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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 (*n*ZnO) 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 *n*ZnO and other chemicals rather than individual *n*ZnO. Toxicity of *n*ZnO 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 *n*ZnO toxicity of *n*ZnO-chemicals mixtures. In the present study, the binary mixture toxicity of *n*ZnO/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 *n*ZnO 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 *n*ZnO-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 *n*ZnO-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 *n*ZnO toxicity in the mixtures of *n*ZnO-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 *n*ZnO-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 (*n*ZnO), 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 *n*ZnO, with 30,000 tons worldwide in 2010 [3]. As a result, the amount of *n*ZnO entering the environment is increasing and the occurrences of *n*ZnO, 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 *n*ZnO [6].

To date, the studies of toxic effects for *n*ZnO were mostly focused in the field of individual pollution, and the results demonstrated that *n*ZnO can produce toxic effect upon bacterial, crustaceans, earthworms and mammalian cells [7-8]. Adams et al. [9] determined toxicity of *n*ZnO, nano-titanium dioxide ($n TiO_2$) and nano-silicon dioxide ($n SiO_2$) upon the *Escherichia coli* and found that *n*ZnO was the most toxic nanoparticle. Furthermore, some studies were also performed for the purpose of understanding the toxicity mechanism of *n*ZnO [10]. *n*ZnO 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 *n*ZnO [12]. Moreover, a variety of studies proved that the toxicity of *n*ZnO 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. [15] proved the toxic difference of various *n*ZnO particles mainly depended on their dissolution.

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 (*n*ZnO) with nano-copper oxide (*n*CuO) was identified in the mixture toxic effects upon *Scenedesmus obliquus* [24]; the antagonistic effect between *n*ZnO with Pb was observed in the mixture toxic effects upon *Leucaena leucocephala* seedling [25]. However, the joint effects between *n*ZnO 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 *n*ZnO [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 *n*ZnO toxicity of the binary mixtures containing *n*ZnO and chemicals with different MOA, (2) evaluate the joint effects of *n*ZnO and tested chemicals, and (3) investigate the predictive powers of CA and IA models for the mixture toxicities of *n*ZnO 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. *n*ZnO (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.

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

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

$$Y = A_2 + \frac{A_2 - A_1}{1 + 10^{(\log C_0 - C) \times P}} \quad \text{(Eq. 2)}$$

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 (*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.

$$EC_{i,M} = \frac{C_A + C_B}{\frac{C_A}{EC_{i,A}} + \frac{C_B}{EC_{i,B}}} \qquad \text{(Eq. 3)}$$

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

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:

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

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.

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

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:

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

where $\bar{\hat{y}}$ is the mean predicted responses.

$$\text{MAPE}=\frac{1}{M}\sum_{k=1}^{M}\left|\frac{\hat{p}_{ettc}(k)-p_{ettc}(k)}{p_{ettc}(k)}\right|\times 100\%, \quad \text{(Eq. 8)}$$

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

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 *n*ZnO and chemicals with different MOA, the single toxicity of *n*ZnO, 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), *n*ZnO 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: *n*ZnO> 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> *n*ZnO> Zn^{2+}> SPY> AL.

205 *3.2 Mixture toxicity of nZnO and chemicals with different MOA*

206 *3.2.1 Acute toxicity test*

207 Based on the results of the single acute toxicity test, the mixture toxicity of
208 *n*ZnO/Zn^{2+} and 9 tested chemicals with different MOA was determined at their
209 equitoxic ratios (Fig. S2). As shown in Fig. S2, the relationship between the
210 luminescent inhibition ratio and the concentration of these mixtures was good, with R^2
211 ranging from 0.978 to 0.999 for *n*ZnO-chemicals and from 0.982 to 0.993 for
212 Zn^{2+}-chemicals, respectively. The difference between EC_{50M}^{15min} for binary mixture
213 containing *n*ZnO and EC_{50M}^{15min} for binary mixture of Zn^{2+}-chemicals was presented as
214 Fig. 2A. As shown, the difference was not significant for tested chemicals (P> 0.05).
215 To further verify the results, the acute mixture toxicity of *n*ZnO/Zn^{2+} combined with
216 SMZ (a reactive compound) and AL (a narcotic compound) was subsequently
217 determined at non-equitoxic ratios (Fig. S3). Fig. 2B indicated that in acute toxicity
218 test, the difference between EC_{50M}^{15min} for *n*ZnO-SMZ and EC_{50M}^{15min} for Zn^{2+}-SMZ was still
219 not significant (P> 0.05). Furthermore, the same conclusion can be obtained for
220 *n*ZnO-AL and Zn^{2+}-AL at their non-equitoxic ratios (P> 0.05, Fig. 2C).

221

222 **Fig. 2** Comparison of mixture toxicity between Zn^{2+}-chemicals with *n*ZnO-chemicals.
223 (A) Zn^{2+}-chemicals and *n*ZnO-chemicals at equitoxic ratios
224 (B) in acute test, (B) Zn^{2+}-SMZ and *n*ZnO-SMZ at non-equitoxic ratios in acute test,
225 (C) Zn^{2+}-AL and *n*ZnO-AL at non-equitoxic ratios in acute test, (D) Zn^{2+}-chemicals
226 and *n*ZnO-chemicals at equitoxic ratios in chronic test, (E) Zn^{2+}-SMZ and *n*ZnO-SMZ

227 at non-equitoxic ratios in chronic test, (F) Zn^{2+}-AL and *n*ZnO-AL at non-equitoxic
228 ratios in chronic test
229
230 *3.2.2 Chronic toxicity test*
231 Based on the results of the single chronic toxicity, the mixture toxicity of
232 *n*ZnO/Zn^{2+} and these chemicals was determined at their equitoxic ratios (Fig. S4). As
233 shown in Fig. S4, the relationship between the luminescent inhibition ratio and the
234 concentration of these mixtures was good, with R^2 ranging from 0.966 to 0.994 for
235 *n*ZnO-chemicals and from 0.956 to 0.991 for Zn^{2+}-chemicals. In the case of equitoxic
236 ratios for chronic toxicity test, the difference between EC_{50M}^{12h} for binary mixtures
237 containing *n*ZnO and EC_{50M}^{12h} for binary mixtures containing Zn^{2+} was presented as Fig.
238 2D. It can be observed that, the difference was still not significant for tested mixtures
239 (P> 0.05). To further verify above results, the chronic mixture toxicity of *n*ZnO/Zn^{2+}
240 combined with SMZ (a reactive compound) and AL (a narcotic compound) was
241 determined at their non-equitoxic ratios (Fig. S5). The results of Fig. 2E and Fig. 2F
242 consistently indicated that in chronic toxicity test, the difference between EC_{50M}^{12h} for
243 binary mixtures of *n*ZnO-SMZ/AL with EC_{50M}^{12h} for binary mixtures of Zn^{2+}-SMZ/AL at
244 their non-equitoxic ratios was still not significant (P> 0.05).
245 Consequently, dissolved Zn^{2+} mainly accounted for the *n*ZnO toxicity in the
246 mixtures of *n*ZnO-reactive chemicals and in the mixtures of *n*ZnO-narcotic chemicals,
247 not only in acute toxicity test but also in chronic toxicity test.

248 *3.3 Joint effects of nZnO and chemicals with different MOA*

249

250 **Fig. 3** Joint effects of *n*ZnO-chemicals at equitoxic ratios (A) and at non-equitoxic
251 ratios (B)

252

253 Based on the mixture toxicity results, the joint effects of mixtures of *n*ZnO and
254 tested chemicals were analyzed according to Eq. 3. In the case of acute toxicity test, it
255 can be observed from Fig. 3A that, TU values for the mixtures of *n*ZnO-chemicals at
256 equitoxic ratios ranged from 0.75 to 1.77. Fig. 3B showed that TU values for
257 *n*ZnO-SMZ and *n*ZnO-AL at their non-equitoxic ratios were ranging from 0.93 to
258 1.25 and from 0.99 to 1.88, respectively. According to the study of Broderius et al.
259 [37], those results indicated that in acute toxicity test, (1) the joint effects of *n*ZnO and
260 chemicals with different MOA were mainly additivity or antagonism, but rarely
261 synergism. For example, the TU value lower than 0.80 can only be obtained in the
262 mixture of *n*ZnO-NAL and the joint effect was viewed as synergism; and (2) the joint
263 effects for *n*ZnO-SMZ and *n*ZnO-AL were consistent additivity in the acute test at
264 non-equitoxic ratios. In the case of the mixture of *n*ZnO-SMZ, for example, the TU
265 values were 0.86-1.15.

266 As for chronic toxicity test, it can be observed from Fig. 3A that TU values for
267 *n*ZnO and tested chemicals at equitoxic ratios ranged from 0.47 to 2.45, indicating the
268 joint effects of *n*ZnO and chemicals with different MOA were additivity or
269 antagonism or synergism. The synergism can only be obtained in one mixture

(*n*ZnO-HQ), and the joint effects for other mixtures were mainly additivity or antagonism. Furthermore, Fig. 3B suggested that for the mixtures of *n*ZnO-SMZ and *n*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*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*ZnO and chemicals with different MOA were mainly additivity or antagonism. Similar results were obtained for the joint effects of *n*ZnO combined with propiconazole by Hackenberger et al. [41]. Zhang et al. [42] also found the binary joint effects of Zn^{2+} and 11 nitro-substituted benzenes to *Photobacterium phosphoreum* were mainly antagonism.

3.4 Mixture toxicity of nZnO-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*ZnO-9 chemicals at equitoxic ratios, (B) *n*ZnO-SMZ at non-equitoxic ratios, (C) *n*ZnO-AL at non-equitoxic ratios

Based on the mixture toxicity results of *n*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 when the difference between the concentrations of the components was large (Fig. 4B, Fig.

293 4C). When lg n/m was -2.5 or 2.5 for all test mixtures, for example, the values of
294 MAPE and RMSE were mostly ranging from 0.13 to 0.24, 0.00302 to 0.00319 for CA
295 model and were from 0.17 to 0.30, 0.00307 to 0.00340 for IA model, respectively; (2)
296 the prediction accuracy of CA and IA models was poor in the equitoxic mixtures when
297 the joint effects were antagonism or synergism (Fig. 4A). For example, the MAPE
298 values were 1.26 for CA model and 1.97 for IA model in the mixture of *n*ZnO-AL in
299 chronic test; and (3) overall, the prediction accuracy of IA model was better than that
300 of CA model, not only in the mixtures at equitoxic ratios but also in the mixtures at
301 non-equitoxic ratios, as proved by the MAPE values of 0.105 to 2.506 and 0.108 to
302 2.242 for CA and IA model, respectively. It is well known that CA and IA models
303 were used to predict the toxicity of mixture based on the theoretical assumption that
304 chemicals in the mixture do not interact with each other, therefore both models may
305 underestimate or overestimate the joint effects of binary mixtures [43]. CA model was
306 used by Azevedo et al. [44] to predict the mixture toxicity of *n*ZnO and nano-silver
307 (*n*Ag), the antagonism effect was observed and the mixture toxicity was
308 overestimated; Wang et al. [45] reported the CA model was unsuitable to predict the
309 mixture toxicity of Zn^{2+}-sodium dodecyl benzene sulfonate at equivalent-effect
310 concentration ratio on *Vibrio-qinghaiensis* sp. Q67.

311 *3.5 The mixture toxicity mechanism of nZnO-chemicals*

312 By now, the mixture toxicity of engineered nanomaterials (ENMs) with
313 chemicals is of great interest in the field of toxicology. The mechanisms for the
314 mixture toxicity can be mainly classified into the following types (Fig. 5): (1) ENMs

315 effectively affect bioavailability of pollutants either positively or negatively by
316 adsorption, complexation and degradation [46], (2) the toxicokinetics of pollutants,
317 including the process of uptake, biotransformation, distribution and elimination of
318 pollutants in test organism, can be affected by ENMs by modifying the structure and
319 function of cellular membrane, changing the metabolism pathways and altering the
320 chemical species of pollutants [47]; and (3) ENMs influence the toxicodynamics of
321 pollutants by interfering with the interactions of a toxicant with a biological target and
322 its biological effects [48]. For example, ENMs may ease the entering and transport of
323 pollutants in organisms via "Trojan horse effect" [49], because of the high surface to
324 volume ratio [50]; Carbon nanotubes enter cells through damaging the cell membrane,
325 which subsequently facilitated the entry of pollutants and induced a synergistic
326 toxicity [51]; De La Torre-Roche et al. [52] indicated the dissolved Ag^{+} from silver
327 nanoparticles (AgNPs) inhibited the activity of aquaporin and decreased the uptake of
328 *p,p′*-DDE, and therefore, reducing the bioaccumulation of *p,p′*-DDE in test organisms.
329
330 **Fig. 5** The mechanisms underlying the toxicities of NMs and pollutant
331
332 In the case of single toxicity, studies proved that the released Zn^{2+} mainly
333 accounted for the *n*ZnO toxicity upon *V. fischeri* and *Escherichia coli*, respectively
334 [27, 53]. In the field of mixture toxicity, the role of released Zn^{2+} in *n*ZnO toxicity
335 remains controversial. The work of Yi et al. [54], for example, indicated no significant
336 difference between the toxicity of *n*ZnO-triphenyltin and Zn^{2+}-triphenyltin to

337 *Tigriopus japonicas*. While Lakshmi Prasanna et al. [55] reported that the surface
338 defects of *n*ZnO induced antibacterial activity via reactive oxygen species generation
339 but not dissolved Zn^{2+}. The possible reason for the above difference can be concluded
340 as following: the main toxicity mechanism of *n*ZnO may be different for the
341 divergence of exposure condition, because the species of dissolved zinc ion could be
342 changed by the components in the nature [56].

343 In the present study, the difference of the joint effects between *n*ZnO-chemicals
344 and Zn^{2+}-chemicals was not significant ($P > 0.05$), not only in acute toxicity test but
345 also in chronic toxicity test, suggesting that the toxicity of *n*ZnO on *V. fischeri* was
346 due mainly to the dissolved Zn^{2+}. Thus, the joint effects of the *n*ZnO and tested
347 chemicals can be explained by the interactions between the dissolved Zn^{2+} and these
348 chemicals. The joint effects of tested mixtures were mainly antagonism and additivity
349 (Fig. 3A). The possible reasons for the antagonistic effect can be explained as follows:
350 firstly, metal ions interact with organic compounds which reduce the effective dose of
351 the pollutants in organism. In case of antibiotics, interactions between metal ions and
352 the functional groups of antibiotics are the main mechanism for decreasing the
353 mixture toxicity [57]. The work of Kim et al. [58], for example, revealed that the
354 complexation reactions between Cu^{2+} and the phenolic compounds (narcotic
355 compounds) played an important role in reduction of the Cu^{2+} concentrations and
356 therefore decreased toxicity of the binary mixtures. Secondly, the components of a
357 binary mixture may compete for binding sites [59]. Hackenberger et al. [41] found
358 that two compounds in the treatment canceled the effect of one another in the

359 mixtures of *n*ZnO-propiconazole and Zn^{2+}-propiconazole. The only synergism effect
360 on *V. fischeri* occurred in the *n*ZnO-HQ binary experiment. It was well known that
361 phenols can cause damage to cell membrane [60]. Consequently, we speculated that
362 the HQ disrupted the cell membrane integrity of *V. fischeri*, which facilitated the entry
363 of released Zn^{2+} and increased the mixture toxicity.

364 **5 Conclusions**

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

373 **6 List of abbreviations**

No.	Full name	Abbreviation
1	Zinc oxide nanoparticle	*n*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
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.

7.5 Funding

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7.6 Authors' contributions

Conceptualization: Xiaoming Zou, Lingling Rong and Mi Li.

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

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

Writing: Xiaoming Zou, Ligui Wu.

All authors read and approved the final manuscript.

7.7 Acknowledgements

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Figures

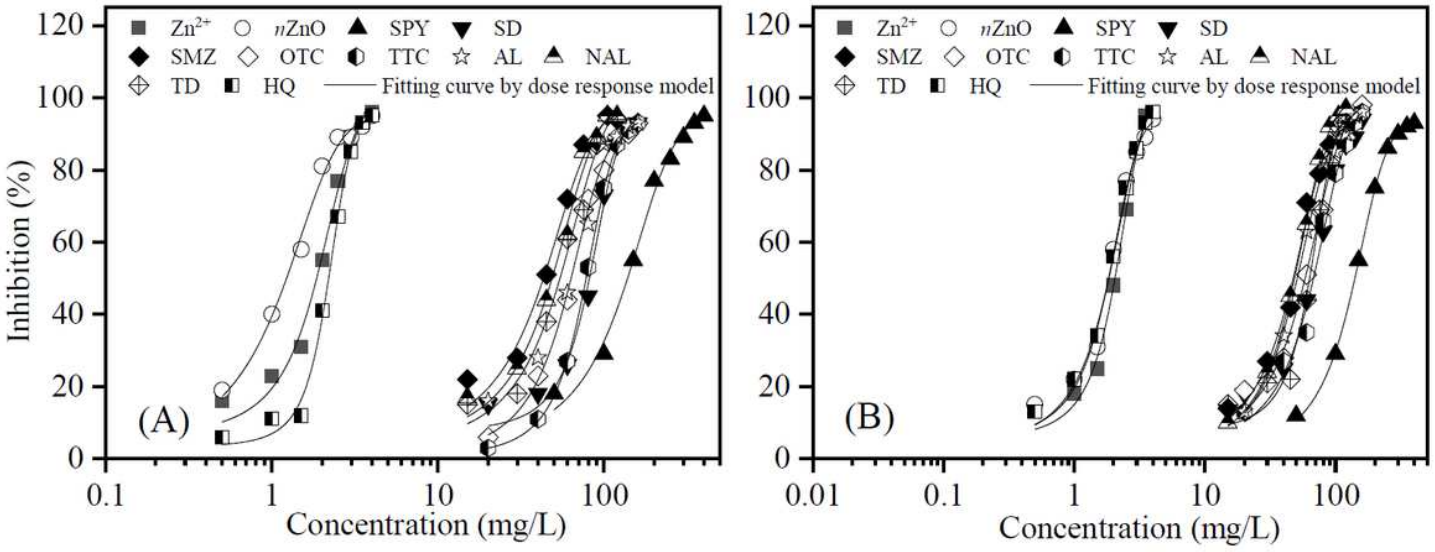

Figure 1

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

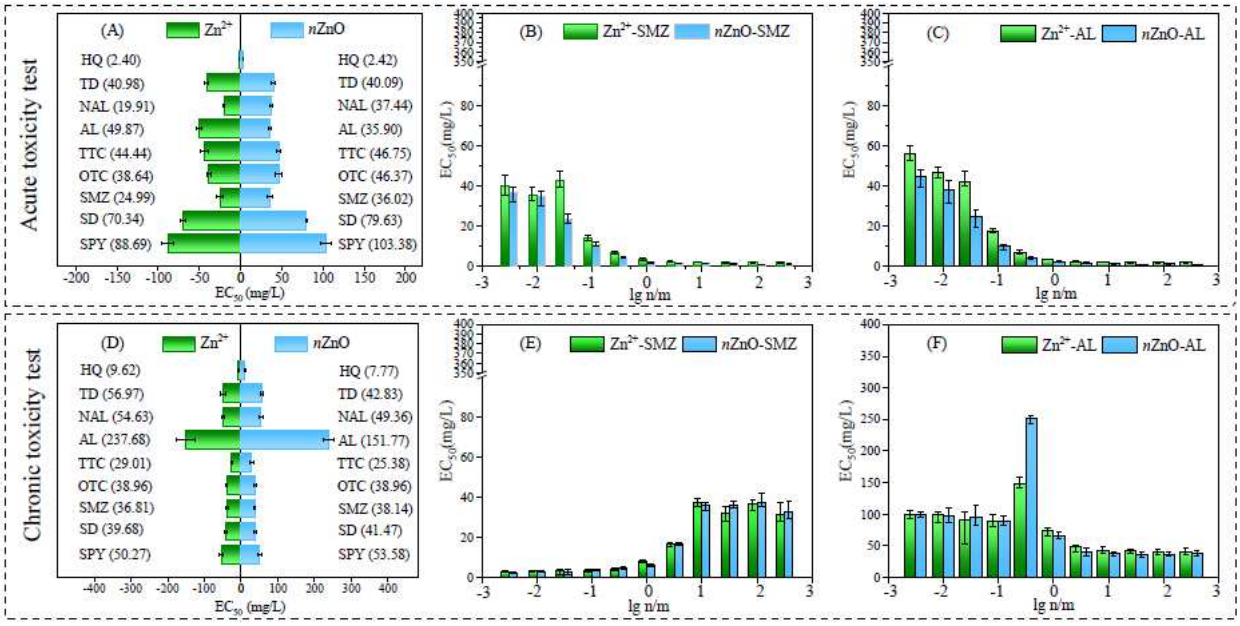

Fig. 2

Figure 2

Comparison of mixture toxicity between Zn2+-chemicals with nZnO-chemicals. (A) Zn2+-chemicals and nZnO-chemicals at equitoxic ratios in acute test, (B) Zn2+-SMZ and nZnO-SMZ at non-equitoxic ratios in acute test, (C) Zn2+-AL and nZnO-AL at non-equitoxic ratios in acute test, (D) Zn2+-chemicals and nZnO-chemicals at equitoxic ratios in chronic test, (E) Zn2+-SMZ and nZnO-SMZ at non-equitoxic ratios in chronic test, (F) Zn2+-AL and nZnO-AL at non-equitoxic ratios in chronic test

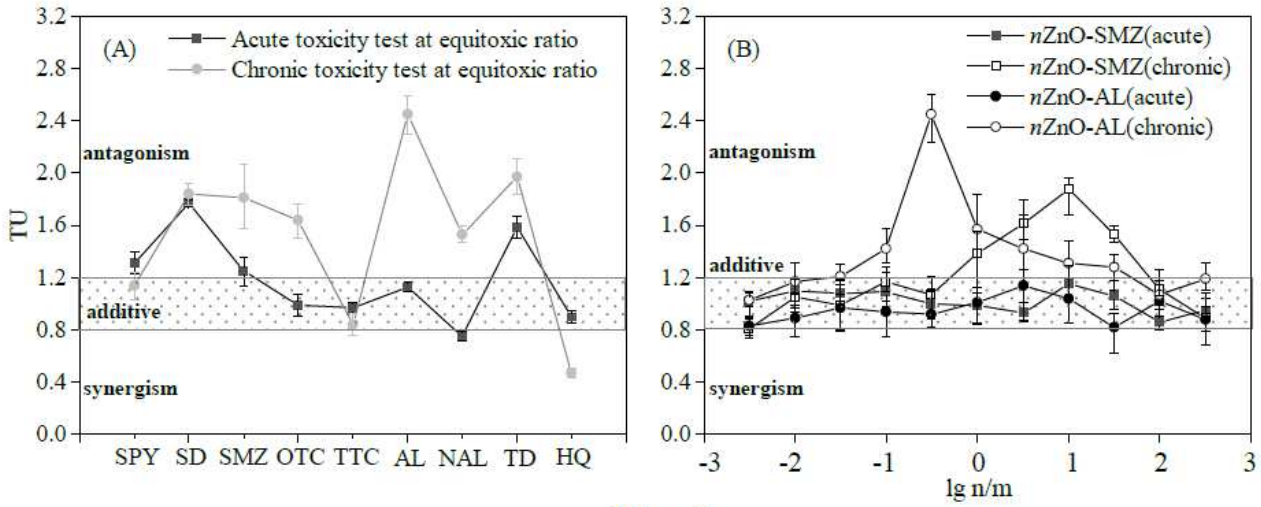

Fig. 3

Figure 3

Joint effects of nZnO-chemicals at equitoxic ratios (A) and at non-equitoxic ratios (B)

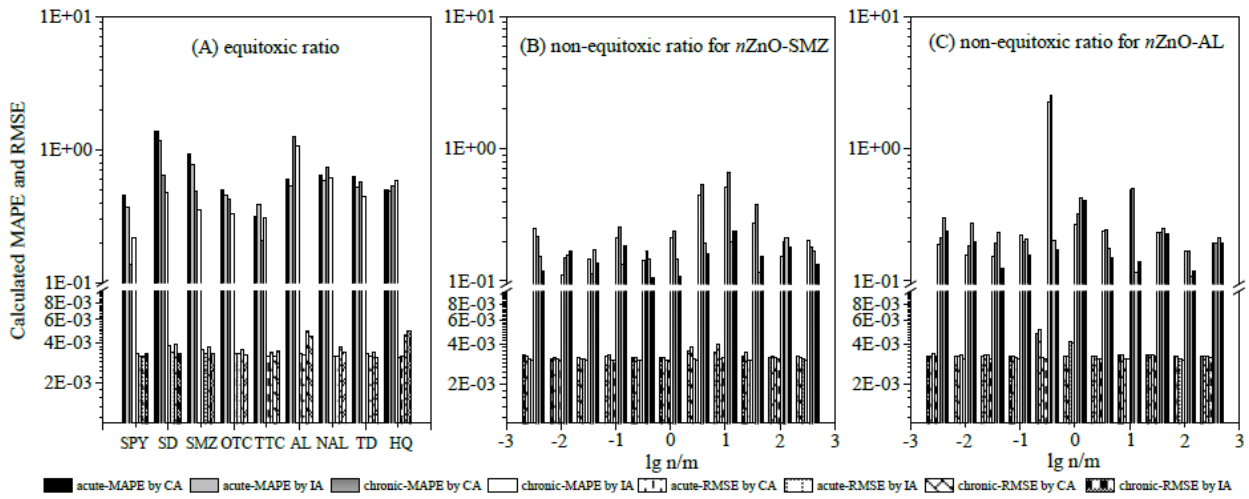

Fig. 4

Figure 4

The results of MAPE and RMSE values for CA and IA models from binary mixtures. (A) nZnO-9 chemicals at equitoxic ratios, (B) nZnO-SMZ at non-equitoxic ratios, (C) nZnO-AL at non-equitoxic ratios

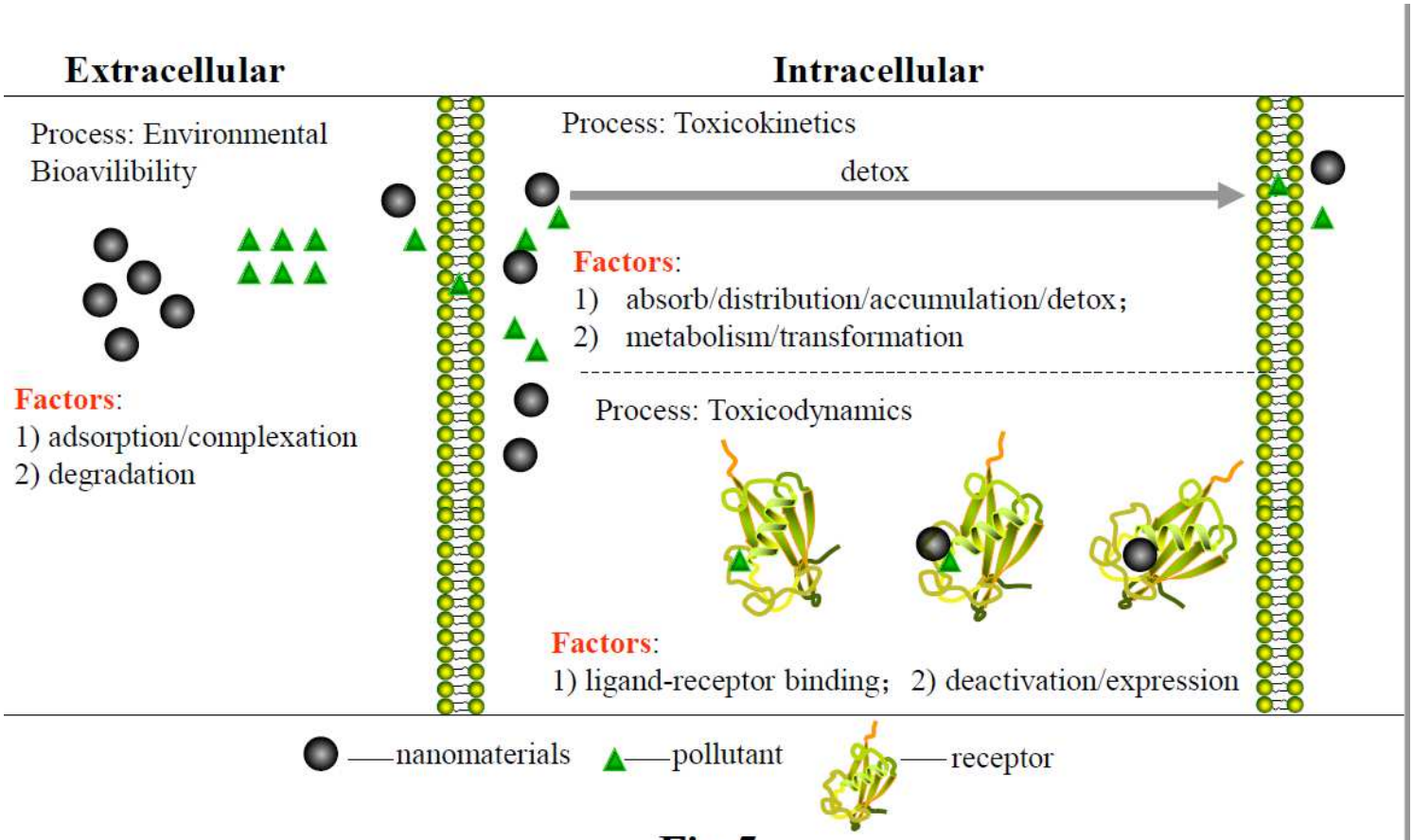

Fig.5

Figure 5

The mechanisms underlying the toxicities of NMs and pollutant

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