Australasian Bulletin of Ecotoxicology and Environmental Chemistry

The Official Bulletin of the Australasian Chapter of the Society of Environmental Toxicology and Chemistry – Asia Pacific

Volume 5

2019



AUSTRALASIAN BULLETIN OF ECOTOXICOLOGY & ENVIRONMENTAL CHEMISTRY

Editor-in-Chief

Dr Reinier Mann, Department of Environment and Science, GPO Box 2454, Brisbane Qld 4000, Australia. email: reinier.mann@qld.gov.au.

Associate Editor

Dr Anne Colville, Centre for Environmental Sustainability, University of Technology, Sydney, Ultimo, NSW 2007, Australia. email: anne.colville@uts.edu.au.

Call for Papers

The Bulletin welcomes Original Research Papers, Short Communications, Review Papers, Commentaries and Letters to the Editors.

Guidelines for Authors

For information on Guidelines for Authors please contact the editors.

AIMS AND SCOPE

The Australasian Bulletin of Ecotoxicology and Environmental Chemistry is a publication of the Australasian Chapter of the Society of Environmental Toxicology and Chemistry – Asia Pacific (a geographic unit of the Society of Environmental Toxicology and Chemistry). It is dedicated to publishing scientifically sound research articles dealing with all aspects of ecotoxicology and environmental chemistry. All data must be generated by statistically and analytically sound research, and all papers will be peer reviewed by at least two reviewers prior to being considered for publication. The Bulletin will give priority to the publication of original research that is undertaken on the systems and organisms of the Australasian and Asia-Pacific region, but papers will be accepted from anywhere in the world. As well as scientific papers, the Bulletin will contain short communications, to allow the publication of original data generated in small-scale projects, and letters to the Editor are most welcome. The Editor will commission and publish reviews from time to time. Authors interested in publishing review articles are invited to contact the Editors. Titles of completed PhD and MSc theses will also be published.

Material published in the Journal represents the opinions of the authors, and not necessarily those of the Editors or the Society.

COPYRIGHT

© Australasian Chapter of the Society of Environmental Toxicology and Chemistry – Asia Pacific

It is the condition of publication that manuscripts submitted to this Journal have not been, or will not be, published elsewhere. Submission of a manuscript implies agreement that the authors transfer the copyright to the publisher if and when the manuscript is accepted for publication (unless the organisation for which the author[s] work does not permit such transfer). Copyright covers the exclusive rights to reproduce or distribute any parts of this journal in any form of reproduction. No part of this publication may be reproduced or utilised in any form or by any means, electronic or mechanical, including photocopying, recording or by any information storage and retrieval system, without written permission of the holder of the copyright. Copyright Act 1968 (Cth) Australia applies. USA Copyright law applies to users in USA.

RESEARCH PAPER

THE EFFECTS OF THE MIXTURES OF THREE MICRO-CONTAMINANTS COMMONLY FOUND IN BIOSOLIDS ON EARTHWORM REPRODUCTION

Louis A Tremblay[1,2*], Lynn Booth[3], Jo-Anne E. Cavanagh[3], Olivier Champeau[1], Grant L Northcott[4], Nina Cedergreen[5]

[1] Cawthron Institute, 98 Halifax Street East, Nelson 7010, New Zealand.

[2] School of Biological Sciences, University of Auckland, Auckland 1142, New Zealand.

[3] Landcare Research, Lincoln 7640, New Zealand.

[4] Northcott Research Consultants Limited, 20 River Oaks Place, Hamilton 3200, New Zealand.

[5] Department of Basic Sciences and Environment, Faculty of Life Science, University of Copenhagen, Thorvaldsensvej 40 DK-1871 Frederiksberg C., Denmark.

ABSTRACT

The risks associated with the presence of organic contaminants represent a barrier to the application of biosolids as a soil amendment. The characterisation of the risk of complex mixtures to receiving soil ecosystems remains challenging. In this study, triclosan, carbamazepine, and bisphenol A were selected as micro-contaminants frequently found in biosolids. Chemicals were added to soil and tested individually and in mixtures to characterise any interactions that could result in a non-additive response. The soil-dwelling earthworm 56-day reproduction chronic test was used to assess the impact of these chemicals on soil ecosystems. The joint toxicity of the three chemicals tested, both in binary and ternary mixtures was partially described by both the Concentration Addition and Independent Action models. This research provided preliminary information to address the knowledge gap on the effects of chemical mixtures on soil biota. Further studies considering different modes of action and better characterisation of chemical fate are required to assess the risk of land application of biosolids to soil biota and derive robust Predicted No Effect Concentration (PNEC) values.

Keywords: Triclosan; Carbamazepine; Bisphenol A; Concentration Addition (CA); Independent Action (IA); *Eisenia andrei.*

INTRODUCTION

Improving and maintaining the fertility of soil is essential for meeting the demands for food production (Meena et al. 2016). The benefit of biosolids as a fertiliser and soil amendment for improving low fertility soils and reclamation of degraded land is widely recognised (Larney and Angers 2012). However, biosolids can contain organic contaminants that have not been removed or degraded during wastewater or sludge treatment (Clarke and Smith 2011). Several studies have characterised the presence of organic contaminants in biosolids destined for land application (Eriksson et al. 2008). The results from a US study indicated that the nine biosolid products investigated were highly enriched with organic contaminants (as mass-normalised concentrations) when compared to effluents or effluent-impacted water (Kinney et al. 2006). A review of peer-reviewed publications and government reports found that out of 516 different organic contaminants

* Author for correspondence, email: louis.tremblay@cawthron.org.nz

in sewage sludge, 83% were not on the US-EPA established list of priority pollutants (Harrison et al. 2006). In the UK, a range of contaminants including trace metals, pharmaceuticals, polycyclic aromatic hydrocarbons (PAHs), emerging and regulated organic pollutants were identified in most sludge samples from 28 wastewater treatment plants (Jones et al. 2014). It has been demonstrated that biologically active chemicals can persist in biosolids and as a consequence, the reuse of biosolids through land application can be an important route for introducing xenobiotic compounds into terrestrial environments (Kinney et al. 2012).

The characterisation of the toxic effects to assess the risks associated with multiple stressors in receiving soil ecosystems represents a major challenge for environmental scientists, risk assessors and regulators (Spurgeon et al. 2010). The risks of complex mixtures of contaminants in soils are often poorly quantified as the chemicals responsible for causing the toxicity are seldom characterised. In addition, compared to studies in the aquatic environment, relatively few studies explored possible interactions between chemicals in soil compared to the aquatic environment. If the chemicals present in the soil do not interact, standard models for predicting mixture effects such as Concentration Addition (CA) and Independent Action (IA) can be used, if knowledge of their presence and toxicity exists (Cedergreen et al. 2012). However, if the chemicals interact in a synergistic manner, enhancing the effect compared to what was predicted, caution is warranted in assessing risk based on the standard mixture models. In general, severe interactions are more likely to occur when a few chemicals dominate the overall toxicity in complex mixtures (Cedergreen 2014).

For this study, triclosan (TCS), carbamazepine (CBZ) and bisphenol A (BPA) were selected as organic wastewater contaminants commonly found in biosolids (Citulski and Farahbakhsh 2010; Kinney et al. 2006; Mohapatra et al. 2014). Triclosan is the most common antimicrobial agent present in domestic wastewater, and 50% or more of TCS mass entering wastewater treatment plants partitions into biosolids (Verlicchi and Zambello 2015; Waria et al. 2011; Ying and Kookana 2007). The anticonvulsant CBZ is becoming a priority for the government agencies and regulatory agencies and the general public because of its persistence and potential chronic effects within receiving environments (Mohapatra et al. 2014). Bisphenol A is a widely used plasticiser commonly present in biosolids and a recognised endocrine disrupting chemical (Citulski and Farahbakhsh 2010; Clara et al. 2004).

The aim of this study was to characterise the combined toxicity of TCS, CBZ and BPA using the chronic earthworm reproduction assay. The three chemicals were added to soil and their effects tested individually and in mixtures. The toxicity results were analysed by the CA and IA reference models to assess whether they can predict the interactions. The earthworm model was selected as it is well recognised for soil toxicity studies due to its consumption of organic matter in soil and direct contact with soil constituents and correlation between laboratory and field conditions (Kinney et al. 2012).

MATERIAL AND METHODS

Chemicals and reagents

Triclosan (TCS) and methyl-triclosan (m-TCS) were supplied by Dr Ehrenstorfer GmbH Germany. Deuterated triclosan-d3 (TCS-d3) and methyl-triclosan-d3 (m-TCS-d3) were supplied by CDN Isotopes Quebec, Canada. Bisphenol-A (BPA), deuterated bisphenol-A-d16 (BPA-d16), bisphenol-C (BPC), carbamazepine and 10,11-dihydrocarbamazepine were from Sigma Aldrich NZ Ltd. Acetone (HPLC Grade, Fisher Chemicals), ethyl acetate (Optima Grade, Fisher Chemicals) and hexane (95%, Mallinckrodt Chrom AR grade) were from Thermo Fisher Scientific (Auckland, NZ). Ortho-phosphoric acid (AR Grade), glacial acetic acid (J.T. Baker A.C.S regent grade), diatomaceous

earth sorbent (hydromatrix) from Dionex, cellulose filters, and Ottawa sand were from Thermo Fisher Scientific (Auckland, NZ). A.C.S reagent grade sulfuric acid and N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) were supplied by Sigma-Aldrich NZ Ltd (Auckland, NZ). Sodium hydrogen carbonate, sodium sulfate anhydrous, and sodium hydroxide (all AnalaR) were supplied by Merck-BDH NZ Ltd (Auckland, NZ).

Soil preparation

The exposure followed the procedure described by the OECD guideline 222 (OECD 2004) with variations. The OECD standard soil was prepared at the following ratios: 70% sand, 20% kaolin clay, 10% *Sphagnum* peat, and calcium carbonate to adjust pH to 6.0-7.5. Soil was spiked using a method previously described (Brinch et al. 2002) which entails incorporating the spike into a subsample of soil, and then mixing this into the greater bulk of soil. The moisture content of a 75% portion of dry soil was adjusted to 60% of the water holding capacity with distilled water and left to equilibrate for 7 to 14 days before use (Brinch et al. 2002).

Stock solutions were prepared by dissolving each test compound in acetone. For TCS and BPA, stock solutions of 50 $mg.mL^{-1}$ were prepared, but for CBZ, which has a lower solubility, a stock solution of 12.5 $mg.mL^{-1}$ was prepared. The remaining 25% portion of dry soil was spiked at the rate of 80 mL $acetone.kg^{-1}$ total soil, mixed homogeneously and left to evaporate overnight in a fume cupboard. The spiked soil was then combined with the wet soil and the moisture content adjusted. Soil was thoroughly mixed by hand until it was uniform in colour and texture, and then evenly distributed among 1-L glass jars with a 105-cm^2 cross section and covered with lids. Only 250 g of soil was used to allow enough headspace in the jar and air renewal in the soil.

Earthworms and exposure conditions

The earthworm *Eisenia andrei* was used as a test organism and cultured in a 50% mix of peat and horse manure, at 20°C. Seven days prior to initiating experiments, the earthworms were removed from the bedding mix and transferred to the OECD soil used in the trials to acclimate.

For each treatment, three replicates of 10 adult clitellate worms (total weight of 300-600 mg) were weighed without depuration and placed in the prepared jars (t=0). They were monitored at day one to ensure burrowing into the soil and 5 g of dried horse manure was placed on the surface of the soil as a food source. Food intake, as determined by the observation of the disappearance of the manure from the soil surface, was monitored during the experiment and manure was replenished when previously added food was fully consumed. Moisture was checked weekly for the first four weeks, then at 28 and 56 days of exposure by weighing the jars and maintained throughout the experiment by adding double-distilled water. Experiments were conducted at 20°C in a 16:8 light/dark cycle.

After 28 days of exposure, the soil from each jar was tipped onto a tray and adults were hand-sorted from the soil, counted and weighed without depuration. The soil was returned to the jars, 5 g of dried horse manure was added and carefully mixed in for a further 28-d incubation. After the additional 28 days, the soil was hand sorted to remove cocoons (hatched and unhatched), and the number of juveniles was determined.

Mixture experiments

The exposure dose range comprised four or five concentrations selected based upon the results of range finding tests (data not shown) for each chemical tested. Treatments covered a range of concentrations to assess EC50 for reproductive effects (number of juveniles produced). The concentrations used were Bisphenol A (100, 180, 320, 560 $mg.kg^{-1}$), triclosan (18, 100, 180, 320,

560 mg.kg^{-1}), and carbamazepine (180, 320, 560, 1000 mg.kg^{-1}). In addition, controls with and without solvent were tested. The simplex-centroid design was used for assessing the toxicity of mixtures as previously described (Scheffé 1963). The design points correspond to all combinations of the pure chemical alone (as EC50), in binary mixtures (as EC50) and one ternary mixture (as EC50), as summarised in Table 1.

Chemical analysis

To determine the fate of the test compounds, soil samples were collected from each of the exposure jars (n=3) on days 0 and 28, stored in plastic bags and frozen at -80°C until analysis. The different chemical properties of the test compounds necessitated the application of different extraction methods to recover them from the test soil samples.

Extraction and derivatisation of bisphenol-A and triclosan

Bisphenol-A and TCS were extracted from soil using a variation of a solvent ultrasonication extraction method developed to analyse a wide range of organic contaminants, including phenols, from sediment, soil and biota (Holland et al. 1993).

An aliquot of soil equivalent to approximately 2.5 g dry weight was weighed into a glass Oak Ridge centrifuge tube (Kimax 50 mL) and spiked with an acetone solution (0.025 to 0.10 mL) containing the recovery compounds methyl-triclosan-d3, triclosan-d3 and bisphenol-C. After spiking, 50 µL of concentrated ortho-phosphoric acid was added to the soil to reduce the pH and enhance the recovery of TCS and BPA during solvent extraction. Quality control samples consisting of a solvent blank, blank control soil, and control soil spiked with the target analytes were prepared with each batch of extracted samples. The soil was shaken by hand to mix, and 15 mL of a binary solution of acetone:hexane (2:3 v/v) was added. The centrifuge tubes were sealed with Teflon-lined screw caps, shaken by hand to mix, and extracted by ultrasonication for 20 min at 25°C (Bandelin Sonerex Digital 10P) followed by shaking at 200 rpm on a horizontal shaker (IKA KS501 Digital) for 30 min. Thirty mL of purified laboratory water (Millipore Milli-Q Integral 5) was added to each centrifuge tube, which was capped and gently inverted six times to mix the contents. The centrifuge tubes were then centrifuged at 2500 g (Hettich Rotanta 460R) to break down any emulsion and assist the partitioning of hexane from the lower acetone/water layer.

A predetermined aliquot of the upper hexane extract (based on the expected concentration of analyte) was removed from each sample tube and transferred into a labelled 2-mL GC vial. An aliquot of

Table 1. Experimental outline of the simplex-centroid design. Carbamazepine (CBZ), triclosan (TCS) and bisphenol A (BPA) were added at respective fractions of their effective concentrations (EC50) after 56 days (EC50 based on nominal concentrations).

Mixture treatment	CBZ (mg.kg^{-1})	TCS (mg.kg^{-1})	BPA (mg.kg^{-1})
Water control	0	0	0
Solvent control	0	0	0
CBZ alone	EC50	0	0
TCS alone	0	EC50	0
BPA alone	0	0	EC50
CBZ + BPA	EC50	0	EC50
CBZ + TCS	EC50	EC50	0
TCS + BPA	0	EC50	EC50
TCS +BPA + CBZ	EC50	EC50	EC50

internal standard in acetone (Bisphenol-A-d16) was added to each vial and the solution was gently blown to dryness under a stream of nitrogen gas. The dry residue was re-dissolved in 25 µL of ethyl acetate, 30 µL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) added and the vial capped. The contents were mixed by vortex, and left to react at room temperature for 30 min. At the completion of the derivatisation reaction the final volume of the solutions was adjusted to 1 mL with ethyl acetate and the vials stored at 4°C until analysis.

Extraction of carbamazepine

Residues of CBZ were extracted and purified using a modification of the method previously described (Duran-Alvarez et al. 2009). Soil (2.5 g dry weight equivalent) was thoroughly mixed with 2.5 g of hydromatrix and transferred into 11-mL stainless steel Accelerated Solvent Extraction (ASE) cells containing two cellulose filters in the base. The sample mixture was gently packed and the remaining free volume in the cell filled with solvent-extracted Ottawa sand. Quality control samples consisting of a solvent blank, blank control soil and a spiked control soil were similarly prepared for each batch of extracted samples. The spiked control soil was spiked with a solution of carbamazepine in acetone before the addition of the Ottawa sand. A cellulose filter was inserted into the top of the extraction cells, which were sealed with stainless steel caps.

The sample cells were extracted with a Dionex ASE 200 using a solvent mixture of acetone:hexane:acetic acid (50:50:2 v/v/v) at a temperature of 100°C and a pressure of 1000 psi. The cells were subjected to two sequential cycles of static extraction for 5 minutes followed by a 50% flush volume. The extraction solvent was collected in 60-mL glass collection vials and evaporated under nitrogen at 30°C to a volume of 2 to 2.5 mL using a Zymark Turbovap, 20 mL of MilliQ water was added, and the aqueous solution mixed by vortex.

The sample solutions were passed through previously-conditioned Oasis HLB cartridges (0.5 g, 6 mL) at a flow rate of 1 to 2 mL.minute^{-1}. The sample vials were rinsed with two 2-mL individual Milli-Q water rinses that were similarly passed through the Oasis cartridge, followed by a final wash with Milli-Q water (10 mL). Carbamazepine was eluted from the Oasis cartridge with 15 mL of a binary solution of 40:60 (v/v) acetone:0.10 M sodium hydrogen carbonate buffer (adjusted to pH 10 with 1 M sodium hydroxide) and collected in glass tapered test tubes. This solution was evaporated to approximately 10 mL to remove the greater proportion of acetone, acidified to pH 2 by the addition of sulfuric acid, and extracted with three individual 2-mL aliquots of diethyl ether which were dried by passing through a small column of anhydrous sodium sulfate. Aliquots of the ethyl acetate solutions were transferred to glass Reacti-Vials (Alltech NZ Ltd, Auckland NZ) and the internal standard 10,11-dihydrocarbamazepine (250 ng) added. The contents of the Reacti-Vials were evaporated to dryness under a stream of nitrogen, reconstituted in ethyl acetate (25 µL) and MTBSTFA (50 µL) added, capped and incubated at 60°C for 30 min. After returning to room temperature the volume of the derivatised solutions was adjusted to 1 mL with ethyl acetate and the solutions transferred into GC vials and stored at 4°C until analysis.

GC-MS analysis

A total of eight calibration standards containing the target compounds and internal standards and covering a concentration range from 5.0 to 1000 ng.mL^{-1} were derivatised with each batch of individual samples prepared for analysis by gas chromatography mass-spectrometry (GC-MS). The resulting tert-butyldimethylsilyl esters were analysed by high resolution gas chromatography mass-spectrometry using an Agilent 7000C Triple Quadrupole GC/MS coupled to an Agilent 7890 Gas Chromatograph and 7693 automated liquid sampler.

Samples were injected (1 µL) into an Agilent Split/splitless inlet operating at a temperature of 280°C using pressure pulse injection mode. The initial inlet pressure was held at 45 psi for 0.75 minutes with a split flow of 50 mL.min^{-1} at 1.5 min, reducing to 15 mL.min^{-1} at 3 min. Target analytes were separated on an Agilent HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm) using helium as a carrier gas at a constant flow of 1 mL.min^{-1}.

For the analysis of BPA, BPA-d16, BPC, TCS, TCS-d3, m-TCS, and m-TCS-d3, the oven temperature was held at 90°C for 1 min, then increased at 40°C.min^{-1} to 230°C, followed by 4°C.min^{-1} to 270°C and 30°C.min^{-1} to 320°C with a 5 min hold. For the analysis of CBZ and dihydrocarbamazepine, the oven temperature was held at 75°C for 1 minute, then increased at 15°C.min^{-1} to 280°C with a 5-minute hold.

The mass spectrometer was operated in Electron Impact (EI) mode with a source temperature of 230°C, quadrupole temperatures (MS 1 and 2) of 150°C, and interface temperature of 280°C. Instrument grade helium was used as a quench gas and instrument grade nitrogen as collision gas at flow rates of 2.25 mL.min^{-1} and 1.25 mL.min^{-1}, respectively.

Mass spectral data were acquired in single ion monitoring (SIM) mode using mass ions specific to the corresponding tert-butyldimethylsilyl esters of the target, surrogate, and internal standard compounds.

Compound-specific mass fragments were obtained using single ion monitoring and were used in combination with retention time matching for positive identification. The concentration of the target analytes was determined by extracting compound-specific mass ions and comparing the relative abundance of the three mass ions against those obtained from pure compound standards. Quantification of target analytes was completed by internal standard quantitation using Agilent Mass Hunter Workstation Quantitative Analysis Software.

Statistical analysis

The numbers of juveniles produced per adult as a function of soil concentrations of the three individual chemicals were described with a three parameter log-logistic concentration response model using the statistical program R (R Core Team 2017) and the add-on package *drc* (Ritz and Streibig 2005):

Equation 1

$$Response = \frac{d}{1+(x/e)^b}$$

Where the response is the number of juveniles per jar, d is the maximal response corresponding to the control treatment, x is the nominal soil concentration at the beginning of the exposure, e is the concentration reducing the response by 50% (EC50) and b is proportional to the slope of the concentration response curve around e.

The mixture experiments were evaluated in relation to both Concentration Addition (CA) and Independent Action (IA) (Cedergreen et al. 2013; Jonker et al. 2005). The experimental design was made so all the mixtures should reduce the response by 50% (using the concentration response parameters of the number of juveniles as a basis). Hence, any significant deviation from 50% of the control value in the mixture experiment would indicate either synergism (response significantly reduced below 50% of the controls) or antagonism (response significantly increased above 50% of the control) relative to CA. The 95% CIs were calculated using R with the 'drc' package (Ritz and Streibig 2005) from logged concentrations. Significance was assessed as 95% confidence limits not

overlapping the 50% control value (Wheeler et al. 2006). Differences between the treatments were tested by an Analysis of Variance (ANOVA) followed by a Tukey HSD-test using the software R 3.4.2 (R Core Team 2017). The IA predictions were made based on the dose-response curves from the first experiment on the individual chemicals, calculating the product of the relative responses (R) of the concentrations used in the mixture and multiplying the predicted fraction of produced juveniles (R_{mix}) with the control value of the water control of the mixture experiment:

Equation 2

$$E(c_{Mix}) = \prod_{i=1}^{n}[E(c_i)]$$

RESULTS AND DISCUSSION

Quality assurance outcomes

No residues of TCS, methyl-TCS, BPA or CBZ were detected in the quality assurance solvent blank or blank control soil samples above their respective method detection limits (Table 2). The mean percentage recoveries and 95% confidence intervals obtained for the surrogate compounds methyl-triclosan-d3, triclosan-d3 and bisphenol-C spiked into all QA and soil treatment samples were 96% ± 4%, 98% ± 6%, and 90% ± 6%, respectively.

The mean percentage recoveries of TCS, BPA and CBZ spiked onto quality assurance spike control soil samples were 96% ± 5%, 92% ± 6%, and 87% ± 6%, respectively.

The high mean percentage recoveries and narrow confidence intervals obtained for the surrogate standards methyl-triclosan-d3, triclosan-d3 and bisphenol-C spiked into each extracted sample, combined with the high percentage recoveries obtained for TCS, BPA and CBZ spiked into quality assurance spiked control soils, were excellent and demonstrated that the extraction and analysis methods were robust and fit for purpose.

Table 2. Nominal and measured concentrations (mean ± standard deviation, *n*=3) of the test chemicals added in soil samples at the beginning of the exposure (day 0) and after 28 days.

	Concentration (mg.kg^{-1} dry weight)			% Reduction at 28 days
	Day 0		Day 28	
Chemical	Nominal	Measured	Measured	
Triclosan	0	<MDL*	<MDL	
(MDL = 0.005 mg.kg^{-1})	18	13.6 ± 1.9	7.9 ± 1.4	42
	100	90.9 ± 8.5	51.3 ± 4.8	44
	180	233.5 ± 57.8	70.6 ± 3.9	70
Bisphenol A	0	<MDL	<MDL	
(MDL = 0.010 mg.kg^{-1})	180	146.5 ± 23.3	4.1 ± 1.4	97
	320	282 ± 31.2	21 ± 14.5	92
Carbamazepine	0	<MDL	<MDL	
(MDL = 0.010 mg.kg^{-1})	180	110.7 ± 6.7	101.7 ± 2.2	8
	560	301 ± 24.3	257.3 ± 10.1	15
	1000	547.7 ± 50.5	475 ± 21.9	13

MDL: minimum detection limit.

This study provided laboratory observations as suggested by the EU SOLUTIONS project to improve the assessment of the impacts of contaminant mixtures (Altenburger et al. 2012). The chemical analysis showed that after 28 days approximately 50% and 90% of the TCS and BPA were degraded, respectively, while CBZ was more persistent with only a reduction of 10-15% (Table 2). Bisphenol A has been reported to be readily biodegradable (Staples et al. 2010). A conservative approximation of the half-life of TCS was estimated at 100 d in biosolids-amended soils, but this period is highly dependent on soil properties (Higgins et al. 2011; Waria et al. 2011). The persistence of CBZ has been reported and was suggested to be due to its inherent low biodegradability in the environment (Wu et al. 2010).

The fate of the three chemicals follows that which was reported in a study that investigated the effects of land application of biosolids on earthworms (Kinney et al. 2012). These authors studied the fate of a range of micro-pollutants including CBZ, BPA and TCS in biosolids amended to soil and their bioaccumulation in the earthworm *E. fetida*. In that study, the concentrations of TCS and BPA in biosolids applied to soil were 3240 and 533 $\mu g.kg^{-1}$ respectively, but reduced to less than their respective detection limits 8 weeks after the biosolids were incorporated into soil field plots. However, both chemicals bioaccumulated in the earthworms. The concentration of carbamazepine in the biosolids was comparatively much lower at 22 $\mu g.kg^{-1}$ but the residues of carbamazepine were relatively persistent and remained detectable in the biosolid-amended soil after 28 weeks (which agrees with the low rate of degradation measured in the present study), and did not accumulate in the earthworms (Kinney et al. 2012).

All tests complied with the OECD validity criteria (adult mortality <10%, each replicate producing ≥30 juveniles, coefficient of variation for reproduction ≤30% in control replicates) (Clarke and Smith 2011; OECD 2004).

The parameters for choosing the mixture toxicity ratios and for making the IA predictions are summarised in Table 3 from the fitted models in Figure 1. Bisphenol A was the most potent of the three compounds tested with an EC50 value of 274 mg BPA kg^{-1} soil. Bisphenol A has an estimated predicted no effect concentration in soil ($PNEC_{soil}$) of 3.7 $mg.kg^{-1}$ which was derived from data from a range of species not including *E. andrei* (Staples et al. 2010). The concentration of BPA measured in soil after 28 days was marginally above this PNEC value, suggesting the residues of BPA will continue to present a risk to soil organisms. For TCS, the number of juveniles decreased with increasing concentrations, with EC50 values around 340 mg TCS kg^{-1} soil. This EC50 is well over the PNEC estimated at 0.0006 to 0.06 mg TCS kg^{-1} using the factorial approach and *E. andrei* (Amorim et al. 2010). Carbamazepine was the least toxic of the three chemicals tested with EC50 values around 1000 mg CBZ kg^{-1} soil. It is difficult to explain why BPA was more toxic than TCS and additional information on the fate of these chemicals over time would be required to characterise the fate of these chemicals in soil.

Table 3. Concentration response curve parameters (mean ± standard error) for bisphenol A (BPA), triclosan (TCS) and carbamazepine (CBZ) on the number of juveniles of the earthworm *Eisenia andrei.*

Chemical	Slope ± SE *b*	Upper limit ± SE (Number per jar) *d*	EC50 ± SE (mg.kg-1 DW soil) *e*
BPA	5.47 ± 1.17	172 ± 6	274 ± 14
TCS	1.05 ± 0.19	165 ± 8	342 ± 53
CBZ	1.32 ± 0.52	171 ± 10	1074 ± 166

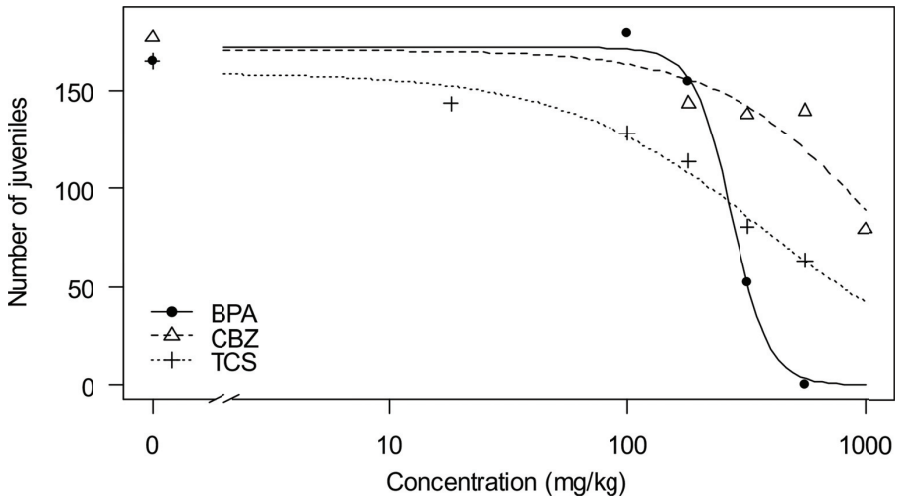


Figure 1. Fitted curves from log-logistic model to means of number of juveniles at each treatment concentration. (BPA, Bisphenol A; CBZ, carbamazepine; TCS, triclosan)

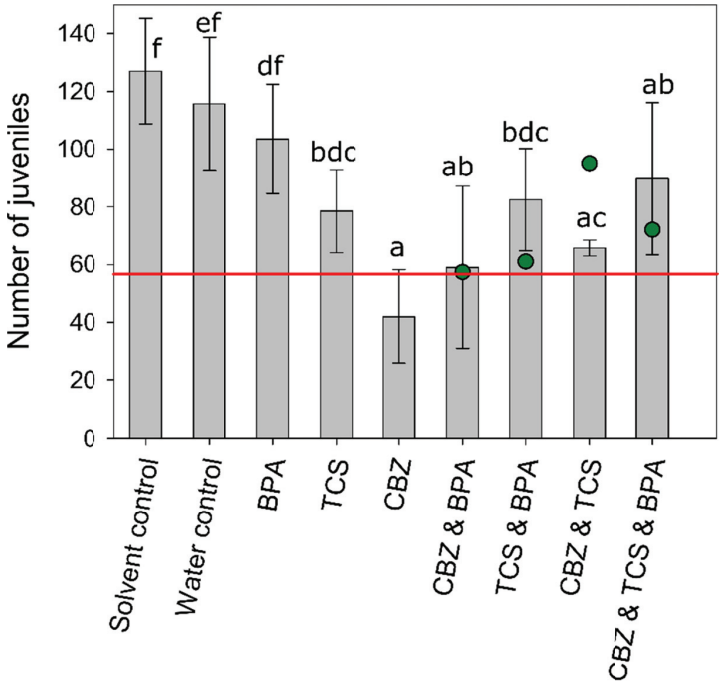


Figure 2. Number of juveniles (mean ± 95% confidence limits, n=3) of the earthworms *Eisenia andrei* produced when exposed to single chemicals or mixtures of bisphenol A (BPA), triclosan (TCS) and carbamazepine (CBZ) in laboratory reproduction tests. Red line and green circles represent the mixture predictions in accordance with Concentration Addition and Independent Action models, respectively. The concentrations of the chemicals are provided in Table 1. Significantly different treatments (ANOVA followed by a Tukey-test, $p < 0.05$) are indicated with different letters.

The results of the mixture experiments are shown in Figure 2. There was no significant difference for numbers of juveniles produced between water and solvent controls (*t*-test: $p > 0.05$; data not shown).

Individual chemicals were tested at the concentrations corresponding to their estimated EC50 values (Table 1). Both BPA and TCS produced more juveniles than expected, i.e. 50% of the number of juveniles in controls, while CBZ produced on average approximately 30% of the control juveniles (Figure 2). This could be explained partly by the differences in the degradation of these compounds between the two experiments. However, the EC50s were estimated under the same conditions, so the degradation of these compounds should be similar between experiments. More detailed temporal degradation of the chemicals for use in a fate model would be required to better explain those discrepancies. The results from the binary mixtures were closer to the expected 50% reduction in juveniles while there was a greater number present in the ternary mixture.

The CA and IA mixture toxicity predictions mostly fell within the 95% confidence intervals of the observed number of juveniles produced. The exception was the CBZ + TCS mixture that was more toxic than the IA prediction. Hence, both models could suitably predict the toxicity. Both the CA and the IA models can provide an initial mathematical basis for the prediction of mixture effect but have limitations with chemicals and mixtures with unknown or unpredictable modes of action (Spurgeon et al. 2010). Further investigations are required to assess the risk from chemical mixtures, including chemicals like TCS, associated with the land application of biosolids (Verlicchi and Zambello 2015)

CONCLUSIONS

The joint toxicity of the three chemicals tested in binary and ternary mixtures was partially described by both the CA and IA models. These results demonstrate that the independent action and the concentration addition models can serve as a protective approach in risk assessment of mixtures of organic contaminants in soil. However, further studies considering different modes of action and chemical fate are required to suitably assess the risk of land application of biosolids to soil biota and derive robust PNEC values. This research provided preliminary information to address the knowledge gap on the effects of chemical mixtures on soil biota.

ACKNOWLEDGEMENTS

This research was funded by the New Zealand Ministry Business Innovation and Employment project Closing the loop - rebuilding our soils with biowastes (contract C03X0902).

REFERENCES

Altenburger R, Scholz S, Schmitt-Jansen M, Busch W and Escher BI. 2012. Mixture toxicity revisited from a toxicogenomic perspective. *Environmental Science and Technology* **46**, 2508-2522.

Amorim MJB, Oliveira E, Soares A and Scott-Fordsmand JJ. 2010. Predicted No Effect Concentration (PNEC) for triclosan to terrestrial species (invertebrates and plants). *Environment International* **36**, 338-343.

Brinch UC, Ekelund F and Jacobsen CS. 2002. Method for spiking soil samples with organic compounds. *Applied and Environmental Microbiology* **68**, 1808-1816.

Cedergreen N. 2014. Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. *PloS ONE* **9**(5), e96580.

Cedergreen N, Svendsen C and Backhaus T. 2012. Toxicity prediction of chemical mixtures. In *Encyclopedia of Environmental Management*. Jorgensen SE (Ed), Taylor and Francis, New York. pp 2572-2581.

Cedergreen N, Svendsen C and Backhaus T. 2013. Chemical mixtures: concepts for predicting toxicity. In *Encyclopedia of Environmental Management*, Vol IV. Jorgensen SE (Ed), Taylor and Francis, New York. pp 2601-2610.

Citulski JA and Farahbakhsh K. 2010. Fate of endocrine-active compounds during municipal biosolids treatment: A review. *Environmental Science and Technology* **44**, 8367-8376.

Clara M, Strenn B, Saracevic E and Kreuzinger N. 2004. Adsorption of bisphenol-A, 17ß-estradiole and 17α-ethinylestradiole to sewage sludge. *Chemosphere* **56**, 843-851.

Clarke BO and Smith SR. 2011. Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. *Environment International* **37**, 226-247.

Duran-Alvarez JC, Becerril-Bravo E, Castro VS, Jimenez B and Gibson R. 2009. The analysis of a group of acidic pharmaceuticals, carbamazepine, and potential endocrine disrupting compounds in wastewater irrigated soils by gas chromatography-mass spectrometry. *Talanta* **78**, 1159-1166.

Eriksson E, Christensen N, Schmidt JE and Ledin A. 2008. Potential priority pollutants in sewage sludge. *Desalination* **226**, 371-388.

Harrison EZ, Oakes SR, Hysell M and Hay A. 2006. Organic chemicals in sewage sludges. *Science of the Total Environment* **367**, 481-497.

Higgins CP, Paesani ZJ, Chalew TEA, Halden RU and Hundal LS. 2011. Persistence of triclocarban and triclosan in soils after land application of biosolids and bioaccumulation in *Eisenia foetida*. *Environmental Toxicology and Chemistry* **30**, 556-563.

Holland PT, Hickey CW, Roper DS and Trower TM. 1993. Variability of organic contaminants in inter-tidal sandflat sediments from Manukau Harbor, New Zealand. *Archives of Environmental Contamination and Toxicology* **25**, 456-463.

Jones V, Gardner M and Ellor B. 2014. Concentrations of trace substances in sewage sludge from 28 wastewater treatment works in the UK. *Chemosphere* **111**, 478-484.

Jonker MJ, Svendsen C, Bedaux JJ, Bongers M and Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. *Environmental Toxicology and Chemistry* **24**, 2701-2713.

Kinney CA, Campbell BR, Thompson R, Furlong ET, Kolpin DW, Burkhardt MR, . . . Hay AG. 2012. Earthworm bioassays and seedling emergence for monitoring toxicity, aging and bioaccumulation of anthropogenic waste indicator compounds in biosolids-amended soil. *Science of the Total Environment* **433**, 507-515.

Kinney CA, Furlong ET, Zaugg SD, Burkhardt MR, Werner SL, Cahill JD and Jorgensen GR. 2006. Survey of organic wastewater contaminants in biosolids destined for land application. *Environmental Science and Technology* **40**, 7207-7215.

Larney FJ and Angers DA. 2012. The role of organic amendments in soil reclamation: A review. *Canadian Journal of Soil Science* **92**, 19-38.

Meena MD, Joshi PK, Narjary B, Sheoran P, Jat HS, Chinchmalatpure AR, . . . Sharma DK. 2016. Effects of municipal solid waste compost, rice-straw compost and mineral fertilisers on biological and chemical properties of a saline soil and yields in a mustard-pearl millet cropping system. *Soil Research* **54**, 958-969.

Mohapatra DP, Brar SK, Tyagi RD, Picard P and Surampalli RY. 2014. Analysis and advanced oxidation treatment of a persistent pharmaceutical compound in wastewater and wastewater sludge-carbamazepine. *Science of the Total Environment* **470**, 58-75.

OECD. 2004. *OECD Guidelines for the Testing of Chemicals, Section 2 - Effects on Biotic Systems - Test No. 222: Earthworm Reproduction Test* (Eisenia fetida/Eisenia andrei). 18 pp.

R Core Team. 2017. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing., Vienna, Austria.

Ritz C and Streibig JC. 2005. Bioassay Analysis using R. *Journal of Statistical Software* **12**, 1–22.

Scheffé H. 1963. The simplex-centroid design for experiments with mixtures. *Journal of the Royal Statistical Society Series B (Methodological)* **25**, 235-263.

Spurgeon DJ, Jones OAH, Dorne J, Svendsen C, Swain S and Sturzenbaum SR. 2010. Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. *Science of the Total Environment* **408**, 3725-3734.

Staples C, Friederich U, Hall T, Klecka G, Mihaich E, Ortego L, . . . Hentges S. 2010. Estimating potential risks to terrestrial invertebrates and plants exposed to bisphenol A in soil amended with activated sludge biosolids. *Environmental Toxicology and Chemistry* **29**, 467-475.

Verlicchi P and Zambello E. 2015. Pharmaceuticals and personal care products in untreated and treated sewage sludge: Occurrence and environmental risk in the case of application on soil — A critical review. *Science of the Total Environment* **538**, 750-767.

Waria M, O'Connor GA and Toor GS. 2011. Biodegradation of triclosan in biosolids-amended soils. *Environmental Toxicology and Chemistry* **30**, 2488-2496.

Wheeler MW, Park RM and Bailer AJ. 2006. Comparing median lethal concentration values using confidence interval overlap or ratio tests. *Environmental Toxicology and Chemistry* **25**, 1441-1444.

Wu C, Spongberg AL, Witter JD, Fang M, Czajkowski KP and Ames A. 2010. Dissipation and leaching potential of selected pharmaceutically active compounds in soils amended with biosolids. *Archives of Environmental Contamination and Toxicology* **59**, 343-351.

Ying GG and Kookana RS. 2007. Triclosan in wastewaters and biosolids from Australian wastewater treatment plants. *Environment International* **33**, 199-205.