Epidemiology-based wastewater monitoring for ecological risks of anti-tuberculosis drugs mixture effects

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Abstract

First-line anti-tuberculosis (TB) drugs are commonly used to treat TB worldwide, leading to more contaminated wastewater being widely discharged into aquatic environments. However, studies of mixture interactions of anti-TB drugs and their residues in aquatic environments are scarce. This study aimed to determine the toxic interactions of anti-TB drugs—isoniazid (INH), rifampicin (RMP), and ethambutol (EMB)—in binary and ternary mixtures on Daphnia magna and used the epidemiology of TB history to construct epidemiology-based wastewater monitoring for assessing the environmental release of residues and related ecological risks. The acute immobilization of median effect concentrations (EC50) was 25.6 mg L⁻¹ for INH, 80.9 mg L⁻¹ for RMP, and 188.8 mg L⁻¹ for EMB, as toxic units (TUs) for assessing mixture toxicity. The ternary mixture exhibited the lowest TUs at 50% effects with 1.12, followed by 1.28 for RMP+EMB, 1.54 for INH+RMP, and 1.93 for INH+EMB, indicating antagonistic interactions. Nevertheless, the combination index (CI) was used to examine the mixture toxicity in response to immobilization, revealing that the ternary mixture of CI ranged from 1.01 to 1.08, tending to have a nearly additive effect when suffering more than 50% effect (at high concentration levels). The forecasted environmentally relevant concentrations of anti-TB drugs have been on downward trends with ng L⁻¹ level from 2020 to 2030 in Kaohsiung, Taiwan. Although ecotoxicological risks from the wastewater treatment plant and receiving water in the field were slightly greater than the prediction from epidemiology-based wastewater monitoring, there were no risk concerns. Here, we achieved the establishment of evidence that anti-TB drug mixtures’ interaction and epidemiological-based monitoring support a systematic approach, resolving the absence of the mixture toxicity information for anti-TB mixture risk assessment in aquatic environments.

Keywords: anti-tuberculosis drugs; mixture toxicity; Daphnia magna; epidemic models; ecotoxicological risk
1. Introduction

A 2019 World Health Organization (WHO) report documented an estimated 10 million people infected with tuberculosis (TB) worldwide (WHO, 2020). First-line anti-TB drugs, such as isoniazid (INH), rifampicin (RMP), ethambutol (EMB), and pyrazinamide (PZA), are the most commonly used TB treatments. In particular, the effective treatment of drug-susceptible TB utilizes a combination of anti-TB drugs for six months or longer. Anti-TB drugs are not completely metabolized, and up to 70% of antimicrobials are excreted in urine and feces in unchanged form (Sweetman, 2007; Magwira et al., 2019). INH and EMB are not completely degraded in wastewater and pose toxic effects on activated sludge bacteria (Vaňková, 2011; Sanderson and Thomsen, 2009), persistent in aquatic environments. Aquatic organisms living in aquatic environments are thus continuously exposed to anti-TB drug mixtures; consequently, these organisms are at risk of exposure to mixture toxicity or drug resistance in aquatic environments.

Studies on the toxic effects of individual anti-TB drugs on aquatic organisms are limited. Fu et al. (2017) have shown low RMP toxicity in the growth and yield of the algae *Pseudokirchneriella subcapitata* because of its low RMP bioavailability. INH has been shown to significantly increase alanine transaminase and aspartate aminotransferase as well as induced oxidative stress, leading to liver atrophy and injury in zebrafish (Jia et al., 2019; Zhang et al., 2017). INH has also been shown to significantly decrease the locomotor capacity and larvae development of zebrafish (Zou et al., 2017). However, most studies have focused on aquatic toxicity of INH. For example, median lethal concentrations (LC50s) have been evaluated through quantitative structure-activity relationship (OSAR) and bioassays of *Artemia* marine
shrimp, freshwater species (*Daphnia magna* and *Streptocephalus proboscideus*),
*Brachionus calyciflorus* plankton, and fish (Calleja et al., 1994; Lilius et al., 1994, Sanderson and Thomsen, 2009). Most evidence has shown that anti-TB drugs cause adverse events in mice, rats, and humans and appear to have similarities and differences in modes of action, such as INH-posed neurotoxicity, EMB-posed ocular nerve toxicity, and RMP-posed nephritis (Buznego and Pérez-Saad, 2006; Vilholm et al., 2014; Garg et al., 2015; Katz and Lor, 1986; Rekha et al., 2005), resulting in additive, synergistic, or antagonistic interactions in the mixtures. Regarding anti-TB drug mixture exposure, binary INH+RMP and the co-administration of INH, RMP, EMB, and PZA have been shown to cause antagonistic or synergistic interactions on the liver injury of mice and pose testicular dysfunction (Yue et al., 2009; Skakum and Shman’ko, 1985; Awodele et al., 2013; Shayahmetova et al., 2012). To date, knowledge and data regarding anti-TB drug mixture toxicity and EMB in aquatic environments are scarce. It is thus necessary to fill this data gap on the mixture toxicity of anti-TB drugs and the associated deleterious ecotoxicological effects.

Wastewater epidemiology is a novel quantitative tool that can be used to evaluate rates of drug abuse, infected populations, and the spread of infectious diseases by detecting human metabolic concentrations or biomarkers from wastewater. For example, regarding concentrations of narcotics, this method can be used to evaluate the levels of drug use on university campuses. Meanwhile, the concentration of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA has been used to assess the number of coronavirus disease 2019 (COVID-19) patients (Gushgari et al., 2018; Peccía et al., 2020). In contrast, environmental antimicrobial or anti–infective agent concentrations can be monitored by surveying or predicting the infected populations in communities—a process defined as epidemiology-based wastewater
The conventional method for monitoring antimicrobial residues in environments is chemical analysis, which is expensive and cannot detect residues in real time. Environmental levels of anti-TB drugs are highly correlated to the state of the TB-infected population and population density in the area. The environmentally relevant concentration of anti-influenza virus drugs, oseltamivir and its metabolites, have been estimated based on the number of influenza-infected populations in the UK (Singer et al., 2011), Japan (Azuma et al., 2015), and Taiwan (Chen et al., 2014; 2020). The modes of influenza-infected populations are highly dependent on the seasonal pattern of influenza occurrence (Lofgren et al., 2007; Coletti et al., 2018). Strong evidence has also shown that TB transmission exists seasonally across different regions throughout the world (Manabe et al., 2019; Wubuli et al., 2017; Margalit et al., 2016; Andrews et al., 2020; Willis et al., 2012). For the precise monitoring of environmental anti-TB drug concentrations, the seasonality of TB must be incorporated into the epidemic transmission model (Tedijanto et al., 2018) to accurately estimate the number of infected populations treated with anti-TB drugs, especially in regions with a high incidence of TB. Therefore, we aim to develop epidemiology-based wastewater monitoring with a seasonal epidemiological model to provide real-time forecasting for anti-TB drug residues in aquatic environments. Using epidemiology-based monitoring, this study aimed to determine the toxicity of the interaction mixture of the binary and ternary anti-TB drug in *D. magna* and to assess whether environmentally relevant residues pose risks in aquatic environments.

### 2. Materials and methods

#### 2.1 Individual and mixture toxicity tests
This study obtained the experimental animals *Daphnia magna* from the Freshwater Bioresources Center, National Chiayi University, Taiwan. The test animals were acclimated over five generations in our laboratory. The acclimated condition followed the OECD Test Guidelines for Chemicals 202 (OECD 202; OECD, 2004) and the Environmental Analysis Laboratory for the *Daphnia* static test NIEA B901.14B (Taiwan EPA, 2013). *Daphnia* were acclimated in dechlorinated tap water at 25°C with a 16-h light cycle; feed and medium were renewed three times per week. We mixed powders of *Chlorella* sp., or *Spirulina*, and yeast as the *Daphnia* feed. The cultural density was controlled at less than 30 *Daphnia* per 1-L glass beaker.

INH (> 99% purity) was purchased from Sigma–Aldrich, and RMP (> 97.35% purity) and EMB (> 98% purity) were purchased from APExBIO as test substances. Because limited studies have focused on the effects of RMP and EMB on aquatic organisms, we performed a range-finding test to determine the range of exposure concentration for the acute toxicity tests of individual anti-TB drugs except for INH. We applied the designations of INH exposure concentrations to the acute toxicity tests of *Daphnia* published by Calleja et al. (1994) and Lilius et al. (1994).

One control and five concentrations were used individually or in mixtures for anti-TB drug exposure tests. The exposure concentrations for individual anti-TB drugs were as follows: (i) INH: 12.5, 25, 50, 100, and 200 mg L\(^{-1}\); (ii) RMP: 25, 50, 100, 200, and 400 mg L\(^{-1}\); and (iii) EMB: 50, 100, 200, 300, and 400 mg L\(^{-1}\). The combination of anti-TB drugs used for mixture exposure included (i) binary mixture exposure—INH+RMP, INH+EMB, RMP+EMB—and (ii) ternary mixture exposure—INH+RMP+EMB. We evaluated each anti-TB drug exposure test to identify the median effective concentration (EC50), which was done to determine the
range of mixture exposure concentrations used in the mixture toxicity tests. The range of mixture exposure concentrations was designed based on the EC50 fractions of individual anti-TB drugs, including 1/4-, 1/2-, 1-, 2-, and 4-fold EC50s.

Aged less than 24 h, *D. magna* were released from the brood beakers and transferred to a test medium for 48-h anti-TB drug exposure. Twenty *Daphnia* were divided into four 50-ml beakers for each treatment without feeding and water renewal. We performed acute immobilization tests on the *Daphnia* following the OECD 202 (OECD, 2004), including environmental conditions and the number of test concentrations and excluding water temperature. All tests were performed in triplicate. The cultural temperature of 25°C conformed to our weather environment. Water quality parameters during exposure periods were as follows: pH: 7.7–8.5; DO: 5.2–8.8 mg L⁻¹; and hardness: 164–237 mg L⁻¹. The number of immobile and dead *Daphnia* after 24-h and 48-h exposures were observed to evaluate the 48-h EC50 or median lethal concentration (LC50).

### 2.2 Epidemiology-based wastewater monitoring

This study considered the seasonality of TB outbreak events to estimate environmentally relevant concentrations of anti-TB drugs in water environments. We performed the epidemiology-based wastewater monitoring and incorporated the epidemiological data to evaluate the number of TB cases at the stage of infection progression to estimate the quantities of anti-TB drugs used in Kaohsiung, Taiwan. The number of TB patients who use anti-TB drugs highly contributes to the concentrations of anti-TB drug residues in receiving waters. We adopted the TB epidemiological data, municipal population, and abridged life table and life expectancy in 2015 from the Taiwan Tuberculosis Control Report and Department of
Statistics Ministry of the Interior, Taiwan. We incorporated the seasonality of new TB cases into the epidemic transmission model to estimate the total TB-infected population during the endemic period due to the Taiwanese Centers for Disease Control (CDC)’s reporting of the new confirmed TB cases. The seasonal regression model fitted the monthly new TB cases to characterize the seasonality of TB (Supplementary Information Table S1). The seasonal regression of TB cases was performed to generate 95% CIs using R (version 3.6.1; The R Foundation for Statistical Computing, 2019). The Akaike information criterion (AIC) was used to determine a suitable seasonal model for characterizing the seasonality of TB. The AIC can be expressed as $AIC = 2k - 2\ln(\text{log-likelihood})$, where $k$ is the number of parameters. We have presented the $\Delta AIC$ to compare the relative difference between the best-fitting model and each model. We used the mean absolute percentage error (MAPE) as the goodness-of-fit index to assess the model performance. The best-fitting model coefficients were used to predict the monthly new TB cases in the following years.

To describe TB population transmission dynamics, we used ordinary differential equations based on the natural history of TB as a disease, in which we considered the unexposed “susceptible” population, “latently” infected population (unobserved), and “infected” population. The epidemic Susceptible–Latent–Infected (SLI) model (Table S1) corresponds to Figure S1. The SLI model can also be used to estimate the number of monthly new TB cases ($I_N$) as follows:

$$I_N(t) = p \beta I(t)S(t) + qL(t), \quad (1)$$

where $p$ is the proportion of new infections that develop into TB within one year (–), $\beta$ is the transmission rate (ind$^{-1}$ month$^{-1}$), $I(t)$ is the infectious individuals with TB, $S(t)$ is the susceptible individuals, $q$ is the progression rate to TB (month$^{-1}$), and $L(t)$ is
the latently infected individuals. We linked the seasonal regression model to the SLI
model based on the transmission rate to estimate the seasonal transmission rate as
follows:
\[
\beta(t) = \frac{I_N(t) - qL(t)}{pI(t)S(t)},
\]
(2)
The seasonality of TB, new confirmed cases, and municipal population in the endemic
area were incorporated into the SLI model to develop a dynamic seasonal TB
transmission model to simulate total TB infection population dynamics. The seasonal
TB transmission modeling was performed and optimized using by the Monte Carlo
(MC) simulation implemented in R language with the GNC MCSim software (version
6.1.0). There were 10,000 interactions to ensure the stability of the results for
evaluating the total TB population.

This study assumed that all infected populations were treated with anti-TB drugs.
The TB patient population contributed to environmentally relevant concentrations of
anti-TB drugs in receiving water environments. The prediction of environmentally
relevant anti-TB drug concentrations is essentially based on drug excretion and the
dilution of environmental release into wastewater treatment plants. We considered the
pharmacokinetics of anti-TB drugs concerning drug dosage and the fraction of drug
metabolized in urine and feces. Then, we used the dilution rate concerning the
average residential water consumption to evaluate the predicted environmentally
relevant concentration:
\[
PEC_i = \frac{I \cdot D_i \cdot M_i \cdot RE_i}{W \cdot N_w},
\]
(3)
where \(PEC_i\) is the predicted environmental concentration of anti-TB drug \(i\) in water
environments (mg L\(^{-1}\)), \(I\) is the total infected population (ind), \(D_i\) is the daily average
treatment dosage for one confirmed case (mg ind\(^{-1}\) d\(^{-1}\)), \(M_i\) is the fraction metabolized
of the anti-TB drug (–), \( R_{E_i} \) is the removal efficiency of the anti-TB drug (–), \( W \) is the average per capita water use in the endemic area (L d\(^{-1}\)), and \( N_w \) is the population with water service (ind). TB treatment regimens involve weight-based dosing; we assumed the dosage used for a body weight of more than 55 kg in TB patients. Ellard and Gammon (1976) indicated that the metabolism process of INH can be distinguished as including both a slow and rapid metabolized rate (slow: 17%; rapid: 34%). There was individual difference based on the N-acetyltransferase 2 (NAT2) gene, and the Taiwanese population (76.8%±5.4%) had a rapid metabolite rate for INH. The parameter values of dosage and metabolized rate are summarized in Table S4.

### 2.3 Effluent acute toxicity tests

The effluent samples were collected from two wastewater treatment plants (WWTPs) in Kaohsiung, Taiwan, in June 2022. The WWTP-A is the primary wastewater treatment and source of effluent discharge into the marine, whereas the WWTP-B is the secondary wastewater treatment near the Fengshan River. These two WWTPs treat wastewater produced by more than half the municipal population in Kaohsiung. We also collected effluent water discharged into the Fengshan River (receiving water) for toxicity testing and chemical characteristics. All samples were stored in PE containers with ascorbic acid at 4\(^{\circ}\)C until further analysis and toxicity testing. We measured the water temperature, pH, DO, chemical oxygen demand, hardness, and chemical analyses of INH, RMP, and EMB. The effluent toxicity tests were carried out in accordance with the USEPA (2004) and OECD 202 test guidelines for \( D. magna \); the water temperature was maintained at 25.0\(^{\circ}\)C. The \( Daphnia \) were exposed to 100% receiving water of WWTP-B and effluent concentrations of 6.25%, 12.5%, 25%, 50%, and 100% from WWTP-A and WWTP-B. The toxicity tests
consisted of a blank control, a negative control (ascorbic acid), and treatments in three replicates.

The concentrations of INH, RMP, and EMB were determined by high-performance liquid chromatography (HPLC, Agilent 1260 Infinity II)/triple quadrupole mass spectrometry (MS/MS, Agilent 6470A Triple quadrupole LC/MS²).

The water sample was passed through the PP centrifuge and centrifuged for 15 min; we then added 0.05 mL of 1-mg L⁻¹ internal standard into the 100-mL resultant supernatant. We used an ammonia solution to adjust the pH ranging from 10–10.5 for INH and EMB, and the centrifuge was washed with 6 mL of methanol, 6 mL of 0.5N ammonia solution, and 6 mL of deionized water. The water samples were passed over the SPE column at a flow rate of 0.5 drop sec⁻¹, and the column was washed with 6 mL of deionized water at 0.5 drop sec⁻¹. Elution was performed using 4.5 mL of 0.5M HCl at one drop sec⁻¹, and 5 mL of the eluate was collected. For RMP, we used formic acid to adjust the pH to 4, and the centrifuge was washed with 4 mL of 2% ammonia in methanol, 4 mL of methanol, and 6 mL of 0.1% formic acid in water. Water was passed over the sample SPE column at 0.5 drop sec⁻¹, which was subsequently vacuumed to dryness for 15 min. Elution was performed by 10 mL of 2% ammonia in methanol at one drop per two seconds and collected into a PP tube with 5 mL of eluate. The recovery rates of INH, RMP, and EMB were 99.26%, 107.21%, and 105.82%, respectively, and the limit of detection (LOD) was 2.5 ng L⁻¹.

2.4 Data analysis

The acute toxicity data were analyzed to construct a concentration-effect relationship. The relationship of percentage of adverse effects (Eᵢ) endpoints in the exposure regime of anti-TB drugs (Cᵢ) using the logistic model as the concentration-
effect and toxic unit-effect functions that expressed as below, to assess the hazard of
individual anti-TB drugs or mixture on daphnia,

\[ E(t, C_i) = \frac{E_{\text{max}}(t)}{1 + \left(\frac{EC_{50}(t)}{C_i}\right)^n(t)}, \quad (4) \]

\[ E(TTU) = \frac{E_{\text{max}}}{1 + \left(\frac{TU50}{TTU}\right)^n}, \quad (5) \]

where \( E(t, C_i) \) is the concentration of anti-TB drug \( i \)'s dependent adverse effect at a
given time \( t \) (–), and \( E(TTU) \) is the total toxic unit of exposure-dependent
immobilization. \( E_{\text{max}} \) is the time-specific maximum effect (–), and \( n \) is the time-
dependent slope factor (–) which is a measure of cooperativity between anti-TB drug
and adverse effect; \( n \geq 1 \) represents positive cooperativity. The concentration-effect
function can determine the concentration caused \( x \) fraction of adverse effect \( (EC_x) \), no
observed effect concentration (NOEC), the lowest observed effect concentration
(LOEC), and EC50 included, whereas the toxic unit function determines the toxic
units resulting in 50% immobilization (TU50). The 48-h concentration-effect curves
were used to assess anti-TB drug interaction based on the Chou-Talalay (1984)
method, combination index (CI), and is described as,

\[ CI = \sum_{i=1}^{j} f_i \cdot \frac{EC_{x,\text{mix}}}{EC_{x,i}}, \quad (6) \]

where \( f_i \) is the fraction concentration of anti-TB drug \( i \) in the mixture concentration,
and \( j \) is the number of the drug combination in mixture exposure. The quantitative
definition of CI for antagonism, additive effect, and synergism was \( CI > 1.1 \), \( CI = 0.9-1.1 \), and \( CI < 0.9 \), respectively (Chou, 2006).
We used the TableCurve 2D package (AISN Software Inc., USA) to perform model fitting for the concentration-effect relationship. The predicted environmental concentration and combination index were performed to generate 95% confidence intervals (CIs) with Monte Carlo simulation implemented in Crystal Ball® (Decisioneering, Inc., Denver, CO, USA). We also used the one-way ANOVA with Tukey’s test ($p < 0.05$) to examine statistical differences in immobilization and mortality among exposure concentrations of the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC).

2.5 Risk assessment

The deterministic risk quotient approach was used to assess ecological risk caused by individual anti-TB drugs or mixtures in water environments. We integrated analyses from exposure modeling and toxicity data; the predicted no-effect concentration of $i$ anti-TB drug ($PNEC_i$) was compared to the predicted environmental concentration as follows:

$$RQ = \frac{PEC_i}{PNEC_i},$$

(7)

where $PNEC_i$ is estimated by the division of NOEC for immobilization toxicity with appropriate assessment factors (AF), and the risk quotient (RQ) is performed by Monte Carlo simulation for uncertainty. In order to understand the risk of environmentally relevant exposure, the whole-mixture approach was used to assess the ecological risk of anti-TB drugs in mixtures. We used mixture toxicity data that was assessed by testing anti-TB drugs in mixtures. The predicted no-effect concentration in mixtures ($PNEC_{mix}$) was derived by the LOEC_{mix} from the ternary mixture exposure test and the application of AF.
3. Results

3.1 Mixture interaction determination of anti-TB drugs

The immobilization and mortality of *Daphnia* in response to individual anti-TB drugs at 48 h are shown in Figure S2. All effects increased in response to the increasing concentration of anti-TB drugs. The logistic regression model had the best fit for immobilization and mortality data to estimate the median effect concentration ($r^2 = 0.90–0.99$). Table 3 shows the estimated EC50s—25.59±1.08 (mean±SE) mg L$^{-1}$ for INH ($p < 0.001$), 80.85±13.81 mg L$^{-1}$ for RMP ($p < 0.01$), and 188.78±23.40 mg L$^{-1}$ for EMB ($p < 0.01$). The fitted slope factor ($n$) values in three anti-TB drug treatments were greater than one, indicating positive cooperativities on immobilization and mortality. As toxic units, the EC50s of individual anti-TB drugs were used to design the mixture exposure concentration for mixture toxicity tests. The EC50 and LC50 for anti-TB drugs in mixtures were estimated with the best fit ($r^2 = 0.95–0.99$), as shown in Table 1 and Figure S3. The estimated LC50s for individuals and mixtures of anti-TB drugs are presented in Table S5. The binary mixtures of INH+RMP had the lowest EC50s with 80.73±3.85 mg L$^{-1}$ compared with other anti-TB drug mixtures. The EC50 values of the binary mixtures aside from INH+EMB were close to individual anti-TB drugs, with the highest EC50 in this combination—higher than the other anti-TB drugs. This revealed potential antagonism in anti-TB drug mixtures.

We determined the NOEC and LOEC of immobilization and mortality for individuals and mixtures of anti-TB drugs compared with the control treatment (Table 1). INH showed the lowest threshold values (NOEC: 12.5 mg L$^{-1}$; LOEC: 25 mg L$^{-1}$), indicating it had the highest toxic effect compared to RMP and EMB. EMB had the lowest toxic effect on immobilization and mortality (NOEC:100 mg L$^{-1}$; LOEC: 200
mg L\(^{-1}\)). For anti-TB drug mixtures, the NOEC was 26.25 mg L\(^{-1}\) and 112.5 mg L\(^{-1}\) for INH+RMP and INH+EMB, respectively. There were no observed NOECs of the RMP+EMB and INH+RMP+EMB mixture exposure, whereas the LOECs were 70 mg L\(^{-1}\) and 76.26 mg L\(^{-1}\), respectively.

To determine if the immobilization of *Daphnia* in the anti-TB mixture was additive, we used the total toxic unit exposure to examine the interaction of the mixture toxicity. If the toxic units resulting in 50% immobilization (TU50) equaled one, it indicated additive; less than or greater than additive were synergistic and antagonistic, respectively. The estimated TUs of the binary and ternary mixtures were greater than one (\(p < 0.001, r^2 = 0.95–0.99\)), as shown in Figure 1 and Table 1, indicating antagonistic interaction. Furthermore, we used the approach of combination index to examine the mixture toxicity in response to immobilization levels (Fig. 2). The combination indices of the binary mixtures were higher than 1.1, and the RMP+EMP ranged from 1.27 to 1.33, showing moderate antagonism. Other binary mixtures were higher than the RMP+EMP that presented antagonism. In contrast to the binary mixtures, the ternary mixture tended to have a nearly additive effect when suffering more than 50% immobilization (high concentration exposure).

### 3.2 Predicted anti-TB drugs released into wastewater environments

The seasonal TB regression modeling showed that the new TB cases in Kaohsiung in 2015–2019 had a slow downward trend (\(r^2 = 0.662; p < 0.001\); Fig. 3A). The optimally fitted coefficients of the seasonal regression model are listed in Table S3. For the accuracy of the seasonal TB transmission model to forecast the total TB population, the observed parameters of TB natural history from representative studies and estimated model parameters with probability distribution to generate the
likelihood performed by MC simulation are summarized in Table 2. The forecasting results showed a downward fluctuation trend in the monthly total TB population of 2015–2030, from a population of 321±39.5 to 64±7.2, varying by 80% in size across the period (Fig. 3B). The seasonal TB transmission projection showed that the seasonal peak of TB infection was during the summer and at the end of autumn.

Environmental releases of anti-TB drugs have substantially increased due to the epidemic use of anti-TB drugs. The projection of the release of anti-TB drugs indicates that the environmentally relevant concentration decreased from 2020 to 2030 (Fig. 3C). That is, the median concentrations of INH decreased from 6.82 ng L$^{-1}$ (95%CI: 6.00 – 7.70 ng L$^{-1}$) to 1.71 ng L$^{-1}$ (1.24 – 2.36 ng L$^{-1}$). RMP decreased from 29.46 ng L$^{-1}$ (27.14 – 31.99 ng L$^{-1}$) to 7.36 ng L$^{-1}$ (5.44 – 10.04 ng L$^{-1}$). EMB decreased from 90.11 ng L$^{-1}$ (83.00 – 97.85 ng L$^{-1}$) to 22.51 ng L$^{-1}$ (16.63 – 30.72 ng L$^{-1}$). The EMB had the highest residues in the environmental releases, whereas the INH had the lowest.

3.3 Effluent toxicity and ecotoxicological risk

In acute toxicity tests with effluents of WWTPs, not only did the effluent of WWTP-B and its receiving water not cause immobilization, but neither did the diluted concentrations of WWTPs-A (Table S7). By contrast, the effluent of WWTP-A had a significant impact ($p < 0.001$) that caused 100% immobilization. The effluent of WWTP-A seems not to have been caused by anti-TB drugs; the effluent level from both WWTPs was ng L$^{-1}$, especially the INH of WWTP-B (513.62 ng L$^{-1}$), which was much higher than that of WWTP-A (lower than the limit of detection, LOD). Table S6 shows the water quality in influent and effluent from WWTPs and receiving waters. We observed high COD and low DO in the effluent of WWTP-A, which is unsuitable...
for *D. magna* living and causes immobilization.

We calculated the PECs from epidemiology-based wastewater monitoring and concerted it into the ecotoxicological risk of anti-TB drugs in individuals and mixtures that would decrease from 2020 to 2030 (Fig. 4), RQs were lower than 1.

Individual anti-TB drugs and anti-TB mixtures presented similar RQ patterns in temporal dynamics, showing no risk concerns. INH caused higher toxicity than other anti-TB drugs, whereas there was the lowest ecotoxicological risk from INH exposure. On the contrary, EMB presented the highest risk, although it had the lowest PNEC, reflecting its high PEC. Moreover, the PEC of EMB highly contributed to mixture risk in the aquatic environments. In comparison with the detected anti-TB drug concentration from WWTPs-A and -B and receiving water, a similar observation was found for EMB risk, while INH and ternary mixture risks of WWTP-B and receiving water were higher than our predictions. Although both detection and prediction risks were low, the contribution of the individual drug might influence the level of mixture toxicity and risk.

### 4. Discussion

#### 4.1 Mixture toxicities of anti-TB drugs

Our results indicated that with the knowledge of the toxic effects in individual and mixtures of anti-TB drugs, anti-TB drug mixtures present antagonistic interactions. INH presented the most significant immobilized toxicity; our results of an estimated 48-h EC50 and LC50 of INH were similar to the bioassay and the OSAR estimated for *Daphnia* (Rodrigues-Silva et al., 2022; Sanderson and Thomsen, 2009).

Fu et al. (2017) obtained RMP EC50 on a yield inhibition of 25.43 mg L\(^{-1}\) and growth inhibition of 96.69 mg L\(^{-1}\) in algae; our estimated value was also within this range.
Our estimation of the EC50 value and NOEC of EMB in aquatic organisms was tested for the first time, which showed a less immobilized toxicity than other anti-TB drugs. Both the toxic units and combination index revealed that the binary and ternary mixtures of anti-TB drugs were less harmful than individual drugs alone (antagonism). INH and EMP are notable for inducing neurotoxic action, specifically peripheral neuropathy (Badrinath and John, 2022; Sjoerdsma et al., 1996; Prasad et al., 2008), and increasing oxidative stress (Jia et al., 2019; Ni et al., 2020; Kinoshita et al., 2012). The oxidant potential of INH and EMB binds to the active sites of superoxide dismutase (SOD) and may contribute to optic nerve or retina damage (Şahin et al., 2013). INH and EMB seem to have a similar mode of action for neurotoxicity. The mixture of INH+EMB inhibited antioxidant defense by the total antioxidant capacity, and SOD and increased oxidative stresses; however, Uzar et al. (2014) merely observed a significantly increased malondialdehyde in the brains and sciatic nerve of rats compared to INH. Though the mixture of INH and EMB suppressed the protective effect on neurotoxicity and ocular toxicity (Şahin et al., 2013; Uzar et al., 2014), the additive effect was not observed. RMP diminished the hydroxyl radical and against oxidative stress to neurotoxicity (Tomiyama et al., 1996; Kilic et al., 2004; Oida et al., 2006), opposite to INH and EMB in the mode of action. The lower mixture toxicities of the combined RMP with INH or EMB were elucidated via Daphnia immobilization. On the other hand, there are few inconsistent studies on the mixture of INH and RMP to hepatotoxicity. RMP eased INH-induced cytochrome P450s (CYPs) and free radical generation in rats (Yue et al., 2009; Shen et al., 2008), whereas others found RMP synergistically increased the hepatotoxicity of INH through the induction of CYPs and down-regulating sodium taurocholate cotransporting polypeptide and bile salt...
export pump (Skakum and Shman’ko, 1985; Guo et al., 2015). Shen et al. (2008) observed RMP increased hepatotoxicity in human hepatocytes but not in rat hepatocytes, indicating that the inconsistent mixture toxicity of INH and RMP may attribute to species differences. In addition to INH+RMP+EMB, the CI value showed that its mixture toxicity was antagonistic but tended to be additive because the RMP could not effectively protect Daphnia against the mixture of INH- and EMB-induced cellular oxidative stress at high concentration levels.

4.2 Fluctuation trend in tuberculosis incidence-associated PEC

According to TB epidemiology-based wastewater monitoring, the PECs had similar levels to the detected effluent concentrations of EMB and RMP from WWTPs and INH from WWTP-A, other than the INH concentration from WWTP-B, showing reasonable predictions. Mhuka et al. (2020) showed that INH had a high detection frequency from WWTP effluent in South Africa, with a mean concentration of 12.62 ng L$^{-1}$ (max 27.77 ng L$^{-1}$), which was similar to our forecasted and detected results from WWTP-A.

Our predicted environmentally relevant anti-TB drug concentrations had a downward trend in annual fluctuations. The fluctuating trends of the PEC of anti-TB drugs were highly dependent on TB incidence. Since the TB incidence rate in the Kaohsiung season fluctuates, the seasonal regression showed TB activity in Kaohsiung peaks in summer, similar to the national level of Taiwan, Japan, Korea, and Hong Kong (Lin and Liao, 2014; Manabe et al., 2019; Kim and Bae, 2018; You et al., 2016). On the contrary, China, Israel, South Africa, and the US peak at the beginning of spring (Wubuli et al., 2017; Margalit et al., 2016; Andrews et al., 2020; Willis et al., 2012). However, the seasonal variation of TB was associated with
temperature, sunshine hours, human vitamin D level, latitudes, and aging (Thorpe et al., 2004; Koh et al., 2013; Narula et al., 2015; MacLachlan et al., 2012; Bars et al., 2014; Lau et al., 2021). Vitamin D deficiency and aging populations are more susceptible to TB, and limited sunlight leads to vitamin D deficiency; South Africa had the highest TB incidence during the nadir of sunshine hours (Visser et al., 2013). This supports a possible geographical explanation for seasonal TB variation. Across these studies, higher TB incidence occurs in spring or summer; there is strong evidence that TB transmission exists seasonally across different regions throughout the world.

We forecasted the trends of PEC corresponding to the TB population in Kaohsiung would show a fast downward trend from 2015 to 2030. The WHO proposed the End TB strategy to reduce the global TB epidemic by 2035; the annual TB incidence pattern has since decreased in India, China, Taiwan, and even globally (Dhamnetiya et al., 2023; Liu et al., 2019; Wu et al., 2023; GBD Tuberculosis Collaborators, 2018). Adolescents and young adults presented a rapid decline in TB incidence compared to the elderly in Taiwan (Chen et al., 2019). While aging is a global issue in human populations, aging with low immune function increases TB incidence (Dhamnetiya et al., 2021; Cui et al., 2020), which is an area of future concern. Considering the patterns of contact among different age strata, the TB transmission model could be used to understand the impacts of aging on the TB transmission dynamics and environmental burden (Arregui et al., 2018). We did not consider the aging trend in this proposed seasonal SLI model, possibly underestimating the TB population and drug residues in water environments in the subsequent decades. Although we introduced all improvements into the model, there was no exemption from the limitation of the model parameters and data sources to
affect the uncertainty of the forecasted results. Importantly, updating significant
model parameters and data to calibrate the developed model leads to accurate
predictions of realistic environmental situations (Dowdy et al., 2013; Arregui et al.,
2018).

4.3 Ecological risk of antimicrobial mixtures

The predicted environmentally relevant anti-TB drug concentrations to
ecotoxicological risks showed that there was different highest contributor of
individual anti-TB drug to the mixture risk comparing to the field detected results.
Our forecasting showed EMB was the dominant anti-TB contributor in the water
environment for the anti-TB drug mixture ecotoxicological risk. The daily dosage and
fraction of urine excretion in ENB were higher than INH and RMP (WHO, 2002;
Sweetman, 2007; www.drugbank.ca/drugs/DB00951;
www.drugbank.ca/drugs/DB01045), showing possible high levels of residue released
into the aquatic environment. However, the detected INH concentration highly
contributed to the mixture risk since the INH concentration from WWTP-B was a
hundred-fold more than our forecasted concentration and WWTP-A. For cases with
high levels of INH residue in the WWTP-A, the PEC from epidemiology-based
monitoring must validate the field data to assess mixture ecotoxicological risk
accurately.

Although the estimated ecotoxicological risks of anti-TB drugs are relatively
low, anti-TB drug residues in water environments comprise a potential reservoir for
developing antimicrobial resistance genes (Martinez, 2008). The anti-TB drug-
resistance genes associated with INH, RMP, and EMB were detected in WWTP
effluents and surface water (Mtetwa et al., 2021; Su et al., 2020). The water
environment with high antimicrobial residues proliferated antimicrobial resistance
genes and bacteria. At the same time, long-term exposure of bacteria to low levels of
antimicrobial residues can also widely proliferate and spread antimicrobial resistance
(Gullberg et al., 2011). Otherwise, the antimicrobial selective pressure can accelerate
the evolution of antimicrobial resistance bacteria. Bengtsson-Palme and Karssib
(2016) estimated PNEC for resistance selection, with INH of 125 ng L⁻¹, RMP of 64
ng L⁻¹, and EMB of 2,000 ng L⁻¹. The antimicrobial resistance risk might be neglected
according to forecasted environmentally relevant anti-TB drug concentrations.
However, the detected INH level in WWTP-B effluent presents high potential for
leading to antimicrobial resistance. At this point, wastewater monitoring for anti-TB
drug residues and regulation for assessing antimicrobial resistance in water
environments are needed to prevent the environmental risks of anti-TB drug mixtures.

5. Conclusion

We here present the first study to test the mixture toxicity of anti-TB drugs, and
the estimates predicted no effect concentrations in aquatic organisms, indicating
antagonistic interactions in anti-TB drug mixtures. We highlight that the ternary
mixture toxicity of anti-TB drugs displayed additive interactions at higher effect
levels. Epidemiology-based wastewater monitoring and risk assessment demonstrated
that the RQs of anti-TB drug mixtures were less than the threshold value of 1 in
WWTPs and receiving water in Taiwan, indicating that mixture toxicity risk was
negligible for aquatic organisms. Generally, our study shows crucial progress in the
establishment of epidemiology-based wastewater monitoring to predict the
environmental residues of anti-TB drugs, resolving the limitation of exposure
assessment on the lack of real-time tracking of on-site pharmaceuticals in water. The
evidence establishment of anti-TB drug mixtures’ interactions and epidemiological-based monitoring support a systematic mixture risk assessment, resolving the absence of the mixture toxicity information for chemical mixture risk assessment in aquatic environments.

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