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The time-dependent synergism of the six-component mixtures of substituted phenols, pesticides and ionic liquids to *Caenorhabditis elegans*

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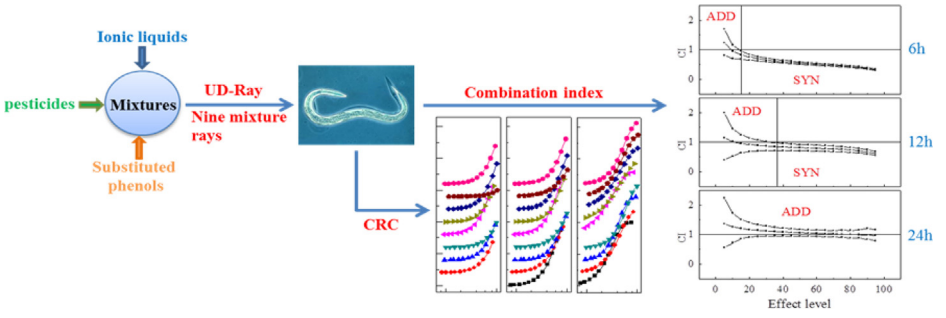
HIGHLIGHTS

- Combined toxicities of SBs, pesticides and ILs were assessed.
- Toxicities of single SBs, pesticides and ILs excepted for GLY increase with time.
- Many mixture rays rationally describe the composition diverses in mixtures.
- Toxicities of all mixture rays with different mixture ratios increase with time.
- Eight of nine rays exhibit synergism and the synergism decrease with time.

GRAPHICAL ABSTRACT



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ABSTRACT

Traditional environmental risk assessment rarely focused on exposures to multi-component mixtures which may cause toxicological interactions and usually ignored that toxicity is a process in time, which may underestimate the environment risk of mixtures. In this paper, six chemicals belonging to three categories, two substituted phenols, two pesticides and two Ionic liquids, were picked to construct a six-component mixture system. To systematically examine the effects of various concentration compositions, the uniform design ray method was employed to design nine mixture rays with nine mixture ratios and for every mixture ray 12 concentration levels were specified by the fixed ratio ray design. The improved combination index was used to evaluate the combined toxicities of the mixtures to *Caenorhabditis elegans* (*C. elegans*) in the exposure times of 6, 12 and 24 h. It was shown that the mixture rays display time-dependent synergism, i.e. the range of synergistic effect narrows and the strength of synergism runs down with exposure time, which illustrates that the mixture toxicity of some chemicals is not a sum of individual toxicities at some exposure times and it is necessary to consider the toxicological interaction in mixtures.

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1. Introduction

Human exposure to environmental contaminants is omnipresent. With the progress of the society and the increasing application of various chemicals, human are always exposed to complex mixtures. Different types of contaminants can compose various mixtures [1–3]. For example, more and more researchers pay attention to pesticides and substituted phenols because of their widespread application. It was found that some combinations of pesticides and phenols can cause toxicological interactions [4–7], which increases the potential risk of the chemicals. Moreover, some new chemicals such as ionic liquids (ILs) also can cause toxicological interactions [8]. Although there is little study on the concentrations of ILs in the environment, it doesn't mean that ILs don't exist [9]. Continued development and further use of these new chemicals may lead accidental discharge and contamination, and may compose diverse mixtures with the chemicals (such as pesticides and substituted phenols) which already existed in the environment. Nevertheless, traditional effect and risk assessment have been routinely focused on exposures to single chemicals and additive behaviors of multiple compounds, which may underestimate the real environment risk of complicated mixtures [4,10–12]. Meanwhile, studies in ecotoxicology usually focus on a single time point and ignore the fact that toxicity is a process in time, which can also lead to severe bias in environmental risk assessment [13,14]. For example, Green and Walmsley found that there are different toxicological interactions in different times [15]. Thirdly, present mixture studies focus on binary mixtures (the bulk) [4,16,17] and ternary mixtures [7,18], only minority involve in multi-component mixtures (MCMs). For complicated multi-component mixtures (MCMs), the absolute majorities of researchers [19–21] used the fixed mixed ratio ray design (FRRD) to design a EC_{50} ratio ray to simply describe the MCM system where there are in fact diverse concentration compositions including many rays with different mixture ratio and a lot concentration levels [8,18]. Therefore, it is necessary to examine the effect of concentration levels, mixture ratios, and exposure times on the mixture toxicity and toxicological interaction in MCM study.

Caenorhabditis elegans (*C. elegans*) is an extremely well-characterized organism which has been proven as a sensitive bioindicator of environmental toxicants [19–24]. A number of toxicity studies were conducted with the aid of lethal endpoint for different chemicals [18,25–30]. In addition, the genome of *C. elegans* has approximately 72% similarity to humans [22] and it is found that toxicity of toxicants in *C. elegans* is similar to that observed in mammals [31], implying that the toxicological studies performed in *C.elegans* will closely reflect the effects to be observed in mammalian models for most compounds tested. So, we chose the *C. elegans* as a model organism, the lethality of *C. elegans* as the endpoint to determine the mixture toxicity of three types of chemicals and their mixtures.

Currently, in combined toxicity field, almost all studies on MCM focus on the mixtures designed by the equivalent effect concentration ratio (EECR) procedure which is a special example of FRRD [32,33]. Obviously, the EECR mixtures are just a small part of complex mixtures and their toxicities cannot explain the nature of toxicities in the MCMs. In order to simulate various concentration compositions for mixtures in real environment, more mixture rays with different mixture ratios should be designed [34]. The uniform design ray method (UD-ray) [35–37] developed by our laboratory is the most suitable method to rationally investigate the combined toxicity in MCMs [38–40]. In this study, we use the UD-ray to design the basic concentration compositions (BCCs) in six-component mixtures.

Two classical additive reference models, the concentration addition (CA) and the independent action (IA) [41] were widely used to evaluate toxicological interaction in mixtures, but are limited to the toxic modes of action (MOA) of MCM system. Based on the mass-action law and median-effect equation, Chou [42,43] developed a combination index (CI) method which is independent of the toxic MOA and can quantitatively describe the interactions in complicated MCM systems. Liu et al. [44] introduced the confidence intervals [45] into the CI to rationally describe the uncertainty originated from experimental errors and fitting deviation from concentration response curve and called the improved index as CI_{imp}. In this study, we apply the CI_{imp} to evaluate toxicological interactions in various mixtures.

To examine the combined toxicity of different types of chemicals, we chose two types of widespread pollutants (two substituted phenols and two pesticides) and one type of emerging contaminants (two ILs) as mixture components to construct a six-component mixture system. Nine mixture rays with different concentration ratios were designed by UD-Ray. The lethal toxicity of various components and mixture rays to *C. elegans* in different times (6, 12, and 24 h) were determined by the method developed by Tang [18]. Through the analysis on the combined toxicity of various six-component mixtures, we try to reveal the toxicological interaction in the mixtures.

2. Materials and methods

2.1. Chemicals

Two ILs (1-butylpyridinium bromide ([bpy]Cl) and 1-butylpyridinium chloride ([bpy]Br)) and two pesticides (dichlorvos (DIC) and glyphosate (GLY)) were purchased from Sigma (USA). Two substituted phenols (4-chlorophenol (4-CP) and 4-nitrophenol (4-NP)) were purchased from CATO (USA). The molecular structures of the chemicals were shown in Fig. S1. Their physical properties and the concentrations of stock were listed in Table 1. Stock solution of each chemical was prepared in Milli-Q water and stored at 4 °C.

2.2. Nematode culture and lethal toxicity test based on microplate and autoscaling

Wild-type strains (N2) of *C. elegans* and its food, *E. coli OP50*, were obtained from the Institute of Medicine, Tongji University. The procedures of *E. coli OP50* culture, nematode culture, subculture, age synchronization, blank and treatment group design, and lethality autoscaling are the same as the literatures [18,46]. To explore the effect of different exposure times on lethality, we in this work set three different exposure times of 6, 12, and 24 h.

2.3. Six-component mixture design

In order to rationally and effectively analyze the combined toxicity of mixtures with diverse concentration compositions in real environment, we have to select some representative mixtures to do toxicity test and analyze their toxicity interaction. The UD-Ray method [35,36] was used to select the representative mixtures from a lot of mixtures in the six-component mixture system of [bpy]Cl, [bpy]Br, 4-CP, 4-NP, DIC, and GLY. Firstly, the uniform table, $U_9(9^6)$ where the subscript 9 refers to the number of mixture rays, 9 to the number of concentration levels of various components, and the superscript 6 to the factor/component number, was employed to design the basic concentration compositions (BCCs) of nine mixture rays (R1, R2, ⋯, R9) in the six-component mixture system (see Table 2(a) in Section 3.2). Based on the BCCs, the mixture ratios