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Antibiotic resistance in triclosan tolerant fecal coliforms isolated from surface waters near wastewater treatment plant outflows (Morris County, NJ, USA)

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ABSTRACT

Triclosan (TCS) is a common antimicrobial agent that has been detected in wastewater treatment plant (WWTP) effluent outflows. A link between TCS exposure and increased antibiotic resistance in microbes has been postulated. The purpose of this study was to evaluate whether fecal coliforms (FC) isolated from surface waters located near (WWTP) outflows display TCS resistance and, if so, whether such organisms exhibit increased resistance to antibiotics. Water samples were collected at two streams in Morris County, NJ that receive WWTP effluent: Loantaka Brook and the Whippany River. Water samples were collected at three sites within each location near the WWTP effluent outflow. Abiotic river parameters were measured and FCs were enumerated for each sample. River parameters were analyzed to determine if TCS or antibiotic resistance was correlated to water quality. Triclosan resistance levels were determined for individual isolates, and isolates were screened against seven classes of antibiotics at clinically relevant levels to assess cross-resistance. At Loantaka Brook, 78.8% of FC isolates were resistant to TCS with an average minimum inhibitory concentration (MIC) of 43.2 $\mu g\ ml^{-1}$. In addition, 89.6% of isolates were resistant to four classes of antibiotics and all were identified as *Citrobacter freundii*. There was a significant effect of stream location on mean TCS MIC values in the Loantaka Brook, with effluent isolates maintaining significantly higher MIC values compared to upstream isolates. At Whippany River sites, TCS resistant isolates were detected on 94% of sampling dates with a significant relationship between TCS resistance and multiple antibiotic resistances ($\geq$ three antibiotic classes, $p<0.001$). TCS resistant isolates were significantly more resistant to chloramphenicol ($p=0.007$) and to nitrofurantoin ($p=0.037$) when compared to TCS sensitive isolates. Environmental FC isolates resistant to high level TCS included species of *Escherichia*, *Enterobacter*, *Serratia* and *Citrobacter*. There was no correlation between river water quality and resistance of isolates to TCS. Presence of isolates not resistant to TCS, but resistant to other antibiotics, were significantly correlated to increased river flow, precipitation, and decreased nutrient levels, suggesting that observed resistance is due to run-off events. This study demonstrates that TCS resistant FC are common in river systems receiving WWTP effluent and display multiple drug resistance.

1. Introduction

Treated wastewater effluent serves as an important reservoir for environmental antibiotic resistant bacteria (Baquero et al., 2008; Kümmerer, 2009). In wastewater treatment plants (WWTPs) antibiotic resistant bacteria, tolerant bacteria, the genetic elements of horizontal gene transfer (plasmids, transposons), and anthropogenic chemicals, including antimicrobials and biocides, intermingle in the aqueous environment (Costa et al., 2006; Ferreira da Silva et al., 2007; Schlüter et al., 2007; Watkinson et al., 2007; Kelly et al., 2009; Zhang et al., 2009). Within the WWTP, bacteria from human and animal sources interact while under selective pressure from discarded waste chemicals. The spread of antibiotic resistance determinants is strongly favored in such a milieu (Summers, 2006; Kelly et al., 2009). The WWTP effluent is then released and disseminated to receiving waters including rivers and streams.

Triclosan (TCS, 2,4,4′ trichloro-2′-hydroxydiphenyl ether) is a synthetic chlorinated phenylether bisphenol with broad-spectrum antimicrobial properties (Bester, 2003). At high concentrations it functions as a biocide while at lower concentrations it is bacteriostatic (Jones et al., 2000; Russell, 2004; M. Gomez Escalada et al., 2005). In recent years triclosan (TCS) has become

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the most widely used antibacterial agent in the US and is now a common ingredient in toothpastes, soaps, deodorants, and other hygiene products at concentrations up to 0.3% (Bhargava and Leonard, 1996; Levy, 2001; Schweizer, 2001; Singer et al., 2002; Russell, 2004). Greater than 95% of all consumer products containing TCS enter wastewaters by disposal of the product down the drain (McAvoy, et al., 2002; Reiss, et al., 2002). Triclosan has been detected in entering wastewater, discharge effluents, sediment and sewage sludge of wastewater treatment plants (WWTPs). The majority of TCS is removed from wastewater by degradation or adsorption to sludge (95%–99%) while the remainder passes through the treatment plant and is discharged as effluent (McAvoy et al., 2002; Singer, et al., 2002; Waltman, et al., 2005). The remaining 5% of TCS continually inoculates aquatic ecosystems, creating a chronic exposure for organisms. Triclosan has been detected in rivers and streams in the US at concentrations ranging between 0.1 and 1.0 $\mu g\,L^{-1}$ (Kolpin et al., 2002; Halden and Paull, 2005; Phillips and Chalmers, 2009).

At high concentrations TCS damages bacterial cell membranes and interferes with protein and lipid synthesis while at lower concentrations TCS inhibits the synthesis of fatty acids in *Escherichia coli* by blocking the action of the enzyme enoyl reductase (Fab I) (McMurry et al., 1998a; M.G. Gomez Escalada et al., 2005; Cottell et al., 2009). *E. coli* strains with a mutant Fab I enzyme exhibit increased TCS resistance compared with wild-type strains. *E. coli* strains that overexpress *marA* allow the up-regulation of *acrAB*, a multidrug efflux pump which effectively removes TCS from the bacterium before cellular damage can be inflicted (McMurry et al., 1998b; Schweizer, 2001). It now appears that multiple mechanisms of action may be responsible for increased tolerance to TCS (Gilbert and McBain, 2003; Saleh et al., 2011).

It has been suggested that exposure to TCS in the environment may select for bacterial strains tolerant to the biocide and exhibiting increased resistance to antibiotics through co- or cross-resistance mechanisms (Chuanchuen, et al., 2001; Maillard, 2007; Cottell et al., 2009). Several researchers have demonstrated cross-resistance between TCS tolerance and low-level resistance to antibiotics including β-lactams, aminoglycosides, chloramphenicol, fluoroquinones, ampicillins and tetracyclines (Randall et al., 2004; Braoudaki and Hilton, 2004a,b; Tkachenko et al., 2007). Some researchers have failed to demonstrate such cross-resistance (Lambert, 2004; Lear et al., 2006; Stickler and Jones, 2008) while other researchers have noted increased susceptibility to specific aminoglycosides in TCS tolerant strains of *E. coli* (Cottell et al., 2009).

Biological water quality is commonly assessed through fecal coliform analysis (US EPA, 2011). The coliform group, species which comprise the normal human and animal intestinal flora, include the genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* (Guentzel, 1996). This group includes opportunistic pathogens responsible for a variety of infections. A subgroup, the fecal coliforms (FC) are characterized by growth at elevated incubation temperature (44.5 °C). Currently, the presence of FC in water is used as an indicator of fecal contamination (Carrero-Colon et al., 2011). *Escherichia coli* is normally a commensal organism but can be a significant pathogen associated with gastrointestinal and urinary tract infections (Donnenberg, 2005). The recent *E. coli* outbreak in Germany (2011) is a reminder of the extreme genome plasticity of these bacteria and the potential for the emergence of new virulent strains (Moriel et al., 2012).

Emerging antibiotic resistance is a well established public health concern (Levy, 2001; Fraise, 2002; Braoudaki and Hilton, 2004b; Brusselaers et al. 2011; de Kraker et al., 2011). The availability of efficacious chemotherapeutic agents is critical to the successful treatment of a wide variety of infections and effective antibiotic therapy could be significantly compromised with the proliferation of antibiotic resistant strains. Therefore, biocide agents with the potential to induce antibiotic cross-resistance in the environment require careful evaluation.

The purpose of this study was to determine the presence and prevalence of environmental strains of thermotolerant fecal coliforms exhibiting tolerance to TCS in local surface waters near WWTPs and to examine whether such strains demonstrate increased resistance to antibiotics. In addition, abiotic river parameters were analyzed to determine if TCS and/or multiple drug resistance were correlated to river water quality.

2. Materials and methods

2.1. Water sampling

Water samples were collected from local surface waters in two different river systems in Morris County, NJ, USA. At each location, three sites near wastewater treatment plant (WWTP) outflows were selected: Site 1, approximately 190 m upstream from the WWTP outflow; Site 2, near outflow; Site 3, approximately 190 m downstream from the WWTP outflow. In 2010, samples from Loantaka Brook near the Woodland WWTP (40° 46′ 36″N, 74° 44′ 55″W) were collected on seven sampling dates. In 2011 samples from the Whippany River near the Butterworth WWTP (40° 46′ 68″N, 74° 29′ 49″W) were collected on seventeen sampling dates. All grab water samples were collected from approximately 0.4 m below the water surface from near the center of the brook or river. Water samples for microbiological analysis were collected aseptically in sterile wide-mouth 500 ml clear glass sampling bottles and held at 4 °C until processed; all samples were processed within 4 h of collection. Water samples for water quality analysis were collected in 1 L brown glass bottles and held at 4 °C in the dark for no more than 12 h before being analyzed.

2.2. Water quality analysis

Woodland WWTP releases 1.5×10^6 gal d^{-1} of treated effluent into Loantaka Brook while Butterworth WWTP releases 3.3×10^6 gal d^{-1} of treated effluent into the Whippany River. Both WWTPs are located in Morris Township, NJ and are tertiary, activated sludge A/O (aerobic/oxic), treatment plants that utilize anaerobic and aerobic bacterial digestion. These systems internally recycle a portion of the A/O effluent containing microorganisms while sending the remaining effluent to final clarification tanks for BOD (biological oxygen demand), TSS (total suspended solid), and P (phosphorous) reduction. Prior to release, final effluent is sand filtered to further decrease suspended solids and is UV sterilized. Residence time for the entire treatment process ranges between 24 and 28 h (M. Howarth, Water Pollution Control Utility (WPCU), Morris Township, personal communication). The average final effluent parameter values include: NH_4= 0.22 mg L^{-1}, BOD=1.4 mg L^{-1} (99% removal), fecal coliforms ≤ 1 colony, TSS= 2.1 mg L^{-1} (99% removal), and P=0.4 mg L^{-1} (Woodland) & 0.8 mg L^{-1} (Butterworth) (J. Morrison and M. Howarth, WPCU, Morris Township, personal communication). More stringent water quality standards (P levels) are in place for WWTPs (including Woodland) that discharge into streams feeding the Great Swamp National Wildlife Refuge.

Water quality parameters were evaluated at each site on each sampling date. Stream velocity was measured (m s^{-1}) using a digital flow meter (Global Water FP111), while dissolved oxygen (D.O.), pH, and temperature were quantified with a digital multiparameter unit (YSI Pro Plus). Dissolved nutrient quantification was analyzed within 2 h of sample collection. Dissolved nitrates (NO_3), nitrites (NO_2), phosphates (PO_4), and hardness (Mg+Ca), in mg L^{-1}, were quantified using Hach® chemical reagents with a Hach® spectrophotometer (DR/890).

2.3. Isolation and enumeration of bacterial isolates

Water samples (1–50 ml) were filtered through 0.45 μ membrane filters (Millipore, Billerica, MA), and placed on a filter pad saturated with mFC broth (base supplemented with 1% roseolic acid) (Becton, Dickinson, Sparks, MD), a selective medium specifically formulated for the enumeration of fecal coliforms by the membrane filtration method without prior enrichment. All samples were incubated at 44.5 °C for 18–24 h. From each water sample representative unique isolates, including a range of blue colonies, were randomly selected from the mFC plates. Selected isolates, classified by this selection process as total thermotolerant fecal coliforms (FC), were enumerated and reported as means of triplicate samples (total CFU 100^{-1} ml). For 2010 samples up to eighteen isolates were selected while up to 24 isolates were selected from 2011 samples; a lesser number of isolates was evaluated only when fewer unique colonies than the protocol number were present on mFC isolation plates. Unique FC isolates (4–24 per sample) were selected to microtitre plates containing 180 μl mFC broth and incubated at 44.5 °C