Mixture toxicity of zinc oxide nanoparticle and chemicals with different mode of action upon *Vibrio fischeri*

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Abstract

Background: Zinc oxide nanoparticle (nZnO) and chemicals with different mode of action (MOA, i.e., narcotic and reactive) were frequently detected in the Yangtze River. Organisms are typically exposed to mixtures of nZnO and other chemicals rather than individual nZnO. Toxicity of nZnO is caused by the dissolution of Zn$^{2+}$, which has been proved in the field of single toxicity. However, it is still unclear whether the released Zn$^{2+}$ plays a critical role in the nZnO toxicity of nZnO-chemicals mixtures. In the present study, the binary mixture toxicity of nZnO/Zn$^{2+}$ and chemicals with different MOA was investigated in acute (15 min) and chronic (12 h) toxicity test upon Vibrio fischeri (V. fischeri). The joint effects of nZnO and tested chemicals were explored. Moreover, two classic models, concentration addition (CA) and independent action (IA) were applied to predict the toxicity of mixtures.

Results: The difference of toxicity unit (TU) values between the mixtures of Zn$^{2+}$-chemicals with those of nZnO-chemicals was not significant ($P > 0.05$), not only in acute toxicity test but also in chronic toxicity test. The antagonistic or additive effects for nZnO-chemicals can be observed in most mixtures, with the TU values ranging from 0.75-1.77 and 0.47-2.45 in acute toxicity test and chronic test, respectively. We also observed that the prediction accuracy of CA and IA models was not very well in the mixtures where the difference between the toxicity ratios of the components was small (less than about 10), with the mean absolute percentage error (MAPE) values ranging from 0.14-0.67 for CA model and 0.17-0.51 for IA model, respectively.

Conclusion: We found that the dissolved Zn$^{2+}$ mainly accounted for the nZnO toxicity in the mixtures of nZnO-chemicals, and the joint effects of these mixtures were mostly antagonism and additivity. CA and IA models were unsuitable for
predicting the mixture toxicity of nZnO-chemicals at their equitoxic ratios.

Key words: Zinc oxide nanoparticle; Zn$^{2+}$; mixture toxicity; Vibrio fischeri.

1 Introduction

The nanoparticles (NPs) have been increasingly manufactured in industry because of the well-known characteristics such as high reactivity, electromagnetic properties and high antibacterial property [1]. Zinc oxide nanoparticle (nZnO), one of the most popular manufactured metal oxide nanomaterial, has unique properties (i.e., surface area and reactive sites) due to the extremely small size and is increasingly used in a range of products, such as sunscreens, cosmetics and antibacterial ointments [2]. The wide applications have caused a rapid increase in the production of nZnO, with 30,000 tons worldwide in 2010 [3]. As a result, the amount of nZnO entering the environment is increasing and the occurrences of nZnO, in the range 1-10 μg/L or higher, have been commonly reported in natural water and sediments [4-5]. Consequently, there is a critical need to investigate the toxic effect and the potential health risk of nZnO [6].

To date, the studies of toxic effects for nZnO were mostly focused in the field of individual pollution, and the results demonstrated that nZnO can produce toxic effect upon bacterial, crustaceans, earthworms and mammalian cells [7-8]. Adams et al. [9] determined toxicity of nZnO, nano-titanium dioxide (nTiO$_2$) and nano-silicon dioxide (nSiO$_2$) upon the Escherichia coli and found that nZnO was the most toxic nanoparticle. Furthermore, some studies were also performed for the purpose of understanding the toxicity mechanism of nZnO [10]. nZnO can cause damage to the organ and change osmoregulatory of Oreochromis niloticus [11]; The phosphodiester bond of L-R-phosphatidylethanolamine in Escherichia coli can be broken by nZnO [12]. Moreover, a variety of studies proved that the toxicity of nZnO is related to the
dissolution of Zn$^{2+}$ [13]. For instance, $n$ZnO caused the cytotoxicity by means of interfering with the homeostasis of Zn$^{2+}$ [14]; Zhang et al. proved the toxic difference of various $n$ZnO particles mainly depended on their dissolution [15].

Organisms are typically exposed to multiple mixtures of pollutants rather than single chemicals [16]. In the process of transportation and disposal, it is conceivable that NPs are able to form nanoparticle-toxin complexes due to their high surface area and large aggregates [17]; thus, there are ongoing concerns on evaluating the environmental risk for the mixtures containing NPs. Recently, the toxic effects of $n$ZnO combined with other chemicals were investigated in few studies [18]. The joint effects of the $n$ZnO and surfactants, for example, were investigated at equitoxic mixtures in acute toxicity test, which showed that the joint effects can be explained by the interactions between the Zn$^{2+}$ and the surfactants [19]. In the field of toxicology, chemicals are classified as narcotic or reactive compounds on the basis of their mode of action (MOA) for a better mechanistic understanding of interactions in the mixture toxicity [20]. A variety of studies proved that mixtures of compounds exerting only one MOA (narcotic or reactive) can be assumed as additive behaviour, whereas the interactions of differently acting compounds tends to yield a less or more mixture toxicity [21]. Unfortunately, the toxic effects of $n$ZnO combined with other chemicals were rarely investigated, leading to the fact that in the mixture pollution, it is still unclear whether the dissolved Zn$^{2+}$ also mainly accounts for the $n$ZnO toxicity in the mixtures of $n$ZnO-chemicals.

In the field of mixture toxicology, the interactions of chemicals always cause the changes in the different joint effects, including synergism, antagonism and additivity [22]. For instance, the joint effects of the $n$ZnO and pollutants were investigated in acute toxicity test, and the results showed antagonism [23]; the additive effect
between (nZnO) with nano-copper oxide (nCuO) was identified in the mixture toxic effects upon Scenedesmus obliquus [24]; the antagonistic effect between nZnO with Pb was observed in the mixture toxic effects upon Leucaena leucocephala seedling [25]. However, the joint effects between nZnO and chemicals with different MOA have been rarely investigated, and the predictive powers of the concentration addition (CA) and independent action (IA) models have not been verified, although CA and IA models were extensively employed to predict the toxic effects of mixtures [26].

V. fischeri, the marine bacterium, has been widely used as the test organism for investigating the toxicity of pollutants, including nZnO [27], antibiotics [26, 28], and heavy metals [29]. In recent years, reactive compounds (i.e., antibiotics) and narcotic compounds (i.e., lignin phenols) were frequently detected in Yangtze River Basin and reported in many previous studies [30-31]. In addition, researches have suggested that organisms are exposed to the metal NPs and metal ions in the Yangtze River [32-33]. Therefore, the purpose of this study is to (1) explore the role of Zn\(^{2+}\) in nZnO toxicity of the binary mixtures containing nZnO and chemicals with different MOA, (2) evaluate the joint effects of nZnO and tested chemicals, and (3) investigate the predictive powers of CA and IA models for the mixture toxicities of nZnO and tested chemicals.

2 Materials and Methods

2.1 Test materials

The freeze-dried marine bacterium, V. fischeri, was supplied by the Institute of Soil Science, Chinese Academy of Sciences, Nanjing PRC. nZnO (30±10 nm), ZnSO\(_4\) (Zn\(^{2+}\)), four narcotic compounds (aniline (AL), 2-nitroaniline (NAL), p-toluidine (TD) and hydroquinone (HQ) and five reactive compounds
(sulfamethoxazole (SMZ), sulfapyridine (SPY), sulfadiazine (SD), tetracycline hydrochloride (TTC) and oxytetracycline hydrochloride (OTC) were purchased from Aladdin Reagent Company (Shanghai, China, www.aladdin-e.com) and were used without further purification. The detail information of 9 organic chemicals was listed in the supplementary information (SI, Table S1).

2.2 Toxicity test

2.2.1 Single toxicity test

The single toxicity test was performed following our previous methods [28]. That is, a 3% NaCl solution was used as the diluant and the bioluminescence of *V. fischeri* was recorded over a range of chemical concentrations by a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, California). The exposure time of tested chemicals with *V. fischeri* for acute and chronic toxicity test was 15 min and 12 h, respectively. The inhibition of the tested chemicals towards bioluminescence was calculated as Eq. 1. Based on the decrease in light emission, the obtained concentration relationship data were fitted using dose response model (Eq. 2) [34] and reported in unit of mg/L. Detail information about single toxicity test was presented in Fig. S1.

\[ Y = \frac{I_{\text{control}} - I_s}{I_{\text{control}}} \times 100\% \] (Eq. 1)

where \( Y \) is the inhibition ratio or response, \( I_{\text{control}} \) and \( I_s \) are the average relative light units of *V. fischeri* exposed to the controls and test chemicals, respectively.

\[ Y = A_2 + \frac{A_2 - A_1}{1 + 10^{(\log C_0 - C)P}} \] (Eq. 2)
where $A_1$ and $A_2$ are bottom inhibition and top inhibition, respectively. $C$ is the concentration of the tested chemical, and $C_0$ is the value of $C$ at 50% of the inhibition ratio, $P$ is the parameter of slope for the concentration response relationship curve.

2.2.2 Mixture toxicity test

The binary mixtures, including mixtures at equitoxic ratios and the mixtures at non-equitoxic ratios based on the single toxicity results ($EC_{50}$), were prepared in ratios $(1:10^{2.5}, 1:10^2, 1:10^1.5, 1:10, 1:10^{0.5}, 1:1, 10^{0.5}:1, 10:1, 10^{1.5}:1, 10^2:1, 10^{2.5}:1)$ of the individual concentration ($n$ ZnO or Zn$^{2+}$):m (chemicals), mg/L. The binary mixture toxicity tests were conducted in a same method as analysis of individual toxicity test. Mixture toxicity data was fitted and described as $EC_{i,M}$ (Eq. 3) [35]. The joint effects of the mixtures were represented as the sum of toxic units (TU) [36], as shown in Eq. 4.

$$EC_{i,M} = \frac{C_A + C_B}{C_A \cdot EC_{i,a} + C_B \cdot EC_{i,b}} \quad (Eq. 3)$$

$$TU = \frac{C_A}{EC_{50A}} + \frac{C_B}{EC_{50B}} \quad (Eq. 4)$$

where $EC_{50A}$ and $EC_{50B}$ are median effective inhibition concentrations of components A and B. $EC_{i,M}$ is the effective concentration of the mixtures. $C_A$ and $C_B$ are the concentrations of the individual chemical in mixtures at median inhibition. Simple additivity is characterized by $1.2 > TU > 0.8$, while $TU > 1.2$ represents antagonism and $TU < 0.8$ indicates synergism [37].

2.3 Toxicity prediction

Concentration addition (CA) and independent action (IA) are two classical models for mixture toxicity prediction and are widely used to predict the joint effect
of mixtures [38]. CA and IA models are expressed mathematically as Eq. (5) and Eq. (6), respectively:

$$EC_{x,m} = \left( \frac{P_A}{EC_{x,A}} + \frac{P_B}{EC_{x,B}} \right)^{-1},$$  \hspace{1cm} \text{(Eq. 5)}

where $EC_{x,m}$ is the concentration of the mixture eliciting x% effect, $P_A$, $P_B$ are the concentration ratios of A and B component in the mixture, $EC_{x,A}$ and $EC_{x,B}$ denote the concentration of the A and B component that elicit an x% effect.

$$E(c_m) = 1 - (1 - E(c_A)) \times (1 - E(c_B)), \hspace{1cm} \text{(Eq. 6)}$$

where $E(c_m)$ is the toxic effect of mixture, $E(c_A)$ and $E(c_B)$ are the effect from analyte A, B if applied singly at an exposure concentration of A and B, respectively.

### 2.4 Statistics

SPSS 25.0 software (SPSS Inc.) was used to test the significant difference of the results and $P<0.05$ was considered to be statistically significant. The statistic quality of linear models was evaluated by determination coefficients ($R^2$), the formula as shown Eq. 7 [39]. The parameters of mean absolute percentage error (MAPE) (Eq. 8) and root mean square error (RMSE) (Eq. 9) were applied to measure the prediction accuracy of CA and IA models [40]. These indices were obtained by the following equations:

$$R^2 = \frac{\left( \sum_{i=1}^{n} (y_i - \bar{y})(\hat{y}_i - \bar{y}) \right)^2}{\left( \sum_{i=1}^{n} (y_i - \bar{y})^2 \sum_{i=1}^{n} (\hat{y}_i - \bar{y})^2 \right)^{\frac{1}{2}}}, \hspace{1cm} \text{(Eq. 7)}$$

where $y$ is the mean predicted responses.
MAPE = $\frac{1}{M} \sum_{k=1}^{M} \left| \frac{\hat{p}_{ettc}(k) - p_{ettc}(k)}{p_{ettc}(k)} \right| \times 100\%$, \hspace{1cm} (Eq. 8)

RMSE = $\sqrt{\frac{1}{M} \sum_{k=1}^{M} \left( \frac{\hat{p}_{ettc}(k) - p_{ettc}(k)}{p_{ettc}(k)} \right)^2}$, \hspace{1cm} (Eq. 9)

where $M$ is the number of sample intervals.

3 Results and discussions

3.1 The single toxicity of tested chemicals

Fig. 1 The single toxic effects of chemicals upon V. fischeri and the fitted dose response curves. (A) The acute toxicity test results, (B) the chronic toxicity test results.

To investigate the mixtures toxicity of nZnO and chemicals with different MOA, the single toxicity of nZnO, Zn$^{2+}$ and other 9 chemicals to V. fischeri was determined in acute and chronic toxicity test. The data was fitted by the model of dose response and obtained curves were presented in Fig. 1. The values of $R^2$ suggested a good fitting (0.977-0.999). In the case of acute toxicity (Fig. 1A), nZnO and Zn$^{2+}$ presented higher toxic effects than other tested chemicals, EC$_{50}$ values for tested chemicals were ranging from 1.17 mg/L to 319.24 mg/L, and the order of acute toxicity was as follows: nZnO > Zn$^{2+}$ > HQ > SMZ > NAL > TD > AL > OTC > TTC > SD > SPY. As far as chronic toxicity test (Fig. 1B), results showed that OTC presented higher toxic effects than other tested chemicals, EC$_{50}$ values for tested chemicals were ranging from 1.17E-2 mg/L to 100.64 mg/L, and the order of chronic toxicity was as follows: OTC > TTC > HQ > SMZ > NAL > SD > TD > nZnO > Zn$^{2+}$ > SPY > AL.
3.2 Mixture toxicity of nZnO and chemicals with different MOA

3.2.1 Acute toxicity test

Based on the results of the single acute toxicity test, the mixture toxicity of nZnO/Zn$^{2+}$ and 9 tested chemicals with different MOA was determined at their equitoxic ratios (Fig. S2). As shown in Fig. S2, the half effective concentration for mixture in acute toxicity test ($EC_{50M}^{15min}$) were well fitted by dose response model, with $R^2$ ranging from 0.978 to 0.999 for nZnO-chemicals and from 0.982 to 0.993 for Zn$^{2+}$-chemicals, respectively. The difference between $EC_{50M}^{15min}$ for binary mixture containing nZnO and $EC_{50M}^{15min}$ for binary mixture of Zn$^{2+}$-chemicals was presented as Fig. 2A. As shown, the difference was not significant for tested chemicals ($P>0.05$).

To further verify the results, the acute mixture toxicity of nZnO/Zn$^{2+}$ combined with SMZ (a reactive compound) and AL (a narcotic compound) was subsequently determined at non-equitoxic ratios (Fig. S3). Fig. 2B indicated that in acute toxicity test, the difference between $EC_{50M}^{15min}$ for nZnO-SMZ and $EC_{50M}^{15min}$ for Zn$^{2+}$-SMZ was still not significant ($P>0.05$). Furthermore, the same conclusion can be obtained for nZnO-AL and Zn$^{2+}$-AL at their non-equitoxic ratios ($P>0.05$, Fig. 2C).

Fig. 2 Comparison of mixture toxicity between Zn$^{2+}$-chemicals with nZnO-chemicals.
(A) Zn$^{2+}$-chemicals and nZnO-chemicals at equitoxic ratios in acute test, (B) Zn$^{2+}$-SMZ and nZnO-SMZ at non-equitoxic ratios in acute test, (C) Zn$^{2+}$-AL and nZnO-AL at non-equitoxic ratios in acute test, (D) Zn$^{2+}$-chemicals and nZnO-chemicals at equitoxic ratios in chronic test, (E) Zn$^{2+}$-SMZ and nZnO-SMZ at...
non-equitoxic ratios in chronic test, (F) Zn$^{2+}$-AL and nZnO-AL at non-equitoxic ratios in chronic test

3.2.2 Chronic toxicity test

Based on the results of the single chronic toxicity, the mixture toxicity of nZnO/Zn$^{2+}$ and these chemicals was determined at their equitoxic ratios (Fig. S4). As shown in Fig. S4, the half effective concentration for mixture in chronic toxicity test ($EC_{50M}^{12h}$) was well fitted by dose response model, with $R^2$ ranging from 0.966 to 0.994 for nZnO-chemicals and from 0.956 to 0.991 for Zn$^{2+}$-chemicals. In the case of equitoxic ratios for chronic toxicity test, the difference between $EC_{50M}^{12h}$ for binary mixtures containing nZnO and $EC_{50M}^{12h}$ for binary mixtures containing Zn$^{2+}$ was presented as Fig. 2D. It can be observed that, the difference was still not significant for tested mixtures ($P > 0.05$). To further verify above results, the chronic mixture toxicity of nZnO/Zn$^{2+}$ combined with SMZ (a reactive compound) and AL (a narcotic compound) was determined at their non-equitoxic ratios (Fig. S5). The results of Fig. 2E and Fig. 2F consistently indicated that in chronic toxicity test, the difference between $EC_{50M}^{12h}$ for binary mixtures of nZnO-SMZ/AL with $EC_{50M}^{12h}$ for binary mixtures of Zn$^{2+}$-SMZ/AL at their non-equitoxic ratios was still not significant ($P > 0.05$).

Consequently, dissolved Zn$^{2+}$ mainly accounted for the nZnO toxicity in the mixtures of nZnO-reactive chemicals and in the mixtures of nZnO-narcotic chemicals, not only in acute toxicity test but also in chronic toxicity test.
3.3 Joint effects of nZnO and chemicals with different MOA

**Fig. 3** Joint effects of nZnO-chemicals at equitoxic ratios (A) and at non-equitoxic ratios (B)

Based on the mixture toxicity results, the joint effects of mixtures of nZnO and tested chemicals were analyzed according to Eq. 3. In the case of acute toxicity test, it can be observed from Fig. 3A that, TU values for the mixtures of nZnO-chemicals at equitoxic ratios ranged from 0.75 to 1.77. Fig. 3B showed that TU values for nZnO-SMZ and nZnO-AL at their non-equitoxic ratios were ranging from 0.93 to 1.25 and from 0.99 to 1.88, respectively. According to the study of Broderius et al. [37], those results indicated that in acute toxicity test, (1) the joint effects of nZnO and chemicals with different MOA were mainly additivity or antagonism, but rarely synergism. For example, the TU value lower than 0.80 can only be obtained in the mixture of nZnO-NAL and the joint effect was viewed as synergism; and (2) the joint effects for nZnO-SMZ and nZnO-AL were consistent additivity in the acute test at non-equitoxic ratios. In the case of the mixture of nZnO-SMZ, for example, the TU values were 0.86-1.15.

As for chronic toxicity test, it can be observed from Fig. 3A that TU values for nZnO and tested chemicals at equitoxic ratios ranged from 0.47 to 2.45, indicating the joint effects of nZnO and chemicals with different MOA were additivity or antagonism or synergism. The synergism can only be obtained in one mixture
and the joint effects for other mixtures were mainly additivity or antagonism. Furthermore, Fig. 3B suggested that for the mixtures of $n\text{ZnO}$-SMZ and $n\text{ZnO}$-AL, the joint effects were additivity in the mixtures where the difference between the concentrations of the components is large (e.g., $\lg (n/m) = -2.5, 2, 2.5$), whereas the joint effects were antagonism in the mixtures where the difference between the concentrations of the components is small. In the case of the mixture of $n\text{ZnO}$-AL, for example, the TU values was 1.57, the corresponding $\lg n/m$ was 0.

Consequently, it can be concluded that for both acute toxicity test and chronic toxicity test, joint effects of $n\text{ZnO}$ and chemicals with different MOA were mainly additivity or antagonism. Similar results were obtained for the joint effects of $n\text{ZnO}$ combined with propiconazole by Hackenberger et al. [41]. Zhang et al. [42] also found the binary joint effects of $\text{Zn}^{2+}$ and 11 nitro-substituted benzenes to $\text{Photobacterium phosphoreum}$ were mainly antagonism.

3.4 Mixture toxicity of $n\text{ZnO}$-chemicals predicted by CA and IA models

Fig. 4 The results of MAPE and RMSE values for CA and IA models from binary mixtures. (A) $n\text{ZnO}$-9 chemicals at equitoxic ratios, (B) $n\text{ZnO}$-SMZ at non-equitoxic ratios, (C) $n\text{ZnO}$-AL at non-equitoxic ratios

Based on the mixture toxicity results of $n\text{ZnO}$ and chemicals, the validity and applicability of CA and IA models was further verified (Fig. 4). Results indicated that, (1) the prediction accuracy of CA and IA models was satisfied in the mixtures where the difference between the concentrations of the components was large (Fig. 4B, Fig.
When \( \log n/m \) was -2.5 or 2.5 for all test mixtures, for example, the values of MAPE and RMSE were mostly ranging from 0.13 to 0.24, 0.00302 to 0.00319 for CA model and were from 0.17 to 0.30, 0.00307 to 0.00340 for IA model, respectively; (2) the prediction accuracy of CA and IA models was poor in the equitoxic mixtures where the joint effects were antagonism or synergism (Fig. 4A). For example, the MAPE values were 1.26 for CA model and 1.97 for IA model in the mixture of \( n \)ZnO-AL in chronic test; and (3) overall, the prediction accuracy of IA model was better than that of CA model, not only in the mixtures at equitoxic ratios but also in the mixtures at non-equitoxic ratios, as proved by the MAPE values of 0.105 to 2.506 and 0.108 to 2.242 for CA and IA model, respectively. It is well known that CA and IA models were used to predict the toxicity of mixture based on the theoretical assumption that chemicals in the mixture do not interact with each other, therefore both models may underestimate or overestimate the joint effects of binary mixtures [43]. CA model was used by Azevedo et al. [44] to predict the mixture toxicity of \( n \)ZnO and nano-silver (nAg), the antagonism effect was observed and the mixture toxicity was overestimated; Wang et al. [45] reported the CA model was unsuitable to predict the mixture toxicity of \( \text{Zn}^{2+} \)-sodium dodecyl benzene sulfonate at equivalent-effect concentration ratio on Vibrio-qinghaiensis sp. Q67.

3.5 The mixture toxicity mechanism of \( n \)ZnO-chemicals

By now, the mixture toxicity of engineered nanomaterials (ENMs) with chemicals is of great interest in the field of toxicology. The mechanisms for the mixture toxicity can be mainly classified into the following types (Fig. 5): (1) ENMs
effectively affect bioavailability of pollutants either positively or negatively by adsorption, complexation and degradation [46], (2) the toxicokinetics of pollutants, including the process of uptake, biotransformation, distribution and elimination of pollutants in test organism, can be affected by ENMs by modifying the structure and function of cellular membrane, changing the metabolism pathways and altering the chemical species of pollutants [47]; and (3) ENMs influence the toxicodynamics of pollutants by interfering with the interactions of a toxicant with a biological target and its biological effects [48]. For example, ENMs may ease the entering and transport of pollutants in organisms via “Trojan horse effect” [49], because of the high surface to volume ratio [50]; Carbon nanotubes enter cells through damaging the cell membrane, which subsequently facilitated the entry of pollutants and induced a synergistic toxicity [51]; De La Torre-Roche et al. indicated the dissolved Ag$^+$ from silver nanoparticles (AgNPs) inhibited the activity of aquaporin and decreased the uptake of $p,p'$-DDE, and therefore, reducing the bioaccumulation of $p,p'$-DDE in test organisms [52].

**Fig. 5** The mechanisms underlying the toxicities of NMs and pollutant

In the case of single toxicity, studies proved that the released Zn$^{2+}$ mainly accounted for the nZnO toxicity upon *V. fischeri* and *Escherichia coli*, respectively [27, 53]. In the field of mixture toxicity, the role of released Zn$^{2+}$ in nZnO toxicity remains controversial. The work of Yi et al., for example, indicated no significant
difference between the toxicity of $n$ZnO-triphenyltin and Zn$^{2+}$-triphenyltin to *Tigriopus japonicas* between [54]. While Lakshmi Prasanna et al. reported that the surface defects of $n$ZnO induced antibacterial activity via reactive oxygen species generation but not dissolved Zn$^{2+}$ [55]. The possible reason for the above difference can be concluded as following: the main toxicity mechanism of $n$ZnO may be different for the divergence of exposure condition, due to the species of dissolved zinc ion could be changed by the components in the nature [56].

In the present study, the difference of the joint effects between $n$ZnO-chemicals and Zn$^{2+}$-chemicals was not significant ($P > 0.05$), not only in acute toxicity test but also in chronic toxicity test, suggesting that the toxicity of $n$ZnO on *V. fischeri* was due mainly to the dissolved Zn$^{2+}$. Thus, the joint effects of the $n$ZnO and tested chemicals can be explained by the interactions between the dissolved Zn$^{2+}$ and these chemicals. The joint effects of tested mixtures were mainly antagonism and additivity (Fig. 3A). The possible reasons for the antagonistic effect can be explained as follows: firstly, metal ions interact with organic compounds which reduce the effective dose of the pollutants in organism. In case of antibiotics, interactions between metal ions and the functional groups of antibiotics are the main mechanism for decreasing the mixture toxicity [57]. The work of Kim et al., for example, revealed that the complexation reactions between Cu$^{2+}$ and the phenolic compounds (narcotic compounds) played an important role in reduction of the Cu$^{2+}$ concentrations and therefore decreased toxicity of the binary mixtures [58]. Secondly, the components of a binary mixture may compete for binding sites [59]. Hackenberger et al. found that
two compounds in the treatment canceled the effect of one another in the mixtures of $n\text{ZnO}$-propiconazole and $\text{Zn}^{2+}$-propiconazole [41]. The only synergism effect on $V.\ fischeri$ occurred in the $n\text{ZnO}$-HQ binary experiment. It was well known that phenols can cause damage to cell membrane [60]. Consequently, we speculated that the HQ disrupted the cell membrane integrity of $V.\ fischeri$, which facilitated the entry of released $\text{Zn}^{2+}$ and increased the mixture toxicity.

5 Conclusions
Our results indicated that no significant difference of the toxicity between $n\text{ZnO}$ and $\text{Zn}^{2+}$ was observed not only in single-component system but also in mixture systems of $n\text{ZnO}/\text{Zn}^{2+}$ and chemicals with different MOA, suggesting that $n\text{ZnO}$ toxicity was mainly caused by released $\text{Zn}^{2+}$. Furthermore, the joint effects of $n\text{ZnO}$ and chemicals at equitoxic ratios were mainly antagonism and additivity, while the joint effects of $n\text{ZnO}$-SMZ or $n\text{ZnO}$-AL were additivity at non-equitoxic ratios. Moreover, the prediction accuracy of CA and IA models was not very well in binary mixtures at equitoxic ratios.

6 List of abbreviations

| No. | Full name                  | Abbreviation |
|-----|----------------------------|--------------|
| 1   | Zinc oxide nanoparticle    | $n\text{ZnO}$ |
| 2   | *Vibrio fischeri*          | $V.\ fischeri$ |
| 3   | Aniline                    | AL           |
| 4   | 2-Nitroaniline             | NAL          |
| 5   | p-Toluidine                | TD           |
| 6   | Hydroquinone               | HQ           |
| 7   | Sulfamethoxazole           | SMZ          |
| 8   | Sulfapyridine              | SPY          |
| 9   | Sulfadiazine               | SD           |
| 10  | Tetracycline hydrochloride | TTC          |
|   | Term                                      | Abbr  |
|---|-------------------------------------------|-------|
| 11 | Oxytetracycline hydrochloride              | OTC   |
| 12 | Concentration addition                    | CA    |
| 13 | Independent action                        | IA    |
| 14 | Toxic units                               | TU    |
| 15 | Mean absolute percentage error            | MAPE  |
| 16 | Root mean square error                    | RMSE  |
| 17 | Mode of action                            | MOA   |
| 18 | Engineered nanomaterials                  | ENMs  |

7 Declarations

7.1 Ethics approval and consent to participate

Not applicable.

7.2 Consent for publication

Not applicable.

7.3 Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

7.4 Competing interests

The authors declare that they have no competing interests.

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7.6 Authors' contributions

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Methodology: Xiaoming Zou, Ligui Wu.

Toxicity test: Ligui Wu, Fen Chen, Xiaoyu Xiao and Lingling Rong.

Data curation: Xiaoming Zou, Ligui Wu, Mi Li.

Writing: Xiaoming Zou, Ligui Wu.

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Not applicable.

8 References

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