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Global concentration additivity and prediction of mixture toxicities, taking nitrobenzene derivatives as an example

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ABSTRACT

The toxicity of a mixture depends not only on the mixture concentration level but also on the mixture ratio. For a multiple-component mixture (MCM) system with a definite chemical composition, the mixture toxicity can be predicted only if the global concentration additivity (GCA) is validated. The so-called GCA means that the toxicity of any mixture in the MCM system is the concentration additive, regardless of what its mixture ratio and concentration level. However, many mixture toxicity reports have usually employed one mixture ratio (such as the EC_{50} ratio), the equivalent effect concentration ratio (EECR) design, to specify several mixtures. EECR mixtures cannot simulate the concentration diversity and mixture ratio diversity of mixtures in the real environment, and it is impossible to validate the GCA. Therefore, in this paper, the uniform design ray (UD-Ray) was used to select nine mixture ratios (rays) in the mixture system of five nitrobenzene derivatives (NBDs). The representative UD-Ray mixtures can effectively and rationally describe the diversity in the NBD mixture system. The toxicities of the mixtures to *Vibrio qinghaiensis* sp.-Q67 were determined by the microplate toxicity analysis (MTA). For each UD-Ray mixture, the concentration addition (CA) model was used to validate whether the mixture toxicity is additive. All of the UD-Ray mixtures of five NBDs are global concentration additive. Afterwards, the CA is employed to predict the toxicities of the external mixtures from three EECR mixture rays with the NOEC, EC_{30}, and EC_{70} ratios. The predictive toxicities are in good agreement with the experimental toxicities, which testifies to the predictability of the mixture toxicity of the NBDs.

1. Introduction

Organisms are usually exposed to numerous different chemicals in the environment. To estimate the risk of mixtures in the environment, it is important to know how to evaluate or predict the toxicity of a multiple component mixture (MCM). Many scientists have been devoted to this field (Altenburger et al., 2013; Cedergreen, 2014; Neale et al., 2015; Pohl et al., 2012). Two classical additive reference models for the assessment of combined toxicity are the concentration addition model (CA) and the independent action model (IA) (Baldwin and Roling, 2009; Larsson et al., 2014; Martin et al., 2015). The CA and IA were widely used to evaluate the combined toxicity of mixtures. However, there are few reports on how to select effectively the representative mixtures to describe rationally the concentration diversity of MCMs. The reason for the scant progress is that it is particularly difficult to evaluate the combined toxicity of various mixtures with many mixture ratios and concentration levels. Optimal experimental designs (OED) currently applied in the mixture design need many experimental toxicity tests. Most OED studies are not good or sufficiently comprehensive in an MCM system containing more than three components (Liu et al., 2016b).

Recently, the equivalent effect concentration ratio design (EECR, usually EC_{50} ratio) or fixed-ratio ray design (FRRD) were usually used to design a series of mixtures (Stork et al., 2007; Yeatts et al., 2010) and attempted to describe the diversity of MCMs. Several researchers used NOEC (Rajapakse et al., 2002) and 0.25% of the EC_{50} (Deneer et al., 1988) to design FRRD mixtures to simulate the composition of poisons in the environment. However, a mixture system with a definite chemical composition comprises many mixture rays with different mixture ratios, and each ray consists of many mixtures with different concentration levels. All of the concentration points of the mixtures designed by the EECR or FRRD are located on one ray in the concentration space. One ray could not represent the whole mixture system and certainly cannot predict the mixture toxicity of other rays with different ratios (Liu et al., 2016a, 2016b).

Increasing numbers of studies have shown that the combined

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toxicity of mixture or interaction is dependent on the concentration level and the concentration ratio (Jonker et al., 2005; Liu et al., 2016a; Nascimento et al., 2016; Zhang et al., 2015). For example, Jonker et al. (2005) found that the antagonism in a binary mixture system be caused primarily by toxicant 1, whereas synergism can be caused mainly by toxicant 2. Dou et al. (2011) found that the interactions of the mixture ray DIC-IL1-R3 with the EECR of EC_{50} at all effect levels are less than those of the DIC-IL1-R4 with the ratio of $2EC_{50,DIC}$:$EC_{50,IL1}$ (another mixture ray). Furthermore, the CA and IA based on the FRRD mixtures can only assess but not predict the combined toxicity (Liu et al., 2016a). For an MCM system with a definite chemical composition, the mixture toxicity can be predicted only if the global concentration additivity (GCA) is validated. The so-called GCA confirms that the toxicity of any mixture is concentration additive, regardless of its mixture ratio/concentration ratio and concentration level. The EECR mixtures cannot simulate the concentration diversity and mixture ratio diversity of mixtures in the real environment; therefore, it is impossible to validate the GCA. Thus, it is necessary to develop a method that can accurately summarize the different mixture compositions with various ratios and concentration levels (Liu et al., 2016b).

MCM is a complex chemical system. It is impossible to test the toxicities of all mixtures in the MCM system, and it is necessary to use an OED procedure to select a small number of mixtures (Liu et al., 2016a, 2016b). Factorial design and orthogonal design are two typical methods for the design of chemical experiments (Groten et al., 1996). Nevertheless, the two OEDs are not suitable for the MCM system with more than three components or with more concentration levels because there are many experiments to be done in these situations. In our previous study, we combined the uniform design (Fang and Zhang, 2000) with FRRD and developed the uniform design ray (UD-Ray) procedure (Fang and Zhang, 2000; Liu et al., 2016b, 2012). The UD-Ray proved to be a suitable method to study the combine toxicity of the MCM system (Ge et al., 2014; Zhang et al., 2010).

Nitrobenzene derivatives (NBDs, including nitrobenzene) are a group of toxic chemicals that are widespread in the environment due to their worldwide use. NBDs are important chemical materials and intermediate products in the production of explosives, drugs, dyes, fungicides, and other industrial compounds (Health et al., 1989). Although NBDs are mostly priority pollutants and thus are banned using in many countries because of their toxicity to living organisms, they are still detected in the environment. The toxicity of exposure to NBDs in different aquatic biota, such as luminescent bacteria, *Scenedesmus obliquus*, *Daphnia magna*, and stingrays, has been widely studied (Limin et al., 2002; Liu and Lang, 1995; Thomulka and Lange, 1997). The combined toxicities of some mixtures containing NBDs have been reported and observed to be concentration additive (Ge et al., 2015; Liu et al., 2009; Liu, 2009; Xiao et al., 2008).

In this study, nine representative mixture rays in the mixture system of five NBDs were designed by the UD-Ray, and the toxicities of various UD-Ray mixtures were determined by a microplate toxicity analysis (MTA). For each ray, 12 concentration levels (points) were set by the FRRD method. The combined toxicities of various five-component NBD mixtures were evaluated by taking the CA as an additive reference. Next, the predictive ability for the toxicity of unknown mixtures was tested by 36 mixtures (points) in three EECR rays, which are different from the UD-Ray mixtures.

2. Materials and methods

2.1. Chemicals

Three nitrobenzene derivatives, 4,6-dinitro-*o*-cresol (NB1), 4-nitrophenol (NB4) and 2-nitrophenol (NB3), were purchased from Dr. Ehrenstorfer Corp. (Germany). The other derivatives, 2,4-dinitrotoluene (NB2) and nitrobenzene (NB5), were purchased from Cato Research Chemicals Inc. (U.S). The derivatives' physical properties are listed in Table 1.

Table 1
Certain physical properties, such as CAS registry number (CAS RN), molecular weight (M.W) and stock solutions, of five nitrobenzene derivatives (NBDs).

No.	Chemical	CAS RN	M.W.	Purity (%)	Stock (mol/L)	Dilution factor
NB1	4,6-dinitro-o-cresol	534-52-1	198.15	99.6	1.11E−03	0.57530
NB2	2,4-dinitrotoluene	121-14-2	182.13	99.5	1.32E−03	0.66893
NB3	2-nitrophenol	88-75-5	139.11	98.2	2.60E−03	0.64257
NB4	4-nitrophenol	100-02-7	139.11	99.3	3.08E−03	0.64257
NB5	Nitrobenzene	98-95-3	123.11	99.5	6.04E−03	0.79433

All stock solutions were prepared with Milli-Q water and dimethyl sulfoxide (DMSO). The final DMSO concentration in the solution was 1% (v/v). The molecular structures of the five NBDs are shown in Fig. S1.

2.2. Bacterial culture

The freeze-dried luminescent bacterium *Vibrio qinghaiensis* sp.-Q67 (*V. qinghaiensis*) was purchased from Beijing Hamamatsu Corp. Ltd. (Beijing, China). The complete liquid culture medium consisted of 13.6 mg KH_2PO_4, 35.8 mg $Na_2HPO_4{\cdot}12H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.61 g $MgCl_2{\cdot}6H_2O$, 33.0 mg $CaCl_2$, 1.34 g $NaHCO_3$, 1.54 g NaCl, 3.0 g glycerin, 5.00 g yeast extract, 5.00 g tryptone, and 1000 mL distilled water, and the pH was adjusted to 8.5–9.0 (Ma et al., 1999; Zhang et al., 2008). By adding 2% agar to the above solution, it becomes a solid culture medium. Next, 50 mL culture medium was added into 100-mL conical flasks, which were occluded with a brown paper and sterilized with high pressure steam for 20 min at 121 °C. The culture medium was cooled to room temperature and stored at 4 °C. Before each test, the bacteria were inoculated from a stock culture, which was maintained on a *V. qinghaiensis* culture medium agar at 4 °C, to a fresh agar and were cultured at 22 ± 1 °C for 24 h. The bacteria were further grown in a liquid culture medium by shaking (120 r/min) at 22 ± 1 °C for 6–12 h to reach the logarithmic growth phase (Liu, 2009; Liu et al., 2009; Yu et al., 2014; Zhang et al., 2012).

2.3. Microplate toxicity analysis

The MTA was used to determine the effects of five nitrobenzene derivatives and their mixtures on *V. qinghaiensis* (Liu, 2009; Liu et al., 2009; Zhang et al., 2008). The experiments were carried out in 96-well standard opaque plates (Corning Corp., USA), and the setup of controls and treatments are as follow: First, 100 μL water (with 1% (v/v) DMSO) was added to 12 wells of the first row in a microplate as controls, and 100 μL solutions of the tested chemicals with twelve gradient concentrations according to a geometric dilution factors (Liu et al., 2012) were added to 12 column wells from the second to the fourth rows. Next, 100 μL of the *V. qinghaiensis* suspension in the logarithmic growth phase was added into each well to reach the final volume of 200 μL. Each microplate test was repeated at least three times.

The relative light unit (RLU) of each well was determined on the Power-Wave microplate spectrophotometer (American BIO-TEK Company) at 22 ± 1 °C after 15 min of exposure. The toxicity or effect of an NBD or a mixture on *V. qinghaiensis* was expressed as the inhibition ratio (Ge et al., 2011; Zhang et al., 2012) according to the following formula:

$$E = \frac{I_0 - I}{I_0} \times 100\% \quad (1)$$

where I is the average RLU of *V. qinghaiensis* exposed to the test NBDs or mixtures (3 parallels), and I_0 refers to the average RLU of *V. qinghaiensis* exposed to the controls (24 parallels).