Preliminary study of cellular metal accumulation in two Antarctic marine microalgae – implications for mixture interactivity and dietary risk

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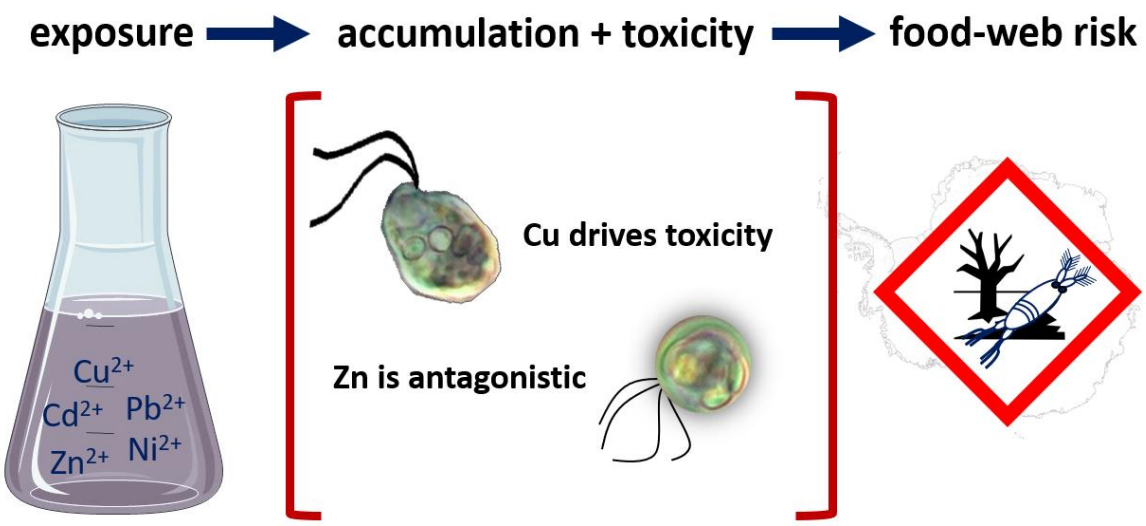

Abstract

Localised sites in Antarctica are contaminated with mixtures of metals, yet the risk this contamination poses to the marine ecosystem is not well characterised. Recent research showed that two Antarctic marine microalgae have antagonistic responses to a mixture of five common metals (Koppel et al., 2018a). However, the metal accumulating potential and risk to secondary consumers through dietary exposure are still unknown. This study investigates cellular accumulation following exposure to a mixture of cadmium, copper, nickel, lead, and zinc for the Antarctic marine microalgae, *Phaeocystis antarctica* and *Cryothecomonas armigera*. In both microalgae, cellular cadmium, copper, and lead concentrations increased with increasing exposures while cellular nickel and zinc did not. For both microalgae, copper in the metal mixture drives inhibition of growth rate with R^2 values >-0.84 for all cellular fractions in both species, while antagonism was likely caused by zinc competition, having significantly positive partial regressions. Metal accumulation to *P. antarctica* and *C. armigera* is likely to be toxic to consumer organisms, with low exposure concentrations resulting in cellular concentrations of 500 and 1400 $x10^{-18}$ mol Zn $cell^{-1}$ and 160 and 320 $x10^{-18}$ mol Cu $cell^{-1}$, respectively.

Capsule

Metal accumulation from mixtures in two Antarctic marine microalgae has the potential to cause dietary toxicity to secondary consumers in the Southern Ocean food web. Extra and intracellular partitioning shows that zinc is protective of copper toxicity at low effect concentrations.

Introduction

Antarctica is known to have contaminated sites in areas close to research stations which predominately occupy the scarce ice-free coastline (Hughes, 2010). Metals and other contaminants have been shown to leach from these sites into the marine environment during the summer melt (Palmer et al., 2010; Stark et al., 2006) and cause disturbances to the near-shore ecosystem (Cunningham et al., 2005). The contaminants can be exposed to organisms by dissolved (Larner et al., 2006) or particulate and dietary exposure routes (Cabrita et al., 2017; Hill et al., 2009).

To manage the risk of metals in the environment, environmental quality standards like water quality guidelines are used, for example, the Australian and New Zealand water quality guidelines (ANZG, 2018), the United States of America water quality criteria (Stephen et al., 1985), and the European Union environmental quality standards (European Parliament and Council, 2008). However, these are typically based on dissolved single metal exposures and fail to consider the risk of dietary exposure or metal mixture interactivity.

Exposure to metal mixtures may cause unexpected toxicities compared to single metal exposures. These include antagonism, where there is less toxicity than expected, and synergism, where there is more toxicity than expected (Cedergreen, 2014). In both cases, the toxicity of these metals is dependent on their ability to interact with cellular processes in the organisms. This may be observed as changes to metal accumulation and fractionation (Franklin et al., 2002; Nugroho et al., 2017; Saibu et al., 2018); however, too few studies have investigated this topic to draw general conclusions. Understanding the extra and intracellular metal partitioning of contaminants gives insight to their cellular regulation (Duval et al., 2015), toxicity (Lavoie et al., 2014; Zeng et al., 2009) and the dietary risk they pose within the food web (Luoma and Rainbow, 2005).

Recent research has highlighted the importance of testing contaminant mixtures, with complex mixture interactivity shown in two Antarctic microalgae, *Phaeocystis antarctica* and *Cryothecomonas armigera* (Koppel et al., 2018a). In that study, copper was expected to be driving toxicity to both microalgae, but could not explain the observed mixture interactivity (Koppel et al., 2018a). These microalgal species occupy different niches in the Antarctic marine environment: *P. antarctica* is a mucogenic prymnesiophyte (Alderkamp et al., 2007),

found in the open ocean and near-shore marine environment (Kang et al., 2001), while *C. armigera* is a heterotrophic protist (phylum Cercozoa) that is associated with sea-ice and feeds on other plankton (Thaler and Lovejoy, 2012; Thomsen et al., 1991). As microalgae, they are the primary producers in the marine ecosystem and are an important food source to a variety of Antarctic organisms. For example, the Antarctic krill *Euphausia superba* is known to graze on sea-ice microalgae as juveniles (Kohlbach et al., 2017) and on *P. antarctica* as adults (Haberman et al., 2003). Whether microalgae accumulate metals at concentrations which can cause toxicity in the Southern Ocean food web is not known; however, in temperate and tropical environments dietary exposure to metals is a significant source of toxicity to many secondary and tertiary consumers (DeForest and Meyer, 2015; Luoma and Rainbow, 2005).

This study investigated the cellular accumulation of five common metals (cadmium, copper, nickel, lead, and zinc) in two Antarctic marine microalgae *P. antarctica* and *C. armigera* from single and mixed metal exposures. It compares the extra and intracellular metal partitioning of metals in the context of understanding how cellular metal concentrations relate to previously reported toxicity endpoints for these microalgae. It also aims to investigate the risk this cellular metal accumulation poses to secondary consumers in the Southern Ocean food web.

Methods

Algal toxicity test protocol

Laboratory equipment and reagents

Glassware used in culturing and toxicity tests were nitric acid washed (10% v/v HNO_3 AR grade, Merck) for ≥24 h and rinsed with ultrapure water (Milli-Q®, 18 MΩ cm; 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 ultrapure water from metal salts, $CuSO_4$, $3CdSO_4.8H_2O$, $PbCl_2$, $NiSO_4.6H_2O$, and $ZnSO_4$ acidified to 0.1% v/v HCl.

Microalgae culturing

Two Antarctic strains of microalgae, *Phaeocystis antarctica* and *Cryothecomonas armigera* (strain numbers AAD 133 and AAD 139), were obtained from the Australian Antarctic Division, Kingston, Australia, and cultured 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^{-2} s^{-1} photosynthetically active radiation (Table 1).

Microalgae test protocol

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 by centrifugation to remove culture growth medium and resuspended in cold filtered seawater three times. Test flasks were inoculated with this algal concentrate at the start of tests at a density of 1-3 x10^3 cells mL^{-1} (day 0). The toxicity test was conducted for 10 and 24 days for *P. antarctica* and *C. armigera*, respectively, to achieve a 16-fold increase in cell density accounting for their different growth rates.

Population growth rate changes were assessed by flow cytometric analysis of the cellular population over time (Koppel et al., 2017) are reported in a previously published study

(Koppel et al., 2018a), and provided in the accompanying dataset to this paper (Koppel et al., 2018b).

Seawater

Filtered natural seawater was used as the control and diluent waters for all experiments. Seawater was filtered to 0.22 or 0.45 µm, depending on the test species (Table 1), after collection from Cronulla, New South Wales, Australia (34°04'13.4"S 151°09'24.5"E), and stored 4 °C in the dark. Background metal concentrations in the seawater were below limits of detection, reported in the Metal Analysis section. Salinity (salinity and conductivity meter, model 30/10 FT; YSI), pH (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.

Exposure treatments

Filtered seawater was used as the control and diluent water in toxicity tests. Metal exposure treatments were prepared in 80 mL filtered seawater, supplemented with 0.15 mg PO_4^{3-} L^{-1} and 1.5 mg $NO_3^-L^{-1}$ (as KH_2PO_4 and $NaNO_3$, respectively) to maintain exponential growth of the algae during tests (Franklin et al., 2001).

Two metal mixtures were tested, an equitoxic mixture and an environmental ratio mixture (Koppel et al., 2018a): (i) the equitoxic mixture was a combination of Cd, Cu, Ni, Pb, and Zn at concentrations equivalent to the population growth rate inhibition EC10 values for each alga determined from single metal tests (Gissi et al., 2015; Koppel et al., 2017). For *P. antarctica* the equitoxic mixture was tested at 0.5, 1, and 2 times the ratio. For *C. armigera* it was tested at 1 times the ratio; (ii) the environmental mixture reflects the dissolved metal concentrations in Brown Bay, a historically-contaminated marine site near Australia's Casey Station in East Antarctica at approximately 8 nmol Cu L^{-1}, 2 nmol Cd L^{-1}, 5 nmol Ni L^{-1}, 1 nmol Pb L^{-1}, and 69 nmol Zn L^{-1} (Larner et al., 2006). To produce a concentration gradient of metals fixed at an environmentally realistic ratio, the environmental mixture tested at set multiples of the original mixture ratio of metals. For *P. antarctica* the environmental mixture was tested at multiples of 5, 10, and 20 and for *C. armigera* 10, 20, 30, 40, 60, and 80.

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^{-2} s^{-1}, white 5050 SMD type LED	
Light Cycle	20:4 h light:dark	
Culture media	*P.* antarctica: 1/5 strength G media with added selenium[a] *C. armigera*: 1/2 strength F media[b]	
Toxicity test conditions[c]		
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 x10^3 cells mL^{-1}	
Test endpoint	Population growth rate inhibition	
Test acceptability	16-fold increase in control cell density (OECD, 2011)	
	P. antarctica	***C. armigera***
Test duration	10 d	24 d
Age of culture[d]	8 - 12 d	25 - 30 d
Diluent water	0.45 µm-filtered seawater[ae]	0.22 µm-filtered seawater[be]

[a] As per Gissi et al. (2015)

[b] As per Koppel et al. (2017)

[c] Toxicity test conditions were equivalent to the culture conditions except for the use of culture media

[d] To ensure cells were in exponential growth phase for the start of test

[e] Supplemented with 0.15 mg PO_4^{3-} L^{-1} and 1.5 mg $NO_3^-L^{-1}$

Determination of metal fractions

The cellular metal fractions referenced in this study are operationally defined as: (i) extracellular, the fraction of metal bound to the cell liberated by an EDTA solution after a rinse with clean seawater, (ii) intracellular, the remaining metal accumulated in the cell (i.e. not liberated by an EDTA solution) following acid digestion, and (iii) total, the sum of extra and intracellular concentrations in units of mol $cell^{-1}$. The exposure concentrations are defined as the mean of the start and end dissolved (0.45 μm-filterable) metal concentration.

Extra and intracellular metal concentrations were determined at the end of the bioassay using methods modified from Levy et al. (2008). The exposure solution was filtered through a hydrophilic polypropylene membrane (GH Polypro, Pall Corporation) in a glass filtration unit, and the filtrate was subsampled as the end-of bioassay dissolved fraction. The filtrate was discarded, and 30 mL of cold clean seawater was filtered through the filtration unit to remove metals loosely bound to the cells and minimise contamination. The rinse was discarded. Once filtered onto a filter paper, the algal cells were well adhered as a layer on the paper throughout the rinsing and filtering process.

The filter paper with the accumulated algal cells was filtered dry and transferred into a Teflon tube with 15 mL of 0.01 M EDTA for 20 minutes in an insulated cooler with ice. After 20 minutes, the filter paper was retrieved from the EDTA solution, placed in a new clean glass filtration unit (with adhered algal cells facing upwards), and the EDTA solution was filtered through to recapture any lost cells from the rinse step. The EDTA rinse solution was retained for metal analysis as the extracellular metal fraction. A 30 mL volume of cold clean seawater was then filtered through the filtration unit to remove excess EDTA solution. This second rinse was discarded.

The filter paper was moved to a plastic tube with 2 mL of 50% HNO_3 (v/v) for ≥24 h and microwave-heated for 1 h (MARS 5, CEM, programmed room temperature to 60 °C, 12 min; 60–65 °C, 10 min; 65–70 °C, 10 min; 70 °C for 10 min) before dilution to 10% final acid content and metal analysis for intracellular metal fraction (Remaili et al., 2016).

All solutions were kept on ice in insulated boxes during this process. Acid-washed plastic or glass vacuum filtration units were used with a hand vacuum pump. Glass filtration units were coated in a silanising solution (Coatasil, Ajax) to minimise metal adsorption to the

glass. Blank EDTA wash solutions were analysed for metal content (Supplementary Table 1). The average blank EDTA wash solution metal concentration was subtracted from the measured metal concentrations in the EDTA wash solutions from metal treatments. Process blanks (n=3) were analysed in each experiment using diluent seawater instead of an exposure solution. The measured intracellular digest concentrations in process blanks were subtracted from the intracellular digest measurements from treatments (Supplementary Table 2). The efficacy of the acid digest was confirmed by analysis of DOLT-3 dogfish liver digestion certified reference material (National Research Council of Canada, Ottawa, Canada), which had recoveries of 93% Cd, 98% Cu, 96% Ni, and 105% Zn, with Pb below detection limit. Control (seawater only exposure) cell accumulation was also assessed and is provided in Supplementary Table 3.

Metal analysis

Subsamples of the exposure solutions, EDTA rinse solution (extracellular fraction), or acid-digested cells (intracellular fraction) were analysed by inductively coupled plasma – atomic emission spectrometry (ICP-AES; Varian 730-ES) or inductively coupled plasma – mass spectrometry (ICP-MS, Agilent 7500CE) depending on the limits of detection required.

All subsamples were analysed using matrix-matched calibration standards, e.g. subsamples of the EDTA solutions were analysed using seawater calibration standards matrix-matched to 0.2 % acidity and salinity of 35 PSU and the intracellular fraction was analysed using calibration standards of 10% HNO_3 in ultrapure water. Analytical performance of the EDTA solution was confirmed by analysis of the EDTA solution spiked with a range of concentrations of a multi-element metal standard (QCS27; Analytical West Inc). For seawater and EDTA solutions, a 200 µg L^{-1} multi-element drift standard (QCS27; Analytical West Inc.) was used to correct for measurement drift over time.

Calculations and statistical analysis

Dissolved metal concentrations decreased or increased over the duration of the bioassay, possibly from cellular accumulation, adsorption or desorption from the glass vials, precipitation or dissolution, or instrument variability – particularly at low concentrations (Levy et al., 2008). The percent change in dissolved metal concentrations for all experiments was cadmium 21% to- 23%, copper 45% to -42%, nickel 23% to -55%, lead 96% to -43%, and

zinc 17% to -49%. The large percentage changes arise from small concentration changes in low concentration treatments (i.e. ± 1 to 2 µg L^{-1}). Reported dissolved metal concentrations are the average of the concentrations at the start and end of the toxicity tests, which were used in all further calculations.

Extra and intracellular metal concentrations on a per cell basis were calculated using the number of cells filtered from each treatment and the metal content of the EDTA wash solution or nitric acid digest, respectively. The number of cells filtered from each treatment was calculated as the product of the final cell density in each treatment, measured by flow cytometry (FACSVerse, BD, Koppel et al. (2017)). The volume of treatment solution filtered was determined gravimetrically by weighing the flask before and after the exposure solution was filtered. A seawater density of 1.027 g mL^{-1} was then used to calculate the volume.

Of the six tests conducted, cadmium contamination in extra or intracellular metal measurements was observed in 8 of 189 exposures. This is discussed in Supplementary Information S1.

Extracellular concentrations were normalised to cell surface area and intracellular concentrations were normalised to the volume of *P. antarctica* and *C. armigera*, respectively. For these calculations, microalgae were measured by phase-contrast microscopy. *P. antarctica* was assumed to be a sphere with a diameter of 10 µm and *C. armigera* was assumed to be an ellipsoid with a diameter of 30 µm and lengths of 7.5 µm for its two other axes. This translated to a surface area and volume of 314 $µm^2$ and 523 $µm^3$ for *P. antarctica* and 1484 $µm^2$ and 4691 $µm^3$ for *C. armigera*, respectively.

Multiple linear regression was used to investigate how cellular metal fractions affect microalgal population growth rate (provided in Supplementary Table 4, taken from Koppel et al., (2018a)). All metals of a cellular fraction (extra and intra), and the total metal accumulation concentration were used in the initial multiple linear regression. This was then optimised by removing metals until the fewest possible parameters gave the best model fit (determined by the smallest AIC). All statistical analyses that investigated significance used an alpha level of 0.05. This was performed by a stepwise model selection function, stepAIC, in the MASS package of R (R Core Team, 2016). All figures were created using the package ggplot2 (Wickham, 2009).

Results

Cellular accumulation

Both *P. antarctica* and *C. armigera* in the control treatment (no metal supplemented) had similar total cellular metal concentrations; 1.5 ± 1.2 and 2.1 ± 1.1 $x10^{-16}$ mol $cell^{-1}$ (mean ± standard deviation of the sum of five cellular metal concentrations), respectively (Fig. 1, Supplementary Table 3). Both microalgae accumulated greater total cellular concentrations in the metal mixture treatments compared to controls (Fig. 1). The exposure concentration and extra and intracellular metal concentrations for both species from single and mixture experiments are given in Table 1 and Supplementary Tables 4-6.

Phaeocystis antarctica

Individual exposure to each metal's EC10 concentration resulted in cellular accumulation with zinc having the highest total cellular concentration, followed by lead, copper, cadmium, and nickel. This was despite nickel having the highest exposure concentration followed by zinc, cadmium, lead and copper. Intracellular concentrations of cadmium, copper, and lead were lower than extracellular concentrations, while nickel and zinc had equal concentrations.

Exposure to the equitoxic mixture changed the accumulation of individual metals when compared to corresponding single-metal exposures (Table 2). The total cellular metal concentrations for cadmium, copper, and nickel increased in the mixture compared to the single-metal exposure while total lead and zinc concentrations decreased. In the mixture, *P. antarctica* accumulated 8.4 $x10^{-16}$ mol $cell^{-1}$, approximately 30% more than the sum of metal accumulated from individual exposures (6.5 $x10^{-16}$ mol $cell^{-1}$). Zinc had the highest total cellular concentration, followed by lead, copper, nickel, and cadmium. The difference between total concentrations from single EC10 exposures and the equitoxic mixture is due to an increase in extracellularly bound metals of 2.2 $x10^{-16}$ mol $cell^{-1}$, the majority of which is zinc, which was offset by a reduction of intracellular zinc.

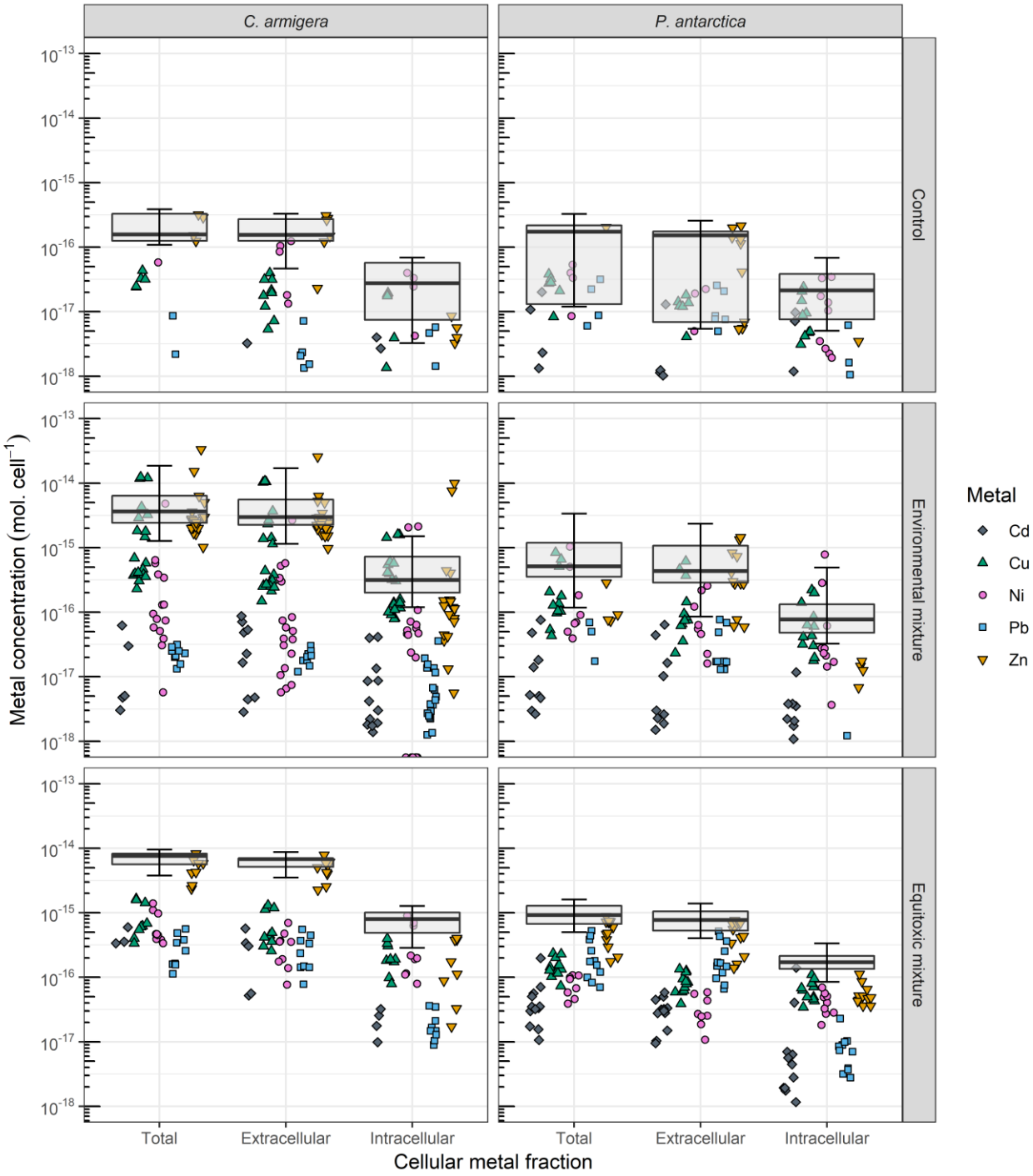

Figure 1. Total, extra, and intracellular metal concentrations for *Cryothecomonas armigera* and *Phaeocystis antarctica* exposed to control, environmental and equitoxic mixture treatments. Boxplots are calculated as the sum of the five metals measured, shown individually as in the legend. Filled black circles above the boxplots are outliers, defined as >1.5x the interquartile range of the sum of the five metals measured. All data above detection limits are shown, including the environmental mixture which had a range of mixture multiples, and were different for each microalga. Average metal concentrations for each mixture are given in Supplementary Table S3. Position of the individual metal points on the x-axis within cellular fraction group is arbitrary.

Cryothecomonas armigera

Single metal exposure at each metal's EC10 concentration resulted in total cellular accumulation where zinc had the highest total cellular concentration followed by copper, nickel, lead, and cadmium. This was despite nickel having the highest exposure concentration followed by zinc, cadmium, lead, and copper.

Exposure to the equitoxic mixture led to increases in total cellular copper, and nickel concentrations, decreases in zinc concentrations, and equivalent cadmium and lead concentrations compared to cellular accumulation from individual exposures to metal's EC10 concentrations. For *C. armigera,* zinc in the equitoxic mixture still had the highest total cellular concentration, followed by copper, nickel, cadmium and lead, which together summed to 85 $x10^{-16}$ mol $cell^{-1}$. This is approximately a 20% reduction in accumulated metal when compared to the sum of metals from single-metal EC10 exposures (104 $x10^{-16}$ mol $cell^{-1}$). This difference is mostly due to a 23 $x10^{-16}$ mol $cell^{-1}$ reduction in extracellularly bound metals, of which the majority was a decrease in zinc of 36 $x10^{-16}$ mol $cell^{-1}$ with increases in other metals offsetting the difference.

Algal comparison

A greater concentration of metals was accumulated by *C. armigera* than *P. antarctica* on a moles per cell basis (Fig. 1). However, when extra and intracellular metal concentrations were normalised to cell surface area and volume, respectively, their concentrations were generally similar: both microalga's extracellular concentrations ranged from 10^{-20} – 10^{-17} mol μm^{-2} and were approximately 10 times higher than intracellular concentrations, which ranged from 10^{-21} to 10^{-18} mol μm^{-3}.

Two treatments allow direct comparison of microalgal metal adsorption and uptake, the environmental mixture at a ratio of 10 (i.e. the treatment for both microalgae had equal exposure concentrations) and the equitoxic mixture (i.e. both treatments had equal theoretical toxicity).

The environmental mixture at a multiple of 10 led to similar extra and intracellular concentrations for both algae (Table 2), but *C. armigera* had a higher molar total of 18 $x10^{-16}$ mol $cell^{-1}$ compared to *P. antarctica* at 7.3 $x10^{-16}$ mol $cell^{-1}$. When normalised to cell surface

301 area and volume, the *P. antarctica* had significantly higher extracellular cadmium and lead
302 and intracellular nickel concentrations. No other extra or intracellular metal fraction had
303 significant differences.

304 The equitoxic mixture resulted in population growth rates of 64 ± 8% and 76 ± 17% for *P.*
305 *antarctica* and *C. armigera*, respectively (Supplementary table 4), which were not
306 significantly different. When extra and intracellular metal fractions were normalised to
307 surface area and volume, respectively, *C. armigera* had significantly greater extracellular
308 nickel and zinc while *P. antarctica* had significantly greater extracellular lead concentration.
309 Of the intracellular fraction, *P. antarctica* had greater intracellular copper and zinc
310 concentrations.

311 For *C. armigera*, cadmium and lead were the only metals to have clear differences in total
312 cellular concentrations as a result of the different mixture exposures (Fig. 3, bottom panel).
313 For *P. antarctica*, exposure to either mixture resulted in a similar range of cellular metal
314 concentrations (Fig. 2, bottom panel). This was despite dissolved metal concentrations being
315 different between the two mixtures, with the equitoxic mixture having a much higher molar
316 total of dissolved metals than the environmental mixture.

Table 2. ***Phaeocystis antarctica*** **and** ***Cryothecomonas armigera*** **cellular metal concentrations from: single metal tests at population growth rate EC10 concentrations, the equitoxic mixture, and the environmental mixture at a multiple of 10. Values are mean ± standard deviation in amol cell^{-1} (10^{-18} mol cell^{-1} extra and intracellular and total concentrations), unless otherwise indicated. EC10 (95% CI) indicates the single-metal concentration that reduces population growth rate by 10% (and 95% confidence interval) previously determined (Gissi et al., 2015; Koppel et al., 2017).**

Phaeocystis antarctica		**Cd**	**Cu**	**Ni**	**Pb**	**Zn**
	EC10 (95% CI)	**1.5 (0 - 3.3) µM**	**0.044 (0.035 - 0.052) µM**	**>15 µM**	**0.72 (0.3 - 1.2) µM**	**3.3 (1.2 - 5.4) µM**
Single (EC10)	Exposure (µM)	1.29 ± 0.07	0.042 ± 0.005	17.4 ± 0.1	0.93 ± 0.08	3.030 ± 0.004
	Extracellular	22 ± 5	60 ± 60	10 ± 3	200 ± 100	160 ± 30
	Intracellular	5 ± 5	20 ± 10	13 ± 5	5 ± 3	160 ± 30
	Total	27 ± 8	80 ± 70	23 ± 1	200 ± 100	320 ± 50
Equitoxic Mixture	Exposure (µM)	1.35 ± 0.02	0.044 ± 0.007	4 ± 3	1.0 ± 0.1	6.5 ± 0.3
	Extracellular	31 ± 2	75 ± 13	32 ± 25	170 ± 50	360 ± 220
	Intracellular	9 ± 16	55 ± 13	37 ± 11	6 ± 3	54 ± 18
	Total	39 ± 15	130 ± 20	69 ± 36	180 ± 50	420 ± 230
Environmental mixture x 10	Exposure (µM)	0.0094 ± 0.0003	0.088 ± 0.002	0.062 ± 0.003	0.009 ± 0.0001	0.55 ± 0.03
	Extracellular	2.3 ± 0.4	100 ± 36	19 ± 5	16 ± 2	500 ± 300
	Intracellular	1.2 ± 0.9	59 ± 28	23 ± 5	0.3	<LOD
	Total	3.4 ± 1.1	160 ± 53	45 ± 8	18	500 ± 300

Cryothecomonas armigera		**Cd**	**Cu**	**Ni**	**Pb**	**Zn**
	EC10 (95% CI)	**4.0 (2.0 - 6.1) µM**	**0.35 (0.28 - 0.41) µM**	**21 (19 - 23) µM**	**0.73 (0.38 - 1.4) µM**	**5.6 (0.63 - 11) µM**
Single (EC10)	Exposure (µM)	1.01 ± 0.02	0.28 ± 0.01	14.91 ± 0.04	0.415 ± 0.004	1.36 ± 0.02
	Extracellular	290 ± 30	450 ± 40	240 ± 20	330 ± 30	8300 ± 700
	Intracellular	7 ± 1	230 ± 90	230 ± 50	31 ± 3	380 ± 70
	Total	300 ± 40	690 ± 90	470 ± 40	360 ± 40	8600 ± 800
Equitoxic Mixture	Exposure (µM)	4.42 ± 0.01	0.35 ± 0.01	20.7 ± 0.1	0.75 ± 0.01	5.3 ± 0.1
	Extracellular	400 ± 200	1200 ± 100	400 ± 300	300 ± 200	5000 ± 800
	Intracellular	25 ± 7	350 ± 50	700 ± 100	31 ± 8	30 ± 10
	Total	400 ± 200	1600 ± 100	1200 ± 200	300 ± 200	5000 ± 1000
Environmental mixture x 10	Exposure (µM)	0.012 ± 0.002	0.093 ± 0.001	0.038 ± 0.005	0.012 ± 0.003	0.62 ± 0.01
	Extracellular	4 ± 1	220 ± 60	10 ± 4	16 ± 3	1400 ± 300
	Intracellular	0.3 ± 0.1	100 ± 20	<LOD	2.1 ± 0.8	70 ± 30
	Total	4 ± 1	320 ± 80	<LOD	18 ± 4	1400 ± 400

Phaeocystis antarctica

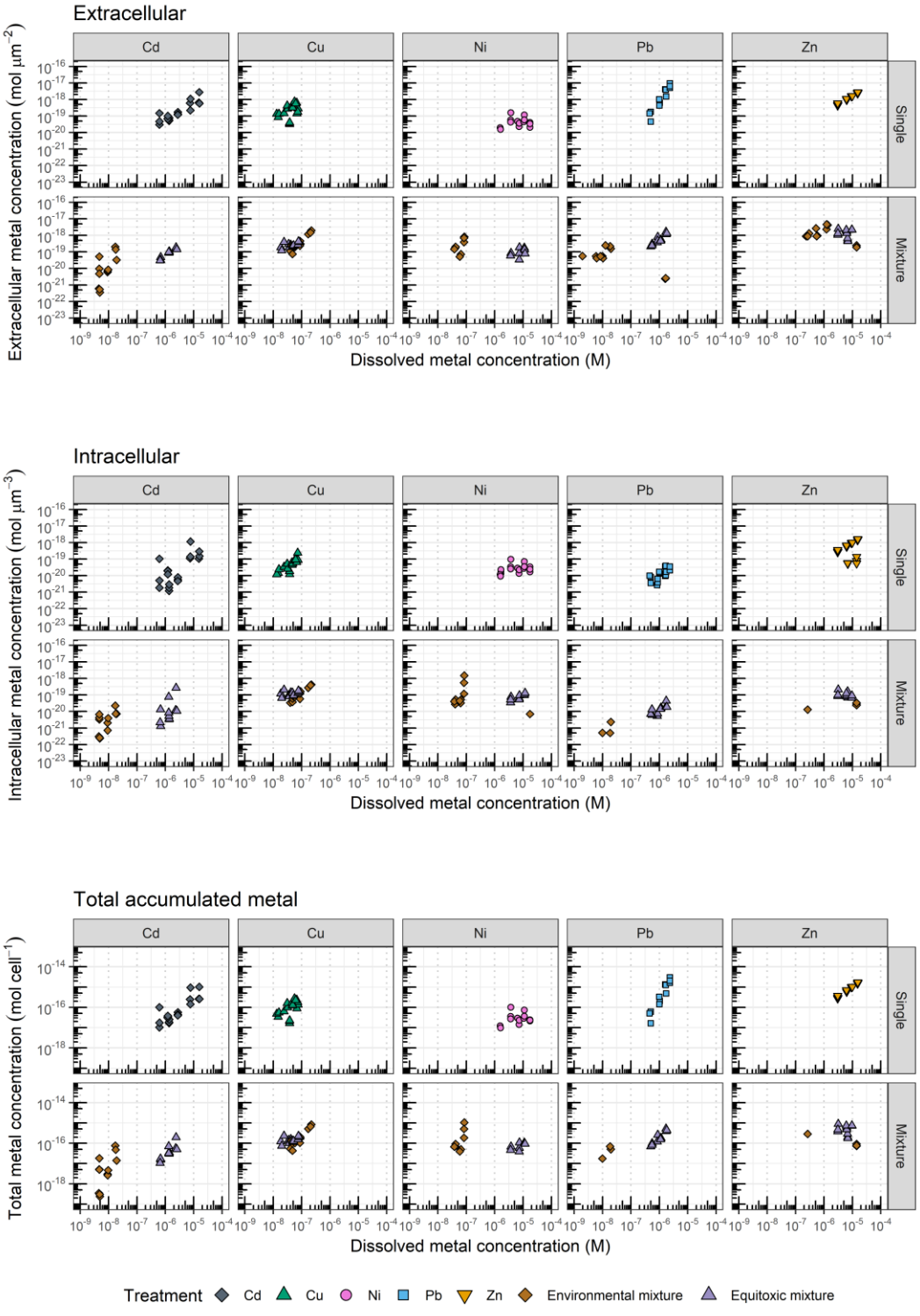

Figure 2. The relationship between extracellular, intracellular, and total metal concentrations and dissolved metal concentrations for *Phaeocystis antarctica* in single and metal mixture exposures. Data is the aggregate of all exposure concentrations. Extra and intracellular concentrations have been normalised to cell surface area and volume.

Cryothecomonas armigera

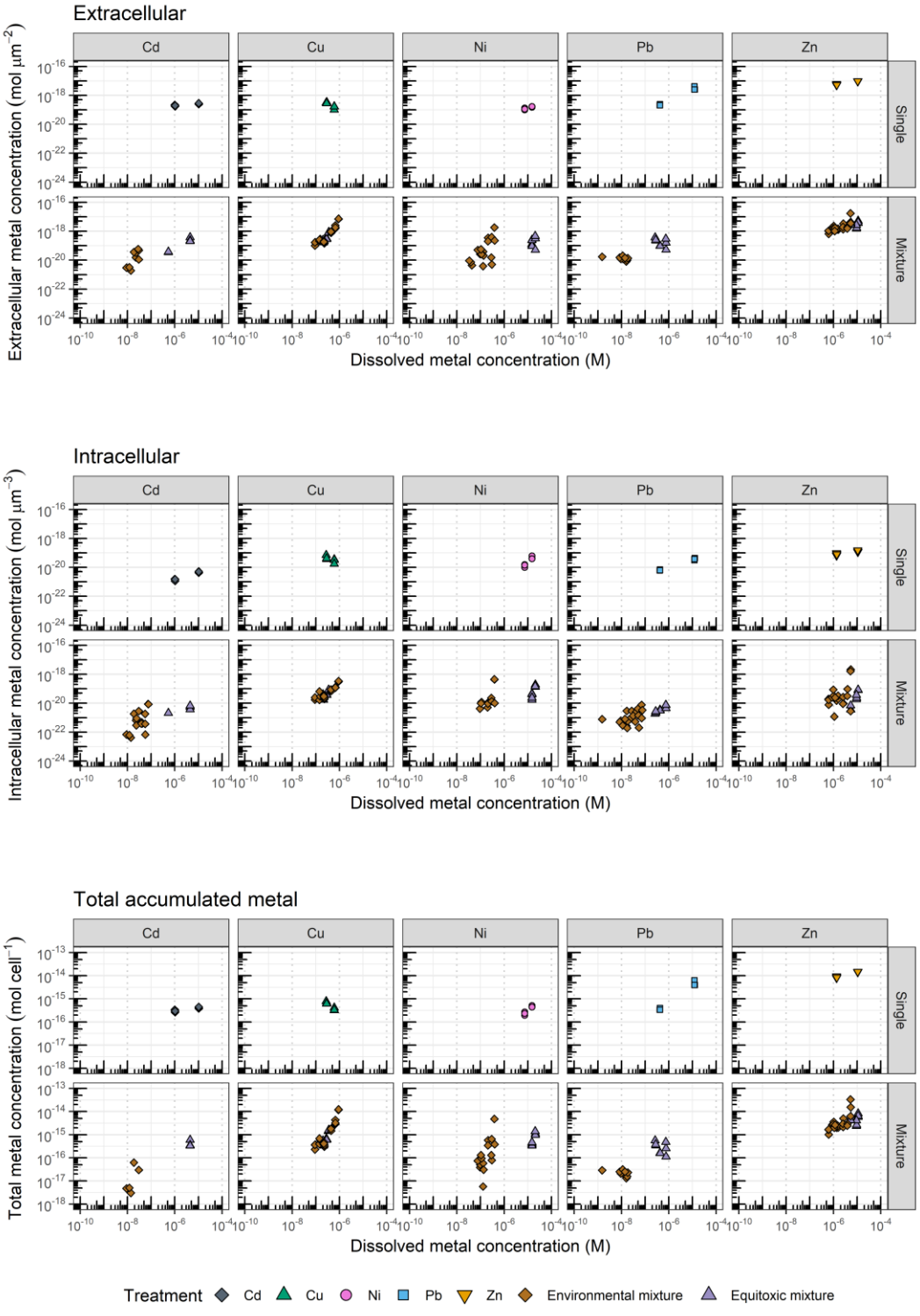

Figure 3. The relationship between extracellular, intracellular, and total cellular metal concentrations, and dissolved exposure concentrations for *Cryothecomonas armigera* in single and metal mixture exposures. Data is the aggregate of all exposure concentrations. Extra and intracellular concentrations have been normalised to cell surface area and volume.

Drivers of mixture toxicity

Population growth rate

In single-metal exposures, cadmium and copper cellular metal fractions strongly correlated, and lead weakly correlated, to population growth rate in *P. antarctica* (Fig. 4 a). Following exposure to the environmental mixture, all fractions of all metals except zinc had strong negative correlations to the population growth rate of *P. antarctica*. Zinc had different correlations for the different fractions – total and intracellular zinc concentrations had a positive while extracellular had a negative correlation to growth rate (Fig. 4 b). The multiple linear regression incorporated all metals of a cellular fraction (i.e. extra, intra, or total cellular) to examine metals' interactions and relationship with population growth rate. Of the extracellular fraction lead was the only significant component of the model, with a negative partial regression coefficient. Of the intracellular fraction, zinc had a positive while copper had a negative partial regression coefficient (Fig. 4 c).

In exposures of the environmental mixture to *C. armigera*, copper had the strongest correlations across all metal fractions to population growth rate. Other metals were more variable. In the multiple linear regression model, all cellular copper fractions correlated to population growth rate with high levels of significance (Fig. 4 c). The partial regression of the intracellular lead fraction was negative while the extracellular lead fraction and intracellular zinc fractions were positive.

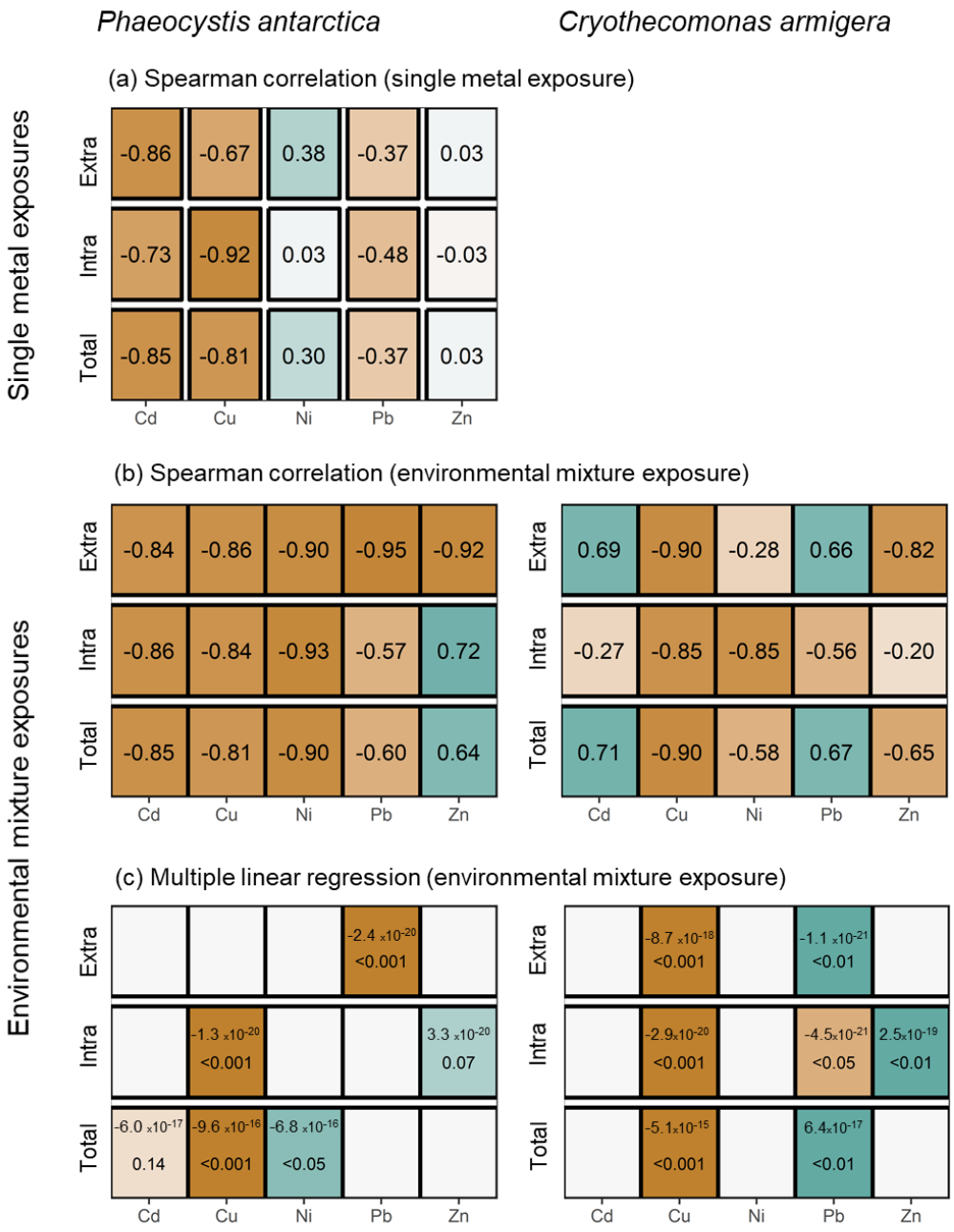

Figure 4. Correlation matrix of extra, intra, and total cellular metal fractions as predictors of population growth rate for *Phaeocystis antarctica* and *Cryothecomonas armigera*. (a) Single metal-exposures showing Spearman correlation coefficients for individual metal fractions. (b) Exposures of the environmental mixture showing Spearman correlation coefficients. (c) the significant metal components for multiple linear regression, as determined by step-wise optimisation by AIC, showing their partial regression coefficient (top number) and significance of its interaction (p-value, bottom number). No single-exposure correlation coefficients for *C. armigera* (a) could be calculated due to lack of data. Note the positive (aqua) and negative (brown) coefficients, with intensity of colour, indicting the strength of the correlation.

Discussion

Metal accumulation

Dissolved metal concentrations were generally a poor predictor of cellular metal concentrations, with metal-, microalgal-, and cellular-fraction specific differences observed. The lack of clear trends in absorption or uptake suggests that these processes are confounded in this study by factors which could include metal competition for binding ligands and regulation of detoxification or uptake pathways. Together, these factors more complicated than what could be explained by a simple equilibrium-based models (Duval, 2016; Hassler et al., 2004). However, there were some trends observed.

C. armigera, the larger cell, accumulated greater extra, intra, and total cellular concentrations than *P. antarctica* (sum of all metals as moles per cell, Table 2 and Fig. 1). This was consistent with the theory that cellular metal binding capacity is proportional to cell size, as greater surface areas are expected to have a greater concentrations of biotic ligands (Paquin et al., 2002). However, some metals had roughly equivalent cellular concentrations between microalga in similar exposures. For example, there were greater or similar total cellular concentrations of nickel, lead, and cadmium in *P. antarctica* and *C. armigera* following exposure to the environmental mixture at a multiple of 10 (i.e. equivalent exposure concentration), despite *P. antarctica* being a smaller cell. Extracellular zinc accumulation on *C. armigera* was the main contributor to the difference between total cellular concentrations of the microalgae (Table 2). This is important to consider as secondary consuming organisms will have different sensitivities to metals from dietary exposure (Hook and Fisher, 2002).

Toxicity and mixture interactivity

Simple regression

Metals in the environmental mixture were covariate to one another because their exposure concentrations were fixed at a ratio and increased by multiples to give a concentration series. This confounds the interpretation of simple correlations, i.e. Fig. 4 b, where nearly all metals are negatively correlated to the growth rate of *P. antarctica* despite only copper and possibly zinc being at a concentration that could cause toxicity (Gissi et al., 2015).

Furthermore, their correlation coefficients share little similarity with single-metal exposures (Fig 4. a). Therefore, the negative correlations of cadmium, nickel, and lead could be explained by them accumulating to each cellular fraction proportionally to copper which overwhelmingly contributes to toxicity in the environmental mixture. Interestingly, zinc accumulation in *P. antarctica* did not follow this trend, with a negative extracellular and positive intracellular correlation.

Correlations between cellular metal fractions and growth rate in *C. armigera* showed different patterns compared to *P. antarctica* (Fig. 4 b). Cadmium and lead had positive correlations in their extra and total cellular fractions, but not to their intracellular fractions. Copper was the only metal with consistently strong negative correlations across all fractions in this species.

Multiple linear regression

The multiple linear regression, to some extent, accounts for the covariate nature of a fixed-ratio metal exposure which otherwise confounds the simple correlation analysis of Fig. 4 b. Metal fractions that did not independently provide a significant improvement to the regression were dropped as a parameter (Fig. 4 c, as defined by optimisation of the AIC parameter). Following this optimisation, copper and zinc appear to have similar effects to both microalgae.

Copper drives toxicity in the presence of other metals in the mixture. Total and intracellular copper concentrations were significant predictors of algal growth rate inhibition of *P. antarctica*, while all copper fractions showed significant negative partial regressions to growth rate. This shows that intracellular copper is proportional to observed toxicity in the presence of other metals, which agrees with previous studies showing the intracellular sequestration of copper in microalgae (Adams et al., 2016), including those particularly sensitive to copper (Levy et al., 2008).

For both microalgae, the intracellular zinc concentration had a positive partial regression coefficient with growth rate. This means that, if all other metal concentration were constant, increasing intracellular zinc concentrations increased growth rate. This result is consistent with previous metal mixture studies (Franklin et al., 2002; Lavoie et al., 2012; Nagai and Kamo, 2014; Versieren et al., 2016).

The protectiveness of zinc was different in each microalga. When *P. antarctica* is exposed to the environmental mixture, every 1.3 $x10^{-20}$ mol Cu μm^3 decreases growth rate by 1% and every 3.3 $x10^{-20}$ mol Zn μm^3 increases growth rate by 1%. When *C. armigera* is exposed to the environmental mixture, every 2.9 $x10^{-20}$ mol Cu μm^3 reduces growth rate by 1% but every 25 $x10^{-20}$ mol Cu μm^3 increases growth rate by 1%. Therefore, while the potency of intracellular copper is similar (this is despite the microalgae having overall different sensitivities to dissolved copper), zinc is less protective in *C. armigera* than *P. antarctica*.

Extracellular lead on *C. armigera* from the environmental mixture had a significant and positive partial regression, meaning that that population growth rate increased with increasing extracellular lead concentrations. This could indicate that lead is outcompeting an otherwise toxic metal at the cell surface leading to antagonism (Lavoie et al., 2014, 2012; Volland et al., 2014). This is supported, for example, by studies showing that copper and lead share a cellular uptake transporter (Sánchez-Marín et al., 2014). The positive partial regression of total cellular nickel concentrations in *P. antarctica* agrees with observed stimulation in single-metal exposures (Gissi et al., 2015).

Exposure to low concentrations of the environmental mixture led to antagonism in both *P. antarctica* and *C. armigera* (Koppel et al., 2018a). These data suggest that intracellular copper is driving toxicity with zinc likely causing this antagonism. The mechanism behind this antagonism is unknown but could be based on competition for shared uptake pathways or binding sites on the cell surface, particularly as zinc was present at a ratio to copper of 4.5 to 1. However, changes in metal regulation by up- or down-regulating detoxification or uptake mechanisms, respectively, could have an influence.

These results, while somewhat inconclusive in explaining all observed toxicity or mixture interactivity, highlight the confounding nature of cellular regulation, detoxification, and mixture interactivity to predictions of equilibrium models like the free ion or biotic ligand models (Levy et al., 2007; Slaveykova and Wilkinson, 2005). Previous studies have found that intracellular metal concentrations for some microorganisms correlate better to toxicity than do dissolved concentrations (Franklin et al., 2002; Lavoie et al., 2014; Wilde et al., 2006; Zeng et al., 2009). While this was generally true for copper for both microalgae, it is subject to complicated metal regulation mechanisms (Rüdel et al., 2015). To better identify

specific metal interactions, a full factorial experimental design would be needed (e.g. Deruytter et al. (2017) or Nys et al. (2016)).

Predicted risk to the Southern Ocean food web

Few studies have investigated the risk of dietary metal exposure within the Southern Ocean food web. However, chromium, copper, lead, and zinc have been reported to have the potential to accumulate through marine food webs (Cabrita et al., 2017). While no studies have investigated the dietary toxicity of metal-laden microalgae to Antarctic organisms, comparison to other fresh or seawater organisms may inform their possible risk.

The environmental mixture at a multiple of 10 has dissolved concentrations are like what has been reported from other contaminated Antarctic near-shore marine sites (Table 2). In this exposure, *C. armigera* had total cellular zinc concentrations of 1400 ± 400 amol cell^{-1}, approximately 4x higher than the next highest metal which was copper at 320 ± 80 amol cell^{-1}. *P. antarctica* also had higher zinc concentrations at 500 ± 300 amol Zn cell^{-1} than copper concentrations at 160 ± 50 amol Cu cell^{-1}, even with intracellular zinc concentrations below the limit of detection. Both algae had nickel and lead concentrations under 50 amol cell^{-1} and cadmium concentrations under 5 amol cell^{-1} (Fig. 1). These concentrations, when compared to previous studies of dietary toxicity, suggest that toxicity to secondary consumers is likely.

For example, dietary exposure of the marine diatom *Thalassiosira pseudonana* laden with Cu (10 amol cell^{-1}), Ni (7.5 amol cell^{-1}), or Zn (0.24 amol cell^{-1}) to the marine copepod *Acartia tonsa* resulted in a 20% decrease to reproduction (Bielmyer et al., 2006). Both *C. armigera* and *P. antarctica* had Cu and Ni concentrations 20 times and Zn concentrations >1000 times these values when exposed to the environmental mixture (Table 2). The freshwater *Chlorella pyrenoidosa* exposed to cadmium concentrations of 0.01 µM had cellular concentrations of 2.5 amol Cd cell^{-1} which, when fed to the saltwater cladoceran *Moina monogolica*, led to reproductive toxicity (Wang et al., 2009). The cadmium concentration on *C. pyrenoidosa* was less but like what was found on *P. antarctica* or *C. armigera* exposed to the environmental mixture at a multiple of 10 (Table 2).

These comparisons suggest that even the low metal concentrations reported in contaminated sites around the near-shore Antarctic marine environment (Cabrita et al.,

2017; Stark et al., 2006) may pose a risk to the Southern Ocean food web via microalgae ingestion. Experiments to investigate this link, particularly with important calanoid or euphausiids warrants further investigation to enable more accurate predictions of risk.

Conclusion

The risk of metals to the Antarctic near-shore marine ecosystem is affected by metal mixture interactions and sub-lethal metal accumulation in microalgae. Two common microalgae species, *Phaeocystis antarctica* and *Cryothecomonas armigera* showed similar metal uptake and regulation processes. Copper was found to be the driver of observed toxicity in an environmental mixture of metals, while zinc likely acts as an antagonist. This study demonstrates that *P. antarctica* and *C. armigera* accumulate metal contaminants at sub-lethal, environmentally realistic, exposure concentrations which may in turn cause bioaccumulation of metals and or toxicity to microalgae-grazing plankton the Southern Ocean food web.

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692 **Supplementary information**

693 **Preliminary study of cellular metal accumulation in two Antarctic marine**

694 **microalgae – implications for mixture interactivity and dietary risk**

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Supplementary information S1.

Quality assurance and quality control: metal concentrations from blank EDTA wash solution (Supplementary Table 1); metal concentrations from process blank acid digestions (Supplementary Table 2); measured extra and intracellular metal concentrations from control (seawater only) exposures for *Phaeocystis antarctica* and *Cryothecomonas armigera* (Supplementary Table 3). Blank cells indicate measurements below detection limit which were on average: 2 nM Cd, 13 nM Cu, 28 nM Ni, 14 nM Pb, and 14 nM Zn for ICP-AES and 0.03 nM Cd, 1.8 nM Cu, 1.2 nM Ni, 0.6 nM Pb, and 4.2 nM Zn for ICP-MS.

Contamination

One experiment with *P. antarctica* had cadmium contamination in two of the three control replicates in the extra and intracellular metal fraction, but not the dissolved fraction. This was likely a result of dust falling into the filtration unit during the extracellular washing step, which was carried through to the intracellular digestion. Similar contamination was found in one experiment with *C. armigera* where one replicate of the 20x and two replicates of the 30x multiple of the environmental mixture had intracellular cadmium concentrations 100 – 1000x greater than other replicates of that treatment. Outlier measurements were removed from the dataset for analyses but can be found in the data for this study (Koppel et al., 2018b).

Supplementary Table 1. Metal concentrations from blank EDTA wash solution.

	Cd	Cu	Ni	Pb	Zn
Average (μg L^{-1})	0.003	0.6	2.6	0.2	2.0
Standard deviation (μg L^{-1})	0.002	0.4	0.8	0.0	1.0
Count (n)	3	9	6	3	16

Supplementary Table 2. Metal concentrations from process blank acid digestions.

	Cd	Cu	Ni	Pb	Zn
Average (μg L^{-1})	0.2	0.4	0.8	0.6	1.1
Standard deviation (μg L^{-1})	0.4	0.3	0.7	1.0	1.1
Count (n)	13	15	13	10	14

Supplementary Table 3. Measured extra and intracellular metal concentrations (mean ± standard deviation, amol cell^{-1}) from control (seawater only) exposures for *Phaeocystis antarctica* and *Cryothecomonas armigera*.

Metal	*P. antarctica*		*C. armigera*	
	Intracellular	Extracellular	Intracellular	Extracellular
Cd	5 ± 5	2 ± 5	2 ± 2	1 ± 1
Cu	1.1 ± 0.8	12 ± 5	12 ± 9	20 ± 10
Ni	10 ± 10	10 ± 10	30 ± 20	70 ± 50
Pb	2 ± 3	13 ± 8	3 ± 2	2 ± 2
Zn	3	100 ± 80	5 ± 2	200 ± 100

Supplementary information S2.

Cellular metal concentrations (mean ± standard deviation) of *Phaeocystis antarctica* and *Cryothecomonas armigera* following exposure to metal mixtures. Measured extra- and intra-cellular metal concentrations from control (seawater only) exposures (Supplementary Table 4). Dissolved metal concentrations from mixture exposures (Supplementary Table 5), extracellular metal concentrations from mixture exposures (Supplementary Table 6), and intracellular metal concentrations from mixture exposures (Supplementary Table 7).

The multiple of the equitoxic (EC) and environmental (EM) mixture are denoted after the mixture abbreviation. Where measurements from the EDTA wash or acid digest were below detection limits, the instrument limit of detection was used to calculate the limit of detection for each treatment (as amol cell^{-1}, accounting for the different number of cells at the end of each treatment).

Supplementary Table 4. Dissolved metal concentrations and population growth rate inhibition for *Phaeocystis antarctica* and *Cryothecomonas armigera* from mixture exposures. Data for individual flasks is provided in the accompanying dataset provided by Koppel et al. (2018b).

Microalgae	Mixture	Population growth rate (% of control)	Exposure (nM)				
			Cd	Cu	Ni	Pb	Zn
P. antarctica	EM x5	89 ± 9	5.0 ± 0.2	48 ± 6	33 ± 8	5 ± 2	280 ± 20
	EM x10	76 ± 6	10 ± 3	88 ± 2	62 ± 3	9 ± 1	550 ± 30
	EM x 20	35 ± 9	20 ± 1	200 ± 20	83.5 ± 0.8	17 ± 4	1.27 ± 0.07
	EC x0.5	70 ± 12	670 ± 20	21 ±3	3680 ± 60	530 ± 30	3100 ± 700
	EC x1	64 ± 8	1350 ± 20	44 ± 7	4000 ± 4000	1000 ± 100	6500 ± 400
	EC x2	53.9 ± 0.3	2400 ± 100	79 ± 5	11300 ± 300	1800 ± 500	9580 ± 80
C. armigera	EM x 10	107 ± 5	9 ± 1	108 ± 2	48.9 ± 0.4	15 ± 4	589 ± 1
	EM x 20	91 ± 4	21 ± 3	190 ± 30	96 ± 9	19 ± 6	1000 ± 100
	EM x 30	100 ± 5	27 ± 2	228 ± 7	129 ± 7	22.1 ± 0.3	1500 ± 200
	EM x 40	70 ± 4	41 ± 2	441 ± 5	205 ± 6	37 ± 5	2560 ± 50
	EM x 60	79 ± 2	56 ± 1	669 ± 2	294 ± 8	52 ± 3	3910 ± 70
	EM x 80	49 ± 8	40 ± 4	911 ± 6	389 ± 4	73 ± 3	5280 ± 60
	EC x1	76 ± 17	2000 ±	280 ± 60	17000 ± 3000	500 ± 200	9000 ± 3000

747 **Supplementary Table 5. Extracellular metal concentrations from mixture exposures**

		Extracellular metal concentration (amol cell^{-1})				
Microalgae	**Mixture**	**Cd**	**Cu**	**Ni**	**Pb**	**Zn**
P. antarctica	Env x5	4 ± 6	60 ± 20	55 ± 9	8 ± 8	200 ± 200
	Env x10	2.2 ± 0.4	100 ± 40	19 ± 5	16 ± 2	500 ± 300
	Env x20	40 ± 30	500 ± 100	200 ± 70	70 ± 10	1200 ± 400
	EC x0.5	1.2 ± 0.3	70 ± 50	23 ± 6	80 ± 20	500 ± 200
	EC x1	31 ± 2	70 ± 10	30 ± 20	170 ± 50	400 ± 200
	EC x2	51 ± 7	110 ± 20	40 ± 10	440 ± 80	790 ± 10
C. armigera	Env x10	4 ± 1	220 ± 60	10± 4	16 ± 3	1400 ± 300
	Env x20	40 ± 20	320 ± 80	60 ± 20	26 ± 4	2400 ± 600
	Env x30	60 ± 40	270 ± 50	30 ± 30	19 ± 4	2000 ± 200
	Env x40	<18	1300 ± 100	400 ± 200	<250	3000 ± 2000
	Env x60	<36	2900 ± 700	200 ± 300	<490	2600 ± 300
	Env x80	<91	10600 ± 200	2000 ± 2000	<1200	10000 ± 10000
	EC x1	300 ± 200	700 ± 400	300 ± 200	300 ± 200	5000 ± 2000

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749 **Supplementary Table 6. Intracellular metal concentrations from mixture exposures**

		Intracellular metal concentration (amol cell^{-1})				
Microalgae	**Mixture**	**Cd**	**Cu**	**Ni**	**Pb**	**Zn**
P. antarctica	Env x5	1 ± 1	40 ± 20	20 ± 10	<3	13 ± 4
	Env x10	1.2 ± 0.9	60 ± 30	23 ± 05	0.27	<70
	Env x20	6 ± 5	190 ± 40	400 ± 400	0.7 ± 0.7	<300
	EC x0.5	3 ± 4	70 ± 40	30 ± 10	5 ± 2	70 ± 40
	EC x1	10 ± 20	60 ± 10	40 ± 10	6 ± 3	50 ± 20
	EC x2	50 ± 80	80 ± 10	59 ± 9	14 ± 7	44 ± 7
C. armigera	Env x10	0.27 ± 0.06	100 ± 20	<5	2.1 ± 0.8	70 ± 30
	Env x20	4 ± 3	150 ± 80	20 ± 30	7 ± 6	200 ± 100
	Env x30	13	120 ± 40	<30	2 ± 1	110 ± 40
	Env x40	2.0 ± 0.2	380 ± 50	40 ± 10	7 ± 5	70 ± 50
	Env x60	4 ± 4	580 ± 30	80 ± 20	9 ± 9	300 ± 200
	Env x80	40.7 ± 0.9	1540 ± 90	1000 ± 1000	20 ± 20	6000 ± 5000
	EC x1	20 ± 10	200 ± 100	300 ± 300	20 ± 40	200 ± 200

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