Predicting the long-term toxicity of five-antibiotic mixtures to *Vibrio qinghaiensis* sp. Q67

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Abstract: Concentration addition (CA) is commonly used as a standard additive reference model to predict the short-term toxicity for most chemical mixtures. Whether CA can predict the long-term toxicity of antibiotic mixtures was investigated. The long-term toxicity of five antibiotics including apramycin sulfate, paromomycin sulfate, tetracycline hydrochloride, chloramphenicol and streptomycin sulfate and their mixtures to a photo bacterium Q67 were detected by the long-term toxicity microplate analysis procedure. Seven five-antibiotic mixtures with various concentration ratios and concentration levels were designed by employing uniform design ray method. The long-term mixture toxicity was predicted by CA based on the toxicity data of single antibiotics. The results showed that Weibull or Logit function fit well with the long-term toxicity data of all the components and their mixtures (R>0.98 and $RMSE$<0.07). According the toxicity index, the negative logarithm of mean effect concentration, the long-term toxicity of the five antibiotics differs greatly and is higher than their short-term toxicity. The predicted values by CA model conformed to the experimental values of mixtures, which implies CA can predict reliable results for the long-term toxicity of antibiotic mixtures.

Keywords: Antibiotics; mixtures; concentration addition; long-term toxicity.

INTRODUCTION

Antibiotics play a major role in our modern life, which used them as therapeutics, growth promoters, feed additives and antiseptics. However, a large proportion of the used antibiotics is excreted without metabolizing and is dispersed in all places, eventually reaching surface waters (Nilsson and Webster, 2014). The adverse effect of antibiotics has been elucidated due to the increasing amount of these substances in the environment, which has received little notice till now. Therefore, the investigation on the hazards and risks of antibiotic substances may pose to non-target organisms should be undertaken (Gonzalez-Pleiter *et al*., 2013).

Often, most of the toxicity test methods including the standard ecotoxicological bioassay with *Vibrio fischeri* focus on the short-term toxicity data. However, articles published indicate that the short-term toxicity results may underestimate the toxicity of some pollutants (Newman and McCLoskey 1996). Thus, the long-term toxicity observing the toxicity over the entire lifetime of the exposure organisms for the assessment of hazards and risks by antibiotics is vital important.

Various antibiotics have been detected at the same time in environmental compartments (Ambili *et al*., 2013). Therefore, the organisms may be exposed to various antibiotic mixtures with different concentrations and forms. Though the concentrations of individual antibiotics in environments may be very low or undetectable, the mixture concentrations could result in obviously effect to the exposure organisms (Backhaus *et al*., 2011). Therefore, to accurately evaluate the potential toxic effects and quantifying the possible risks associating with the exposure to antibiotics, antibiotic mixtures should be taken into appoint.

Applying or developing a model helps to throw a light on the combined toxicity of chemicals. Many models including the concentration addition (CA), were developed to predict and evaluate the toxic effects of chemical mixtures (Spurgeon, *et al*., 2010). CA has been successfully used to predict the toxicity of most chemical mixtures in the past years (Liu *et al*., 2013). Unfortunately, the predictions by CA for most chemical mixtures are based on short-term toxicity of single chemicals. Whether the CA model can predict the long-term mixture toxicity still remains untouched.

Therefore, the main purpose of the present study is to test whether CA can predict the long-term toxicity of antibiotic mixtures. To do so, the long-term toxicity of five antibiotics as well as their mixtures to a freshwater photobacterium *Vibrio qinghaiensis* sp.-Q67 (Q67) was investigated by using the long-term toxicity micro plate analysis (L-MTA) method. The prediction values of long-term toxicity of antibiotic mixtures by CA based on the single concentration-response curves (CRCs) were compared to the experimental values.

MATERIAL AND METHODS

Test materials

Five antibiotics including apramycin sulfate (APR), paromomycin sulfate (PAR), tetracycline hydrochloride (TET), chloramphenicol (CHL) and streptomycin sulfate

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(STR) were purchased from Dr. Company (Germany) (table 1). The purities of the five antibiotics were all above 90.0%. The stock solutions were prepared by dissolving antibiotics in milli-Q water and stored in the dark at 4°C.

Microplate toxicity analysis

To compare the long-term toxicity to the short-term toxicity of the five antibiotics, the short-term toxicity was also performed by using the S-MTA procedure. The freeze-dried Q67 was purchased from Beijing Hamamatsu photon techniques Inc. The process of cell culture and microplate design were same as those in the literature (Zhu *et al*., 2009).

The long-term toxicity of the five antibiotics and their mixtures was determined by using the L-MTA method developed in our previous work (Zhu *et al*., 2009). Thirty-six peripheral wells were filled with 200uL distilled water to avoid possible edge effects. Twenty-four wells in the columns of No. 2, 3, 7 and 11were filled with 100uL distilled water as the controls. Twelve different test concentrations prepared in according to a dilution factor were added into the remaining wells. Then, 100uL of the precultured Q67 in the complete medium were added into the control and treatment wells. Another two duplicates were operated simultaneously. All the microplates prepared were incubated at 22±1°C for 12 h and the final relative light units (RLUs) were determined by using Spectra Max M5.

The calculation formula of toxicity/effect of toxicants, expressed as a percent inhibition on the luminescence of Q67, was same as that in the literature (Zhang *et al*., 2008; Zhang *et al*., 2011). The obtained toxicity data was fitted to Logit and Weibull, two-parameter nonlinear functions. The higher correlation coefficient (*R*) and lower root mean square error (*RMSE*) are, the better fitting is. The formulae of the two functions were same as those in the references.

Mixture design

All the antibiotic mixtures under study were designed by using uniform design ray method. Seven different antibiotic mixture rays with various concentration ratios (p_is) (U1, U2,…, U7) were given in table 2. On each mixture ray, 12 concentration-effect points were arranged.

CA prediction model

CA is selected as a reference model and is written as follows (Berenbaum, 1985):

$$EC_{x,mix} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x,i}} \right)^{-1}$$

where $EC_{x,\ mix}$ is the effect concentration of the mixture eliciting *x*% effect, $EC_{x,i}$ denotes the concentration of the *i*th component when exists individually and elicits the same effect (*x*%) as the mixture, p_i is the molar concentration ratio of the *i*th component in the mixture.

RESULTS

The short-term and long-term toxicity of the individual antibiotics

Both of short-term and long-term concentration-response data were fitted to the two two-parameter functions and the results were given in table 1. All the CRCs were plotted in fig. 1.

From table 1, Weibull or Logit can well describe the short- and long-term concentration-response data of the five anbiotics to Q67 with R>0.89 and $RMSE \leq 0.16$. According to the pEC_{50} (the negative logarithm of EC_{50}),

Table 1: The physiochemical properties, CAS numbers, stock solutions, short-term (S) and long-term (L) toxicity statistics of five antibiotics

Antibiotics	CAS RN	M.W.	Stock ($mol \cdot L^{-1}$)	Toxicity type	F	α	β	R	*RMSE*	pEC_{50}
Apramycin sulfate (APR)	65710-07-8	637.6	2.20E-02	S	Weibull	11.84	5.81	0.8857	0.163	2.08
				L	Weibull	14.37	2.71	0.9950	0.032	5.44
Paromomycin sulfate (PAR)	1263-89-4	713.7	1.09E-03	S						NA
				L	Weibull	12.47	2.14	0.9973	0.024	6.00
Tetracycline hydrochloride (TET)	64-75-5	480.9	1.44E-03	S	Weibull	13.23	3.80	0.9969	0.027	3.58
				L	Weibull	11.52	2.04	0.9966	0.025	5.83
Chloramphenicol (CHL)	56-75-7	323.1	3.38E-03	S	Weibull	2.99	1.31	0.9735	0.027	2.49
				L	Logit	19.96	3.00	0.9977	0.025	6.65
Streptomycin sulfate (STR)	3810-74-0	1457.4	1.54E-03	S						NA
				L	Logit	23.76	3.36	0.9910	0.049	7.07

Note: MW- molecular weight, F- the fitted functions, pEC_{50}- negative logarithm of mean effect concentration and NA- not available within the limited concentrations.

Table 2: The concentration ratios (p_i), fitted functions and some statistics of seven five-antibiotic mixture rays

Ray	p_{APR}	p_{PAR}	p_{TET}	p_{CHL}	p_{STR}	F	α	β	R	RMSE	pEC_{50}
U1	4.22E-01	1.40E-01	3.07E-01	8.11E-02	5.02E-02	W	13.22	2.24	0.9963	0.021	6.06
U2	3.44E-01	1.39E-01	4.94E-01	1.09E-02	1.21E-02	W	11.49	2.02	0.9884	0.042	5.87
U3	5.09E-01	3.46E-01	8.50E-02	5.54E-02	5.39E-03	L	17.25	2.97	0.9960	0.024	5.81
U4	6.15E-01	2.69E-02	3.11E-01	1.82E-02	2.84E-02	W	12.80	2.24	0.9700	0.065	5.88
U5	8.11E-01	8.23E-02	3.08E-02	6.36E-02	1.28E-02	W	14.52	2.56	0.9878	0.044	5.82
U6	7.41E-01	1.29E-01	1.09E-01	1.55E-02	4.96E-03	W	12.55	2.27	0.9950	0.029	5.69
U7	5.66E-01	1.56E-01	2.31E-01	3.44E-02	1.32E-02	W	13.72	2.42	0.9964	0.023	5.82

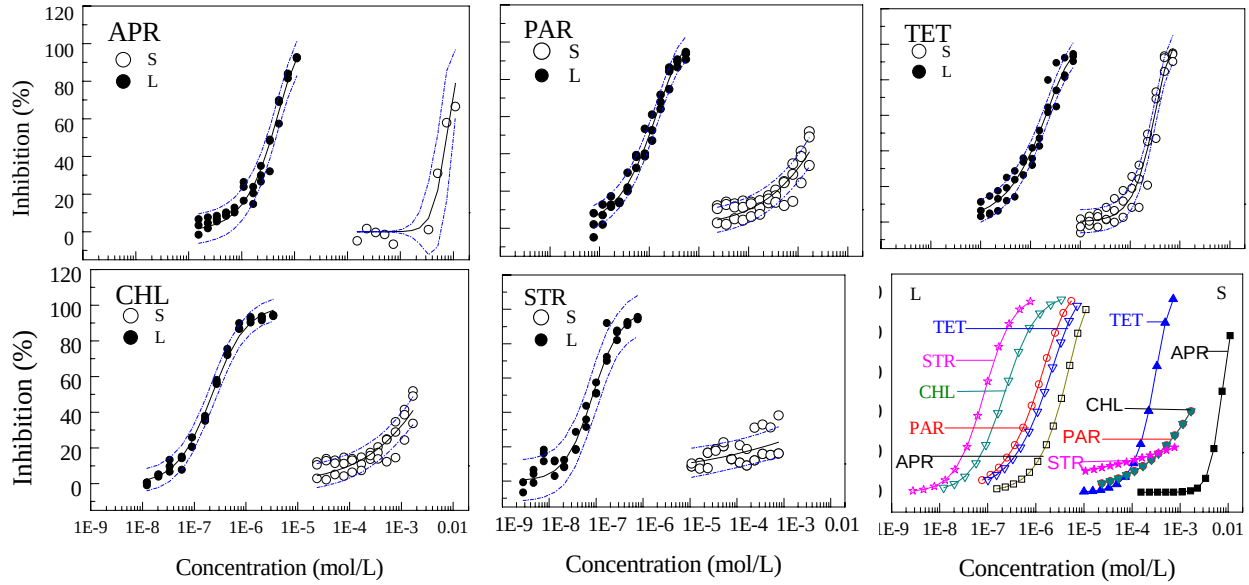

Fig. 1: Comparison between short-term toxicity (S) and long-term toxicity (L) CRC profiles of five antibiotics. The black scared points, black solid lines and blue dash dot lines in figure represent the experimental values, fitted lines and 95% confidence intervals, respectively.

a toxicity index, the long-term toxicity of the five antibiotics are higher than their short-term toxicity. PAR and STR exhibit almost not available short-term toxicity but obviously long-term toxicity to Q67. The long-term toxicity of the five antibiotics differ greatly and the toxicity order is STR>CHL>PAR>TET>APR (table 1). The long-term toxicity of STR is the highest which shows no available short-term toxicity. Fig. 1 also shows that all the short-term CRCs of the five antibiotics lie on the right side of the long-term CRCs.

Long-term toxicity of seven five-antibiotic mixtures

The long-term CRCs of seven pentabasic antibiotic mixture rays were shown in fig. 2 and the fitted functions as well as some statistics were given in table 2. From table 2, Weibull or Logit function can also well describe the long-term concentration-response curves of seven mixture rays (R>0.98 and $RMSE$<0.07). The long-term toxicity of the seven mixture rays with different p_is differs little. From fig. 2, all the CRCs-CA almost overlaps with the observed CRCs of the seven mixture rays and clear deviations do not be significant though they have different p_is.

Prediction of the toxicity of five-antibiotic mixtures by CA

All of the CRCs predicted by CA (CRCs-CA) of the seven mixture rays were plotted in fig. 2. All the CRCs-CA of the seven mixture rays completely lies in the confidential intervals of the observed CRCs and exhibit additive action. Therefore, CA can accurately predict the long-term toxicity of the five-antibiotic mixture rays.

DISCUSSION

Due to the continued release and long-time existing in the environment, antibiotics are considered to be “pseudopersistant” pollutants. The short-term toxicity has overlooked the fact that toxicity can be influenced by both exposure intensity and duration (Zhu *et al*., 2009). In this paper, the long-term toxicity of the five antibiotics is obviously higher than their short-term toxicity. Bioluminescence is a complex phenomenon in photo bacteria. As for the antibiotics, the significant increase of toxicity in the L-MTA may be attributed to the change of energy status of the bacteria, which regulate the growth and reproduction of Q67.

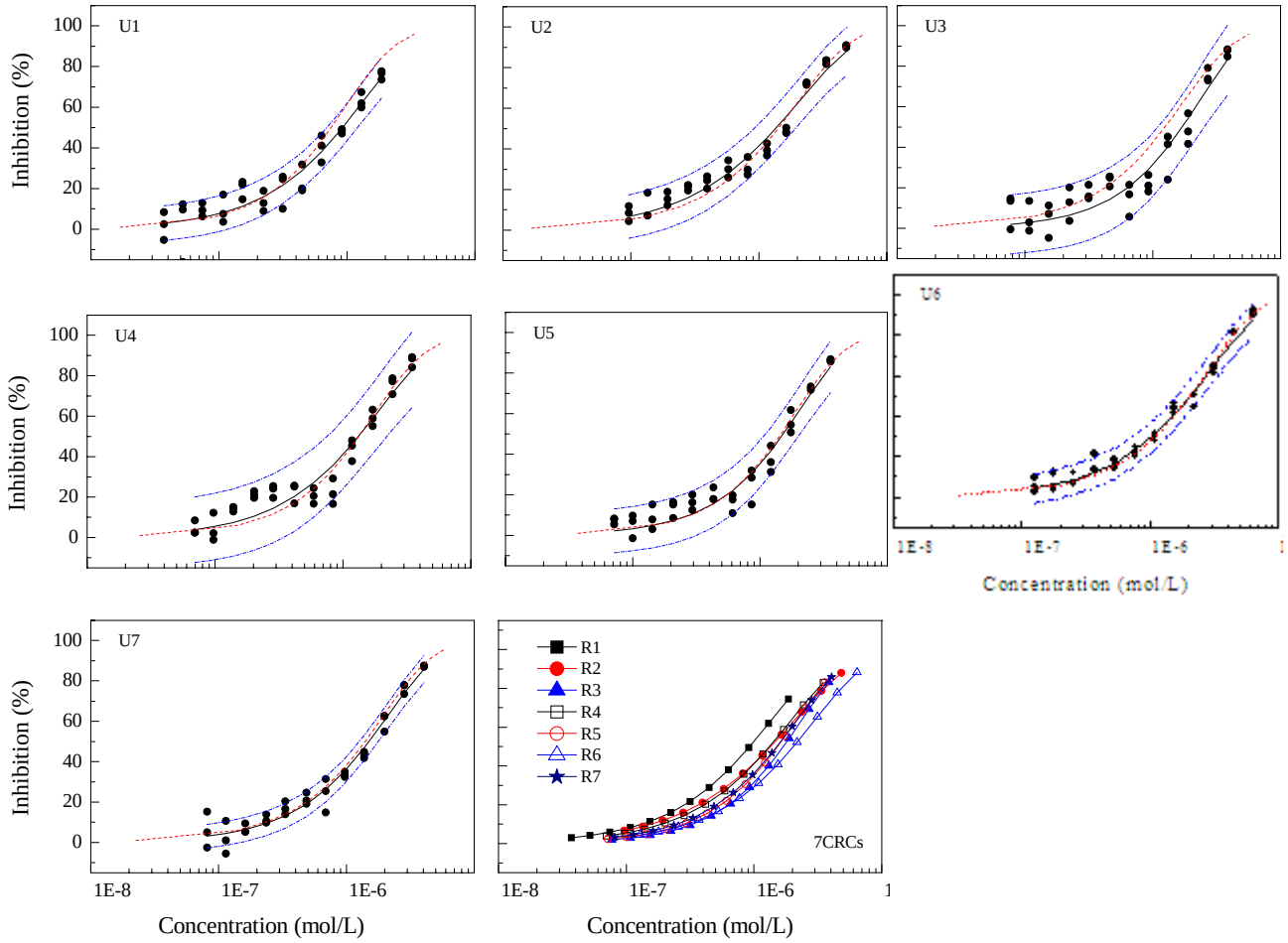

Fig. 2: The long-term toxicity CRC profiles of seven antibiotic mixture of five antibiotics. The red dash line in fig. represent the predicted values by CA.

CA can be used to predict the possible combined toxicity between mixture components (Liu *et al*., 2013). The CRCs of the seven mixture rays almost overlapped and obviously difference does not exist although they have different p_is. All of the CRCs predicted by CA in fig. 2 lie in the confidential intervals of the observed CRCs of seven mixture rays exhibiting additive action. The results indicate that CA can have a good prediction of the long-term mixture toxicity of the five antibiotics though they may different mode of action. The possible reason may be the same toxicity mechanism of antibiotics on Q67 in the L-MTA as stated above. Therefore, the model CA can also be suggested to predict the long-term toxicity of antibiotic mixtures.

CONCLUSIONS

The long-tem toxicity data of the five antibiotics and their mixtures can be described well by Weibull or Logit functions (R>0.98 and $RMSE$<0.07). Selecting the pEC_{50} value as a toxicity index, the long-term toxicity of the five antibiotics to Q67 is higher than their short-term toxicity. The long-term toxicity of the five antibiotics differs greatly and the toxicity order of the five antibiotics is STR>CHL>PAR>TET >APR. The long-term toxicity of the seven five-antibiotic mixture rays with different p_is differs little. CA can well predict the long-term toxicity of the five-antibiotic mixture rays with different p_is exhibiting additive action. CA can also be suggested to predict the long-term toxicity of antibiotic mixtures.

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