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# Chronic toxicity of an environmentally relevant and equitoxic ratio of five metals to two Antarctic marine microalgae shows complex mixture interactivity

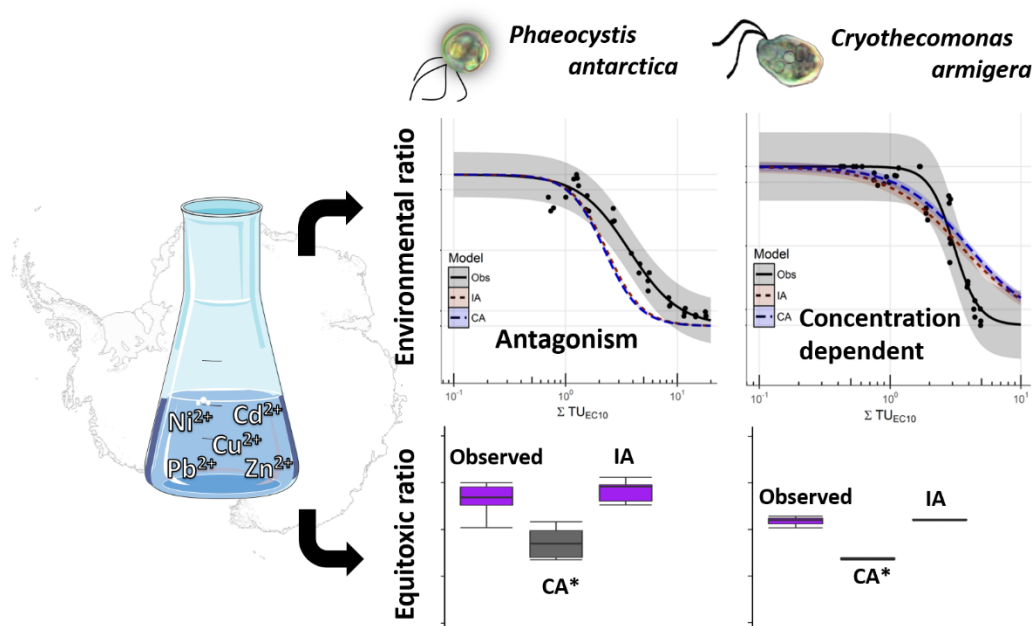
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## Graphical abstract



## Highlights

- Mixture models using EC10 values can predict toxicity from 5 metals to 2 microalgae
- An equitoxic mixture was non-interactive by IA and antagonistic by CA to both algae
- The environment mixture elicited different mixture interactions to both algae
- Cellular biomarkers may explain the mechanisms behind mixture interactions
- Mixture models can help manage complex metal mixtures in marine environments

## **Abstract**

Metal contaminants are rarely present in the environment individually, yet environmental quality guidelines are derived from single-metal toxicity data. Few metal mixture studies have investigated more than binary mixtures and many are at unrealistically high effect concentrations to freshwater organisms. This study investigates the toxicity of five metals (Cd, Cu, Ni, Pb, and Zn) to the Antarctic marine microalgae *Phaeocystis antarctica* and *Cryptothecomonas armigera*. Two mixtures were tested: (i) an equitoxic mixture of contaminants present at their single-metal EC10 concentrations, and (ii) an environmental mixture based on the ratio metal concentrations in a contaminated Antarctic marine bay.

Observed toxicity, as chronic population growth rate inhibition, was compared to Independent Action (IA) and Concentration Addition (CA) predictions parameterised to use EC10 values. This allowed for the inclusion of metals with low toxicities. The biomarkers chlorophyll a fluorescence, cell size and complexity, and intracellular lipid concentrations were assessed to investigate possible mechanisms behind metal-mixture interactions.

Both microalgae had similar responses to the equitoxic mixture: non-interactive by IA and antagonistic by CA. Toxicity from the environmental mixture was antagonistic by IA to *P. antarctica*; however, to *C. armigera* it was concentration-dependent with antagonism at low toxicities and synergism at high toxicities by both IA and CA. Differences in dissolved organic carbon production and detoxification mechanisms may be responsible for these responses and warrants further investigation. This study shows that mixture toxicity interactions can be ratio, species, and concentration dependent. The responses of the microalgae to different mixture ratios highlight the need to assess toxicity at environmentally realistic metal ratios. Parameterising IA and CA reference models to use EC10s allowed for the inclusion of metals at low effect concentrations, which may otherwise be ignored.

Reference mixture models are generally suitable for predicting chronic toxicity of metals to these marine microalgae at environmentally realistic ratios and concentrations.

## **Capsule**

Toxic metal-mixture interactions were found to be concentration, ratio, and species dependent in exposures to two Antarctic marine microalgae.

## Introduction

Localised areas of Antarctica are contaminated as a result of historical and ongoing anthropogenic activities, particularly around research stations (Tin et al., 2009). Former waste disposal sites, abandoned work sites and stations, and fuel spills contribute to an estimated 1 million m<sup>3</sup> of uncontained contaminated pollution (Snape et al., 2001). Some of this pollution is mobilised from soils to the near-shore marine environment, generally by particle entrainment in summer meltwaters (Stark et al., 2006). This has resulted in elevated concentrations of a mixture of contaminants, including the metals Cd, Cu, Ni, Pb, and Zn in near-shore marine environments (Fryirs et al., 2015; Larner et al., 2006), causing toxicity to marine organisms (Cunningham et al., 2005).

To better understand how contaminants affect the Antarctic marine ecosystem, recent research has developed toxicity test protocols for a range of Antarctic and subantarctic organisms (Duquesne and Liess, 2003; Gissi et al., 2015; Hill et al., 2009; Holan et al., 2016; King and Riddle, 2001; Lewis et al., 2016; Marcus Zamora et al., 2015; Runcie and Riddle, 2007; Sfiligoj et al., 2015). These studies have only investigated the toxic response of organisms to single contaminants, and do not assess interactive effects of mixtures, as would be found in the Antarctic marine environment.

The toxicity of single metals may not reflect their toxicity in a mixture. Competition at cellular binding sites, changes to uptake pathways, and upregulation of detoxification mechanisms can influence the exposure and the response of an organism to metal mixtures (Duval et al., 2015). Recent efforts to understand and model the toxicity of metal mixtures has focused on freshwater organisms. We are only aware of two metal mixture studies conducted on a marine organisms – a marine mussel and a sea urchin (Deruytter et al., 2017; Manzo et al., 2010). There is therefore a deficiency in our knowledge about the application of metal-mixture models to marine and Antarctic organisms. This has left a significant gap in our understanding of metal mixture toxicity in marine systems.

Two reference models are widely accepted for the determination of metal mixture toxicity: Independent Action (IA) and Concentration Addition (CA) (Berenbaum, 1985; Jonker et al., 2005). Both models assume that the toxicity of the mixture components are non-interactive; but, have different approaches to combine individual contaminants' toxicities. IA calculates the toxicity of a mixture as the product of the individual contaminant's toxicity, making the

assumption that they have dissimilar modes of action. The CA model expresses each contaminant of the mixture as a toxic unit (TU), calculated as the concentration of the contaminant in the mixture divided by a measure of its potency, such as a 10% effect concentration (EC10). CA assumes that contaminants within a mixture have a similar mode of action, and that contaminants are dilutions of each other. Both models rely on single-metal toxicity data and assume no interactions between contaminants. However, deviations from model predictions are often observed. These interactions are described as: (i) synergism, where observed is greater than predicted toxicity, and; (ii) antagonism, where observed toxicity is less than predicted (Cedergreen, 2014). Mixtures are described as non-interactive where observed toxicity is predicted by the model.

The use of EC10 values as sensitivity estimates in ecotoxicology is growing because of its preferred use in the derivation of environmental quality guidelines (Batley et al., 2014; Warne et al., 2014). This shift towards the use of EC10s may be because EC50 effects are too high to tolerate under a precautionary principle or EC50s are typically not environmentally realistic concentrations. Previous research has found that mixtures of contaminants at  $\leq$ EC10 may produce mixture interactions (Nys et al., 2017b). Yet, only a few studies have investigated environmentally relevant concentrations in chronic ecotoxicology studies (Nys et al., 2017b; Versieren et al., 2016).

Mixture models could help guide environmental assessment and decision making for sites impacted by contaminant mixtures in Antarctica. Under the Protocol on Environmental Protection to the Antarctic Treaty System, nations operating in Antarctica have a responsibility to ensure effective remediation of historical waste and to prevent further contamination (ATCM, 1991). Yet little progress in the remediation of historical waste has been made (Filler et al., 2015), with some exceptions (Errington et al., 2018; Snape et al., 2001; Stark et al., 2006), and routine monitoring of contamination and impacts is lacking (Braun et al., 2012; Hughes, 2010). A Clean-Up Manual has been developed to provide practical advice to national Antarctic programs about environmental management practices (CEP, 2013). However, this manual is yet to include environmental quality guidelines and lacks recommendations around the risk of contaminant mixtures. For temperate environments, CA is recommended as a good 'first tier' assessment of sites impacted by contaminant mixtures due to its generally conservative nature (Nagai and De

Schamphelaere, 2016), which therefore tends to overestimate the toxicity of a mixture (Backhaus and Faust, 2012; Hochmuth et al., 2014; Versieren et al., 2016). Before existing contaminant mixture models can be applied to polar marine environments, however, research is needed to validate their suitability to polar marine organisms.

With only two metal mixture studies using marine species (using two invertebrates) reported to date, this study utilises organisms from an additional trophic level; marine microalgae. Microalgae are important components of the Antarctic marine ecosystem. *Phaeocystis antarctica* (Phylum *Haptophyta*) is a common marine microalga associated with open marine waters and is known to be mucogenic (Rousseau et al., 2007). *Cryptothecomonas armigera* (Phylum *Cercozoa*) is a single-celled flagellated heterotrophic protist found in both Arctic and Antarctic marine waters, commonly associated with sea ice, and known to produce significant intracellular lipid stores (Kühn et al., 2000; Thaler and Lovejoy, 2012). These species are both important components as primary producers in Antarctic marine food webs, and have different sensitivities to single metal contaminants (Gissi et al., 2015; Koppel et al., 2017).

This study aims to: (i) investigate the toxicity of Cd, Cu, Ni, Pb, and Zn mixtures as chronic population growth rate inhibition to the Antarctic microalgae *P. antarctica* and *C. armigera* using an equitoxic and environmental mixture. Cellular biomarkers including chlorophyll *a* fluorescence intensity, changes to cell size and complexity, and relative intracellular lipid concentrations will also be investigated. (ii) Assess the applicability of two reference models, IA and CA, to predict chronic toxicity as microalgae population growth rate inhibition, parameterised to use EC10 values. (iii) Investigate metal-mixture interactivity to *P. antarctica* and *C. armigera* by IA and CA from the equitoxic and environmental mixture.

## Methods

### Laboratory techniques

Glassware used in culturing and toxicity tests were nitric acid washed (10% v/v HNO<sub>3</sub> AR grade, Merck) for ≥24 h and rinsed with high purity water (Milli-Q®, 18MΩ.cm<sup>-1</sup>; Merck). Borosilicate 250 mL conical flasks used in tests were coated in a silanising solution (Coatasil, Ajax) to prevent metal adsorption. Plastic containers and consumables used were either new or acid-washed.

All chemicals were analytical grade or higher. Metal stock solutions were prepared in high purity water from metal salts, CuSO<sub>4</sub>, 3CdSO<sub>4</sub>.8H<sub>2</sub>O, PbCl<sub>2</sub>, NiSO<sub>4</sub>.6H<sub>2</sub>O, and ZnSO<sub>4</sub> acidified to 0.1% v/v HCl.

### Seawater

Seawater was collected from Oak Park, Cronulla on the east coast of Australia. Seawater was filtered to 0.22 or 0.45 µm, depending on the test species (Table 1) and stored in the dark at 4 °C. Background metal concentrations in the seawater were measured by inductively coupled plasma – mass spectrometry (see Metal analysis) and were 0.013 ± 0.002 µg Cd.L<sup>-1</sup>, 0.88 ± 0.03 µg Cu.L<sup>-1</sup>, 0.47 ± 0.02 µg Ni.L<sup>-1</sup>, 0.82 ± 0.02 µg Pb.L<sup>-1</sup>, and 3.9 ± 0.1 µg Zn.L<sup>-1</sup>. Dissolved organic carbon concentrations were analysed by total organic carbon analyser after 0.45 µm filtration and found to be 1.4 ± 0.3 mg C.L<sup>-1</sup>. Salinity (salinity and conductivity meter, model 30/10 FT; YSI), pH (measured at the start and end of each test, model 420, probe ROSS 815600; Thermo Fischer Scientific), and dissolved oxygen saturation (Oximeter 330; WTW) were measured with instruments calibrated as per the manufacturer's instructions.

### Algal culture

*Phaeocystis antarctica* (strain number AAD 133) and *Cryptothecomonas armigera* (strain number AAD 139) were obtained from the Australian Antarctic Division, Kingston, Tasmania. Cultures of *P. antarctica* and *C. armigera* were maintained as per Gissi et al. (2015) and Koppel et al. (2017), respectively. Cultures were grown in a temperature-controlled incubator at 2 ± 2 °C, with a 20:4-h light:dark ratio and light intensity of 70 ± 20 µmol.m<sup>-2</sup>.s<sup>-1</sup> photosynthetically active radiation.

**Table 1. Culture and toxicity test conditions for the Antarctic microalgae *Cryothecomonas armigera* and *Phaeocystis antarctica*.**

Culturing conditions		
Temperature	2 ± 2 °C	
pH	7.9 ± 0.2	
Salinity	35 ± 1 ‰	
Light Intensity	70 ± 20 μmol.m <sup>-2</sup> .s <sup>-1</sup> , white 5050 SMD type LED	
Light Cycle	20:4 h light:dark	
Toxicity test conditions <sup>a</sup>		
Test type	Static/non-renewal; flasks swirled twice a week to promote gas exchange	
Test chamber	Erlenmeyer glass flasks, silanised Polycarbonate lids	
Initial bioassay cell density	1-3 x10 <sup>3</sup> cells.mL <sup>-1</sup>	
Test endpoints	<ul style="list-style-type: none"><li>• Growth rate inhibition</li><li>• Cell size and complexity</li><li>• Chlorophyll <i>a</i> fluorescence</li><li>• Cellular lipid concentration (BODIPY 493/503)<sup>b</sup></li></ul>	
Test acceptability	16-fold increase in control cell density (OECD, 2011)	
	<i>P. antarctica</i>	<i>C. armigera</i>
Test duration	10 d	24 d
Age of culture <sup>c</sup>	8 - 12 d	25-30 d
Diluent water	0.45 μm filtered seawater <sup>d</sup>	0.22 μm filtered seawater <sup>e</sup>

<sup>a</sup> Toxicity test bioassay conditions also incorporated the culture conditions

<sup>b</sup> BODIPY 493/503 was only applicable to *Cryothecomonas armigera*

<sup>c</sup> To ensure cells were in exponential growth phase for the start of test

<sup>d</sup> As per Gissi et al. (2015)

<sup>e</sup> As per Koppel et al. (2017)



### **Toxicity test protocol**

Filtered seawater was used as the control/diluent water in toxicity tests. Metal exposure treatments were prepared in 80 mL of seawater, supplemented with 0.15 mg.L<sup>-1</sup> PO<sub>4</sub><sup>3-</sup> and 1.5 mg.L<sup>-1</sup> NO<sub>3</sub><sup>-</sup> (as KH<sub>2</sub>PO<sub>4</sub> and NaNO<sub>3</sub>, respectively) to maintain exponential growth of the algae during tests.

Two metal mixtures were tested: an equitoxic mixture and an environmental mixture. The equitoxic mixture is a combination of Cd, Cu, Ni, Pb, and Zn at concentrations equivalent to the population growth rate inhibition EC10s for each alga determined from single metal tests (Gissi et al., 2015; Koppel et al., 2017), given in Table 2 and 3. The environmental mixture reflects the dissolved metal concentrations in Brown Bay, a historically-contaminated marine site near Australia's Casey Station in East Antarctica (Larner et al., 2006). The environmental mixture was tested at increasing multiples to produce a concentration gradient of metals fixed at an environmentally realistic ratio.

Algal cells in exponential growth phase (8-12 days for *P. antarctica* and 25-30 days for *C. armigera* (Gissi et al., 2015; Koppel et al., 2017)), were washed to remove culture growth medium by centrifugation and resuspension in cold filtered seawater three times. The algal concentrate was then used to inoculate flasks at the start of tests at a density of 1-3 x10<sup>3</sup> cells.mL<sup>-1</sup> (day 0).

### **Measures of toxicity**

A BD-FACSVerse flow cytometer was used with a 488 nm excitation laser and multiple detectors to quantify cell density and the fluorescence and light scattering of individual cells in solution following methods described in Franklin et al. (2005), with specific modifications for *P. antarctica* and *C. armigera* outlined by Gissi et al. (2015) and Koppel et al. (2017), respectively.

Population growth rate was determined by a linear regression of cell densities over multiple time points (at least 4) during the exposure. The population growth rate (specific growth rate,  $\mu$ ) was calculated from the regression of a plot of log<sub>10</sub> (cell density) versus time (hours) and was converted to equivalent population doublings per day).

Biomarkers of algal health were determined by fluorescence and light scattering of individual cells, including: cell size (forward-angle scattering <15°), cell complexity (side-

angle scattering 90°), chlorophyll *a* fluorescence intensity (700 ± 27 nm band filter), and relative intracellular lipid concentrations (green fluorescence from the intracellular stain BODIPY 493/503 measured with a 527 ± 16 nm band filter) (Kerker, 1983). For each parameter, the fluorescence intensity of exposed cell populations was compared to control cell populations by establishing three regions on a one-dimensional plot of the parameter of interest (e.g. forward-angle scatter, red 700 nm fluorescence). Control cells were used to define a healthy region, R2, which contained >95% of the control populations. Non-overlapping regions of increased (R3) and decreased (R1) fluorescence intensity were established to monitor shifts in fluorescence in metal-exposed cell populations. Results are presented as percent of the cell population in the R2 region (Koppel et al., 2017).

### **Metal analysis**

Subsamples (5 mL) of test solutions were taken from each flask at the start and end of each test (Table 1), filtered to <0.45 µm, acidified to 0.2% HNO<sub>3</sub>, and analysed for dissolved metal analysis using an inductively coupled plasma-atomic emission spectrometry (ICP-AES; Varian 730-ES) or inductively coupled plasma – mass spectrometry (ICP-MS; Agilent 7500CE) depending on detection limits required. Calibration standards were matrix-matched to 0.2% acidity and 35‰ salinity. Matrix-matched blanks were used to determine detection limits, which were on average: 0.3 µg Cd.L<sup>-1</sup>, 1.1 µg Cu.L<sup>-1</sup>, 1.6 µg Ni.L<sup>-1</sup>, 3.3 µg Pb.L<sup>-1</sup>, and 1.7 µg Zn.L<sup>-1</sup> for ICP-AES and 0.001 µg Cd.L<sup>-1</sup>, 0.007 µg Cu.L<sup>-1</sup>, 0.005 µg Ni.L<sup>-1</sup>, 0.003 µg Pb.L<sup>-1</sup>, and 0.05 µg Zn.L<sup>-1</sup> for ICP-MS. For samples in a seawater matrix, a 200 µg. L<sup>-1</sup> multi-element drift standard (QCS27; Analytical West Inc.) was used to correct for measurement drift over time. Reported metal concentrations for each test treatment are the mean of initial and final dissolved metal concentrations.

### **Statistical analysis**

Measures of toxicity were expressed as a relative effect (RE, Equation 1) as a percentage of control response, where *y* is the response of the exposure, and  $\bar{x}_{y \text{ control}}$  is the mean of the control response (Nys et al., 2016).

**Equation 1** 
$$RE (\% \text{ of control}) = \frac{y}{\bar{x}_{y \text{ control}}} * 100$$

The analysis of the toxicity of metal mixtures uses previously published single-metal toxicity data for *P. antarctica* (Gissi et al., 2015) and *C. armigera* (Koppel et al., 2017). Data for *P.*

*antarctica* was reanalysed to determine EC10 values for population growth rate, cellular chlorophyll *a* fluorescence, size, and complexity using the statistical approach in the R environment outlined in Koppel et al. (2017).

Two reference models of mixture toxicity were tested to predict the toxicity of the metal-mixture ratios:

**(i) Independent Action**

Independent Action was determined by Equations 2-5, where:  $y_i$  is the response of the alga to metal  $i$  if it were exposed to a single metal at the same concentration found in the mixture.  $y_i$  was determined by log-logistic model described by Equation 2, where  $x_i$  is the concentration of metal<sub>*i*</sub>,  $\beta$  is the slope parameter, and EC50 is the concentration of metal<sub>*i*</sub> which causes a 50% reduction in the response variable  $y_i$ .

**Equation 2** 
$$y_i = \frac{100}{1 + \left(\frac{x_i}{EC50}\right)^\beta}$$

This study used EC10 values, rather than EC50s to calculate  $y_i$ . To do this, EC50 parameters were converted to EC10s by Equation 3 (Nys et al., 2017b), giving Equation 4.

**Equation 3** 
$$EC50_i = EC10_i * 9^{\frac{1}{\beta_i}}$$

**Equation 4** 
$$y_i = \frac{100}{1 + \left(\frac{x_i}{EC10_i * 9^{\frac{1}{\beta_i}}}\right)^{\beta_i}}$$

Independent Action response predictions were then determined as the product of the expected response of individual metals, shown by Equation 5.

**Equation 5** 
$$y_{IA} = 100 * \prod_{i=1}^n \frac{1}{1 + \left(\frac{x_i}{EC10_i * 9^{\frac{1}{\beta_i}}}\right)^{\beta_i}}$$

**(ii) Concentration Addition**

Concentration Addition predictions ( $y_{CA}$ ) were determined by Equations 6 and 7 where  $x_i$  is the concentration of metal  $i$  in the mixture, EC10<sub>*i*</sub> is the concentration of metal<sub>*i*</sub> that causes a 10% inhibition to population growth rate, and  $\sum TU_{EC10}$  is the sum of the toxic unit contribution for each metal where each toxic unit is equivalent to a 10% reduction in

population growth rate.

**Equation 6** 
$$\sum TU_{EC10} = \sum_{i=1}^n \frac{x_i}{EC10_i}$$

**Equation 7** 
$$\sum_{i=1}^n \frac{x_i}{(EC10_i * 9^{\frac{1}{\beta_i}}) * (\frac{100-y_{CA}}{y_{CA}})^{\frac{1}{\beta_i}}} = 1$$

The model parameters (i.e.  $EC_{10i}$  and  $\beta_i$  values for each metal) used in the reference mixture models were optimised prior to predicting metal-mixture toxicities as per the method given by Hochmuth et al. (2014). A random normal distribution of 40,000 sets of model parameters were generated about the means of each parameter. The mean and standard deviations of each  $EC_{10i}$  and  $\beta_i$  parameter were taken from the previously determined log-logistic model fits of single metal concentration-response curves for each microalga. Each parameter set was used to predict single-metal toxicity from existing single-metal toxicity datasets of *P. antarctica* and *C. armigera*. The parameter set that gave the lowest sum of squared error (SSE) was chosen to then predict the metal-mixture toxicities. The best parameters and SSE for each model are given in Supplementary information S1. The model predictions for single-metal concentration-response curves are given in Supplementary information S2 and S3 for *P. antarctica* and *C. armigera*, respectively.

### ***Determination of significant mixture interactivity***

To determine if there were significant synergistic or antagonistic deviations from the reference models, the IA and CA models were extended to include a deviation parameter 'a'. These are termed IASA and CASA and described by Equation 8 and 9, respectively (Hochmuth et al., 2014). To test if the deviation is significant, a nested F-test was conducted to determine whether the extended model provides for a significantly better fit than the reference model (Hochmuth et al., 2014; Jonker et al., 2005).

**Equation 8** 
$$y = 100 * \Phi(\Phi^{-1}(\prod_{i=1}^n \frac{1}{1 + (\frac{x_i}{EC10_i * 9^{\frac{1}{\beta_i}})}^{\beta_i})} + \frac{a * (\prod_{i=1}^n TU_i)}{(\sum_{i=1}^n TU_i)^2})$$

**Equation 9** 
$$\sum_{i=1}^n \frac{x_i}{(EC10_i * 9^{\frac{1}{\beta_i}}) * (\frac{100-y}{y})^{\frac{1}{\beta_i}}} = exp(\frac{a * (\prod_{i=1}^n TU_i)}{(\sum_{i=1}^n TU_i)^2})$$

An example R script for this approach with *C. armigera*, adapted from Hochmuth et al. (2014), can be found with all data in this study at the Australian Antarctica Data Centre

(Jolley et al., 2018). The parameter pairs used in the reference models (IA and CA) and their extensions (IASA and CASA) are given in Supplementary information S1.

## **Results and Discussion**

### **Metal mixtures**

#### ***Equitoxic mixture***

The equitoxic mixture tested 5 metals at their population growth inhibition EC10 concentrations, to *P. antarctica* and *C. armigera*, respectively (Tables 2 and 3). Nickel was not toxic to *P. antarctica* in single-metal exposures but was included in this mixture to observe competition effects. The EC10 is increasingly used in place of the EC50 as a basis for environmental quality guidelines for single contaminants (Warne et al., 2014). For some contaminants the reliability of higher effect measures (such as EC50s) may be confounded by the solubility limit of metals in marine waters. For example, at a concentration of 1,800  $\mu\text{g Pb.L}^{-1}$ , which is approximately the Pb solubility limit in seawater, the population growth rate of *C. armigera* was 75% of the control (Koppel et al., 2017). In such cases, these contaminants may contribute to toxicity or interactivity when present in a mixture and using their EC10 value then allows them to be included in reference models. This has been demonstrated as necessary, as mixtures with concentrations  $\leq \text{EC10s}$  have demonstrated mixture interactivity, such as synergism and antagonism (Nys et al., 2017b; Versieren et al., 2016).

#### ***Environmental mixture***

The environmental mixture was based on measurements from marine waters in the historically contaminated Brown Bay near Australia's Casey Station. This site had a metal ratio of 1 Cu : 0.1 Cd : 0.3 Ni : 0.3 Pb : 4.5 Zn (multiple of 1, in  $\mu\text{g.L}^{-1}$ ). While these concentrations are low, it is expected that concentrations will be much higher immediately adjacent to contaminant point sources or closer to the sediment-water interface (Amato et al., 2015, 2014). For example, freshwater melt pools in the Thala Valley tip, which drains into Brown Bay, had concentrations of 443  $\mu\text{g Cu.L}^{-1}$ , 1476  $\mu\text{g Pb.L}^{-1}$ , and 3045  $\mu\text{g Zn.L}^{-1}$  prior to any clean-up or remediation efforts (Snape et al., 2001).

The environmental mixture has a high Cu concentration relative to Cd, Ni, Pb, and Zn when

compared against individual toxicity, i.e. Cu is the most toxic metal to both microalgae by 1 to 2 orders of magnitude with EC10s of 2.8 and 22  $\mu\text{g.L}^{-1}$  to *P. antarctica* and *C. armigera*, respectively (Gissi et al., 2015; Koppel et al., 2017). Therefore, it was expected that Cu would be the main contributor to observed toxicity. Zinc was present at the highest concentration of all the metals in the ratio. However, it generally had a low toxicity to both algae in single-metal exposures, with EC10s of 217 and 366  $\mu\text{g.L}^{-1}$  to *P. antarctica* and *C. armigera*, respectively.

### ***Phaeocystis antarctica***

#### ***Growth rate inhibition***

The equitoxic mixture reduced population growth rate to  $67 \pm 8\%$ , relative to the control treatment (Fig. 1, Table 2). Nickel was not toxic in single-metal exposures but was included in the equitoxic mixture at  $420 \pm 70 \mu\text{g.L}^{-1}$  to investigate any contribution to mixture interactivity. There were no significant differences in the population growth rate, cellular chlorophyll fluorescence, or cellular size or complexity when compared to the equitoxic mixture treatments that excluded Ni (Supplementary information S4). Therefore, equitoxic mixture exposures with and without Ni were pooled for the analysis. The environmental mixture tested multiples of the ratio from 5 to 60 (Table 2). This mixture was toxic to population growth rate in a concentration-dependent manner, decreasing population growth rate to 10% at a multiple of 40.

#### ***Cellular chlorophyll a fluorescence***

The equitoxic ratio resulted in an approximately equal proportion of the cell population with an increased and decreased fluorescence intensity; however, 81% of the cell population remained unaffected. The environmental mixture increased fluorescence intensity in a concentration-dependent manner (Fig. 1 B). In single-metal exposures to *P. antarctica*, Cu and Ni increased chlorophyll *a* fluorescence intensity, Pb decreased fluorescence intensity, and Cd and Zn caused greater variability (i.e. approximately equal increases and decreases). The different fluorescence shifts suggest different modes of action of individual metals. However, in single-metal exposures these were generally low responses; <30% difference to the response in control populations at all concentrations tested. The increased variability observed in response to the equitoxic mixture could suggest non-interactive toxicity, but the

relative contributions of each metal and underlying mechanism is unknown. The environmental mixture reflects a Cu-only response with a greater magnitude of shift e.g. 70% population shift at a multiple of 20 with a Cu concentration of 14  $\mu\text{g.L}^{-1}$  which was the chlorophyll *a* fluorescence EC50 in single-metal exposures (Supplementary information S5).

### ***Cell size and complexity***

The equitoxic mixture increased the size of cells in 17% of the cell population and decreased complexity in 11% of the population. The environmental mixture increased the variability of cell size at multiples  $\geq 5$ , which was coupled with a concentration-dependent increase to complexity in 100% of the cell population (Fig 1. C and D).

In single metal exposures, the complexity and size of *P. antarctica* cells were affected by Cd and Cu, but not by Ni, Pb, or Zn. Exposure to Cu  $\geq 6 \mu\text{g.L}^{-1}$  increased the range of cell sizes observed and increased complexity in a maximum of 50% of the cell population (Supplementary information S5). The single-metal Cu response is consistent with the response to the environmental mixture; however, the magnitude of the response is much greater in the environmental mixture at comparable single Cu exposure concentrations.

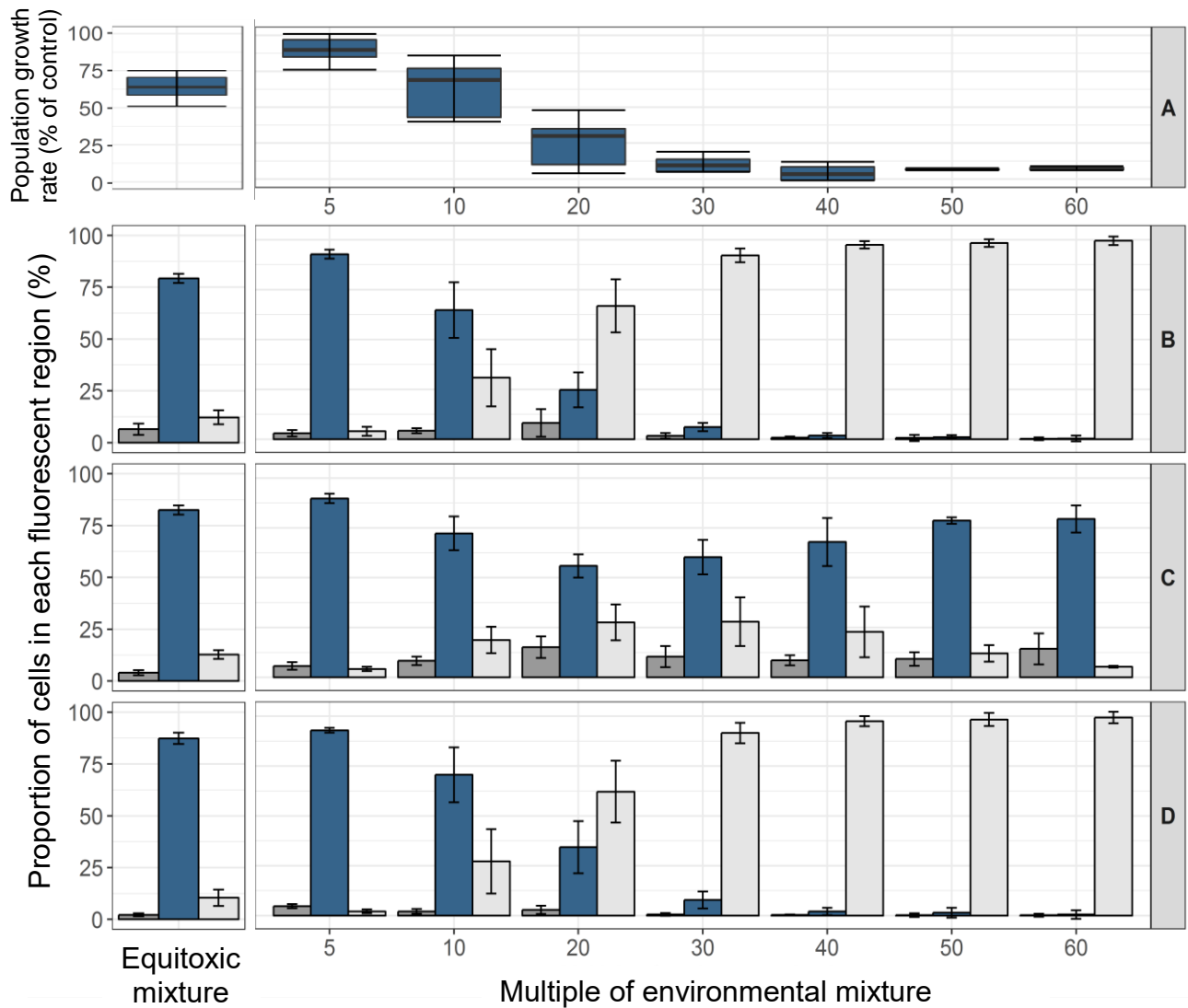
Table 2. Metal mixture toxicity test exposure concentrations and test results for the Antarctic microalgae *Phaeocystis antarctica*. Measurements are means  $\pm$  standard deviations. (a) Metal concentrations of metal mixture treatments; (b) Toxicity of metal mixtures; (c) Independent Action (IA) and Concentration Addition (CA) reference model predictions.

Phaeocystis antarctica									
Metal	Nominal population growth rate EC10 mean (95% CIs)	Equitoxic mixture	Environmental mixture <sup>a</sup>						
			5	10	20	30	40	50	60
(a) Metal concentration (µg.L <sup>-1</sup> )									
Cd	163 (0-373)	160 ± 10	0.6 ± 0.1	1.1 ± 0.1	2.3 ± 0.2	3.5 ± 0.1	4.7 ± 0.2	5.3 ± 0.1	6.5 ± 0.1
Cu	2.8 (2.2 - 3.3)	2.7 ± 0.4	3.1 ± 0.3	6.2 ± 0.7	13 ± 1	21.7 ± 0.3	29.7 ± 0.4	37.4 ± 0.2	45.9 ± 0.3
Ni	>1070	420 ± 70	2.1 ± 0.6	3.7 ± 0.3	6 ± 1	10.4 ± 0.7	12 ± 1	15 ± 1	19.8 ± 0.5
Pb	150 (61 - 240)	180 ± 50	0.8 ± 0.5	1.3 ± 0.9	3.6 ± 0.8				3.7 ± 0.9
Zn	217 (77 - 356)	350 ± 120	18 ± 1	38 ± 3	83 ± 3	127 ± 3	172 ± 4	213 ± 2	263 ± 3
(b) Observed toxicity (% of control) <sup>b</sup>									
Population growth rate		67 ± 8	89 ± 9	76 ± 6	35 ± 9	15 ± 3	9 ± 3	7 ± 1	7 ± 2
Chlorophyll <i>a</i> fluorescence		81 ± 4	93 ± 3	76 ± 9	31 ± 7	7 ± 2	3 ± 1	1.0 ± 0.4	0.3 ± 0.6
Cellular size		83 ± 4	90 ± 2	79 ± 5	60 ± 5	67 ± 2	77 ± 6	79 ± 1	79 ± 3
Cellular complexity		89 ± 3	93 ± 2	82 ± 7	44 ± 10	11 ± 3	3 ± 2	1 ± 1	0.5 ± 0.9
(c) Model predictions (% of control)									
CA		52 ± 6	88 ± 5	60 ± 18	15 ± 5	3.8 ± 0.1	1.8 ± 0.1	0.97 ± 0.01	0.58 ± 0.01
IA		66 ± 9	87 ± 6	56 ± 18	14 ± 5	3.6 ± 0.1	1.68 ± 0.05	0.95 ± 0.01	0.56 ± 0.01

<sup>a</sup> Numbers 5 to 60 indicate nominal multiples of the environmental mixture used as the concentration gradient in experiments with *P. antarctica*

<sup>b</sup> Note that the observed toxicity for relative chlorophyll *a* fluorescence, cellular size, and cellular complexity represents the percent similarity to the control cell population. It does not show the direction (increase or decrease in fluorescence intensity), which can be seen in Fig. 1.





**Figure 1.** The toxicity of the equitoxic and environmentally relevant metal mixtures to the Antarctic microalgae *Phaeocystis antarctica*. Measures of toxicity are: (A) population growth rate inhibition, (B) cellular chlorophyll *a* fluorescence intensity, (C) cellular size, and (D) cellular complexity, all expressed as percent of control cell populations  $\pm$  95% confidence intervals. Boxplots indicate mean (central line), upper and lower quartiles (upper and lower shaded box, respectively).

## ***Cryothecomonas armigera***

### ***Growth rate inhibition***

The equitoxic mixture reduced population growth rate to  $54 \pm 3\%$ . For the environmental mixture, the population growth rate was unaffected at multiples below 30; above this there was a concentration-dependent decrease of the growth rate to  $4 \pm 5\%$  at a multiple of 100 (Table 3, Fig. 2 A).

### ***Cellular chlorophyll *a* fluorescence***

The equitoxic mixture showed approximately equal proportions of the cell population with increased and decreased fluorescence intensity, with a combined  $26 \pm 4\%$  of the population affected. The environmental mixture increased chlorophyll *a* fluorescence in up to 30% of the population until a multiple of 50. At multiples greater than this, 62% of the cell population had decreased fluorescence at a multiple of 100 (Table 3, Fig. 2, B).

In single-metal exposures, Cd, Cu, Ni, and Pb increased fluorescence intensity, while Zn caused a decrease. By single-metal responses, the concentrations present in both mixtures suggests only a small proportion of the cell population should be affected (see chlorophyll *a* fluorescence EC10 values, Supplementary information S5). The response to the equitoxic mixture agrees with this expectation. However, the toxicity of the environmental mixture was unexpected. At multiples up to 50, chlorophyll *a* fluorescence was increased, suggesting Cu toxicity. Above this, fluorescence decreased, suggesting Zn toxicity. Furthermore, the population shift was greater than what could be explained solely by single-metal toxicity.

### ***Cell size and complexity***

The equitoxic mixture had a small effect on cell size and complexity, with 10% of the cell population affected (Table 3, Fig. 2 C and D). In exposures to the environmental mixture, size increased in 20% of the population and complexity decreased in 30% of the population (Fig. 2 C and D).

Single-metal treatments (except Cd) affected the size of *C. armigera*, with Ni, Pb, and Zn reducing size in a maximum of 10% of the population, while Cu increased cell size in 70% of the population (Koppel et al., 2017). However, only Cd reduced cell complexity, with an EC10 of  $269 \mu\text{g.L}^{-1}$  (Supplementary information S5). The equitoxic mixture had concentrations of Cd, Cu, and Ni that were expected to reduce cell size, and a concentration

of Cd expected to reduce cell complexity based on single-metal exposures. However, this was not observed in the equitoxic mixture. Exposure to the environmental mixture increased size in a population of cells roughly equivalent to the population with decreased complexity. These changes could be related, as an increased size in the absence of intracellular changes could lead to a perceived decreased complexity. The mechanism behind the change in size is unknown but could be due to differences in osmotic pressure resulting from changes to membrane permeability (Jamers et al., 2009).

### ***Intracellular lipid concentrations***

The equitoxic mixture resulted in a reduction of intracellular lipid concentrations, with  $27 \pm 11\%$  of the exposed population having decreased green fluorescence compared to control populations (Fig. 2 E). Despite having lower metal concentrations, the environmental mixture resulted in a greater reduction of lipid concentrations. However, this was not concentration dependent with 40-60% of the cell population having reduced lipids at multiples >10.

In single-metal exposures,  $Pb > Cu > Cd$  elicited the greatest effect on lipids, with EC10s of 11, 33, and  $89 \mu\text{g.L}^{-1}$ , respectively. Changes to lipid concentrations were a more sensitive measure of toxicity than changes in growth rate for Pb and Cd. The response to the equitoxic ratio was less than expected based on the concentrations of Cd, Cu, and Pb. Curiously, there were no differences to lipid concentrations in exposures of equitoxic mixtures where Cd was excluded or included at  $50 \mu\text{g.L}^{-1}$  or  $500 \mu\text{g.L}^{-1}$  (Supplementary information S6), despite Cd showing high lipid-reducing toxicities at these concentrations in single-metal exposures (Koppel et al., 2017).

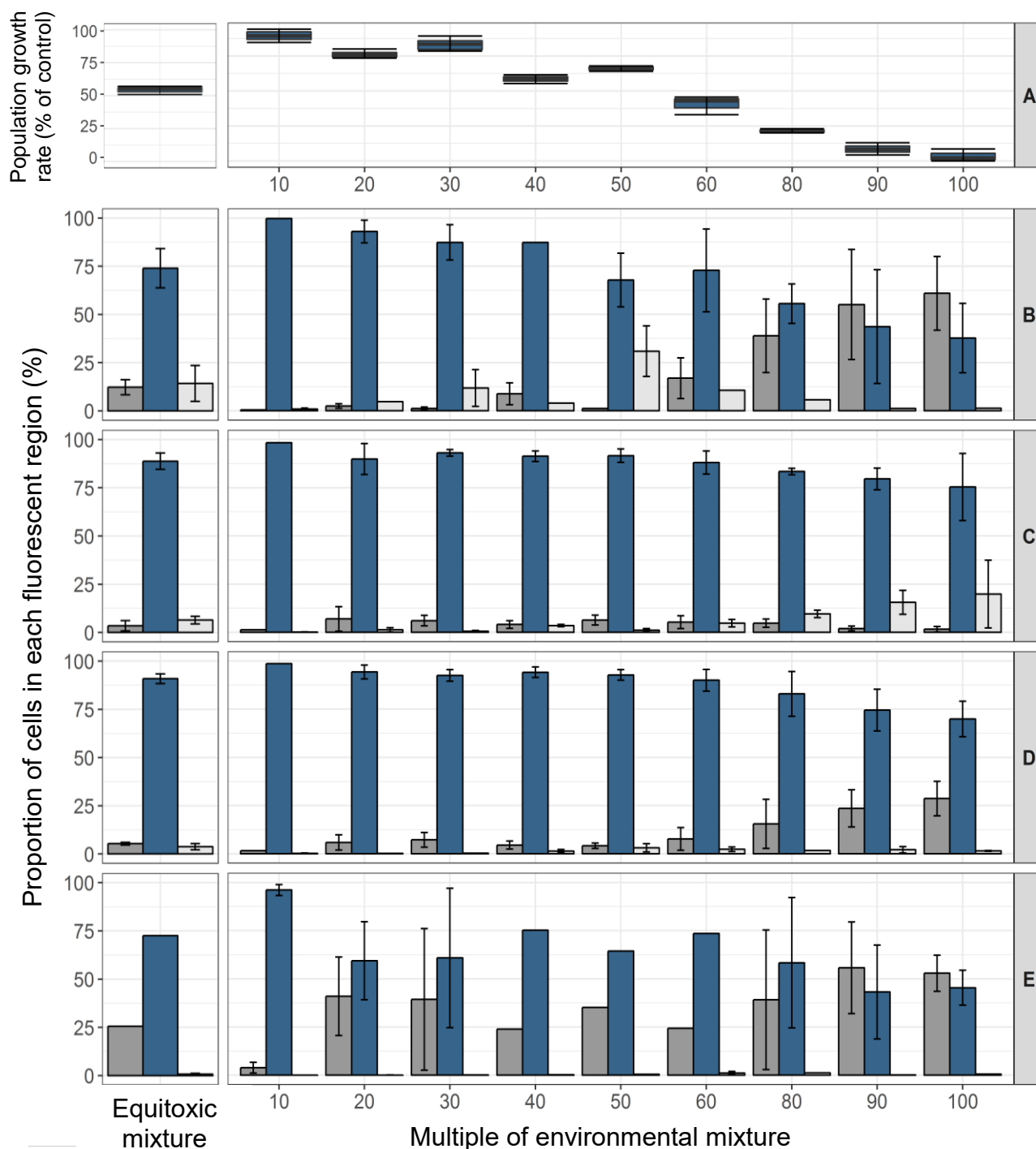
The environmental mixture elicited unexpected results. At a multiple of 50, only Cu and Zn concentrations are equivalent to their single-metal intracellular lipid EC10 (i.e., no toxicity to lipid concentrations was expected at lower multiples, and less toxicity was expected in the 50x multiple compared to the equitoxic mixture). This may be explained by cellular detoxification and division. Where cell division is not greatly inhibited, lipids may be diluted as cells divide, especially when coupled with energetically expensive detoxification mechanisms (Farese and Walther, 2009; Lavoie et al., 2016). That is, at low toxicities (normal growth rates) lipid concentrations may be most affected. However, this wasn't observed, with variable but roughly equivalent reductions at all multiples >10 (Fig. 2 E).

Table 3. Metal mixture toxicity exposures and results for the Antarctic microalgae *Cryothecomonas armigera*. Measurements are means  $\pm$  standard deviation. (a) Metal concentrations of metal mixture treatments; (b) Toxicity of metal mixtures; (c) Independent Action (IA) and Concentration Addition (CA) reference model predictions.

Cryothecomonas armigera											
Metal	Nominal population growth rate EC10 mean (95% CIs)	Equitoxic mixture	Environmental mixture <sup>a</sup>								
			10	20	30	40	50	60	80	90	100
(a) Metal concentration (µg.L <sup>-1</sup> )											
Cd	454 (225 - 682)	497 ± 2	1.2± 0.4	2.7 ± 0.4	3.7 ± 0.5	4.6 ± 0.2	5.7 ± 0.6	6.3 ± 0.1	8.5 ± 0.2	9.5 ± 0.3	10.4 ± 0.2
Cu	22 (18 - 26)	22 ± 1	7 ± 1	12 ± 2	20 ± 5	28.0 ± 0.3	40.6 ± 0.6	42.5 ± 0.1	57.8 ± 0.4	65.6 ± 0.5	72.5 ± 0.1
Ni	1220 (1107 - 1335)	1220 ± 10	2.6 ± 0.5	5.9 ± 0.7	8.2 ± 0.7	12.0 ± 0.3	13.8 ± 0.2	17.3 ± 0.4	22.8 ± 0.3	26.1 ± 0.4	29.3 ± 0.4
Pb	152 (78 - 300)	156 ± 2	3 ± 1	4 ± 1	7 ± 4	8 ± 1	19 ± 1	10.7 ± 0.6	15.0 ± 0.6	17 ± 1	18.1 ± 0.8
Zn	366 (41 - 691)	347 ± 5	40 ± 1	77 ± 5	125 ± 6	167 ± 3	230 ± 1	256 ± 5	345 ± 4	388 ± 6	441 ± 4
(b) Observed toxicity (% of control) <sup>b</sup>											
Population growth rate		54 ± 3	107 ± 5	91 ± 4	100 ± 5	70 ± 4	79 ± 2	49 ± 8	26 ± 2	10 ± 5	4 ± 5
Chlorophyll <i>a</i> fluorescence		74 ± 4	100 ± 2	93 ± 25	87 ± 9	87 ± 5	68 ± 6	73 ± 9	56 ± 4	44 ± 12	38 ± 7
Cellular size		89 ± 2	98 ± 2	90 ± 5	93 ± 2	91 ± 1	92 ± 1	88 ± 2	83 ± 1	80 ± 2	75 ± 7
Cellular complexity		91 ± 1	99 ± 2	94 ± 5	92 ± 3	94 ± 1	93 ± 1	90 ± 2	83 ± 5	75 ± 4	70 ± 4
Relative lipid concentration		73 ± 11	96 ± 3	59 ± 29	61 ± 34	75 ± 17	64 ± 15	74 ± 12	58 ± 14	43 ± 10	45 ± 4
(c) Model predictions (% of control)											
CA		31.4 ± 0.8	95.3 ± 0.6	92 ± 1	86 ± 4	77.7 ± 0.4	62.6 ± 0.7	60.1 ± 0.2	43.7 ± 0.4	37.1 ± 0.4	32.0 ± 0.1
IA		58.1 ± 0.4	93.3 ± 0.6	90 ± 1	84 ± 5	75.9 ± 0.4	60.1 ± 0.7	58.4 ± 0.2	41.5 ± 0.4	34.7 ± 0.4	29.5 ± 0.1

<sup>a</sup> Numbers 10 to 100 indicate nominal multiples of the environmental mixture used as the concentration gradient in experiments with *C. armigera*

<sup>b</sup> Note that the observed toxicity for relative chlorophyll *a* fluorescence, cellular size, cellular complexity, and relative lipid concentration represents the percent similarity to the control cell population. It does not show the direction (increase or decrease in fluorescence intensity), which can be seen in Fig. 2.



**Figure 2.** The toxicity of the equitoxic and environmental metal mixtures to the Antarctic microalgae *Cryothecomonas armigera*. Measures of toxicity are: (A) population growth rate inhibition, (B) cellular chlorophyll *a* fluorescence intensity, (C) cellular size, (D) cellular complexity, and (E) relative intracellular lipid concentrations, all expressed as percent of control cell populations  $\pm$  95% confidence interval. Boxplots indicate mean (central line), upper and lower quartiles (upper and lower shaded box, respectively).

## Mixture interactivity

### *Equitoxic mixture*

The equitoxic mixture had metals at concentrations that summed to  $\Sigma TU_{EC10}$  of  $4.5 \pm 0.8$  for *P. antarctica* and  $6.63 \pm 0.04$  for *C. armigera*, which resulted in population growth rates of  $67 \pm 8\%$  for *P. antarctica* and  $54 \pm 3$  for *C. armigera*, Fig. 3. Both microalgae had similar responses to the equitoxic mixture; observed toxicity was well predicted by IA while CA overestimated toxicity, i.e. was antagonistic. The antagonism as measured by CA was significant for both algae, but the power of the Tukey's HSD test for *C. armigera* was low due to the low number of replicates,  $n=3$ . IA calculates mixture toxicity from individual contaminants under the assumption of independent modes of action, whereas CA considers contaminants to have the same mode of action. The equitoxic mixture specifically investigated how metals may interact at concentrations where they are known to exert equivalent toxicity. That IA better predicted toxicity than CA suggests that the metals have a dissimilar mode of action, rather than joint modes of toxicity (Berenbaum, 1985).

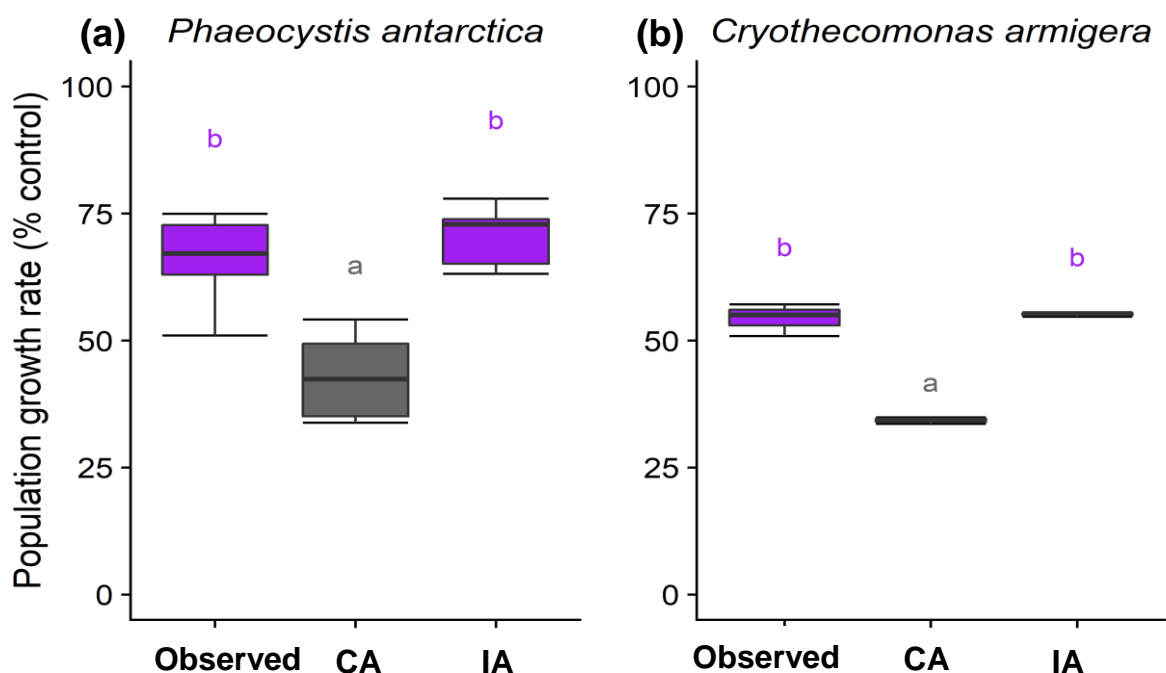


Figure 3. Toxicity of the equitoxic metal mixture to Antarctic microalgae: (a) *Phaeocystis antarctica*, and (b) *Cryothecomonas armigera*. Population growth rate presented as observed toxicity and predicted toxicity based on the Concentration Addition (CA) and Independent Action (IA) model. The observations or model prediction were compared to each other by Tukey's Honest Significant Difference. Different letters represent significant differences ( $P < 0.05$ ).

### ***Environmental mixture***

Observed and predicted toxicities from the environmental mixture to the population growth rates of *P. antarctica* and *C. armigera* was concentration-dependent and well described by a log-logistic model (Fig. 4). Both models gave similar predictions to both algae; however, each alga had a different response. Antagonism to *P. antarctica* and concentration-dependent mixture interactivity to *C. armigera*, where there was antagonism at low and synergism at high effect concentrations (Fig. 4 A and B, respectively).

The only significant mixture interaction ( $p < 0.05$ ) for the environment metal mixture ratio was antagonism measured by IA to *P. antarctica* ( $p = 3.37 \times 10^{-5}$ , Supplementary information S1). No other interaction was significant for either model or algae. Antagonism by CA to *P. antarctica* was not significant despite its predictions being very similar to IA.

The response to *C. armigera* was not significant, possibly because the mixture interactivity was concentration-dependent. That is, significant mixture interactivity at high or low concentrations is masked when the response across the whole concentration range is assessed (Fig. 4 B). In such cases, a full factorial experimental design (rather than individual ratios) or a different statistical approach assessing concentration-dependent mixture interactivity may be needed (Deruytter et al., 2017; Nys et al., 2015). While the synergism by IA and CA to *C. armigera* at high toxicities was not determined to be significant, similar responses are well documented with freshwater organisms (Cedergreen, 2014; Nys et al., 2017b, 2015). However, this is not always the case, with Ni recently found to induce synergism at low concentrations and antagonism at high concentrations when in a binary mixture with Cu (Deruytter et al., 2017).

It was expected that Cu would cause the most toxicity, given it is the most toxic single metal to both microalgae and present in the ratio at the second highest concentration. Zinc was less toxic to both algae but was present at 4.5 times the concentration (in  $\mu\text{g.L}^{-1}$ ) of Cu. To both algae, exposures  $\geq 20$  of the environmental mixture had Zn concentration within the 95% confidence interval of the single-metal Zn EC10. To *P. antarctica* this caused antagonism, but to *C. armigera* this led to concentration-dependent interactions with antagonism up to a 50% effect, beyond which synergism was observed.

Cellular biomarkers give clues to the mechanisms behind these results. The changes to

chlorophyll *a* fluorescence in *C. armigera* were coincident with the mixture interactivity shift from antagonism to synergism in population growth rate. The fluorescence shifts suggest toxicity from Cu at multiples <50 and Zn at multiples >50, thus Zn may be antagonist at low concentrations but synergistic at high concentrations. However, changes to cell size and complexity suggest Cd toxicity, possibly affecting membrane permeability. So, although Cu is the most toxic and Zn the most abundant, the influence of Cd, Ni, and Pb cannot be discounted. Especially considering in the equitoxic mixture they were non-interactive by IA and antagonistic by CA.

The antagonism observed to *P. antarctica* may be due to the production of dissolved organic carbon or detoxification mechanisms as inferred from cellular biomarkers. *P. antarctica* is known to produce dissolved organic carbon to increase Fe and Zn uptake in the Southern Ocean (Alderkamp et al., 2012; Saito and Goepfert, 2008). Dissolved organic carbon is known to bind strongly to Cu, effectively reducing its toxicity (Wood et al., 2011). This may explain the observed antagonism in the environmental mixture. In contrast, less is known about the dissolved organic carbon production of *C. armigera*, but it is not known to be mucogenic.

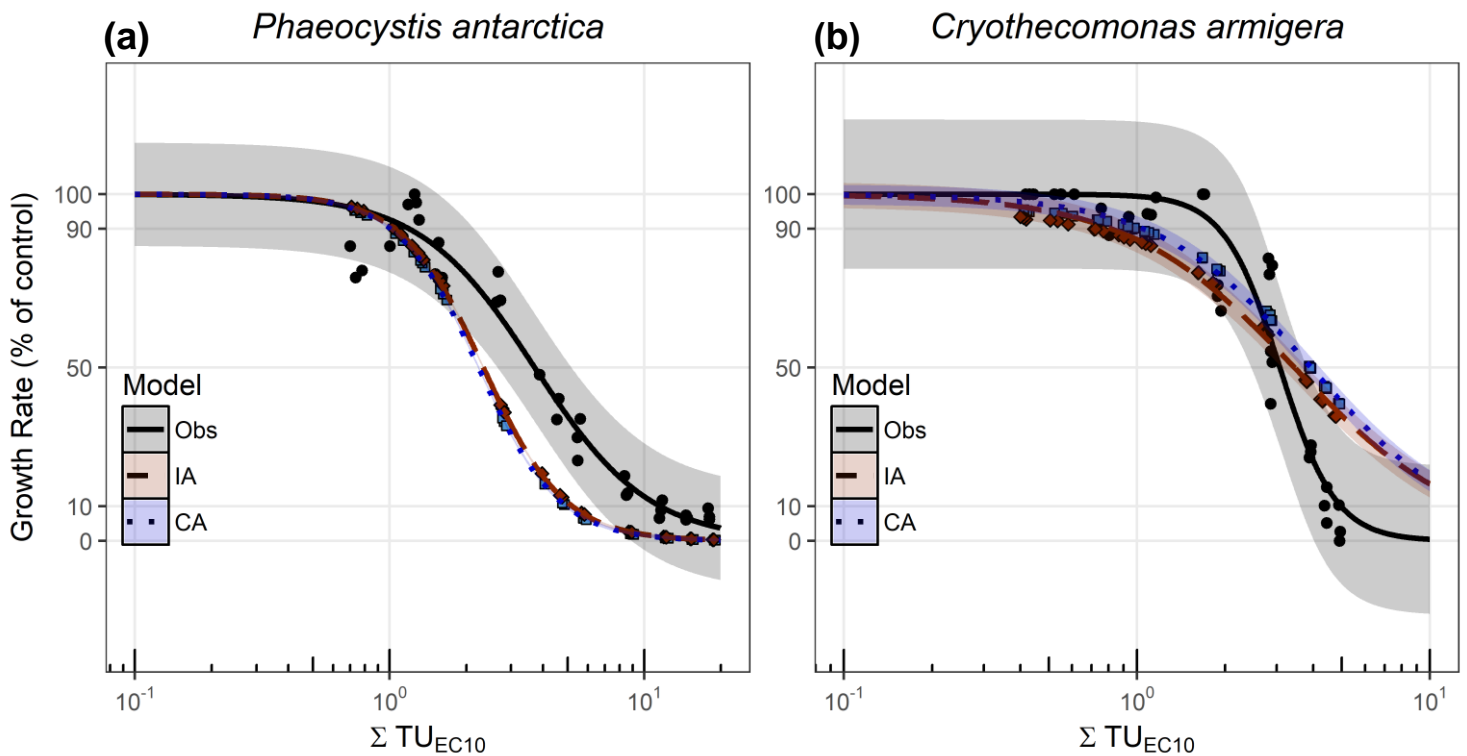
A large proportion of *P. antarctica* had increased cellular complexity at low concentrations of the environmental mixture, compared to *C. armigera* (Fig. 1 D compared to Fig. 2 D). This could indicate detoxification of metals by intracellular sequestration in *P. antarctica*.

Previous research has shown Cu can be sequestered by polyphosphate bodies or phytochelatins to intracellular bodies, which would be observed as an increase to cell complexity (Adams et al., 2016; Levy et al., 2008). Such a response wasn't observed in *C. armigera*, suggesting different detoxification mechanisms (Fig. 2 D).

Different chlorophyll *a* fluorescence changes in the microalgae suggest different metals are causing toxicity. *P. antarctica* had a greater proportion of cells affected than *C. armigera*, and the fluorescence shift was different. *P. antarctica* had a consistent increase, while *C. armigera* had an increase up to multiples of 50 followed by a decrease in fluorescence intensity. The increase in fluorescence intensity suggests *P. antarctica* and *C. armigera* had an impaired electron transport chain in the photosynthesis pathway (Guo and Tan, 2015), possibly a result of Cu toxicity. The decrease in fluorescence intensity in *C. armigera* suggests Zn toxicity, possibly impairing the light harvesting apparatus of chlorophyll



moieties (Küpfer and Andresen, 2016). These changes in biomarkers indicate different physiological responses to metal mixtures and give insight into mechanisms behind these differences.



**Figure 4.** The observed and modelled toxicity of increasing multiples of the environmental mixture to the population growth rate of Antarctic microalgae (a) *Phaeocystis antarctica* and (b) *Cryothecomonas armigera*. Observed toxicity is represented by black circles, Independent Action (IA) predictions by red diamonds, and Concentration Addition (CA) by blue squares. A log-logistic model was fitted to observed toxicities (solid black line) and IA (red dashed line) and CA (dotted blue line) predictions. Ribbons represent the 95% confidence interval of the models.

## Metal mixture modelling for environmental management

Studies of the toxicity of metal mixtures are predominately freshwater based, with few studies investigating mixture toxicity to marine organisms, such as the mussel *Mytilus edulis* (Deruytter et al., 2017) or sea urchin *Paracentrotus lividus* (Manzo et al., 2010). The response of freshwater organisms may not be comparable to those in seawater; however, the metal-organism interactions that cause toxicity may be similar. These include complex specific- and non-specific uptake processes, metal-specific modes of toxicity, specific or general detoxification mechanisms, and rapid homeostatic regulation of cellular physiology (Cedergreen, 2014; Versieren et al., 2017). The mixture interactivity found in this study is largely within the range observed in freshwater microalgae.

Most metal-mixture studies only investigate binary metal mixtures. For example, in freshwater studies to green microalga Zn has shown to protect against Cd to *Chlamydomonas reinhardtii* (Lavoie et al., 2014) and Pb to *Micrasterias denticulate* (Volland et al., 2014). While Cd has been shown to be synergistic in the presence of Cu to *Chlorella* sp. (Franklin et al., 2002). This study found that the equitoxic mixture was non-interactive by IA and antagonistic by CA and the environmental mixture was found to elicit more complicated interactions which were microalgae dependent. More research is needed to investigate more complicated metal mixtures, which may better represent environmental contamination.

Previous studies consistently find that CA tends to overestimate toxicity (Nys et al., 2018). This has been observed both in metal mixture studies (Nagai and De Schamphelaere, 2016; Nys et al., 2017a, 2015) and in studies with organic contaminants (Cedergreen et al., 2008). As a result, CA is recommended as a conservative first-tier screening model for the environmental management of contaminant mixtures (Cedergreen, 2014; Nys et al., 2018). This trend was observed in the equitoxic mixture, but not in the environmental mixture, where both models gave largely equivalent predictions to the environmental mixture (see SSEs in Supplementary information S1). At low concentrations (<1 TU), the environmental mixture has a more realistic ratio and metal concentrations than the equitoxic mixture. Thus, either IA or CA should be suitable to predict toxicity to *P. antarctica* or *C. armigera* in environmentally realistic conditions.

## Conclusion

This study demonstrated that mixture interactivity of five metals could be ratio, concentration, and microalgae specific. Adapting IA and CA mixture reference models to use EC10 values as parameters allowed for the inclusion of metals which individually only exhibited low toxicities. This will be beneficial for future studies as the use of low-effect toxicity values such as EC10s, particularly in environmental quality guideline derivation, grows.

The equitoxic mixture was non-interactive by IA and antagonistic by CA to both *P. antarctica* and *C. armigera*. This implies a greater likelihood that the contaminants have dissimilar modes of action. The environmental mixture was antagonistic by IA and CA to *P. antarctica*, with IA being a significant interaction. To *C. armigera*, the environmental mixture was concentration-dependent with antagonism at low effect concentrations and synergism at high effect concentrations by both models. Mixture interactions in both *P. antarctica* and *C. armigera* were observed concurrent to changes in biomarkers, including chlorophyll *a* fluorescence, cell complexity, and lipid concentrations. These biomarkers give insights into toxicity and detoxification mechanisms.

Frameworks designed to manage the environmental risk of metal mixtures typically recommend a 'first-tier' screening using a CA model (Backhaus and Faust, 2012; Farley et al., 2015). This study showed either models are suitable at environmentally realistic ratios and concentrations of metal mixtures, where there were equivalent predictions from both models to both microalgae. However, a better understanding of mixture interactivity to other Antarctic marine organisms is still needed guide environmental management.

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<http://dx.doi.org/10.4225/15/5ae93ff723ff8>

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## **Supplementary Information**

### **Chronic toxicity of an environmentally relevant and equitoxic ratio of five metals to two Antarctic marine microalgae shows complex mixture interactivity**

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## Supplementary information S1

Parameter estimates used in Independent Action (IA) and Concentration Addition (CA) reference models, and their extensions (designated CASA and IASA) used to assess the significance of mixture interactivity. The original parameter estimates were derived from log-logistic regressions from single-metal concentration-response curves for *Phaeocystis antarctica*<sup>8</sup> and *Cryptothecomonas armigera*<sup>38</sup>. From each of the original parameter estimates, 40,000 new parameters were generated about a random normal distribution and tested to determine which combination gave the lowest sum of squared error (SSE).

<i>P. antarctica</i>	$\beta$ Cd	E Cd	$\beta$ Cu	E Cu	$\beta$ Pb	E Pb	$\beta$ Zn	E Zn	sum of logs	SSE	a	p
Original	1.1 ± 0.2	216 ± 52	3.0 ± 0.3	2.8 ± 0.3	1.6 ± 0.4	150 ± 45	1.4 ± 0.3	217 ± 69	-	-	-	-
CA	1.0	211	2.9	2.7	1.5	138	1.4	247	322	23679		
CASA	1.1	196	3.0	2.7	1.5	153	1.4	271	320	23695	128	1
IA	1.1	230	3.0	2.7	1.9	175	0.8	178	348	26571		
IASA	1.1	201	2.9	2.5	1.1	124	1.9	380	315	24892	86	3.37 x10 <sup>-5</sup>

<i>C. armigera</i>	$\beta$ Cd	E Cd	$\beta$ Cu	E Cu	$\beta$ Ni	E Ni	$\beta$ Pb	E Pb	$\beta$ Zn	E Zn	sum of logs	SSE	a	p
Original	0.4 ± 0.2	440 ± 220	2.1 ± 0.2	22 ± 2	9 ± 1	1221 ± 56	0.31 ± 0.06	61 ± 33	0.5 ± 0.1	366 ± 161	-	-	-	-
CA	0.4	434	1.9	19	7.9	1166	0.4	89	0.7	495	236	11309		
CASA	0.4	408	1.9	20	8.5	1195	0.3	65	0.6	412	246	11262	76	0.30
IA	0.5	560	2.0	21	7.8	1153	0.4	88	0.6	444	233	11348	-	
IASA	0.3	366	2.0	21	7.8	1154	0.3	53	0.5	397	238	11312	157	0.36

$\beta$  = slope parameter of the log-logistic model fitted to the single-metal toxicity data

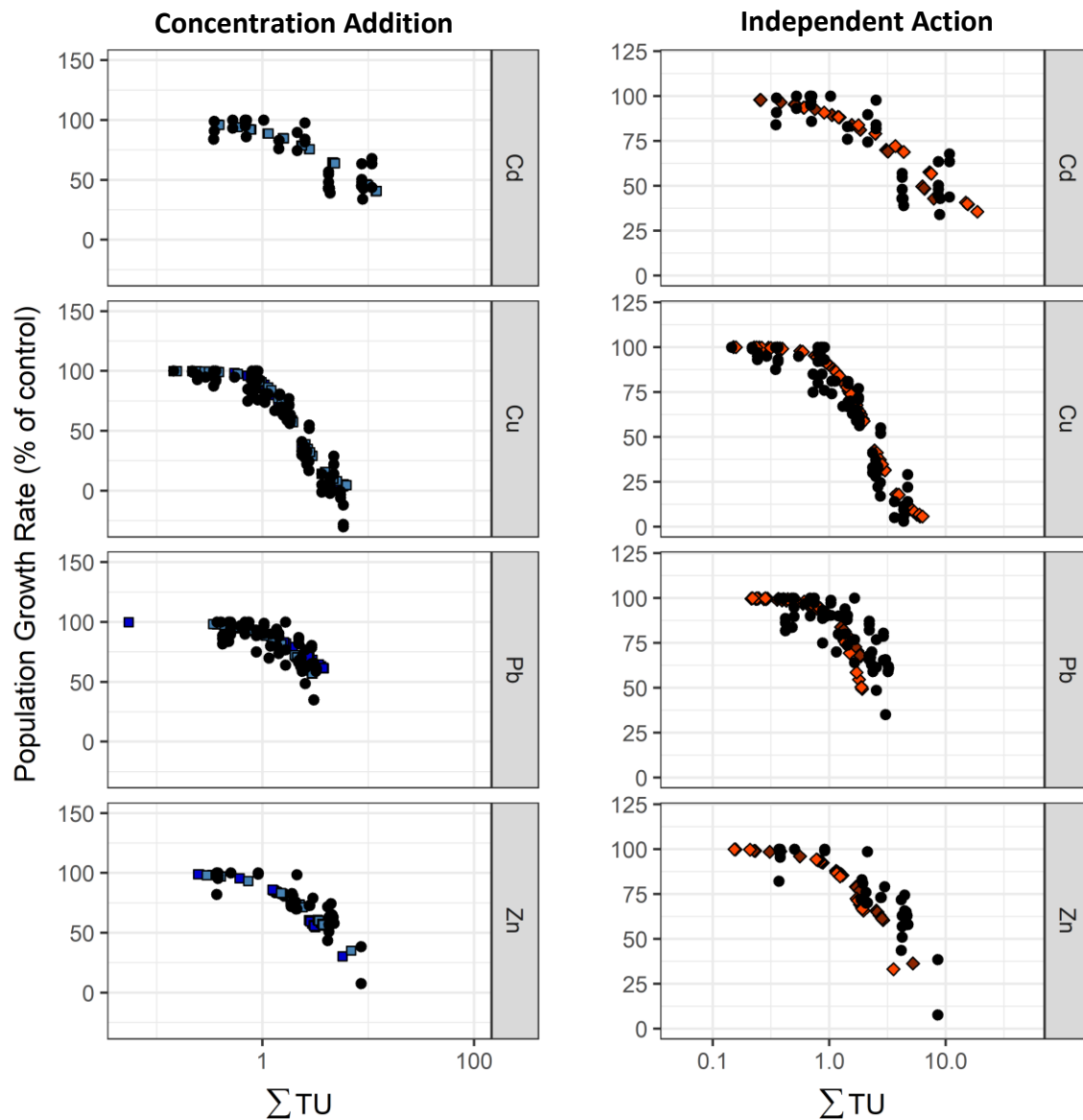
E = EC10 parameter used in lieu of the EC50 parameter of the log-logistic model fitted to the single-metal toxicity data

a = deviation parameter used to extend the IA and CA models to allow for mixture interactivity

p = significance of nested F-test between the extended and non-extended IA and CA models.

## Supplementary Information S2

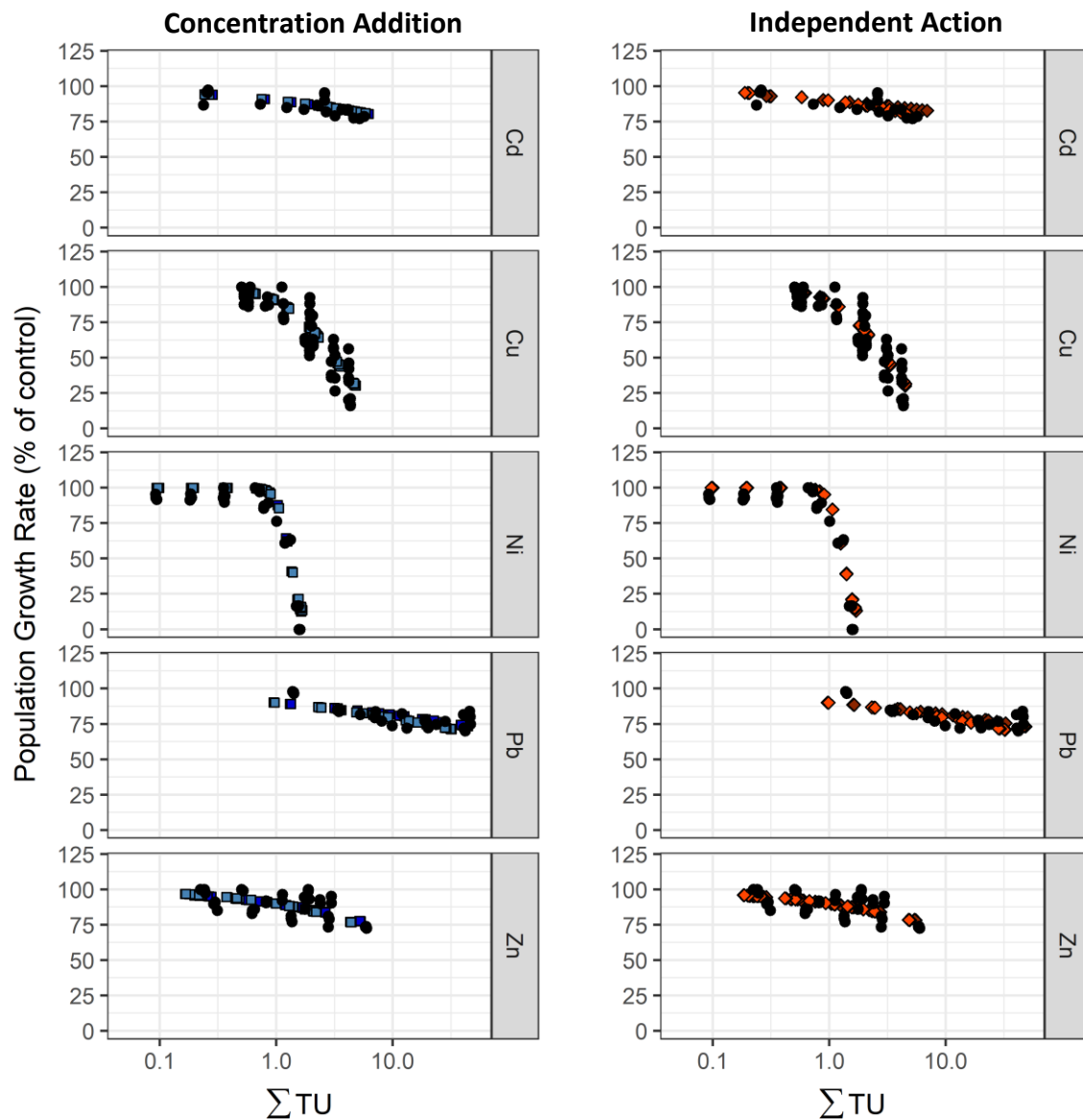
Toxicity and model predictions of single metals (Cd, Cu, Pb, and Zn) to Antarctic microalgae *Phaeocystis antarctica*. Observed toxicity for metals are given by black circles. Concentration Addition and its extension are given in the left column (dark and light blue squares, respectively) Independent Action and its extension are given in the right column (dark and light red diamonds, respectively). Observed toxicity data taken from Gissi et al. (2015).  $\Sigma TU$  = sum of toxic units in the exposure.



### Supplementary Information S3

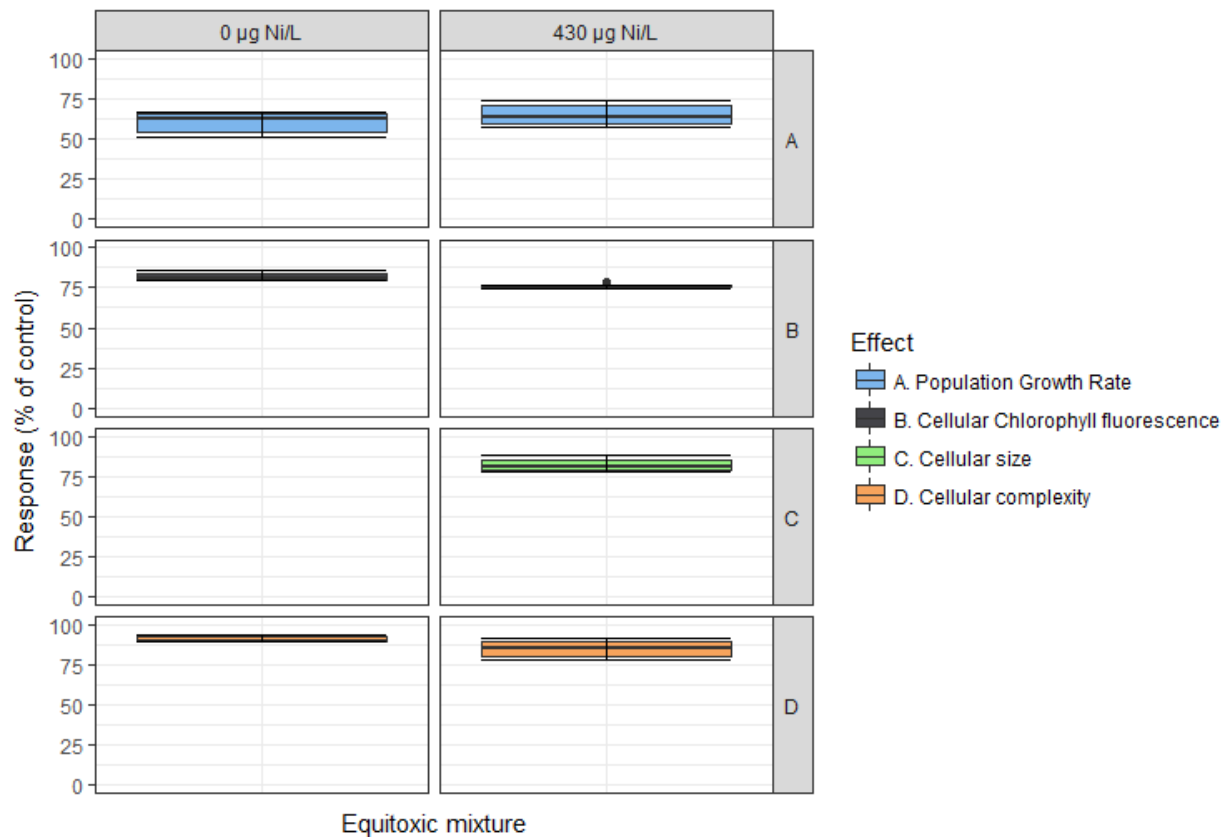
Toxicity and model predictions of single metals to *Cryptosporidium parvum*. Observed toxicity for Cd, Cu, Ni, Pb, and Zn are given by black circles. Concentration Addition and its extension are given in the left column (dark and light blue squares, respectively)

Independent Action and its extension are given in the right column (dark and light red diamonds, respectively). Observed toxicity data taken from Koppel et al. (2017).  $\Sigma TU$  = sum of toxic units in the exposure.



#### Supplementary Information S4

Comparison of the toxicity of the equitoxic mixture of metals (Cd, Cu, Pb, Zn) to *Phaeocystis antarctica* with and without Ni at 430  $\mu\text{g}\cdot\text{L}^{-1}$ . Nickel was included in mixtures to assess if it contributes to mixture interactivity. There was no significant difference in responses to the equitoxic mixture with and without Ni, so all treatments were pooled.



## Supplementary Information S5

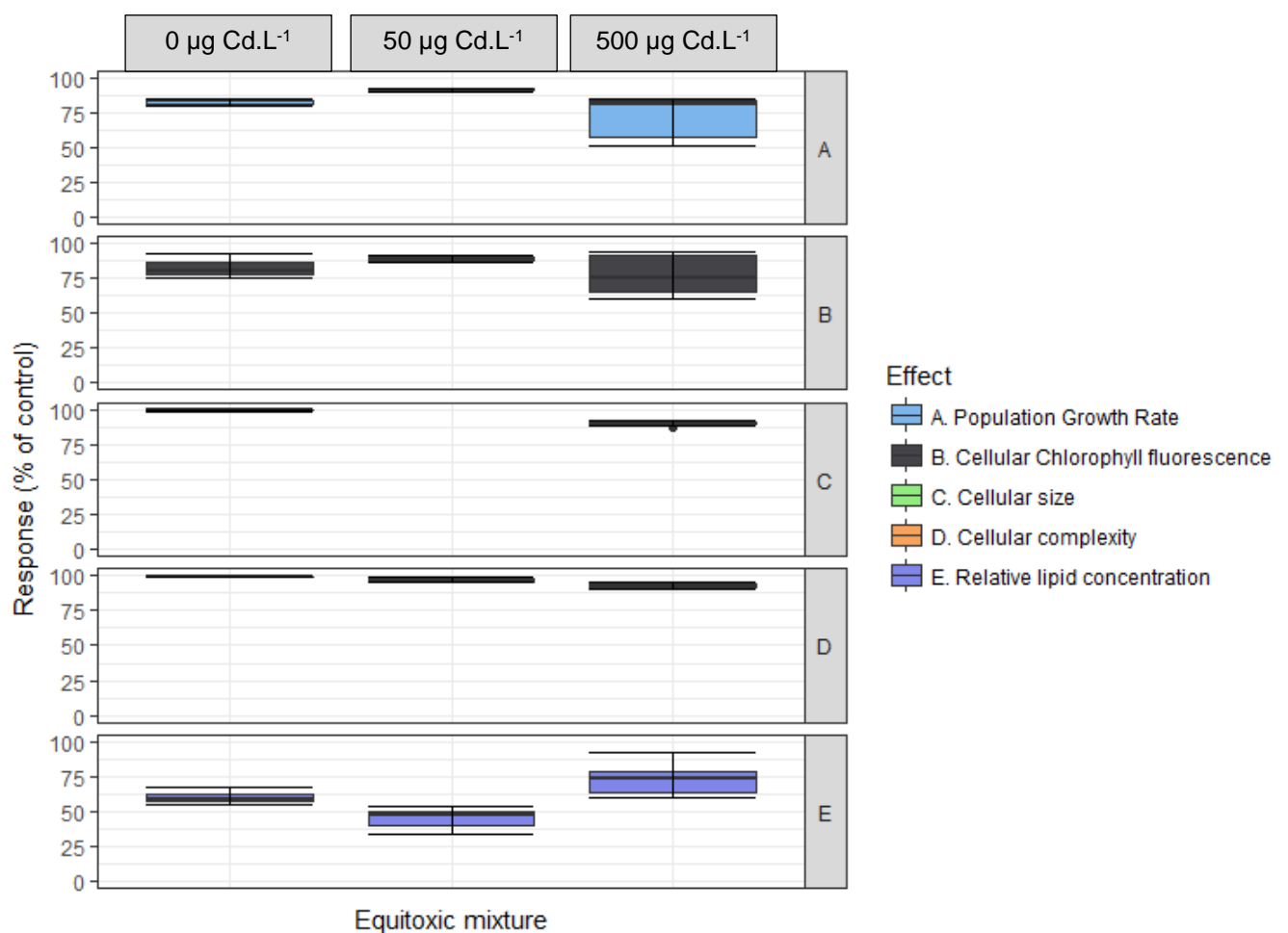
Metal toxicity to the Antarctic microalgae *Phaeocystis antarctica* and *Cryptothecomonas armigera*. EC10 and EC50 for population growth rate inhibition, cellular chlorophyll *a* fluorescence intensity, cellular complexity, cellular size, and cellular lipid content (for *C. armigera* only) are reported in  $\mu\text{g}\cdot\text{L}^{-1}$ .

<i>Phaeocystis antarctica</i>					
		Population growth rate	Cellular chlorophyll <i>a</i> fluorescence intensity	Cell complexity	Cell size
Cd	EC10	163 (0-373)	177 (84 - 270)	206 (86 - 326)	ND
	EC50	1430 (670 - 2200)	2400 (1580 - 3230)	2060 (1360 - 2760)	ND
Cu	EC10	2.8 (2.2 - 3.3)	2.8 (1.1 - 4.5)	2.6 (0 - 5.1)	4.5 (0 - 10.7)
	EC50	5.8 (5.3 - 6.2)	14.1 (11.8 - 16.4)	163 (0 - 401)	>16
Ni	EC10	>1070	196 (122 - 269)	73 (0 - 159)	726 (234 - 1220)
	EC50	>1070	>1070	>487	>1070
Pb	EC10	150 (61 - 240)	286 (0 - 603)	91 (0 - 239)	249 (0 - 821)
	EC50	574 (458 - 690)	>487	>1860	>487
Zn	EC10	217 ( 77 - 356)	536 (0 - 1260)	269 (178 - 359)	27.4 (0 - 199)
	EC50	1100 (888 - 1310)	>1860	1130 (1000 - 1260)	>1860

<i>Cryptothecomonas armigera</i>						
		Population growth rate	Cellular chlorophyll <i>a</i> fluorescence intensity	Cell complexity	Cell size	Cellular lipid concentration
Cd	EC10	454 (225 - 682)	1036 (179 - 1890)	269 (178 - 359)	>2530	89 (59 - 118)
	EC50	>2530	>2530	1130 (1000 - 1260)	>2530	280 (229 - 330)
Cu	EC10	10 (6 - 15)	>93	>93	37.9 (32.7 - 108)	33 (0 - 68)
	EC50	22 (18 - 26)	>93	>93	>93	>93
Ni	EC10	1220 (1107 - 1335)	1460 (1380 - 1550)	>1950	681 (0 - 2680)	894 (641 - 1150)
	EC50	1570 (1500 - 1630)	2000 (1950 - 2050)	>1950	>1950	1055 (100-1100)
Pb	EC10	152 (78 - 300)	807 (365 - 1250)	>2830	2140 (0 - 5950)	11 (0 - 31)
	EC50	>2830	>2830	>2830	>2830	163 (60 - 266)
Zn	EC10	366 (41 - 691)	987 (0 - 2000)	>2190	1340 (0 - 3340)	241 (0 - 948)
	EC50	>2190	>2190	>2190	>2190	>2190

## Supplementary Information S6

Comparison of the toxicity of the equitoxic mixture of metals (Cd, Cu, Ni, Pb, and Zn) to *Cryptomonas armigera* with Cd concentrations of 0, 50, and 500  $\mu\text{g.L}^{-1}$ . The concentration of Cd was adjusted to investigate how Cd influences cell size, complexity, and lipid concentration. In single metal exposures, Cd reduced the intracellular lipid concentrations of 50% of the cell population at a concentration of 280 (229 – 330)  $\mu\text{g.L}^{-1}$ . It also caused a coincident reduction in cellular complexity. Interestingly, there was no clear trend in intracellular lipid concentrations at increasing Cd concentrations, nor was there a change in cellular complexity which was expected from single-metal toxicity<sup>38</sup>.

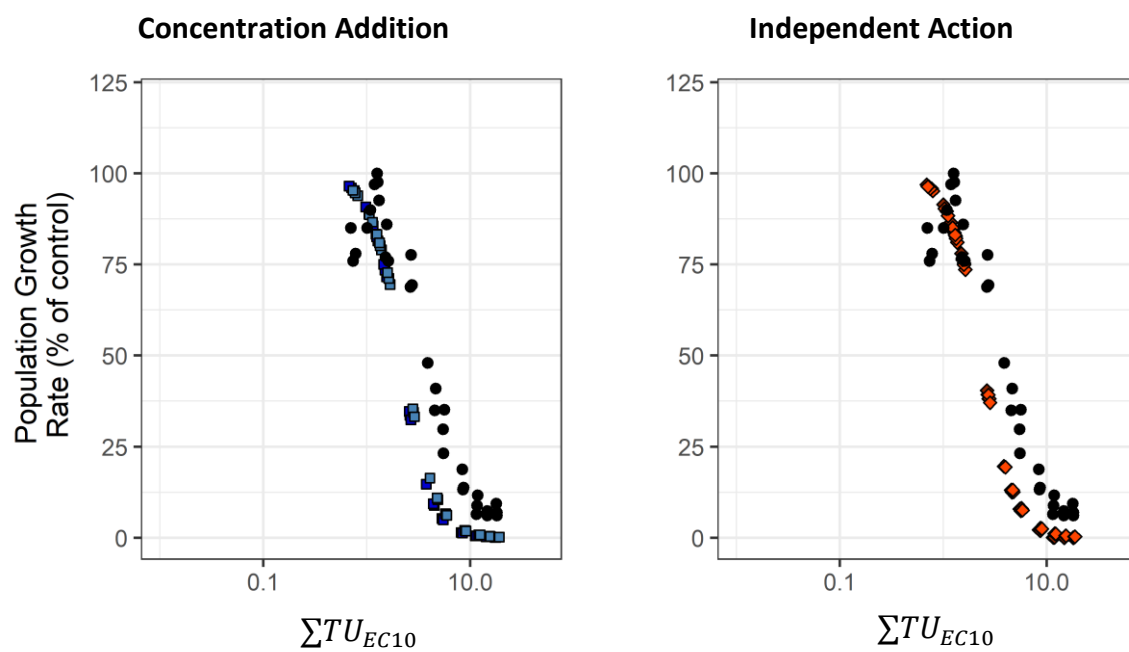




### Supplementary Information S7

Observed toxicity (black circles) and model predictions of the environmental mixture to A. *Phaeocystis antarctica* and B. *Cryothecomonas armigera*. Concentration Addition (CA) reference model and its extension are given in the left column (dark and light blue squares, respectively), Independent Action (IA) reference model and its extension are given in the right column (dark and light red diamonds, respectively).  $\sum TU$  = sum of Toxic Units in the exposure.

(a) *Phaeocystis antarctica*



(b) *Cryptothecomonas armigera*

