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Novel approach to predicting hormetic effects of antibiotic mixtures on *Vibrio fischeri*

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HIGHLIGHTS

- Different hormetic effects were determined in antibiotics and their mixtures.
- CA or IA models cannot predict the hormetic effects of interactive mixtures.
- Six-point approach can successfully predicted the hormetic effects of mixtures.

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ABSTRACT

The determination of the hormetic effects of a mixture is quite difficult because of the moderate simulation and the complexity of measurement in low doses. In the present study, two typical models for mixture toxicity prediction, concentration additive (CA) and independent action (IA), were used to predict the hormetic effects of mixtures. The predictive power of those models was validated by the hormetic effects (24-h exposure) of antibiotic's binary mixtures to *Vibrio fischeri*. The results showed that CA and IA were unable to predict the hormetic dose-response of mixture, especially those of the interactive mixtures. As an alternative, a novel model, which was named as "six-point" and developed based on the quantitative features in the determined dose-response curve and on the Quantitative Structure Activity Relationships (QSARs) approach, was proposed for predicting the hormetic effects of mixtures in low dose. The results indicated that the "six-point" model can accurately predict the mixture hormetic effects in low dose, not only for non-interactive mixtures but also for interactive mixtures. Therefore, the "six-point" model is a powerful tool to predict the mixture hormetic effects at low dose, and may offer an important approach in the environment risk assessment of mixtures.

1. Introduction

Hormesis is an interesting phenomenon that was characterised by low-dose stimulation and high-dose inhibition (Calabrese and Baldwin, 2002). A growing body of reports proved that the phenomenon of hormesis were frequently encountered in toxicity assays as well as in the natural environment (Calabrese and Baldwin, 2003; Calabrese, 2008). As is well-known, organisms are commonly exposed to mixtures, rather than single chemicals (Yang et al., 1998), and thus there is a critical need to investigate the hormetic effects in mixture and assess its potential toxic effects in environmental risk assessment (Chapman, 2002; Kaiser, 2003). However, the determination of mixture hormetic effects is quite difficult mainly because of the typical low-magnitude simulator and the complexity of the measurement in low doses (Calabrese and Baldwin, 2003; O'Brien and Dietrich, 2004). Therefore, it is essential to explore a novel approach to predict the hormetic effects of mixtures.

Most of the hormetic studies, however, focused on the quantitative features of individual chemicals. In an earlier study (Calabrese and Baldwin, 1997), a quantitative approach based on the features

Abbreviations: TU, toxicity units; MOA, mode of action; CA, concentration additive; IA, independent action; CA and IA, CA (IA); *M*, the concentration of maximal stimulatory effect; *ZEP*, the concentration of zero equivalent point; Y_{max}, maximal stimulatory effect; $Y_{h\text{-}ZEP}$, the stimulatory effect at half the concentration of the zero equivalent point; *RMSE*, root-mean-square-error; TMP, trimethoprim; SMZ, sulphamethoxazole; SMP, sulphamethoxypyridazine; SCP, sulphachloropyridazine; TCC, tetracycline·HCl; OTC, oxytetracycline·HCl; QSARs, Quantitative Structure Activity Relationships.

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of dose-response curve was proposed to evaluate the hormetic effects of chemicals (e.g., hormesis magnitude, reproducibility). The results indicated that 53% of the experiments were ranked in the "low" evidence category. Then, the researchers further founded that the maximum stimulation for hormetic response appeared to be 30–60% greater than the control group (Calabrese and Baldwin, 1998). Lately, hormesis quantitative parameters, including the width of stimulatory effects, the magnitude of hormesis, were demonstrated to be relate to the chemical toxic effects (Calabrese and Blain, 2005; Nascarella and Calabrese, 2009; Nascarella et al., 2009).

On the other hand, only a small number of studies investigated the hormetic features of mixtures (Ohlsson et al., 2010), although the hormetic effects was reported in some mixtures, such as heavy metals, PCBs, and persistent organic pollutants (Gennings et al., 2002; Love et al., 2003; Gregoraszczuk et al., 2008). In the field of mixtures toxicity, a number of approaches, especially the concentration addition (CA) model and the independent action (IA) model (Bliss, 1939; Plackett and Hewlett, 1952), were extensively employed to predict the toxic effects of mixtures (Backhaus et al., 2004). Recently, the CA and IA models were also employed in predicting the hormetic effects of mixtures in a few studies. Ge et al. (2011) proved the hormetic effects of ionic liquids can be predicted by the CA model. The study of Belz et al. (2008) found that the concentration of maximal stimulatory effect (*M*) and the zero equivalent point (*ZEP*) could be forecasted by the CA model. However, other studies showed that the hormetic effects of mixtures were inaccurately predicted by CA and IA models, especially when the toxicity of dissimilarly acting chemicals was investigated (Backhaus et al., 2004). Therefore, when predicting the hormetic effects of mixtures, it still remains unclear what the application domain (e.g., non-interactive or interactive mixtures) of the CA and IA models is. If the CA and IA models can only be applied to predict the hormetic effects in the limited number of mixtures, the general approach to predict the mixture hormetic effects should be explored because most mixtures do not conform to the simple CA or IA model (McCarty and Borgert, 2006).

Recently, several researchers have raised concerns about the risk assessments on antibiotics and their mixtures (Kümmerer, 2009). When discharged into the environments like water and soil (Kolpin et al., 2002), antibiotic can cause toxicity to a number of organisms like bacteria, algae, invertebrates, and mammals (Kümmerer, 2009; Aristilde et al., 2010; Bruce et al., 2010). Furthermore, the toxicity of antibiotic mixtures was also addressed in previous reports (Zou et al., 2012), because of the frequent combination therapies (Bushby, 1973). The hormetic effects of antibiotic mixtures, however, were rarely investigated, even though the hormetic effects of antibiotics in low doses were observed as early as the 1880s (Calabrese and Baldwin, 2000). What is more, a number of studies have proved that antibiotics and their metabolites were typically detected at trace levels (Hernando et al., 2006; Benotti et al., 2008). Consequently, the hormetic effects of antibiotic mixtures at low dose should be investigated.

In the present study, the toxic effects of antibiotics on *V. fischeri* (a classical bacterium that has been widely used in the determination of the toxic effect of chemicals), individually and in binary mixtures, were determined. The purposes of this study are as follows: (1) to determine the hormetic (toxic) effects on *V. fischeri* (24 h exposure) for single antibiotics and binary mixtures; (2) to verify the predict power of CA and IA models for hormetic effects of mixtures; and (3) to develop a general approach to predict the hormetic effects in binary mixtures based on the quantitative features of the dose-response curve of the individual chemicals.

2. Materials and methods

2.1. Single toxicity test

The following test antibiotics were purchased from Sigma Co., Ltd. (purity ⩾ 99%): Trimethoprim (TMP), three sulfonamides (SAs) (sulphamethoxazole (SMZ), sulphamethoxypyridazine (SMP), sulphachloropyridazine (SCP)), and two TCs (tetracycline·HCl (TCC), oxytetracycline·HCl (OTC)). The stabilities of the test antibiotics were obtained by determining their concentrations by HPLC at 0 h and 24 h (Table 1), and detailed information concerning the stability test is shown in Supplementary information.

The single toxicity test was performed according to the methods of Backhaus et al. (1997). The derived concentration relationship data for no hormetic effects chemicals were fitted with Logistic-model (Eq. (1)) (Finney, 1971), whereas the concentration relationship data for hormetic effects chemicals were fitted with Brain–Cousens Model (Eq. (2)) (Brain and Cousens, 1989). The higher the square of the correlation coefficient (R^2) and the lower of the root-mean-square error (*RMSE*), the better fit.

$$Y = \delta + \frac{\alpha - \delta}{1 + \exp(\beta \ln(x/EC_{50}))}, \quad (1)$$

$$Y = \delta + \frac{\alpha - \delta + \gamma \times x}{1 + \left(1 + \frac{2\gamma \times EC_{50}}{\alpha - \delta}\right) \times \exp(\beta \ln(x/EC_{50}))}, \quad (2)$$

where α is the response at indefinitely large concentrations, δ is the expected response of the control group, γ denotes the rate of increase in the response at low-concentrations, EC_{50} denotes the concentration causing 50% inhibition of the control response, and β denotes the rate of change around EC_{50}.

In the case of the hormetic dose-response another three parameters were determined (Fig. 1A): Y_{max} is the maximal stimulatory effect, *M* denotes the concentration of maximal stimulatory effect, and *ZEP* is the concentration of zero equivalence point (Nascarella and Calabrese, 2009).

2.2. Mixture toxicity test

Based on the single chemical toxicity results (EC_{50}), the binary mixtures were designed in the ratio of individual EC_{50} ($1{:}10^{2.5}$, $1{:}10^{2.0}$, $1{:}10^{1.5}$, 1:10, $1{:}10^{0.5}$, 1:1, $10^{0.5}{:}1$, 10:1, $10^{1.5}{:}1$, $10^{2}{:}1$, $10^{0.5}{:}1$). The mixture toxicity was fitted and described as EC_{50}^{mix}. The sum of toxicity unit (TU_{mix}) was derived basing on the results of EC_{50}^{mix} (Backhaus et al., 2004), and the detailed information concerning TU_{mix} is shown in Supplementary information.

Table 1
Stability results and hormetic dose response parameters for single antibiotics.

Chemical	CAS	Solutes loss (%)	α	δ	γ	β	$x0$ (EC_{50}) (M)	R^2 (Adj)	*RMSE*	*M* (M)	*ZEP* (M)	Y_{max}
SMZ	723-46-6	−0.99	17.771	98.597	−1.773E−7	5.812	1.855E−5	0.990	0.0645	9.094E−6	1.494E−5	−112.804
SMP	80-35-3	−0.81	4.528	97.913	−3.591E−6	6.357	4.395E−5	0.987	0.0774	2.343E−5	3.686E−5	−66.411
TMP	738-70-5	−0.42	11.896	105.089	−2.448E−7	7.065	6.160E−6	0.975	0.0575	3.575E−6	5.163E−6	−60.867
TCC	64-75-5	−8.58	8.373	100.998	–	1.693	2.383E−8	0.974	0.0522	–	–	0
OTC	2058-46-0	−10.10	3.393	101.012	–	1.598	2.166E−8	0.972	0.0442	–	–	0