker throughout this work, and is consistent with that observed in studies performed by others (Bordas, et al., 1997; Brady and Katz, 1992; Kleiner, et al., 2007).

The MIC values for samples collected on 05/01/07 and 05/02/07 from the Raritan River where identical to the controls except for duplicate samples (RR10-2) collected on 5/2/07. The MIC values in the sample exceeded the control for Amp (1936 times control), Ery (256 times control), Kan (4 times control), Nor (8 times control), Tet (16 times control), and Met (32 times control). The MIC values for the corresponding duplicate sample were identical to the control. Van was not assayed for its MIC value in this assay.

To further evaluate the results and discrepancies between duplicate determinations observed in the samples run on 05/02/07, samples were re-assayed for their MIC values on 06/01/07. Comparison of relative results for samples that showed an increase in the MIC value versus the control (i.e. MIC at least 2 wells greater than control) were very similar. Results are shown in Table 20a and are summarized in Table 20c. As a result, only the MIC values from the initial assay were used in final data summaries.

Table 20: The MIC values for Delaware Raritan Canal (DRC), New Brunswick tap water (NBW), and Raritan River at Bound Brook (RR) samples. Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. Duplicate assay results are shown as multiple values in the appropriate cell in the table.

Sample Date	5/1/07	5/2/07	5/1/07	5/2/07	5/2/07	5/1/07	5/2/07	
Assay Date 05/21/07 Compound	DRC 10 – 1	DRC 10-2	DRC NB-1	DRC NB-2	NBW	RR 10 – 1	RR 10 – 2	Control
Amp	5.32 0.011	0.011 0.011	0.011 0.011	21.3 21.3	21.3 0.011	0.011 0.011	21.3 0.011	0.011 0.011
Ery	0.25 0.25	0.25 0.25	0.25 0.25	64 64	0.25 0.25	0.25 0.25	64 0.25	0.5 0.25
Kan	8 8	8 8	8 8	64 64	8 16	8 8	64 16	16 16
Nor	0.5 0.5	0.25 1	0.25 0.50	4 16	0.5 0.50	0.5 0.5	8 0.5	0.5 1
Sul	256 256	256 256	256 256	256 256	256 256	256 256	256 256	256 256
Tet	8 0.25	0.25 0.25	0.25 0.125	8 16	4 0.25	0.25 0.25	4 0.25	0.25 0.25
Met	64 2	2 2	2 2	64 64	64 64	2 2	64 2	2 2

Table 20a: The MIC re-assay values for samples from the Delaware Raritan Canal (DRC), New Brunswick tap water (NBW), and Raritan River at Bound Brook (RR). Values represent the concentration (ug/ml) of antibiotic/antimicrobial at which the MIC was observed in each sample. MIC assay results for duplicate exposures are shown as multiple entries into the appropriate cell in the table.

Sample Date	**5/1/07**	**5/2/07**	**5/2/07**	**5/2/07**	**5/2/07**	
Assay Date 6/1/2007						
Compound	**DRC 10-1**	**DRC 10-2**	**RR 10 – 2**	**DRC NB 2**	**NBW**	**Control**
Amp	2.65 0.0025	0.0025 0.0025	1.33 0.0025	21.3 21.3	10.65 0.0025	0.005
Ery	0.125 0.125	0.125 0.125	16 0.125	64 32	0.25 0.125	0.125
Kan	4 4	4 4	32 4	32 32	4 4	4
Nor	0.25 0.25	0.25 0.25	4 0.25	4 2	0.25 0.25	0.25
Sul	256 32	32 16	256 16	256 64	256 16	64
Tet	8 0.125	0.125 0.125	2 0.125	8 2	2 0.125	0.125
Met	16 0.5	1 1	64 1	64 16	64 0.125	1

Table 20b: MIC value scoring for samples collected from the Delaware Raritan Canal (DRC), the Raritan River (RR), and the city of New Brunswick tap water (NBW Samples were assayed on two different days to verify results. A + indicates an increase in the MIC of at least two wells greater than control. A – indicates an MIC value between one well greater, equal to, or any number of wells less than the control. Duplicates are samples exposed to SA in duplicate. NA - Not assayed.

Assay Date 5/21/07 Compound	DRC 10-1	Duplicate DRC 10-1	DRC 10-2	Duplicate DRC 10-2	RR 10-1	RR 10-2	Duplicate RR 10-2	DRC NB-2	Duplicate DRC NB-2	NBW	Duplicate NBW
Amp	+	-	-	-	-	+	-	+	+	+	-
Ery	-	-	-	-	-	+	-	+	+	-	-
Kan	-	-	-	-	-	+	-	+	+	-	-
Nor	-	-	-	-	-	+	-	+	+	-	-
Sul	-	-	-	-	-	-	-	+	+	-	-
Tet	+	-	-	-	-	+	-	-	-	+	-
Met	+	-	-	-	-	+	-	+	+	+	+
Assay Date 6/1/2007 Compound											
Amp	+	-	-	-	NA	+	-	+	+	+	-
Ery	-	-	-	-	NA	+	-	+	+	-	-
Kan	-	-	-	-	NA	+	-	+	+	-	-
Nor	-	-	-	-	NA	+	-	+	+	-	-
Sul	+	-	-	-	NA	+	-	+	-	+	-
Tet	+	-	-	-	NA	+	-	+	+	+	-
Met	+	-	-	-	NA	+	-	+	+	+	-

General Discussion of Results

This study evaluated the ability of environmental waters to induce antibiotic resistance in a laboratory Gram-positive bacterial strain, *Staphylococcus aureus ATCC* 9144 (*SA*). This strain of *SA* has been utilized previously in studies evaluating the ability of various natural and synthetic compounds and mixtures to induce antibiotic resistance (Kliener, et al., 2007; Bordas, et al., 1997; Brady and Katz, 1992). Moreover, this strain of *SA* is known to be sensitive to a wide spectrum of antibiotic classes including beta lactams, macrolides, aminoglycosides, tetracyclines, glycopeptides, fluoroquinolones, and sulfonamides. Consequently, this strain of *SA* is considered a viable tool to screen environmental waters for their ability to induce antibiotic resistance.

The antibiotics employed in this evaluation and their respective classifications and modes of action are shown in Table 21.

Table 21: Summary of the antibiotic/antimicrobial used in the MIC assay for *Staphylococcus aureus* that had been exposed to environmental water samples.

Compound	**Classification**	**Mechanism of Action**	**Targets**
Ampicillin (Amp)	Beta lactam	Bactericidal Inhibits cell wall synthesis	Gram-positive Some Gram-negative
Erythromycin (Ery)	Macrolide	Bacteriostatic Inhibits protein synthesis	Gram-positive
Kanamycin (Kan)	Aminoglycoside	Bactericidal Inhibits protein synthesis	Gram-negative Some Gram-positive
Norfloxacin (Nor)	Fluoroquinolone	Bactericidal Inhibits protein synthesis	Gram-positive
Sulfamethoxazole (Sul)	Sulfonamide	Bacteriostatic Inhibits DNA synthesis	Gram-positive
Tetracycline (Tet)	Tetracycline	Bacteriostatic Inhibits protein synthesis	Gram-positive/Gram-negative
Vancomycin (Van)	Glycopeptide	Inhibits cell wall synthesis	Gram-positive
Methicillin (Met)	Beta Lactam (penicillin class)	Inhibits cell wall synthesis	Gram-positive

Development of antibiotic resistance was observed subsequent to exposure of *SA* to samples from most of the environmental water classes examined (ambient fresh water, sewage influent, sewage effluent, finished drinking water), but not to all environmental waters within a class. Table 22 provides a summary of results for the MIC value determinations for each class of environmental water. Antibiotic resistance was not detected after exposure of *SA* to either of the two pharmaceutical manufacturing plant effluents. Within the classification of ambient freshwaters examined, samples were collected from rivers, and drinking water source waters. Drinking water sources were contributing streams and downstream reservoirs that supply potable water to the City of New York; the Delaware Raritan Canal, which supplies potable water to the City of New Brunswick, NJ; and the Raritan River, which supplies drinking water to the Town of Bound Brook, NJ. Results for MIC value determinations of drinking water sources are summarized in Table 23.

Eight of thirty-five ambient water samples exhibited multi-antibiotic resistance. One sample (Kensico M) exhibited resistance to tetracycline and no other antibiotic in one sample from duplicate exposures. Resistance was seen to each compound included in the MIC assay panel, though resistance to each was not observed in every sample. One of three sewage influent samples exhibited multi-antibiotic/antimicrobial resistance. The effluent sample collected from the same facility also exhibited multi-antibiotic/antimicrobial resistance to the same panel of antibiotic/antimicrobial markers. The magnitude of resistance, when observed, and the antibiotics/antimicrobials to which resistance in *SA* developed were the same in both influents and effluents collected from a given facility, suggesting that the agent of resistance (presence of antibiotic/antimicrobial,

genetic material, or other agent in the wastewater) was persistent.

A summary of results categorized by antibiotic/antimicrobial compound class are shown in Table 24. Resistance of exposed *SA* to ampicillin and tetracycline were the most commonly observed, and occurred in all samples where resistance was demonstrated, except for one of the duplicate exposures for sample Kensico M which was only positive for tetracycline,. The data strongly implies that a combined beta lactamase and active efflux activity in each of the resistant SA cultures. Methicillin resistance was nearly as prevalent, but at a slightly reduced frequency, suggesting a somewhat reduced incidence of transmission or induction of the factors generally associated with resistance to this antibiotic. The mec loci that codes for the various specific penicillin binding proteins associated with methicillin resistance has been identified to co-occur with erythromycin, tetracycline, and fluoroquinolone resistance in community acquired MRSA strains in the U.S. (Tenover, et al., 2006). Resistance was also observed to vancomycin in 4 of the multi-antibiotic/antimicrobial resistant samples in this study, and has been shown in clinical strains of methicillin and vancomycin resistant *SA* (Tenover, et al., 2001). This is believed to be associated with vancomycin trapping by over-expressed membrane bound penicillin binding proteins. The relatively reduced frequency and magnitude of resistance profiles to other antibiotics evaluated suggests that the mechanisms of resistance may be less robust (e.g. non specific efflux) or that specific factors are not strongly expressed. Preliminary experiments performed with this research suggested that exposure to ug/ml concentrations of ampicillin, tetracycline, and methicillin only resulted in low increases in the MIC value, if any. The magnitude of antibiotic/antimicrobial resistance observed in the determinations provided here were variable, but maximum increases in the MIC value of greater than

1000 times the control for ampicillin and up to 64 times control for methicillin ambient fresh water is reason for serious concern regarding dermal and ingestion related exposure of humans.

Table 22: Summary of the MIC assay results for environmental water samples.

Environmental Water Sample	**Number of samples analyzed**	**Number of MIC Assays of environmental samples***	**Number of samples capable of inducing resistance (MIC ≥ 2 wells > control MIC)****	**Compounds for which antibiotic/antimicrobial Resistance Observed (MIC ≥ 2 wells > control MIC)**
Ambient Fresh Water	35	63	9	Amp, Ery, Kan, Nor, Sul, Tet, Van, Met,
Sewage Influent	3	15	1	Amp, Ery, Sul, Tet, Met
Sewage Effluent	3	14	1	Amp, Ery, Sul, Tet, Met
Pharmaceutical Effluent	4	7	0	
Finished Drinking Water	2	2	1	Amp, Sul, Tet, Met

* Includes all MIC assays of the same sample. Does not include assay of control samples

** Does not include split (co-located) samples

Table 23: Summary of the MIC assay results for drinking water source samples.

Drinking Water Source	**Number of Samples**	**Number of Samples with Antibiotic/Antimicrobial resistance**	**Antibiotics/ Antimicrobials**
New York City Reservoir and Tributaries	14	3	Amp, Ery, Nor, Sul, Tet, Van, Met
Delaware Raritan Canal	4	2	Amp, Ery, Kan, Nor, Sul, Tet, Met
Raritan River at Bound Brook	2	1	Amp, Ery, Kan, Nor, Sul, Tet, Met

Table 24: Summary of the MIC assay results by antibiotic class for 10-day exposures.

Compound	Antibiotic/Antimicrobial Class	Frequency	Frequency of Multi-resistance with Amp Resistance
Amp	Beta Lactam	10/51	10/10
Ery	Macrolide	8/51	8/8
Kan	Aminoglycoside	3/49	3/3
Nor	Fluoroquinolone	5/51	5/5
Sul	Sulfonamide	8/51	8/8
Tet	Tetracycline	11/51	10/11
Van	Glycopeptide	4/40	4/4
Met	Beta Lactam	9/51	9/9

The widespread occurrence of antibiotics and antibiotic resistant bacteria in the environment is cause for concern due to the potential that further resistance may be conferred to non-resistant pathogens by either exposure to antibiotics or by incorporating genetic material from resistant bacteria already present. This work is the first known to demonstrate that antibiotic resistance can be induced in a laboratory strain of pathogenic Gram-positive bacteria, *Staphylococcus aureus*, by exposure to filter sterilized environmental water *in vitro*. Moreover *S. aureus* is associated with widespread morbidity and mortality both in the United States and other countries directly related to resistance in *S. aureus* to the antibiotic methicillin. This work is the first identified to have demonstrated that factors that convey resistance to methicillin are present in environmental waters. Moreover, resistance to vancomycin in the majority of samples analyzed for both methicillin and vancomycin resistance exhibited multi-antibiotic resistance to both. This finding is of importance, since vancomycin is considered one of the final therapeutic defenses against MRSA infections. The factors that convey the MAR operon to bacteria appear to coexist in the environment, and may indicate that vancomycin resistant MRSA will continue its emergence as a common pathogen, with possible widespread increases in mortality occurring as a result.

The multiple antibiotic resistances exhibited by samples assayed in this investigation indicate that one or more mechanisms may be associated with the observed resistances. Evidence for this potential was suggested when two sub-colonies from a single exposure culture were assayed for their respective MIC values to the test panel of antibiotic/antimicrobial. The MIC values for each subculture were highly reproducible, yet each exhibited unique resistance profiles. Possibilities for the observed variability include

acquisition of more than one resistance factor (e.g. plasmid or integron containing variable resistance cassettes) by *SA* from the environmental water, loss of a factor as a result of excision or recombination, or potential point mutation of some but not all bacteria in the culture. The observed result is, in all cases, a mixed bacterial culture with respect to the resistance phenotype. Each of the potential mechanisms had been described in the literature with respect to observed resistance phenotypes, and none can be discounted here.

The relative abundance of methicillin resistant *Staphylococcus aureus* (MRSA) in the environment is, at present, unknown. The results of this investigation suggests that the presence of MRSA in the environment may be substantial. Such presence may alone be associated with some of the observed public health consequences, or may emerge as a future source of MRSA with potential risks to public health. Moreover, 18% of the assayed samples, and 1/3 of the methicillin resistant samples, exhibited a co-resistance with vancomycin. This number may be greater since vancomycin was not assayed in 4 samples evaluated in this study that induced methicillin resistance. Vancomycin and methicillin multiple-antibiotic resistance has been described in clinical settings, but not in the environment (Schaff, et al. 2002). This suggestion of multiple-antibiotic resistance to both vancomycin and methicillin is consequently of concern since the results suggest that mechanisms are present in the environment for conveyance of the deduced co-resistance.

The mechanism(s) that are responsible for the observed antibiotic resistance remain to be investigated. Filter sterilization of all environmental waters evaluated in this study indicate that transmittal of resistance was not associated with conjugation. The potential that point mutations induced the observed multiple antibiotic resistance appears unlikely in

accordance with reports of the low frequency of mutation that induce resistance to even a single antibiotic. Notwithstanding, mutation enhancement of the frequency of mutation *in vitro* has been reported to be an important factor in development of vancomycin resistance in *S. aureus* (Schaff, et. al, 2002). However, enhancement of resistance to other antibiotics assayed was not observed. Integrons that integrate cassettes conveying multiple-antibiotic resistance to up to 4 antibiotics of distinct classes have been described, and such integrons have been observed both as free non plasmid genetic material, free plasmids or phage plasmid associated, and integrated into phage genomes. Any or all of these are mechanistic candidates for induction of the observed resistance profiles. Some evidence for a particle associated conveyance of resistance was obtained in the failure to establish a dose dependency for a sample for which a high resistance activity was repeatedly observed. It is possible that the factor(s) that conveyed resistance existed in a highly dilute state, but the effects were either magnified or the probability of factor transmittal increased with exposure to fresh sample over the course of multiple days.

Summary

The work presented here is the first demonstration of *in vitro* induction of antibiotic resistance in either a Gram-positive or Gram-negative bacterium after exposure to ambient environmental waters, wastewaters, and finished drinking water. Forty-three environmental water samples were assayed for their ability to induce antibiotic resistance in *Staphylococcus aureus* strain 9144. Samples consisted of WWTP influent and effluents, pharmaceutical manufacturing effluents, ambient waters, and finished drinking water. Antibiotic resistance was assayed by determining the Minimum Inhibitory

Concentration (MIC) for compounds representing the beta lactam, macrolide, tetracycline, aminoglycoside, glycopeptides, and sulfonamide classes of antibiotics. MICs were generally determined after 10-day exposures of *Staphylococcus aureus* to each of the environmental water samples.

Environmental water samples increased the MIC values versus the controls in 11 of the 51 (22%) of post exposure *Staphylococcus aureus* samples assayed. Increases in the MIC values for all of the assayed antibiotic/antimicrobial compounds assessed were observed in more than one sample. Antibiotic resistance, observed as increases in the MIC value greater than 2 wells (4-fold) from the control MIC value, was observed most frequently for the tetracycline (22%), the beta lactam ampicillin (20%), and the beta lactam methicillin (18%). The aminoglycoside kanamycin increased the MIC values least frequently (6%). Methicillin was co-resistant with ampicillin and tetracycline in all of the samples exhibiting methicillin resistance. For other antibiotics assayed for their MIC values in environmental water exposed *Staphylococcus aureus,* all displayed multi-resistance with ampicillin and tetracycline resistance suggesting a common origin or assembly of resistance traits. Vancomycin resistance was present in 10% of the exposure samples, and coexisted with methicillin in 75% of the vancomycin resistant samples.

Increases in the MIC values were associated with ambient surface water, WWTP influent and effluent, and finished drinking water. The results are consistent with findings of studies that investigated the occurrence of antibiotics in water samples. The results also parallel the isolation of antibiotic resistant bacteria or genes conferring antibiotic resistance from environmental water samples. The two pharmaceutical manufacturing effluents evaluated did not induce antibiotic resistance. However, the types of pharmaceuticals

manufactured at the sampled facilities, whether they manufacture antibiotics, and wastewater treatment processes employed are unknown.

Variability was evident with respect to the MIC values determined from water samples from which duplicated exposures were assayed. In some cases, the MIC value results from duplicate exposures show very poor agreement. To assess this, samples were re-assayed for MIC. Re-assay results generally confirmed the initial MIC assay results indicating that the discrepancies were attributable to differences in the exposed cultures and not variability in the assay.

Sulfamethoxazole MIC values tended to be difficult to interpret. The cutoff point for determining the MIC was an increase in absorbance to twice that of the previous well in a 96-well assay plate. For the many sulfamethoxazole MIC value assays, the cutoff point was not definitive; measured absorbance tended to slowly increase, eventually reaching a plateau at the level of the associated control samples. Moreover, the absorbance observed for high concentration sulfamethoxazole MIC titers were generally two to three times greater than those of other antibiotics assayed. Absorbance measured for sulfamethoxazole alone was found not to contribute to the observed increase in absorbance in the MIC value assay. While the reason for the observed effect is unknown, it is important to note that in general the MIC value cutoff for sulfamethoxazole was not clear cut, and the interpreted MIC value may overstate the actual MIC value.

The mechanism associated with the observed increases in antibiotic resistance in *SA* after exposure to environmental waters remains unknown and to be determined. The established presence of antibiotics in many ambient waters and wastewaters suggests that selective pressures for antibiotic resistant phenotypes is widespread. However, the

possibility that point mutation alone is responsible appears negligible in light of observation relating to the frequency of mutation alone, and the observations that exposures to environmental waters induced multi resistance phenotypes to antibiotics with dissimilar mechanisms of action and consequent mechanisms of antibiotic resistance.

The mechanism associated with the observed increases in antibiotic resistance in *SA* after exposure to environmental waters remains unknown and to be determined. Corresponding to the results of others, it appears likely that the observed induction of multi-antibiotic resistance results from transfer transduction and/or transformation. Phages or plasmids would be expected to pass through 0.2 u filters. However, bacteria would be excluded, arguing against transfer between the *SA* utilized for exposures and antibiotic resistant bacteria residing in the experimental environmental waters. The relatively long term incubation time (10-days) could permit sufficient time to uptake of DNA from the medium, thereby inducing resistance during exposure. Confirmed discrepancies in results obtained in some cases, for exposures to the same environmental water, suggests that the nature of the samples and their ability to induce antibiotic/antimicrobial resistance in the *SA* used in these studies was indeed variable.

The variability in the antibiotic resistance profiles obtained also suggests one or more mechanisms conveying the observed resistance phenotypes. Resistance to multiple antibiotics of several classes was ubiquitous in samples that demonstrated antibiotic resistance. However, results were not consistent with respect to the observed resistance profiles in a given sample. For example, the WWTP sample Effluent A exhibited relatively high levels of resistance to multiple antibiotics, but did not display resistance to vancomycin. Vancomycin resistance was observed to occur in the majority of samples

that exhibited antibiotic resistance.

Considerable variability in the magnitude of resistance was also observed, but does not appear related to the multi-antibiotic resistance profile. This could be the result of different molecular mechanisms of resistance that affect the actual mode of action of a post translational element as observed for various beta lactamases, penicillin binding proteins, and methicillin resistance *mec* gene products, or transcriptional control by endogenous or exogenous promoters.

Notwithstanding, the public health implications of the ability of environmental waters to induce antibiotic resistance in pathogenic bacteria are substantial. Waters used for direct contact recreation, wild animal or livestock maintenance, and for non-disinfected potable uses could all be reservoirs of various antibiotic resistant bacteria. *Staphylococcus aureus* has recently been detected in environmental fresh and marine waters, with up to 45% of the freshwater and 54% of the marine isolates being resistant to one or more antibiotics (Harakeh, et al., 2006). The potential for infection of humans or animals due to contact or ingestion and the consequent difficulty to employ effective treatments could substantially increase in patient care needs with the high associated costs, as well as potential mortality among susceptible populations including the young, elderly, and immunocompromised individuals. Economic impacts could include reductions in recreational water use, and increased costs of wastewater and drinking water treatment. Moreover, the observed multi-antibiotic resistance profiles obtained demonstrated that exposure to environmental waters induces resistance to methicillin, and in a high proportion of samples exhibiting methicillin resistance, to vancomycin. This observation implies that the potential for resistance to both of the principal antibiotics for addressing *S.*

aureus infections is widespread, and may be of concern in cases where domestic or recreational water use induces infection. Environmental exposure and consequent dissemination of community acquired methicillin and vancomycin resistant *S. aureus* (MVRSA) resulting from this exposure may not be far behind.

The 10-day time generally used for exposure of *SA* to environmental waters to assess induction of resistance may seem extensive from perspective of suspended bacteria in the water column. However, observations of bacterial interactions in surface waters suggests that bacterial attachment to immobile phases is common. Following immobilization on sediments or other materials, bacteria may be exposed for long periods.

The results suggest that the presence of antibiotics and/or antibiotic resistant bacteria in the environment pose a threat to human health. Active control of the release of antibiotics to the environment should be actively undertaken. Various measures have been proposed to reduce the discharge of unused pharmaceuticals, including antibiotics, to waters (Daughton, 2003). While discharge of unmetabolized antibiotics in urine is inherently more difficult, and perhaps impossible to control, it is evident that reducing exposures to humans and animals to pathogenic bacteria could mitigate at least some of the need to employ antibiotics as a first line of defense.

The variability in the observed resistance profiles combined with observations that selected antibiotics alone or in combination did not result in substantial increases in antibiotic resistance in *SA* suggests that the factors underlying the observed resistance are associated with the nature of the environmental water to that the *SA* were exposed. Whether this is a result of inducible resistance due to endogenous traits, or transfer of resistance factors is not discernable. Inducement of antibiotic resistance resulting from

activation of an operon has been observed for induction of multi-antibiotic resistance in *Bacteroides fragilis* when exposed to benzene and benzene derived compounds; for regulation of the multiple antibiotic resistance (MAR) operon for salycilate induced multi-antibiotic resistance in *E. coli;* and for salycilate induction of multi-antibiotic resistance in *S. aureus* (Pumbwe**,** et al., 2007; Cohen, et al., 1993; Riordan, et al., 2007). Since this is the first demonstration that multi-antibiotic resistance could be induced in bacterial by exposure to environmental waters, any suggestions of the underlying mechanism are speculative, at best.

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Curriculum vitae

Dennis J. McChesney

Education

1979-1983	B.S. Biology	University of San Francisco, San Francisco, CA.
1988-1991	M.S. Environmental Sciences	Rutgers University, New Brunswick, NJ. .
1988-1991	M.B.A.	Fairleigh Dickinson University, Rutherford, NJ

Employment History

2005-present: Chief - Groundwater Compliance Section, U.S. EPA Region 2, New York, NY.
Responsible for the federal Underground Storage Tank (UST), Leaking Underground Storage Tank (LUST), and Underground Injection Control (UIC) Regulatory and Enforcement Programs in EPA Region 2.

2004-2005 Environmental Scientist, Pesticides and Toxic Substances Branch/ Toxics Section, U.S. EPA Region 2, Edison, NJ.
Responsible for approvals of federal remedial actions being performed under the TSCA PCB regulations (40 C.F.R. 761.61) in EPA Region 2.

1998-2004 Hydrologist, Monitoring and Assessment Branch, U.S. EPA Region 2, Edison, NJ.
Responsible as the EPA Region 2 Quality Assurance Officer for state water quality programs (New York, Puerto Rico, Virgin Islands) including ambient and drinking water programs and drinking water laboratory certifications. Develop, plan, and implement large scale monitoring projects including groundwater assessments, and fish/ shellfish, water and sediment assessments of the Peconic Estuary in NY, and the San Juan Bay Estuary in Puerto Rico. Coordinated water monitoring assessments for the World Trade Center response activities.

1995-1998 Hydrologist, Groundwater Management Section/Freshwater Protection Section, U.S. EPA Region 2, New York, NY.
Technical expert on Superfund program, groundwater contamination, and aquifer protection. Provided technical and policy support to Superfund program for pre-remedial, remedial, and post remedial investigations; remedial action technologies; remedial design; project scoping and cost analysis; cost recovery; litigation; negotiations with responsible parties, and federal, state, and local government entities; provided support at public meetings.

1992–1995 Environmental Scientist, Groundwater Management Section, U.S. EPA Region 2, New York
Supported Superfund program, groundwater contamination, and aquifer protection. Provided technical and policy support to Superfund program for pre-remedial, remedial, and post remedial investigations; remedial action technologies; remedial design; project scoping and cost analysis; cost recovery; litigation; negotiations with responsible parties, and federal, state, and local government entities; provided support at public meetings.

1986–1987 Research Physiologist, Department of Physiology, University of California – San Francisco, San Francisco, CA.
Developed and utilized novel assays for detection of mammalian hormone receptors expressed in *Xenopus* oocytes. Designed and conducted experiments, coordinated multi-laboratory research project effort.

1983–1986 Research Assistant, Cell Biology Research Laboratory, Mt. Zion Hospital and Medical Center, San Francisco, CA.
Applied immunological and biochemical techniques for: production and analysis of monoclonal antibodies to hormone receptors; receptor isolation and purification; physiological response of gastrointestinal hormones; characterization of brain hormone receptors.

Invited Lectures and Presentations

Halley, M., McChesney, D. J. (2008). Urban Pollution from Funeral Homes. National Groundwater Association Eastern Conference, Ronkonkoma, NY (Abstract submitted for June 2008 invitational presentation).

Schlotter, N, Borst, M., McChesney, D. J. (2008). Organic Carbon as Inhibitor to SVOC and Metal Migration in Stormwater Drywells. National Groundwater Association Eastern Conference, Ronkonkoma, NY (Abstract submitted for June 2008 invitational presentation).

Brock, F. C., Jr., McChesney, D. J. (2008). Underground Injection Control: Requirements for Authorization to Inject. National Groundwater Association Eastern Conference, Ronkonkoma, NY (Abstract submitted for June 2008 invitational presentation).

Ferri, R., Brock, F. C, Jr., McChesney, D. J. (2008). Regulation of Carbon Dioxide Injection to Mitigate Climate Change – Technical Challenges and EPA's Approach under the Underground Injection Control Program. National Groundwater Association Eastern Conference, Ronkonkoma, NY (Abstract submitted for June 2008 invitational presentation).

Halley, M., McChesney, D. J. (2008). Geothermal Wells: Regulatory and Technical

Requirements under EPA's UIC Program. National Groundwater Association Eastern Conference, Ronkonkoma, NY (Abstract submitted for June 2008 invitational presentation).

McChesney, D. J., (2007). EPA Region 2 Contractor UST Inspections to Support State Compliance with EpAct. Presentation at Association of State and Territorial Waste Management Officials Meeting, November 27– 28, 2007, Arlington, VA.

McHale, M. R., McChesney, D. (2004). Comparability of Laboratories Conducting Phosphorus Analyses for Natural Waters in the NYC Reservoir Watershed. 2nd Annual New York City Watershed Science and Technical Conference. September 21- 22, 2004.

McChesney, D. J. (2004). Can the Laboratory Meet My Monitoring DQO's? Methods for Assessment of Laboratory Capabilities and Data Quality Analysis. Presented at 2004 National Water Quality Monitoring Conference, Chattanooga, TN, May 17 – 19, 2004.

McChesney, D. J. (2004). The Accuracy and Precision of Laboratories Analyzing Water Quality Samples for Phosphorus: An Evaluation in the Catskill Mountains of New York State. Presented at 2004 National Water Quality Monitoring Conference, Chattanooga, TN, May 17 – 19,

McChesney, D. J. (2000). Seminar on DNAPL Remediation of Groundwater, University of Taiwan, Taipei, Taiwan, July, 2000.

McChesney, D. J., Smeiszek, E. J. (2000). Sensitivity and Vulnerability Assessments of Public Water Supply Ground Water Wells. Sixth Drinking Water Seminar, American Water Works Association, Rio Mar, Puerto Rico, May, 2000.

McChesney, D. J. (1996). Application of New Approaches for Site Investigations. Graduate Seminar given at Drexel University Department of Environmental Science and Engineering, Philadelphia, PA, October, 1996.

McChesney, D. J. (1996). State of the Science Approaches to Modeling Contaminated Groundwater. Presentation at the U. S. Department of Defense Groundwater Modeling Workshop, Albuquerque, NM, August, 1996.

McChesney, D. J. (1993). *In situ* Bioremediation of Soils and Aquifers. Lecture to graduate level Pollution Microbiology students, Rutgers University, New Brunswick, NJ. Fall 1993.

Course Taught

Groundwater Hydrogeology and Contamination: Hunter College, Graduate School of Environmental./Public Health Sciences., New York, NY.
Designed curriculum, taught 3 credit graduate course.

Peer Reviewed Publications

McHale, M. R., McChesney, D. (2007). Phosphorus Concentrations in Stream-Water and Reference Samples - An Assessment of Laboratory Comparability. USGS Open-File Report 2007-1267.

Litten, S., McChesney, D. J., Hamilton, M.C., Fowler, B. (2003). The World Trade Center Disaster and PCBs, TCDD/F's, TBDD/F's and Polybrominated Diphenyl Ethers in Water, Sediment, and Sewage Sludge. Environmental Science and Technology, 37(24), 5502-5510.

Diamond, J., Eaton, A., Annis, C., Brass, H., Keith, L., Strong, A., McChesney, D., Shockey, M. (2001). Towards a Definition of Performance- Based Laboratory Methods. National Water Quality Monitoring Council Technical Report 01-02, 13pp.

Williams, J. A., McChesney, D. J., Calayag, M. C., Lingappa, V. R., Logsdon, C. D. (1988). Expression of receptors for cholecystokinin and other Ca2+-mobilizing hormones in *Xenopus* oocytes. Proceedings of the National Academy of Sciences, 85, 4939-4943.

Burnham, D. B., McChesney, D. J., Thurston, K.C, Williams, J. A. (1984). Interaction of cholecystokinin and vasoactive intestinal polypeptide on function of mouse pancreatic acini *in vitro*. Journal of Physiology, 349; 475-482

Book Chapters

Howard, G., Jahnel, J., Frimmel, F. H., McChesney, D., Reed, B., Schijven, J., Braun-Howland, E. 2006. Human excreta and sanitation: Potential hazards and information needs. *In:* O. Schmoll, G. Howard, J. Chilton and I. Chorus (eds.) Protecting Groundwater for Health. IWA Publishing (WHO drinking-water quality series), London, pp. 275-308.

Howard, G., Reed, B., McChesney, D., Reed, B., Taylor, R., (2006). Human excreta and sanitation: Control and Protection. *In:* O. Schmoll, G. Howard, J. Chilton and I. Chorus (eds.) Protecting Groundwater for Health. IWA Publishing (WHO drinking-water quality series), London, pp. 587-612.

Advisory Committees and Workgroups

1994 – 1995 Chariman – U.S. EPA Region 2 Groundwater Sampling Workgroup.
1994 – 1996: EPA Region 2 Liaison - Rutgers Natural Bio-Attenuation Project in New Jersey.
1995 – 1996 Co-Chair – U.S. EPA National Groundwater Protection Technical Forum.
1999 – 2004: Alternate Co-chair: Methods and Data Comparability Board (a workgroup

of the National Water Monitoring Council/President's Advisory Commission on Water Information).

EPA Awards

1993 Special Achievement Award - Environmental Education Grants Review
1993 Outstanding National EPA Volunteer - Education of America's Youth (Wetway)
1993 Bronze Medal Award - NYC Water Supply Filtration Avoidance Decision
1994 Bronze Medal Award (from EPA Region 3) - Review of Philadelphia International Airport Development Project
1995 Bronze Medal Award - Low-Flow Sampling Workgroup
2001 Bronze Medal Award - Peconic Estuary CCMP
2002 World Trade Center Response Team Recognition