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Effects of prevalent freshwater chemical contaminants on in vitro growth of *Escherichia coli* and *Klebsiella pneumoniae*

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Using a microtiter plate assay, E. coli and Klebsiella bacteria were exposed to a panel of common chemical pollutants of fresh water; only ethylene glycol and 2,4-D inhibited bacterial replication.

Abstract

Many surface and ground waters in the continental US are contaminated with a variety of chemical pollutants, which are usually present in concentrations in the ppm and ppb range. The effects of these pollutants on coliform bacteria, which are prominent members of the aquatic flora, are poorly understood. Using a microtiter plate assay, isolates of *Escherichia coli* (from chicken intestine and fresh water), and an isolate of *Klebsiella pneumoniae* (from bovine milk) were exposed to varying concentrations of common pollutants over a 24 h period. The herbicides/pesticides simazine, atrazine, and diazinon; the VOCs trichloroethene and MTBE; the estrogens estradiol and estrone; and caffeine, all failed to inhibit bacterial growth at ppm levels. Only ethylene glycol, and the herbicide 2,4-D, significantly inhibited bacterial growth compared to controls. These results suggest that the replication of coliform bacteria in fresh waters is not adversely impacted by many common pollutants. Published by Elsevier Ltd.

Keywords: Coliforms; *E. coli*; *Klebsiella*; VOC; Pesticides; Estrogens

1. Introduction

Many surface and ground waters in industrialized countries are contaminated with a variety of chemicals of anthropogenic origin. For example, in the United States, a nationwide survey of 98 aquifers found volatile organic compounds (VOC) in 90 of them; 90% of the positive samples were at concentrations of <1 μg/L. The mostly commonly encountered VOC included: solvents, such as trichloroethene; trihalomethanes (THM), such as chloroform; refrigerants, such as dichlorofluoromethane; and gasoline additives such as methyl *tert*-butyl ether (MTBE) (Zogorski et al., 2006).

Pesticides and pesticide degradation products also are prevalent in US surface and ground waters; according to the National Water Quality Assessment (NAWQA) Program, pesticides were detected in >90% of water samples obtained from streams located in agricultural, urban, and mixed land-use areas. Atrazine, simazine, and diazinon were the most frequently detected pesticides in all three types of watershed. Insecticides that have been banned from usage for several decades, such as DDT, dieldrin, and chlordane, persist in stream water and sediments associated with agricultural operations (Gilliom et al., 2006; Scribner et al., 2006).

In addition to pesticides and VOC, various other chemicals have attracted interest, either for reasons of public health, or as indicators of water quality. Among these chemicals are caffeine, which may approach concentrations of 300 μg/L in wastewater, and which may have value for purposes of 'source tracking' the introduction of pollutants into affected streams (Standley et al., 2000). Another class of chemicals of public health interest are the estrogens 17β estradiol (E2), estrone

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(E1), and 17α ethinylestradiol (EE2), which can mediate endocrine disruption of aquatic vertebrates. High concentrations of estrogens can be detected in stream water downstream of waste treatment plants in many industrialized countries, and there is evidence that agricultural operations, such as livestock rearing, may play a role in depositing these chemicals into waterways (Burnison et al., 2003; Falconer et al., 2006; Khanal et al., 2006; Matthiessen et al., 2006). In addition to estrogens, livestock operations (particularly concentrated animal feeding operations or CAFO) may be responsible for the deposition of a variety of antibacterial and antiparasitical compounds into freshwaters (Boxall et al., 2004).

Historically, studies on the effects of such chemicals on aquatic life have focused on macroscopic organisms, with particular emphasis on vertebrates (for example, Hayes et al., 2006). However, we note that bacteria represent important members of the fresh water flora and are presumably vulnerable to alterations in their growth and reproduction due to exposure to chemical contaminants. Studies conducted in a variety of countries indicate that <1 to $>16 \times 10^6$ bacterial cells may be present in 1 mL of stream water. Whether all of the cells forming this considerable proportion of stream biomass are viable is unclear; typically, heterotrophic plate counts result in the culture of <1 to $>36 \times 10^4$ colony-forming units (CFU) per mL of water (Lemke and Leff, 2006). Thus, ~1% of the cells in a milliliter of stream or river water are capable of replicating in the laboratory setting in the presence of abundant concentrations of nutrients.

Despite the substantial contribution of bacteria to the aquatic biomass, the influence (or lack of it) of the abovementioned chemicals on bacterial viability at concentrations expected to be encountered in fresh water is poorly understood. Most of the available literature tends to focus on examining mutagenic effects of such chemicals using bacteria-based assay systems in conjunction with those for algae, crustaceans, and vertebrates, rather than investigating inhibition of bacterial replication per se. For example, Kado et al. (1998) conducted in vitro genotoxicity testing for MTBE on *Salmonella*; the highest concentration used, 7400 μg in a culture tube, had a toxic effect on both tester strains TA98 and TA100. In a study expressly designed to investigate the effect of a common water pollutant on the replication of enteric bacteria, Koutsotoli et al. (2005) exposed *Escherichia coli* and *Enterococcus faecalis* to atrazine at concentrations of 0.1, 1, 10, and 100 μg/L for up to 80 days and observed phenotypic and morphologic changes in the bacteria. These authors also reported that replication of the bacteria in the presence of atrazine exceeded that of non-exposed controls. In another study involving a commonly used herbicide, Balague et al. (2001) reported abolition of *E. coli* growth at 2 mM concentration of 2,4-dichlorophenoxyacetic acid (2,4-D).

In an effort to add to our understanding of the effects of anthropogenic chemicals on the survival of bacteria in the freshwater environment, we carried out a series of in vitro experiments in which the coliform bacteria *E. coli* and *Klebsiella pneumoniae* were exposed to varying concentrations of the more commonly reported contaminants. The experimental protocols we used were designed to measure changes in the growth profiles of these bacteria over the short term (i.e., 24 h) when they are actively replicating, and thus (presumably) vulnerable to deleterious effects of chemical contaminants.

2. Materials and methods

2.1. Bacteria

Two isolates of *E. coli* were used; the first originated from the polluted freshwater stream Gwynn's Run (GR) (Higgins et al., 2005), located in metropolitan Baltimore, Maryland. The isolate was made in Fall 2003 from a grab sample of 100 mL. The second isolate originated from the caecal mucosa of a chicken and was provided by Dr R. Joerger of the University of Delaware in 2005. The *K. pneumoniae* isolate originated from cow milk collected in 2005, and was provided by Dr W. Hare of the Beltsville campus of the US Department of Agriculture. All isolates were maintained as glycerol stocks at −70 °C. Prior to use in an experiment they were thawed and grown to stationary phase (aerobically) in LB (Luria–Bertani) broth at 37 °C; the concentration of cells, as determined by light microscopy, was $\sim 10^7$–10^8 cells per mL. Three microliter aliquots ($\sim 3 \times 10^5$–3×10^6 cells) of these 'starter' stationary phase cultures, warmed to 37 °C, were used to inoculate wells of the 96-well plates used for exposure assays (below). Starter cultures were kept at 4 °C for no longer than 5 days before used in experiments.

2.2. Chemicals

Caffeine, 17β estradiol, estrone, ethylene glycol, and methyl-*tert*-butyl ether were purchased from Sigma/Fluka (St Louis, MO). Simazine, diazinon, atrazine, 2,4-D (2,4-dichlorophenoxyacetic acid), and trichloroethene were purchased from Chem Service (West Chester, PA). Chemicals were diluted in ethanol or sterile water and maintained at room temperature in the dark in glass vials with Teflon-lined caps.

The concentrations of chemicals used in the microtiter plate exposure assays (below) were informed by published data on their concentrations per unit volume (usually 1 L) in streams and/or aquifers. For example, if atrazine is present in some agricultural streams at a concentration of 50 ng/L (Gilliom et al., 2006) this equates to 50 fg/μL; therefore, a microtiter plate well destined to receive atrazine at a concentration equivalent to 50 ng/L received 50 fg/μL × 200 μL DMEM (Dulbecco's minimal essential medium) per well $= 10^4$ fg, or 10 pg, atrazine per well. Efforts were made to minimize the volume of chemical added to wells to ≤5 μL (the exceptions were 50 mM ethylene glycol at 10 μL). For those chemicals for which dilutions were made in ethanol, we attempted to use the minimal volume of diluent so that wells on the microtiter plate received ≤5 μL ethanol. Assays were carefully observed for the formation of precipitates within the wells (as opposed to clumping of bacterial cells); if precipitates were noted, the assay was discontinued and subjected to an alternative protocol for chemical deposition in which the chemical (in water or ethanol) was pipetted into the empty wells of the plate and briefly heated at 60 °C to dry the liquid onto the interior of the well. Medium then was added to the wells with vigorous pipetting used to solubilize the dried material.

2.3. Exposure assays

The culture assay was adapted from that used by Sheik et al. (2001) for the quantitation of biofilm formation by enteroaggregative pathotypes of *E. coli*. The culture medium was DMEM supplemented with glucose at 4.5 g/L (catalogue No. 12-917F, Cambrex, Walkersville, MD) and L-glutamine (2 mM). (This formulation of DMEM lacks phenol red and thus has a neutral coloration). For all assays but those involving MTBE, 200 μL aliquots of warm (37 °C) DMEM were placed into the wells of a polystyrene 96-well microtiter plate (Falcon, Becton Dickson Labware, Franklin Lakes, NJ). Chemicals (warmed to room temperature) were then added to the wells, followed by the addition of bacteria, after which the plates were kept under aerobic conditions in a 37 °C incubator and removed at given intervals for absorbance readings