Evaluation of the combined toxicity of compound pollutants of azole fungicides to Raphidocelis subcapitata

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Abstract. In order to investigate the combined toxicity of compound contaminated azole fungicides on alage. Systematic investigation of Difenoconazole, Penconazole, Metconazole, Tetraconazole, Flutriafol, Fuberidazole, Triflumizole, Terrazole, HyMexazol, 9 azole fungicides as the components of the mixture. Thirty-five binary mixture systems with 173 mixture rays were designed by direct equipartition ray method. The growth inhibition toxicity of binary mixtures to *Raphidocelis subcapitata* was determined by 96-well microplates. Toxic interactions were qualitatively analyzed by Concentration addition (CA) and Independent Action (IA) models. Model Deviation Ratio (MDR) was used to quantitatively evaluate the toxic interaction of binary mixtures of azole fungicides, as well as the equivalent graph method, Toxic Unit (TU) method, additivity Index method (AI), Toxicity Index (MTI) and Combination Index (CI) methods were used to analyze the combined toxicity and toxic interaction of the mixture. The results showed that the frequency of synergistic effect was higher when mixed with Difenoconazole, Penconazole and Triflumizole, and the binary mixing with Flutriafol, Fuberidazole and Metconazole could reduce the toxicity of the mixture, showing additive and antagonistic effects. The toxicity of the mixture ranged from 3.053 to 6.415. In addition, at ambient concentrations, the mixture interaction is dominated by addition.

1 Introduction

Triazole fungicides are a widely used class of chemical substances in agriculture, effective in controlling a variety of fungal diseases affecting vegetables, fruits, and food crops. However, due to their bioaccumulative and persistent nature in the environment, these fungicides can gradually accumulate in the food chain, raising potential risks to both environmental health and human safety.

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Green algae are a type of organism commonly found in aquatic environments, known for their rapid growth and reproductive capabilities. Their sensitivity to pollutants makes them an effective indicator for monitoring water quality and assessing the degree of water pollution[1].

While studies have focused on the impact of triazole fungicides on green algae, the presence of various triazole fungicide mixtures in the actual environment could result in diverse toxic effects on different organisms, including antagonistic, additive, or synergistic effects.

The research aims to evaluate the toxic effects of different triazole fungicide mixtures on *Raphidocelis subcapitata*. Dual hybrid systems can improve control effectiveness, reduce the risk of resistance, and expand control coverage, while also helping to reduce environmental risks and improve stability. By designing representative binary mixture systems to explore the overall toxicity of these mixtures, the study seeks to assess and predict the toxic effects of triazole fungicide mixtures and investigate potential toxicity mechanisms. This endeavor contributes to a more comprehensive understanding of the impact of triazole fungicides in the environment, thereby aiding in better management and mitigation of their potential risks.

2 Materials and methods

2.1 Algae and pharmaceuticals

The experimental algae species was *Raphidocelis subcapitata* ([FACHB]-271) (purchased from the Freshwater Algal Strain Bank (FACHB) of Wuhan Institute of Hydrobiology, Chinese Academy of Sciences). The method of testing algae was referred to the OECD 201 algal growth inhibition experimental method [2], and sterilization BG-11 nutrient medium was used for continuous culture. The light intensity of the algae was 2500lx, the culture temperature was 22℃, and the light/dark period was 12h/12h. From 10 to 11 am, algal cell metabolism was the most vigorous stage, and the algae seeds were diluted by the upper algal fluid at a rate of 1:4 every 4-6 days, and the exponential growth algal cells with an initial cell density of about $8×10^5$ cells· ml^{-1} were inoculated into the sterilized medium before expanding the culture toxicity test. The experimental experiment was carried out after 4 to 5 days of culture.

The 9 pollutants include: Difenoconazole, Penconazole, Metconazole, Tetraconazole, Flutriafol, Fuberidazole, Triflumizole, Terrazole, HyMexazol (HYM; All of these compounds are analytically pure with a purity of over 95%. Store in 4° refrigerator for later use. The EquRay design was used for binary mixing of multiple target pollutants compound design.

2.2 Analysis of toxic interaction of mixtures

Different methods may reveal the characteristics of the interaction from different angles or levels, helping to verify and strengthen the credibility of the conclusions. In order to obtain more comprehensive and reliable results, the study evaluated interactions using a variety of methods. Qualitative analysis was performed using concentration addition (CA) independent action (IA) and equivalent line graph method , and through Model Deviation Ratio (Model Deviation Ratio, MDR for quantitative analysis of toxic interactions of mixtures.

The CA and IA models do not take into account the potential interactions between pollutants in the mixture [3], as well as the uncertainties brought about by experimental errors. The Toxic Unit (TU)[4], Additive Index (AI)[5], Mixture Toxicity Index (MTI), Combination Index (Combination Index) were introduced. CI) [6] [7] respectively evaluated the toxicity combination mode of action, and on this basis, the uncertainty of toxicity experimental data was characterized by Observation based confidence interval (OCI) [8].

3 Results and analysis

3.1 Combined toxic effects of mixtures of azole fungicides on *Raphidocelis subcapitata*

The toxicity of single azole fungicides to *Raphidocelis subcapitata* varied greatly, and the pEC_{50} values ranged from 3.664 to 4.899, reflecting the difference in their toxicity levels. In the presence of 9 single azole fungicides, the toxicity distribution of diazole mixture to *Raphidocelis subcapitata* is shown in Figure 1. It can be seen from the figure that the pEC_{50} range of the mixture is from 3.053 to 6.415. The diazole mixtures containing FLU, TER and HYM showed low toxicity, with the highest toxicity pEC_{50} being 5.530, 5.296 and 5.346, and the lowest toxicity pEC50 being 3.053, 3.222 and 3.527, respectively. The diazole mixtures containing MET and FUB showed the highest pEC_{50} toxicity of 6.415 and 6.140, and the lowest pEC_{50} toxicity of 4.021 and 3.876, respectively.

The toxicity of the binary mixture of azole fungicides is related to its component and corresponding concentration ratio. For example, the toxicity of DIF (pEC_{50}=4.899) and TRI (pEC_{50}=3.867) mixed in a certain proportion has a pEC_{50} of 6.140, and the toxicity is enhanced. The results showed that the toxicity of PEN and FLU was synergistic. When PEN (pEC_{50}=4.242) and FLU (pEC_{50}=4.892) were mixed in a certain proportion, the toxicity was weakened, and the pEC50 was 3.052. In this case, the toxicity of PEN and FLU was antagonistic.

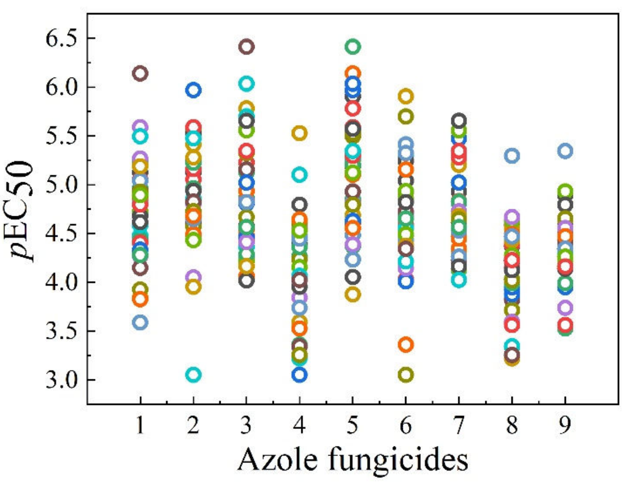

Fig. 1. Toxicity of biazole fungicide mixture.Note: Horizontal coordinates 1 to 9 represent 9 azole fungicides, respectively:Difenoconazole, Penconazole, Metconazole, Flutriafol, Fuberidazole, Tetraconazole, Triflumizole, Terrazole, HyMexazol

3.2 CA and IA models were used to qualitatively evaluate toxic interactions

As shown in Figure 2, CA was used as the reference model to calculate the proportion of toxic synergies produced by the binary mixture. The proportion of antagonistic and additive effects of the mixture accounted for 31.74% and 66.72%, while the proportion of synergies accounted for only 1.64%. IA was used as a reference model to calculate the proportion of toxic synergies produced by the binary mixture, and the proportion of antagonism, addition and synergy of the mixture was 29.81%, 68.25% and 2.03%, respectively, which was consistent with the observation of Maj Britt et al. [9].

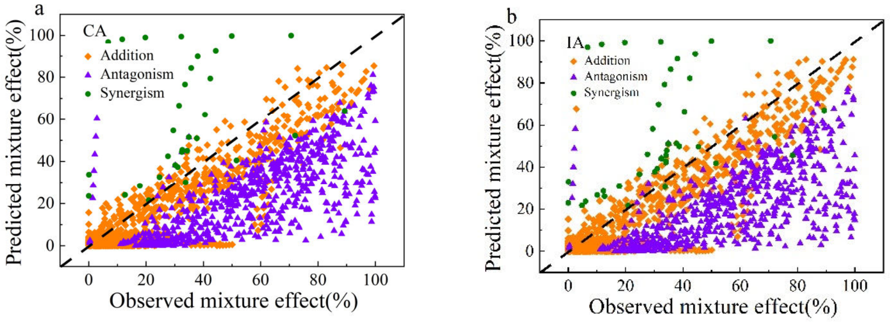

Fig. 2. CA and IA predictions of toxic interactions of binary mixtures on *Raphidocelis subcapitata*.

3.3 Evaluation of toxic interaction of azole fungicides on *Raphidocelis subcapitata* based on equivalent map

The toxicity prediction results of some binary mixture systems analyzed and evaluated based on CA and IA models are shown in Figure 3. The toxic interaction of the mixture was evaluated according to the positions of CA and IA models and the mixture rays (R1~R5). The equivalent lines of CA and IA toxicity of diazoles mixed fungicides, such as DIF - PEN, TET-TRI, and TRI-TER mixed systems, were all higher than the observed values. These results indicated that the five rays of these mixed systems were all synergistic, which indicated that these mixtures were more toxic to *Raphidocelis subcapitata*. However, the toxicity prediction lines of some rays of mixed systems such as DIF-FLU, DIF-TET, and FUB-FLU were between CA and IA toxicity equivalence lines, indicating that the mixture with this composition ratio had no toxic interaction and showed an additive effect. Taking the mixture of DIF-FLU as an example, the toxic interaction of mixture rays R1~R5 on the algae was gradually weakened with the decrease of the mixture concentration of DIF, and the synergistic effect changed to additive effect. Some rays (R1, R2, R4) of the mixture of MET-FUB and PEN-MET were higher than the toxicity equivalent lines of CA and IA, but in the region surrounded by the additive equivalent lines of CA and IA and the independent equivalent lines. The toxicity to the mixture of *Raphidocelis subcapitata* showed the form of synergistic → additive → partial additive. In the mixed systems of DIF-TER and PEN-HYM, the mixture rays were larger than the CA and IA toxicity equivalent lines and showed additive or antagonistic effects, indicating that the toxicity of the mixture was less or no toxic interaction was observed. The farther the mixture rays were from the CA and IA equivalent lines, the less toxic they were.

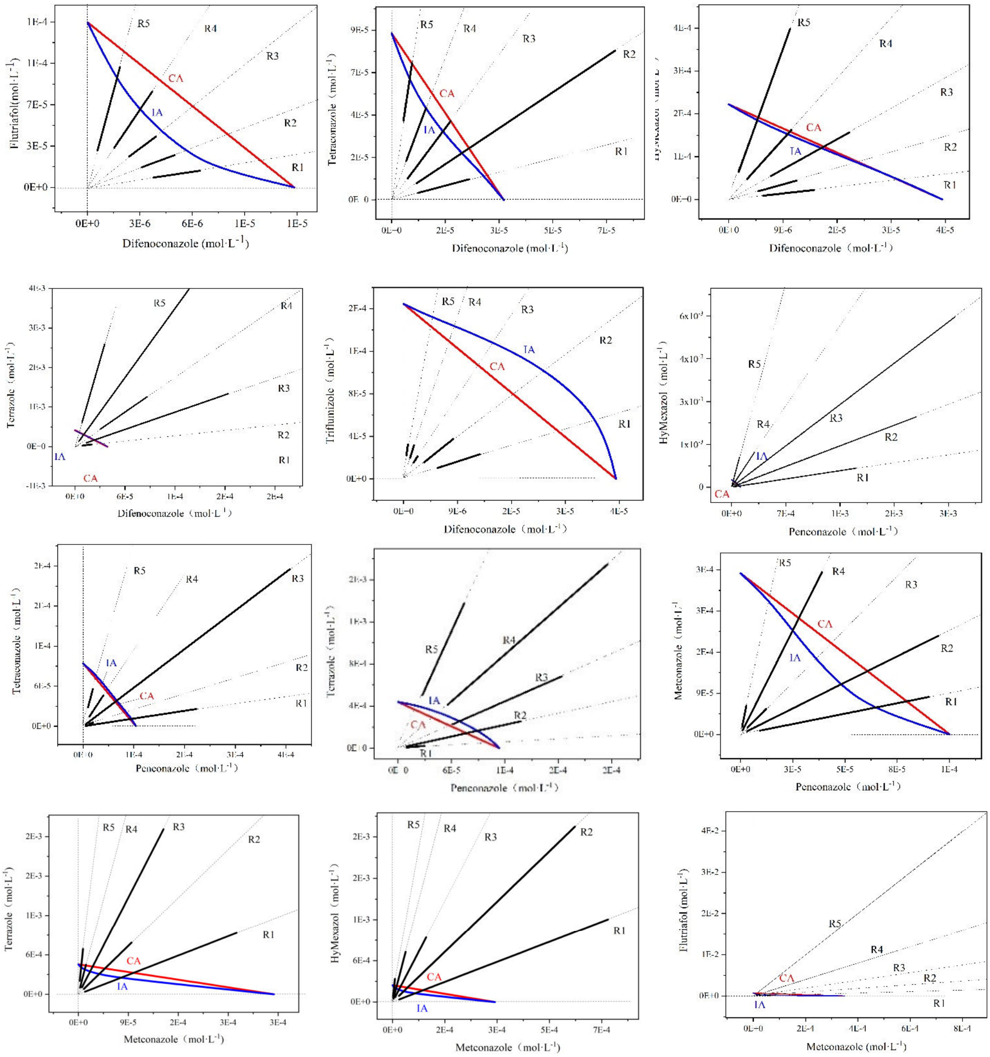

Fig. 3. Equivalent diagram of binary hybrid system based on CA and IA models.

3.4 Quantitative evaluation of toxic interactions based on model deviation

On the basis of 36 mixed systems of azole fungicides, 173 mixed curves were designed by EquRay method, and toxicity data under different exposure concentrations were fitted with logit or Weibull function. The CRCs (Concentration-effect curve) of 173 mixed rays under different exposure concentrations were quantitatively characterized by MDR method based on 95%OCI. The results showed that MDR data showing 10 to 90 percent effects revealed the variation of toxic effects. Figure 4 shows that the antagonistic frequency of the biazole mixture predicted by the CA model is 19.91%, the additive frequency is 38.69%, and the cooperative frequency is 41.40%, while the antagonistic frequency predicted by the IA model is 20.50%, the additive frequency is 41.47%, and the cooperative frequency is 38.03%, with little difference between the two. At different concentrations (EC_{10}, EC_{30}, EC_{50}), the antagonistic effect was stronger at low concentrations, and the toxic effect changed from additive to synergistic with the increase of concentration. MDR predictions for 10% to 90% effects indicate that TET-TER and TRI-HYM show synergistic effects under some effects, but with the increase of effects and concentrations, the form of toxic effects gradually changes from synergistic to additive.

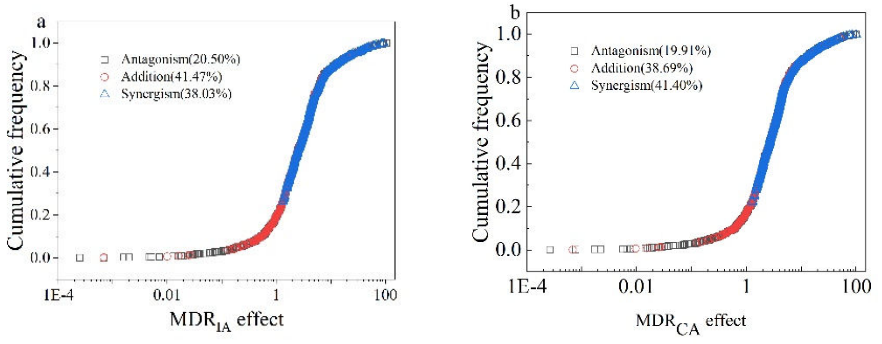

Fig. 4. Cumulative frequency of deviation ratio of azole fungicides.

3.5 The toxic interaction of mixtures was evaluated by index method

In the study of the combined toxic effects of the mixture of azole fungicides on the algae, the types of the combined toxic effects were quantitatively analyzed by the toxic unit method (TU), the additive index method (AI), the mixed toxicity index (MTI) and the combined index (CI). The results showed that there were differences in the evaluation methods of ECx for different mixtures, and the toxic interaction between the mixtures of azole fungicides and *Raphidocelis subcapitata* was mainly synergistic. For example, the evaluation results of TET-TRI-R3 in the EC_{10}~EC_{90} effect range were consistent through four methods, and all showed synergic effects. On the other hand, with the increase of the specified effect, the toxic interaction of PEN-TER-R4 was gradually weakened, and the results of MTI and AI showed different intensity antagonism. The evaluation results of FUB-TRI-R4 in the EC_{60}-EC_{90} range showed that TU and MTI had the same synergistic effect, while AI showed from synergistic to antagonistic effect, and CI showed additive effect. From EC_{10} to EC_{90}, the evaluation of PEN-FLU-R4 showed that the toxic interaction changed from antagonistic to additive and finally synergistic. On the whole, the results of TU and MTI were consistent, indicating that the toxicity of PEN-FLU-R4 gradually decreased with the increase of the specified effect. These results reveal the complexity of toxic interactions of mixtures of azole fungicides on *Raphidocelis subcapitata*, and emphasize the importance of mixture concentration and effect level in the type of toxic interaction.

4 Conclusion

The toxic interaction of the mixture of azole fungicides on *Raphidocelis subcapitata* may depend on the concentration level of the mixture or the concentration ratio of the toxic components of the mixture. The toxic interactions between mixtures with low concentration are mainly synergistic and additive effects. With the increase of exposure concentration or the increase of specified effects, the toxic effects will weaken and gradually change to additive or antagonistic effects. Compared with CA and IA models, MDR model quantification based on 95%OCI can accurately and intuitively evaluate the type of toxic mixed toxic interaction. An exponential evaluation of the interaction between mixture toxicity showed that most of the azole binary mixtures had a synergistic effect on the survival of *Raphidocelis subcapitata*. Among them, the evaluation results of TU, MTI and CI are consistent. MDR, TU, MTI, AI and CI may be different when evaluating the effect level toxicity interaction of azole fungicides on *Raphidocelis subcapitata* within a certain range. However, the overall trend of the interaction, that is, the change of the status of the agent of the toxic effect with the increase of the specified toxic effect in the 96h acute toxicity test, is consistent. When MDR_{CA} predicted the effect of 10%, the combination of TET, TRI and MET had the greatest synergic effect on the combined toxicity, among which the combination of TET and FLU had the greatest synergic effect (MDR_{CA}=1023.047), and the synergic effect of the mixture decreased from low concentration to high concentration. The synergic potential of azoles combined with MET,PEN and FUB increased, while the synergic potential of mixed with TET,HYM and FLU decreased.

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