REMOVAL OF PHARMACEUTICALS FROM WATER USING NANO-ENGINEERED POROUS CERAMIC MEDIA AND EXPRESSION OF ANTIBIOTIC RESISTANCE OF *E.COLI* EXPOSED TO TETRACYCLINES

By

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ABSTRACT

REMOVAL OF PHARMACEUTICALS FROM WATER USING NANO-ENGINEERED POROUS CERAMIC MEDIA AND EXPRESSION OF ANTIBIOTIC RESISTANCE OF *E.COLI* EXPOSED TO TETRACYCLINES

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Highly porous ceramic media can be effective to remove contaminants from agricultural waters. In this study, these sorbent media were tested for their efficiency to remove three representative pharmaceuticals (i.e., lincomycin, sulfamethazine and tetracycline) from water. Results reveal that the tested media demonstrated relatively effective sorption for the selected pharmaceuticals from water. Tetracycline manifested the greatest sorption, followed by lincomycin and sulfamethazine. A granular medium was identified to have great removal efficiency for the selected pharmaceuticals, and could be potentially employed to remove pharmaceuticals from agricultural drainage water.

The widespread tetracyclines in the environment have been considered to be responsible for the development of antibiotic resistance in microorganisms. At contaminated sites multiple tetracyclines are commonly found, and could collectively impact microbial populations. The uptake of different types of tetracyclines by *Escherichia coli* exerted varying levels of selective pressure on the bacteria to express antibiotic resistance, following the order of tetracycline > chlortetracycline > oxytetracycline. Linear relations between promoter activity and intracellular tetracycline concentration were observed except for anhydrotetracycline. The mixture of tetracyclines generally demonstrated additive effects on the *E.coli* bioreporter for expression of antibiotic resistance. These results suggest that the risk levels of antibiotic resistance invoked by exposure to tetracycline mixtures could be additive.

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REMOVAL OF PHARMACEUTICALS FROM WATER USING NANO-ENGINEERED POROUS CERAMIC MEDIA

INTRODUCTION

Agricultural drainage has been widely applied to crop production systems in the United States (Pavelis, 1987). However, agricultural drainage water discharged to recipient surface waters may adversely impact water quality due to contaminants carried in the drainage water. Typical contaminants commonly present in agricultural drainage water are nutrients (e.g., nitrogen and phosphorous), heavy metals (e.g., As, Se, Cd, Pb, Cu, and Zn) and organic chemicals (e.g., pharmaceuticals, hormones, and pesticides). Thus, the treatment of agricultural drainage water may be needed to prevent contamination of surface waters. In this study we attempt to test the removal of pharmaceuticals from water using recently developed novel porous ceramic media.

Veterinary pharmaceuticals are commonly administered to animals for disease control and improving feeding efficiency, livestock growth, and animal health (Song et al,. 2010). These pharmaceuticals are widely used in concentrated animal feeding operations to enhance the production of meat and dairy and thus the farmers' profit. In the U.S., the annual domestic sales and distribution of antimicrobials approved for use in food-producing animals were approximately 14.6 million kilograms (FDA, 2014). Large fractions of the antimicrobials used in animal feeding operation are excreted to animal manures either as parent compounds or as bioactive metabolites (Jacobsen et al., 2004; Kay et al., 2004; Aga et al., 2005). These animal manures contain certain levels of pharmaceuticals; after a short period of storage/treatment (3-6 months), they are commonly land-applied as fertilizers for crop production. As a result, pharmaceuticals are introduced to agricultural ecosystems (Kolpin et al., 2002; Hamscher et al., 2005; Snow et al., 2008), which can potentially enter crop or vegetable produce.

In order to remove the pharmaceuticals from agricultural drainage water, sorption process can be a viable treatment option. In the past, many filter media have been used to remove phosphorus from water, such as recently developed highly porous ceramic media by MetaMateria. These materials are expected *a priori* to strongly adsorb pharmaceuticals, and hence reduce the contamination in agricultural surface waters. MetaMateria media are characterized as unique and highly porous ceramic adsorbents that provide exceptional performance and cost-effective removal approach for contaminants in wastewater. These media have been shown to be able to effectively remove phosphorus from drainage water. The large surface areas of the porous media could be modified with either loading reactive nanomaterials or hosting beneficial bacterial colonies in order to achieve effective chemical or biological treatments of contaminated water. The hierarchical pore structure helps maintain high water flow rate into or through the media, and the surfaces can be engineered to provide the desired functionality for adsorbing targeted contaminants from wastewater. For example, the surfaces could be modified via coating polymers, anionic or cationic surfactants iron oxide (e.g. FeOOH), manganese oxide (MnO_2), zinc oxide (ZnO) and silver (Ag) nanomaterials on the surfaces (Boujelbena et al., 2008).

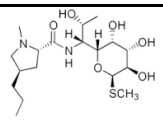
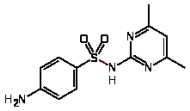
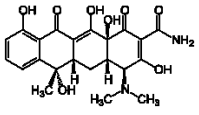
OBJECTIVE

The objective of this study was to test if the sorbent media (provided by MetaMateria) could effectively remove selected pharmaceuticals from water. The goal was to identify the appropriate sorbent media that could achieve the high efficiency of pharmaceuticals removal from agricultural drainage water.

MATERIALS AND METHODS

Lincomycin hydrochloride (purity≥90%), sulfamethazine (purity≥99%) and tetracycline chloride (purity≥95%) were obtained from Sigma-Aldrich Chemical Company. The sorbent media were provided by MetaMateria Technologies. The media included hybrid media (hybrid#1, 2, 3, 13A, 14A, 15A, 16A, 17A, 17B, 17C, 17D and 22), granular media from 1" column (without and with hydrophobic coatings), large-surface-area media (HSA-0), hydrophilic iron-modified media (new and old), and phosphorus-saturated iron-modified media. In addition, granular activated carbon (FILTRASORB 300, coal based, 0.8-1.0 mm) was obtained from Calgon Carbon Corporation. The selected properties of the three pharmaceuticals are listed in Table 1.1.

Table 1.1 Properties of selected three pharmaceuticals

Pharmaceuticals	Molecular Weight ($g\ mol^{-1}$)	Chemical Structure	Water Solubility ($mg\ L^{-1}$)	pK_a	$logK_{ow}$
Lincomycin	406.54		927	7.6	0.2
Sulfamethazine	278.34		1500	2.6, 7.6	0.14
Tetracycline	444.43		231	3.3, 8.3, 10.2	-1.37

From TOXNET database: http://toxnet.nlm.nih.gov/index.html

Pharmaceutical sorption by MetaMaterial media was measured using a single- or multi-point batch sorption experiment method. For the multi-point sorption isotherm experiments, 50.0 mg of sorbent media was weighed to glass centrifuge tubes containing 5.0 mL of pharmaceutical solution with the initial concentrations of 50, 100, 200, 400, 600, 800, and 1000 μg/L. The glass tubes were wrapped with Al-foil to prevent the potential of photodegradation. Then the centrifuge tubes were shaken on a platform shaker at 20 rpm for 48 hours for lincomycin and sulfamethazine, and 4 hours for tetracycline. The shorter shaking time (4 hours) selected for tetracycline was tested to be sufficient to approach sorption equilibration. After approaching sorption equilibration, the tubes were centrifuged at 3500 rpm for 20 min. The supernatant pH value was measured with a pH meter. The pharmaceutical concentration in the supernatant was analyzed with a Shimadzu Prominence high-performance liquid chromatograph coupled to an Applied Biosystems Sciex 3200 triple quadrupole mass spectrometer (LC-MS/MS). Control experiments free of MataMaterial sorbents were also conducted, and the sorbate loss during experiment was negligible. Sorbed pharmaceutical concentration by sorbent media was calculated as:

$$S = \frac{(C_0 - C)V}{M} \qquad (1.1)$$

where S is the sorbed pharmaceutical concentration by media (μg/kg), C_0 is the initial pharmaceutical concentration in the aqueous phase as measured in the media-free control treatment (μg/L), C is the pharmaceutical concentration in the aqueous phase after equilibration (μg/L), V is the solution volume (mL), and M is the mass of sorbent media (kg).

The sorption isotherms for lincomycin and sulfamethazine were fitted with the Freundlich model to estimate sorption parameters. The Freundlich sorption isotherm is mathematically expressed as:

$$\log S = logK_f + nlogC \qquad (1.2)$$

In this equation, K_f and n are fitting constants for a given sorbate and sorbent combination at a given temperature (room temperature in this study). The sorption efficiencies of tetracycline by all tested media approached to 99% at the majority of the initial concentrations. Thus, it is inappropriate to generate sorption isotherms for tetracycline. Instead, the removal percentage at the initial concentration of ≈ 600 μg/L was reported.

In the single-point sorption experiment, pharmaceutical solutions (5.0 mL) of the initial concentration of 500 μg/L were prepared, and mixed with approximately 50.0 mg of sorbent media in the experiments with lincomycin and sulfamethazine, and 10.0 mg of sorbent media for tetracycline. Before placing the tubes on the platform shaker, hydrochloric acid (HCl) was added to adjust the final pH range of 6-7. The experimental protocol was the same to that previously described. The pharmaceutical removal percentage by sorbent media was calculated as:

$$Removal\ (\%) = \frac{C_0 - C}{C_0} \times 100\% \qquad (1.3)$$

RESULTS AND DISCUSSION

The selected properties of the sorbent media provided by MetaMateria are shown in Table 1.2. The surface area of the sorbent could reach 90 m^2/g after hydrophobic modification. These materials are shown to have reasonably good retention for water and for hydrophobic organic compounds (e.g. toluene).

Table 1.3 shows the removal percentage of pharmaceuticals by MataMetrial media using the one-point sorption experiment. The corresponding results are also presented in Figures 1.1. The removal percentage ranged from 32.1 % to 97.4 % for lincomycin, 13.2 % to 86.4 % for sulfamethazine, and 48.7 % to 98.0 % for tetracycline. Among these media tested, the granular media from "1-inch column" consistently demonstrated the highest pharmaceutical removal percentage, i.e. 97.4 % for lincomycin, 86.4 % for sulfamethazine, and 97.7 % for tetracycline. This media performed better than other media including those with surface-hydrophobic-modification media (hybrid media). For activated carbon, the removal rate was 98.2 % for lincomycin, 99.9 % for sulfamethazine, and 96.9 % for tetracycline. The performance of the granular media from "1-inch column" could achieve a removal percentage close to that of activated carbon (Table 1.3). Among the hybrid media, hybrid #22 overall demonstrated better performance than other hybrid media, with 92.3 % for lincomycin, 60.0 % for sulfamethazine, and 95.7 % for tetracycline.

The pharmaceutical removal rate was found to depend on solution pH for lincomycin and sulfamethazine. Greater removal percentage was achieved in slightly acidic solution than that in alkaline pH (Table 1.3 and Table 1.4). For the example of Hybrid#3, at pH 6.3-6.5 the removal percentage was measured at 83.7 % for lincomycin and 59.6 % for sulfamethazine, but 38.8 % for lincomycin and 17.4 % for sulfamethazine at pH 10.1-10.5. However, this pH effect was not apparent for tetracycline since all tested sorbent media manifested a very high removal percentage (> 95.1 % for all tested media expect HAS-0). The strong sorption of tetracycline could be due to the fact that Al or Fe hydrous oxides are the major components of these media. Formation of tetracycline-Fe/Al complexes could substantially enhance sorption of tetracycline by the media from water.

Table 1.2 Selected properties of sorbent media

Sorbents	Surface area (m^2/g), as prepared	Surface area (m^2/g), after modification	Surface area (m^2/g), after hydrophobic coating	Water retention	Toluene retention
Hybrid#1	20.5	77.1	91.2	123%	108%
Hybrid#2	19.8	80.4	97.9	86%	98%
Hybrid#3	18.1	68.1	66.8	94%	85%
Hybrid#13A	23.2	67.5	72.5	115%	97%
Hybrid#14A	24.3	57.4	65.3	111%	97%
Hybrid#15A	39.4	70.2	89.6	140%	98%
Hybrid#16A	26.2	44.8	74.8	108%	105%
Hybrid#17A	37.6	92.6	72.5		
Hybrid#17B	36.5	91.2	65.3		
Hybrid#17C	18.4	60.1	89.6		
Hybrid#17D	21	69.9	74.8		
Hybrid#22	150	-	-		
Granules from 1" column - without hydrophobic coating	-	-	-		
Granules from 1" column - with hydrophobic coating	-	-	-		
High Surface Area - 0 (HSA-0)	-	-	-		
Iron-modified media (new)	-	-	-		
Iron-modified media (old)	-	-	-		
P-saturated Iron-modified media	-	-	-		

Table 1.3 Pharmaceutical removal percentage by selected sorbent media

Sample Name	Lincomycin		Sulfamethazine		Tetracycline	
	Removal percentage (%)	Final pH	Removal percentage (%)	Final pH	Removal percentage (%)	Final pH
Hybrid#3	83.7	6.3	59.6	6.4-6.5	97.9	5.9
Iron-modified media (new)	79.9	6.0	67.5	6.1-6.2	95.6	5.4-5.5
P-saturated Iron-modified media (old)	32.1	6.1-6.2	22.0	6.2-6.3	97.2	5.7-5.8
Hybrid#17A	56.7	6.1-6.2	43.2	6.0-6.2	95.1	6.1
Hybrid#17B	64.1	6.3-6.4	44.8	6.3-6.4	96.6	6.0-6.1
Hybrid#17C	64.0	6.4-6.5	41.4	6.5-6.6	98.0	5.9
Hybrid#17D	68.7	6.3	44.3	6.3-6.4	97.8	5.8-5.9
Hybrid#22	92.3	5.7-5.8	60.0	5.9-6.2	95.7	5.5-5.6
Granules from 1" column - without hydrophobic coating	97.4	5.9	86.4	5.9	97.7	5.6-5.7
Granules from 1" column - with hydrophobic coating	92.3	5.9-6.0	53.1	5.9-6.0	94.4	5.5-5.7
HAS-0	32.2	9.6	13.2	9.9-10.0	48.7	3.0-3.2
Activate Carbon	98.2	7.3-7.5	99.9	7.0-7.1	96.9	6.6-6.7

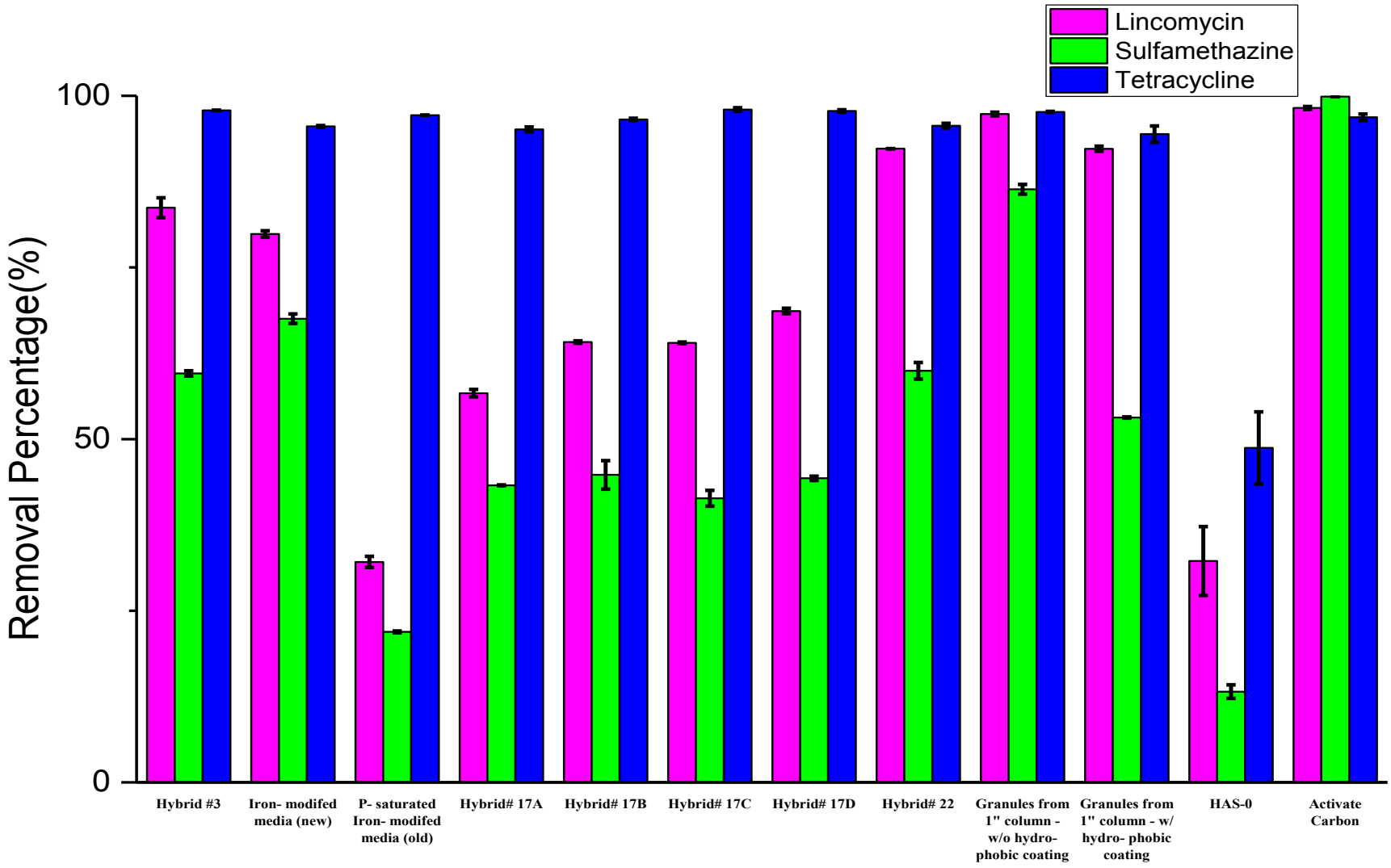


Figure 1.1 Pharmaceutical removal percentage by selected sorbent media

Table 1.4 Pharmaceutical removal percentage by sorbent media (initial concentration ≈ 600 μg/L).

Sorbents	Lincomycin		Sulfamethazine		Tetracycline	
	Final pH	Removal percentage (%)	Final pH	Removal percentage (%)	Final pH	Removal percentage (%)
Hybrid#1	9.7-9.9	31.6	9.6-10.0	12.8	9.0-9.5	99.4
Hybrid#2	9.0-9.9	31.3	9.8-10.0	9.3	8.6-9.1	99.0
Hybrid#3	10.1-10.5	38.8	10.2-10.5	17.4	9.0-9.7	97.7
Hybrid#13A	10.1-10.5	29.0	10.1-10.5	12.7	8.8-9.8	99.5
Hybrid#14A	8.7-10.0	28.2	9.6-10.0	25.9	8.5-9.0	99.8
Hybrid#15A	9.2-9.7	35.2	9.4-10.0	13.9	8.0-8.5	99.8
Hybrid#16A	8.8-9.0	35.1	8.8-9.4	11.5	7.8-8.0	99.9
Iron-modified media (new)	8.1-9.4	38.7	8.4-8.9	35.7	8.7-9.6	99.9
Iron-modified media (old)	7.2-8.3	25.5	7.3-8.3	28.5	7.2-8.2	99.8
P-saturated Iron-modified media (old)	5.9-6.4	25.8	6.2-6.5	18.4	5.8-6.4	99.8

Table 1.5 Fitting Freundlich isotherm parameters for sorption of lincomycin and sulfamethazine by sorbent media.

Sorbents	Lincomycin			
	Final pH	K_f [a]	n	R^2
Hybrid#1	9.7-9.9	214	0.76	0.988
Hybrid#2	9.0-9.9	155	0.80	0.995
Hybrid#3	10.1-10.5	225	0.80	0.990
Hybrid#13A	10.1-10.5	34.8	1.01	0.981
Hybrid#14A	8.7-10.0	23.7	1.08	0.982
Hybrid#15A	9.2-9.7	34.8	1.08	0.990
Hybrid#16A	8.8-9.0	14.5	1.16	0.753
Iron-modified media (new)	8.1-9.4	264	0.77	0.985
Iron-modified media (old)	7.2-8.3	200	0.71	0.984
P-saturated Iron-modified media (old)	5.9-6.4	329	0.66	0.951
	Sulfamethazine			
	Final pH	K_f [a]	n	R^2
Hybrid#1	9.6-10.0	65.4	0.78	0.845
Hybrid#2	9.8-10.0	27.9	0.86	0.842
Hybrid#3	10.2-10.5	84.1	0.79	0.927
Hybrid#13A	10.1-10.5	254	0.49	0.738
Hybrid#14A	9.6-10.0	50.9	0.87	0.885
Hybrid#15A	9.4-10.0	143	0.55	0.852
Hybrid#16A	8.8-9.4	174	0.53	0.614
Iron-modified media (new)	8.4-8.9	103	0.90	0.960
Iron-modified media (old)	7.3-8.3	117	0.79	0.871
P-saturated Iron-modified media (old)	6.2-6.5	50.1	0.88	0.936

[a] Unit of K_f is $\mu g/kg\ (\mu g/L)^{-n}$.

Sorption isotherms were measured for lincomycin and sulfamethazine by MetaMaterial media in relatively alkaline solution (Figures 1.2 to 1.21). The Freundlich fitting results are reported in Table 1.5. For lincomycin the sorption isotherms appeared relatively nonlinear and linear, with n values range from 0.66 to 1.16 (Figures 1.2 to 1.11). At the aqueous concentration at 400 μg/L, the estimated sorption concentration (using

Freundlich fitting results) was 20322.6 μg/kg for Hybrid#1, 18705.9 μg/kg for Hybrid#2, 27153.8 μg/kg for Hybrid#3, 14779.5 μg/kg for Hybrid#13A, 15309.9 μg/kg for Hybrid#14A, 22480.4 μg/kg for Hybrid#15A, 15127.2 μg/kg for Hybrid#16A, 26618.9 μg/kg for iron-modified media (new), 14076.4 μg/kg for iron-modified media (old) and 17161.5 μg/kg for P-saturated iron-modified media (old). For sulfamethazine, the isotherms demonstrated essentially nonlinear sorption with n values from 0.49 to 0.9 (Figures 1.12 to 1.21). At 400 μg/L, the estimated sorption was 7001.4 μg/kg for Hybrid#1, 4823.7 μg/kg for Hybrid#2, 9559.2 μg/kg for Hybrid#3, 4784.6 μg/kg for Hybrid#13A, 9343.5 μg/kg for Hybrid#14A, 3858.9 μg/kg for Hybrid#15A, 4165.2 μg/kg for Hybrid#16A, 22630.3 μg/kg for iron-modified media (new), 13298.8 μg/kg for iron-modified media (old) and 9764.5 μg/kg for P-saturated iron-modified media (old). Overall the sorption capacity for lincomycin and sulfamethazine was modest, which could be utilized for pharmaceutical removal in agricultural drainage system, but may not be the best sorbent used for this purpose.

Since the MetaMaterial media have been successfully applied for phosphorus removal from agricultural drainage water, we compared sorption of pharmaceuticals by P-saturated iron-modified media vs. iron-modified media (Figure 1.10 vs. Figure 1.11 for lincomycin, and Figure 1.20 vs. 1.21 for sulfamethazine). The results reveal that P-saturated iron-modified media did not substantially reduce sorption by the media for lincomycin and sulfamethazine. These results indicate that P-adsorption on this medium did not affect sorption of the tested pharmaceuticals, which could be due to the fact that phosphorus and lincomycin/sulfamethazine have different sorption sites on the medium surfaces.

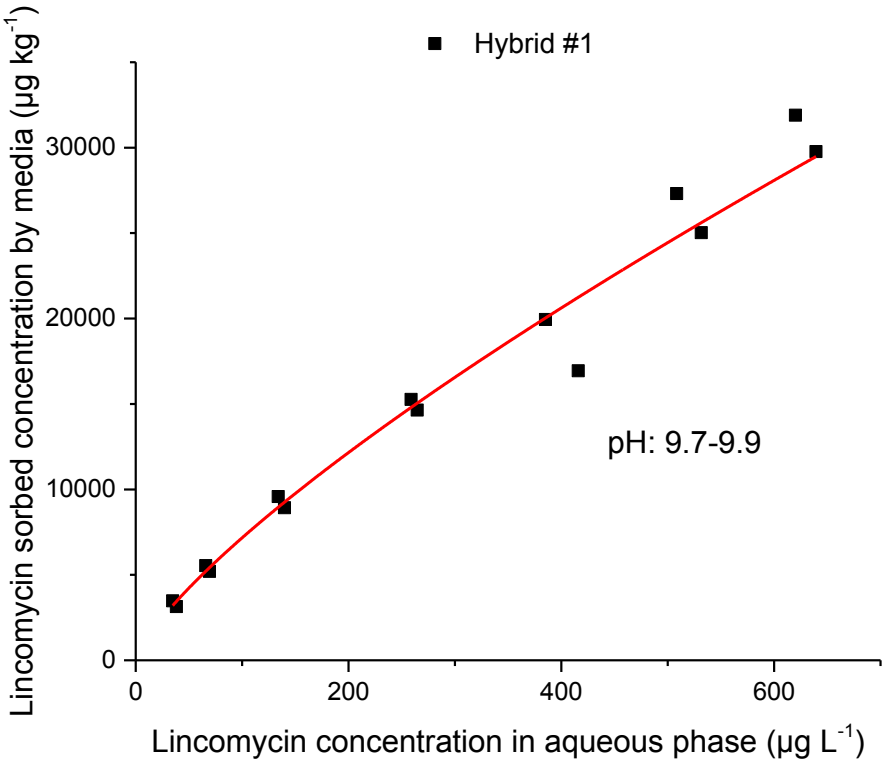


Figure 1.2 Sorption isotherm of lincomycin by hybrid media #1

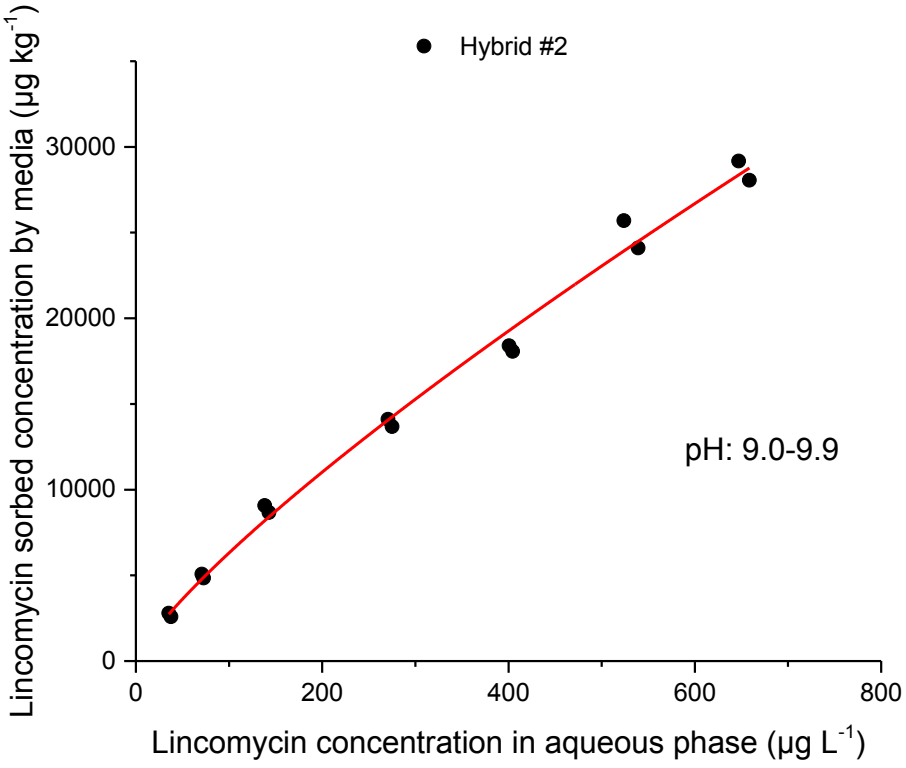


Figure 1.3 Sorption isotherm of lincomycin by hybrid media #2

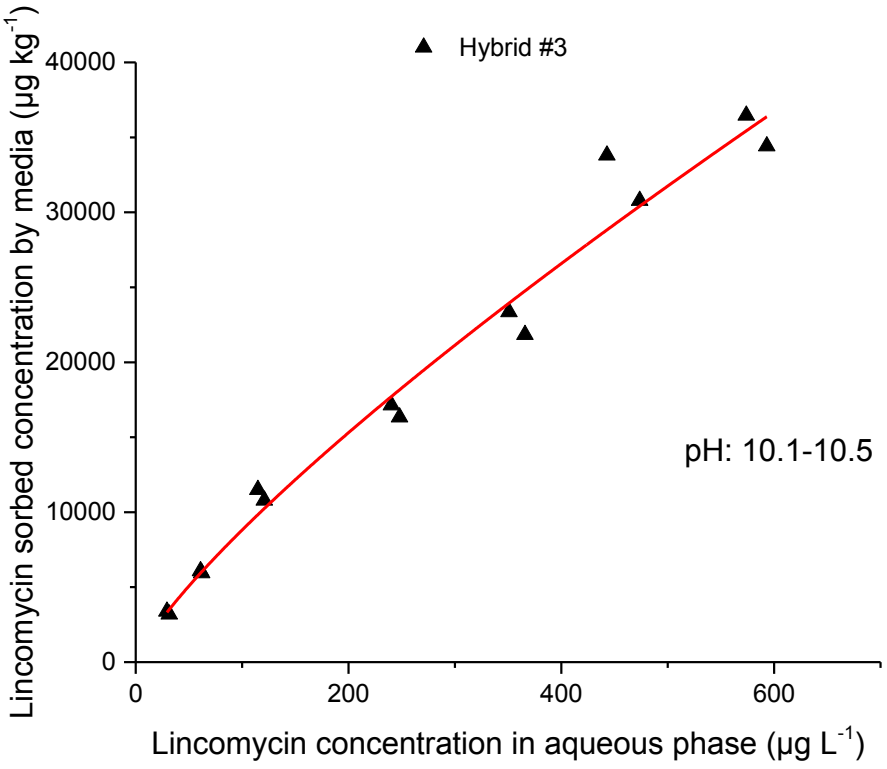


Figure 1.4 Sorption isotherm of lincomycin by hybrid media #3

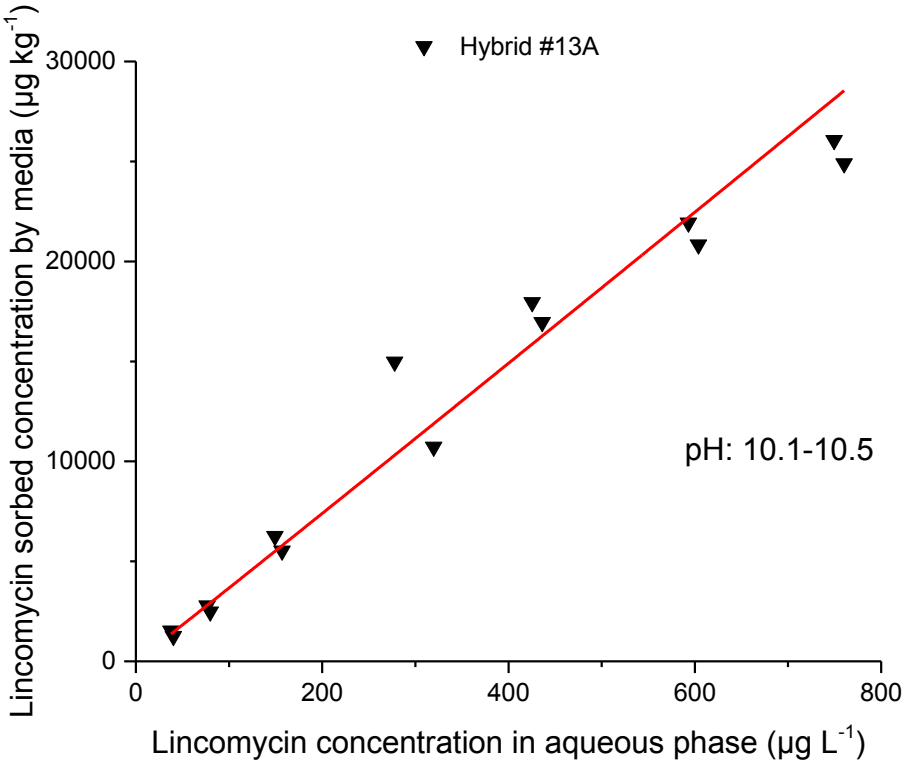


Figure 1.5 Sorption isotherm of lincomycin by hybrid media #13A

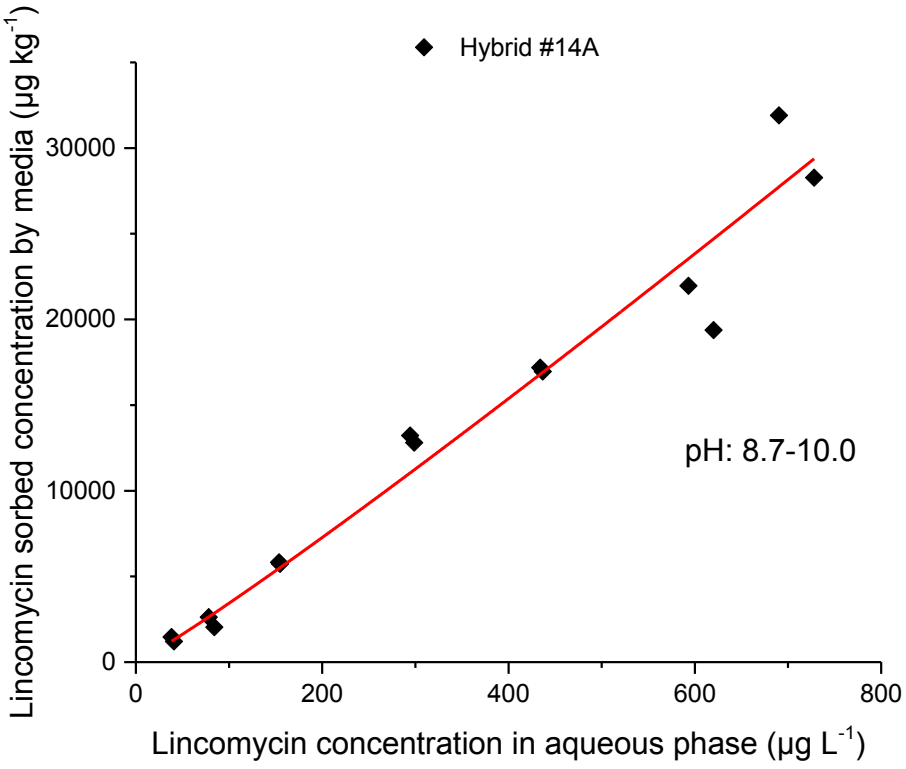


Figure 1.6 Sorption isotherm of lincomycin by hybrid media #14A

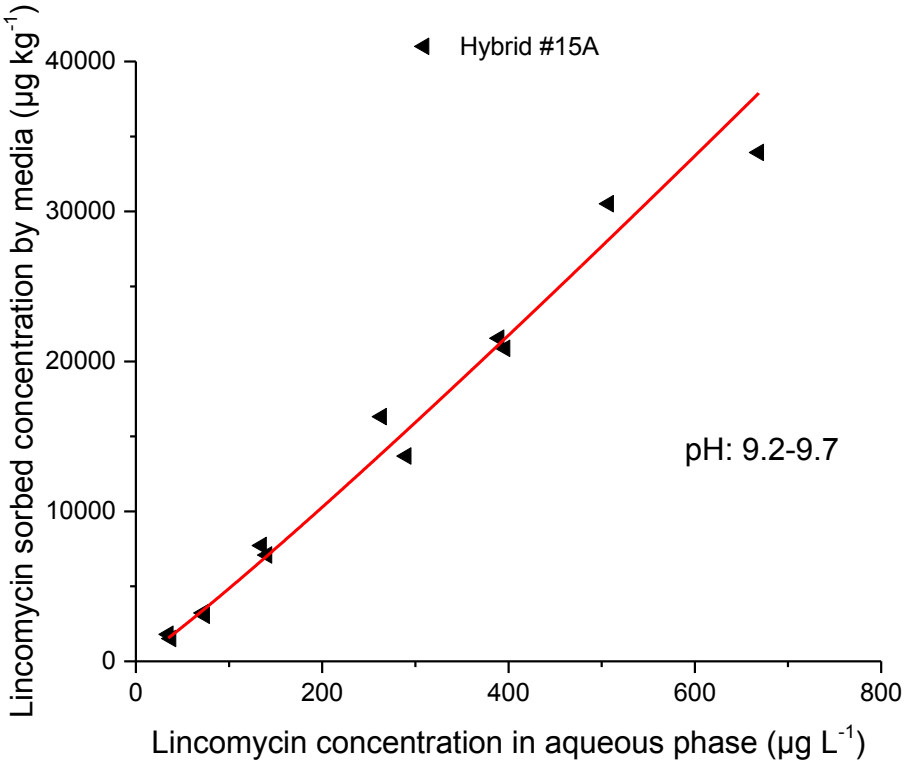


Figure 1.7 Sorption isotherm of lincomycin by hybrid media #15A

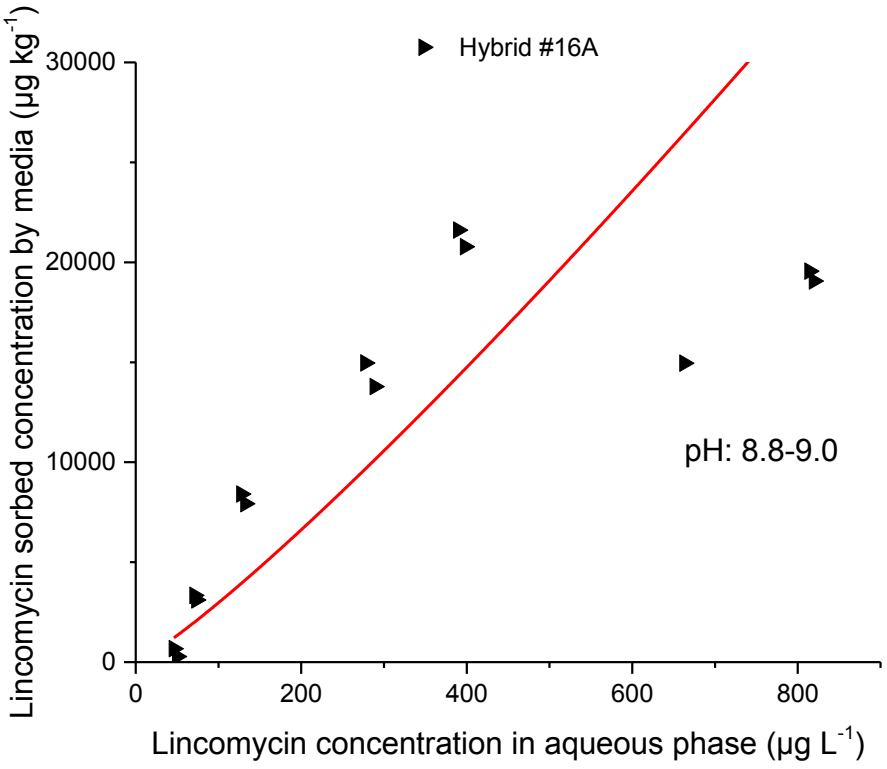


Figure 1.8 Sorption isotherm of lincomycin by hybrid media #16A

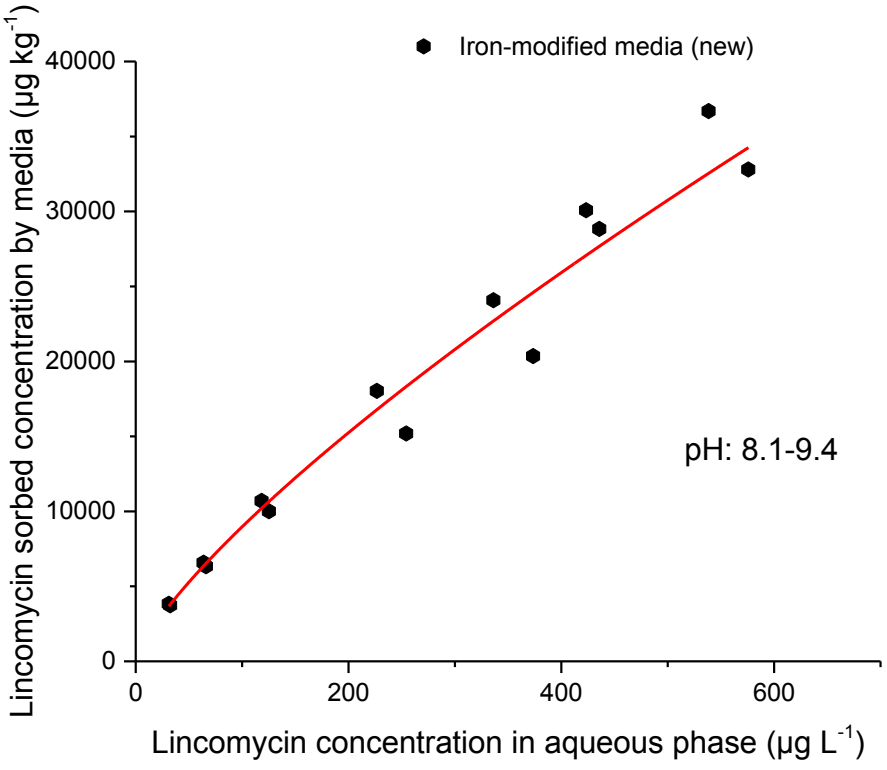


Figure 1.9 Sorption isotherm of lincomycin by iron-modified media (new)

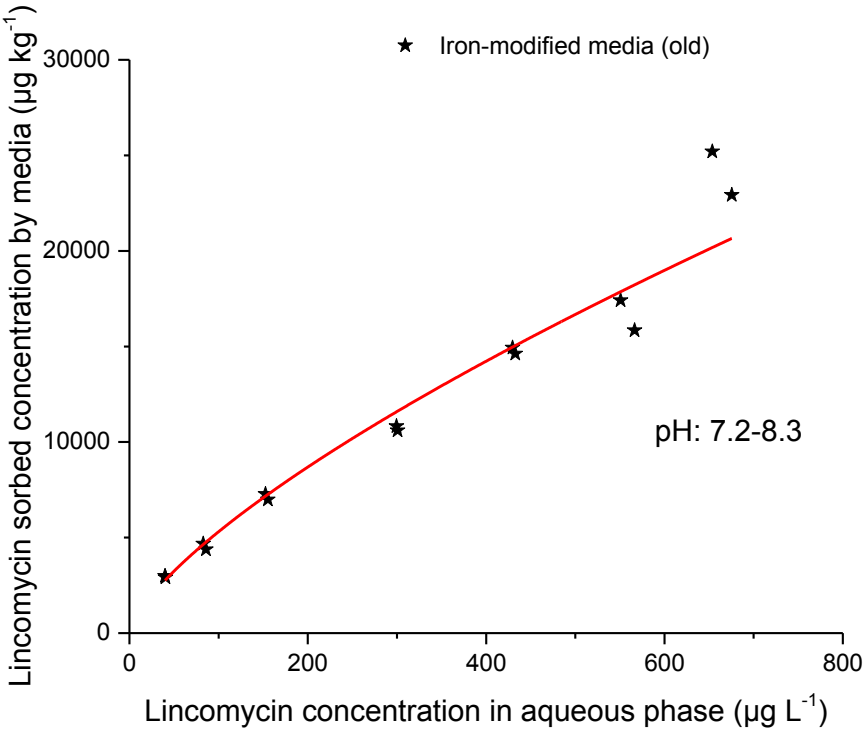


Figure 1.10 Sorption isotherm of lincomycin by iron-modified media (old)

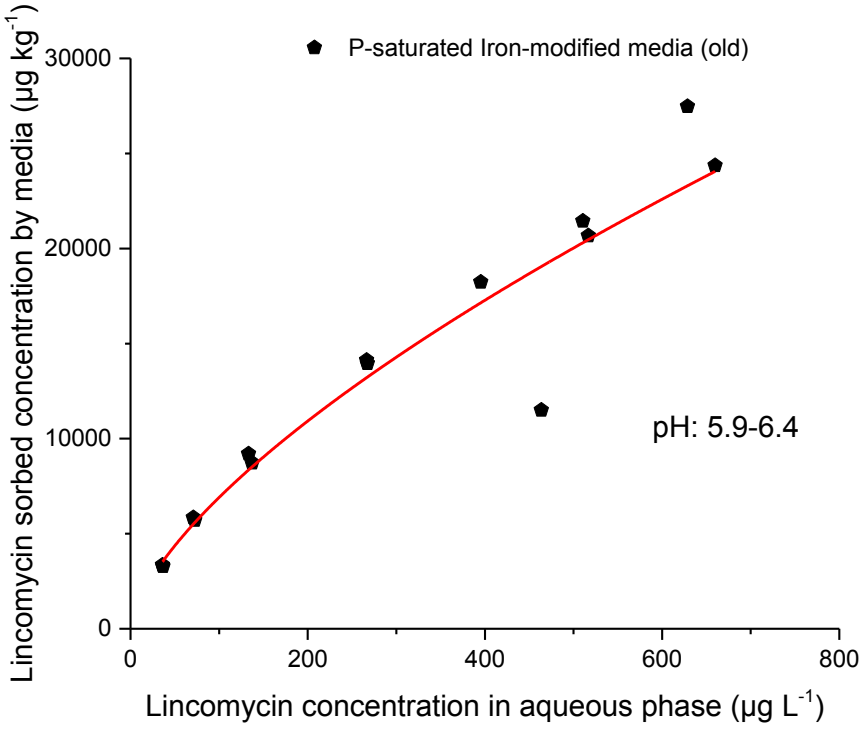


Figure 1.11 Sorption isotherm of lincomycin by phosphorus-saturated iron-modified media (old)

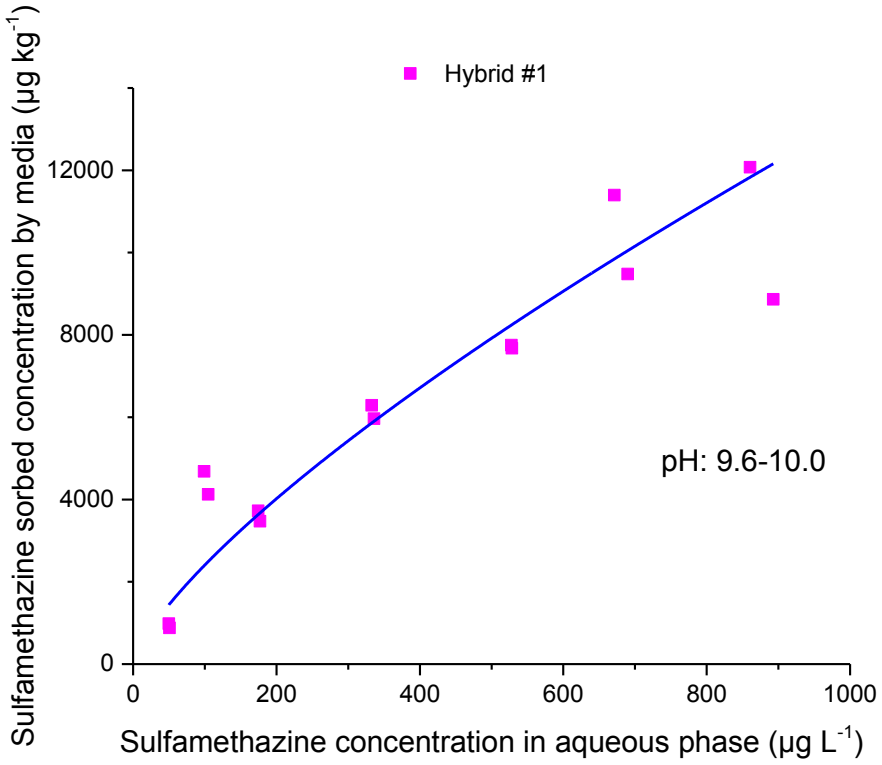


Figure 1.12 Sorption isotherm of sulfamethazine by hybrid media #1

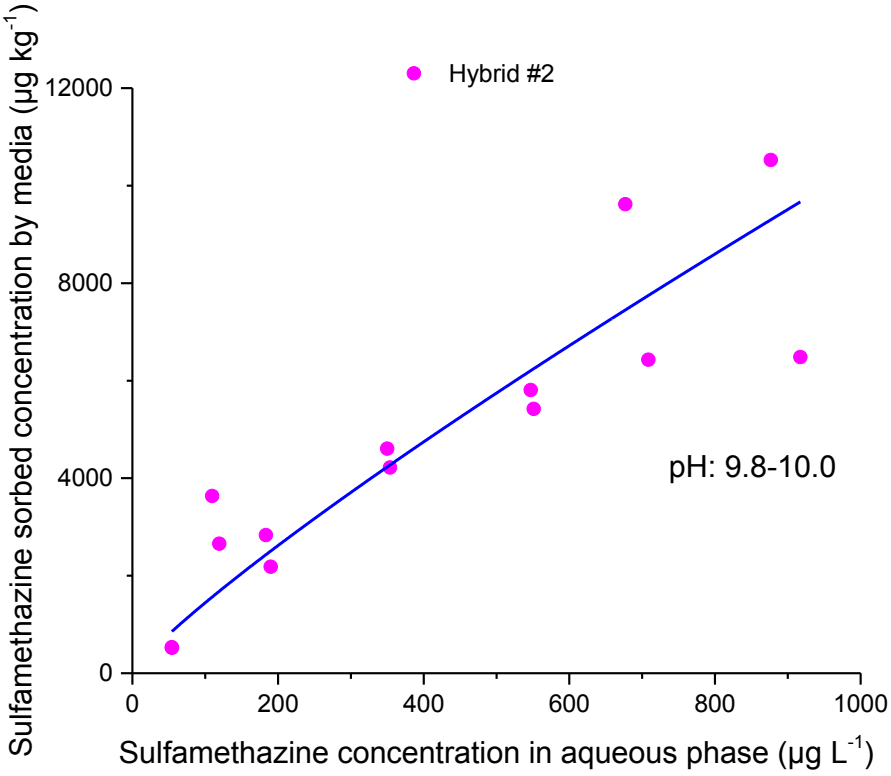


Figure 1.13 Sorption isotherm of sulfamethazine by hybrid media #2

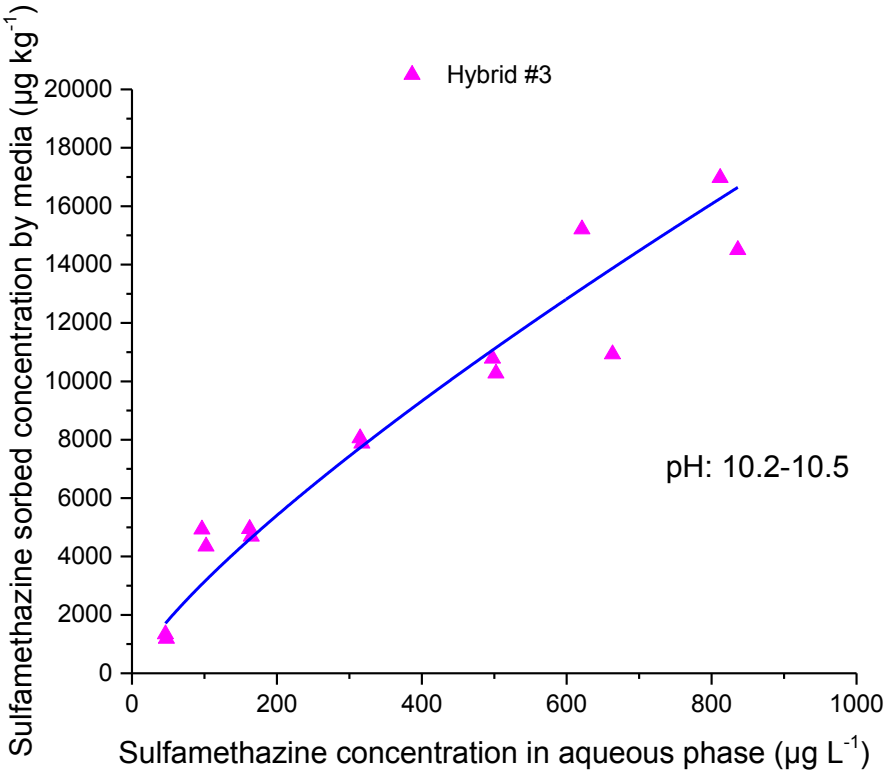


Figure 1.14 Sorption isotherm of sulfamethazine by hybrid media #3

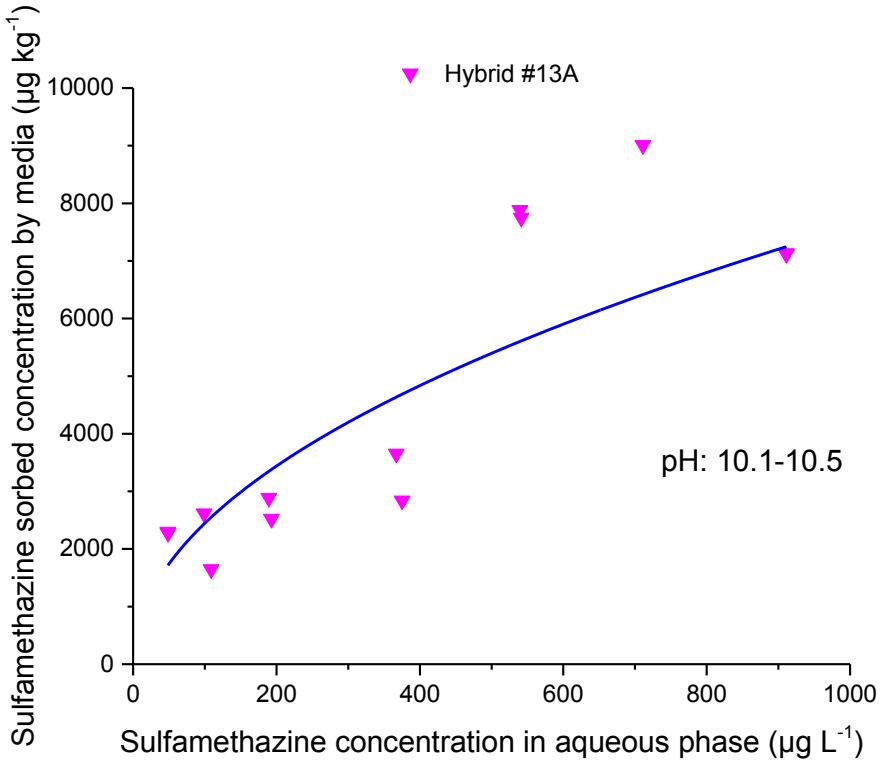


Figure 1.15 Sorption isotherm of sulfamethazine by hybrid media #13A

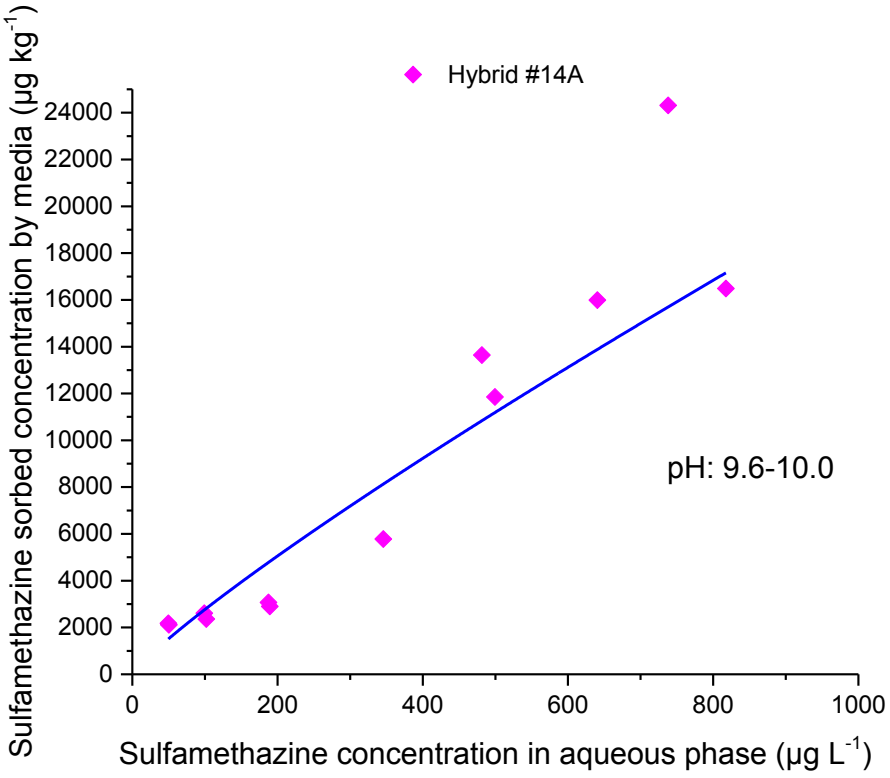


Figure 1.16 Sorption isotherm of sulfamethazine by hybrid media #14A

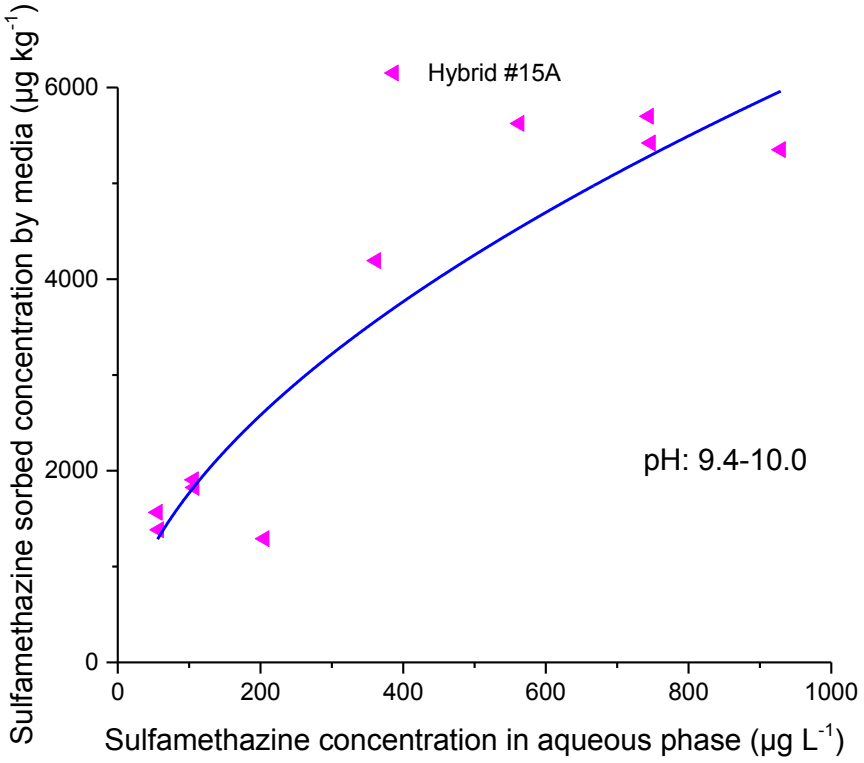


Figure 1.17 Sorption isotherm of sulfamethazine by hybrid media #15A

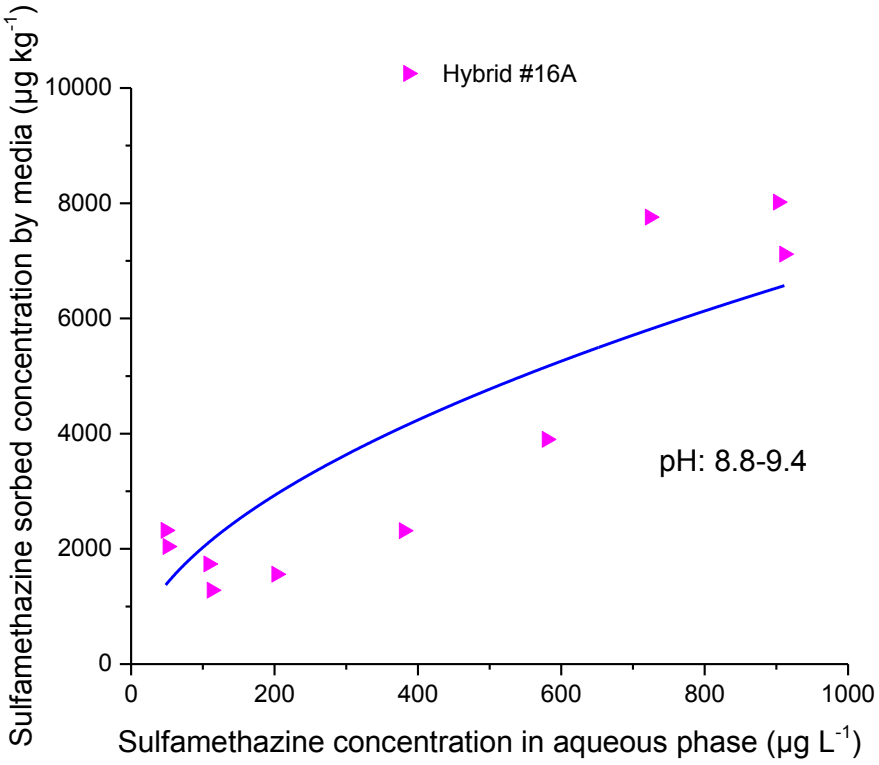


Figure 1.18 Sorption isotherm of sulfamethazine by hybrid media #16A

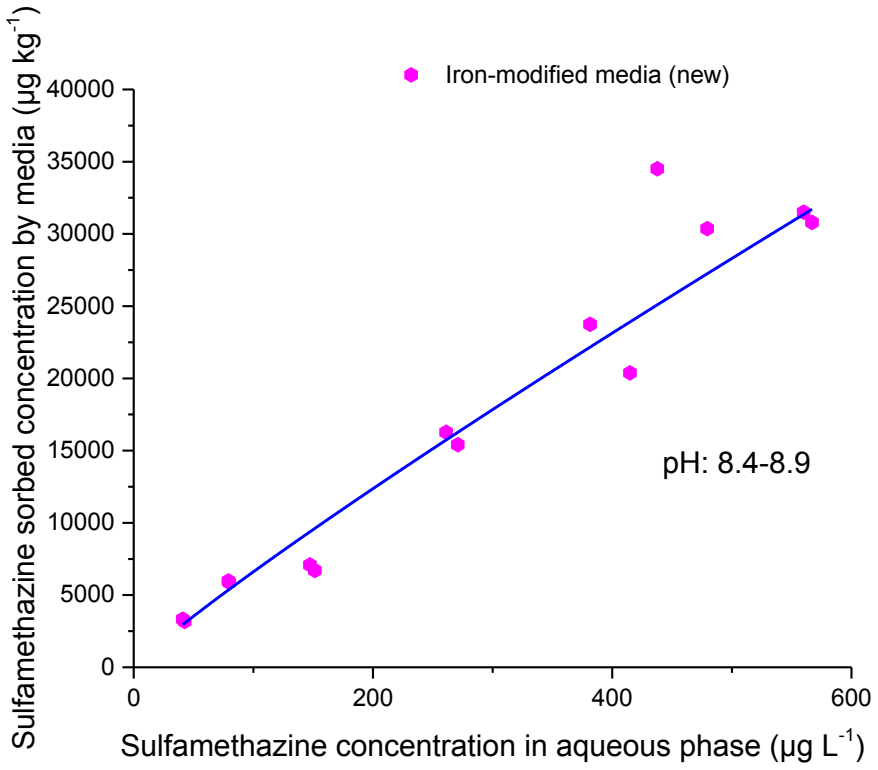


Figure 1.19 Sorption isotherm of sulfamethazine by iron-modified media (new)

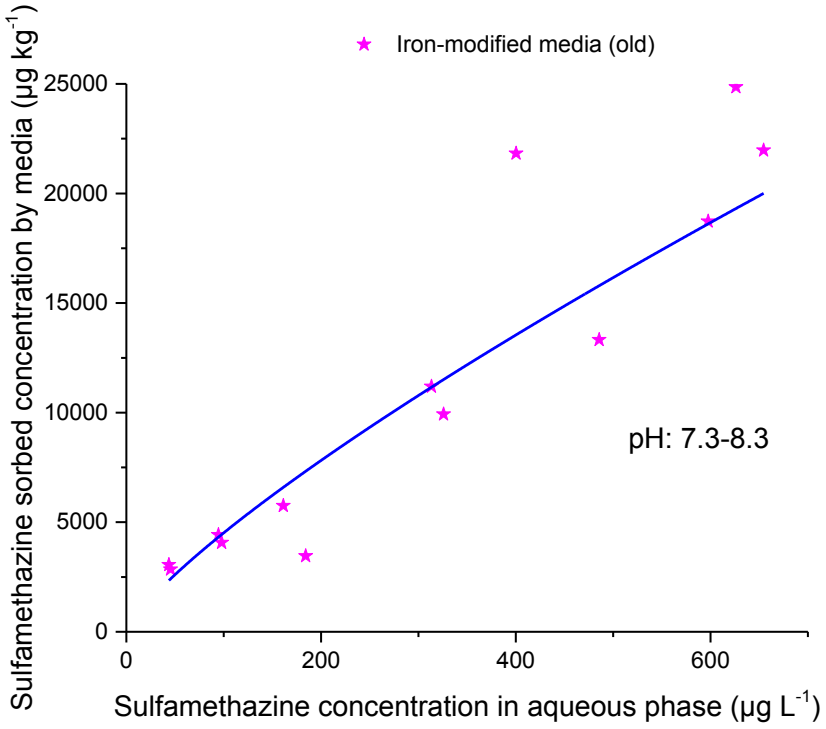


Figure 1.20 Sorption isotherm of sulfamethazine by iron-modified media (old)

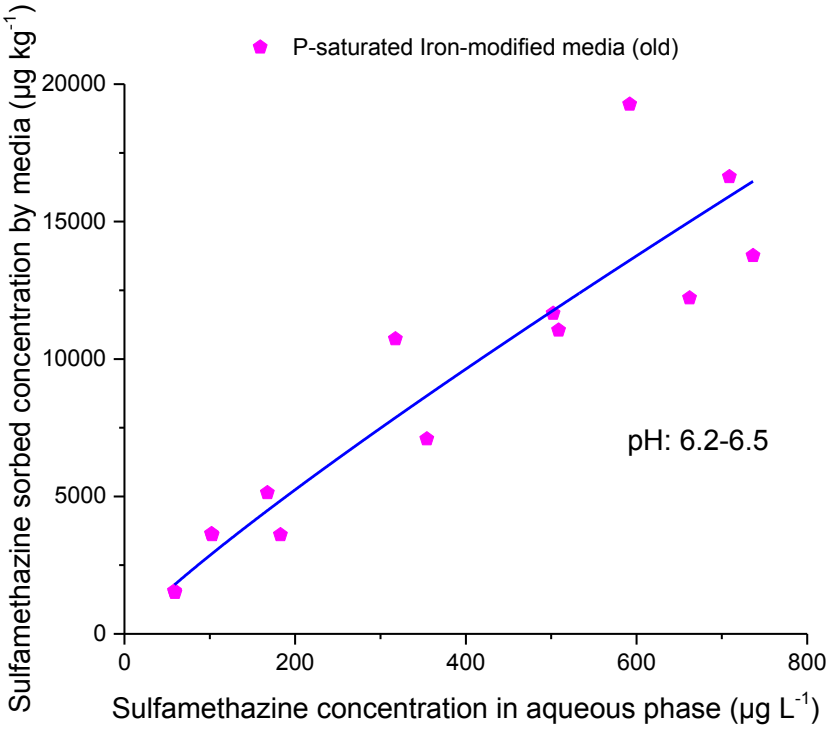


Figure 1.21 Sorption isotherm of sulfamethazine by phosphorus-saturated iron-modified media (old)

CONCLUSIONS

Overall, the MetaMaterial media demonstrated relatively effective sorption for the selected pharmaceuticals from water. Tetracycline manifested the greatest sorption, followed by lincomycin and then sulfamethazine. Granular medium from the 1-inch column manifested the greatest removal efficiency for the selected pharmaceuticals, and the sorption was comparable to that by activated carbon. This medium could potentially employed to removal pharmaceuticals from agricultural drainage water, along with the major function of phosphorus removal. Among the modified hybrid media, hybrid #22 overall demonstrated a higher sorption for the selected pharmaceuticals. Sorption of the selected pharmaceuticals in neutral or slight acidic solution (pH from 6 to 7) was greater than that in alkaline solution. The surface areas of the MetaMaterial media fell within the same order of magnitude; modification on the surface properties could plausibly improve sorption of pharmaceuticals from water.

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REFERENCES

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CHAPTER II

EXPRESSION OF ANTIBIOTIC RESISTANCE OF *E.COLI* EXPOSED TO TETRACYCLINES

INTRODUCTION

Antibiotics have been widely used in animal production to treat diseases and to improve feed efficiency (Allen, 2014; Durso, et al., 2014). They have been used extensively in livestock industries since the 1950s (Barton, 2014; Tilman, et al., 2002). The long-term utilization of antimicrobial drugs in animal production and microbial exposure to low-dosed antibiotics in the environment contribute to the development of antimicrobial resistance in microorganisms, and recently draw substantial attention to this global healthy issue (Khachatourians, 1998; Jensen, et al., 2014; Smalla, et al., 2014). Tetracyclines are a class of a broad-spectrum polyketide antibiotics, and are among the most commonly used antibiotics in animal feeding operations and disease control. In 2012, approximately 5.9 million kilograms of tetracyclines were used for both food-producing animals (e.g., cattle and swine) and nonfood-producing animals (e.g., dogs and cats) in United States, which accounted for 40.7% of the total antibiotics use for animals (FDA, 2012). Tetracycline, chlortetracycline and oxytetracycline are the main active members in the tetracycline family. Since large amount of tetracyclines are used in agricultural livestock production, tetracyclines have been frequently detected in soils, surface waters, and even in groundwater (Hu et al., 2010; Jacobsen et al., 2004; Batt and Aga, 2005; Christian et al., 2003; Kolpin et al., 2002; Luo et al., 2011; Wei et al., 2011 Chee-Sanford et al., 2001; Gottschall et al., 2012).

The presence of antibiotics such as tetracylines in the environment has been related to the emergence and ever-increasing abundance of antibiotic resistance genes (ARGs) in natural microbial populations. In several recent studies, tetracycline ARGs were more frequently detected in the sites where tetracyclines were present, compared to the sites

free of tetracyclines (Pei, et al., 2006; Storteboom, et al., 2010; Knapp et al., 2010). Thus, the concurrent presence of tetracyclines in the environment and the increasing abundance of tetracycline ARGs suggests that tetracyclines might pose selective pressure on the exposed microbial communities for the development and proliferation of antibiotic resistance.

In order for tetracyclines to exert selective pressure and other biological effects on bacteria, they should first become available to bacterial for uptake into the cells. Previous studies have demonstrated that zwitterionic tetracycline is the most effective species to enter *E. coli* from aqueous solution, resulting in the expression of antibiotic resistance genes (Zhang, et al., 2014). However, in the environment it is more common that multiple types of tetracyclines are present at the contaminated sites. However, little is known about how these tetracyclines collectively contribute to the uptake and resistance gene expression of tetracyclines by the bacteria. In this study, we aimed to investigate the expression of antibiotic resistance genes of *E. coli* exposed to different combinations of tetracyclines. The goal was to examine any potential synergistic or antagonistic effects among the combinations of common tetracyclines on the *E. coli* for expression of antibiotic resistance. An *E. coli* bioreporter was used for quantifying ARGs responses exposed to tetracycline. The bioreporter was *E. coli* strain MC4100 containing the plasmid pTGM with a transcriptional fusion between tetracycline inducible promoter and fluorescence-assisted cell sorting optimized *gfp* gene. Tetracycline uptake by the bioreporter (determined as intracellular tetracycline concentration) was quantified by using a high-performance liquid chromatography integrated with a tandem mass spectrometer (LC-MS/MS). The degree of expression of antibiotic resistance genes was

quantified through promoter activity by integrating bacterial growth rate and fluorescence emitted from the *E. coli* bioreporter at a steady state.

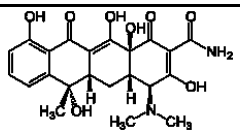
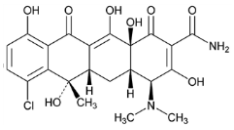
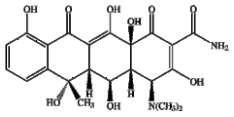
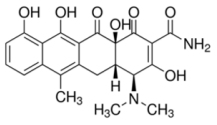
MATERIALS AND METHODS

Tetracycline chloride (purity≥95%), chlortetracycline hydrochloride (purity≥97%), oxytetracycline hydrochloride (purity≥95%), anhydrotetracycline hydrochloride (purity≥98%), methanol, and 3-(N-morpholino) propanesulfonic acid (MOPS, buffer range 6.5−7.9) were obtained from Sigma-Aldrich Chemical Company. The selected properties of the four tetracyclines are listed in Table 2.1. Sodium chloride, potassium chloride, ethylenediaminetetraacetic acid (EDTA), citric acid, formic acid, sodium phosphate dibasic, and potassium phosphate monobasic were obtained from J.T. Baker. Bacto tryptone and Bacto yeast extracts were obtained from Becton, Dickinson and Company. Acetonitrile (HPLC grade) and hydrochloric acid were obtained from EMD Chemicals.

The *E. coli* strain MC4100/pTGM used as bacterial bioreporter was provided by Dr. S. J. Sørensen at the University of Copenhagen. The *E. coli* strain was constructed by inserting *tet* (M) gene (encoding tetracycline resistance by ribosomal protection) into plasmid pTGM, which contained a transcriptional fusion between a *tet*R-regulated promoter and flow cytometry-optimized *gfp* gene (*gfp*mut3) encoding green fluorescence protein (GFP). As tetracyclines enter the *E. coli* bioreporter cell, they deactivate the *tet*R repressor protein in the P_{tet} promoter and activate *gfp* gene transcription. The pTGM construct contains tetracycline resistance gene *tet* (M) that inhibits tetracyclines from killing the cells, and maintains the intracellular tetracycline concentration. Meanwhile, the GFP translated from the expression of *gfp* gene emits the fluorescence signal with the

intensity proportional to the P_{tet} activity that drives antibiotic resistance gene expression in natural settings.

Table 2.1 Properties of selected four tetracyclines

Pharmaceuticals	Molecular Weight (g mol^{-1})	Chemical Structure	Water Solubility (mg L^{-1})	pK_a	$logK_{ow}$
Tetracycline	444.43		231	3.3, 8.3, 10.2	-1.37
Chlortetracycline	478.88		630	3.3,7.4,9.3	-0.62
Oxytetracycline	460.43		313	3.6, 7.5, 9.4	-0.9
Anhydrotetracycline	426.42		2299[a]	3.2, 6.0, 8.5[b]	0.63[a]

From TOXNET database: http://toxnet.nlm.nih.gov/index.html
[a] from database: http://www.chemspider.com/Chemical-Structure.20117965.html
[b] from reference: João et al., 1994

The *E. coli* bioreporter was cultured in a low-salt lysogeny broth (LB) medium, and pH was adjusted to 7.0 using 50 mM of MOPS buffer. The LB medium was autoclaved at 121 °C for 30 min; *E. coli* bioreporter was inoculated and cultivated in 25.0 mL of LB medium amended with 100 mg L^{-1} ampicillin. The culture was incubated on a horizontally moving shaker at 150 rpm and at 30℃. When the bacterial culture grew to

the mid-log phase as indicated by optical density at 600 nm (OD_{600}) approaching ~0.7, 0.5 mL of the culture was diluted 100 fold in 50.0 mL of freshly prepared LB media amended with 100 mg L^{-1} of ampicillin. The LB media were prepared to contain single tetracycline, chlortetracycline, oxytetracycline and anhydrotetracycline at the concentration of 0, 50, 100, 200, 300 and 400 nmol L^{-1}, binary tetracyclines (tetracycline + chlortetracycline, and tetracycline + oxytetracycline) with the concentration of 0, 50, 100, 150, 200 nmol L^{-1} for each tetracycline, and trinary tetracyclines (tetracycline + chlortetracycline + oxytetracycline) with the concentration at 0, 66.7, 100, 133.3 nmol L^{-1} for each. All culture samples were prepared in triplicate. One milliliter of the culture sample was collected every 30 min for each treatment, and measured the emitted fluorescence (*gfp*mut3 excitation wavelength = 488 nm, emission wavelength = 511 nm) using a SpectraMax M2 spectrofluorometer.

The expression of antibiotic resistance is quantified as the promoter activity of *tet* (M) in the *E. coli* bioreporter at a steady state according to the model developed by Leveau and Lindow (2001).

$$P = f_{ss} \times \mu \times (1 + \mu/m) \qquad (2.1)$$

where P is promoter activity (relative unit of immature GFP per OD unit per hour, RU OD^{-1} h^{-1}), f_{ss} represents the fluorescence at the steady state during bacterial growth (relative unit of fluorescent mature GFP per OD unit, RU OD^{-1}). μ (h^{-1}) is bacterial growth rate, and m (1.54 h^{-1} for *gfp*mut3) is the maturation constant for GFP to develop into fluorescent GFP. The f_{ss} value is obtained from a plot of fluorescence against OD_{600}. The μ value was determined from the slope of linear plot of natural logarithm of OD_{600} values against time (h).

$$OD_{600} = OD_{600,0}\, e^{(\mu \times t)} \qquad (2.2)$$

where t is culture time (h), and OD_{600} and $OD_{600,0}$ are the measured optical density at 600 nm at time t and t = 0, respectively. Integration of fluorescence at the steady state and the growth rate to obtain the promoter activity can circumvent the effects of dilution of GFP contents and GFP maturation during the growth of bacteria, which allows comparisons of antibiotic resistance responses among different experimental settings. For the tetracycline-free controls, a small intensity of fluorescence could be measured with the averaged promoter activity of 181 RU OD^{-1} h^{-1}. These promoter activity values were relative constant with standard deviation 27.4 RU OD^{-1} h^{-1} (n = 10). Therefore, the reported promoter activity values in this study were corrected by subtracting the promoter activity of tetracycline-free control as background.

When the OD_{600} value of the *E. coli* bioreporter cultures approached ~0.5, the bacteria were separated from the culture media by centrifugation at 15000 g and 4 °C for 15 min. After the centrifugation, the bacterial cell pellets were rinsed twice with 20 mL of 10-time diluted phosphate-buffered saline solution (PBS solution pH 7.4). Then 10 mL of McIlvain buffer (12.9 g of citric acid monohydrate, 10.9 g of Na_2HPO_4 and 37.2 g of EDTA dissolved in 1 L of water) was used to suspend the cell pellets and remove tetracycline from the cells. Then mixture was vortexed for 1 min, sonicated for 15 min, and centrifuged at 3000 g for 15 min. Hydrophilic−lipophilic balanced (HLB) cartridge (Waters Corporation, Milford, MA) was used in solid phase extraction to extract tetracycline from aqueous phase. The cell extract containing tetracycline (20 mL) was passed through the preconditioned HLB cartridge. The cartridges were then washed with 1:9 (v/v) methanol/water solution (5 mL). Tetracycline retained on the HLB cartridges

was eluted with 1:1 (v/v) methanol/water solution (5.0 mL) containing 150 mg L^{-1} of EDTA, then with additional 5.0 mL of methanol containing 1% (v/v) formic acid. The tetracycline concentration was analyzed with a Shimadzu Prominence high-performance liquid chromatograph coupled to an Applied Biosystems Sciex 4500 triple quadrupole mass spectrometer (LC-MS/MS). To obtain tetracyclines concentration in bacteria, the measured amount of tetracycline was normalized on the basis of dry bacterial biomass to the intracellular concentration (μmol g^{-1}).

The dry bacterial biomasses were estimated using OD_{600} values. The relationship between bacterial biomass and optical density was reported by Zhang et al. (2014). The weights of bacterial biomass in culture suspensions obtained by freeze drying were plotted against the corresponding OD_{600} values. The linear relationship (Figure 2.1) was utilized to estimate the bacterial biomass present in culture suspension from the measured OD_{600} values.

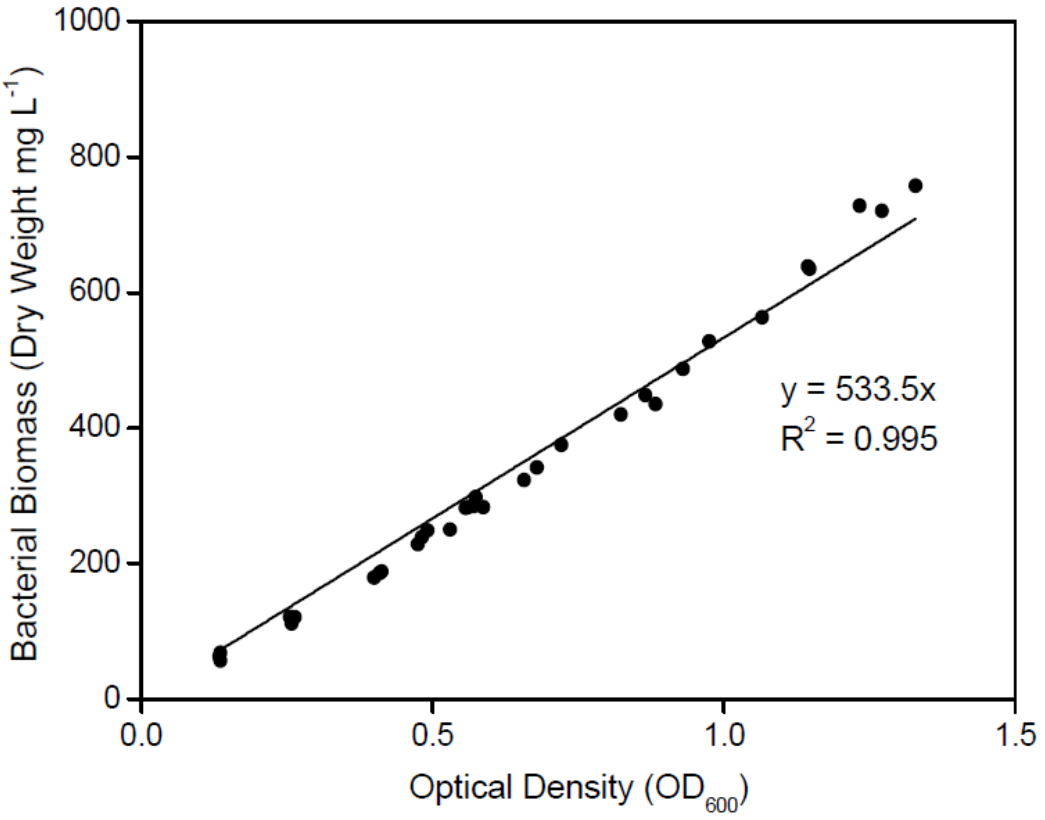


Figure 2.1 Relationship between bacterial biomass in culture suspension and optical density measured at 600 nm.

(Figure from Zhang et al. 2014, Supporting Information Figure S2)

RESULTS AND DISCUSSION

Tetracycline can enter the *E. coli* bioreporter and evoke the expression of antibiotic resistance genes. With increasing initial tetracycline concentrations, the measured intracellular tetracycline concentrations increased, along with the increasing intensity of fluorescence as indicated by the promoter activity values. For example, when the *E. coli* bioreporter exposed to aqueous tetracycline concentration at 50, 100, 200, 300 and 400 nmol L^{-1}, the intracellular tetracycline concentration was 24.8 ± 1.8, 56.3 ± 2.2, 96.8

±1.2, 151.8 ± 29.1 and 206.2 ± 9.5 nmol g^{-1}, respectively. Correspondingly, the estimated promoter activity values increased proportionally yielding 164.6 ± 5.9, 531.7 ± 23.6, 1683.5 ±54.5, 2047.1 ± 259.8 and 2458.3 ± 110.4 RU OD^{-1} h^{-1} , respectively. Similar results were also observed for the bioreporter exposed to oxytetracycline and chlortetracycline, but with varying levels of intracellular uptake and expression of antibiotic resistance genes.

For tetracycline, oxytetracycline and chlortetracycline, the promoter activity expressed in the *E. coli* bioreporter generally manifested a positive linear relation with intracellular tetracycline concentration (Figures 2.2, 2.3 and 2.4). These results suggest that the amount of tetracyclines entering the *E. coli* bioreporter is the determinant for the evoked antibiotic resistance. A previous study indicates that tetracycline zwitterion species in solution is responsible for the uptake by the *E. coli*; and the same aqueous tetracycline concentration may not result in the similar selective pressure on the expression of antibiotic resistance depending on the fractional tetracycline zwitterion (Zhang et al., 2014). Therefore, the intracellular concentration is the appropriate parameter to be used for predicting the expression of antibiotic resistance genes.

Different types of tetracyclines could vary their uptake by the *E. coli* bioreporter. In general, under the exposure of the same initial concentration, the uptake by the *E. coli* bioreporter followed the order of oxytetracycline > chlortetracycline > tetracycline. When exposed to 300 nmol L^{-1} of tetracyclines, the measured intracellular concentrations were 288.3 ± 36.5 nmol g^{-1} for oxytetracycline, 214.4 ± 14.0 nmol g^{-1} for chlortetracycline, and 151.8 ± 29.1 nmol g^{-1} for tetracycline.

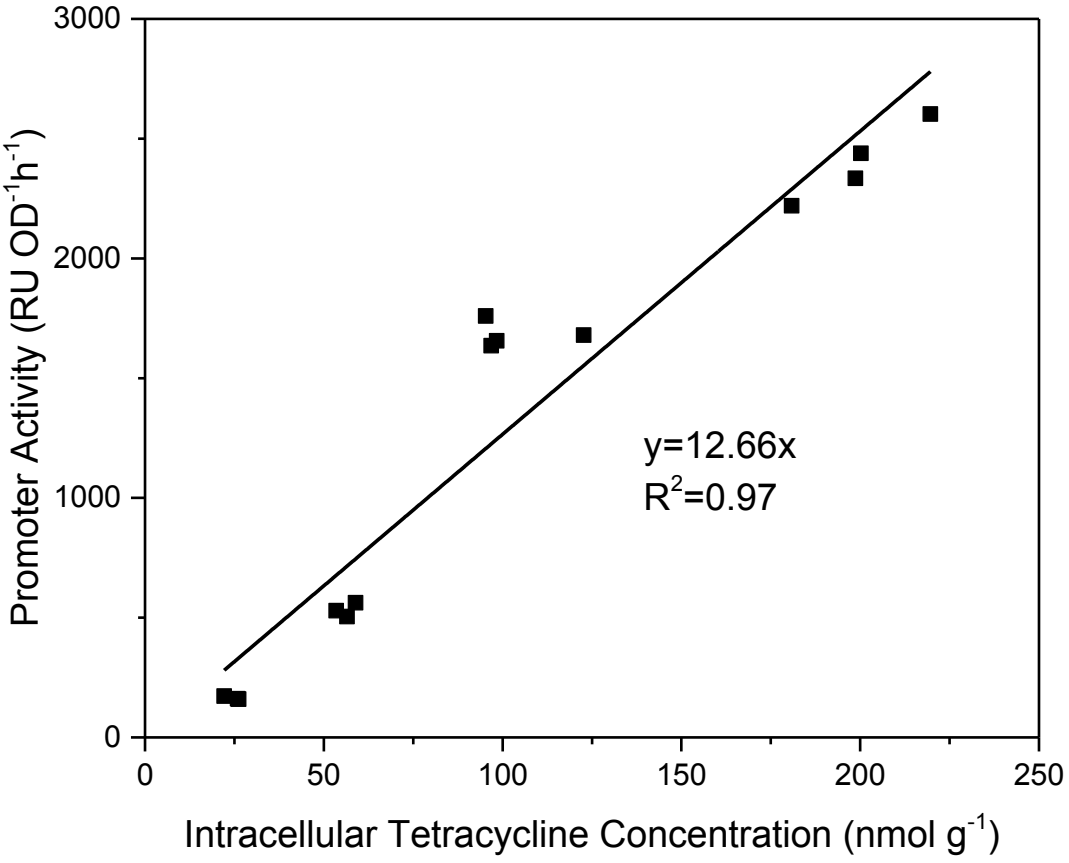


Figure 2.2 Relationship between intracellular tetracycline concentration and the expressed promoter activity of the *E. coli* bioreporter.

In Figures 2.2, 2.3 and 2.4, the slope of the linear fitting represents the increase of promoter activity on the increase of per unit of intracellular concentration. The slope value was 12.66 for tetracycline, 8.90 for chlortetracycline, and 6.33 for oxytetracycline, respectively. This result indicates that under per unit of increasing antibiotic intracellular concentration, tetracycline could exert higher selective pressure on the *E. coli* bioreporter to develop antibiotic resistance, followed by chlortetracycline and oxytetracycline. This

result implies that at the similar intracellular concentration, bacteria exposed to tetracycline plausibly develop more intensity of antibiotic resistance compared to that exposed to chlortetracycline or oxytetracycline.

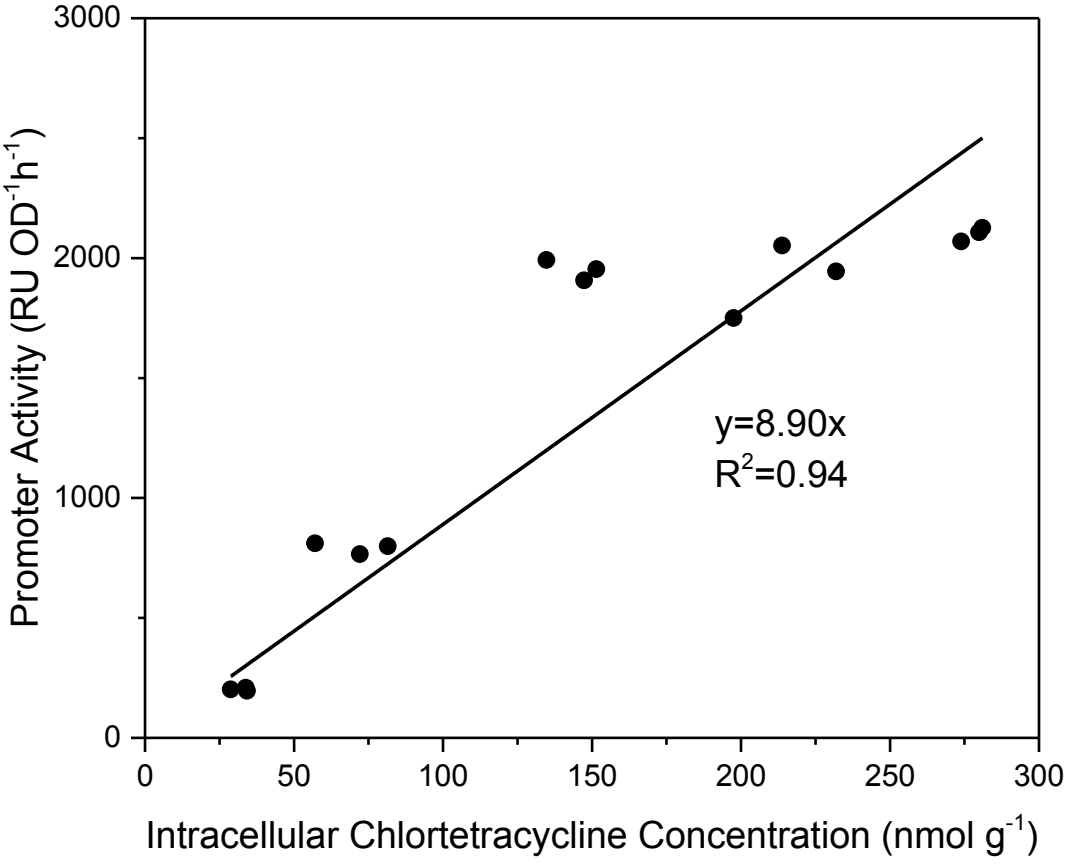


Figure 2.3 Relationship between intracellular chlortetracycline concentration and the expressed promoter activity of the *E. coli* bioreporter.

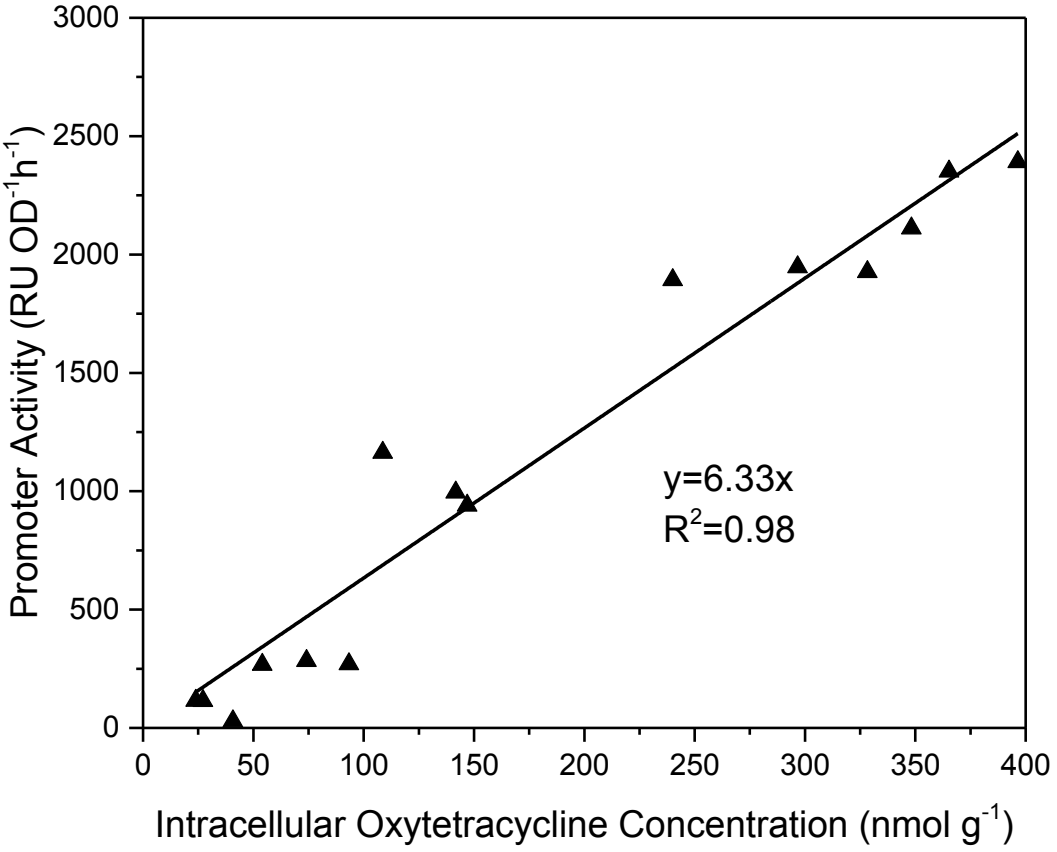


Figure 2.4 Relationship between intracellular oxytetracycline concentration and the expressed promoter activity of the E. coli bioreporter.

In contrast to tetracycline, oxytetracycline and chlortetracycline, the increasing uptake of anhydrotetracycline did not demonstrate enhanced promoter activity (Figure 2.5). The evoked promoter activity approached a relative high level even at a low intracellular concentration. At the intracellular anhydrotetracycline concentration of 100 nmol g^{-1}, the promoter activity was estimated at 2100 RU OD^{-1} h^{-1}. At the similar intracellular concentration the promoter activity values were 1200 RU OD^{-1} h^{-1} for tetracycline, 800

RU OD^{-1} h^{-1} for chlortetracycline, and 500 RU OD^{-1} h^{-1} for oxytetracycline. In Figure 2.6, the promoter activity of anhydrotetracycline reached the highest level even at the lowest initial concentration (50 nmol L^{-1}). Comparing with tetracycline, anhydrotetracycline can more efficiently evoke tetracycline resistance gene. Anhydrotetracycline is a degradation product of tetracycline formed by photolysis under acidic condition (Halling-Sorensen et al., 2002; Hasan et al., 1985; Oka et al., 1989). The formed degradation product anhydrotetracycline has been detected in animal manure composting (Wu et al., 2011) and manure-amended soil (Aga et al., 2005). Although anhydrotetracycline demonstrates reduced antibacterial activity compared to tetracycline, it can bind to tetracycline repressor protein (*TetR*) in bacteria with 500-fold higher affinity than tetracycline (Scholz et al., 2000), which could enhance the activation of resistance genes associated with *TetR*. In our study, the *E. coli* bioreporter consists a *TetR*-regulated tetracycline promoter which is more sensitive to anhydrotetracycline than tetracycline. As a result, the bioreporter manifested very strong promoter activity when exposed to anhyrotetracycline.

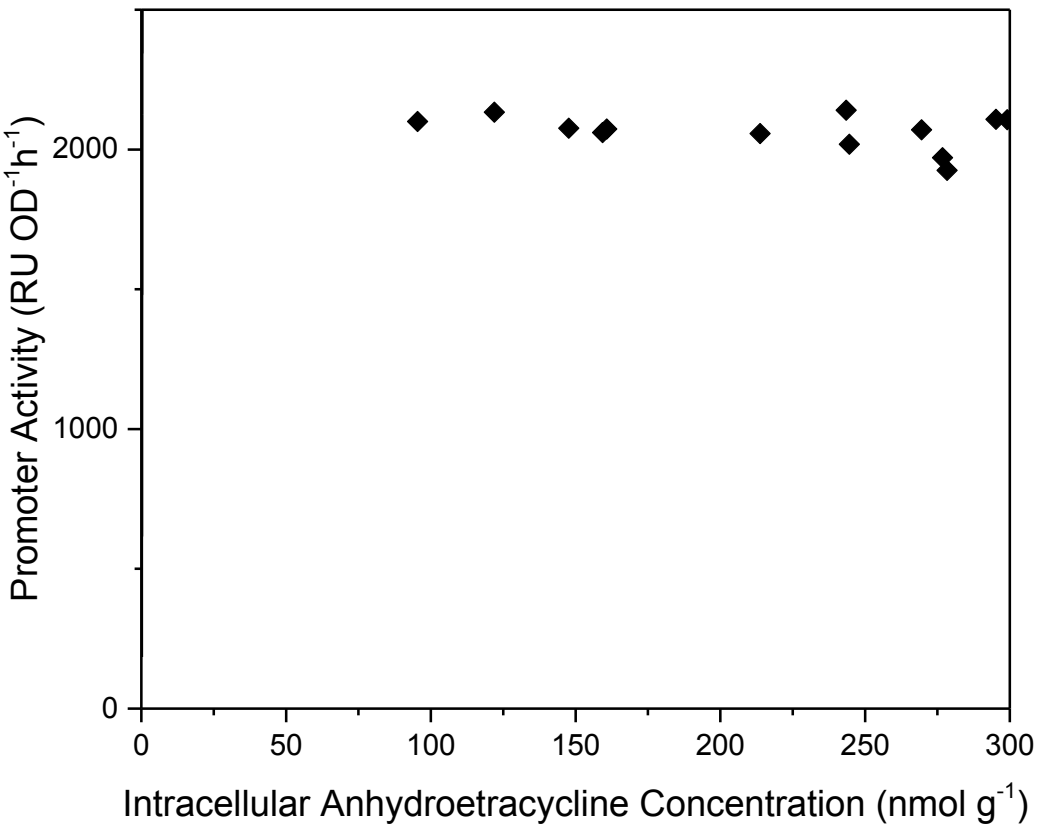


Figure 2.5 Anhydrotetracycline uptake by *E.coli* bioreporter and calculated promoter activity.

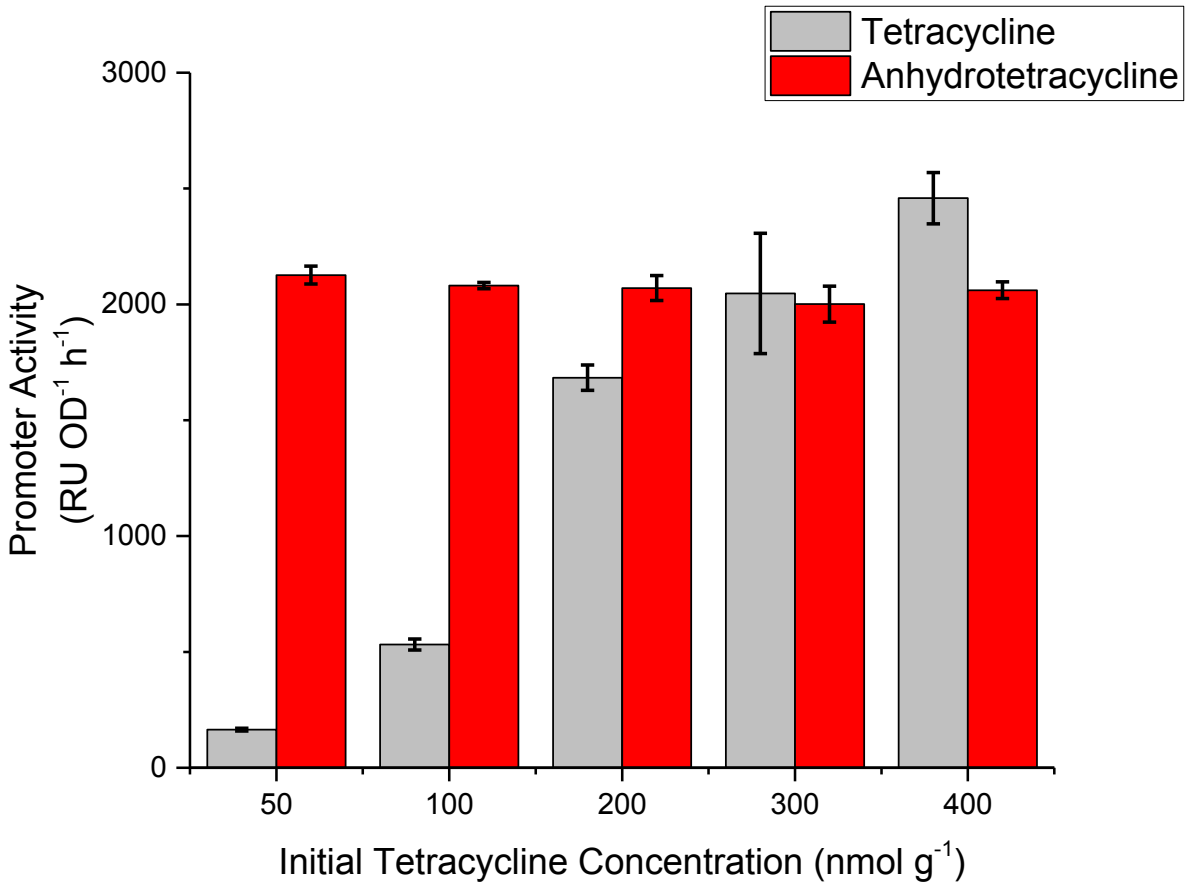


Figure 2.6 Comparison between tetracycline and anhydrotetracycline uptake by *E.coli* bioreporter associated promoter activity.

To examine the biological effects of tetracycline mixtures, binary tetracycline and trinary tetracycline systems were prepared to measure the invoked promoter activity of the *E. coli* bioreporter. In the binary experiments, molar equivalent initial concentrations of 0, 50, 100, 200 nmol L^{-1} for each tetracycline (tetracycline + chlortetracycline; tetracycline + oxytetracycline) and the total initial tetracyclines concentrations were prepared at 0, 100, 200, 300 and 400 nmol L^{-1}. The intracellular concentration for each type of tetracycline was quantified using LC-MS/MS. The promoter activity was estimated using equation (2.1).

The predicted promoter activity for each tetracycline was estimated using the measured intracellular concentration and the linear fitting equations shown in Figures 2.2, 2.3 and 2.4. The overall predicted promoter activity in binary and trinary systems was obtained by summing up the individually estimated promoter activity. For example, in the binary tetracycline + chlortetracycline system, at the total initial concentration of 300 nmol L^{-1} (150 nmol L^{-1} for each), the measured intracellular concentration was 82.4 nmol g^{-1} for tetracycline and 73.6 nmol g^{-1} for chlortetracycline. The estimated promoter activity was 1043.7 RU OD^{-1} h^{-1} from tetracycline (tetracycline: promoter activity = 12.66 × intracellular concentration, Figure 2.2) and 655.2 RU OD^{-1} h^{-1} from chlortetracycline (chlortetracycline: promoter activity = 8.90 × intracellular concentration, Figure 2.3). The sum of 1698.9 RU OD^{-1} h^{-1} was the predicted promoter activity assuming that these two tetracyclines function individually and the resultant biological effects are additive in the *E.coli* cells. Table 2.2 summarizes the measured and predicted promoter activity values in the binary tetracycline +chlortetracycline system. P ($T \leq t$) is the statistical p-value which is used to test a statistical hypothesis from Student's t-test method. The results shows statistically significant difference with the significant levels of 0.05 or 0.01 (Table 2.2). At $P < 0.05$, the measured and predicted promoter activities demonstrated no statistically significant difference for the higher total initial concentrations (300 and 400 nmol L^{-1}), but manifested significant difference for the systems containing lower levels of the total initial concentration (100 and 200 nmol L^{-1}). When selecting $P < 0.01$, the tetracycline + chlortetracycline system was considered as no significant difference at the total initial concentration of 200, 300 and 400 nmol L^{-1}.

Table 2.2 Measured Promoter Activity and Predicted Promoter Activity in the Binary Tetracycline + Chlortetracycline System

Tetracycline + Chlortetracycline					
Initial Total Tetracyclines Concentration (nmol L^{-1})	Measured Promoter Activity (RU OD^{-1} h^{-1})	Predicted Promoter Activity (RU OD^{-1} h^{-1})	P ($T \leq t$)	Statistically Significant Difference ($P < 0.05$)	Statistically Significant Difference ($P < 0.01$)
100	480.6	379.0	0.002	Yes	Yes
	487.3	400.7			
	508.3	357.2			
200	1227.2	424.0	0.022	Yes	No
	1140.0	584.1			
	1199.2	730.5			
300	1473.9	1698.9	0.359	No	No
	1506.3	1396.3			
	1489.6	2134.0			
400	1577.5	1781.0	0.090	No	No
	1590.8	2395.2			
	1519.2	2301.7			

Table 2.3 summarizes the analytical results of the binary tetracycline + oxytetracycline system. Among the four datasets of the total initial concentration, only the system with 200 nmol L^{-1} displayed statistically significant difference. At the other three initial concentration levels, all p-values are > 0.05 indicating that there is no significant difference between the measured promoter activity values and the predicted values. The tetracyclines in the bacterial cells function individually and the antibiotic response effects are additive.

Table 2.3 Measured Promoter Activity and Predicted Promoter Activity in Binary Tetracycline + Oxytetracycline System

Tetracycline + Oxytetracycline					
Initial Total Tetracyclines Concentration (nmol L^{-1})	Measured Promoter Activity (RU OD^{-1} h^{-1})	Predicted Promoter Activity (RU OD^{-1} h^{-1})	$P(T \leq t)$	Statistically Significant Difference ($P<0.05$)	Statistically Significant Difference ($P<0.01$)
100	319.9	305.6	0.385	No	No
	288.7	795.4			
	305.9	338.2			
200	1157.9	576.7	0.001	Yes	Yes
	1142.8	461.6			
	1078.4	410.9			
300	1924.2	1537.4	0.574	No	No
	1929.5	2047.4			
	1750.3	1716.0			
400	2216.0	1925.6	0.803	No	No
	2141.2	2744.3			
	2301.6	2197.4			

In the trinary system (tetracycline + chlortetracycline + oxytetracycline), the initial concentration was 0, 66.7, 100, 133.3 nmol L^{-1} for each type of tetracycline, and the total concentration was 0, 200, 300, 400 nmol L^{-1}. The predicted promoter activity was also estimated by the summation of individual promoter activity contributed from tetracycline, chlortetracycline and oxytetracycline (Figures 2.2, 2.3 and 2.4). The statistical analysis results indicate no significant difference between the measured and predicted promoter activity in the trinary systems. The three tetracyclines presented in the system functioned individually to contribute to the evoked antibiotic resistance.

Table 2.4 Measured Promoter Activity and Predicted Promoter Activity in Trinary Tetracycline + Chlortetracycline + Oxytetracycline System

Tetracycline + Chlortetracycline + Oxytetracycline					
Initial Total Tetracyclines Concentration (nmol L^{-1})	Measured Promoter Activity (RU OD^{-1} h^{-1})	Predicted Promoter Activity (RU OD^{-1} h^{-1})	P(T<=t)	Statistically Significant Difference (P<0.05)	Statistically Significant Difference (P<0.01)
200	1122.3	950.7	0.068	No	No
	1231.4	1005.0			
	1046.9	755.9			
300	1604.6	1288.4	0.137	No	No
	1662.3	1577.3			
	1597.8	1331.1			
400	1738.1	2090.6	0.755	No	No
	1814.2	1436.2			
	1781.1	1594.0			

Taken together, we plotted all data from binary and trinary systems with the measured promoter activity as x-axis and the predicted values as y-axis. The linear fitting obtain the slope of 0.99 (r^2 = 0.90) indicting the ratio of predicted to measured promotor activity is nearly 1:1. In the prediction, we assume that when tetracyclines enter the *E. coli* bioreporter, they function individually with the antibiotic resistance genes, and the resultant effects could be additive. The statistical analysis and comparison of measured vs. predicted promoter activity suggest that the combined effects of tetracyclines to *E. coli* could be considered as additive effects; neither major synergistic nor antagonistic effects is applicable to the tetracyclines in evaluating their combined impact to the expression of antibiotic resistance genes in the *E.coli* bioreporter.

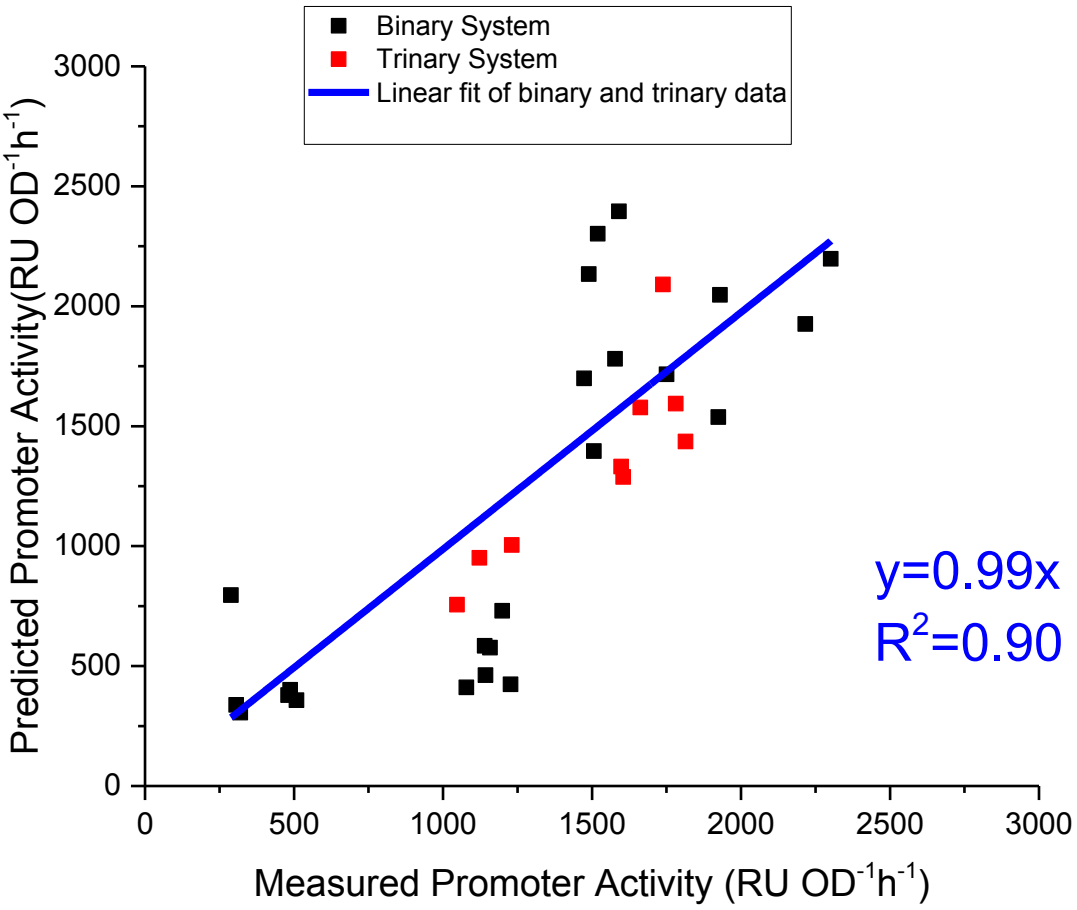


Figure 2.7 Measured Promoter Activity vs Predicted Promoter Activity in binary and trinary systems and linear fitting.

CONCLUSION

In this study, different tetracycline species show different capacity to expression of antibiotic resistant gene for *E.coli* bioreporter. A linear relationship is showed between promoter activity and intracellular tetracycline concentration for tetracycline, chlortetracycline and oxytetracycline. The antibiotic resistance response is in the order of tetracycline > chlortetracycline > oxytetracycline. Bacterial uptake amount is in the order

of oxytetracycline > chlortetracycline > tetracycline. Anhydrotetracycline has the highest antibiotic resistance response although it did not show a linear relationship between promoter activity and intracellular concentration.

In the environment, multiple tetracyclines are commonly found at the contaminated sites which could influence the microbial populations collectively. At the same uptake by *E. coli* different tetracyclines exerted varying levels of selective pressure on the bacteria for expression of antibiotic resistance, with the order of tetracycline > chlortetracycline > oxytetracycline. Linear relations between promoter activity and intracellular tetracycline concentration were observed for tetracycline, chlortetracycline and oxytetracycline, but not for anhydrotetracycline, one of metabolites of tetracycline in the environment. Anhydrotetracycline evoked a very high antibiotic resistance response of the *E. coli* bioreporter due to the fact that it could strongly interact the tetR in the bacteria. The mixture of tetracyclines generally demonstrated additive effects on the *E.coli* reporter for expression of antibiotic resistance. These results suggest that the risk levels of antibiotic resistance invoked by exposure to tetracycline mixtures in the environment could be additive. The effects of formed metabolite from tetracycline such as anhydrotetracycline should be included in the assessment of potential risks of antibiotic resistance to microbial populations.

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