APPLICABILITY OF STANDARD ANTIBIOTIC TOXICITY TESTS TO THE AMBIENT AQUATIC ENVIRONMENT

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ABSTRACT

Antibiotics enter the aquatic environment via wastewater and other sources, where they may promote selection of resistant bacteria, and thus add to the global reservoir of antibiotic resistance. Ambient concentrations typically are several orders of magnitude below the lowest observed effect concentration (LOEC) or minimum inhibitory concentration (MIC), which suggests this is unlikely. However, the dissolved organic matter (DOM) concentration in conventional MIC laboratory assays is typically three orders of magnitude higher than in the ambient aquatic environment. Partitioning of antibiotics on DOM could affect their bioavailability making the laboratory MIC values inapplicable to the ambient environment. This question was investigated using laboratory experiments with *E. coli*, tetracycline and DOM varied over six orders of magnitude. For the DOM concentrations that were able to support significant growth, the calculated MIC endpoint was 1 mg/L. No media effect was observed, which suggests that sorption to MIC test media is insignificant and that the laboratory-determined MIC values are applicable to the ambient environment.

Keywords: Antibiotic, tetracycline, bioavailability, sorption

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1. INTRODUCTION

Antibiotics, used extensively for human medicine and agriculture, enter the aquatic environment via wastewater and other sources, where they have been found at measurable concentrations [1,2]. There may be adverse effects on non-target organisms (i.e. not bacteria) [3,4]. Also, there is concern that the antibiotics may promote selection of resistant bacteria and thus add to the global reservoir of antibiotic resistance [5,6]. This paper is concerned with the effect of antibiotics on bacteria.

Ambient environmental concentrations of antibiotics typically are far below their effect concentration established using the common minimum inhibitory concentration (MIC) laboratory assay. For tetracycline, for example, ambient surface water concentrations are typically ≤0.11 µg/L [1,7], although values as high as 6.8 µg/L have been observed in more heavily impacted systems [8]. MIC values are about 3,000 µg/L for clinical pathogens [9] and 2,000 µg/L for environmental isolates [10].

The conditions in the environment are quite different from those of the conventional MIC test. Specifically, the DOM concentration in the ambient aquatic environment typically is about 6 mgC/L, whereas MIC tests (liquid broth or solid agar) generally are done in growth medium at DOM concentration of about 6,000 mgC/L [11]. Toxicity tests for environmental bacteria also generally add growth media at high concentrations [10,12,13]. Antibiotics, including tetracycline, absorb on DOM [14], which may affect bioavailability.

Accounting for bioavailability is a well-established concept in environmental toxicology. For hydrophobic organic compounds, it is generally accepted that the truly dissolved (i.e. not bound to solids or DOM) form of the compound is bioavailable [15,16].

The truly dissolved concentration can be estimated using a partitioning calculation. Sorption of tetracycline on DOM involves a number of mechanisms (e.g. cation exchange, [17]), but their quantification requires information on solution chemistry and (more importantly) DOM properties, which are not available. Therefore, a simple partition coefficient is used here, as was done previously in models of tetracyclines in the aquatic environment [18,19] and soil [16]. The freely dissolved concentration C_{fd} (µg/L) is [20]:

$$C_{fd} = C \frac{1}{1 + K_{d,DOM}\left[\mathrm{DOM}\right]} \quad (1)$$

where C (µg/L) is the total concentration, $K_{d,DOM}$ (L/kgC) is the DOM partition coefficient and [DOM] (kgC/L) is the DOM concentration. Partitioning of tetracycline to growth media has not been investigated, but $K_{d,DOM}$ for various environmental DOMs (e.g. humic acid, natural organic matter) are available (Table 1). Using the above equation with $K_{d,DOM} = 10^{4.2}$ L/kgC (Table 1), a total concentration of C = 1,000 µg/L corresponds to a freely dissolved concentration of C_{fd} = 10 µg/L in the MIC test ([DOM] = 6,000 mgC/L) and 900 µg/L in the ambient aquatic environment ([DOM] = 6 mgC/L). This suggests that the potency of tetracycline may be increased by two orders of magnitude in the ambient aquatic environment.

Limited studies have explored the effect of environmental factors on the toxicity of tetracycline. Garrett and Miller [21] observe no significant effect of media concentration on growth rates, but concentrations were only varied by a factor of two and other parameters (salt) were different as well. Chander et al. [22] found reduced toxicity in a soil-water mixture with higher tetracycline affinity. These experiments do not cover the 1,000-fold difference in DOM concentration but they are generally consistent with the partitioning mechanism.

We were concerned that the bioavailability in the MIC test and ambient environment are very different, and that the MIC toxicity values may not be applicable to the ambient environment. Specifically, we hypothesized that the high DOM concentration in the MIC test may significantly reduce the bioavailability of antibiotics, and that the environmental potency of antibiotics is much higher in the ambient environment. This is an important question that needs to be answered to properly address the environmental impacts of antibiotics. Based on our literature review, this issue has not been addressed.

We performed a number of growth experiments with *E. coli* and tetracycline at various DOM concentrations to determine the effect of DOM concentration. At DOM concentrations high enough to support significant growth (1.2 - 6,000 mgC/L, almost four orders of magnitude), no media effect was observed, suggesting that sorption to MIC test media is negligible and that MIC values are applicable to the ambient aquatic environment.

2. MATERIALS AND METHODS

Escherichia coli K-12 MG 1655 was cultured using standard methods (details in [11]). Experiments with exponential and stationary phase cells were performed to cover a range of conditions. Bacterial colony-forming units (CFU) were ~4.0×10^8 CFU/100mL and ~1.5×10^{11} CFU/100mL after 2 hr (exponential phase) and 22 hr (stationary phase) of incubation, respectively. For growth medium, Luria-Bertani broth (TEKNOVA) and Mueller-Hinton II cation-adjusted broth (TEKNOVA) were used. The organic carbon concentration of LB medium was measured to be approximately 0.3 gC/gLB [11]. Thus, 20g LB/L corresponds to 6,000 mgC/L, and 22 gMHB/L corresponds to 6,600 mgC/L, and concentrations of dilutions are calculated from these values. Tetracycline hydrate (99%) (Aldrich) was dissolved and diluted in deionized water in 15 mL Falcon tubes wrapped in aluminum foil to prevent light degradation.

Table 1 Tetracycline partitioning to dissolved organic matter (DOM)

log $K_{d,DOM}$ (L/kgC)	DOM	Reference
3.2	Elliot soil humic acid (ESHA)(a)	Gu and Karthikeyan [24]
3.6-4.2	ESHA(b)	Gu et al. [17]
4.4-4.7	Aldrich HA(c)	Sithole and Guy [25]
4.8-5.4	River and wetland NOM	Verma et al. [26]

(a) 0.01 M *I*, estimated from data in reference.
(b) sorption and desportion, 0.01 M NaCl, , estimated from data in reference.
(c) Fit to linear portion below Ceq = 5 µM, assumed foc = 0.34.

For the toxicity experiments, LB or MHB medium and tetracycline were added to phosphate buffer solution (PBS, [23]) in 250 mL Pyrex wide-mouth flasks. The flasks were wrapped with aluminum foil, covered with cotton swabs, stirred at 400 rpm, and kept at 20 °C. Experiments with and without equilibration were performed to cover a range of conditions. For equilibration, bacteria were added after 24 hours. For no equilibration, bacteria were added after 10 min. Cell densities were counted using membrane filtration [11]. A sample was filtered through 0.45 μm filters, put on LB agar plates, incubated at 37°C for 24 hours, and colonies were counted visually.

The minimum inhibitory concentration (MIC), the lowest observed effect concentration (LOEC), is calculated as follows. For each experiment, the growth rate is calculated as the regression slope of the natural logarithms of the cell densities vs. time. The MIC for each set of experiments is taken as the minimum concentration for which the growth rate is significantly lower (α = 1%) than that of the corresponding experiments with lower tetracycline concentration.

3. RESULTS AND DISCUSSION

The results are presented in Figure 1, which shows the time course of cell densities for experiments with different tetracycline and DOM concentrations. The growth media serves as substrate for growth and it may (as hypothesized) serve as a partitioning medium. Therefore, the growth rate is expected to be higher at higher DOM concentration due to two factors: higher nutrients and less bioavailable tetracycline. For example, in the experiments with MHB media, the growth rate for the 1,000 μg/L tetracycline treatment is higher at 6,600 than at 120 mgC/L DOM (yellow line in panels B1 and B2). Is this due to higher substrate or less available tetracycline? The effect of DOM on the bioavailability of tetracycline cannot be judged by differences in the growth rates of experiments with different DOM concentration.

To interpret the results, we first examine differences between experiments with various tetracycline concentrations for a given DOM type and concentration (i.e. within each panel in Figure 1). Specifically, we determine the minimum inhibitory concentration (MIC) for each panel by comparing the growth rates. Then we compare the MIC for different DOM concentrations. An increase in MIC with increasing DOM is consistent with a reduction in bioavailability due to partitioning.

Visual examination of the data in Fig. 1 suggests growth inhibition for the 1,000 and 10,000 μg/L tetracycline concentrations for all experiments, except those with the lowest DOM concentration (panel A4). For this set of experiments, no significant differences are evident and densities for all tetracycline concentrations are relatively close (note y-axis scale). We attribute this to the low DOM available for growth. For the lower tetracycline concentrations (0, 1, 10 and 100 μg/L) there is no consistent pattern for the effect of tetracycline on growth. For example, the highest growth rate in panels A2 and A3 are for 0 and 10 μg/L tetracycline, respectively. These differences are likely due to experimental variability, as illustrated by the experiments with 0 μg/L tetracycline in panel A1 (identical experimental conditions). The calculated MIC (see Methods section) is 1,000 μg/L for all panels in Fig. 1 (except panel A4).

For the experiments with sufficient media concentration to support growth, the MIC end point is 1,000 μg/L. This is consistent for various experimental conditions, including type of media (LB vs. MHB), growth phase of cells (exponential vs. stationary) and sorption equilibration (equilibration vs. no equilibration). There is no correlation between MIC and DOM. The experiments covered DOM concentrations from those of pure growth media (6 gC/L) down to those far below those typical of surface waters (12 μgC/L). No media effect is observed, which suggests no significant sorption to MIC test media, and laboratory-derived MIC values should be applicable to the ambient aquatic environment.

These results are for tetracycline, and the situation may be different for other antibiotics. Of course, the bioavailability in the aquatic environment will also be affected by partition to natural DOM and solids, which has to be considered [16].

We previously presented a model of tetracycline in the Poudre River [19]. In that study, we used a partitioning coefficients based on environmental DOMs to calculate partitioning to MIC test media (see Introduction section), which resulted in a significant increase in potency. This led to selection of antibiotic resistant bacteria in the river. The results presented here suggest that there is no significant sorption to MIC test media, and therefore the presence of tetracycline-resistant bacteria in the Poudre River cannot be explained by the effect of the antibiotic (i.e. see Models 3B2 and 3C2, Fig. S4, ref. [19]).

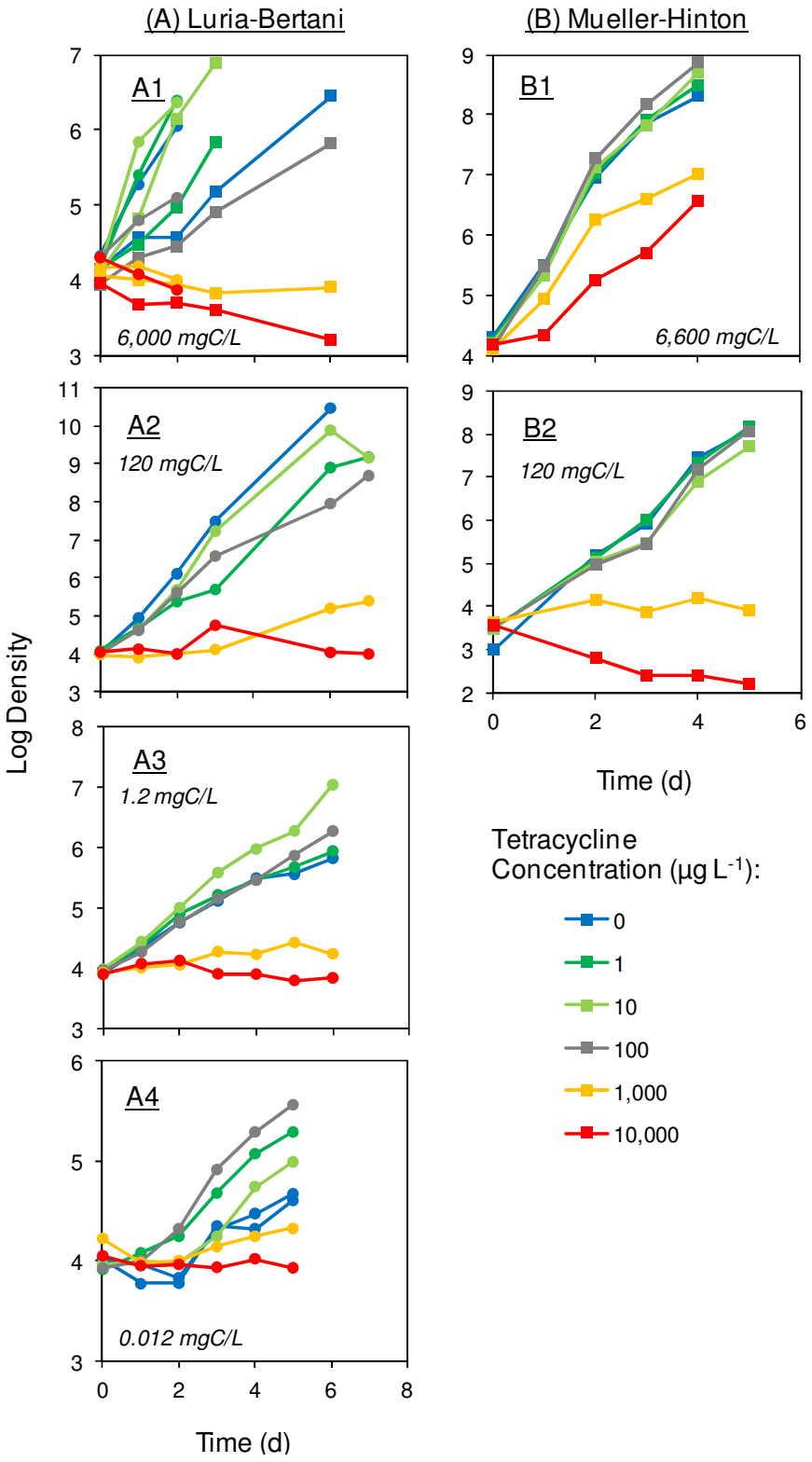

Figure 1 Time course of bacteria densities in water with various tetracycline and dissolved organic matter (DOM) concentrations. Log_{10} of density in CFU/100mL. A: Luria-Bertani broth, stationary phase cells. B: Mueller-Hinton broth, exponential phase cells. Number in italics shows DOM concentration. Circles (squares) are experiments without (with) equilibration.

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