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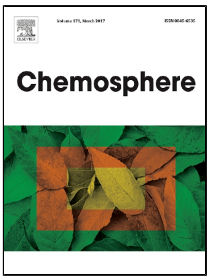

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Applying mixture toxicity modelling to predict bacterial bioluminescence inhibition by non-specifically acting pharmaceuticals and specifically acting antibiotics

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Abstract

Pharmaceuticals and antibiotics co-occur in the aquatic environment but mixture studies to date have mainly focused on pharmaceuticals alone or antibiotics alone, although differences in mode of action may lead to different effects in mixtures. In this study we used the Bacterial Luminescence Toxicity Screen (BLT-Screen) after acute (0.5 h) and chronic (16 h) exposure to evaluate how non-specifically acting pharmaceuticals and specifically acting antibiotics act together in mixtures. Three models were applied to predict mixture toxicity including concentration addition, independent action and the two-step prediction (TSP) model, which groups similarly acting chemicals together using concentration addition, followed by independent action to combine the two groups. All non-antibiotic pharmaceuticals had similar EC_{50} values at both 0.5 and 16 h, indicating together with a QSAR (Quantitative Structure-Activity Relationship) analysis that they act as baseline toxicants. In contrast, the antibiotics' EC_{50} values decreased by up to three orders of magnitude after 16 h, which can be explained by their specific effect on bacteria. Equipotent mixtures of non-antibiotic pharmaceuticals only, antibiotics only and both non-antibiotic pharmaceuticals and antibiotics were prepared based on the single chemical results. The mixture toxicity models were all in close agreement with the experimental results, with predicted EC_{50} values within a factor of two of the experimental results. This suggests that concentration addition can be applied to bacterial assays to model the mixture effects of environmental samples containing both specifically and non-specifically acting chemicals.

Keywords: antibiotics; bacterial toxicity; concentration addition; independent action; pharmaceuticals

1. Introduction

Bacterial assays based on bioluminescence inhibition are widely applied tools to evaluate the effect of individual chemicals, chemical mixtures and environmental samples (Altenburger et al., 2000; Katsoyiannis and Samara, 2007; Escher et al., 2008; Tang et al., 2013). The advantages of such assays include ease of use, sensitivity and speed, with sample exposure times typically between 15 to 30 minutes (Parvez et al., 2006). Further, Kaiser (1998) found a good correlation between a range of aquatic *in vivo* endpoints and observed effect in marine bacteria *Aliivibrio fischeri*, more commonly referred to as the Microtox assay. While acute bacterial assays are suitable for non-specifically acting compounds, their applicability to specifically acting chemicals, such as antibiotics, is questionable, with several studies finding low or no effect in the Microtox assay after exposure to different classes of antibiotics (Isidori et al., 2005; van der Grinten et al., 2010). Antibiotics can have specific effects on different bacterial species, including inhibition of protein synthesis and inhibition of DNA synthesis (Kohanski et al., 2010), thus the typical short exposure times are not sufficient to account for the specific effects of antibiotics. Consequently, previous studies have shown that antibiotic effects in bacterial assays can increase by several orders of magnitude with longer exposure periods (Thomulka et al., 1993; Backhaus et al., 1997; Froehner et al., 2000; Zou et al., 2012).

While there are increasing concerns about the presence of antibiotics in the aquatic environment (Kummerer, 2009), environmental waters can contain a wide range of chemicals, including pharmaceuticals, pesticides and industrial compounds (Loos et al., 2013; Neale et al., 2015). Consequently, it is important to consider the potential mixture effects that can occur between chemicals. Mixtures that contain chemicals that share a common mode of action in a particular organism can be predicted using the concentration addition model, while chemicals that act according to different modes of action can be described by independent action (Backhaus and Faust, 2012). While environmental samples typically contain a large number of chemicals with diverse modes of action, the concentration addition model is considered to be suitable for hazard assessment as it provides a worst-case prediction of mixture toxicity in most cases (Backhaus et al., 2000). An alternative approach is the two-step prediction (TSP) model proposed by Junghans (2004), which groups similarly acting compounds together using the concentration addition model and then applies independent action to combine the predicted effects of the individual groups. This has previously been applied to successfully predict mixture toxicity in *Daphnia magna* (Ra et al., 2006) and algal assays (Tang and Escher, 2014).

In this study the Bacterial Luminescence Toxicity Screen (BLT-Screen) using bioluminescent bacteria *Photobacterium leiognathi* was applied to single compounds and mixtures containing both non-specifically acting pharmaceuticals, referred to as non-antibiotic pharmaceuticals, and specifically acting antibiotics after both acute and chronic exposure. Backhaus et al. (1997) showed that the ratio of acute to chronic effects in bioluminescent bacteria can provide information about the mode of action of studied chemicals, with an increased acute to chronic ratio observed for specifically acting chemicals. In the current study 0.5 h was used for acute exposure as this is the typical sample exposure period in the assay (van de Merwe and Leusch, 2015), while 16 h was used for chronic exposure. The studied compounds have all been detected previously in wastewater and surface water in the ng/L to µg/L concentration range (Kolpin et al., 2002; Watkinson et al., 2009; Hughes et al., 2013; Loos et al., 2013). Effect concentrations inhibiting 50% of the bioluminescence output (EC_{50}) were determined at both 0.5 and 16 h for individual compounds, with equipotent mixtures of non-antibiotic pharmaceuticals only, antibiotics only and both non-antibiotic pharmaceuticals and antibiotics prepared based on the ratio of the experimental EC_{50} values. The observed effects were compared with predicted effects based on the concentration addition, independent action and TSP models.

2. Materials and methods

2.1 Chemicals

Five non-antibiotic pharmaceuticals, carbamazepine, diclofenac, fluoxetine, gemfibrozil and naproxen, and five antibiotics, doxycycline, monensin, sulfamethizole, sulfamethoxazole and tetracycline, were selected for this study. Properties of the studied chemicals are provided in Table 1. Pentachlorophenol (PCP) was used as the positive reference compound for the BLT-Screen. PCP is an weak acid uncoupler and exhibits a specific effect in the assay (Schultz and Cronin, 1997). Individual chemical stocks and chemical mixtures were prepared in HPLC grade methanol. All chemicals were purchased from Sigma Aldrich (Castle Hill, Australia) or Novachem Pty Ltd (Collingwood, Australia).

2.2 Bioanalysis

The BLT-Screen was run according to van de Merwe and Leusch (2015), with some modifications. Briefly, the chemical stocks, along with positive control PCP and solvent control methanol, were added to white 96 well plates and serially diluted in phosphate buffer (pH 4) using either a 1:3 or 1:5 dilution series, with a final volume of 200 µL. The final concentration of methanol in the assay did not exceed 0.8% (v/v). While many bacterial assays are conducted at pH 7, the BLT-Screen was run at pH 4 to increase assay sensitivity to organic contaminants (van de Merwe and Leusch, 2015).

Following the serial dilution, a cryopreserved *Photobacterium leiognathi* stock was thawed and diluted 1:6 in growth medium, with 5 µL of growth medium added to each well. Luminescence was measured at 0.5 and 16 h using a Fluostar Omega plate reader (BMG Labtech, Ortenberg, Germany). Between readings, the plates were stored at 22°C and gently shaken at 90 RPM. Percent luminescence inhibition was calculated using Equation 1 based on sample luminescence, provided in relative light units (RLU), (RLU_{sample}) and the average luminescence of the solvent control ($RLU_{control}$). All samples were run in duplicate on the same plate, with each sample run independently two to three times. $RLU_{control}$ was reasonably stable between experiments (coefficient of variance <20%), with $RLU_{control}$ typically decreasing by approximately 35% from 0.5 to 16 h. The effect concentration causing 50% effect (EC_{50}) at 0.5 and 16 h was calculated using log-logistic concentration-effect curves (Equation 2), where EC_i is the concentration at a defined percent effect y. The slope was fitted from the experimental data.

$$\% \text{ Inhibition} = \left(1 - \frac{RLU_{sample}}{RLU_{control}}\right) \cdot 100\% \quad (1)$$

$$\% \text{ effect (y)} = \frac{1}{1 + 10^{\text{slope} \cdot (\log EC_{50} - \log EC_i)}} \quad (2)$$

2.3 Mixture toxicity modelling

Using the experimental EC_{50} values for the individual chemicals, equipotent mixtures were prepared for non-antibiotic pharmaceuticals only (5 components), antibiotics only (5 components) and all chemicals (10 components) for both 0.5 and 16 h time points. All components contribute equally to the mixture effect in equipotent mixtures and the fraction of each chemical included in the mixtures is provided in Table 2. The experimental results were compared with concentration addition, independent action and TSP mixture toxicity predictions. EC_{50} based on the concentration addition predictions ($EC_{50,CA}$) was calculated using Equation 3, where p_i is the fraction of component i (i = 1 to n) in the mixture and $EC_{y,i}$ is the EC_y of component i at any effect level y. As standard error is symmetrical on a log scale, Equation 3 was expanded to Equation 4 to calculate log $EC_{y,CA}$. The effect predicted based on independent action (E_{IA}) was determined using Equation 5, where E_i is the effect of component i in the mixture.

$$EC_{y,CA} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{EC_{y,i}}} \quad (3)$$

$$\log EC_{y,CA} = \log\left(\left(\frac{p_1}{EC_{y,1}}\right) + \left(\frac{p_2}{EC_{y,2}}\right) + \left(\frac{p_3}{EC_{y,3}}\right) + \ldots\right) \quad (4)$$

$$E_{IA} = 1 - \prod_{i=1}^{n}(1 - E_i) \quad (5)$$

The variability associated with the mixture toxicity model predictions was estimated using error propagation. For concentration addition, Equation 2 was rearranged to give Equation 6, with the error associated with log $EC_{y,i}$, $\sigma \log EC_{y,i}$, calculated for each component i using Equation 7.

$$\log EC_{y,i} = \log EC_{50,i} - \frac{1}{slope} \cdot \log\left(\frac{1 - y}{y}\right) \quad (6)$$

$$\sigma \log EC_{y,i} = \sqrt{\sigma \log EC_{50,i}^{2} + \left(\log \frac{1 - y}{y}\right)^2 \cdot \left(\frac{\sigma slope}{slope^2}\right)^4} \quad (7)$$

The error associated with the concentration addition prediction, $\sigma \log EC_{y,CA}$, was estimated using Equation 8, with $\frac{\sigma \log EC_{y,CA}}{\sigma \log EC_{y,i}}$ calculated using Equation 9.

$$\sigma \log EC_{y,CA} = \sqrt{\sum_{i=1}^{n} \frac{\sigma \log EC_{y,CA}^{2}}{\sigma \log EC_{y,i}} \cdot \sigma \log EC_{y,i}^{2}} \quad (8)$$

$$\frac{\sigma \log EC_{y,CA}}{\sigma \log EC_{y,i}} = \frac{p_1 \cdot 10^{-\log EC_{y,1}}}{p_1 \cdot 10^{-\log EC_{y,1}} + p_2 \cdot 10^{-\log EC_{y,2}} + p_3 \cdot 10^{-\log EC_{y,3}} + \ldots} \quad (9)$$

For independent action, the error associated with $effect_i$, $\sigma effect_i$ was calculated for each component i using Equation 10. The required parameters for Equation 10 were calculated using Equations 11 to 13, where C is the concentration and ln is the natural logarithm.

$$\sigma E_i = \left(\frac{\sigma E_i}{\sigma slope_i}\right)^2 \cdot \sigma slope_i^{\,2} + \left(\frac{\sigma E_i}{\sigma logEC_{50,i}}\right)^2 \cdot \sigma logEC_{50,i}^{\,2} + \left(\frac{\sigma E_i}{\sigma logC}\right)^2 \cdot \sigma logC^2 \quad (10)$$

$$\frac{\sigma E_i}{\sigma slope_i} = \frac{10^{slope \cdot (logEC_{50,i} - logC)} \cdot (logEC_{50,i} - logC) \cdot ln10}{\left(1 + 10^{slope \cdot (logEC_{50,i} - logC)}\right)^2} \quad (11)$$

$$\frac{\sigma E_i}{\sigma logEC_{50,i}} = \frac{10^{slope \cdot (logEC_{50,i} - logC)} \cdot slope \cdot ln10}{\left(1 + 10^{slope \cdot (logEC_{50,i} - logC)}\right)^2} \quad (12)$$

$$\frac{\sigma E_i}{\sigma logC} = \frac{10^{slope \cdot (logEC_{50,i} - logC)} \cdot slope \cdot ln10}{\left(1 + 10^{slope \cdot (logEC_{50,i} - logC)}\right)^2} \quad (13)$$

The error associated with the independent action predicted effect, σE_{IA}, was calculated with Equation 14.

$$\sigma E_{IA} = \left((1 - E_{i,2}) \cdot (1 - E_{i,3}) \cdot \sigma E_{i,1}\right)^2 + \left((1 - E_{i,1}) \cdot (1 - E_{i,3}) \cdot \sigma E_{i,2}\right)^2 + \left((1 - E_{i,1}) \cdot (1 - E_{i,2}) \cdot \sigma E_{i,3}\right)^2 \quad (14)$$

The CA prediction was calculated for a range of effect levels y to draw a complete concentration-effect curve for CA, while the IA prediction was calculated for a range of concentrations C to draw a complete concentration-effect curve for IA. For the ten-component mixtures, the TSP model was also applied by predicting concentration addition for the non-antibiotic pharmaceutical group and antibiotics group separately and then combining the two groups together using the independent action model.

3. Results and discussion

3.1 Individual chemicals

The EC_{50} values at 0.5 and 16 h are provided for all individual chemicals in Table 3, with the full concentration-effect curves shown in Figure 1. There was little change in EC_{50} over time for the studied non-antibiotic pharmaceuticals, with the ratio of EC_{50} at 0.5 h to EC_{50} at 16 h ranging from 1.0 to 4.4 (Table 3). This is also shown in Figure 2, where the difference in log EC_{50} for all non-antibiotic pharmaceuticals at 0.5 and 16 h falls within 0.5 log units. As the effect remained relatively constant over time, this indicates that the studied non-antibiotic pharmaceuticals were baseline toxicants and did not exhibit a specific effect on *Photobacterium leiognathi*. In contrast, the effect of the antibiotics increased with time, with the EC_{50} values decreasing by up to three orders of magnitude (Table 3). The ratio of EC_{50} at 0.5 h to EC_{50} at 16 h ranged from 7.2 to 995. The two most potent antibiotics, doxycycline and tetracycline, inhibit protein synthesis, and previous studies also found that this class of antibiotics were among the most toxic to *Aliivibrio fischeri* (Backhaus and Grimme, 1999). In contrast, the sulphonamides, sulfamethoxazole and sulfamethizole, which are inhibitors of folic acid, had the lowest decrease in EC_{50} values over time. This has been observed previously (Zou et al., 2012) and it has been hypothesised that the presence of bacteria growth media components, such as yeast extract, may be a source of folic acid for the bacteria (Backhaus and Grimme, 1999). Yeast extract is composed of autolyzed *Saccharomyces cerevisiae* cells, with *Saccharomyces cerevisiae* strains previously shown to contain total folate concentrations ranging from 4 to 14.5 mg per 100 g of dried yeast extract (Hjortmo et al., 2005). The growth media contained 3 g/L of yeast extract (van de Merwe and Leusch, 2015), with 5 µL of growth media added to 200 µL sample volume in the BLT-Screen giving a final volume of 205 µL, thus the potential concentration of folate in the assay was 2.9 to 10.6 µg/L.

The results were compared with the baseline toxicity Quantitative Structure-Activity Relationship (QSAR) developed by Tang et al. (2013) for the Microtox assay based on 0.5 h exposure. The QSAR was developed based on the liposome-water partition coefficient (K_{lipw}) of neutral chemicals, but was shown to be applicable to ionisable organic chemicals when K_{lipw} was replaced with the speciation-corrected distribution ratio (D_{lipw}) (Tang et al., 2013). While octanol-water partition coefficients (K_{ow}) are often used for QSARs, previous studies have shown that liposome-water partition coefficients (K_{lipw}) are a suitable descriptor for both polar and nonpolar chemicals (Vaes et al., 1997) and have been successfully applied to predict toxicity using QSAR models (Tang et al., 2013; Klüver et al., 2016). As many of the studied chemicals are charged at pH 4, D_{lipw} was used in the QSAR to account for speciation (Table 1). Therefore, while the QSAR was developed based on

the experimental K_{lipw} of neutral compounds, D_{lipw} could be applied to correct for speciation at pH 4. Figure 3 indicates that the majority of non-antibiotic pharmaceuticals fit well with the QSAR predictions based on the Microtox assay, with the ratio of the QSAR predicted EC_{50} to the experimental EC_{50}, also known as the toxic ratio, less than 10, suggesting that the non-antibiotic pharmaceuticals are baseline toxicants (Tang et al., 2013). The exception was naproxen, which had a toxic ratio from 70 (0.5 h) to 250 (16 h). While a toxic ratio above 10 usually indicates that a compound has a specific mode of action, the acute to chronic ratio for naproxen was 3.6, which suggests that it is having a non-specific effect on *Photobacterium leiognathi*. Thus, the dissimilarity may be related to differences in sensitivity of the applied bacterial strain (*Aliivibrio fischeri* compared to *Photobacterium leiognathi*). van de Merwe and Leusch (2015) also found a similar EC_{50} value for naproxen in the BLT-Screen (Figure 3). In contrast, all antibiotics at both 0.5 and 16 h deviated significantly from the baseline toxicity QSAR, which was also observed by Tang et al. (2013).

3.2 Mixture toxicity modelling

Experimental EC_{50} values for the separate equipotent non-antibiotic pharmaceutical and antibiotics mixtures were compared with EC_{50} values predicted using concentration addition and independent action (Table 4). Concentration addition was more conservative than independent action for the non-antibiotic pharmaceutical-only mixtures at both 0.5 and 16 h, with the concentration addition predicted concentration-effect curves agreeing well with the experimental results (Figure 4A-B). However, the independent action EC_{50} predictions were only a factor of two higher than the experimental EC_{50} values. For the antibiotics, independent action yielded a lower EC_{50} at 0.5 h (Figure 4C), while both concentration addition and independent action provided close agreement with the experimental EC_{50} value at 16 h (Figure 4D). The suitability of the independent action model at 16 h may be due to the fact that the different antibiotics have different modes of action on bacteria, such protein synthesis inhibition and folic acid inhibition, which is not apparent after only 0.5 h exposure (Froehner et al., 2000). Independent action was also shown to be the most applicable model to predict mixture toxicity of dissimilarly acting compounds using *Aliivibrio fischeri* (Backhaus et al., 2000). However, concentration addition was overall the most suitable model for the five-component mixtures, with predictions within a factor of 1.1 to 1.4 of the experimental results, meaning it is suitable for use in the TSP model.

The effect of the ten-component mixture containing both non-antibiotic pharmaceuticals and antibiotics was predicted using concentration addition, independent action and the TSP model (Table 4, Figure 4E-F). For the TSP model, the concentration addition predictions of the five-

component mixtures of either non-antibiotic pharmaceuticals or antibiotics were integrated using independent action, with p_i representing the fraction of each group in the ten-component mixture. This was possible as the ratio of similarly acting components within each group remained the same in both the five and ten-component mixtures. All three models gave a good agreement with the experimental data, with all predictions within a factor of two of the experimental EC_{50} values. The TSP model tended to fall between the concentration addition and independent action predictions, indicating that the TSP model is an appropriate model to predict the effect of specific and non-specific chemicals in bioluminescent bacterial assays. However, given the similarity of all predictions, this still supports the application of concentration addition as a suitable model for compounds whose mode of action is unknown, given it tends to be more conservative, particularly at the chronic 16 h exposure. This suggests that the bioanalytical equivalent approach, which assumes that chemicals act according to concentration addition (Neale et al., 2015), can be applied to predict the mixture toxicity of environmental samples containing both antibiotics and non-specifically acting chemicals in bacterial assays.

4. Conclusions

In this study the effect of two groups of commonly detected water pollutants, pharmaceuticals and antibiotics, were assessed in the BLT-Screen after acute (0.5 h) and chronic (16 h) exposure periods, with equipotent mixtures prepared. Despite using a different bacterial species, the EC_{50} values for most non-antibiotic pharmaceuticals fit well with previously published baseline toxicity QSAR predictions; however, the QSAR was clearly unsuitable for specifically acting antibiotics, with the observed effect two to seven orders of magnitude higher than predicted. Thus, while the bioluminescence inhibition of bacteria is widely applied to test the baseline toxicity of chemical and environmental mixtures (Altenburger et al., 2000; Tang et al., 2013; Di Nica et al., 2016; Vethaak et al., 2016), antibiotics appear to be outliers. The applied mixture toxicity models of concentration addition, independent action and TSP all gave a good agreement with the experimental data. While independent action and TSP may have given closer predictions, concentration addition is still recommended for mixture toxicity modelling of dissimilarly acting compounds. Therefore, it is still possible to apply this assay to environmental mixtures, such as water samples, even if they contain substantial fractions of antibiotics because the overall mixture effect can be satisfactorily modelled by concentration addition.

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420

421 **Table 1**: Selected properties of the studied non-antibiotic pharmaceuticals and antibiotics.

Chemical	CAS No	Molecular weight (g/mol)	Methanol stock solution (M)	Log K_{ow}[a]	Log K_{lipw}[b]	Log D_{lipw} (pH 4)
Carbamazepine	298-46-4	236.27	0.10	2.45	2.57	2.57[c]
Diclofenac	15307-86-5	318.13	0.10	4.51	4.67	4.45[d]
Fluoxetine	56296-78-7	345.79	0.05	4.05	4.20	3.84[e*]
Gemfibrozil	25812-30-0	250.33	0.10	4.77	4.94	4.83[c]
Naproxen	22204-53-1	230.26	0.01	3.18	3.31	3.21[c]
Doxycycline	24390-14-5	512.94	0.01	-0.02	0.05	0.01[c]
Monensin	22373-78-0	692.85	0.01	1.62	1.72	1.56[c]
Sulfamethizole	144-82-1	270.33	0.05	0.54	0.62	0.62[c]
Sulfamethoxazole	723-46-6	253.28	0.01	0.89	0.98	0.98[c]
Tetracycline	64-75-5	482.92	0.01	-1.30	-1.26	-1.33[c]

422 [a]US EPA (2008); [b]Calculated based on Vaes et al. (1997); [c]Calculated based onEscher et al. (2011); [d]Avdeef
423 et al. (1998); [e]Neuwoehner et al. (2009)

424 *D_{lipw} calculated at pH 7, but fluoxetine speciation is fully charged at pH 4 and 7.

425 **Table 2**: The fraction of each chemical used in the different equipotent mixtures.

	Non-antibiotic pharmaceutical t=0.5	**Non-antibiotic pharmaceutical t=16**	**Antibiotic t=0.5**	**Antibiotic t=16**	**All t=0.5**	**All t=16**
Carbamazepine	48.6%	66.1%			38.5%	64.6%
Diclofenac	2.2%	2.1%			1.8%	2.0%
Fluoxetine	35.7%	27.2%			28.5%	26.5%
Gemfibrozil	12.8%	4.4%			10.2%	4.2%
Naproxen	0.6%	0.3%			0.5%	0.3%
Doxycycline			10.1%	0.1%	2.1%	0.003%
Monensin			8.5%	4.4%	1.8%	0.1%
Sulfamethizole			26.3%	55.8%	5.4%	1.3%
Sulfamethoxazole			41.8%	39.3%	8.6%	0.9%
Tetracycline			13.4%	0.4%	2.7%	0.01%

426

427 **Table 3**: Experimental log EC_{50} (M) and slope of individual chemicals at 0.5 and 16 h ± standard error (SE), with the 0.5 h to 16 h ratio.

	Chemicals	t=0.5		t=16		t=0.5/t=16 ratio	Classification
		log EC_{50} ± SE	*slope ± SE*	*log EC_{50} ± SE*	*slope ± SE*		
Non-antibiotic pharmaceuticals	Carbamazepine	-3.68 ± 0.02	0.97 ± 0.05	-3.67 ± 0.05	1.82 ± 0.30	0.99	Baseline toxicant
	Diclofenac	-4.92 ± 0.01	6.35 ± 0.59	-5.24 ± 0.08	6.00 ± 0.60*	2.11	Baseline toxicant
	Fluoxetine	-3.71 ± 0.02	1.84 ± 0.14	-4.02 ± 0.03	2.54 ± 0.52	2.04	Baseline toxicant
	Gemfibrozil	-4.16 ± 0.01	2.64 ± 0.20	-4.81 ± 0.03	2.22 ± 0.20	4.43	Baseline toxicant
	Naproxen	-5.47 ± 0.02	1.49 ± 0.10	-6.03 ± 0.02	1.95 ± 0.15	3.58	Baseline toxicant
Antibiotics	Doxycycline	-4.92 ± 0.10	0.39 ± 0.04	-7.92 ± 0.02	1.42 ± 0.09	995	Specifically acting
	Monensin	-4.93 ± 0.02	1.74 ± 0.15	-6.44 ± 0.05	1.17 ± 0.13	32.6	Specifically acting
	Sulfamethizole	-4.44 ± 0.03	1.33 ± 0.09	-5.29 ± 0.03	2.48 ± 0.35	7.16	Specifically acting
	Sulfamethoxazole	-4.24 ± 0.02	1.35 ± 0.08	-5.46 ± 0.02	1.16 ± 0.06	16.7	Specifically acting
	Tetracycline	-4.83 ± 0.08	0.48 ± 0.05	-7.51 ± 0.02	1.62 ± 0.16	484	Specifically acting

428 *slope fitted to 6

429 **Table 4**: Experimental EC_{50} (M) with 95% confidence intervals for all mixtures compared to
430 concentration addition and independent action predictions.

	Concentration addition (CA) predictions		Independent action (IA) predictions		Experimental		# Components
	log EC_{50}	EC_{50}	log EC_{50}	EC_{50}	log EC_{50}	EC_{50}	
			Non-antibiotic pharmaceuticals				
t=0.5	-3.99 (-4.00 to -3.97)	1.03×10^{-4} (9.91×10^{-5} to 1.07×10^{-4})	-3.76 (-3.78 to -3.74)	1.76×10^{-4} (1.68×10^{-4} to 1.84×10^{-4})	-4.07 (-4.11 to -4.02)	8.55×10^{-5} (7.73×10^{-5} to 9.47×10^{-5})	5
t=16	-4.18 (-4.23 to -4.13)	6.59×10^{-5} (5.91×10^{-5} to 7.35×10^{-5})	-3.82 (-3.90 to -3.74)	1.52×10^{-4} (1.27×10^{-4} to 1.82×10^{-4})	-4.08 (-4.13 to -4.03)	8.29×10^{-5} (7.38×10^{-5} to 9.32×10^{-5})	5
			Antibiotics				
t=0.5	-4.59 (-4.65 to -4.53)	2.56×10^{-5} (2.23×10^{-5} to 2.94×10^{-5})	-4.96 (-5.06 to -5.86)	1.10×10^{-5} (8.73×10^{-6} to 1.38×10^{-5})	-4.54 (-4.61 to -4.47)	2.86×10^{-5} (2.43×10^{-5} to 3.36×10^{-5})	5
t=16	-5.76 (-5.79 to -5.74)	1.72×10^{-6} (1.61×10^{-6} to 1.83×10^{-6})	-5.64 (-5.67 to -5.61)	2.27×10^{-6} (2.12×10^{-6} to 2.43×10^{-6})	-5.62 (-5.71 to -5.53)	2.40×10^{-6} (1.93×10^{-6} to 2.98×10^{-6})	5
			All components				
t=0.5	-4.20 (-4.27 to -4.12)	6.36×10^{-5} (5.38×10^{-5} to 7.51×10^{-5})	-4.40 (-4.51 to -4.28)	4.03×10^{-5} (3.11×10^{-5} to 5.22×10^{-5})	-4.41 (-4.48 to -4.35)	3.87×10^{-5} (3.33×10^{-5} to 4.49×10^{-5})	10
t=16	-4.46 (-4.50 to -4.41)	3.50×10^{-5} (3.19×10^{-5} to 3.85×10^{-5})	-4.11 (-4.16 to -4.05)	7.85×10^{-5} (6.87×10^{-5} to 8.97×10^{-5})	-4.28 (-4.32 to -4.24)	5.29×10^{-5} (4.84×10^{-5} to 5.79×10^{-5})	10

	Two-step prediction (TSP)		Experimental		
	log EC_{50}	EC_{50}	log EC_{50}	EC_{50}	
t=0.5	-4.27 (-4.29 to -4.26)	5.34×10^{-5} (5.16×10^{-5} to 5.52×10^{-5})	-4.41 (-4.48 to -4.35)	3.87×10^{-5} (3.33×10^{-5} to 4.49×10^{-5})	10
t=16	-4.36 (-4.36 to -4.36)	4.40×10^{-5} (4.38×10^{-5} to 4.41×10^{-5})	-4.28 (-4.32 to -4.24)	5.29×10^{-5} (4.84×10^{-5} to 5.79×10^{-5})	10

431 NB: 95% confidence intervals were calculated based on the derived σlogEC from error propagation.

List of Figures

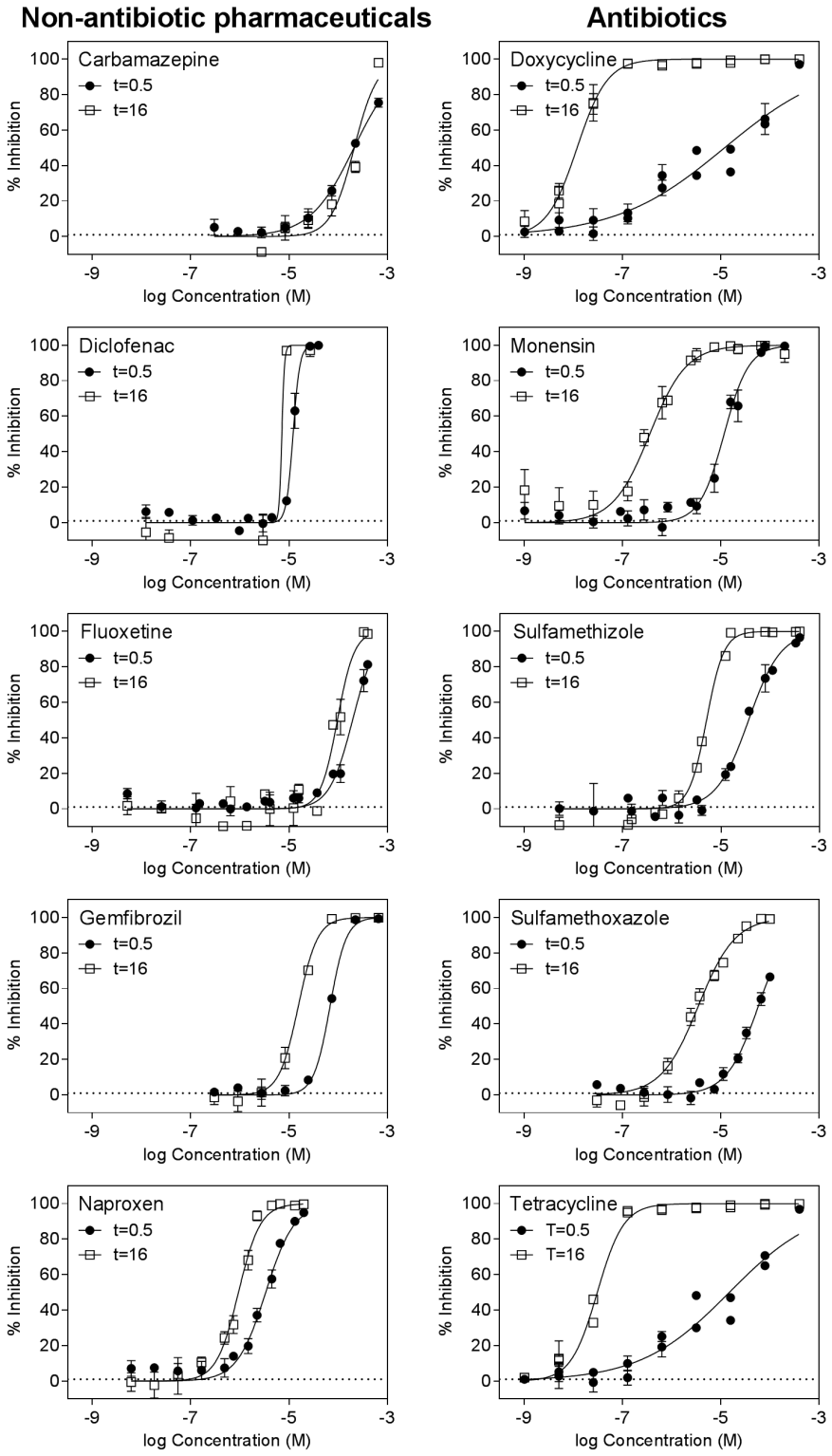
Figure 1: Concentration-effect curves for non-antibiotic pharmaceuticals and antibiotics at 0.5 and 16 h.

Figure 2: log EC_{50} at 0.5 h versus log EC_{50} at 16 h.

Figure 3: Experimental EC_{50} values in the BLT-Screen at 0.5 h (closed black symbols) and 16 h (open black symbols) (pH 4) compared to literature BLT-Screen (0.5 h) EC_{50} values for carbamazepine, gemfibrozil and naproxen (blue closed diamond) (pH 4) (van de Merwe and Leusch, 2015) and literature Microtox EC_{50} values for diclofenac, fluoxetine, gemfibrozil, naproxen, doxycycline, sulfamethoxazole and tetracycline (red closed triangle) (pH 7) (Tang et al., 2013). The experimental EC_{50} values in the present study were also compared to the baseline toxicity QSAR predictions (red line) from Tang et al. (2013), which were corrected for speciation using log D_{lipw} at pH 4.

Figure 4: Concentration addition (CA) and independent action (IA) predictions for the five-component non-antibiotic pharmaceutical mixtures at A) 0.5 h and B) 16 h and the five-component antibiotics mixtures at C) 0.5 h and D) 16 h, with CA, IA and two-step prediction (TSP) for the ten-component non-antibiotic pharmaceutical and antibiotics mixtures at E) 0.5 h and F) 16 h. Experimental results are shown using white squares. Different mixture ratios were used for each time point and are provided in Table 2.

455 **Figure 1**



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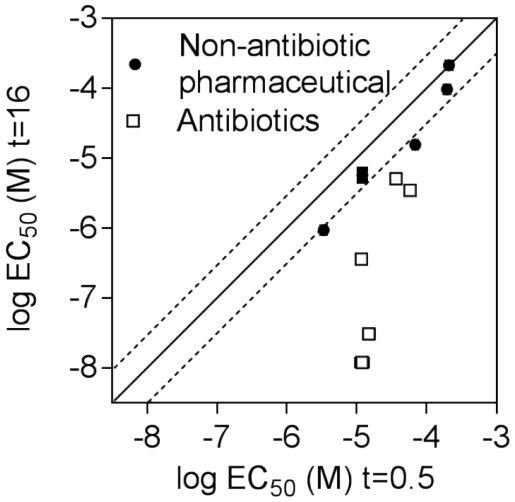
460 **Figure 2**



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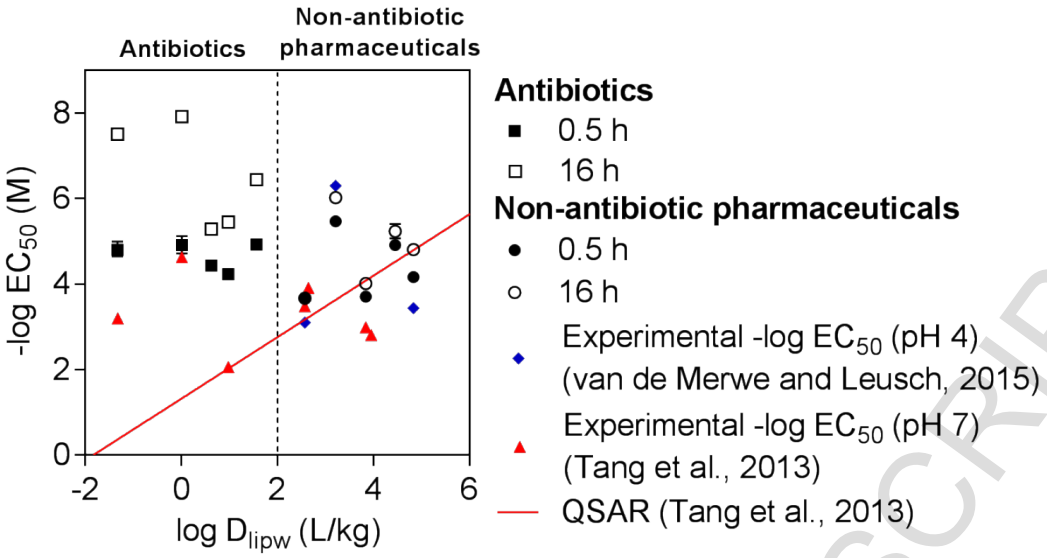
463 **Figure 3**



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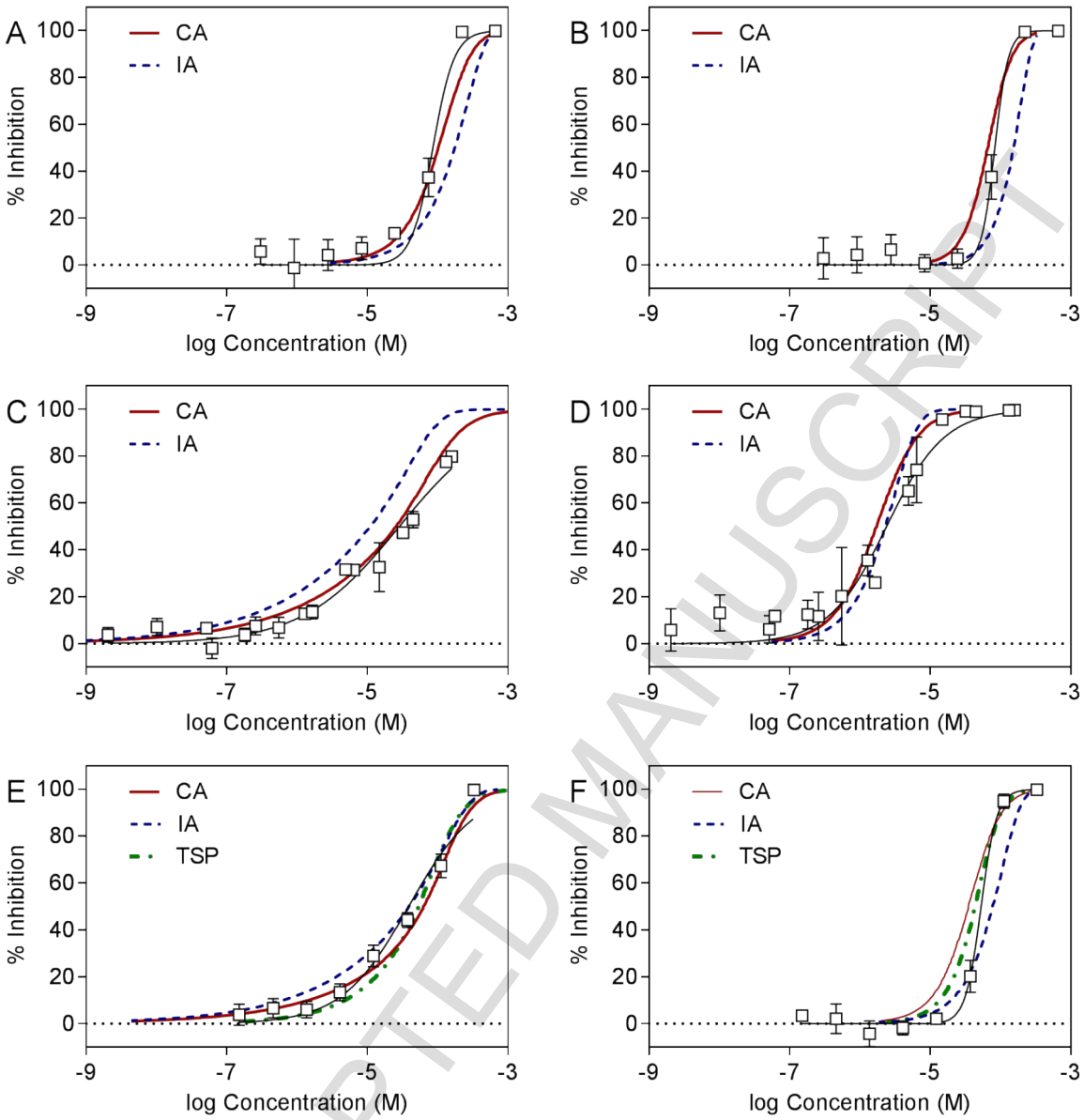
469 **Figure 4**



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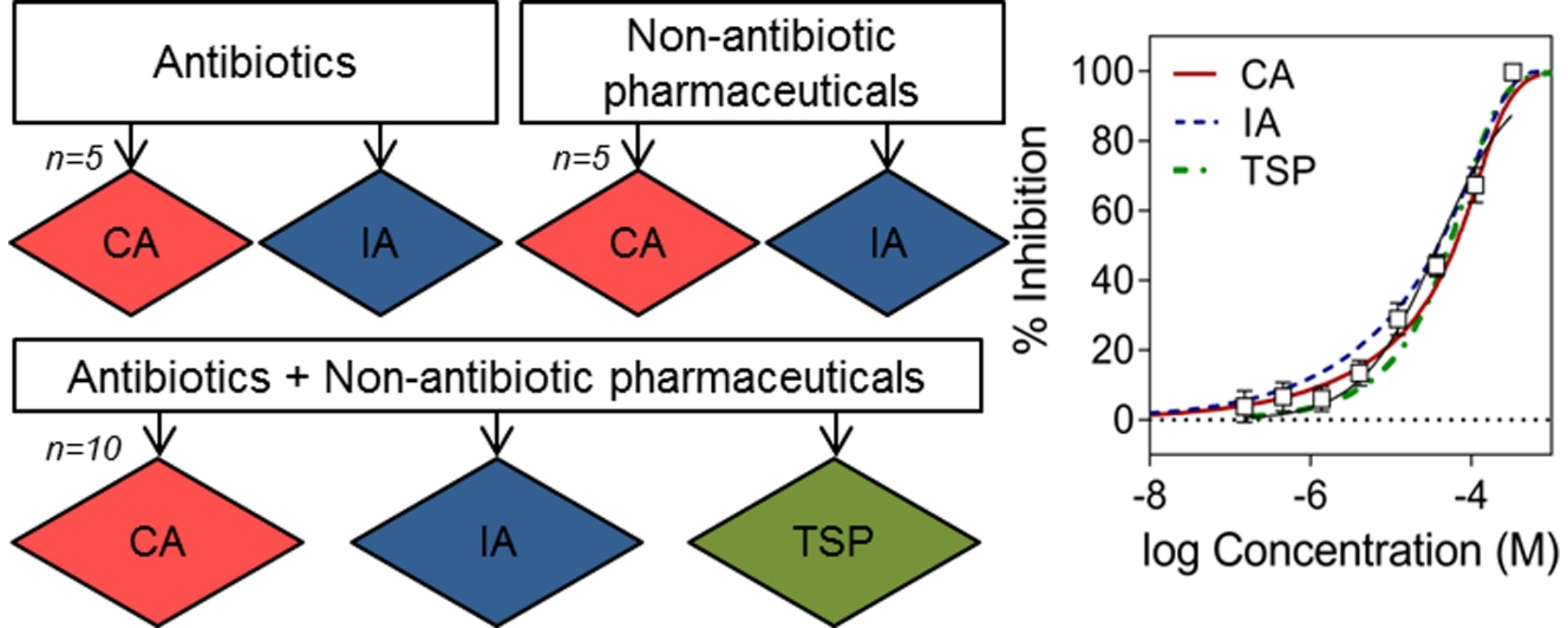
Highlights

- Mixture effects of pharmaceuticals and antibiotics assessed in a bacterial assay
- Acute and chronic exposure considered, with antibiotic effect increasing over time
- Concentration addition, independent action and two-step prediction models applied
- Mixture toxicity modelling in close agreement with experimental results
- CA suitable to model non-specific and specific chemical effects in bacterial assays


Antibiotics
Non-antibiotic pharmaceuticals
n=5
n=5
CA
IA
CA
IA
Antibiotics + Non-antibiotic pharmaceuticals
n=10
CA
IA
TSP
% Inhibition
100
80
60
40
20
0
CA
IA
TSP
-8
-6
-4
log Concentration (M)