RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

RSC Advances

PAPER

Predictability of the time-dependent toxicities of aminoglycoside antibiotic mixtures to *Vibrio qinghaiensis* sp.-Q67

Jin Zhang[a,b], Shu-Shen Liu[b], Xin-Qi Dong[a] and Min Chen[a]

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Whether the toxicities of aminoglycoside (AG) antibiotics in aquatic environment and their mixtures change with time and whether AG antibiotic mixtures exhibit the toxicological interaction are seldom reported. In this paper, four AG antibiotics, apramycin sufate, dihydrostreptomycin, kanamycin sulfate, and neomycin sulfate, were selected as mixture components to construct six binary mixture systems. Five rays with different concentration ratios were designed by using the direct equipartition ray design procedure for each system. The toxicities of single antibiotics and their binary mixtures to *Vibrio qinghaiensis* sp.-Q67 (*V. qinghaiensis*) at five time points, 0.25, 2, 4, 8 and 12 h, were determined by the time-dependent microplate toxicity analysis method. It was found that the AG antibiotics and their binary mixtures have the time-dependent toxicity to *V. qinghaiensis*, i.e., their toxicities increase with time. Taking the concentration addition as an additive reference, we found that the toxicities of all mixtures are concentration additive, in other words, the additivity does not change with time, concentration, and concentration ratio.

Introduction

Antibiotics are often used to control human and animal diseases. As growth promoters, antibiotics have been playing an important role in stock farming and aquiculture since the 1990s. However, the abuse of antibiotics and illegal discharge of drug plant wastewater have caused serious issue in recent decades, especially in developing countries. In addition, antibiotics are incompletely absorbed by humans and animals, so large quantities enter various parts in the environment.[1] Aminoglycoside (AG) antibiotics are a class of potent antibiotics with broad spectrum activity against gram-negative bacteria. AG use in developed countries is widespread in cystic fibrosis patients and premature infants. Worldwide, AGs are more widely used due to their low cost and high efficacy against a variety of severe or recalcitrant bacterial infections, including drug-resistant tuberculosis.[2] The toxicology of single AG antibiotics such as streptomycin is well-studied and several cases of allergic reactions have been reported.[3] Considerable attention has been paid due to the toxicity of the residues to human health and the potential increase of drug resistance in bacteria.[4, 5]

However, many AG antibiotics such as apramycin sufate (APR), dihydrostreptomycin (DIH) which is a derivative of streptomycin, kanamycin sulfate (KAN) and neomycin sulfate (NEO) have high water solubility and easily discharge into water body to contaminate water environment so that harm aquatic organism. To evaluate the environmental risk of these antibiotics entering water body, we have to firstly determine their toxicity to aquatic organism such as photobacteria. There are many reports on the toxicities of antibiotics to aquatic organism[6-9] but few on those of AG antibiotics. On the other hand, chemicals in the environment are rarely found alone, antibiotics are the same, too. These chemicals, occurring usually at low concentration and in mixtures, affect subtle physiological traits in organisms and may directly or indirectly cause long-term adverse ecological effects.[10] In other words, the toxicities of antibiotics may change with the concentration, exposure time, and various combinations. Wollenberger et al.[6] determined the acute and chronic toxicities of nine antibiotics to *Daphnia magna* and found that the acute toxicities of the antibiotics are less than their chronic toxicities. Zhu et al.[11] also found that the long-term toxicities of four antibiotics to *Vibrio qinghaiensis* sp. –Q67 (*V. qinghaiensis*) are obviously stronger than their short-term toxicities. Liu et al. found that two AG antibiotics (APR and NEO) are not toxic to *V. qinghaiensis* in 15 min but very toxic after the exposure of 12 h.[12] Gonzalez-Pleiter et al.[13] reported the toxicities of five antibiotics and their mixtures towards photosynthetic aquatic organisms and revealed the toxicological interactions (synergism) in the whole range of effect levels whether in binary or multicomponent mixtures. Christensen et al.[14] tested the combined toxicity of antibiotics. In their report, the concentration addition (CA) and independent action (IA) models were used to identify the toxicological interaction in antibiotic mixtures. The CA and IA models were widely used to assess the combined toxicity of chemicals.[15-21] Recently, Rodney et al.[22] concluded that the toxicities of pesticide

[a] Key Laboratory of Water Pollution Control and Wastewater Resource of Anhui province, College of Environment and Energy Engineering, Anhui Jianzhu University, Hefei 230601, PR China.
[b] Laboratory of Yangtze River Water Environment, Ministry of Education, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, PR China. Tel: 86-021-65982767, E-mail: ssliuhl@263.net.

†Electronic Supplementary Information (ESI) available: See DOI: 10.1039/x0xx00000x

mixtures can be well characterized using the CA model.

Although it was found that some antibiotics may have stronger toxicity in the long-term exposure and several antibiotic mixtures exist as synergism interaction, it is at present unclear whether the toxicities of AG antibiotics and their mixtures change with time and whether the combined toxicity is additive. In this paper, four AG antibiotics, APR, DIH, KAN and NEO, were selected as mixture components to construct six binary mixture systems. For each system, five mixture rays were designed by using the direct equipartition ray design procedure (EquRay).[23] The time-dependent microplate toxicity analysis (t-MTA)[24] was used to determine the toxicities of single antibiotics and their binary mixtures to *V. qinghaiensis* at five time points, 0.25, 2, 4, 8 and 12 h. The CA model was selected as an additive reference to assess the toxicological interaction. The main purpose is to examine how the toxicity changes with time and whether or not to produce toxicological interaction such as synergism in antibiotic mixtures.

Results and discussion

The time-dependent toxicities of antibiotics

Concentration-inhibition (toxicity) data of four antibiotics at different time points determined by the t-MTA can be well described by the Logit function (Table S1†). The fitted location parameter (α) and shape parameter (β), statistics (correlation coefficient (R) and root mean square error (RMSE)) and two effective concentration (EC_{20} and EC_{50}) as well as their negative logarithm ($-\log_{10}(EC_{20}) = pEC_{20}$ and $-\log_{10}(EC_{50}) = pEC_{50}$) were listed in Table S1.† Fitted concentration-response curves (CRCs) of four antibiotics at five time points were shown in Fig. 1.

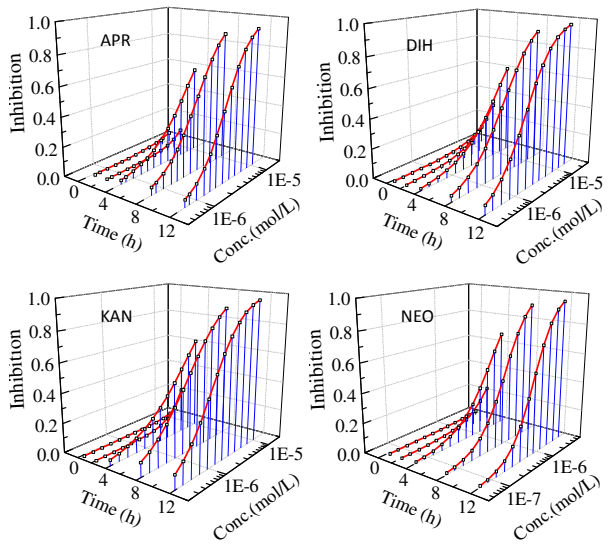


Fig. 1 The fitted concentration-response curves (CRCs) of the four AG antibiotics at five time points.

From Fig. 1, the toxic effects of four antibiotics on *V. qinghaiensis* have time-dependence, which is similar to the results of some antibiotics such as tetracyline hydrochloride chroramphenicol[11] and pesticides such as velpar and aminotriazole[25] and triazine herbicide, metribuzin.[26] The CRCs of the antibiotics gradually rise with exposure time, i.e., the toxicity increases with time. In the first time point (0.25 h), the maximum inhibition effect of four antibiotics is less than 0.2 (20%). If taking pEC_{20} as a toxicity index, all four AG antibiotics have no acute toxicities (at 0.25 h), but are toxic to *V. qinghaiensis* at the other times (2, 4, 8 and 12 h) except for no toxicity of APR at 2 h (Fig. 2a). If taking pEC_{50} as a toxicity index, all antibiotics have no toxicity at starting two time points of 0.25 and 2 h but are toxic at latter three time points of 4, 8 and 12 h (Fig. 2b).

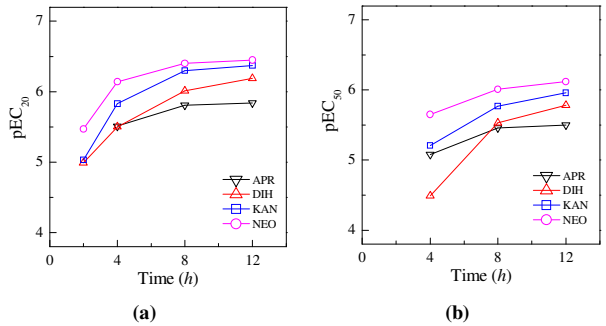


Fig. 2 Plot of pEC_{20} (a) and pEC_{50} (b) of four AG antibiotics versus time.

However, not all chemicals have monotonically incremental toxicities with time. For example, the toxicities of two triazine herbicides (simetry and hexazinone) and three insecticides (imidacloprid, pirimicarb and acetamiprid) do not basically change with time.[25, 27] This may be due to the toxicity mechanism of the five pesitcides on *V. qinghaisensis* being contact toxicity, which causes the test organism die in a short time. The obvious time-dependent toxicity of AG antibiotics with different concentrations to *V. qinghaisensis* may come from their bactericidal mechanism. The AG antibiotics bind to the bacterial ribosomal RNA and increase the probability of accepting a non-cognate tRNA during the translation process, which results in the creation of nonfunctional proteins and eventually leads to death of a bacterial cell.[28, 29] And the bactericidal rate and duration of AG antibiotics positively correlate with their concentrations. Some bacteria can produce AG modifying enzymes (AME) to resist AG actions.[28] Often, the antibiotic resistance produces easily when the concentrations of antibiotics is low, which also testify our results that AG antibiotics are of concentration-dependency and time-dependency.

On the contrary, the acute toxicities (EC_{50} at exposure time of 15 min) of some ionic liquids such as 1-ethyl-3-methylimidazolium chloride is stronger than those at exposure times of 2, 4, 8 and 12 h.[30] So, it is still necessary to unceasingly examine the time-dependent toxicities of pollutants with different chemical structures.

The time-dependent toxicities of antibiotics mixtures

The concentration-inhibition data of all 30 rays in six binary mixture systems (APR-DIH, APR-KAN, APR-NEO, DIH-KAN, DIH-NEO, and KAN-NEO) can be well described by the Logit function. The fitted parameters (α and β), statistics (R and RMSE) and two effective concentrations (EC_{20} and EC_{50}) as well as their pEC_{20} and pEC_{50} were listed in Table S2.† The CRCs of various rays at different time points were shown in Fig. 3. The plots of the pEC_{50} or pEC_{20} versus time were shown in Fig. 4.

From Fig. 3, the CRCs of all mixture rays in six binary mixture systems gradually rise with the exposure time, which illustrates that the toxicities (taking pEC_{20} or pEC_{50} as toxicity index) of the binary mixtures increase with time (Fig. 4). From Fig. 4, it is very clear that the toxicities of all mixtures are monotonically increasing with time during 0 ~ 12 h, which illustrates the toxicity of the AG antibiotic mixture has time-dependence.

Additivity of binary antibiotics mixtures

The CA model (eq. 4 or 5) was used to identify whether the mixture toxicity is additive. The experimental scatters, observed CRCs and their 95% observation-based confidence intervals (OCI) and CRCs predicted by CA were shown in Fig. S2.† From all 5 × 5 × 6 subgraphs in Fig. S2†, 150 CRCs predicted by the CA model are located between the 95% confidence upper and lower limits of the experimental observed CRCs, which testifies that the toxicities of all test mixtures are the concentration additive. In our mixture toxicity test, we determined the toxicities of 360 mixtures, including six binary mixture systems where each system consists of five rays (CRCs) and each ray has 12 mixture concentration levers (points), at five exposure time points of 0.25, 2, 4, 8 and 12 h. The obvious additivity of AG antibiotics indicates that more AG antibiotics coexsited in the waste water system may inhibit some resistant microorganisms and block the antibiotic inactivation. However, this would need more future work to resolve.

The chemical mixtures have complicated diversity due to the difference in composition, concentration ratio and concentration.[31] Only examining some mixtures with equipotent concentration ratio in a definite mixture system is insufficient. Our AG antibiotic mixtures, including all binary combinations (six systems) and various mixture ratios (five rays for each system) as well as various concentration levels (12 concentration points for each ray), are very comprehensive and representative and so the conclusion that concentration addition (no synergism or antagonism) has nothing to do with time, concentration ratio (also called mixture ratio) and concentration level is credible. It should be indicated that our five mixture rays with different mixture ratios are capable of simulating various concentration combinations of pollutants in real environment. Currently used equivalent effect concentration ratio[32, 33] or fixed-ratio ray design (FRRD)[34, 35] is impossible to simulate the mixtures with various concentration ratios.

In recent years, there are many studies on mixture toxicities of antibiotics.[14, 36] Most of studies often focus on one time point (such as acute or chronic toxicity) and one effective concentration (such as EC_{50}). However, the mixture toxicity depends on not only the exposure time and concentration level but also the mixture ratio.[37] Chuensombat et al.[38] examined the cytotoxic effects and antibacterial efficacy of a three-antibiotic combination under 1, 3, 5, and 7 days and found that the concentration of 0.024 mg/mL in all experimental groups generated the highest dental pulp cell or apical pulp cell viability at all time points. In addition, they also examined the cytotoxic change of the combinations at many concentration levels. However, they did not study more combinations with various mixture ratios, which were performed in this study.

Experimental

Chemicals

Four antibiotics (≥ 95% purity), apramycin sufate (APR), dihydrostreptomycin (DIH), kanamycin sulfate (KAN) and neomycin sulfate (NEO), were purchased from Dr. ehrenstorfer (Germany). Their physical properties such as the molecular weight (MW), CAS registration number (CAS RN) and stock concentration were listed in Table 1. All stock solutions were prepared with Milli-Q water and stored at 4 °C.

Table 1 Some physiochemical properties, stock concentration and dilution factor (f)

Name	Abbr.	M.W.	CAS RN	Stock (mol/L)	*f*
Apramycinsulfate	APR	637.6	65710-07-8	2.19E-05	0.68
Dihydrostreptomycin	DIH	784.5	128-46-1	3.44E-06	0.68
Kanamycinsulfate	KAN	582.6	25389-94-0	2.08E-05	0.70
Neomycinsulfate	NEO	908.9	1405-10-3	7.99E-06	0.68

Bacterial culture

The freeze-dried luminescent bacterium *V. qinghaiensis* was purchased from Beijing Hamamatsu Corp., Ltd. (Beijing, China). The manufacture of complete culture medium, solid culture medium and concentrated medium are the same as those described in the literatures.[26, 39] All the culture medium sterilized with high pressure steam for 20 min at 121°C, then cooled down to room temperature and stored at 4°C. Before each test, the bacteria were inoculated from a stock culture, which is maintained on solid culture medium at 4°C, to a fresh agar and were cultured at 22 ± 1°C for 12 h. The bacteria were further grown in complete culture medium by shaking (120 r/min) at 22 ± 1°C for 8~10 h to reach the logarithmic growth phase. The complete medium with *V. qinghaiensis* was mixed with equal amount of the concentrated medium. The mixed suspension was further incubated for 0.5~1 h to make the final relative light unit (RLU) of *V. qinghaiensis* be around 2.0×10^5, which indicated that the bacteria suspension can be used in toxicity tests.[26, 39]

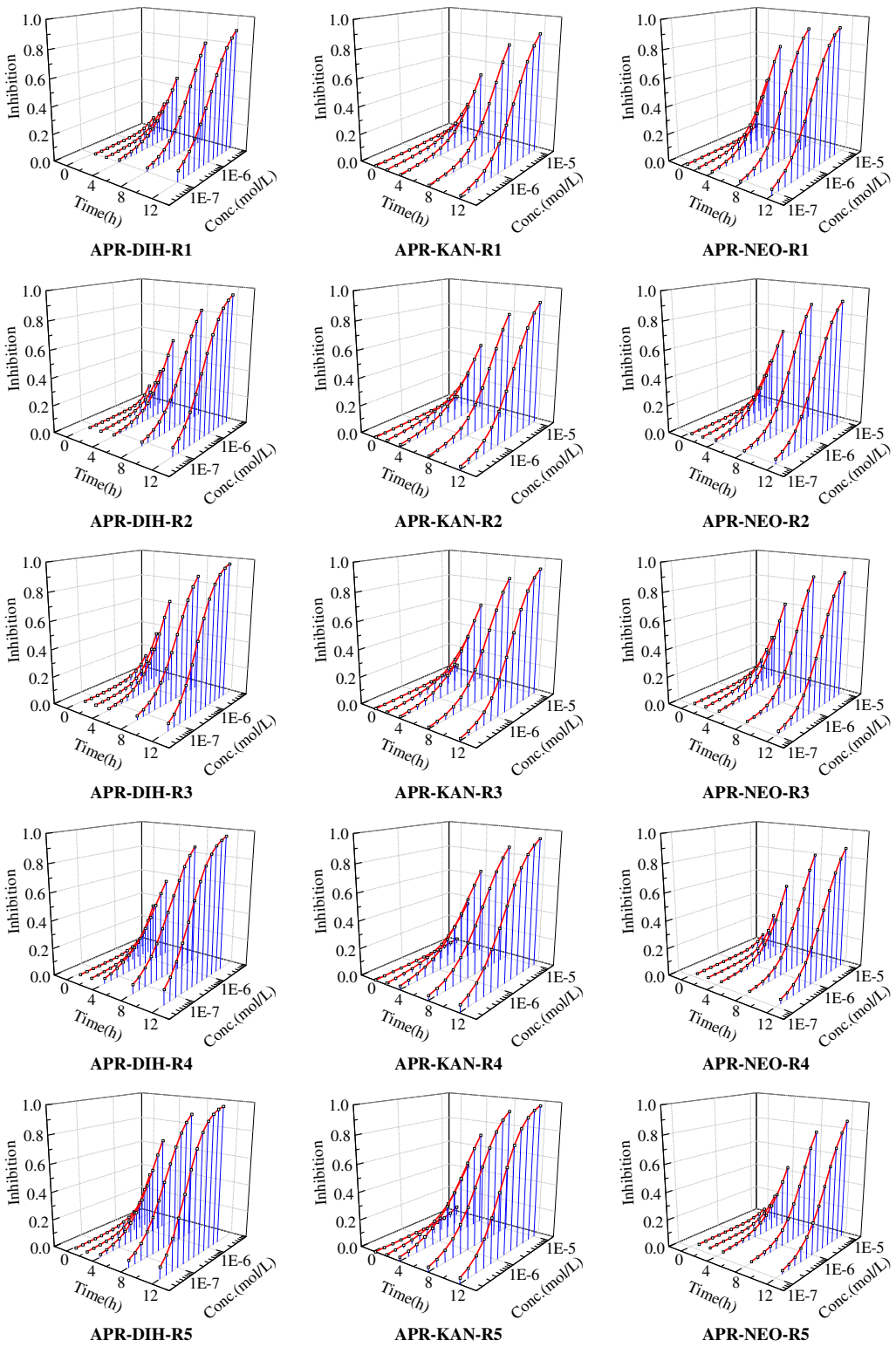


Fig. 3 The fitted CRCs of 30 rays (such as APR-DIH-R1) in six binary mixture systems (APR-DIH, APR-KAN, APR-NEO, DIH-KAN, DIH-NEO, and KAN-NEO) at five time points (0.25, 2, 4, 8, and 12 h).

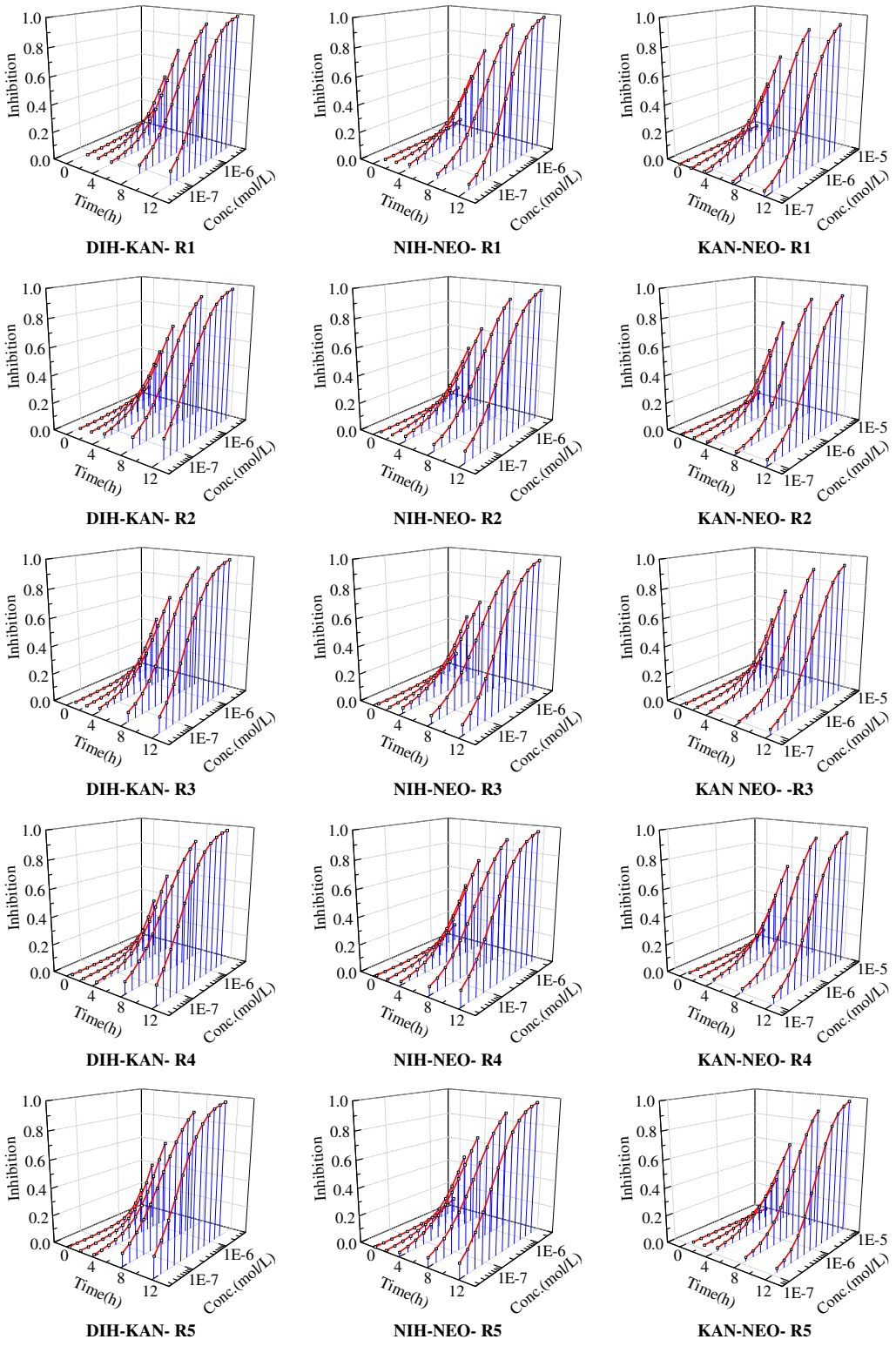


Fig. 3 (continued).

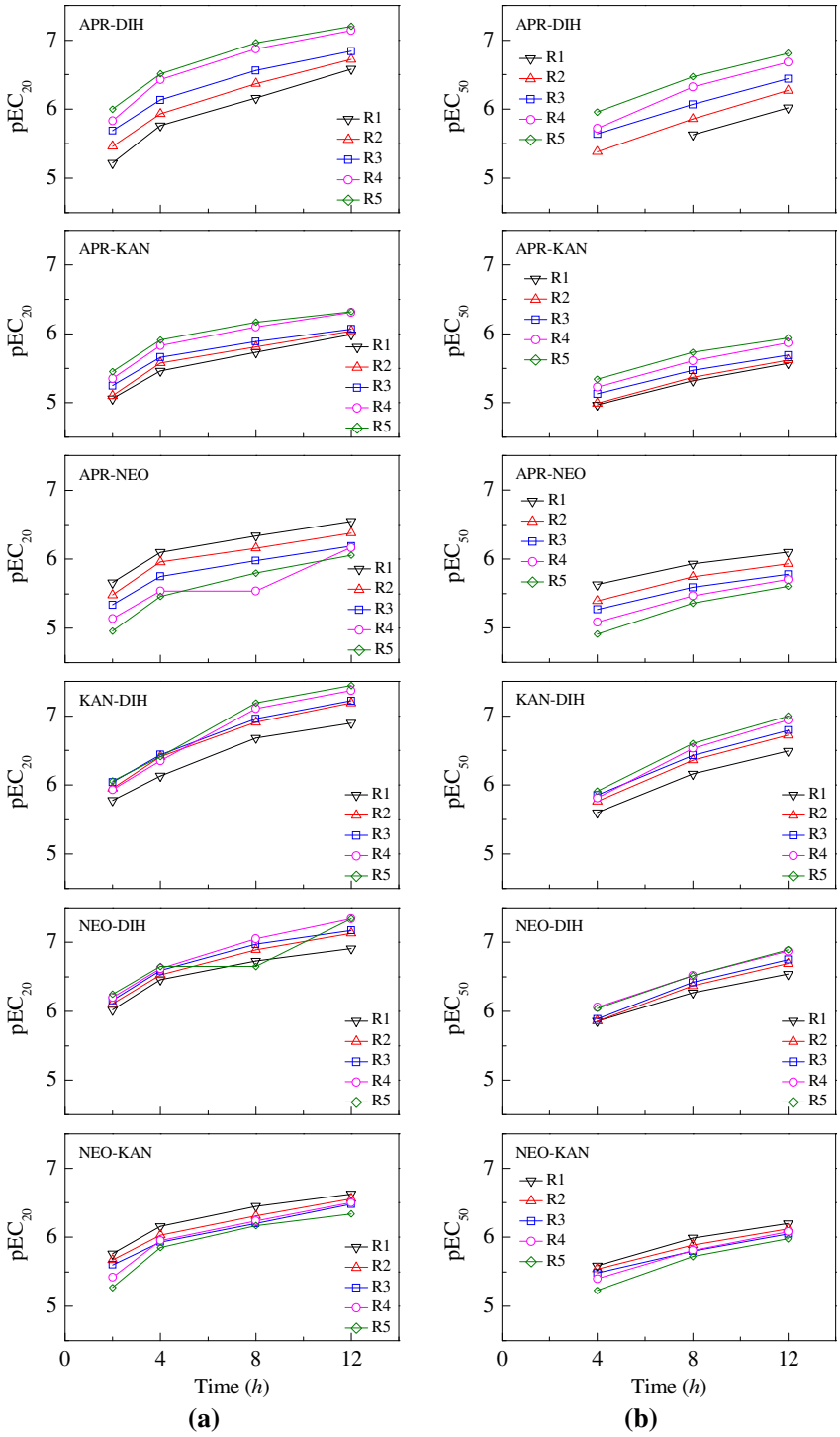


Fig.4 Plot of pEC_{20} (a) and pEC_{50} (b) of various mixture rays versus time for six binary mixture systems.

Time-dependent toxicity test

The time-dependent toxicity test was performed according to the t-MTA developed in literatures.[24, 30,26, 39-40] 12 concentration series in three parallels and 24 controls were arranged in a microplate and the microplate test was repeated three times. The RLU of *V. qinghaiensis* in various wells in the microplate were determined using the PowerWave microplate spectrophotometer (American BIO-TEK company) after 0.25, 2,

4, 8 and 12 h exposure at 22± 1°C. The toxicity of an antibiotic or mixture is expressed as the inhibition value (*x*), which is calculated as follows:

$$x = 1 - \frac{I}{I_0} \quad (1)$$

where *I* is an average of the RLU of *V. qinghaiensis* exposed to the tested chemical and I_0 an average of the RLU exposed to the 24 controls.

Binary mixture design

Taking four antibiotics (APR, DIH, KAN and NEO) as mixture components, we constructed six binary mixture systems, APR-DIH, APR-KAN, APR-NEO, DIH-KAN, DIH-NEO, and KAN-NEO. For each binary mixture system, five mixture rays (R1, R2, R3, R4 and R5) with various concentration ratios (P_is) are designed by using the EquRay.[23] In a two-dimension (X–Y) concentration plane constructed by the effect concentration of the two antibiotics in a binary mixture, the EC_{50} point at X-axis (one component, Comp1) is connected to the EC_{50} point at Y-axis (the other component, Comp2) to form a line segment on which five equidistance points are set. From the origin (O), five rays (R1, R2, R3, R4, and R5) are drawn through five equidistance points. Then, 12 concentration points (x_i, y_i, *i*=1, 2, …, 12) on each of the five rays were designed using a proper dilution factor (*f*). The P_i values of various components (antibiotics) in the rays (R1, R2, R3, R4 and R5) for six mixture systems were listed in Table 2.

Concentration response curve and its confidence intervals

The experimental concentration-inhibition data in different exposure times are fitted to two non-linear functions, Logit (Eq. 2) and Weibull (Eq. 3).[41-43] The Logit (2) and Weibull (3) functions are respectively written as follows:

$$x = 1/(1 + \exp(-\alpha - \beta \log_{10}(c))) \quad (2)$$

$$x = 1 - \exp(-\exp(\alpha + \beta \log_{10}(c))) \quad (3)$$

where α and β are the location and shape parameters to be estimated and c is the concentration.

The delta method is used to construct the observation-based confidence intervals (OCI).[25, 27, 44]

Table 2 The concentration ratios (P_i) of antibiotics in 30 mixture rays of six binary mixture systems.

Ray	P_{APR}	P_{DIH}	Ray	P_{APR}	P_{KAN}
APR-DIH-R1	8.98E-01	1.02E-01	APR-KAN-R1	9.2963E-1	7.0368E-2
APR-DIH-R2	7.78E-01	2.22E-01	APR-KAN-R2	8.4088E-1	1.5912E-1
APR-DIH-R3	6.37E-01	3.63E-01	APR-KAN-R3	7.2544E-1	2.7456E-1
APR-DIH-R4	4.68E-01	5.32E-01	APR-KAN-R4	5.6917E-1	4.3083E-1
APR-DIH-R5	2.60E-01	7.40E-01	APR-KAN-R5	3.4574E-1	6.5426E-1
Ray	P_{APR}	P_{NEO}	Ray	P_{KAN}	P_{DIH}
APR-NEO-R1	4.286E-01	5.714E-01	DIH-KAN-R1	9.824E-01	1.760E-02
APR-NEO-R2	6.522E-01	3.478E-01	DIH-KAN-R2	9.571E-01	4.287E-02
APR-NEO-R3	7.895E-01	2.105E-01	DIH-KAN-R3	9.178E-01	8.221E-02
APR-NEO-R4	8.824E-01	1.176E-01	DIH-KAN-R4	8.481E-01	1.519E-01
APR-NEO-R5	9.494E-01	5.063E-02	DIH-KAN-R5	6.907E-01	3.093E-01
Ray	P_{NEO}	P_{DIH}	Ray	P_{NEO}	P_{KAN}
DIH-NEO-R1	4.836E-01	5.164E-01	KAN-NEO-R1	7.79E-01	2.21E-01
DIH-NEO-R2	3.189E-01	6.811E-01	KAN-NEO-R2	5.85E-01	4.15E-01
DIH-NEO-R3	1.897E-01	8.103E-01	KAN-NEO-R3	4.13E-01	5.87E-01
DIH-NEO-R4	8.564E-02	9.144E-01	KAN-NEO-R4	2.61E-01	7.39E-01
DIH-NEO-R5	7.007E-01	2.993E-01	KAN-NEO-R5	1.24E-01	8.76E-01

Identification of toxicological interaction

Taking the CA model[45, 46] as an additive reference, mixture toxicity is considered to be synergistic when the toxic effect predicted by CA is less than the confidence lower limit of the experimental effect, antagonistic when the predictive effect is greater than the confidence upper limit of the experimental effect, and additive when the predictive effect locates between the upper limit and lower limit of OCI.

Mathematically CA can be formulated as:[42]

$$\sum_{i=1}^{n} \frac{c_i}{EC_{x,i}} = 1 \quad (4)$$

where *n* is the number of mixture components, $EC_{x,i}$ the concentration of the *i*th component that provokes x% effect when applied individually, and c_i the concentration of the *i*th component in the mixture.

The effective concentration of a mixture at certain effect (x) predicted from the CRCs of individual chemicals can be written as follows.

$$EC_{x,mix} = \left(\sum_{i=1}^{n} \frac{P_i}{EC_{x,i}} \right)^{-1} \quad (5)$$

where $EC_{x,mix}$ is the effective concentration of the mixture eliciting x% effect.[43, 47]

Conclusions

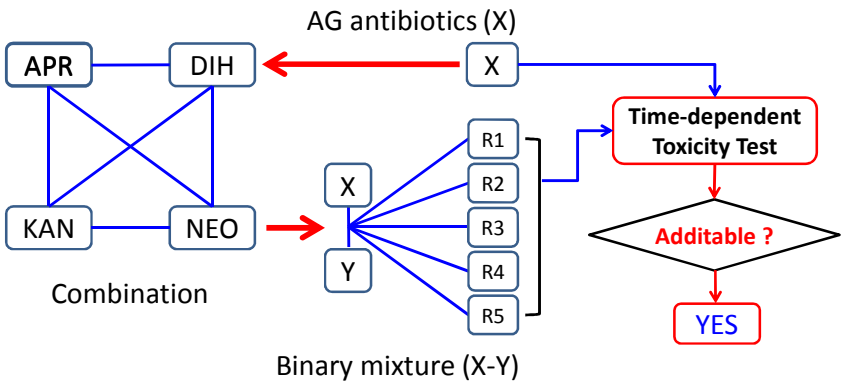
Four AG antibiotics, apramycin sufate (APR), dihydrostreptomycin (DIH), kanamycin sulfate (KAN) and neomycin sulfate (NEO), and their binary mixtures have monotonically increasing time-dependent toxicity to *V. qinghaiensis*, i.e., their toxicities increase with time. 30 binary mixture rays in six systems, APR-KAN, APR-DIH, APR-NEO, DIH-KAN, DIH-NEO and KAN-NEO, were designed by the direct equipartition ray procedure (EquRay) and the toxicities of 360 mixtures at five time points (0.25, 2, 4, 8 and 12) were determined using a time-dependent microplate toxicity analysis (t-MTA). Taking the concentration addition as an additive reference, it was proven that the toxicities of all mixtures are concentration additive, having no time-dependency, concentration ratio-dependency and concentration-dependency.

Acknowledgements

We are thankful to the National Natural Science Foundation of China (21207002, 21177097), Specialized Research Fund for the Doctoral Program of Higher Education (20120072110052), Beijing Key Laboratory for Emerging Organic Contaiminants Control and Doctoral Foundation of Anhui Jianzhu University (2014nzx-094) for their financial support.

Notes and references

1 C. Song, N. Y. Gao and H. W. Gao, Mol Biosyst, 2010, 6, 1901-1910.
2 A. B. Coffin, E. W. Rubel and D. W. Raible, J Assoc Res Otolaryngol, 2013, 14, 645-659.
3 D. A. Bohm, C. S. Stachel and P. Gowik, Food Add Contam: Part A, 2012, 29, 189-196.
4 C. Wang, X. Gu, S. Zhang, P. Wang, C. Guo, J. Gu and J. Hou, Arch Environ Con Tox, 2013, 65, 635-641.
5 F. Xu, W. Jiang, J. Zhou, K. Wen, Z. Wang, H. Jiang and S. Ding, J Agri Food Chem, 2014, 62, 3108-3113.
6 L. Wollenberger, B. Halling-Sorensen and K. O. Kusk, Chemosphere, 2000, 40, 723-730.
7 A. A. Robinson, J. B. Belden and M. J. Lydy, Environ Toxicol Chem, 2005, 24, 423-430.
8 I. Ebert, J. Bachmann, U. Kühnen, A. Küster, C. Kussatz, D. Maletzki and C. Schlüter, Environ Toxicol Chem, 2011, 30, 2786-2792.
9 A. Martins, L. Guimarães and L. Guilhermino, Environ Toxicol Pharm, 2013, 36, 1022-1032.
10 G. R. Scott and K. A. Sloman, Aquat Toxicol, 2004, 68, 369-392.
11 X. W. Zhu, S. S. Liu, Q. Zhang and Y. Liu, Res Environ Sci, 2009, 22, 589-594. (in chinese)
12 L. Ling, L. S. Shen, Y. Mo, Z. Jin and C. Fu, Anal Methods, 2015, 7, 9912-9917.
13 M. González-Pleiter, S. Gonzalo, I. Rodea-Palomares, F. Leganés, R. Rosal, K. Boltes, E. Marco and F. Fernández-Piñas, Water Res, 2013, 47, 2050-2064.
14 A. M. Christensen, F. Ingerslev and A. Baun, Environ Toxicol Chem, 2006, 25, 2208-2215.
15 T. Backhaus, Å. Arrhenius and H. Blanck, Environ Sci Technol, 2004, 38, 6363-6370.
16 N. Cedergreen, A. M. Christensen, A. Kamper, P. Kudsk, S. K. Mathiassen, J. C. Streibig and H. Sorensen, Environ Toxicol Chem, 2008, 27, 1621-1632.
17 S. Bosgra, J. C. van Eijkeren and W. Slob, Crit rev Toxicol, 2009, 39, 418-426.
18 S. Ermler, M. Scholze and A. Kortenkamp, Toxicol Appl Pharm, 2011, 257, 189-197.
19 V. Christen, P. Crettaz, A. Oberli-Schrämmli and K. Fent, Toxicol Appl Pharm, 2012, 259, 169-176.
20 N. Hadrup, C. Taxvig, M. Pedersen, C. Nellemann, U. Hass and A. M. Vinggaard, Plos One, 2013, 8, e70490.
21 M. Scholze, E. Silva and A. Kortenkamp, Plos One, 2014, 9, e88808.
22 S. I. Rodney, R. S. Teed and D. R. Moore, *Hum Ecol Risk Assess*: An Int J, 2013, 19, 1557-1575.
23 R. N. Dou, S. S. Liu, L. Y. Mo, H. L. Liu and F. C. Deng, Environ Sci Pollut R, 2011, 18, 734-742.
24 J. Zhang, S. S. Liu, Z. Y. Yu, H. L. Liu and J. Zhang, *J Hazard Mater*, 2013, **258**, 70-76.
25 X. W. Zhu, S. S. Liu, H. L. Ge and Y. Liu, *Water Res*, 2009, **43**, 1731-1739..
26 M. C. Wang, S. S. Liu and F. Chen, Acta Chim. Sinica, 2014, 72, 56-60.
27 L. Liu, S. S. Liu, M. Yu and F. Chen, Environ Toxicol Phar, 2015, 39, 447-456.
28 M. Kulik, A. M. Goral, M. Jasiński, P. M. Dominiak and J. Trylska, Biophys J, 2015, 108, 655-665.
29 J. Romanowska, N. Reuter and J. Trylska, Proteins: Struct, Func, Bioinf, 2013, 81, 63-80.
30 J. Zhang, S. S. Liu, Z. Y. Yu and J. Zhang, Chemosphere, 2013, 91, 462-467.
31 S. S. Liu, Q. F. Xiao, J. Zhang and M. Yu, Sci Bull, 2015,Doi: 10.1007/s11434-015-0925-6
32 J. Hu, J. Li, J. Wang, A. Zhang and J. Dai, Chemosphere, 2014, 96, 81-88.
33 F. Chen, S. S. Liu, M. Yu, R. Qu and M. C. Wang, Chemosphere, 2015, 132, 108-113.
34 V. C. Moser, M. Casey, A. Hamm, W. H. Carter Jr, J. E. Simmons and C. Gennings, Toxicol Sci, 2005, 86, 101.
35 K. B. Norgaard and N. Cedergreen, Environ Sci Pollut R, 2010, 17, 957-967.
36 J. Y. Ji, Y. J. Xing, Z. T. Ma, M. Zhang and P. Zheng, Chemosphere, 2013, 91, 1094-1098.
37 M. J. Jonker, C. Svendsen, J. J. M. Bedaux, M. Bongers and J. E. Kammenga, Environ Toxicol Chem, 2005, 24, 2701-2713.
38 S. Chuensombat, S. Khemaleelakul, S. Chattipakorn and T. Srisuwan, J Endod, 2013, 39, 813-819.
39 M. Yu, S. S. Liu, M. Wang, F. Chen and H. X. Tang, Chin J Chem, 2014, 32, 545-552.
40 J. Zhang, S. S. Liu and X. W. Zhu, Chemosphere, 2014, 112, 420-426.
41 Y. H. Zhang, S. S. Liu, X. Q. Song and H. L. Ge, Ecotox Environ Safe, 2008, 71, 880-888.
42 S. S. Liu, X. Q. Song, H. L. Liu, Y. H. Zhang and J. Zhang, Chemosphere, 2009, 75, 381-388.
43 S. S. Liu, J. Zhang, Y. H. Zhang and L. T. Qin, Acta Chim Sinica, 2012, 70, 1511—1517. (in chinese)
44 X. W. Zhu, S.-S. Liu, L.-T. Qin, F. Chen and H.-L. Liu, Ecotox Environ Safe, 2013, 89, 130-136.
45 F. Chen, S. S. Liu, X. T. Duan and Q. F. Xiao, Rsc Adv, 2014, 4, 32256-32262.
46 A. Coors, B. Weisbrod, U. Schoknecht, F. Sacher and A. Kehrer, Environ Toxicol Chem, 2014, 33, 382-393.
47 S. S. Liu, L. Liu and F. Chen, Acta Chim Sinica, 2013, 71, 1335-1340.



The combined toxicities of all binary mixtures constructed by four aminoglycoside (AG) antibiotics are concentration additive, which has nothing to do with exposure time, mixture ratio, and concentration level.