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A robust bioassay to assess the toxicity of metals to the antarctic marine microalga Phaeocystis antarctica

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Abstract

Despite evidence of contamination in Antarctic coastal marine environments, no water-quality guidelines have been established for the region because of a paucity of biological effects data for local Antarctic species. Currently, there is limited information on the sensitivity of Antarctic microalgae to metal contamination, which is exacerbated by the lack of standard toxicity testing protocols for local marine species. In the present study, a routine and robust toxicity test protocol was developed using the Antarctic marine microalga Phaeocystis antarctica, and its sensitivity was investigated following 10-d exposures to dissolved copper, cadmium, lead, zinc, and nickel. In comparisons of 10% inhibition of population growth rate (IC10) values, P. antarctica was most sensitive to copper (3.3 µg/L), followed by cadmium (135 µg/L), lead (260 µg/L), and zinc (450 µg/L). Although an IC10 value for nickel could not be accurately estimated, the no-observed-effect concentration value for nickel was 1070 µg/L. Exposure to copper and cadmium caused changes in internal cell granularity and increased chlorophyll a fluorescence. Lead, zinc, and nickel had no effect on any of the cellular parameters measured. The present study provides valuable metal-ecotoxicity data for an Antarctic marine microalga, with P. antarctica representing one of the most sensitive microalgal species to dissolved copper ever reported when compared with temperate and tropical species.

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A robust bioassay to assess the toxicity of metals to the Antarctic marine microalga *Phaeocyctis antarctica*

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ABSTRACT

Despite evidence of contamination in Antarctic coastal marine environments, no water quality guidelines have been established for the region due to a paucity of biological effects data for local Antarctic species. Currently there is limited information on the sensitivity of Antarctic microalgae to metal contamination, which is exacerbated by the lack of standard toxicity testing protocols for local marine species. In the present study, a routine and robust toxicity test protocol was developed using the Antarctic marine microalga *Phaeocystis antarctica*, and its sensitivity investigated following 10-d exposures to dissolved copper, cadmium, lead, zinc, and nickel. In comparisons of IC10 (10% inhibition of population growth rate) values, *P. antarctica* was most sensitive to copper (3.3 µg/L), followed by cadmium (135 µg/L), lead (260 µg/L) and zinc (450 µg/L). While an IC10 value for nickel could not be accurately estimated, the NOEC value for nickel was 1070 µg/L. Exposure to copper and cadmium caused changes in internal cell granularity, and increased chlorophyll *a* fluorescence. Lead, zinc and nickel had no effect on any of the cellular parameters measured. The present study provides valuable metal-ecotoxicity data for an Antarctic marine microalga, with *P. antarctica* representing one of the most sensitive microalgal species to dissolved copper ever reported when compared to temperate and tropical species.

Keywords: Polar ecotoxicology, Water quality guidelines, Metals, Contaminants, Flow cytometry

INTRODUCTION

Polar regions are subject to inputs of anthropogenic contaminants, and some of these inputs are expected to increase in the future [1]. In the Arctic, extraction of oil and gas, mining, disposal of nuclear wastes and human settlements have resulted in contamination of terrestrial and aquatic environments [1]. Contaminant classes detected in the Arctic include polychlorinated biphenyls (PCBs), organochlorine pesticides, petroleum hydrocarbons, persistent organic pollutants (POPs), heavy metals and radionuclides [2]. It is well documented that these contaminants are accumulating in, and causing toxicity to endemic flora and fauna [3-5].

Many of the above anthropogenic activities are not yet permitted in Antarctica, and so the continent is considered to be the last pristine environment on Earth [1]. However, over a century of human activities in the region including scientific research, fishing, and tourism, as well as atmospheric pollution from lower latitudes has impacted the Antarctic environment [1]. Metals, petroleum hydrocarbons, PCBs, POPs and nutrients have been detected all over Antarctica, particularly in regions adjacent to scientific research stations, including Arthur Harbour, McMurdo Sound [6], the Ross and Weddell Seas [7], King George Island [8], and Wilkes Land, East Antarctica [9]. Contaminant sources include sewage discharge, fuel spills, and leachates from abandoned stations and legacy waste tips [9]. In East Antarctica, metals are common contaminants of particular concern as they have been shown to impact on local flora and fauna. Elevated concentrations of copper, cadmium, nickel and lead occur in marine sediments and tissues of benthic fauna in nearshore environments adjacent to Australia's Casey station in East Antarctica [10]. Furthermore, changes in the community composition of local benthic diatoms were directly linked to metal contamination (tin, lead, copper and iron) in sediments adjacent to Casey station [11], whilst Majer et al. [8] observed bioaccumulation of arsenic, cadmium and lead in invertebrates and macroalgae collected from Admiralty Bay, King George Island on the Antarctic Peninsula.

Water quality guidelines (WQGs) for the Antarctic marine environment have not yet been established. As a consequence, ecological risk assessment protocols and the thresholds values necessary to protect endemic flora and fauna, as well as targets for contaminated site remediation, do not exist. While robust ecotoxicity data for temperate marine species is available (e.g. ANZECC/ARMCANZ, OECD, USEPA, Environment Canada), it is not appropriate to apply these to polar regions due to the vast differences in environmental parameters, physical and chemical dynamics, species composition, and the unique physiological characteristics of polar organisms [12]. The absence of guidelines is exacerbated by the lack of routine protocols to assess contaminant effects in Antarctic species, as polar organisms have slower growth rates, longer development times, and slower acute responses associated with the lower temperatures than do related tropical and temperate species [1].

Revisions to the Australian and New Zealand WQGs recommend a minimum of eight species from at least four taxonomic groups for water quality guideline value derivation [13]. Only a handful of studies have assessed the toxicity of metals to marine biota in Arctic regions with investigations focussing primarily on invertebrates including sea urchins and amphipods [14, 15]. Similarly, a limited number of studies have evaluated the toxicity of metals to Antarctic marine biota. Antarctic ecotoxicology protocols exist for sea urchin development [16], amphipod survival [17], polychaete survival [18], and chlorophyll fluorescence in macroalgae [19]. While one recent study screened a number of microalgae species for their potential use in toxicity tests [20], no routine and robust protocols are currently available for Antarctic marine microalgae.

Microalgae are important test organisms in toxicity assessments due to their environmental relevance (as primary producers, the foundation of aquatic food webs, and wide distribution), ease of culturing, rapid growth rates (relative to higher trophic organisms) and as their cell wall is in direct contact with aqueous contaminants, often making them more sensitive than invertebrates or fish [21]. Microalgae are critical to the ecological structure and function within Antarctic marine systems, with many higher order organisms dependent on the seasonal phytoplankton blooms for their nutrition [20]. As important members of Antarctic ecosystems and food webs, microalgae are highly relevant species for WQG development. Chronic toxicity, measured as growth rate inhibition, is the most sensitive and relevant endpoint in microalgal toxicity testing, as it allows the observation of toxicity over several generations of cells [21]. Numerous test protocols exist for a range of temperate and tropical microalgae [22, 23], with some species exhibiting toxic effects at concentrations as low as 1 µg Cu/L [24].

This research investigates the toxic effects of dissolved metals on *Phaeocystis antarctica*. This species is commonly found in coastal Antarctic waters, contributing ~65% to the total annual primary production in the Southern Ocean, and is an important food source for copepods, krill and other zooplankton [25]. The resultant ecotoxicity data from the present study will be used in the future to contribute to the development of WQGs for the Antarctic marine environment.

METHODS

General laboratory techniques and reagents

All plasticware used for analyses was new, and was washed by soaking in 10% (v/v) HNO_3 (AR grade Merck KGaA) for ≥24 h, followed by thorough rinsing with deionized water (Milli-Q, 18 MΩ.cm, Merck). Borosilicate glass Ehrlenmeyer flasks (250-mL) were silanised, i.e. coated with a silanising solution (Coatasil, Ajax Chemicals), to minimise the adsorption of metals to the flask walls. Flasks were thoroughly rinsed with deionised water, and then washed as above prior to use in toxicity tests.

Seawater was collected from the shoreline at Cronulla, NSW, Australia, in high-density polyethylene (HDPE) containers. On return to the laboratory, seawater was immediately filtered through an acid-washed (10% v/v, nitric acid, Merck) 0.45-µm cartridge filter (Sartobran P sterile midicap, Sartorius Stedium Biotech) to produce natural filtered seawater (FSW), and was stored in polyethylene containers in the dark at 4˚C. Following filtration, sub-samples were taken, acidified (0.2% v/v, nitric acid, Merck) and analysed for dissolved metals as per methods below. Dissolved metal concentrations in filtered seawater are presented in supplemental data, Table S1. Salinity (YSI salinity and conductivity meter, model 30/10 FT, YSI), pH (Thermo Orion pH meter model 420, probe ROSS 815600, Thermo Fischer Scientific), and dissolved oxygen saturation (Oximeter 330 WTW) were measured in test solutions using equipment calibrated as per manufacturer's instructions.

All chemicals used were analytical grade or higher. Metal stock solutions were prepared from copper (II) sulfate (A.R grade, AJAX Chemicals); cadmium (II) sulfate octahydrate (Lab reagent, Analar, BDH Chemicals Ltd.), lead chloride (Lab reagent, Ajax chemicals), nickel chloride hexahydrate (Lab reagent, Analar, BDH Chemicals Ltd.) and zinc chloride (ACS reagent, Sigma), then acidified to 1% HCl (Tracepur, Merck).

Microalgae cultures and toxicity tests

Phaeocystis antarctica (Prymnesiophyceae, strain number AAD 133, solitary cell phase) was obtained from the Australian Antarctic Division, Kingston, Tasmania. Cultures were maintained in one fifth strength Gse medium (Gse/5) [26] as described in Table 1, in a temperature controlled room at 0 ± 2˚C, with a 20:4 h light to dark ratio and light intensity of 150-200 µmol photons /m^2/s (cool white 36W/840 globes, Sylvania Lighting, Sydney, Australia), conditions that are broadly representative of those that this alga would experience in its natural environment during the Antarctic summer.

FSW was used as the control/diluent water and was supplemented with nutrients including 1.5 mg NO_3^- /L and 0.15 mg PO_4^{3-}/L ($NaNO_3$ and KH_2PO_4, AR grade, APS Ajax) in order to maintain exponential growth of the algae over the test period [27]. Metal treatments were prepared by adding the appropriate volume of

metal stock solution to FSW, with 3 to 5 concentrations for each metal. Nominal concentrations of metals tested in definitive tests were 1 to 20 µg/L of copper, 100 to 2000 µg/L of cadmium, 10 to 500 µg/L of lead, 100 to 2000 µg/L of zinc and 200 to 1000 µg/L of nickel. Three replicates per treatment and control were used. For each metal, one rangefinder and two to 4 replicate tests were carried out.

Cultured cells in the exponential growth phase (8-12 days old) (Supplemental data, Figure S1) were centrifuged (280 *xg*, 7 min, 1 ± 2˚C) and washed in FSW three times, then counted by flow cytometry (BD-FACSCalibur, Becton Dickenson BioSciences) and inoculated into test flasks at a density of 1-3 x 10^3 cells/mL. Toxicity tests were performed over 10 days, by which time cell densities in the control treatments had increased 16-fold (as per OECD guidelines [22]). The cell densities were determined every second day, and the growth rate (cell division; µ) was calculated as the slope of the regression line from a plot of $\log_{10}$ (cell density) versus time (h) [27]. Growth rates for all treatments were expressed as a percentage of the control growth rates. The pH was measured on days 0 and 10, and sub-samples were taken from test solutions on days 0, 6 and 10, filtered to 0.45 µm then analysed to determine dissolved metals as per Levy et al. [28]. Flasks were swirled and moved to a new random position on the light box daily.

Effects of metals on cellular parameters

Flow cytometry was used to simultaneously measure cell density, cell size, chlorophyll *a* fluorescence intensity, and internal cell granularity/complexity. Using an excitation laser (488 nm), the light scattered by the algal cells is measured by two photomultiplier tubes: the forward-angle light scatter (FSC <15˚) which provides information on cell size, and the side-angle light scatter (SSC 90˚) which provides information on internal cell complexity and granularity. Chlorophyll *a* fluorescence was detected as red fluorescence in > 670 nm long pass filter band (FL3). Parameters were set according to Franklin et al. [27]. Cell density was estimated using fluorescent beads (BD Biosciences TruCount beads) as an internal standard [27]. In brief, 0.15 mL of bead stock was added to 0.5 mL of test solution and mixed well prior to analysis. Healthy (control) cells were identified and displayed in a one-dimensional dot plot (SSC v FL3) using the flow cytometric software package CellQuest Pro. Cells identified and captured in this plot were then analysed in one-dimensional histogram plots of cell number vs FL3 (chlorophyll *a* fluorescence), FSC (cell size) and SSC (internal cell granularity), defining three regions of fluorescent/light scattering states (R1, R2, R3). The R2 region was set to capture a minimum of 95% of healthy control cells. The R1 region was set to the left of R2 to indicate a decrease in each parameter. The R3 region was set to the right of R2, to indicate an increase in each parameter. FlowJo software (version 7/9, Tree Star Inc) was used to depict shifts on the FL3, FSC and SSC axes.

Metal analysis

Dissolved metals (0.45 µm) were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Varian 730-ES). Metal concentrations

were calculated from a matrix-matched calibration curve, using a serial dilution of an internal mixed metal standard (metals included: Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, Tl, V, Zn). A 200 µg/L internal mixed metal drift standard was incorporated into the analysis procedure, in addition to seawater blanks. The ICP-AES detection limits for Cu, Cd, Pb, Ni and Zn were 1, 0.12, 1.7, 1.2 and 0.1 µg/L, respectively. For copper, the lowest concentration tested was the same as the detection limit for the ICP-AES (1 µg/L), and so for these samples, copper was analysed by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500CE), where the detection limit was 0.05 µg/L. All samples were diluted 1:3 with 0.2% nitric acid (Tracepur, Merck) to reduce the salt load on the ICP-MS, which was calibrated with matrix-matched Milli-Q® water standards and an internal standard (indium 115). For quality assurance purposes, acid-digest blanks (10% of samples), replicates (20% of samples), analyte sample-spikes and Environment Canada certified reference materials (SLEW-3 estuarine water and CASS-5 near-shore seawater) were also analysed. Replicates were within 20% of each other and recoveries for spikes and standards were within 85-115% of expected values. The limits of reporting for the various methods were less than 10% of the lowest measured values. For all metals the mean dissolved concentration measured across days 0, 6 and 10 was used in all statistical analyses to derive toxicity values.

Statistical analysis

The specific growth rates (µ) were used to derive toxicity values using Toxcalc (Version 5.0.23, TidePool Scientific Software). Data were tested for normal distribution using Shapiro-Wilk's test ($p > 0.01$); and equal variances using Bartlett's test ($p = 0.09$). The inhibitory concentration which reduced population growth rate by x% (ICx as well as 95% confidence limits - CL) compared to controls was estimated using linear interpolation. The Dunnett's multiple comparison test was used to determine which treatments were significantly different to the control (2 tailed, $p \leq 0.05$) in order to determine the no observable effect concentration (NOEC) and the lowest observable effect concentration (LOEC).

For each metal, data from individual toxicity tests were deemed to be not different due to an overlap in the 95% confidence limits around the IC10/50 values. Therefore data from replicate tests for each individual metal were pooled. Toxcalc is an industry standard software package, widely used in ecotoxicology. While this program was suitable to analyse data from individual toxicity tests, it has limited ability to analyse pooled data sets from multiple tests carried out over time. This is because of inherent limitations associated with the linear interpolation method that this program uses to determine ICx estimates. This method requires that data be monotonically non-increasing (i.e. the mean response at each higher concentration is less than or equal to the mean response at the previous treatment), and that the data follow a piecewise linear response function [29]. As a result, linear interpolation methods distort variable data and fail to adequately account for variability around treatment concentrations. To overcome this limitation, the R studio package program

with the drc method, was used to analyse and to plot pooled data from multiple tests [30]. Seven different models were fitted to all datasets, and based on the residual standard errors and the number of parameters for each model; the log-logistic model (with 3 parameters) was selected as the best model to represent the data in all cases. IC10 and IC50 values and corresponding 95% confidence limits were estimated from the log-logistic model plots.

The statistical software NCSS© (Version 07.1.3), was used to run a one-way analysis of variance with Dunnett's two-sided Multiple Comparison test with control, to determine significant differences (with an alpha value of 0.05) in the percentage of cells fluorescing for chlorophyll *a* fluorescent intensity, cell size and internal cell granularity between treatments.

RESULTS

Chronic growth rate inhibition tests with Phaeocystis antarctica

The control growth rate for *P. antarctica* was reproducible across eight individual toxicity tests, with an average of 0.54 ± 0.16 (2 SD) doublings per day (Supplemental data, Table S2). In all treatments in all toxicity tests, pH did not fluctuate by more than 0.5 units over the 10-d test duration (data not shown). Of all the metals tested, copper was the most depleted from solution after 10 days, decreasing by up to 46% in the lowest exposure concentration (nominal, 1 µg/L) (Supplemental data, Table S3). Lead decreased by 2 to10% at higher concentrations and by 5 to13% in lower exposure concentrations respectively. Dissolved concentrations of cadmium, nickel and zinc decreased by 1-8% after 10 days across all treatments (data not shown).

Each of the metals tested produced different concentration-response relationships, in terms of their toxicity to *P. antarctica* (Figure 1). In general, population growth rate decreased with increasing metal exposure concentration. Copper was the most toxic metal, with the lowest IC10 value of 3.3 µg/L, followed by cadmium (IC10 135 µg/L), lead (IC10 260 µg/L) and zinc (IC10 450 µg/L) (Table 2). There was high variability in responses to nickel exposure, which prevented the accurate estimation of IC10 and IC50 values (Table 2). Some concentrations (60, 90, 510, 1070 µg/L) actually stimulated the population growth rate. For example, at 1070 µg Ni/L, the growth rate was 11% greater than in the control, although this increase was not significant.

After a 10-d exposure, dissolved measured copper concentrations ≥12 µg/L caused complete (100%) inhibition in the growth rate of *P. antarctica* (Figure 1A). Increasing concentrations of cadmium also inhibited the population growth rate of *P. antarctica*, with approximately 60% inhibition at concentrations ≥900 µg/L (Figure

1B). Measured concentrations of dissolved lead ≥100 µg/L significantly decreased growth rates of *P. antarctica*, however complete inhibition was never observed, with only 60% inhibition at the highest concentration tested (450 µg/L; Figure 1C). Dissolved zinc decreased population growth rate, reaching 80% inhibition at 1860 µg/L (Figure 1D). In contrast to the other metals, there was no observed growth rate inhibition due to nickel exposure (Figure 1E).

Changes in cellular parameters of Phaeocystis antarctica *in response to metal exposure*

Changes in cellular parameters in *P. antarctica* following exposure to metals were observed as shifts on the FL3, FSC and SSC axes as depicted in Figure 2. Exposure to copper caused a concentration-dependent increase in chlorophyll *a* fluorescence intensity (Figure 3A). Ten-day exposures to 6 µg Cu/L significantly increased fluorescence in 15% of cells ($p < 0.05$), and inhibited growth rate by ~50% (Figure 1A). The effect of copper on cell size was less significant; at 9 and 15 µg Cu/L, the percentage of cells in the R2 region were significantly less than in the control treatments (Figure 3B). While the percentage of cells fluorescing in the R1 and R3 regions increased, this was not significantly different to the controls. Increasing concentrations of copper also caused an increase in internal cell granularity, with a significant shift of cells into the R3 region on the SSC axis, after 10-d exposure to ≥9 µg Cu/L (Figure 3C).

In contrast to copper, increasing concentrations of cadmium significantly decreased chlorophyll *a* fluorescence intensity ($p < 0.05$); at concentrations ≥910 µg Cd/L, ~40% of cells were fluorescing in the R1 region on the FL3 axis (Figure 4A). A simultaneous decrease in cell size was also observed at ≥910 µg Cd/L with 40% of cells fluorescing in the R1 region (Figure 4B). Cadmium concentrations caused an increase in internal cell granularity, with 50% of cells displaying greater internal complexity after 10-d exposures to ≥910 µg Cd/L (Figure 4C). Exposure to lead, zinc and nickel caused no significant changes in any of the cellular parameters analysed (data not shown).

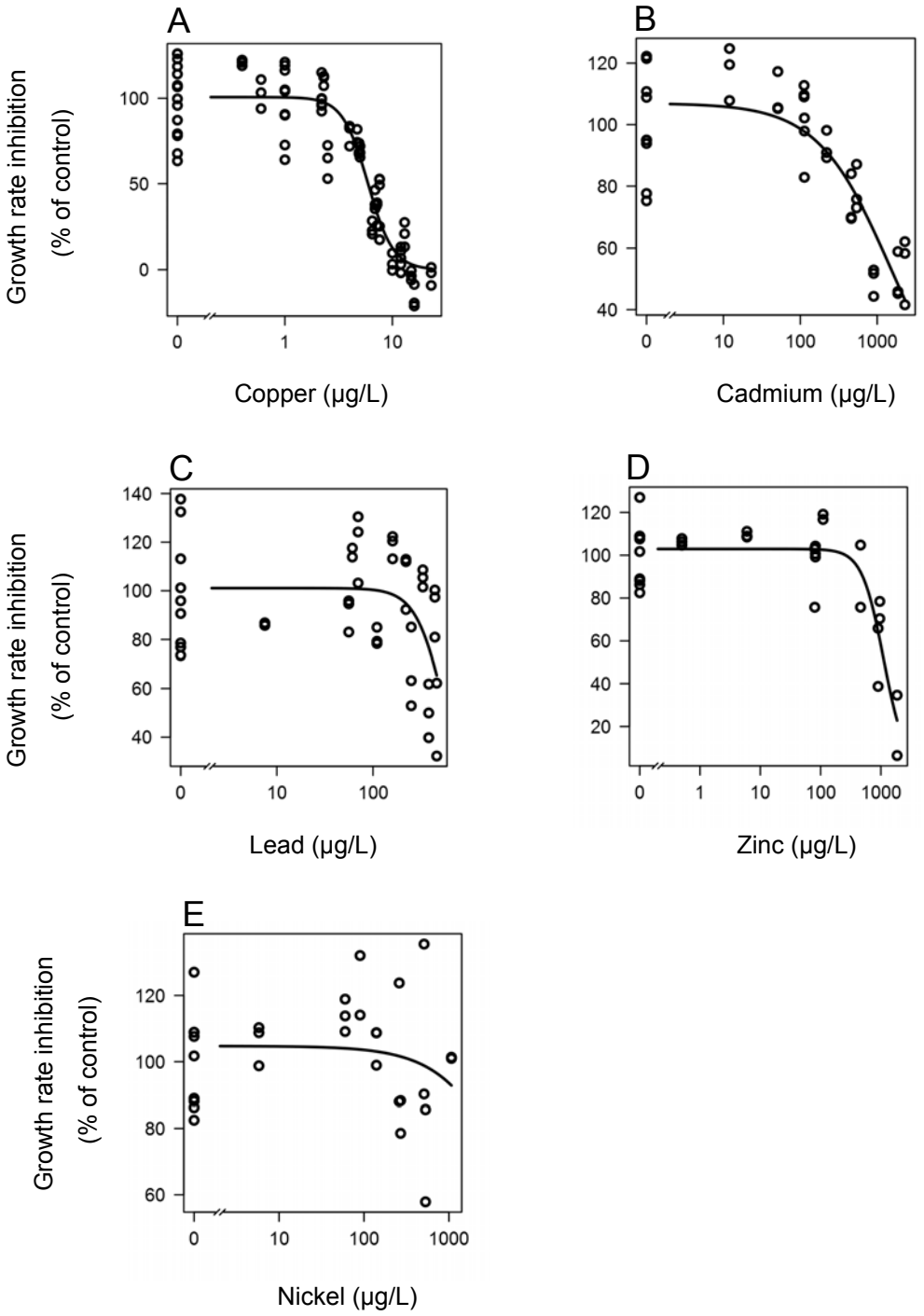

Figure 1 The effect of metals on the growth rate of the Antarctic microalgae *Phaeocystis antarctica* after 10-day exposure to A) copper, B) cadmium, C) lead, D) zinc and E) nickel. Each point represents 1 replicate from an individual toxicity tests.

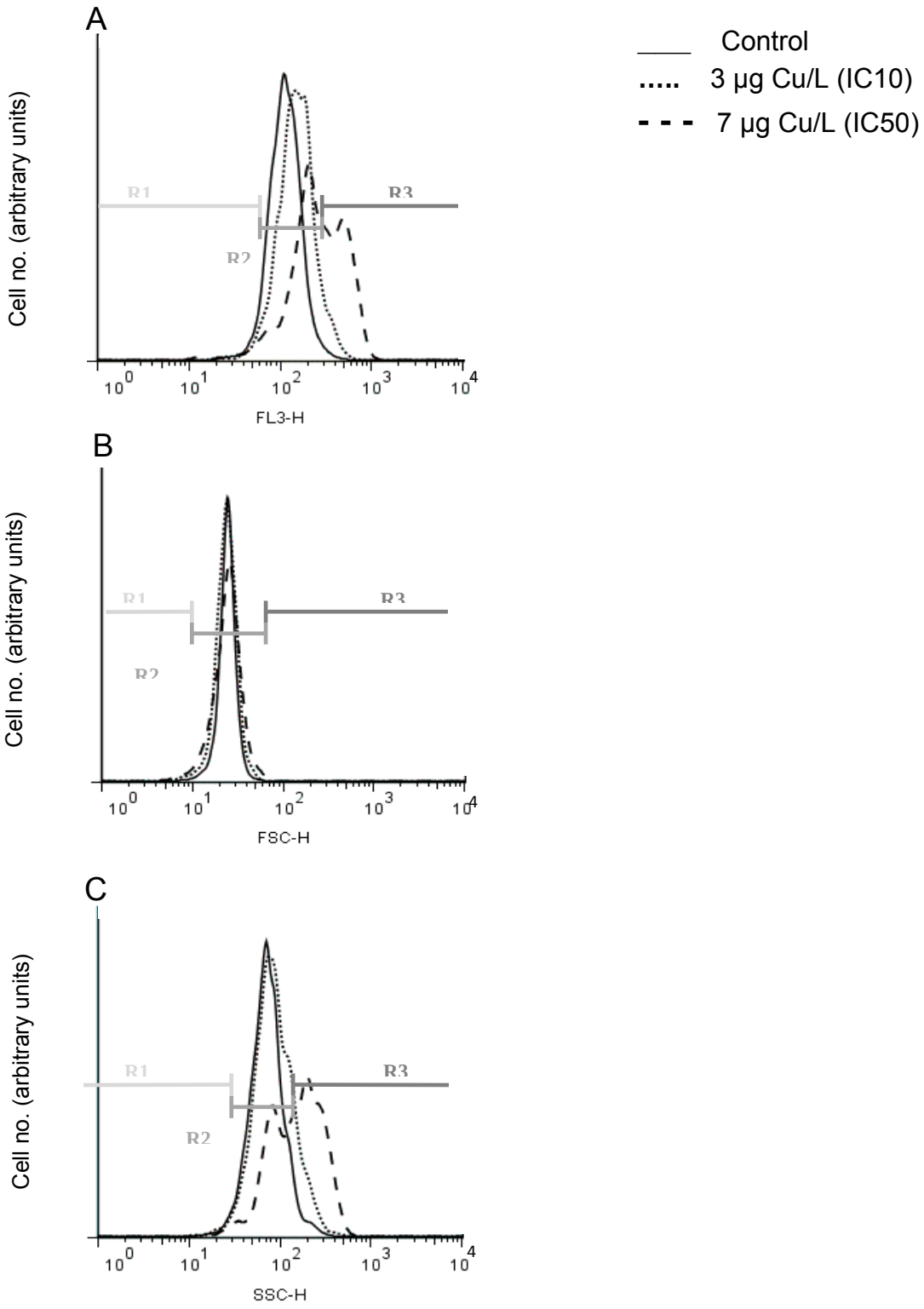

Figure 2 Representative diagram to demonstrate the defined regions (R1, R2, R3) and to depict the shifts described on the FL3, FSC and SSC axes. Each treatment is represented by one replicate from one individual toxicity test, showing the effect of copper on A) chlorophyll *a* fluorescence intensity B) cell size and C) internal cell granularity, in the Antarctic microalgae *Phaeocystis antarctica* after 10-day exposure.

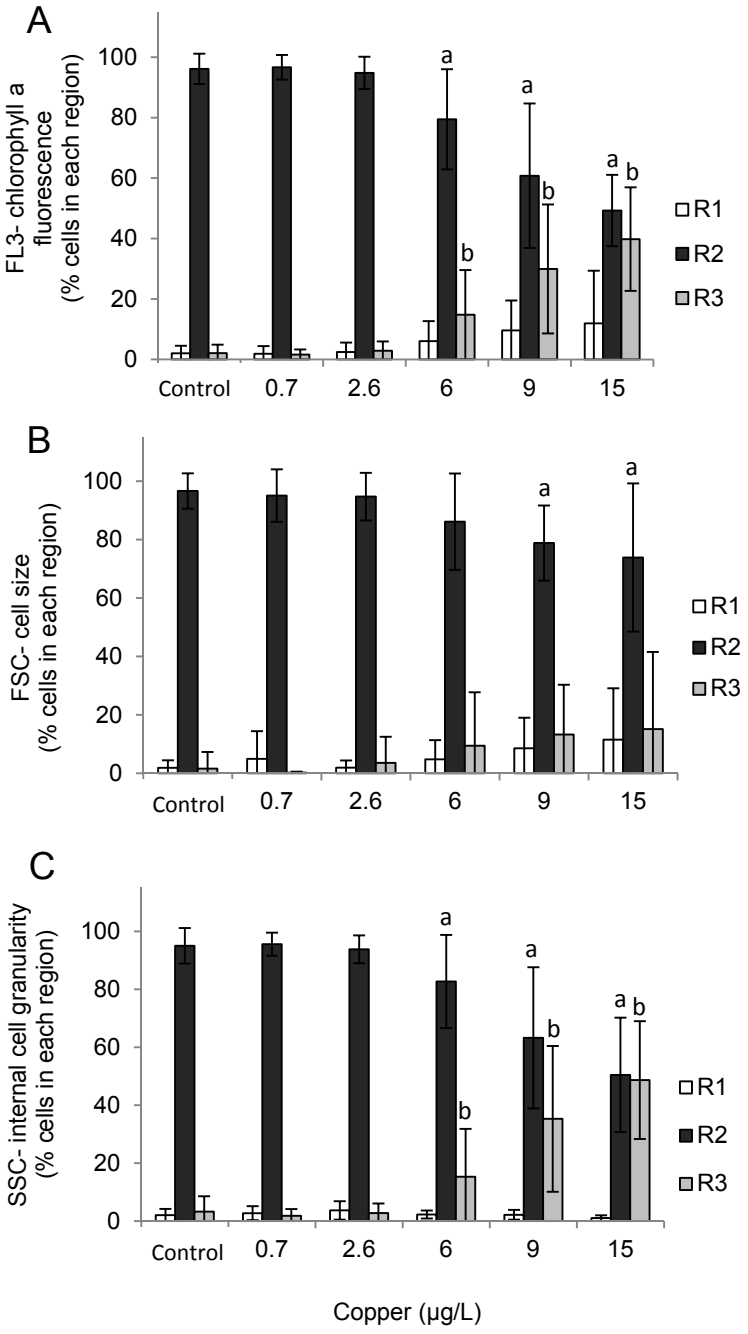

Figure 3 The effect of copper on cellular parameters A) chlorophyll a fluorescence intensity, B) cell size, C) internal cell granularity, in the Antarctic microalgae *Phaeocystis antarctica* after 10-day exposure (mean ± SD, n=5). R1 = decrease in parameter relative to control cells, R2 = parameter is the same as control cells, R3 = increase in parameter relative to control cells. Different letters indicate significant difference to the control ($p \leq 0.05$).

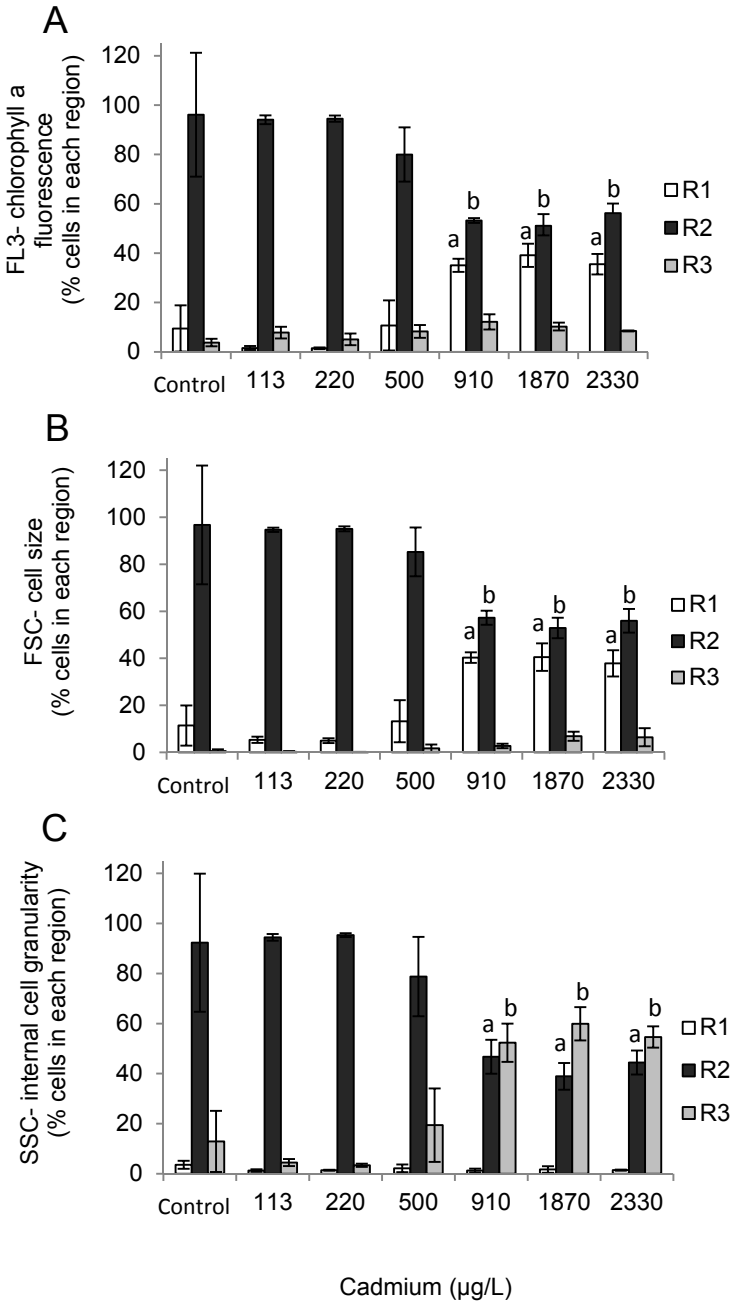

Figure 4 The effect of cadmium on cellular parameters A) chlorophyll a fluorescence intensity, B) cell size, C) internal cell granularity, in the Antarctic microalgae *Phaeocystis antarctica* after 10-day exposure (mean ± SD, n=3). R1 = decrease in parameter relative to control cells, R2 = parameter is the same as control cells, R3 = increase in parameter relative to control cells. Different letters indicate significant difference to the control ($p \leq 0.05$).

DISCUSSION

Development of chronic growth rate inhibition test with Phaeocystis antarctica

In the present study, the growth-rate inhibition bioassay was based on standard toxicity test protocols for temperate and tropical species [22], with modifications to the some test conditions including temperature and test duration. Standard tests with temperate species typically run for 48-96 h, by which time microalgae have reached a minimum 16-fold increase in biomass in the control treatments. The growth rate of *P. antarctica* was approximately three times slower than temperate and tropical microalgal species (Table 3), requiring 10 days to reach a 16-fold increase, likely a result of the low Antarctic water temperatures. Antarctic marine organisms have evolved to suit their extreme environmental conditions, with the constant low temperatures resulting in slower metabolism and growth rates, and longer developmental times [1]. These inherent characteristics indicate that Antarctic organisms may take longer to respond to environmental stressors, and hence longer exposures are required in bioassays to observe a significant toxic effect [12]. This hypothesis was supported by Marcus-Zamora et al. [31] who found that longer exposure durations were required for Antarctic copepods (7 days, in comparison to 2 days for tropical species [32]) in order to generate relevant sensitivity data for metals.

Exposure duration is a confounding factor in toxicity tests with microalgae, causing an underestimation in toxicity due to the depletion of nutrients and metals from the test solution [33], hence this must also be considered when developing growth-rate inhibition tests with Antarctic marine microalgae. We showed that in tests with *P. antarctica*, exponential growth in the control treatments was still occurring up to 10 days (Supplemental data, Figure S2), indicating that nitrate and phosphate were ample in the test solutions.

Decreases in dissolved metal concentrations are likely due to metal adhering to the glass walls of the test flasks, to adsorption to cell surfaces and to internalisation by cells [28]. In the present study, initial copper tests used flasks which had been silanised 12 months prior, and in these tests we observed the greatest loss in copper. Tests conducted later used flasks which were silanised within 1 month of their use in tests. Therefore, it is recommend that newly silanised flasks be used in future studies to reduce the loss of dissolved metals to the glass walls of test flasks. It is difficult to determine why loss of dissolved copper was greater than for other metals. It is possible that copper was adsorbed/absorbed by algal cells; however this cannot be confirmed. Future experiments should also measure metal concentrations accumulated in algal cells.

Relative toxicity of Cu, Cd, Pb, Zn, and Ni to Phaeocystis antarctica

Copper was the most toxic metal to *P. antarctica*. Cadmium, lead, zinc and nickel were less toxic (Table 2), a trend that has been reported elsewhere in the

literature for many other microalgal species (Table 3). The sensitivity of *P. antarctica* to copper (10-d IC50 of 5.9 µg/L) was similar to that of a related tropical alga, *Isochrysis sp.* (3-d IC50 of 4 µg/L) which is also in the Prymnesiophyceae class [24]. When comparing IC50 values for lead, *P. antarctica* (570 µg/L) was more sensitive than another Prymnesiophyte, *Isochrysis galbana* (1340 µg/L) [34].

Our study incorporated current best practices in microalgal toxicity testing methods, i.e. use of low nutrient test media, low initial cell density, and of accurate cell counting methods (flow cytometry) [27]. There are few published studies that have incorporated these aspects into their work that have also investigated the toxicity of cadmium, zinc and nickel to microalgae (Table 3). This makes comparisons of the sensitivity of *P. antarctica* to other species difficult. *Phaeocyctis antarctica* is the least sensitive to zinc out of four marine alga which included three Bacillariophyceae (IC50 range of 65-910 µg Zn/L, Table 3); and the second most sensitive species to cadmium out of three species, two of which are also Bacillariophytes (IC50 range of 350 and 2400 µg/L, Table 3).

Interestingly, the response of *P. antarctica* to nickel differs from responses observed for other marine microalgae. The temperate marine diatom *Ceratoneis closterium* (previously known as *Cylindrotheca closterium* and *Nitzschia closterium*) was more sensitive to nickel, with 72-h IC50 of 250 µg Ni/L [35] (Table 3). In contrast, no growth rate inhibition was observed in *P. antarctica* at concentrations as high as 1070 µg Ni/L. Similar tolerance to nickel has only been shown so far for freshwater green microalgae (including *Scenedesmus quadricauda* and *Chlorella miniata*), which grew in wastewaters containing up to 30 mg Ni/L. These species were even able to successfully sequester nickel from the wastewater, reducing its concentration by up to 97% [36].

The concentrations of metals that inhibited population growth rate in *P. antarctica* are well above concentrations occurring naturally in Antarctic marine waters, and are only likely to be encountered by *P. antarctica* in the most contaminated sites in Antarctica. Background metal concentrations in Antarctic waters have been reported at 1.9 ng Cu/L, 6.5 ng Zn/L , 0.45 ng Cd/L, 2 ng Pb/L and 400 ng Ni/L [37, 38]. However, elevated concentrations do occur at contaminated sites near the coastline including those adjacent to abandoned waste tips and in the vicinity of wastewater outfalls. Sediments collected from bays surrounding Australian research stations are known to be contaminated with copper (10-13500 mg/kg), zinc (20-8300 mg/kg) and lead (50-4890 mg/kg) [39]. These sediments are a source of metal fluxes to the water phase, both in pore waters and in the water column, via bioturbation, mixing by currents and tides, as well as iceberg scour [40]. In addition, during the summer season, meltwater runs through abandoned waste tips, mobilising contaminants and redistributing them into the receiving near-shore environment and coastal waters [39].

Effect of metals on cellular parameters in Phaeocystis antarctica

The effect of exposure to dissolved metals on cellular parameters in *P. antarctica* provided additional information on the potential modes of toxic action of the five metals tested. After 10-d exposure to copper and cadmium, not only was the population growth rate of *P. antarctica* inhibited, there were also significant changes in chlorophyll *a* fluorescence intensity, cell size and internal cell granularity, relative to the controls (Figures 3 and 4). However, zinc, lead and nickel did not cause any significant changes in any of the cellular parameters investigated (data not shown).

Franklin et al. [41] investigated the effects of increasing copper concentration on chlorophyll *a* fluorescence in two freshwater microalgae (*Chlorella sp., Selenastrum capricornutum*) and two marine microalgae (*Phaeodactylum tricornutum, Dunaliella tertiolecta*) exposed for 48-72 h. Copper significantly decreased chlorophyll *a* fluorescence in *Chlorella sp.* at exposures ≥90 µg/L, with some reduction in fluorescence observed in *S. capricornutum* at only 1.6 µg/L. This is interesting as both species have the same 72-h IC50 value (8 ± 2 µg Cu/L). Franklin et al. [41] suggest that this is evidence for an uncoupling of the mechanisms of cell division and photosynthesis. During the exponential phase of microalgal growth, external dissolved metal concentrations, cellular metal uptake rate, intracellular metal concentrations, and specific growth rate are all linked to one another by a series of interconnecting relationships [42]. Toxicity occurs when a metal interferes with any one of these factors. For example it has been shown that copper oxidizes thiols in marine diatoms which leads to a lowering of the reduced glutathione to oxidised glutathione (GSH:GSSG) ratio. This in turn may inhibit spindle formation during mitosis and consequently cell division (or growth rate) [43]. Similar to our results, copper increased chlorophyll *a* fluorescence intensity in 60% of *P. tricornutum* cells after 72-h exposure to ≥11 µg Cu/L, while growth was inhibited at the same concentrations (IC50 10 ± 4 µg Cu/L) [41] . Lelong et al. [44] also found that copper increased fluorescence in two strains of *Pseudo-Nitzschia* (*P. multiseries* and *P.delicatissima*) at concentrations ≥46 µg Cu/L.

Non-essential metals accumulated by microalgal cells can bind to active sites of metalloproteins (for example cadmium can replace manganese) and this can inhibit normal cellular metabolism [42]. In our study, chlorophyll *a* fluorescence intensity decreased significantly ($p < 0.05$) with increasing cadmium concentration (Figure 4A). While inhibition in chlorophyll *a* fluorescence is caused by a suppression of electron flow in Photosystem II (PSII) reaction centre at the donor side, an increase in fluorescence occurs if the inhibition takes place at the acceptor side of PSII [41, 45]. Based on these studies, it can be inferred that exposure to copper increases chlorophyll *a* fluorescence intensity through the inhibition of the PSII at the acceptor side in *P. antarctica* cells. Conversely, the decrease in chlorophyll *a* fluorescence observed in cadmium-exposed *P. antarctica* could be a result of the

metal inhibiting the PSII at the donor side. However, further studies are needed to support or reject these hypotheses.

Increased cell granularity was observed in *Chlorella sp.*, *S. capricornutum* and *P. tricornutum* after 72-h exposures up to 18 µg Cu/L [41] and in *P. delicatissima* at 48 µg Cu/L after 48 h exposures [44]. The SSC parameter indicates changes in internal cell structures of algae; for example lysosomes, vacuoles, starch granules, polyphosphate bodies, nucleus, as well as the cell wall [41, 46] . An increase in the SSC parameter could be correlated to an increase in the number or size of any of these structures; for example polyphosphate bodies, which have also been shown to bind metals in a non-toxic form within the cell [47]. The SSC parameter may also indicate changes in cell size because changes internally (e.g. swollen vacuoles) will lead to an increase in cell size [43]. However, in the present study, it was shown that while copper caused an increase in internal cell granularity in *P. antarctica*, no significant changes in cell size occurred (Figures 3 B and C). In contrast, cadmium caused an increase in cell granularity and a simultaneous decrease in cell size (Figures 4 B and C). Levy et al. [28] also showed that when *Tetraselmis sp.* and *D. tertiolecta* were exposed to elevated concentrations of copper, there were no changes in cell size for either species, but they did observe an increase in the number of vacuoles and their size within cells. They concluded that compartmentalisation of copper may be a detoxification mechanism for these two species [28], and this is perhaps a mechanism also implemented by *P. antarctica* when exposed to copper and cadmium, however further studies would be required to confirm this.

Visviki and Rachlin [46] used electron microscopy to study ultra-structural changes following exposure to increasing concentrations of copper and cadmium in two temperate microalgae *Dunaliella salina* and *Chlamydomonas bullosa*. Both metals caused significant changes in total cell volume, nucleus, starch granules, polyphosphate bodies, lipids, vacuoles and the cell wall. Active chloroplast volume was noticeably reduced when cells were exposed to 380 µg Cu/L and 560 µg Cd/L. It was hypothesised that this reduction in chloroplast volume was an indirect effect brought about by an inhibition of normal cell division and subsequent accumulation of starch granules that compromised the cell's ability to photosynthesise [46]. It is possible that this same effect was occurring in *P. antarctica* following exposure to elevated concentrations of copper and cadmium, as these metals simultaneously decreased growth rate and increased internal granularity/complexity (Figures 1, 3C, and 4C). While there were metal-induced changes inside the cells of *P. antarctica*, accurate conclusions as to the physiological effects of metals on this species are not possible without further investigations. It would be useful to study internal cell changes in response to metal exposure using electron microscopy, electron spectroscopic imaging or radiotracers to determine intracellular metal distributions and to further predict the subsequent physiological changes and toxic mode of action

for copper and cadmium in this species [46]. Studies on bioaccumulation of metals in polar microalgae would contribute to the understanding of metal transfer and biomagnification in Antarctic food webs. It is also apparent that *P. antarctica* is tolerant to dissolved zinc, lead, nickel and cadmium; it would be valuable to understand the tolerance of *P. antarctica* to these metals.

CONCLUSION

While some work has been done previously on screening Antarctic microalgae species to metals [20], standard toxicity test procedures have not been developed, and the present study is the first to establish and describe a robust, routine test protocol with a polar microalgal species, the Antarctic marine alga *P. antarctica*. It was shown that longer exposure times are required for slower growing Antarctic microalgae in order to produce relevant toxicity data, and it is likely that extended exposure durations will be required for tests with other polar microalgal species. A test duration of 10 days was required to achieve suitable growth of *P. antarctica* in control treatments, which is three times longer than that used in most temperate and tropical microalgal toxicity tests. Through the development and application of a toxicity test protocol for *P. antarctica*, ecotoxicity data for Cu, Pb, Zn, Cd and Ni has been produced. Based on growth rate inhibition, copper was the most toxic metal, with *P. antarctica* now realised as one of the most sensitive species to copper out of a range of temperate and tropical species. Cellular parameters provided some insight into potential modes of toxic action for the different metals tested, however further studies would help further elucidate these mechanisms. The protocol developed in the present study can be used in future investigations into the toxicity of other contaminants including other metals, petroleum hydrocarbons, polyaromatic hydrocarbons and POPs. Finally, the present study has provided valuable ecotoxicity data for metals for an important taxonomic group that can be used in the derivation of WQGs for the Antarctic marine environment.

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

Culture/toxicity test conditions	
Temperature	0 ± 2˚C
pH	8.1 ± 0.2
Salinity	35‰
Light intensity/quality	150-200 µmol/m^2/s, cool white 36W/840 globes
Light cycle	20:4 h light:dark
Toxicity test parameters	
Test type	Static/non-renewal. Flasks swirled every 2 days to assist gas exchange
Test duration	10 days
Test chamber	250-mL Erleymer glass flasks, silanised with coatasil solution, glass lids
Test solution volume	50 mL
Age of test organism	8-12 days, exponential growth phase
Initial cell density	1-3 x10^3 cells/mL
No. of replicate chambers per concentration	3
Control/diluent water	0.45-µm filtered seawater
Test endpoint	Growth rate inhibition
Test acceptability	>0.5 growth rate in controls, pH units within acceptable limits <1 unit change in pH

Table 2. Toxicity of metals to the Antarctic microalgae *Phaeocystis antarctica* based on 10-day growth rate inhibition[a] .

Metal	N	IC10[b] (µg/L)	IC50[c] (µg/L)	NOEC[d] (µg/L)	LOEC[e] (µg/L)
Copper	5	3.3 (2.5-4)	5.9 (5.4-6.4)	1.6	4.3
Cadmium	3	135 (30-240)	1500 (920-2080)	300	910
Lead	3	260 (100-420)	570 (300-830)	120	300
Zinc	3	450 (200-700)	1110 (870-1350)	410	1860
Nickel	3	NR	NR	1070	>1070

[a] Pooled data from individual toxicity tests combined and toxicity values estimated using log logistic model in R, values in parentheses are 95% CL, values are based on measured metal concentrations
[b] Concentration to cause 10% inhibition in growth, compared to controls
[c] Concentration to cause 50% inhibition in growth, compared to controls
[d] Geometric mean, No Observable Effect Concentration, highest concentration tested not statistically ($p \leq 0.05$) different to the control
[e] Geometric mean, Lowest Observable Effect Concentration, lowest concentration tested statistically ($p \leq 0.05$) different to the control

Table 3. Inhibition in population growth rate of marine microalgae from different climatic regions after 72-h exposure to metals.

Class	Algal Species	IC50[a] (µg/L) Cu	Zn	Cd	Ni	Pb	Control Cell Division Rates (doublings per day)[b]	Climatic Region	Reference
Chlorophyceae (green algae)	*Duniella tertiolecta*	530 (450-600)					1.39 ± 0.02	Temperate	[24]
Prasinophyceae (green flagellates)	*Tetraselmis* sp.	47 (46-49)					1.37 ± 0.26	Temperate	[24]
	Tetraselmis chuii	330 ± 100				2640 ± 200	NR	Temperate	[34]
Bacillariophyceae (diatoms)	*Nitzschia closterium*[c]	18 (6-30)					1.53 ± 0.16	Temperate	[24]
				350	250	>500	1.4 ± 0.2		[35]
			65						[48]
	Nitzschia closterium[d]	40 ± 4	226 ± 105		>500		1.4 ± 0.1	Tropical	[49]
					>500		0.8 ± 0.07		[35]
	Entomoneis cf punctulata	22	910	2400		>1000	NR	Temperate	[50]
	Phaeodactylum tricornutum	8 (5-8.3)					1.78 ± 0.08	Temperate	[24]
	Minutocellus polymorphus	0.6 (0.5-0.8)					1.77	Temperate	[24]
	Chaetoceros sp.	88 ± 10				105 ± 60	NR	Temperate	[34]
Dinophyceae (Dinoflagellate)	*Heterocapsa niei*	4.8 (3.5-7.2)					0.96	Temperate	[24]
Prymnesiophyceae	*Isochrysis* sp.	4 (3.8-4.2)					1.85	Tropical	[24]
	Isochrysis galbana	58 ± 30				1340 ± 20	NR	Temperate	[34]
	Phaeocystis antarctica[e]	3.8 ± 0.5					0.6	Antarctic	[20]
	Phaeocystis antarctica[f]	5.9 (5.4-6.4)	1110 (870-1350)	1500 (920-2080)	NR	570 (300-830)	0.54 ± 0.08	Antarctic	This study
Cryptophyceae	*Proteomonas sulcata*	4.2 (2.4-7.5)					1.54	tropical	[24]
	Rhodomonas salina	48 ± 10				900 ± 90	NR	Temperate	[34]

[a] IC50, Inhibition Concentration, concentration of contaminant to cause a 50% inhibition in population growth rate, compared to controls. Values in parentheses are 95% confidence limits or ± 1 standard deviation

[b] 1 standard deviation

[c] Currently known as *Ceratoneis closterium*

[d] Tropical strain of *N. closterium*

[e] IC50 value estimated after 7-8 day exposure

[f] IC50 value estimated after 10 day exposure

NR, not reported

SUPPLEMENTARY TABLES and FIGURES

Table S1. Analysis of filtered (0.45 µm) seawater used in all toxicity tests with *Phaeocystis antarctica*, as control and diluent water (means ± SD, n=4).

	Metal (µg/L)										
Collection date	Al 167.019	As 188.980	Cd 228.802	Co 238.892	Cr 205.560	Cu 327.395	Fe 234.350	Mn 257.610	Ni 216.555	Pb 220.353	Zn 213.857
Jul-2013	2.5 ± 1.3	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Oct-2013	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Dec-2013	2.7 ± 1.4	3.0 ± 0.6	<1	<1	<1	<1	<1	<1	<1	4.8 ± 1.7	<1
Feb-2014	2.5 ± 1.4	3.0 ± 1.5	<1	<1	<1	<0.5	1.7 ± 1.4	<1	1.4 ± 0.1	3.0 ± 1.0	2.0 ± 1.3
Apr-2014	2.5 ± 0.36	2.6 ± 1.1	<1	<1	<1	<0.5	2.2 ± 0.77	<1	1.2 ± 0.04	3.5 ± 1.4	1.6 ± 0.48

Table S2. Mean control growth rate of the Antarctic microalgae *Phaeocystis antarctica* in low nutrient seawater[a] over the 10-day test duration (n=3).

Test No.	Control growth rate (doublings/day)	Coefficient of Variation (%)
1	0.52	6
2	0.58	6
3	0.51	9
4	0.46	19
5	0.45	17
6	0.7	1
7	0.53	15
8	0.6	16
Average	0.54	
2 SD	0.16	

[a] Filtered seawater was supplemented with low levels of nitrate (1.5 mg NO_3^- /L) and phosphate (0.15 mg PO_4^{3-} /L)

Table S3. Decrease in dissolved copper concentrations after 10-day toxicity tests with the Antarctic microalgae *Phaeocystis antarctica.*

Test No.		1	2	3	4	5		
Initial (day 0) copper concentration (µg/L)		Loss of copper over 10-d (%)[b]						
Nominal	Measured[a]						Average	SD
1	0.9 ± 0.3	10	50	33	93	42	46	30
4	2.8 ± 1	22	33	8.7	6.1	5.3	15	12
8	6.1 ± 1.4	16	14	10	2.8	12	11	5.1
12	11 ± 5.5	50	18	5.7	2.3	1.5	16	20
20	16 ± 6.7	36	6	1.7	4.0	1.0	10	15

[a] Average (± 1 SD) concentration from 5 individual copper tests, measured as dissolved (0.45 µm) metal
[b] loss of copper as a % of the initial measured copper concentration for each individual test

Table S4. Toxicity of metals to the Antarctic microalgae *Phaeocystis antarctica* measured as 10-day inhibition in population growth rate. All toxicity values were determined using measured dissolved (0.45 µm) metal concentrations.

Metal	Test	IC10[a] (µg/L)	IC50[b] (µg/L)	NOEC (µg/L)	LOEC (µg/L)
Copper	1	2 (0-3.6)	5.7 (5-6.3)	1	4
	2	2.6 (0-4.8)	5.8 (4.3-6.8)	3	7
	3	1.4 (0.7-3.3)	5.5 (5.2-6)	0.7	2.2
	4	2.8 (0.8-3.9)	8.5 (6.7-10)	2.2	4.9
	5	2.8 (2-3.3)	5.7 (5.2-6.4)	2.5	5
	Mean[d]	2.3 ± 1.2	6.2 ± 2.5	1.6[c]	4.3
	Pooled data[e]	3.3 (2.5-4)	5.9 (5.4-6.4)		
Cadmium	1	>113	>113	113	>113
	2	440	>540	540	>540
	3	230 (35-975)	>2330	460	910
	Mean[d]	335 ± 297	>2330	304[c]	910
	Pooled data[e]	135 (30-240)	1500 (920-2080)		
Lead	1	134 (0-276)	432	59	449
	2	146 (0-386)	>380	248	380
	3	189 (0-557)	>440	330	440
	Mean[d]	124 ± 139	432	124[c]	300
	Pooled data[e]	260 (100-420)	570 (300-830)		
Zinc	1	>82	>82	82	>82
	2	286 (0-1616)	>959	959	>959
	3	248 (0-883)	1100 (0-2787)	896	1860
	Mean[d]	267 ± 54	1100	413[c]	1860
	Pooled data[e]	450 (200-700)	1110 (870-1350)		
Nickel	1	>140	>140	140	>140
	2	>510	>510	510	>510
	3	>1070	>1070	1070	>1070
	Mean[d]	NR	NR	1070[c]	>1070
	Pooled data[e]	NR	NR		

[a] Concentration to cause 10% inhibition in growth compared to controls, values in parentheses are 95% CL
[b] Concentration to cause 50% inhibition in growth compared to controls, values in parentheses are 95% CL
[c] Geometric mean, No Observable Effect Concentration, highest concentration tested that is not statistically different to the controls
[d] Mean ± 2 SD of toxicity values (ICx, NOEC) from individual toxicity tests. Mean values excluded > values.
[e] Pooled data from individual toxicity tests combined and toxicity values estimated using log-logistic model in R, values in parentheses are 95% CL
NR – Not reported, values could not be determined

SUPPLEMENTARY FIGURES

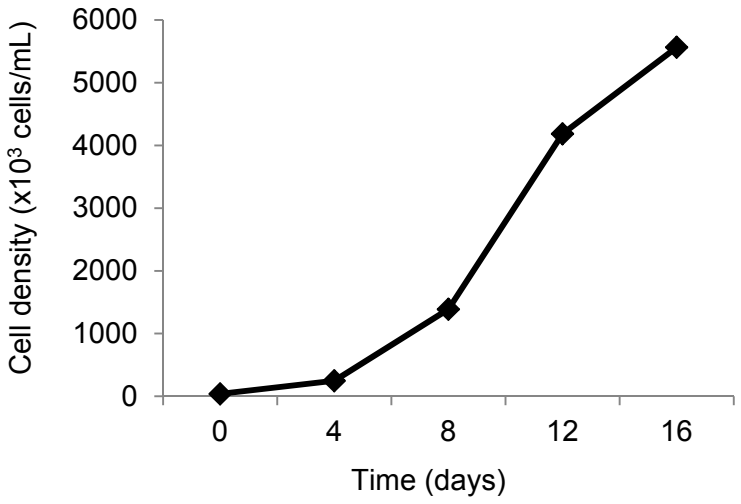

Figure S1 Growth rate of the Antarctic microalgae *Phaeocystis antarctica* in Gse/5 culture medium over 16 days.

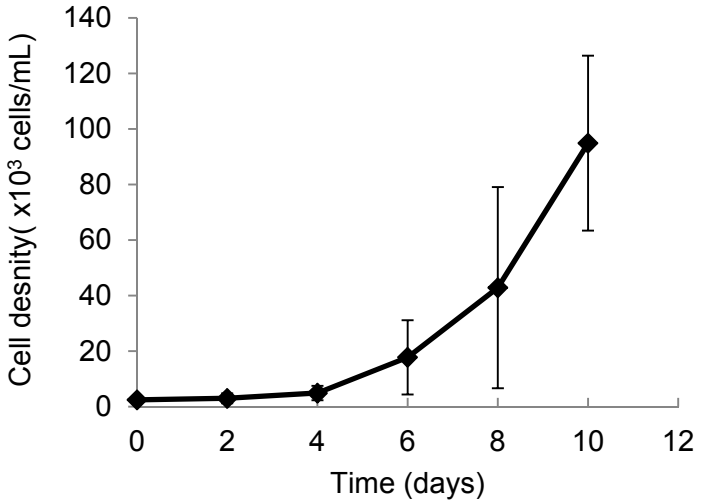

Figure S2 Growth rate of the Antarctic microalgae *Phaeocystis antarctica* in low nutrient seawater (1.5 mg NO_3^- /L, 0.15 mg PO_4^{3-} /L), (n=8 ± 1 SD).