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Measuring and assessing the risk of metal contaminants in the Antarctic nearshore marine environment

Darren J. Koppel
University of Wollongong

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UNIVERSITY
OF WOLLONGONG
AUSTRALIA

**Measuring and assessing the risk
of metal contaminants in the Antarctic
nearshore marine environment**

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This thesis is presented as required
for the conferral of the degree:

Doctor of Philosophy

30th November 2018

School of Chemistry
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DECLARATION OF ORIGINALITY

I, Darren J. Koppel, declare that this thesis is submitted in fulfilment of the requirements for the completion of the conferral of the degree of Doctor of Philosophy, from the University of Wollongong.

The content of my thesis is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

DARREN J. KOPPEL

30th November 2018

Abstract

ANTARCTICA is generally considered to be pristine; however, localised contamination around research stations is causing disturbances in the nearshore marine environment. Mixtures of metal contaminants enter the nearshore marine environment by leaching from historical waste during summer ice-melt events, in station wastewater discharge, and from anthropogenic activities such as fuel burning. To address these and other human impacts, the Protocol on Environmental Protection to the Antarctic Treaty System came into force in 1998 and required more stringent environmental management practices from nations, such as cleaning up historical waste and limiting the impact of anthropogenic activities. Despite being in place for 20 years, very few waste sites have been remediated across the continent and environmental management practices lack defined guidelines and benchmarks.

Hindering good environmental management practices is the lack of Antarctic-specific environmental quality standards and contaminant monitoring tools. Organisms in the Antarctic marine ecosystem have unique adaptations to their cold environment which may influence their sensitivity to contaminants; including, longer developmental times, high lipid contents, and slower metabolic rates. These differences mean that ecotoxicological data needs to be generated using native Antarctic organisms to ensure environmental quality standards will protect the unique ecosystem. These standards also need to consider the risk of contaminants in mixtures, and the potential for toxicity from a variety of exposure pathways. Environmental quality standards should be supported by contaminant monitoring tools that account for the high variability in contaminant concentrations over time as well as environmental factors that may modify contaminant toxicity.

This thesis investigated the toxicity of five common metal contaminants (cadmium, copper, nickel, lead, and zinc), singly and in mixtures, to two Antarctic marine microalgae. *Cryothecomonas armigera* was most sensitive to copper, reducing population growth rate inhibition by 10% (EC10) at a concentration of $22 \mu\text{gL}^{-1}$. The lipid concentrations of *C. armigera* were sensitive to cadmium and lead, with EC10s of 89 and $11 \mu\text{gL}^{-1}$, respectively. *Phaeocystis antarctica* and *C. armigera* were exposed to mixtures of five metals. Two mixture toxicity reference models, independent action

and concentration addition, were investigated for their ability to predict toxicity from metal mixtures. Both *P. antarctica* and *C. armigera* had antagonistic interactions at low effect concentrations following exposure to an environmentally relevant mixture, with *C. armigera* demonstrating synergism at high effect concentrations. Both models gave equivalent toxicity predictions, suggesting either are suitable for contaminant risk assessment.

The cellular metal accumulation and partitioning in both microalgae showed that copper was driving toxicity in the environmental mixture of metals. Antagonism to the mixture was likely caused by zinc competition with copper for cellular binding sites. Both microalgae can accumulate metals from sub-lethal exposure concentrations similar to those found in contaminated Antarctic nearshore marine sites. These concentrations are likely to cause toxicity to grazing organisms, based on extrapolations from previous research.

Diffusive gradients in thin-films (DGT) were established as an effective metal contaminant monitoring tool for use in Antarctic marine conditions. DGT-labile concentrations account for factors modifying toxicity and provide time-averaged concentrations for the duration of their deployment. Empirical diffusion coefficients for cadmium, copper, nickel, lead, and zinc were derived from laboratory experiments, and found to be lower than previously reported theoretical coefficients. Guidance around their use is also provided, including expected method detection limits and recommended deployment times. These guidelines were supported by a field study in Antarctica in which DGT were deployed to sites around an operating and an abandoned research station.

Finally, DGT-labile concentrations were used in a process to assess the risk of mixtures of metal contaminants. In laboratory experiments the DGT-labile and dissolved metal fractions were equivalent and gave equivalent metal-mixture toxicity predictions to both microalgae. In field deployments, DGT-labile concentrations predicted a low risk to organisms in the Antarctic nearshore marine environment.

The findings presented in this thesis will contribute to the generation of Antarctic-specific environmental quality standards by providing high-quality chronic metal toxicity data for single and mixture exposures, and important information about the capacity of Antarctic microalgae to accumulate metals which may cause dietary toxicity to grazing organisms. It also demonstrates a process for metal contaminant monitoring and risk assessment by linking DGT measurements with previously determined ecotoxicological data and mixture toxicity modelling.

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Resume

LIST OF PUBLICATIONS

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2. **Koppel, D. J.**, Adams, M. S., King, C. K., Jolley, D.F., 2018. Preliminary study of cellular metal accumulation in two Antarctic marine microalgae – implications for mixture interactivity and dietary risk in the Southern Ocean food web, *Environmental Science and Technology*, *manuscript under review*.
3. **Koppel, D. J.**, Adams, M. S., King, C. K., Jolley, D.F., 2018. DGT can predict the toxicity of metal mixtures to two microalgae - validation of DGT for environmental monitoring in Antarctic marine conditions, *Environmental Toxicology and Chemistry*, *manuscript under review*.
4. **Koppel, D. J.**, Adams, M. S., King, C. K., Jolley, D.F., 2018. Chronic toxicity of an environmentally relevant and equitoxic ratio of five metals to two Antarctic marine microalgae shows complex mixture interactivity, *Environmental Pollution*, 242 B, 1319 – 1330. doi: 10.1016/j.envpol.2018.07.110
5. **Koppel, D. J.**, Adams, M. S., King, C. K., Jolley, D.F., 2017. Chronic toxicity of five metals to the polar marine microalga *Cryothecomonas armigera* – Application of a new bioassay, *Environmental pollution*, 228, 211 – 221. doi: 10.1016/j.envpol.2017.05.034..

CONFERENCE PRESENTATION

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4. **Koppel, D. J.**, Adams, M. S., King, C. K., Jolley, D.F. *Metal mixture toxicity to two Antarctic marine algae*; Oral presentation at the Annual Chemistry Postgraduate Conference, Springwood, Australia; November, 2016.
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SCIENCE OUTREACH AND MEDIA

Science outreach

1. Research 'Polartox' website and blog, 24,000 views from December 2017 to November 2018.
www.darrenkoppel.com/portfolio/2017-2018-antarctic-field-campaign
2. Presentation to the Rotary Club of Corrimal, Australia; November 2018.
3. Presentation to the Rotary Club of West Wollongong, Australia; September 2018.
4. Presentation to Smithfield Public School Year 3 and 4 students on the environmental management of Antarctic. Smithfield, Australia; April, 2018.
5. Keynote presentation to the 120 science teachers from Curriculum Network Illawarra - Staff Development Day, Wollongong, Australia; April, 2018.
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Media

1. "Measuring metal pollution in the Antarctic environment". *Australian Antarctic Magazine*, Issue 34; June 2018.
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2. "Research and chill: the challenges of working in Antarctica". *CSIROblog*, CSIRO; March, 2018. <https://blog.csiro.au/research-and-chill-the-challenges-of-working-in-antarctica/>
3. "What it takes to do research in Antarctica". *The Stand*, University of Wollongong. February, 2018. www.stand.uow.edu.au/research-in-antarctica/
4. "Great expeditions – cleaning up Antarctica". *CSIROblog*, CSIRO; February, 2018. <https://blog.csiro.au/research-and-chill-the-challenges-of-working-in-antarctica/>
5. Interview with Lindsay McDougall, ABC Illawarra; 27 January, 2017.
www.darrenkoppel.com/2018/01/28/a-quick-call-to-the-antarctic
6. "Penguin jumps aboard a UOW research boat in the Antarctic". Illawarra Mercury, Australia; 18 January, 2017. www.illawarramercury.com.au/story/5192816/penguin-jumps-aboard-a-uow-research-boat-in-the-antarctic
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Common abbreviations

AIC: Akaike information criterion, an estimate of the quality of fit of a model to a dataset - useful for comparing the fit of different models against common data

ATCM: Antarctic Treaty Consultative Meeting, the forum for Antarctic treaty nations to make decisions about Antarctica under the Antarctic Treaty System

Benthic: Relating to the ocean floor

Bioavailability: A relative measure of the extent to which a metal can interact with an organism

CA: Concentration addition

CEP: Committee for environment protection, the advisory body established with as part of the Protocol of Environmental Protection to the Antarctic Treaty System

CI: Confidence interval

DGT: Diffusive gradients in thin-films

Dissolved: Less than 0.45 µm in size

DOC: Dissolved organic carbon

DRM: Dose response model

East Antarctica: The area of Antarctica directly below Australia separated geographically from West Antarctica by the Transantarctic Mountains

EC_x: Effect concentration, the concentration of a contaminant required to produce an x% effect

EDTA: Ethylenediaminetetraacetic acid, a strong chelator of divalent metals

Epontic: Relating to the underside of ice

Environmental quality standards: A policy guideline regulating anthropogenic impact

to the environment, usually by setting limits for concentrations of contaminants or by specifying a maximum allowable degradation of the environment

IA: Independent action

ICP-AES: Inductively coupled plasma – atomic emission spectroscopy

ICP-MS: Inductively coupled plasma – mass spectroscopy

LOD: Limit of Detection

LOEC: Lowest observable effect concentration

MDL: Method detection limit

Mobility: The ability of a chemical to move in its environment

Nearshore: The area of the ocean near the shoreline

NEC: No effect concentration

NOEC: No observable effect concentration

OECD: Organisation for economic cooperation and development

Pelagic: Relating to the open ocean

Phase: The distinct form or environment of a chemical (e.g. solid, liquid, gas)

SSD: Species sensitivity distribution

SSE: Sum of squared errors

TU: Toxic unit

Water Quality Guidelines: Numerical values (such as of contaminant concentrations) or statements used to protect aquatic environments from anthropogenic impact. A type of environmental quality standard.

CHAPTER 1

Introduction



At a time when it's possible for thirty people to stand on the top of Everest in one day, Antarctica still remains a remote, lonely and desolate continent. A place where it's possible to see the splendors and immensities of the natural world at its most dramatic and, what's more, witness them almost exactly as they were, long, long before human beings ever arrived on the surface of this planet. Long may it remain so.

Sir David Attenborough

ANTARCTICA is synonymous with adventure, isolation, and an alien landscape. Its isolation has afforded it protection from exploration and settlement until the “heroic age” of the early 1900s saw nations competing to land, cross, and inhabit the continent. The first structures were wooden huts which made way for whaling and research stations with the lure of Earth’s last frontier driving nations to claim what land they could. Fearing the militarisation of Antarctica amid the political tensions of the Cold War and to de-escalate conflicting territorial claims, the twelve nations active in the International Geophysical Year of 1957 - 1958 signed the Antarctic Treaty in 1959 to ensure Antarctica will be used for peaceful purposes only, ensure freedom of scientific investigation, and to make scientific observations and results free for all (Conference on Antarctica 1959).

The Antarctic Treaty has since expanded to include a conglomerate of agreements and protocols outlining international conduct in Antarctica, known collectively as the Antarctic Treaty System. The Protocol on Environmental Protection to the Antarctic Treaty (herein the “Protocol”) was signed in 1991 and entered into force in 1998. It was created in recognition of the growing threat of pollution to the Antarctic environment and commits signatories to “*designate Antarctica as a natural reserve, devoted to peace and science*”. By its annexes, the Protocol outlines specific requirements for environmental protection, including: (I) the need to conduct environmental impact assessments, (II) the conservation of flora and fauna, (III) waste disposal and management, (IV) prevention of marine pollution from ship discharge, (V) management of protected areas, and (VI) liability for environmental emergencies (ATCM 1991). Despite the adoption of the Protocol, very few sites have been cleaned up, and questions have been raised about our commitment to protect the Antarctic environment from Anthropogenic impacts (Hodgson-Johnston et al. 2017; Hughes 2010).

1.1 The Antarctic environment

Antarctica is a 14×10^6 km² land mass of which 98% is covered in ice with an average depth of 1.9 km, but with areas as deep as 4 km (Fretwell et al. 2013). The 18,000 km coastline is characterised by floating ice shelves (44%), grounded ice walls (38%), glacial outlets (13%), and exposed rock (5%) (Drewry 1982). Its desert climate results from scarce precipitation, averaging 151 mm y^{-1} across the continent, mostly on the coastal fringes (Cullather et al. 1998). The ice-free surfaces, totalling 3.3×10^5 km², are predominately scattered rocky coastlines or exposed mountainous slopes and ridges. Ice-free coastal areas are the ecological refuges in an otherwise icy continent. These areas have been categorised into 16 biologically distinct bioregions (Figure 1.1), and are home to terrestrial flora and fauna, and important breeding grounds for semi-aquatic organisms including birds, penguins, and seals (Terauds and Lee 2016).

Surrounding the Antarctic continent is the Southern Ocean, which links the Pacific, Indian and Atlantic oceans at their southern edges. Although connected, exchange between these oceans is limited by the polar front, a thermocline between 50 and 60 °S (Barnes et al. 2006). Within the Southern Ocean is the easterly flowing Antarctic Circumpolar Current. It is slow moving and driven by winds, travelling $0.25 - 0.4 \text{ m s}^{-1}$ at the surface. However, it transports $100 - 150 \times 10^6 \text{ m}^3 \text{ s}^{-1}$ of seawater, the greatest

of any current in the world. The circumpolar current and steep temperature gradient at the polar front has isolated the Antarctic marine environment, resulting in a unique ecosystem highly adapted to its cold and seasonal conditions (Rintoul 2009).

The Southern Ocean ecosystem is controlled by a dynamic sea-ice environment. The presence of sea ice creates a physically and chemically stabilised marine environment: sea temperatures average 1.8 °C, wind driven water column mixing is inhibited, light is limited (i.e. absorbed by sea ice or reflected by snow), and the water column becomes highly stratified. This provides shelter to pelagic and benthic communities and creates an epontic ecosystem where micro and macroalgal communities form on the under surface of the ice (Lancelot et al. 2009). Antarctic sea ice reaches its maximum extent around September and its minimum around February. At its maximum, it has a surface area greater than Europe and Australia combined, for example $>18 \times 10^6 \text{ km}^2$ in 2017. Salinity in the Southern Ocean is also subject to seasonal variation due to sea ice formation and melting (Morrow and Kestenare 2014). Sea ice formation excludes salts (including nutrients and contaminants), increasing the salinity of underlying waters. The formation of sea ice also captures seawater within the ice, creating brine pockets. The high salinity of the water lowers its freezing point, causing the brine to melt through the ice forming brine channels which feed the underlying epontic community nutrients (Underwood et al. 2010).

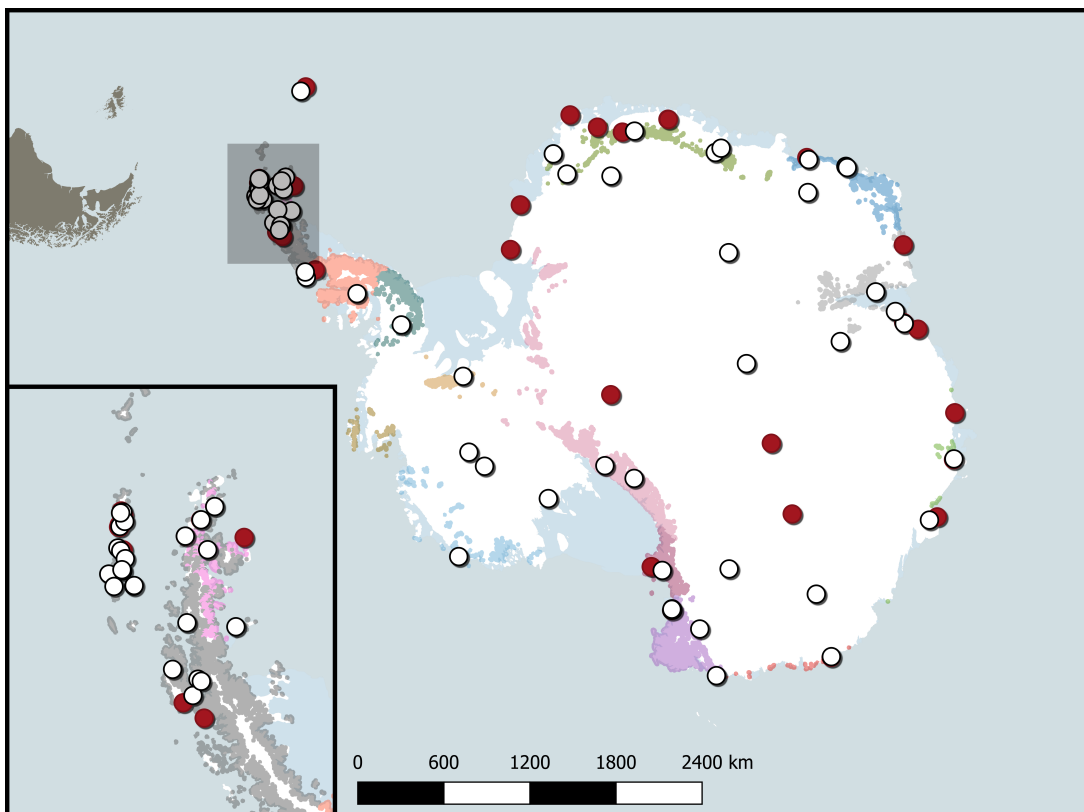


Figure 1.1: Location of Antarctic research stations; filled and open dots are year-round and summer-only stations, respectively. Coloured sections indicate the biogeographic regions of Antarctica which correspond to the ice-free areas of the continent (COMNAP 2017; Terauds and Lee 2016).

The Southern Ocean ecosystem has low productivity in the winter but is highly productive in summer. The Southern Ocean is a “high nutrient low chlorophyll” zone, because of its high macronutrient concentrations (i.e. nitrogen and phosphorus) and low primary productivity. In temperate and tropical marine ecosystems, nitrates are usually the limiting nutrient. In the Antarctic marine ecosystem, nitrate concentrations remain high year round, fluctuating between $28 \mu\text{mol L}^{-1}$ in winter and $15 \mu\text{mol L}^{-1}$ during the summer algal blooms (Grotti et al. 2001). Instead, iron has been shown to be the limiting nutrient for algal growth and ecosystem productivity (Death et al. 2014; Wadley et al. 2014). The coastal marine environment has greater iron concentrations than the open ocean because there are more sources, such as benthic sediment suspension, trace-metal upwelling and diffusion (Jong et al. 2013; Park et al. 1999), aeolian deposition (Fung et al. 2000), continental runoff advection (Bowie et al. 2009), glacial melt (Sheppard et al. 1997), and faunal inputs (Nicol et al. 2010). In winter the sea ice traps iron and other nutrients, such as in brine pockets or from dust deposition, storing it like a reservoir until the summer ice melt (Lannuzel et al. 2006, 2008). During the sea-ice melt, this nutrient stock combines with trapped sea-ice algae, the breakup of the epontic community, and penetrating sunlight to fertilise a bloom in phytoplankton and zooplankton (Merwe et al. 2011; Schlosser et al. 2018) which sustains the Southern Ocean food web, from copepods and krill to penguins, seals, and whales (Murphy et al. 2012).

1.2 The Antarctic nearshore marine environment

The Antarctic nearshore environment is unique because of the seasonal influences on the environment, its physicochemical conditions, and the life that inhabits it (Arrigo et al. 1998; Griffiths 2010). The high latitude of Antarctica means 24 h darkness in winter and 24 h sunlight in summer. Sea ice retreats to a minimum in February during summer and a maximum in September at the start of spring. This meaning that coastal areas may only be ice free for a brief period each year (Arrigo 2014; Vernet et al. 2008).

1.2.1 Physicochemical environment

The physicochemical conditions of the Antarctic nearshore marine environment are relatively stable, particularly in winter. However, they can be affected by seasonal ice and biological cycles related to the summer ice melt. Winter seawater temperatures of $-2 \text{ }^\circ\text{C}$ increase to between -2 to $0 \text{ }^\circ\text{C}$ during December to February. The pH of the nearshore marine environment is more variable than open oceans, ranging between 7.9 to 8.2 (Kapsenberg et al. 2015) compared to 8.0 to 8.1, respectively (Williams et al. 2018). The salinity of the nearshore marine environment is also more variable than the open ocean, ranging from 30 to 35 PSU because of fresh meltwater inputs and sea ice formation and melt (Kim et al. 2015; Sheppard et al. 1997).

Dissolved organic carbon (DOC) is an important part of aquatic environments and is a source of carbon, lipids, and protein (or amino acids) to organisms (Hansell 2013). Microorganisms are the major source of DOC in the Antarctic marine environment, with DOC concentrations correlated to microorganism biomass (Biddanda and Benner 1997; Guglielmo et al. 2004).

DOC can be produced by organic matter degradation in the water column or sea ice by extracellular enzymatic activity (Arrigo 2014; Guglielmo et al. 2004; Wing et al. 2012), exudates of microorganisms (Underwood et al. 2010), faunal inputs (Nicol et al. 2010), and anthropogenic inputs, usually as sewage (Stark et al. 2016). In the sea ice, DOC is especially important. Exudates produced by algae make up 68 to 100% of the total carbohydrate pool, with short chained carbohydrates the most common fraction (Underwood et al. 2010). These exudates control the local environment by moderating brine salinity, and retaining nutrients (Dumont et al. 2009; Ugalde et al. 2014).

1.2.2 Antarctic ecosystem

The coastal terrestrial ecosystem has low biodiversity, but is home to mosses, lichens, and semiaquatic birds and mammals, Appendix A1.1. In comparison, the nearshore marine environment is home to a wide variety of organisms including sponges, soft corals, macroalgae, microalgae, jellyfish, fish, and invertebrates (see Figure 1.2 and De Broyer et al. (2014)). The diversity of these ecosystems can be as high as comparative temperate or tropical ecosystems around the world (Clarke 2008). Productivity in the nearshore marine environment is tied to the seasons. In winter, the sea ice becomes its own habitat while also sustaining organisms in the pelagic and epontic ecosystems (Kohlbach et al. 2018). Brine channels in the sea ice are home to diverse bacteria, microalgae, and small invertebrates. The epontic algae communities support invertebrates and fish, including zooplankton like amphipods and the Antarctic krill *Euphausia superba* (Meyer 2012; Mori et al. 2019). In summer, algal blooms support an intense population increase of higher order organisms from krill to penguins and whales (Murphy et al. 2012).

Life in the nearshore marine environment is highly adapted to the unique Antarctic conditions. Evolutionary adaptations to this environment include longer development times and lifecycles (Chapman and Riddle 2005). Compared to other ocean systems there is rich species diversity for some taxa, e.g. sponges, bryozoans, and amphipods but reduced (e.g. gastropods) or non-existent (e.g. some decapod crustacean groups) for others (Griffiths 2010). The Antarctic food web has few species linking primary producers to tertiary consumers (Murphy et al. 2012), with the Antarctic krill considered the keystone link between primary producers and tertiary consumers (Schmidt et al. 2014). Microalgae are the primary producers of the Antarctic marine food web, with a rich diversity of types supporting environmental niches. For example, diatoms form benthic mats in the nearshore environment (Alderkamp et al. 2012), the heterotrophic protist *Cryothecomonas armigera* is commonly found in sea ice brine channels (Thomsen et al. 1991), and the prymnesiophyte *Phaeocystis antarctica* forms large blooms along the melting sea ice edge (Williams et al. 2016).

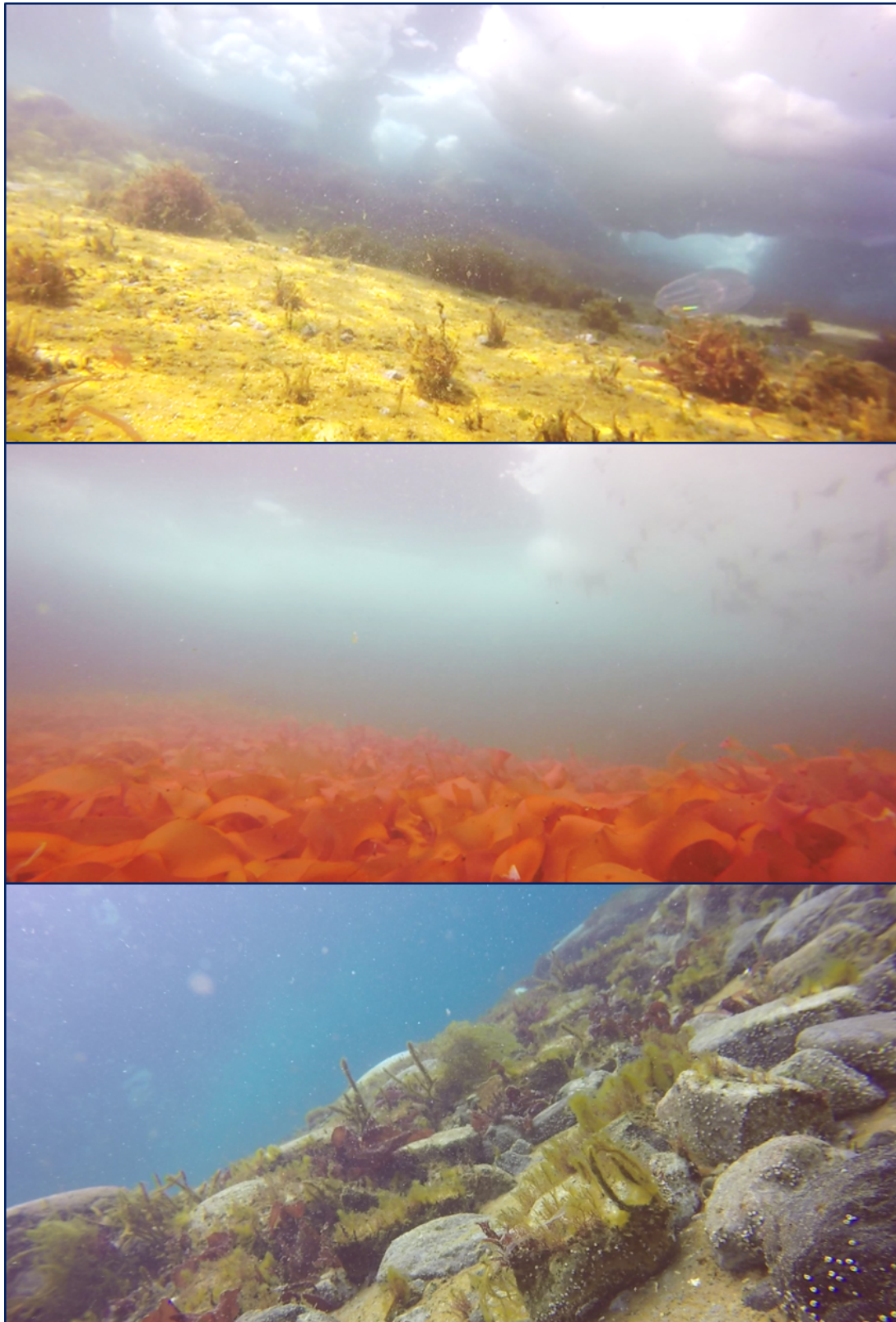


Figure 1.2: Examples of the nearshore marine environment around Australia's Casey Station. Note the large diversity of life including different algae, epontic (under-ice) communities, and invertebrates. Personal photographs, January 2018.

Food web and energy flows

The Antarctic nearshore marine ecosystem has a unique food web because of the harsh conditions and the seasonality of sea ice. In winter, the sea ice ecosystem has an annual primary production contribution of 23.7 Tg C yr⁻¹, approximately 1% of total Southern Ocean primary production (Saenz and Arrigo 2014). While modest, this ecosystem supports microalgae and invertebrates in the ice (Kohlbach et al. 2018; Meiners et al. 2011), and provides constant enrichment to underlying epontic communities through brine drainage (Arrigo 2014; Wing et al. 2012).

The nearshore marine ecosystem is diverse and relies on a variety of primary food sources. For example, areas of inter- and subtidal macroalgae, phyto and zooplankton, organic particulate matter from sediments or detritus in the water column (Norkko et al. 2007; Zenteno et al. 2018). Energy flows are typically linked to habitat type (Corbisier et al. 2004). For example benthic organisms tend to feed on benthic sediment organic matter and micro and macroalgae, while pelagic amphipods and suspensivores graze on pelagic particulate organic matter and microalgae (Zenteno et al. 2018).

Lipid reserves are important to the Southern Ocean food web (Murphy et al. 2016). To survive low-productivity winters, many Antarctic marine organisms such as krill (Kohlbach et al. 2018) and fish (Maes et al. 2006) accumulate lipid reserves. Some organisms, like copepods (Atkinson et al. 2012; Lee et al. 1971), use these reserves to retreat to deeper waters where they pause their development in an aquatic hibernation. Microalgae can be a high energy source of lipids, accumulating intracellular stores observed as droplets, such as in *Cryothecomonas armigera* (Thomsen et al. 1991) and *Chlamydomonas sp.* (Mou et al. 2012).

1.3 Humans in Antarctica

Carsten Egeberg Borchgrevink is attributed with first stepping foot on Antarctica in 1895. The first wooden huts followed shortly after in 1899 on Borchgrevink's return to Cape Adare. These huts gave way to larger structures supporting whaling and eventually permanent scientific stations. International interest in Antarctic science and exploration expanded during the International Geophysical Year of 1957-1958, when 12 nations established over 60 stations. Now, approximately 80 stations are operational with many more closed or abandoned, (Figure 1.1 COMNAP (2017)). Most research stations are built on scarce ice-free rocky areas, within 2 km of the coastline (Figure 1.1), an area representing approximately 0.05% of the continent (Hughes 2010; Hull and Bergstrom 2006). In this area, an estimated 53 stations are active in addition to other waste sites, field camps, and runways.

Tourism to Antarctica began in the 1950s with Chile and Argentina facilitating 500 passengers to the West Antarctic Peninsula on a naval ship. Since then, the number of tourists to Antarctica has steadily increased, with a record high of 51,707 recorded for the 2017-18 season (ATCM 2018). Visitation is expected to increase, with 17 new expedition ships expected by 2021 – a 50% increase in berth capacity (Walton 2018). Tourism is voluntarily governed by the International Antarctic Association of Tour Operators. They have developed policies to manage the environmental impacts of tourism, particularly related to threats of invasive species.

Tour operators also support scientific and conservation efforts by transporting scientists and equipment between stations, providing non-urgent medical evacuations, participating in citizen science programs, and raising funds for conservation efforts (ATCM 2018). However, questions remain about the sustainability of tourism and its impact to the Antarctic environment (Kriwoken and Rootes 2000).

1.4 Contamination in Antarctica

Exploration, tourism, and scientific research have left a potentially toxic legacy of organic and inorganic contamination, particularly around research stations (Bargagli 2008; Tin et al. 2009). A lack of environmental management in the 20th century resulted in waste being burned, discarded to the sea ice and coastal environment, or stored in open waste sites (Crockett and White 1997; Deprez et al. 1999). Contaminants from these sites are mobilised to the coastal environment through processes of dissolution, or particle entrainment from melt waters running through the sites during the summer season (Snape et al. 2001). This has already been shown to affect the nearshore marine ecosystem (Conlan et al. 2010; Cunningham et al. 2005), and is expected to worsen with increasing temperatures associated with climate change (McIvor 2014).

Contamination exists across all of Antarctica, but concentrations are generally lower than temperate or tropical environments due to reduced anthropogenic activity (Guerra et al. 2011). Much of the contamination is a result of environmental management practices prior to the introduction of the Protocol. Approximately $1-10 \times 10^6 \text{ m}^3$ of abandoned waste is estimated to be present in Antarctica (Figure 1.3), in addition to a similar amount of petrochemical contaminated soil. Additional contamination arises from an ongoing anthropogenic presence, such as from station activities like burning diesel fuel for electricity, wastewater discharge (Montone et al. 2010), and waste incineration. Station accidents such as barges overturning, small aircraft crashes, ships sinking, petrochemical spills, and station fires, have also led to contamination (Brooks et al. 2018; Guerra et al. 2013; Jaraula et al. 2008).

1.4.1 Metal contaminants in the nearshore marine environment

The major source of contaminants to the Antarctic nearshore marine environment come from nearby research stations, with only minor inputs from atmospheric transportation from Southern Hemisphere countries. For example, lead deposition to Antarctic terrestrial and marine environments (Van de Velde et al. 2005). Metal contaminant concentrations from these sources are low and not likely to cause contamination. More significant enrichment of organic pollutants in the Antarctic environment has occurred from atmospheric transport (Hao et al. 2019), for example of polychlorinated biphenyls (Bargagli 2008). The toxicity and bioaccumulation potential of organic contaminants are subject to ongoing investigation (Dawson et al. 2018b) and are not considered further in this thesis, which focuses on metal contaminants. Some metal contaminants of concern are routinely reported in the Antarctic nearshore marine environment, including cadmium, copper, nickel, lead, and zinc.



Figure 1.3: Types of historical waste found uncontained near the marine environment in Antarctica including fuel barrels, station supplies, machinery, and building material. Personal photographs, January 2018.

Cadmium is a non-essential metal for life, yet can replace zinc in enzymes with little to no loss of function (Lane and Morel 2000; Sinoir et al. 2012). Cadmium may also share uptake pathways with other essential nutrients, such as calcium in some crustacean (Rainbow 1997). While cadmium is known to be toxic to marine life, at concentrations of about 10 to 100's $\mu\text{g Cd L}^{-1}$ (Echeveste et al. 2014; Gissi et al. 2015; Marcus Zamora et al. 2015). These concentrations are 1-3 orders of magnitude above reported open ocean or nearshore cadmium concentrations (Padeiro et al. 2016; Sañudo-Wilhelmy et al. 2002).

Copper in the Antarctic nearshore marine environment can occur from anthropogenic contamination or geogenic sources like the weathering of volcanic minerals (Yin et al. 2006). The sediment of the contaminated Brown Bay near Casey Station has been reported to have between 10 and 13,500 mg Cu kg^{-1} (Deprez et al. 1999). In contrast, uncontaminated sediment copper concentrations typically range from 10 to 190 mg Cu kg^{-1} , for example in King George Island (Santos et al. 2005), Windmill Island (Gasparon et al. 2007), the Ross Sea (Ianni et al. 2010), and Deception Island (Guerra et al. 2011). Meltwater concentrations around Scott Base, in the Ross Sea, have shown elevated concentrations of copper from the leaching of soils historically contaminated with photographic dark room chemicals. Two small ponds of meltwater recorded total recoverable copper concentrations of 17.1 and 10.5 $\mu\text{g L}^{-1}$, and $<0.45 \mu\text{m}$ filterable copper of 5.5 and 6.7 $\mu\text{g L}^{-1}$. In comparison, meltwaters from uncontaminated areas showed maximum dissolved copper concentrations of 0.63 $\mu\text{g L}^{-1}$.

Sea ice has a controlling influence on copper to the marine environment. Particulate copper can be trapped in sea ice, typically by sediment resuspension during ice formation, which can be released in a pulse during the summer ice melt (Grotti et al. 2005). In winter, copper leaches from sea ice through brine channels to the underlying waters. Copper binds strongly to DOC in marine systems (DePalma et al. 2011), with copper-DOC complexes having reduced bioavailability and therefore toxicity to organisms (Arnold et al. 2010; Rivera-Duarte et al. 2005; Sánchez-Marín et al. 2010). This has also been shown in the Antarctic marine environment, for example Biesuz et al. (2006) found copper to be strongly complexed to DOC in the waters of Terra Nova Bay, near the Italian research station Zucchelli.

Nickel concentrations are elevated around research stations, but has not been shown to be toxic to the Antarctic organisms it has been tested against Table 1.1. However, it has been shown to be toxic to some temperate and tropical marine organisms (Gissi et al. 2016). In uncontaminated Antarctic bays and coastal areas, nickel concentrations range from 2 to 60 mg Ni kg⁻¹ (Guerra et al. 2011). Uncontaminated meltwaters around the McMurdo Sound showed similarly low concentrations, up to 0.41 µg Ni L⁻¹. There is no evidence of nickel toxicity to Antarctic marine organisms. For example, Sfiligoj et al. (2015) found no toxicity to the amphipod, *Orchomenella pinguides* at concentrations up to 1120 µg Ni L⁻¹. Similarly, Gissi et al. (2015) found no toxicity to *Phaeocystis antarctica* at concentrations up to 1070 µg Ni L⁻¹.

Lead contamination is widespread at low concentrations (up to 6 ng kg⁻¹, Wolff and Suttie (1994)) across the terrestrial Antarctic environment from long range atmospheric transport (Bargagli 2008). Analyses of lead in snow cores can identify the start of the industrial revolution, the adoption and abandonment of lead fuel additives, and the move to biofuels in South America (Barbante et al. 1997; Van de Velde et al. 2005; Wolff and Suttie 1994). In the water column, particulate lead (resulting from atmospheric deposition) is rapidly scavenged, and does not contribute to dissolved lead, which fluxes from sediments or is dissolved in metal waters (Frache et al. 2001). For example, meltwater taken from a pond near solid waste containers with stream sources waters running under buildings and across roads showed dissolved lead concentrations of 3.1 µg Pb L⁻¹, compared to below detection limit (<0.09 µg Pb L⁻¹) in uncontaminated meltwaters (Sheppard et al. 1997). Lead has also contaminated Brown Bay, with sediment concentrations up to 4890 mg Pb kg⁻¹ (Deprez et al. 1999); however, more recent measurements have not reported concentrations above 200 mg kg⁻¹ (Cunningham et al. 2005; Scouller et al. 2006), suggesting high spatial variability.

Zinc is an essential nutrient, but can be toxic at high concentrations. Zinc readily binds to natural organic ligands, with studies suggesting only 2 to 5% of zinc in the ocean is present in the dissolved phase (Bruland 1989). Zinc in atmospheric dust quickly dissolves in seawater and can concentrate in sea ice (Thuróczy et al. 2010). Biological cycling of zinc is particularly important for some sea-ice microalgae, which remineralise zinc from sea ice resulting in localised surface water maxima (Croot et al. 2011; Löscher 1999). Dissolved zinc concentrations are usually elevated near research stations compared to open ocean concentrations (0.1 to 0.3 µg L⁻¹, Kim et al. (2015) and Sañudo-Wilhelmy et al. (2002)). For example, bioavailable concentrations up to 17 µg L⁻¹ in Ardley Cove, King George Island, and Brown Bay and O'Brien Bay in the Windmill Islands (Larner et al. 2006; Padeiro et al. 2016).

Sediment zinc concentrations in contaminated sites around research stations are typically not elevated. For example, reference sites have zinc concentrations up to 300 mg kg⁻¹ in the Windmill Islands (Gasparon et al. 2007) compared 150 mg kg⁻¹ at the impacted Brown Bay (Cunningham et al. 2005). Similar concentrations, typically below 100 mg kg⁻¹, are reported around the West Antarctic Peninsula (Guerra et al. 2011; Ianni et al. 2010; Santos et al. 2005).

1.4.2 Contamination associated with research stations

Contamination localised around research stations has been shown to negatively affect the Antarctic nearshore marine environment, (e.g. Conlan et al. (2010), Cunningham et al. (2005), and Stark et al. (2016, 2014, 2003b)). At some of these sites, contamination in water, sediment, or soils has been found at concentrations comparable to industrial ports or harbours (Crockett and White 1997; Kennicutt et al. 1995). When frozen, this contamination is generally not bioavailable or mobile; however, dissolution, entrainment, or displacement by melt water run off or iceberg scour leads to contaminant mobilisation (Stark et al. 2006a).

Few national programs actively report results of environmental monitoring, if it occurs, or the types and distribution of contaminants around their stations (Hughes 2010). The Australian Casey station, Brazilian Ferraz station, and United States McMurdo station are some that actively monitor and report on environmental contamination.

Casey Station

The Australian Casey Station is situated on the ice-free rocky coast of the Windmill islands, and has a population of approximately 20 in winter and 120 in summer. It is the third station to be built in the area, replacing the old Casey Station, which occupied a site 300 m away and was dismantled in 1989, which in turn replaced the abandoned Wilkes Station 2 km north in 1969. Casey station has been a site of considerable clean up and remediation efforts over the past 15-20 years. A disused waste disposal tip in the Thala Valley contains the waste generated between 1969 and 1986 from old Casey Station including laboratory, workshop, and domestic waste. A clean up of Thala Valley removed most of the waste and contaminated soil that was most likely to have caused environmental harm to the adjacent Brown Bay (Stark et al. 2005); however, some contaminated soil remains and no clean up of the sediment occurred (Raymond and Snape 2017). Brown Bay sediments have total metal concentrations of copper, lead, and zinc 1 to 2 orders of magnitude greater than clean reference sites in adjacent bays (Stark et al. 2003a). However, only a small fraction of this contamination has been shown to be bioavailable (Deprez et al. 1999), potentially due to the organic carbon content, or the anoxic conditions of the soils and sediments (Palmer et al. 2010). Nevertheless, biodiversity surveys and *in situ* sediment recruitment studies in Brown Bay have shown that copper, iron, tin, and lead contamination have caused benthic community changes (Cunningham et al. 2005; Palmer et al. 2010).

Additional sources of contamination include fuel spills and wastewater discharge. Both the old and current Casey stations have fuel contamination in soils around their workshops and powerhouses at concentrations up to 47,600 and 92,500 mg fuel kg⁻¹, respectively (Snape et al. 2006). In addition, a wastewater outfall distributes primary

or secondary treated wastewater to the adjacent Shannon Bay. The resulting elevated seawater ammonia, phosphorus and bacteria concentrations have caused a decrease to diatom, sponge, and bryozoan diversity (Stark 2008).

Ferraz Station

Ferraz is a Brazilian research station located on Admiralty Bay on King George Island near the tip of the Antarctic Archipelago. Paint residues, fuel and sewage discharge have led to elevated metal concentrations in nearshore marine sediments adjacent to Ferraz station (Santos et al. 2005). Compared to reference sites, copper, nickel, lead, and zinc concentrations were 90% higher in impacted areas. However, these metal contaminants were shown to have low bioavailability because of the reducing, sulphidic nature of the soils and sediments, as determined by weak acid extractions (Santos et al. 2007). In reducing conditions, hydrogen sulphide (H₂S) is generated from the degradation of organic matter. These sulphide ligands can bind to metal contaminants forming insoluble metal-sulphide complexes which are generally not bioavailable unless oxidised (Huerta-Diaz and Morse 1992).

In seawaters around Ferraz Station, anthropogenic inputs have elevated bioavailable metal concentrations, including copper concentrations of up to 2 µg L⁻¹. These are likely to cause toxicity to the microalga *Phaeocystis antarctica* and the sea urchin *Sterechinus neumayeri*, based on previous ecotoxicological studies (Table 1.1). Phytoplankton exposed to these waters, or collected from these sites, may also have the potential to bioaccumulate chromium, copper, lead, and zinc, possibly posing a risk to the nearshore marine food web (Cabrita et al. 2017; Padeiro et al. 2016).

McMurdo Station

The United States' McMurdo station is located on the southern coastline of Ross Island, adjacent to the McMurdo Sound in the Ross Sea and is the largest permanent research station in Antarctica with a population that can exceed 1,500 in the summer (NSF 2014). Winter Quarters Bay is the former waste disposal site for McMurdo station, and is highly contaminated with debris, PAHs, PCBs, metals, and hydrocarbons (Lenihan 1992; Lenihan et al. 1990), with declining concentrations in a gradient moving away from the source (Kennicutt et al. 1995). This contamination is bioaccumulated by benthic organisms (Negri et al. 2006) and has reduced the abundance and changed the diversity of species, when compared to reference sites (Conlan et al. 2004).

1.4.3 Influence of Antarctic conditions on metal contaminants

Environmental factors will modify metal mobility, speciation, bioavailability, and toxicity. Temperature, salinity, pH, and the presence of DOC drive changes to these factors. The processes behind these factors are well understood for temperate or tropical environments, but less so for the unique conditions of Antarctica. For the purposes of this thesis, mobility is defined as the movement of a metal in its environment, phase is defined as the distinct form of the metal (i.e. solid or aqueous), and bioavailability is a relative measure of the extent to which a metal can interact with an organism at biotic ligands.

Table 1.1: Metal toxicity to Antarctic marine organisms from laboratory and field studies. EC10 and EC50 represent the concentrations of the metal required to cause a 10 and 50% effect to the organism, respectively.

Organism	Species	Conditions	Temp (°C)	pH	Endpoint	Exposure (d)	Metal	EC10 ($\mu\text{g L}^{-1}$)	EC50 ($\mu\text{g L}^{-1}$)	Reference
Sea urchin	<i>Sterechinus neumayeri</i>	Field collected	0 ± 1	8.0 - 8.2	Embryonic development success	6 to 8	Cd	–	6940	King and Riddle (2001)
							Cu	–	11.4	
					Larval development success	20 to 23	Cu	–	2230	
							Zn	–	1.4	
Amphipod	<i>Paramorea walkeri</i>	Field collected	0 ± 0.5	–	Mortality	8	Cd	–	190 (100 - 340)	Duquesne et al. (2000)
							Cu	–	290 (150 - 510)	
Amphipod	<i>Paramorea walkeri</i>	Field collected	-0.9 ± 0.5	7.8 ± 0.3	Behavioural change	21	Cd	108 ± 8	247 ± 61	Sfiligoj (2013)
							Cu	22 ± 4	61 ± 1	
							Ni	268	864	
							Pb	30 ± 29	–	
							Zn	290 ± 98	761 ± 61	
Amphipod	<i>Orchomenella pinguides</i>	Field collected	-1 ± 1	7.99 - 8.05	Mortality	30	Cu	9 (8 - 13)	31 (27 - 36)	Sfiligoj et al. (2015)
							Cd	57 (42 - 94)	168 (137 - 215)	
							Zn	288 (216 - 401)	822 (716 - 957)	
							Pb	42 (26 - 71)	256 (209 - 327)	
Copepod	<i>Paralabidocera antarctica</i>	Field collected	-1 ± 1	7.4 - 8.5	Mortality	7	Cu	$8 \pm 1.9\%$	$20 \pm 2.5\%$	Marcus Zamora et al. (2015)
							Cd	75	$237 \pm 18\%$	
	<i>Stephos longpipes</i>						Cu	$28 \pm 3.1\%$	$56 \pm 3.7\%$	
	<i>Oncaea curvata</i>						Cd	685	1250	
							Cu	$35 \pm 2.5\%$	$61 \pm 2.8\%$	
							Cd	$506 \pm 32\%$	$901 \pm 37\%$	
Phytoplankton	Endemic phytoplankton	Field collected	-1 ± 0.5	–	Population abundance	–	Cd	53 - 139	350 - 913	Echeveste et al. (2014)
							Pb	22 - 46	146 - 304	
							Hg	0.3 - 2.8	–	
Macroalga	<i>Desmarestia menziesii</i>	Field collected	-1	8.2	Fluorescence yield	7	Cu	–	61	Runcie and Riddle (2007)
Microalga	<i>Phaeocystis antarctica</i>	Laboratory culture	0 ± 2	8.1 ± 0.2	Population growth rate inhibition	10	Cd	163 (0 - 373)	1500 (920 - 2080)	Gissi et al. (2015)
							Cu	3.3 (2.5 - 4.0)	5.9 (5.4 - 6.4)	
							Ni	>1070	>1070	
							Pb	260 (100 - 420)	570 (300 - 830)	
							Zn	450 (200 - 700)	1110 (870 - 1350)	

Cadmium, copper, nickel, lead, and zinc are common metal contaminants in the Antarctic nearshore marine environment. They have different physical and chemical properties that affect their speciation in seawater. Copper, lead, and zinc preferentially bind to dissolved organic carbon because of their high binding affinities for thiol, amine, or carboxylate ligands (Hirose 2006; Stumm and Morgan 1996; Turner et al. 1981). In the absence of organic ligands, all metals form inorganic complexes with the major inorganic ligands of seawater, chloride (Cl^-), carbonate (CO_3^{2-}), and sulphate (SO_4^{2-}) (Stumm and Morgan 1996). In model seawater conditions (i.e. surface waters at 25 °C with a pH of 8.2), the major metal species are expected to be cadmium chloride, copper carbonate, nickel split between the free ion, chloride, and carbonate complexes, lead chloride or carbonate, and zinc in the free ion or chloride complexes (Stumm and Morgan 1996; Turner et al. 1981).

Temperature

The low temperatures of Antarctic seawater slow chemical reaction kinetics and reduce the likelihood of reactions with high enthalpies. This has multiple effects on metal mobility and speciation. Generally, less metal will be in the aqueous phase but a greater proportion will exist as a free ion (Byrne et al. 1988).

Metals in Antarctica will be less soluble, because the free energy of dissolution is proportional to temperature (Stumm and Morgan 1996). When dissolved, metals will form inorganic complexes that require lower enthalpies of formation. However, copper, lead, and cadmium have higher enthalpies of formation than nickel and zinc, meaning that the speciation of nickel and zinc is less affected by the cold temperatures. This will result in different proportions of inorganic metal complexes. For example, lead is more likely to form chloride complexes, than carbonate complexes, while inorganic copper species will shift slightly towards free ion complexes, increasing from 5% in warmer to 12% in colder waters (Byrne et al. 1988).

Salinity

Salinity changes may affect bioavailability of metal contaminants by altering their speciation or the physiology of marine organisms (Deruytter et al. 2015). Reduced salinity, such as from ice-melt input to the nearshore marine environment, also reduces pH. For example, a salinity reduction of 5 units (which is typically observed in the Antarctic nearshore environment), reduced pH by approximately 0.1 to 0.2 units (Kim et al. 2015; Marion et al. 2011).

pH

The Antarctic nearshore marine ecosystem has a pH of 7.9 to 8.2 (Kapsenberg et al. 2015). This range is slightly more variable than the open ocean due to the freshening effect of ice melt and localised phytoplankton blooms. A lower pH will affect metal speciation by reducing the proportion of metal-carbonate complexes and increasing the proportion of free ion species (Millero et al. 2009). Organic ligands also have a pH dependent charge due to functional groups, including carboxylic and phenolic acids, involved in metal binding. Hence, their binding capacity for metals is expected to be reduced at a lower pH (Louis et al. 2009).

The impact of these processes to the Antarctic nearshore marine environment is expected to be minor. However, future ocean acidification projections considered in conjunction with the effects of cold seawater temperatures on metal speciation and the freshening of seawater from ice melt in the nearshore environment could see significant rises in the proportion of free ion complexes for metals like copper, nickel, and zinc, increases in the solubility of lead, but only minor changes to the speciation of cadmium (Millero et al. 2009).

Dissolved organic carbon

The bioavailability of metal contaminants is reduced by DOC through the formation of strong bonds which prevent metal binding to biotic ligands on organisms (Blewett et al. 2018; Wood et al. 2011). This is regularly shown to reduce the toxicity of metals like copper, zinc, nickel, and cadmium (Arnold et al. 2010; Deruytter et al. 2014; Nadella et al. 2009). However, DOC can also interact with cellular membranes, altering their permeability (Vigneault et al. 2000), or can stick to membrane surfaces and form ternary complexes with contaminants like lead, increasing bioavailability (Lamelas et al. 2005) and toxicity (Nadella et al. 2013).

DOC can be made up of different sizes of carbon-based molecules. The different characteristics of DOC have been shown to exert different protective effects to metal toxicity (Holland et al. 2018). For example, humic acids are a fraction of DOC that have large, highly aromatic structures. They are shown to be more protective of metal toxicity than shorter chained DOC fractions like carbohydrates and proteins. However, these fractions originate from the degradation of organic matter like leaves and wood, which do not exist in the Antarctic environment (Doval et al. 2002). Instead, Antarctic marine DOC is dominated by proteins and carbohydrates associated with microbial exudates (Alderikamp et al. 2012; Solomon et al. 2003). It is not yet clear how Antarctic-specific DOC will affect metal toxicity.

Exposure pathways

The phase of a contaminant will affect how it is exposed to an organism, which can affect its bioavailability. Most studies investigate the dissolved fraction of metal contaminants, but dietary exposure may also contribute to the risk of a contaminant (Stark et al. 2006b). For example, particulate matter has been shown to cause toxicity to an Antarctic filter feeder (Hill et al. 2009), and dietary exposure of metal-laden algae may cause toxicity in secondary consumers like invertebrates (Bielmyer et al. 2012; Sevilla et al. 2014).

Physiology of Antarctic organisms

Antarctic organisms have longer developmental times than their temperate or tropical counterparts (Marcus Zamora et al. 2015). As early life stages are usually more sensitive to contaminants than later life stages (e.g. King and Riddle (2001)), it is proposed that the longer developmental times of Antarctic organisms may make them more comparatively more sensitive (Chapman et al. 2006). However, this is yet to be demonstrated and the toxicity estimates for tested Antarctic organisms largely fall within the range of temperate organisms (Table 1.1, Chapman and Riddle (2005)).

However, Antarctic organisms may be exposed to the highest concentrations of metal contaminants when they are at their most sensitive. As described above, the spring summer transition leads to the highest pulses of metal contaminants (Stark et al. 2006b). This time is also when the sea ice epontic communities break up and become pelagic or benthic, phytoplankton bloom, and many other organisms reproduce (Rozema et al. 2017). The coincident timing of contamination flux and reproduction events is an unexamined risk to the nearshore ecosystem.

1.5 Assessing the risk of contaminants in Antarctica

The co-occurrence of a concentrated anthropogenic presence in sensitive ice-free ecological niches of an otherwise pristine continent necessitates strong environmental management. A variety of tools exist to interpret the risk that contaminants pose in temperate or tropical environment, including environmental quality standards and other lines of evidence from ecotoxicological studies.

1.5.1 Environmental quality standards

Environmental quality standards have been proposed as an environmental management tool for Antarctica to help national programs assess the impact of existing and ongoing contamination to the environment (Chapman and Riddle 2003; Klein et al. 2018). Many countries have developed frameworks to assess the potential impact of contamination to their local environment. Such as the Australian and New Zealand Guidelines for Fresh or Marine Waters (ANZG 2018), the United States' Water Quality Criteria (Stephen et al. 1985), or the European Environmental Quality Standards Directive (European Parliament and Council 2008).

Different national jurisdictions use different methods to derive environmental quality standards. Where sufficient data exists around a contaminant's toxicity and behaviour in the environment, bioavailability models like the biotic ligand model are used (for example De Schampelaere et al. (2005), Di Toro et al. (2001), Esbaugh et al. (2012), and Nys et al. (2017a)). For most contaminants, toxicological data from a range of environmentally-relevant species across multiple taxonomic groups are used in probabilistic models like species sensitivity distributions. These incorporate individual organisms responses to a contaminant in a distribution and derive concentrations that are likely to affect a proportion of those organisms (Wheeler et al. 2002). The Australian and New Zealand guidelines (ANZG 2018) specify a minimum of 5 species across at least 4 taxonomic groups are needed in these models to derive a low reliability contaminant guideline value; however, data from 8 species is recommended and data from more than 15 species is considered optimal (Warne et al. 2018).

When derived, these guideline values need to be interpreted in a framework that also accounts for environmental factors that can modify the contaminants toxicity, such as the pH, salinity, or DOC. An understanding of both the chemistry of a contaminant and the physiological response is needed to understand the risk of contaminants in the environment (Levy et al. 2007; Paquin et al. 2002).

Extensive ecotoxicological data has been generated for temperate regions for the purposes of environmental management. However, there are significant differences in the physical and chemical environment between the temperate and Antarctic environments and there are also significant differences in organism physiology, which may lead to different responses to contaminants (Chapman and Riddle 2005). These environmental and physiological differences necessitate the need for a better understanding of toxicity to Antarctic organisms for the development of Antarctic-specific guidelines (Chapman et al. 2006; Warne et al. 2014).

To date, only a few studies have quantified metal toxicity to Antarctic marine organisms, including the microalga *Phaeocystis antarctica*, the macroalga *Desmarestia menziesii*, the amphipods *Paramorea walkeri* and *Orchomenella pinguides*, the sea urchin *Sterechinus neumayeri*, and the copepods *Paralabidocera antarctica*, *Stephos longipes*, and *Oncaea curvata* (Table 1.1). These studies have focused on the effects of a range of metals, including cadmium, copper, nickel, lead and zinc, which are of concern at contaminated sites in the region (Stark et al. 2014). More ecotoxicology data are needed (Chapman and Riddle 2003), particularly for under represented species like microalgae (Chu et al. 2018), before environmental quality guidelines can be derived with high confidence. This requires the development of new experimental protocols to test the response of organisms. These data must also be coupled with an understanding of factors that influence metal toxicity as already discussed (e.g. pH, salinity, DOC), and should also consider the response of organisms to contaminant mixtures.

1.5.2 Ecotoxicological studies

Ecotoxicological studies measure the response (toxicity) of an organism to increasing concentrations of a contaminant. Two broad classes of toxic effects can be defined: acute, referring to short-term responses like immobility or death, and chronic, referring to sub-lethal responses over a longer period exposure, like growth rate, morphological changes, or changes to reproduction. Chronic effects are important to understand as they represent contamination effects over time periods more reflective of an organisms life cycle. Chronic toxicity generally occurs at contaminant concentrations lower than acute toxicity, but have similar consequences for the ecosystem. Chronic data is considered more reliable and environmentally relevant for the purposes of deriving guideline values (Warne et al. 2018). However, test protocols need to be developed to account for the longer development times and slower metabolisms of Antarctic organisms. For example, Gissi et al. (2015) found that the microalgae *Phaeocystis antarctica* required a 10-d test to achieve a 16-fold increase in cell density, the standard for OECD test methods compared to 2 to 4 d for temperate or tropical species (OECD 2011). For acute toxicity tests with the Antarctic copepods *Paralabidocera antarctica* and *Stephos longipes*, and the cyclopoid *Oncaea curvata* a minimum 7-d exposure was recommended, rather than 2 to 4 d as is typical for temperate species (Marcus Zamora et al. 2015).

Contaminant mixtures

Contaminants exist as mixtures in the environment; however, environmental quality standards are typically based on single-contaminant toxicity thresholds (ANZG 2018). The toxicity of a mixture of contaminants may not reflect the sum of the individual contaminant's toxicity (Cedergreen 2014; Nys et al. 2017c). Competition at cellular binding sites, changes to uptake pathways, and upregulation of detoxification mechanisms can influence the exposure and the response of an organism to metal mixtures (Duval et al. 2015). Recent efforts to understand and model the toxicity of metal mixtures has focused on freshwater organisms (for example, Cañizares-Villanueva et al. (2000), Franklin et al. (2002b), and Nys et al. (2015, 2016, 2017a,b)), but very few investigate marine organisms, with only a marine mussel and a sea urchin studied so far (Deruytter et al. 2017; Manzo et al. 2010). This leaves a significant gap in our understanding of metal mixture toxicity in marine systems.

Two reference models have been applied to predict mixture toxicity using toxicity estimates from the individual contaminants in the mixture (Ashford 1981): independent action, which calculates the toxicity of a mixture as the product of the individual contaminant's toxicity, making the assumption that they have dissimilar modes of action, and concentration addition, which assumes that contaminants within a mixture have a similar mode of action and are dilutions of each other. Predicted toxicity is calculated as the sum of the quotients (or toxic units) of each contaminant concentration divided by a common measure of potency (Berenbaum 1985). Both models assume that the contaminants do not interact with one another and that the response of the organism to one contaminant does not affect its response to other contaminants (Greco et al. 1995). However, deviations from model predictions are commonly observed (Deruytter et al. 2017; Franklin et al. 2002b; Nagai and De Schamphelaere 2016; Nys et al. 2015). These interactions are described as: (i) synergism, where observed is greater than predicted toxicity, and (ii) antagonism, where observed toxicity is less than predicted (Cedergreen 2014; Jonker et al. 2005).

Reference mixture toxicity models could help guide environmental assessment and decision making for sites impacted by contaminant mixtures in Antarctica. For temperate environments, concentration addition has been recommended as a good 'first tier' assessment of contaminant mixtures (Nagai and De Schamphelaere 2016; Nys et al. 2018), because of its generally conservative toxicity predictions (Backhaus and Faust 2012; Hochmuth et al. 2014; Versieren et al. 2016). Before existing contaminant mixture models can be applied to Antarctic marine environments, research is needed to validate their suitability to Antarctic marine organisms.

1.5.3 Food web effects

Dietary toxicity of metals has been shown to be an important exposure pathway for toxicity to grazing organisms in temperate and tropical environments (DeForest and Meyer 2015; Luoma and Rainbow 2005; Wang et al. 2009). Microalgae, as a primary producer of the Antarctic food web, may be an important source of dietary contaminants to higher order organisms. Recent research has found that some metals including cadmium and zinc may bioconcentrate in the Antarctic food web from microalgae, but there is not enough evidence to draw conclusions around ecosystem risk (Cabrita et al. 2017; Trevizani et al. 2016, 2018).



Figure 1.4: Environmental factors influencing metal mobility in the nearshore Antarctic marine environment. Left, evidence of ice scour in the sediment. Right, terrestrial melt water running into the nearshore marine environment. Personal photographs, January 2018.

1.6 Challenges to the measurement of environmental contaminants

1.6.1 The temporal and spatial variability of metal concentrations

The Antarctic marine environment is subject to dynamic processes that can lead to episodic increases in metal contaminant concentrations. These processes can be physical, such as from ice melt and movement, or biological, such as from algal blooms and the variable anthropogenic presence. Melt water running through contaminated sites is a major source of metals to the nearshore marine environment (Fryirs et al. 2015; Mão de Ferro et al. 2013; Stark et al. 2006b), but is coupled with air temperatures meaning the volume of melt water in summer is highly variable day-to-day (Figure 1.4).

Ice scour of contaminated sediments or nearshore soils can mobilise metal contaminants (Husmann et al. 2012). Antarctic nearshore sediments can be sulphidic (Santos et al. 2007) and anoxic below the sediment-water interface (Palmer et al. 2010), probably from high organic input from the productive benthic environment. When undisturbed, contaminants in these sediments form metal-sulphide complexes rendering them not bioavailable (Smith et al. 2002). When these sediments are disturbed, such as by ice scour (Figure 1.4), metal-sulphide complexes can oxidise, mobilising metals. Bioturbation, such as from polychaete or bivalve burrowing or penguin foraging, can have a similar effect (Amato et al. 2016; Remaili et al. 2016).

Antarctic microalgae produce carbon exudates to make metals like iron more bioavailable (Thuróczy et al. 2012), or to regulate their local environment against high salinity (Underwood et al. 2010). In the sea ice, this carbon and trace metal mixture can leach through brine channels to underlying waters where it can sustain epontic

communities. When the ice melts, this reservoir of carbon and trace metals fertilises algae blooms (Grotti et al. 2005; Meiners et al. 2011). It is not known whether this DOC will make metal contaminants more or less bioavailable.

The anthropogenic presence in Antarctica is also seasonal. The majority of humans travel to Antarctica in the brief summer period between November and February. This influx of humans means an increase in wastewater discharge, waste incineration, and electricity generation from diesel, all of which have been shown to cause nearshore contamination (Bargagli 2008). This occurs concurrent to the environmental processes, described above, which largely increase contaminant mobility.

The temporal and spatial variability of metals in the nearshore marine environment make chemical monitoring particularly challenging. Simple monitoring tools need to be developed to account for this variability to accurately understand the risk of metal contaminants to this environment.

1.6.2 Current approaches to measuring metal contaminants

A variety of techniques exist to measure metal contaminants in the nearshore marine environments, including direct sampling, investigating contaminant concentrations in organisms, and passive samplers. Most sampling is undertaken by direct sampling at a point in time, yet there are a number of drawbacks to this approach. Metal contaminant concentrations in the Antarctic nearshore marine environment are typically low, $<10 \mu\text{gL}^{-1}$ (Larner et al. 2006). This makes the analytical detection of metals challenging, particularly with the high-salt matrix of seawater. These challenges can be overcome by using resins to concentrate metal analytes (Kim et al. 2015) or solvent extractions (Sañudo-Wilhelmy et al. 2002), but both techniques require large volumes of the sampled waters. Furthermore, direct sampling only gives a concentration snapshot in time and place. In the nearshore marine environment contaminant concentrations will fluctuate because of variable inputs linked to physical and biological processes. Repeat sampling regimes can overcome these challenges, but they can be logistically prohibitive and may still miss pulses of contaminants, such as from the breakout of melt water from terrestrial ice.

The metal content of Antarctic organisms in impacted areas is well reported (e.g. Clason et al. (2003), Duquesne and Riddle (2002), Duquesne et al. (2000), Negri et al. (2006), Palmer et al. (2006), Rhodes et al. (2015), Stark et al. (2006a), and Trevizani et al. (2016, 2018)). This provides information on the presence of contaminants, but not a source, the environmental concentration, nor the route of exposure. Accumulated metals may not necessarily cause harm to an organism, as a variety of physiological factors confound relationships between environmental concentrations and tissue concentrations. By virtue of surviving the exposure, bioaccumulating organism must have at least some tolerance to the contaminant. This can be achieved by depurating, excreting, down regulating uptake mechanisms, or storing contaminants in non-metabolically active forms (Rainbow et al. 2015).

Passive samplers are a class of chemical measurement tools that rely on accumulation of a target analyte to a binding resin (Seethapathy et al. 2008). They are typically deployed into the environment (termed *in situ*), giving more realistic environmental concentrations that are time-averaged for their deployment period. A variety of passive

samplers have been developed for environmental measurements, including passive diffusion bag samplers, semi-permeable membrane devices, permeation liquid membrane, and diffusive gradients in thin-films (DGT) (Vrana et al. 2005).

DGT are a type of passive sampler deployed *in situ* to the environment that measures analytes by a process of diffusion. A binding resin is used to selectively bind and preconcentrate the analyte. The binding resin is separated from the environmental matrix by a diffusion gel (typically a hydrogel) and filter paper (Figure 1.5 Davison 2016). As analytes diffuse through the diffusion gel, they are sequestered to the binding resin. This creates a permanent concentration gradient allowing for continuous diffusion (see the yellow arrow of Figure 1.5). The rate of diffusion is defined by Fick's first law of diffusion, which allows for the accurate back calculation of the analyte's concentration in the environment based on the mass of metal accumulated and the time the DGT was deployed to the environment (Zhang and Davison 1995).

More recently, DGT have been used to assess the bioavailability of metal contaminants. The accumulation of metal analytes to DGT with binding resin based on iminodiacetic acid functional groups (known as Chelex-100) has been shown to be a good analogue of metal accumulation to some organisms in complex matrices like natural waters and sediments (Amato et al. 2018; Simpson et al. 2012). For an analyte to bind to the resin of a DGT, it has to pass a filter membrane and dissociate from any ligands it is bound to. This means that particulate, adsorbed, or strongly complexed metals are not bound in the DGT. Similarly, these fractions of metals in the environment are typically considered not bioavailable to organisms (Roulier et al. 2008).

The benefits of DGT as a contaminant monitoring tool for metals include low detection limits from analyte preconcentration, *in situ* assessment, time-averaged concentrations, and concentrations of the labile metal fraction. This makes them particularly attractive for the dynamic Antarctic nearshore marine environment. As such, they are starting to be used with more regularity (Cabrita et al. 2017; Larner et al. 2006; Padeiro et al. 2016; Stark et al. 2006a). However, guidance around their deployment and interpretation of their measurements are still needed.

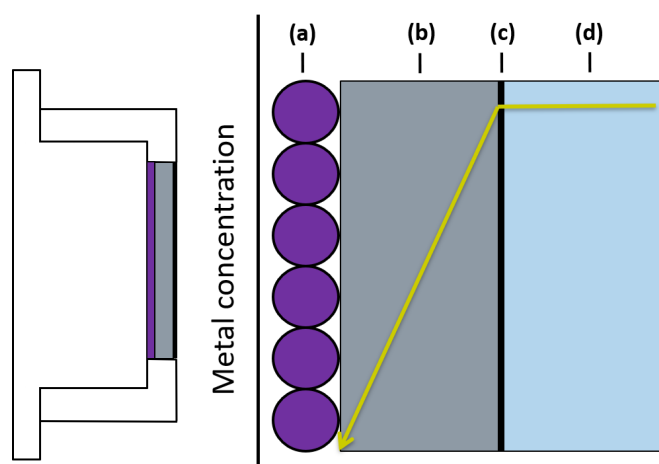


Figure 1.5: A diffusive gradient in thin-film (DGT) sampler showing a side-on profile (left) and a representation of the concentration gradient (yellow arrow) that is created when analytes are bound to the binding resin (a) after diffusing across the diffusion gel (b) and filter paper (c) which is interfaced with an aqueous environmental matrix (d).

1.7 Aims and objectives

The mission of this thesis is to contribute data and techniques that can be used to assess the risk of metal contaminants in the Antarctic nearshore marine environment. This review of literature demonstrates a need to better understand the toxicity of metals to Antarctic marine microalgae and develop a process that can measure metal contaminants in the nearshore marine environment. This process should measure metal contaminants, account for their temporal variability and factors modifying their toxicity, and integrate ecotoxicology data to provide an assessment of their risk to Antarctic organisms. This mission will be achieved with the following aims and objectives.

Aim 1

To further our understanding of metal toxicity in the Antarctic nearshore environment. This aim will be achieved by:

1. contributing toxicity data on metal exposures (singly and in mixtures) to marine microalgae, which are the primary food source in the Antarctic food web (Chapters 3 and 4). These data will be crucial in the derivation and application of Antarctic specific water quality guidelines.
2. exploring the metal-bioaccumulation potential of two Antarctic microalgae to infer possible dietary toxicity risks to their food web (Chapter 5).

Aim 2

Develop a process to measure metal contaminants and assess their risk to Antarctic marine organisms. This aim will be achieved by:

1. validating the performance of time-integrated *in situ* passive samplers (diffusive gradients in thin-films) in polar marine waters to measure metals and their mixtures in laboratory (Chapter 6) and field (Chapter 7) studies.
2. developing a process that uses diffusive gradients in thin-films to assess the risk of metals (as a surrogate for biological testing) in a laboratory setting (Chapter 6) and apply it in a field study (Chapter 7).

The outcomes of this research will provide a better understanding of metal toxicity to Antarctic marine microalgae and provide data that can be used in the derivation of Antarctic-specific water quality guidelines. This research will also demonstrate a process to assess the risk of metal contaminants which incorporates a time-averaged *in situ* passive sampling tool and ecotoxicological data. It is hoped that the data and outcomes of this thesis will be used to derive Antarctic-specific water quality guidelines and incorporated into international environmental guidelines, such as the Committee for Environmental Protection's "Clean-up Manual" and "Guidelines for Environmental Impact Assessment in Antarctica".

CHAPTER 2

General methods



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2.1 General laboratory techniques and reagents

2.1.1 General laboratory equipment and chemicals

All glass and plastic ware used for culturing or toxicity tests were washed in 10% nitric acid for at least 24 h and rinsed with ultrapure water (18.2 M Ω Milli-Q, Merck) before being used. Borosilicate 250 mL conical flasks were coated in a silanising solution (Coatasil; Ajax Chemicals) to prevent metal adsorption to flask walls during toxicity tests. All plastic containers and consumables were new or acid-washed.

All chemicals used were analytical grade or higher. Metal stock solutions were prepared in ultrapure water from copper (II) sulphate (analytical reagent grade; Ajax Chemicals); cadmium (II) sulphate octahydrate (laboratory reagent, Analar; BDH Chemicals), lead chloride (laboratory reagent; Ajax Chemicals), nickel sulfate hexahydrate (laboratory reagent, Analar, BDH Chemicals), and zinc sulfate (American Chemical Society reagent; Sigma) and acidified to between 0.1 and 1% v/v HCl (Tracepur; Merck).

2.1.2 Seawater

Seawater was collected from the east coast of New South Wales, Australia (34°4'13.4'' S 151°9'24.5'' E), in acid-washed high-density polyethylene containers. Seawater was only collected on fine-weather days where there was no precipitation in the previous 48 h. This controlled for the influence of stormwater runoff that may lead to diminished water quality. Collected seawater was immediately filtered to 0.45 μ m with an acid-washed (10% v/v HNO₃; Merck) filter cartridge (Sartobran P sterile midicap; Sartorius), and stored in the dark at 4 °C. If required, 0.45 μ m filtered seawater was further filtered to 0.22 μ m by vacuum filtration (cellulose nitrate membrane; Whatman) using sterilised glass filtration units and aseptic techniques.

Salinity (measured in the unitless practical salinity units, salinity and conductivity meter, model 30/10 FT; YSI), pH (pH meter 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 instruction. Seawater DOC concentrations from this source were typically $1.4 \pm 0.3 \mu\text{g L}^{-1}$ (APHA 5310B).

2.1.3 Metals analysis

Subsamples of test solutions were taken from each flask at the start and end of the toxicity test (Table 2.1), filtered to 0.45 μ m, acidified to 0.2% HNO₃, and 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 detection limits required. Calibration standards were matrix-matched to 0.2% acidity and a salinity of 35. Matrix-matched blanks were used to determine detection limits, which were on average: 0.3 $\mu\text{g Cd L}^{-1}$, 1.1 $\mu\text{g Cu L}^{-1}$, 1.6 $\mu\text{g Ni L}^{-1}$, 3.3 $\mu\text{g Pb L}^{-1}$, and 1.7 $\mu\text{g Zn L}^{-1}$ for ICP-AES and 0.001 $\mu\text{g Cd L}^{-1}$, 0.007 $\mu\text{g Cu L}^{-1}$, 0.005 $\mu\text{g Ni L}^{-1}$, 0.003 $\mu\text{g Pb L}^{-1}$, and 0.05 $\mu\text{g Zn L}^{-1}$ for ICP-MS. For samples in a seawater matrix, a 200 $\mu\text{g L}^{-1}$ multi-element standard (QCS27; Analytical West) was used to correct for measurement drift over time. Reported metal concentrations for each test treatment were the mean of initial and final dissolved metal concentrations.

Table 2.1: Culture and toxicity test conditions for the Antarctic microalgae *Phaeocystis antarctica* and *Cryptothecomonas armigera*.

Culturing conditions		
Temperature	2 ± 2 °C	
pH	7.9 ± 0.2	
Salinity	35 ± 1	
Light intensity	70 ± 20 $\mu\text{mol photons s}^{-1} \text{m}^{-2}$	
Light cycle	20:4 h light:dark	
Culture media	<i>P. antarctica</i> : 1/5 strength G media with added selenium <i>C. armigera</i> : 1/2 strength F media	
Toxicity test conditions ^a		
Test type	Static, non-renewal with flasks swirled twice a week to promote gas exchange	
Test chamber	Silanised 250 mL conical glass flasks	
Starting cell density	$1 - 3 \times 10^{-3}$ cells mL ⁻¹	
Test acceptability	16-fold increase in control cell density (OECD 2011)	
Test endpoints	<ul style="list-style-type: none"> • Population growth rate inhibition • Cellular size and complexity • Chlorophyll <i>a</i> fluorescence • Cellular lipid concentration (BODIPY 493/503)^b • Cellular membrane permeability (SYTOX green) 	
	<i>P. antarctica</i>	<i>C. armigera</i>
Test duration	10 d	24 d
Age of microalgae ^c	8 - 12 d	25 - 30 d
Diluent seawater ^d	0.45 μm filtered	0.22 μm filtered

^a toxicity test conditions were equivalent to the culture conditions except for the use of culture media

^b the analysis of intracellular lipids was only applicable to *C. armigera*

^c to ensure cells were in exponential growth phase for the start of test

^d supplemented with 0.15 mg PO₄³⁻ L⁻¹ and 1.5 mg NO₃⁻ L⁻¹ to maintain algal growth

2.2 Toxicity testing with microalgae

2.2.1 Microalgae cultures

Two Antarctic microalgae were used for toxicity tests, *Phaeocystis antarctica* and *Cryptothecomonas armigera*, following an assessment of their suitability by Gissi (2014). *P. antarctica* (strain number AAD 133) and *C. armigera* (strain number AAD 139) were obtained from microalgal collections at the Australian Antarctic Division, Kingston, Tasmania. Cultures were maintained in $G_{Se}/5$ and $f/2$ media, respectively (Guillard and Ryther 1962), in a temperature-controlled incubator at 2 ± 2 °C, with a 20:4 h light:dark ratio and light intensity of 60-90 $\mu\text{mol photons s}^{-1} \text{m}^{-2}$ photosynthetically active radiation 2.1. Fluorescent globes (cool white 36W/840, Sylvania lighting) were used for the first exposures with copper. Subsequent exposures with copper and other metals used white LED lights to minimise radiated heat (5050-SMD type, Electus Distribution). While LED lights resulted in a significantly greater control growth rate of $0.23 \pm 0.02 \text{ d}^{-1}$ compared to fluorescent lights of $0.20 \pm 0.01 \text{ d}^{-1}$ (mean \pm standard deviation, $n=9$ and 15, respectively), the population growth rate inhibition (as percent of control) of *C. armigera* from copper exposure was equivalent under either LED or fluorescent lights (Appendix A2.1). Algae used for bioassays were cultured in the light conditions in which they were tested.

2.2.2 Microalgae toxicity test protocol

The toxicity test protocol was developed by Gissi (2014) based on the Organisation for Economic Co-operation and Development microalgae growth inhibition toxicity test OECD (2011). Filtered seawater (0.45 and 0.22 μm for *P. antarctica* and *C. armigera*, respectively) was used as the control and diluent water in toxicity tests. Control and metal treatments were supplemented with 0.15 $\text{mg PO}_4^{3-} \text{L}^{-1}$ and 1.5 $\text{mg NO}_3^- \text{L}^{-1}$ (KH_2PO_4 and NaNO_3 , analytical reagent grade; Ajax) to maintain exponential growth of the algae during the toxicity test (Table 2.1). A volume of 80 mL filtered seawater was used in 250 mL conical flasks, with metal treatments prepared by the appropriate addition of metal stocks. The volume of metal stock and nutrient addition accounted for less than 1% of the final volume.

Algal cells in exponential growth phase (8–12 days old for *P. antarctica* and 25–30 days old for *C. armigera* (Gissi et al. 2015; Koppel et al. 2017)), were washed to remove culture growth medium by centrifugation (170 g, 4 min, 1 ± 2 °C) and resuspension in cold filtered seawater three times. The algal concentrate was then used to inoculate flasks at the start of tests at a density of $1\text{--}3 \times 10^3 \text{ cells mL}^{-1}$ (day 0).

2.2.3 Measures of toxicity

Changes to cell density, cellular chlorophyll *a* fluorescence intensity, complexity, size, lipid concentration, and membrane permeability were determined by flow cytometry (BD-FACSCalibur or BD-FACSVerse flow cytometer). A 488 nm excitation laser was paired with multiple detectors to simultaneously assess multiple biomarkers. Forward-angle ($<15^\circ$, FSC) and side-angle (90° , SSC) light scatter detectors provided indicative measurements of relative cell size and complexity, respectively (Kerker 1983). Chlorophyll *a* fluorescence intensity was measured with a $>670 \text{ nm}$ long-pass

band filter (FL3). Green fluorescence (FL1, resulting from SYTOX Green or BODIPY 493/503 stains following excitation with a 488 nm laser) was measured with a 515–545 nm band filter, when using the FACSCalibur flow cytometer. For the FACSVerse flow cytometer, cellular chlorophyll *a* fluorescence was measured at 700 ± 27 nm and green fluorescence was measured at 527 ± 16 nm (Stauber et al. 2002).

The software package CellQuest Pro (BD Bioscience, Australia) was used to determine test endpoints (Table 2.1). Microalgae cells were identified by gating the cell population against FL3 and SSC parameters. Cell density was converted to population growth rate by a linear regression of cell densities over multiple time points (at least 4) determined during the toxicity test. The population growth rate was calculated from the regression of a plot of \log_{10} (cell density) versus time (h) and was converted to equivalent population doublings per day (Franklin et al. 2005).

To quantify changes in cellular biomarkers, control cells were viewed in a 1-dimensional histogram plot of the fluorescence intensity of the parameter of interest. Three non-overlapping regions, R1, R2, and R3, were established on the 1-dimensional histograms from low to high fluorescence intensity. R2 was manually positioned to capture $\geq 95\%$ of healthy control cells. Decreases and increases in fluorescence intensity were observed on the 1-dimensional histogram plot as a cell population shift to R1 (to the left) and R3 (to the right) of R2, respectively Figure 2.1. The measured parameters are reported as a change in the R2 population, i.e. the percent of the treated population that has the same fluorescence intensity (which is related to the parameter of interest) as the control population. The EC10 for these parameters was derived as the metal concentration which caused 10% of the exposed population to show fluorescence intensity different to the control's fluorescence intensity for the given parameter.

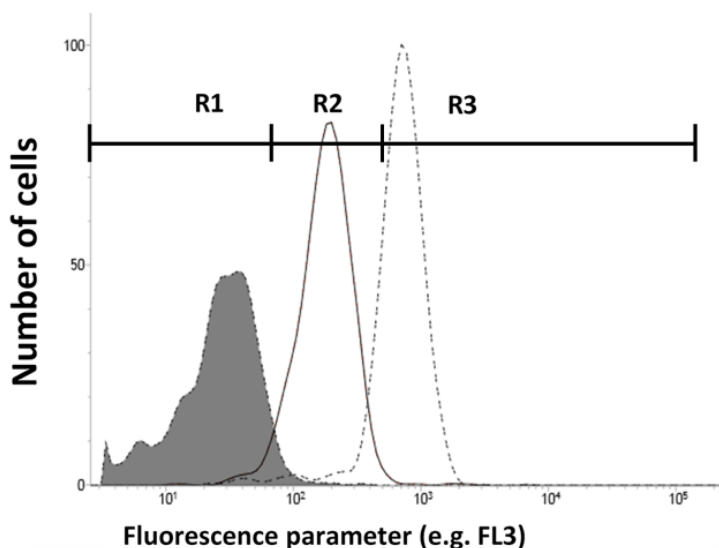
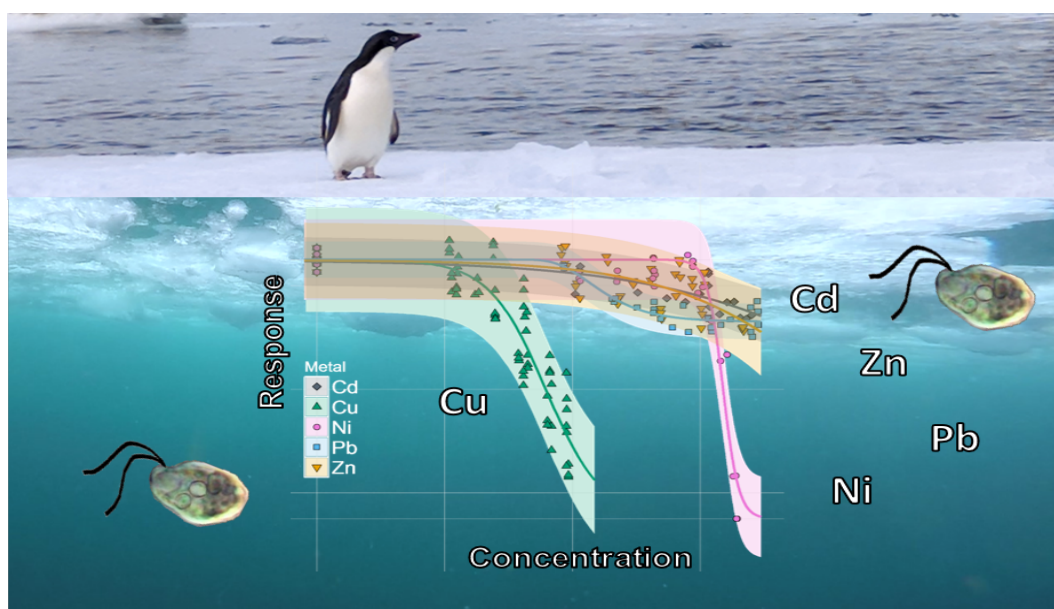


Figure 2.1: Biomarkers were analysed using 1-dimensional plots of the fluorescent marker of interest. Three regions were established (R1, R2, and R3), with R2 being positioned to capture $\geq 95\%$ of the control cell population's fluorescence (solid line histogram). Metal exposures that reduced the fluorescence intensity resulted in a 'left' population shift, increasing the percent of cells in R1 (grey fill). Metal exposures that increased fluorescence intensity resulted in a 'right' population shift increasing the percent of cells in R3 (dashed line).

CHAPTER 3

Single metal toxicity to *Cryothecomonas armigera*



Data from this section is available from doi.org/10.4225/15/5746938EC8C8B

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*yet for a week...the water remained poisonous,
a fact attested by the deaths of goldfish
suspended in cages downstream.*
Rachel Carson

3.1 Introduction

Anthropogenic activities have left a toxic legacy contamination in localised areas, particularly around research stations (Bargagli 2008; Tin et al. 2009). The introduction of the Protocol on Environmental Protection to the Antarctic Treaty in 1998 required signatory nations to the Antarctic Treaty System to commit to the comprehensive protection of the Antarctic environment and associated ecosystems, including the clean-up and remediation of historical waste. The requirement for remediation has been in force for nearly two decades, however, progress has been slow (Hughes 2010). Impediments to remediation efforts include the lack of universally agreed targets, such as water quality guidelines, a lack of applicable environmental monitoring protocols, and a dearth of toxicity data for polar organisms (Hughes 2010; Tin et al. 2009). While extensive toxicological data has been generated for temperate regions, the difference in the physical and chemical environment of polar regions and differences in organism physiology necessitates the need for region-specific ecotoxicological data (Chapman et al. 2006; Chapman and Riddle 2003; Warne et al. 2014).

The derivation of water quality guidelines requires high-quality ecotoxicological data. To date, only a few studies have quantified metal toxicity to Antarctic marine organisms, including the microalga *Phaeocystis antarctica* (Gissi et al. 2015), the macroalga *Desmarestia menziesii* (Runcie and Riddle 2007), the polychaete *Spirorbis nordenskjoldi* (Hill et al. 2009), the amphipods *Paramorea walkeri* and *Orchomenella pinguides* (Duquesne et al. 2000; Sfiligoj et al. 2015), the sea urchin *Sterechinus neumayeri* (King and Riddle 2001), and the copepods *Paralabidocera antarctica*, *Stephos longipes*, and *Oncaea curvata* (Marcus Zamora et al. 2015). These studies have focused on the effects of a range of metals including cadmium, copper, nickel, lead and zinc, which are of concern at contaminated sites in the region (Table 1.1, Stark et al. 2014).

Microalgae are valuable test organisms due to their sensitivity to contaminants (Stauber and Davies 2000), their importance as primary producers in the ocean (Arrigo et al. 1998), and their ease of culturing and testing (Stauber et al. 2002). Standard chronic toxicity test protocols for microalgae have been developed internationally, e.g. OECD (2011), and are primarily based on the endpoint of population growth rate inhibition. Tests investigating biomarkers can provide multiple lines of evidence for contaminant risk assessment. For example, investigating subcellular functionality have been developed, including esterase activity (Franklin et al. 2001), membrane permeability (Gillmore et al. 2016), and relative measures of lipid concentrations (Croxtton et al. 2015). These tests have been developed and optimised to temperate environmental conditions (OECD 2011), but their suitability for Antarctic organisms is yet to be explored.

For environmentally relevant water quality guidelines, robust toxicity testing protocols for a range of species specific to the region must be developed to determine sensitivities to contaminants. To date, only one toxicity test protocol has been developed for toxicity testing with Antarctic microalga, the prymnesiophyte *Phaeocystis antarctica*. It was found to be sensitive to copper (population growth inhibition EC10 of $3.3 \mu\text{gL}^{-1}$) and relatively tolerant to cadmium ($135 \mu\text{gL}^{-1}$), lead ($260 \mu\text{gL}^{-1}$), zinc ($450 \mu\text{gL}^{-1}$), and nickel (no observed toxicity at concentrations up to $1070 \mu\text{gL}^{-1}$, Gissi et al. (2015)). The heterotrophic protist *Cryothecomonas armigera* (phylum Cercoza, Kühn

et al. (2000)) was identified as another suitable microalgae species for toxicity testing (Gissi 2014). It is associated with Arctic and Antarctic sea-ice ecosystems (Scott and Marchant 2005) and occupies a different environmental niche to *P. antarctica*. Lipid droplets have been observed in *C. armigera* (Thomsen et al. 1991), which may be an energy reserve for survival over winter and an important food source to grazing organisms in the Southern Ocean food web.

This chapter investigates the chronic toxicity of dissolved cadmium, copper, nickel, lead, and zinc to the Antarctic microalga *C. armigera* under Antarctic conditions. Endpoints measured include population growth inhibition, and chronic sub-lethal physiological and biochemical effects.

3.2 Methods

3.2.1 Toxicity test protocol

The toxicity test protocol for *P. antarctica* and *C. armigera* is given in Section 2.2.3 and Table 2.1. This protocol was also adapted to investigate cellular membrane permeability and intracellular lipid concentrations using the molecular stains SYTOX Green (Molecular Probes; Life Technologies) and BODIPY 493/503 (Molecular Probes; Life Technologies), respectively.

Cellular membrane permeability was measured using SYTOX Green, which fluoresces green when bound to nucleic acids after crossing a compromised and permeable cell membrane. The incubation concentration and time for staining was determined to be 0.5 μM SYTOX Green (final concentration) for 5 min. Positive controls were created by heat-treating control cells at 60 °C for 5 min to permeabilise the cell membranes. Once cooled to 2 °C, heat-treated cells were added to healthy control cells at a 1:1 ratio and analysed (Appendix A3.1).

Cellular lipid concentrations were determined using BODIPY 493/503, a non-polar lipophilic probe that fluoresces green when bound to intracellular neutral lipids. Reported lipid concentrations in this study refer to a population increase or decrease in BODIPY fluorescence intensity, normalised to the control population. The optimal incubation concentration and time was determined to be 20 μM BODIPY (final concentration) for 5 min (Section 2.2.3).

3.2.2 Statistical analysis

The mean of the start and end dissolved metal concentrations was used for all calculations. This average accounts for any loss in metal concentration in seawater as a result of metal adsorption to glass surfaces, precipitation, or metal accumulation by algae (Angel et al. 2015b). To derive effect concentrations, the Dose Response Curve (Ritz et al. 2015), and Mixed Effect Dose Response Modelling packages of R statistical analysis software were used (Gerhard et al. 2014; R Core Team 2016). The mixed effect dose response model accounts for the variation between and within repeated experiments (and should not be confused with reference toxicity mixture models, discussed in Chapter 4).

The Akaike Information Criterion (AIC) function was used to determine model suitability where multiple models were tested (Pinheiro and Bates 2000). Generally, a 4-parameter log-logistic model provided the best fit. Effect concentrations were calculated after fixing upper and lower model limits to 100 and 0%, respectively, reflecting the possible range of biological response investigated by these models, thus only 2 parameters were estimated. Effect concentrations were calculated from pooled concentration-response data after being normalised to the controls from individual experiments. To account for inter-experimental variability the marginal effect concentrations were calculated from a non-linear mixed-effect model fit by maximum likelihood based on the log-logistic model. No effect concentrations (NEC) were derived using the non-linear dose-response model NEC function in the DRC package (Pires et al. 2002), which assumes a threshold (the “NEC”) below which the response is equal to the control. NEC values could not be derived for nickel and zinc due to the limited response data between 0 and 100 $\mu\text{g L}^{-1}$.

3.3 Results and discussion

3.3.1 Development of a chronic growth inhibition bioassay

This study modified standard temperate toxicity test protocols for microalgae to accommodate the different growth rate and temperature requirements of the polar species *C. armigera*. The test durations and conditions for the toxicity test are given in Table 2.1. Chronic toxicity tests require an exposure period that encompasses a sub-lethal effect on a significant portion of an organism’s life stage. For microalgae the OECD standard methods stipulate a 16-fold increase in control cell biomass, which is typically achieved in 2 to 4 d with temperate microalgae species (OECD 2011). *C. armigera* required approximately 24 days to achieve a 16-fold increase, which reflects the slower metabolic processes in this species in cold water temperatures.

This requirement for extended test duration is common for toxicity tests using Antarctic organisms. For example, the Antarctic microalga *P. antarctica* required a 10 day test duration to achieve the required growth (Gissi et al. 2015). For acute toxicity tests with the Antarctic copepods *Paralabidocera antarctica* and *Stephos longipes*, and the cyclopoid *Oncaea curvata* a minimum 7 day exposure was recommended, rather than 2 to 4 days as is typical for temperate species (Marcus Zamora et al. 2015). A chronic bioassay with the echinoid *Sterechinus neumayeri* investigated larval development to two life-stage endpoints: hatched blastula (6 to 8 day exposure), and development to the 2-armed plutei (22 to 23 day exposure). These exposures were much longer than tests with temperate echinoid species which typically proceed for 5 h and 3 to 7 days, respectively (Table 1.1, King and Riddle 2001).

The control population growth rate of *C. armigera* averaged 0.22 ± 0.02 doublings per day and was consistent throughout the study (i.e. maintained exponential growth rates), suggesting that the macronutrients NO_3^- and PO_4^{3-} were not depleted and did not inhibit growth. The pH in test solutions was measured on days 0 and 24, and was stable at $\text{pH } 7.9 \pm 0.2$, suggesting that no significant change in metal speciation (caused by pH changes) was likely throughout each test.

Dissolved metal concentrations of copper, lead, and zinc were reduced during the test by an average of 5, 5, and 9% of their initial (day 0) concentrations, respectively, with no change in cadmium and nickel. This decrease may be a result of metal binding to glass surfaces (despite the use of silanised glassware), the formation of insoluble metal complexes with seawater constituents, or metals accumulated by cells during the bioassay.

3.3.2 Metal toxicity to population growth rate

Copper was the most toxic metal to *C. armigera*, with a concentration causing a population growth rate inhibition of 10% (EC10) and 50% (EC50) of 21.6 and 63.1 $\mu\text{g L}^{-1}$ respectively (Table 3.1, Figure 3.1 b). Nickel was the only other metal toxic enough at the concentrations tested to derive an EC50 (1570 $\mu\text{g L}^{-1}$). However, nickel was also the least toxic based on the EC10 value (1110 $\mu\text{g L}^{-1}$, Table 3.1, Figure 3.1 c). *C. armigera* was relatively tolerant to cadmium, lead, and zinc resulting in EC10 concentrations of 454, 152, and 366 $\mu\text{g L}^{-1}$, respectively (Figure 3.1 a, d, and e). The EC50 values for cadmium, lead and zinc could not be derived due to the relatively high tolerance of *C. armigera* to these metals, and are instead reported as greater than the measured maximum concentration tested (2530, 2830, and 2190 $\mu\text{g L}^{-1}$ respectively, 3.1). The overall order of dissolved metal toxicity to *C. armigera* based on EC10 values is $\text{Cu} > \text{Pb} > \text{Cd} = \text{Zn} > \text{Ni}$. This was similar to findings for the Antarctica microalga *P. antarctica*, in which a 10-d exposure to dissolved metals resulted in an EC10 toxicity ranking of $\text{Cu} > \text{Cd} > \text{Pb} > \text{Zn} > \text{Ni}$ (Gissi et al. 2015). These results show that *C. armigera* is a metal-tolerant species, with copper sensitivities similar to the temperate green alga *Tetraselmis sp.* (EC50 of 47 $\mu\text{g Cu L}^{-1}$) (Levy et al. 2007). In contrast, *P. antarctica* was 10 times more sensitive to copper with an EC50 of 5.9 $\mu\text{g Cu L}^{-1}$ (Gissi et al. 2015). While the EC10 values for the metals tested were generally greater than reported dissolved metal concentrations in contaminated bays in polar marine ecosystems (Crockett and White 1997; Stark et al. 2006b), it is possible that algae could be impacted by such concentrations in areas adjacent to contaminated sites or near wastewater outfalls.

The toxicity of metals is better correlated to the free metal ion concentrations than to the total dissolved metal concentrations. The speciation of metals in seawater is controlled by various environmental factors, including temperature. Temperature positively correlates to solubility and the stability constants of metal complexes. In polar conditions, solubility is expected to be reduced but a greater proportion of the metal will exist as the free ion (Byrne et al. 1988). This may increase the toxicity of metals in polar marine conditions compared to equivalent dissolved concentrations in temperate or tropical marine environments. However, this will be metal specific with metals that form weak complexes in seawater, such as zinc and nickel predominately existing as the free ion. In contrast, copper, cadmium, and lead predominately exist as carbonate or chloride complexes (Byrne et al. 1988). The concentration ranges tested in this study are likely near their solubility limits for seawater; however, these solubility limits are generally uncharacterised in seawater. Only lead has a reported solubility concentration, of approximately 2000 $\mu\text{g L}^{-1}$ in seawater at 22 °C (Angel et al. 2015a).

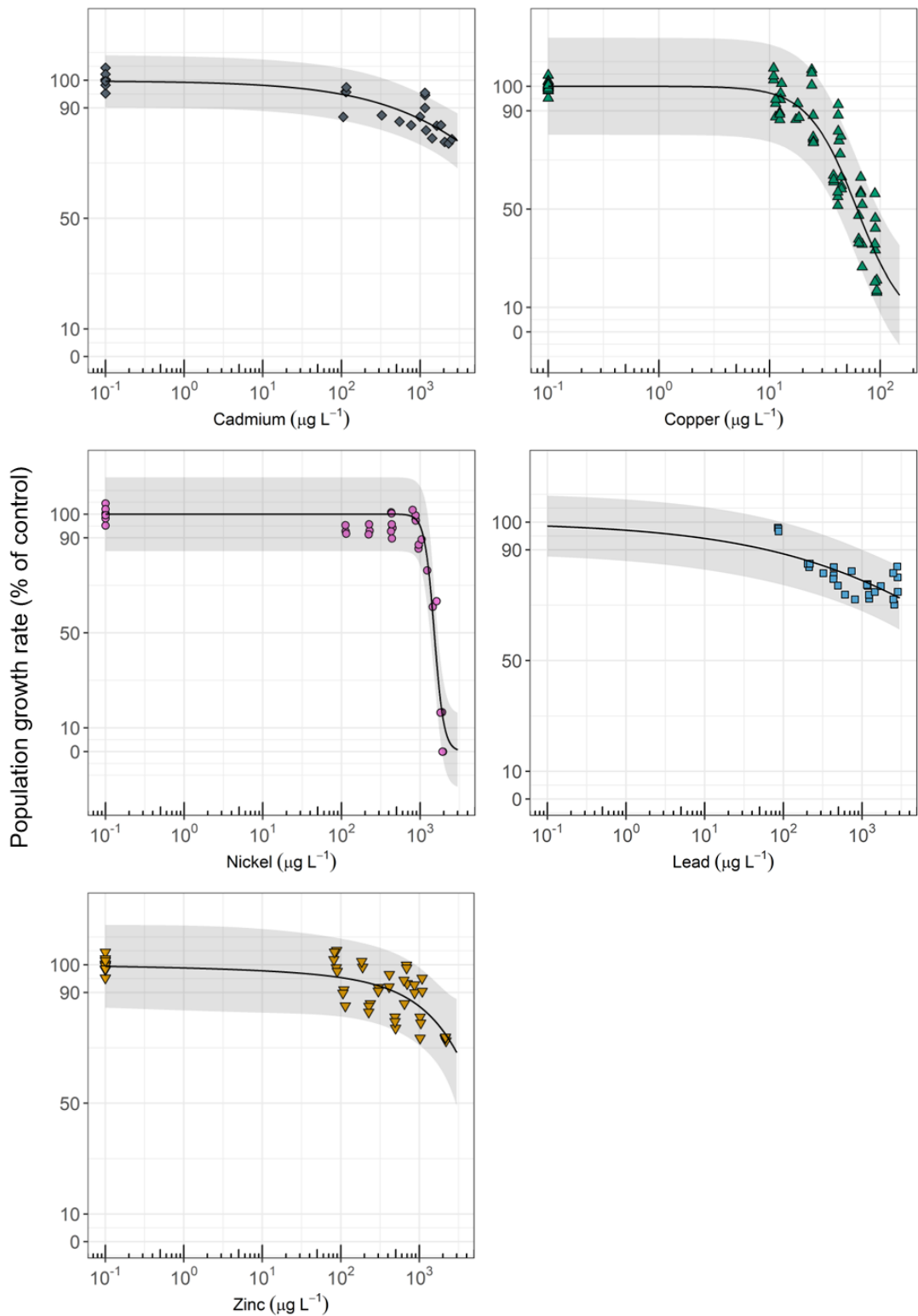


Figure 3.1: The effect of 24-d dissolved metal exposures on the growth rate of the polar microalga *Cryothecomonas armigera* expressed as a percentage of control growth for (a) cadmium, (b) copper, (c) nickel, (d) lead, and (e) zinc. Grey ribbons represent the 95% prediction intervals for the fitted log-logistic models (solid black line).

Table 3.1: Toxicity of metals to the Antarctic microalga *Cryothecomonas armigera* after a 24-d exposure. Metal concentrations are in $\mu\text{g L}^{-1}$ and values in parenthesis are the 95% confidence limits. Direction of arrow indicates the direction of the fluorescence shift of the cellular biomarker. The model used to derive these values and its accompanying parameters are provided in Appendix A3.2.

Parameter		Cadmium	Copper	Nickel	Lead	Zinc
Population growth rate inhibition	NEC	41 (0 - 342)	10 (6 - 15)		105 (86 - 126)	
	EC10	450 (230 - 680)	22 (17 - 26)	1200 (1110 - 1340)	150 (80 - 300)	370 (40 - 690)
	EC50	>2530	63 (59 - 68)	1570 (1500 - 1630)	>2800	>2200
Cellular lipid concentration	EC10	89 ↓ (26 - 118)	33 ↓ (0 - 68)	894 ↓ (641 - 1150)	11 ↓ (0 - 31)	241 ↓ (0 - 948)
	EC50	280 ↓ (229 - 330)	>93	1060 ↓ (100 - 1100)	163 ↓ (60 - 266)	>2190
Chlorophyll <i>a</i> fluorescence	EC10	1036 ↑ (179 - 1890)	>93	1460 ↑ (1380 - 1550)	807 ↑ (365 - 1250)	987 ↓ (0 - 2000)
	EC50	>2530	>93	2000 ↑ (1950 - 2050)	2370 ↑ (1880 - 2870)	>2190
Cell complexity	EC10	269 ↓ (178 - 359)	>93	>1950	>2830	>2190
	EC50	1130 ↓ (1000 - 1260)	>93	>1950	>2830	>2190
Cell size	EC10	>2530	37.9 ↑ (32.7 - 108)	681 ↑↓ ^a (0 - 2680)	2140 ↑↓ (0 - 5950)	1340 ↑↓ (0 - 3340)
	EC50	>2530	>93	>1950	>2830	>2190

^a Values with both up and down arrows indicate approximately equal shifts of the cell population.

3.3.3 Metal toxicity to cellular biomarkers

Metal exposure led to changes in biomarkers of *C. armigera*, including cellular chlorophyll *a* fluorescence, intracellular lipid concentration, cell complexity and size. There were no observed changes to membrane permeability from any exposure.

Cellular chlorophyll *a* fluorescence

Cellular chlorophyll *a* fluorescence intensity refers to the emission intensity of the individual cell's chlorophyll *a* molecules when excited by a 488 nm laser. It is a well-established, non-invasive indicator of metal induced toxicity (Corcoll et al. 2011). All five metals affected the cellular chlorophyll *a* fluorescence intensity of *C. armigera* (Figure 3.2). Cadmium, copper, nickel, and lead increased and zinc decreased fluorescence intensity. Cadmium and copper had the smallest effect, with copper not toxic enough to derive an EC10 and the EC10 for cadmium more than 2 times the growth rate inhibition EC10. The highest cadmium concentration tested $2300 \mu\text{g L}^{-1}$ caused 24% effect. Lead increased cellular chlorophyll *a* fluorescence resulting in an EC10 of $807 \mu\text{g L}^{-1}$ (Figure 3.2), approximately 5 times its growth rate inhibition EC10. Exposure to nickel increased cellular chlorophyll *a* fluorescence at concentrations that also caused population growth inhibition, with the cellular chlorophyll *a* fluorescence EC10 of $1460 \mu\text{g L}^{-1}$, within the range determined for growth rate inhibition EC10 and EC50 values. Zinc was the only metal to decrease chlorophyll *a* fluorescence, with an EC10 value of $987 \mu\text{g L}^{-1}$. However, at $2190 \mu\text{g L}^{-1}$ the effect was also approximately 10%.

The decrease in chlorophyll *a* fluorescence following zinc exposure suggests an impairment of the chlorophyll apparatus, potentially by metal substitution of its magnesium centre (Küpper and Andresen 2016). In comparison, an increase in chlorophyll *a* fluorescence may suggest the inhibition of electron-transport proteins (Guo and Tan 2015). The excitation of the chlorophyll structure is a physical process that relies on available light and a functional chlorophyll apparatus. If electron transport proteins were to be impaired, excited chlorophyll moieties would de-excite by electronic emission resulting in an increase in fluorescence intensity (Silva et al. 2012). This was observed with cadmium, copper, nickel, and lead, suggesting that these metals affect the electron transport chain proteins, rather than the chlorophyll complex.

Lipid concentrations

All tested metals reduced the intracellular lipid concentration of *C. armigera*. Cadmium and lead caused the greatest decreases in intracellular lipid concentrations (Figure 3.2), with 100% of cells having reduced intracellular lipids compared to the control populations following exposure to $1000 \mu\text{g Cd L}^{-1}$ and $500 \mu\text{g Pb L}^{-1}$, respectively. The highest tested concentrations of copper and nickel reduced cellular lipid concentrations in 20 and 40 % of the exposed cell population compared to the control cell population at concentrations of $93 \mu\text{g Cu L}^{-1}$ and $1950 \mu\text{g Ni L}^{-1}$, respectively. This reduction in lipids occurred at concentrations which also caused growth rate inhibition (Figure 3.2).

Lipids are a diverse class of biomolecules that play important roles in cellular structure and function. A significant fraction of total lipids exist as lipid droplets, where nonpolar lipids produced by microalgae are intracellularly stored. Recent studies have shown that lipid droplets are involved in energy storage, membrane synthesis, cellular trafficking, and cellular signalling. Lipid droplets are composed of a triacylglycerol core and phospholipid monolayer, with various inclusions of other constituents (proteins, sterol esters, etc) (Farese and Walther 2009; Goold et al. 2014). The non-polar lipophilic probe BODIPY 493/503 has been shown to bind to lipid droplets with greater specificity than other lipid stains, such as Nile Red, making it a powerful fluorescent probe to investigate these important organelles (Gocze and Freeman 1994).

Interestingly, exposure to dissolved cadmium and lead reduced lipid concentrations with only a minor impact to growth rate. This may be due to the presence of an energy intensive detoxification mechanism, or the ability to repair damage arising from these metals' toxic effects. Lead has been shown to be detoxified by precipitation as $PbO_{(s)}$ and $Pb_3(PO_4)_{2(s)}$ in the green freshwater alga *Chlamydomonas reinhardtii*, as well as through organic complexation thiol-based peptides (Stewart et al. 2015). Cadmium is known to be efficiently sequestered intracellularly by phytochelatins in algae, e.g. *Phaeodactylum tricorutum* (Scarano and Morelli 2002, 2003), *Chlorella vulgaris* (Huang et al. 2009), and *Nitzschia palea* (Figueira et al. 2014), unlike lead (Scarano and Morelli 2002). The exact mechanism of detoxification is unknown in *C. armigera*, but it is apparent that it comes at an energetic cost. Exposure to copper resulted in only a limited decrease in cellular lipid concentration, despite copper being the most toxic metal to population growth rate. This may indicate a limited ability to detoxify copper, and/or an inability to repair damage caused by what is likely to be oxidative stress arising from intracellular Fenton cycling (Smith et al. 2014). Nickel reduced lipid concentrations only at metal concentrations that resulted in population growth inhibition, suggesting that regulation of nickel is not energy intensive. The reduction in cellular lipid concentration that occurred concurrent to population growth inhibition may be a result of cellular repair mechanisms.

The measure of cellular lipid concentrations is an important biomarker, as changes to the energy content of a food source may have broader implications to the food web. These changes may be especially impactful in a polar environment where marine productivity is concentrated to the summer period. As the EC10 values for cadmium, lead, and nickel toxicity to cellular lipid concentrations were lower than their respective EC10 values for population growth rate inhibition (Table 3.1), these results highlight the potential application of BODIPY as a sensitive indicator for metal stress in marine microalga. Further work should quantify the reduction in cellular lipid concentrations and investigate the potential for lipid reductions to cause adverse ecosystem effects.

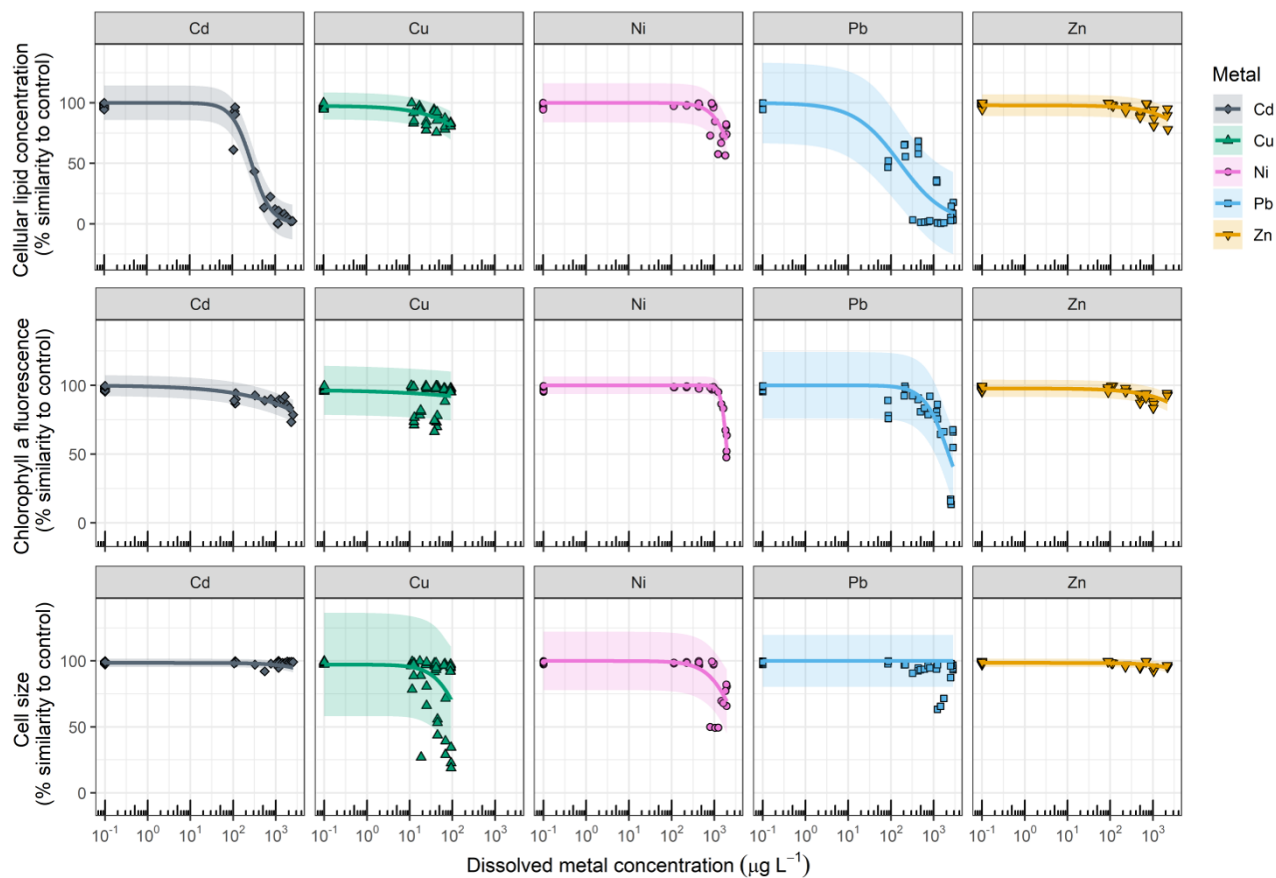


Figure 3.2: The effect of 24-d dissolved metal exposures on the relative lipid concentration, cellular chlorophyll *a* fluorescence, and cell size of *Cryothecomonas armigera* expressed as a percentage of control population's fluorescence intensity. Ribbons represent the 95% prediction interval of the log-logistic model. All metals resulted in a decrease to intracellular lipid concentrations (i.e. the cell population shifted to R1). Chlorophyll *a* fluorescence intensity increased following cadmium, copper, nickel, and lead exposure (i.e. the cell population shifted to R3), whereas the chlorophyll *a* fluorescence decreased following zinc exposure (i.e. the cell population shifted to R1). Copper increased the size of *C. armigera*.

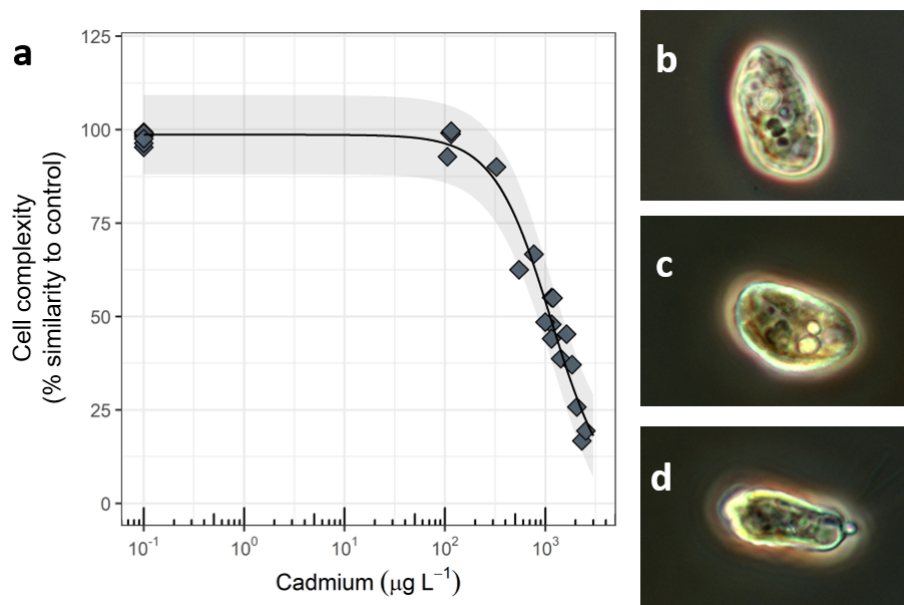


Figure 3.3: The effect of cadmium on the cellular complexity of *Cryothecomonas armigera*: (a) percent of cells with cell complexity equivalent to the control cell population, measured by side-angle light scatter intensity. The grey ribbon represents the 95% prediction interval of the log-logistic model. Light microscopy images of *C. armigera* following (b) control, (c) 2700 $\mu\text{g Pb L}^{-1}$ and (d) 2500 $\mu\text{g Cd L}^{-1}$ exposure. Cells displayed are 35 μm along their longest axis. Note the change in cell morphology after exposure to cadmium. The cadmium treatments reduced the cellular complexity, as measured by side-angle light scatter intensity (i.e. the cell population shifted to R1).

Cellular complexity and size

The cellular complexity of *C. armigera* was only affected by cadmium, with exposures showing a concentration-dependent decrease in internal complexity. This change in cellular complexity was thought to be related to a reduction in cellular lipid concentrations (and therefore the absence of lipid droplets); however, lead reduced lipid concentrations without affecting cellular complexity (see Figure 3.2 and Figure 3.3 c). Subsequent cell analysis by light microscopy of cadmium exposed cells clearly showed variations in cellular morphology including a reduction in cellular complexity and apparent shrivelling (a narrowing and increased outer surface roughness of the cell membrane, Figure 3.3). Interestingly, no changes in the cellular size were observed concurrent to these cellular complexity changes. These results imply that cadmium may affect osmotic regulation. In contrast to these results, Jamers et al. (2009) found that cadmium increased cellular complexity in the freshwater temperate microalga *Chlamydomonas reinhardtii*, which was associated with vacuolisation. This was not observed by light microscopy in *C. armigera*.

Copper exposures increased cell size (Figure 3.2), with an EC10 of 38 $\mu\text{g L}^{-1}$. The variability of the response reflects the different sensitivities of the flow cytometers used. Nickel, lead, and zinc had relatively small, contradictory results between experiments, i.e. the relative size of cells slightly increased in some tests and decreased in others. This may be due to variability in the control population, which the metal-exposed cells are gated against, differences in the sensitivities of the flow cytometer used, or a reflection of variable physiology.

3.3.4 Environmental relevance of observed toxicity to Antarctic ecosystems

The toxicity test protocol outlined in this chapter was developed to be as realistic to the Antarctic marine environment as possible. For example, natural seawater with low nutrient additions was used as the test medium. The low initial algal cell densities, temperature, pH, and light intensity aim to reflect normal polar marine conditions (Table 2.1). Important environmental factors, such as the effect of DOC or the presence of contaminants in mixtures can also affect metal toxicity resulting in different effects from those predicted from standard laboratory bioassays. These effects should be elucidated to better understand the applicability of water quality guidelines in complex environments. *C. armigera* was shown to be moderately metal tolerant relative to other microalgal species. If this is due to intracellular sequestration mechanisms, then it may act as a source of metal contaminants to organisms at higher trophic levels. Similarly, the impact of contaminants to its lipid concentrations may pose an indirect mechanism of toxicity by reducing the energetic value of a food source (Lavoie et al. 2016).

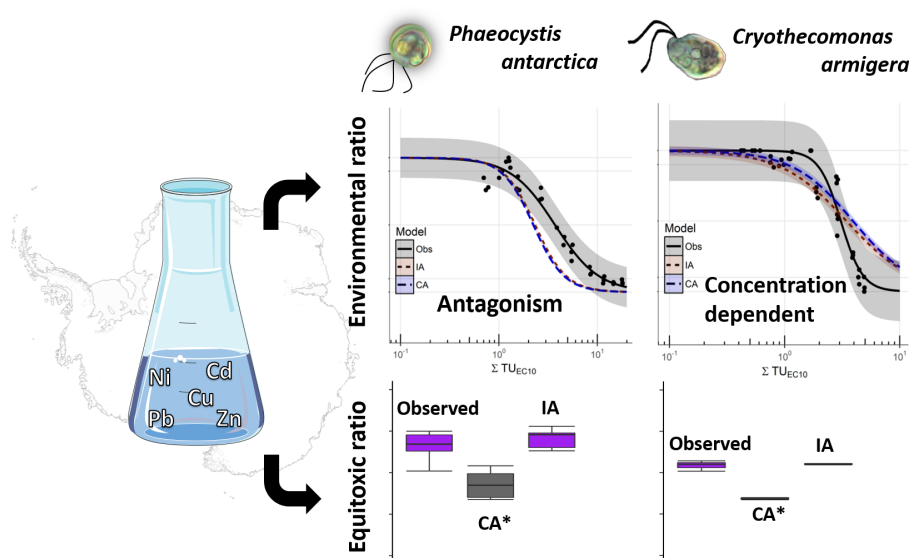
3.4 Conclusion

This chapter established a toxicity test protocol for the assessment of contaminants to the polar marine microalga *C. armigera*. This protocol was applied to determine population growth inhibition, and chronic sub-lethal physiological effects following exposure to five metals. Based on population growth rate inhibition EC10 estimates, copper the most toxic metal followed by lead, cadmium and zinc, and nickel. Cellular lipid concentrations were a sensitive indicator of metal toxicity, with responses to cadmium, nickel, and lead more sensitive than growth rate inhibition response. These results highlight the usefulness of intracellular lipids for understanding metals' mode of action and for assessing indirect impacts of metal toxicity to food webs. Cellular complexity and chlorophyll *a* fluorescence were generally poor indicators of metal toxicity compared to growth rate inhibition; however, they provided insights into metal-specific modes of action. Ecotoxicological data from this study will ultimately contribute towards the development of water quality guideline values for the Antarctic marine environment.

Water quality guidelines are typically based on single-metal toxicities; however, organisms in the Antarctic nearshore marine environment are exposed to mixtures of metal contaminants. Previous research has shown that mixtures of contaminants could result in unexpected toxicities; therefore, mixture interactivity needs to be assessed to better understand the risk of metal contaminants in the nearshore marine environment.

CHAPTER 4

Chronic toxicity of metal mixtures to *Phaeocystis antarctica* and *Cryothecomonas armigera*



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Author Contributions: DJK designed and ran the experiments, analysed the data, prepared all figures and wrote the manuscript. All authors contributed to the experimental design and editing of the manuscript before submission.

One must imagine Sisyphus happy
Albert Camus

4.1 Introduction

Environmental monitoring in Antarctica regularly reports mixtures of contaminants, including the metals cadmium, copper, nickel, lead, and zinc in nearshore marine environments (Fryirs et al. 2015; Larner et al. 2006). The development of toxicity testing protocols for a range of Antarctic marine organisms, discussed in Chapter 3, Table 1.1, provides the means to derive ecotoxicological data for environmental quality standards. However, tests so far have only investigated the toxicity of metals in single-metal exposures.

The toxicity of single metals may not reflect their toxicity in a mixture. Competition at cellular binding sites, changes to uptake pathways, and upregulation of detoxification mechanisms can influence the exposure and the response of an organism to metal mixtures (Duval et al. 2015). Recent efforts to understand and model the toxicity of metal mixtures has focused on freshwater organisms, with only a few mixture studies conducted on marine organisms; including, a marine mussel (Deruytter et al. 2017), a sea urchin (Manzo et al. 2010), and a diatom (Filimonova et al. 2018, albeit with organic and inorganic contaminant mixtures). There is therefore a deficiency in our knowledge about metal-mixture toxicity to marine and especially Antarctic organisms.

Two reference models are widely accepted for the determination of metal mixture toxicity: independent action (IA) and concentration addition (CA) (Berenbaum 1985; Jonker et al. 2005). Both models assume that the toxicity of the mixture components are non-interactive; but, have different approaches to combine individual contaminants' toxicities. IA calculates the toxicity of a mixture as the product of the individual contaminant's toxicity, making the assumption that they have dissimilar modes of action. The CA model expresses each contaminant of the mixture as a toxic unit (TU), calculated as the concentration of the contaminant in the mixture divided by a measure of its potency, such as a 10% effect concentration (EC10). CA assumes that contaminants within a mixture have a similar mode of action, and that contaminants are dilutions of each other. Both models rely on single-metal toxicity data and assume no interactions between contaminants. However, deviations from model predictions are often observed. These interactions are described as: (i) synergism, where observed is greater than predicted toxicity, and; (ii) antagonism, where observed toxicity is less than predicted (Cedergreen 2014). Mixtures are described as non-interactive when observed toxicity is predicted by the model.

The use of the lowest available parametrically derived toxicity estimates (e.g. no effect concentrations or EC10) in ecotoxicology is growing because of their preferred use in the derivation of environmental quality guidelines (Batley et al. 2014; Warne et al. 2014). This may be because higher effect estimates (e.g. EC50 values) represent too much toxicity for environmental protection or that for some contaminants, EC50 values may not be derivable (see Figure 3.1). Previous research has found that mixtures of contaminants at \geq EC10 may produce mixture interactions (Nys et al. 2018). Yet, only a few studies have investigated environmentally relevant concentrations in chronic ecotoxicology studies (Nys et al. 2017c; Versieren et al. 2016).

The Protocol on Environmental Protection to the Antarctic Treaty System requires that nations operating in Antarctica remediate their historical waste and limit their impact to the environment (ATCM 1991). To support this goal, a Clean-up Manual was developed

to provide practical guidance about contaminated site assessment and remediation. However, it provides no recommendations around assessing the risk of contaminant mixtures (CEP 2013). Ideally, contaminant toxicity to Antarctic organisms would be so well understood that integrated ecosystem risk models, such as the tiered approach developed by Nys et al. (2018), can be adopted. Until enough data is generated for Antarctic organisms, however, reference models like CA are recommended as a good ‘first tier’ assessment due to its generally conservative nature (Backhaus and Faust 2012; Hochmuth et al. 2014; Nagai and De Schampelaere 2016; Versieren et al. 2016). Before these contaminant mixture models can be applied to Antarctic marine environments, research is needed to validate their suitability for Antarctic marine organisms.

The toxicity of five metals to two Antarctic marine microalgae, occupying different ecological niches in the marine ecosystem, have recently been assessed. *Phaeocystis antarctica* (phylum Haptophyta) is a common marine microalga associated with open marine waters and is known to be mucogenic (Rousseau et al. 2007). Gissi et al. (2015) found it was sensitive to copper with an EC10 of $3.3 \mu\text{gL}^{-1}$, and relatively tolerant to other metals (Table 3.1). Chapter 3 investigated metal toxicity to *C. armigera*, a single-celled flagellated heterotrophic protist found in both Arctic and Antarctic marine waters, commonly associated with sea ice, and known to produce significant intracellular lipid stores (Kühn et al. 2000; Thaler and Lovejoy 2012). It had a different sensitivity to *P. antarctica* being moderately tolerant to copper and tolerant to other metals (Table 3.1).

This chapter aims to: (i) investigate the toxic effects of cadmium, copper, nickel, lead, and zinc mixtures to the Antarctic microalgae *P. antarctica* and *C. armigera* using an equitoxic and environmental mixture. Measured toxic effects include population growth rate inhibition, and the cellular biomarkers chlorophyll *a* fluorescence intensity, changes to cell size and complexity, and changes to intracellular lipid concentrations; (ii) assess the applicability of two reference models, IA and CA, to predict chronic toxicity as microalgae population growth rate inhibition, parameterised to use EC10 values; and (iii) investigate metal-mixture interactivity to *P. antarctica* and *C. armigera* by IA and CA from the equitoxic and environmental mixture.

4.2 Methods

4.2.1 Toxicity test protocol

The toxicity test protocol and conditions for *P. antarctica* and *C. armigera* are given in Section 2.2 and Table 2.1. Two metal mixtures were tested: an equitoxic mixture and an environmental mixture. The equitoxic mixture was a combination of cadmium, copper, nickel, lead, and zinc at concentrations equivalent to the population growth rate inhibition EC10s determined from single metal tests for *P. antarctica* (Table 1.1, Gissi et al. (2015)) and *C. armigera* (Table 3.1). The environmental mixture reflects the dissolved metal concentrations in Brown Bay, a historically-contaminated marine site near Australia’s Casey Station in East Antarctica (Larner et al. 2006). The environmental mixture was tested at increasing multiples to produce a concentration gradient of metals fixed at an environmentally realistic ratio.

4.2.2 Calculations of mixture toxicity and interactivity

Two reference models of mixture toxicity were tested to predict the toxicity of the metal-mixture ratios, Independent Action (IA) and Concentration Addition (CA). Measures of toxicity were expressed as a relative effect (RE, Equation 4.1) as a percentage of control response, where y is the response of the exposure, and $\bar{x}_{y \text{ control}}$ is the mean of the control response (Nys et al. 2016). The calculation of relative effect was integrated into the mixture toxicity models to harmonise the toxicity prediction process. This allows for algae-specific toxicity predictions with only metal-concentrations as inputs.

$$RE \text{ (\% of control)} = \frac{y}{\bar{x}_{y \text{ control}}} \cdot 100\% \quad (4.1)$$

Single-metal toxicity data for *P. antarctica* were reanalysed to determine EC10s values using the approach outlined in Section 2.2.3 and are given in Table 4.1.

Table 4.1: Single metal toxicity to *Phaeocystis antarctica* reanalysed from Gissi et al. (2015). All values are in μgL^{-1} . Model parameters and their standard errors are provided in Appendix A4.1.

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

Independent Action

IA was determined by Equations 2-5, where: y_i is the response of the alga to metal i if it were exposed to a single metal at the same concentration found in the mixture. y_i was determined by a log-logistic model described by Equation 4.2, where x_i is the concentration of metal i , β is the slope parameter, and EC50 is the concentration of metal i which causes a 50% reduction in the response variable y_i .

$$y_i = \frac{100\%}{1 + \left(\frac{x_i}{EC50}\right)^{\beta_i}} \quad (4.2)$$

This study used EC10 values, rather than EC50s to calculate y_i . To do this, EC50 parameters were converted to EC10s by Equation 4.3 (Nys et al. 2017c), which is derived from Equation 4.2 where y_i and x_i are replaced with 90% and the EC10. This then gives Equation 4.4.

$$EC50_i = EC10_i \cdot 9^{\frac{1}{\beta_i}} \quad (4.3)$$

$$y_i = \frac{100\%}{1 + \left(\frac{x_i}{EC10_i \cdot 9^{\frac{1}{\beta_i}}}\right)^{\beta_i}} \quad (4.4)$$

IA response predictions were then determined as the product of the expected response of individual metals (y_{IA}), shown by Equation 4.5.

$$y_{IA} = 100\% \cdot \prod_{i=1}^n \frac{1}{1 + \left(\frac{x_i}{EC10_i \cdot 9^{\frac{1}{\beta_i}}}\right)^{\beta_i}} \quad (4.5)$$

Concentration Addition

CA predictions (y_{CA}) were determined by Equations 4.6 and 4.7 where x_i is the concentration of metal i in the mixture, $EC10_i$ is the concentration of metal i that causes a 10% inhibition to population growth rate, and ΣTU_{EC10} is the sum of the toxic unit contribution for each metal where each toxic unit is equivalent to a 10% reduction in population growth rate.

$$\Sigma TU_{EC10} = \sum_{i=1}^n \frac{x_i}{EC10_i} \quad (4.6)$$

$$\sum_{i=1}^n \frac{x_i}{\left(EC10_i \cdot 9^{\frac{1}{\beta_i}} \right) \cdot \left(\frac{100\% - y_{CA}}{y_{CA}} \right)^{\frac{1}{\beta_i}}} = 1 \quad (4.7)$$

The model parameters (i.e. $EC10_i$ and β_i values for each metal) used in the reference mixture models were optimised prior to predicting metal-mixture toxicities using Monte-Carlo simulations based on the method of Hochmuth et al. (2014). A random normal distribution of 40,000 sets of model parameters were generated about the means of each parameter. The mean and standard deviations of each $EC10_i$ and β_i parameter were taken from the previously determined log-logistic model fits of single metal concentration-response curves for each microalga (Tables 4.1 and 3.1). Each of the 40,000 parameter sets was used to predict single-metal toxicity with the metal concentrations from the single-metal toxicity tests of *P. antarctica* (Gissi et al. 2015) and *C. armigera* (Chapter 3). For parameter set predictions, the sum of squared errors (SSE) was calculated and the set that gave the lowest sum of squared error (SSE) was chosen to then predict the metal-mixture toxicities. The model predictions for single-metal toxicity data (i.e. what was used to optimise the models) are given in Appendix A4.2 and A4.3 for *P. antarctica* and *C. armigera*, respectively. The best model parameters used to predict toxicity for each model and microalgae are given in Appendix A4.4. This process allows the reference mixture models to incorporate the uncertainty from the single-metal toxicity parameters, train the model by optimising predictions against measured toxicities, and thereby improve the confidence around predictions in unknown mixture treatments.

4.2.3 Determination of significant mixture interactivity

To determine if there were significant synergistic or antagonistic deviations from the reference models, the IA and CA models were extended to include a deviation parameter 'a'. These are termed IASA and CASA and described by Equations 4.8 and 4.9, respectively (Hochmuth et al. 2014). To test if the deviation is significant, a nested F-test was conducted to determine whether the extended model provides for a significantly better fit than the reference model (Hochmuth et al. 2014; Jonker et al. 2005).

$$y = 100\% \cdot \Phi \left(\Phi^{-1} \prod_{i=1}^n \left(\frac{1}{1 + \left(\frac{x_i}{EC10_i \cdot 9^{\frac{1}{\beta_i}}} \right)^{\beta_i}} \right) + \frac{a \cdot (\prod_{i=1}^n TU_i)}{(\sum_{i=1}^n TU_i)^2} \right) \quad (4.8)$$

$$\sum_{i=1}^n \frac{x_i}{\left(EC10_i \cdot 9^{\frac{1}{\beta_i}} \right) \cdot \left(\frac{100\% - y}{y} \right)^{\frac{1}{\beta_i}}} = \exp \left(\frac{a \cdot (\prod_{i=1}^n TU_i)}{(\sum_{i=1}^n TU_i)^2} \right) \quad (4.9)$$

An example of an R script for this approach with *C. armigera*, adapted from Hochmuth et al. (2014), can be found with all data in this study at the Australian Antarctica Data Centre (Jolley et al. 2018). The parameters used in the reference models (IA and CA) and their extensions (IASA and CASA) are given in Appendix A4.4.

4.3 Results and discussions

4.3.1 Equitoxic mixture

The equitoxic mixture tested 5 metals at their population growth inhibition EC10 concentrations, to *P. antarctica* and *C. armigera*, respectively. Nickel was not toxic to *P. antarctica* in single-metal exposures but was included in this mixture to observe any potential competition to biological binding sites. For some contaminants the reliability of higher effect measures (such as EC50s) may be confounded by the solubility limit of metals in marine waters. For example, at a concentration of $1,800 \mu\text{g Pb L}^{-1}$, which is approximately the lead solubility limit in seawater, the population growth rate of *C. armigera* was 75% of the control growth rate (Figure 3.1). In such cases, these contaminants may contribute to toxicity or interactivity when present in a mixture and using their EC10 value then allows them to be included in reference models. This has been demonstrated as necessary, as mixtures with concentrations \leq EC10s have demonstrated mixture interactivity, such as synergism and antagonism (Nys et al. 2017c; Versieren et al. 2016).

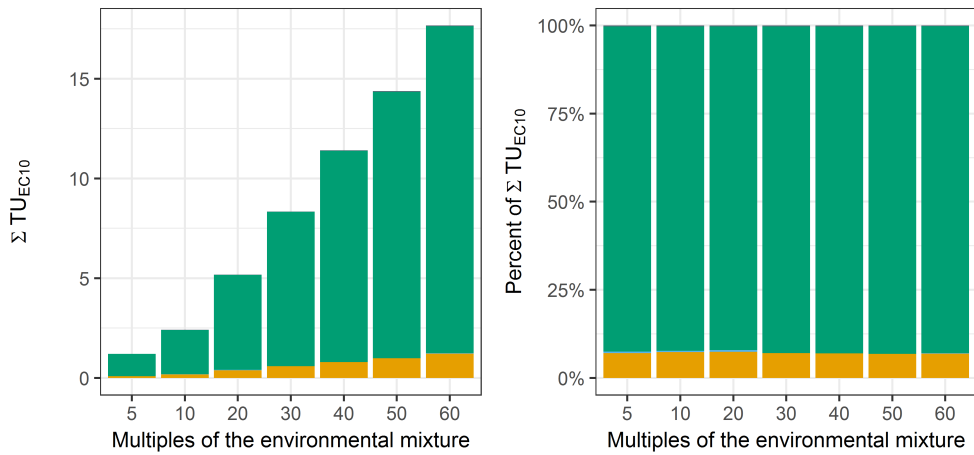
4.3.2 Environmental mixture

The environmental mixture was based on measurements from marine waters in the historically contaminated Brown Bay near Australia's Casey Station. This site had a metal ratio of 1 Cu : 0.1 Cd : 0.3 Ni : 0.3 Pb : 4.5 Zn (multiple of 1, in $\mu\text{g L}^{-1}$). While these concentrations are low, it is expected that concentrations will be much higher immediately adjacent to contaminant point sources or closer to the sediment-water interface (Amato et al. 2015; Birrer et al. 2018). For example, freshwater melt pools in the Thala Valley tip, which drains into Brown Bay, had concentrations of $443 \mu\text{g Cu L}^{-1}$, $1476 \mu\text{g Pb L}^{-1}$, and $3045 \mu\text{g Zn L}^{-1}$ prior to any clean-up or remediation efforts (Snape et al. 2001).

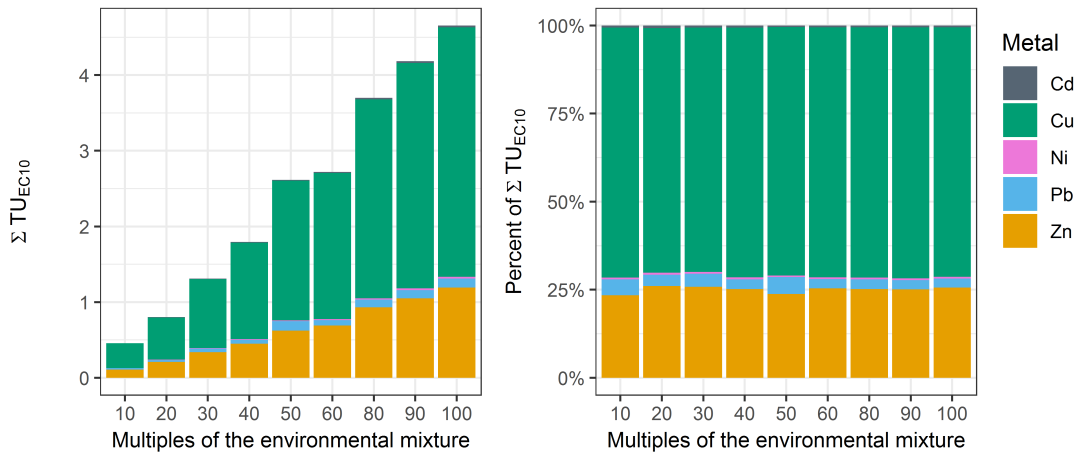
The environmental mixture has a high copper concentration relative to cadmium, nickel, lead, and zinc when compared against individual toxicity, i.e. copper is the most toxic metal to both microalgae by 1 to 2 orders of magnitude with EC10s of 2.8 and $22 \mu\text{g L}^{-1}$ to *P. antarctica* and *C. armigera*, respectively (Tables 3.1 and 4.1). Therefore, it was expected that copper would be the main contributor to observed toxicity. Zinc was present at the highest concentration of all the metals in the ratio. However, it generally had a low toxicity to both algae in single-metal exposures, with EC10s of 217 and $370 \mu\text{g L}^{-1}$ to *P. antarctica* and *C. armigera*, respectively (Tables 3.1 and 4.1). This is shown in Figure 4.1, where for both microalgae copper had the highest TU and represented $\geq 75\%$ of predicted toxicity, with zinc largely making up the balance.

Environmental mixture

Phaeocystis antarctica



Cryothecomonas armigera



Equitoxic mixture

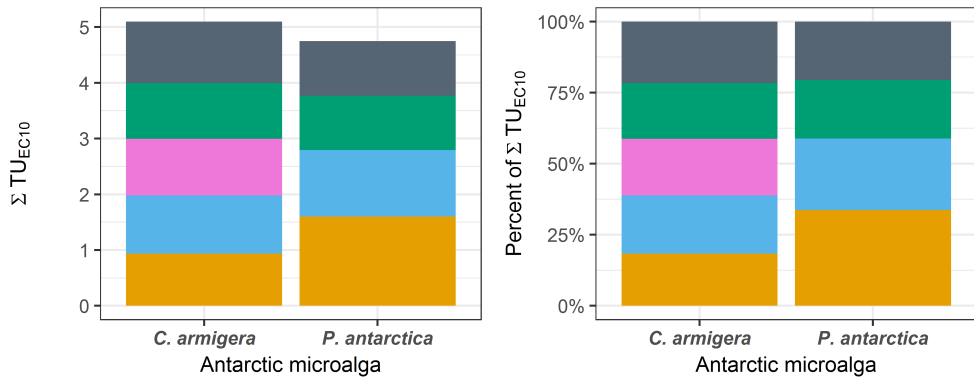


Figure 4.1: Toxic units (ΣTU_{EC10}) and proportional contribution to toxicity for the environmental and equitoxic mixtures for *Phaeocystis antarctica* and *Cryothecomonas armigera*. Toxic units were calculated by Equation 4.6 for the dissolve exposure concentrations of cadmium, copper, nickel, lead, and zinc in the environmental and equitoxic mixtures (top and bottom sets of graphs). Toxic units are expressed as an absolute number and their corresponding proportion (left and right charts, respectively).

4.3.3 Metal mixture toxicity to *P. antarctica*

Growth rate inhibition

The equitoxic mixture reduced population growth rate to $67 \pm 8\%$, relative to the control treatment (Figure 4.2, Table 4.2). Nickel was not toxic in single-metal exposures but was included in the equitoxic mixture at $420 \pm 70 \mu\text{gL}^{-1}$ to investigate any contribution to mixture interactivity. There were no significant differences in the population growth rate, cellular chlorophyll *a* fluorescence, or cellular size or complexity when compared to the equitoxic mixture treatments that excluded nickel (Appendix A4.5). Therefore, equitoxic mixture exposures with and without nickel were pooled for the analysis. The environmental mixture tested multiples of the ratio from 5 to 60 (Table 4.2). This mixture was toxic to population growth rate in a concentration-dependent manner, decreasing population growth rate to 10% at a multiple of 40.

Cellular chlorophyll *a* fluorescence

The equitoxic ratio resulted in an approximately equal proportion of the cell population with an increased and decreased fluorescence intensity; however, 81% of the cell population remained unaffected. The environmental mixture increased fluorescence intensity in a concentration-dependent manner (Figure 4.2 B). In single-metal exposures to *P. antarctica*, copper and nickel increased chlorophyll *a* fluorescence intensity, lead decreased fluorescence intensity, and cadmium and zinc caused greater variability (i.e. approximately equal increases and decreases).

The different fluorescence shifts suggest different modes of action of individual metals. However, in single-metal exposures these were generally low responses; <30% difference to the response in control populations at all concentrations tested. The increased variability observed in response to the equitoxic mixture could suggest non-interactive toxicity, but the relative contributions of each metal and underlying mechanism is unknown. The environmental mixture reflects a copper-only response with a greater magnitude of shift e.g. 70% population shift at a multiple of 20 with a copper concentration of $14 \mu\text{gL}^{-1}$ which was the chlorophyll *a* fluorescence EC50 in single-metal exposures (Table 4.1).

Cell size and complexity

The equitoxic mixture increased the size of cells in 17% of the cell population and decreased complexity in 11% of the population. The environmental mixture increased the variability of cell size at multiples ≥ 5 , which was coupled with a concentration-dependent increase in cell complexity in 100% of the cell population (Figure 4.2 C and D). In single metal exposures, the complexity and size of *P. antarctica* cells were affected by cadmium and copper, but not by nickel, lead, or zinc. Exposure to copper $\geq 6 \mu\text{gL}^{-1}$ increased the range of cell sizes observed and increased complexity in a maximum of 50% of the cell population (Table 4.1). The single-metal copper response was consistent with the response from the environmental mixture; however, the magnitude of response was much greater in the environmental mixture at comparable single copper exposure concentrations.

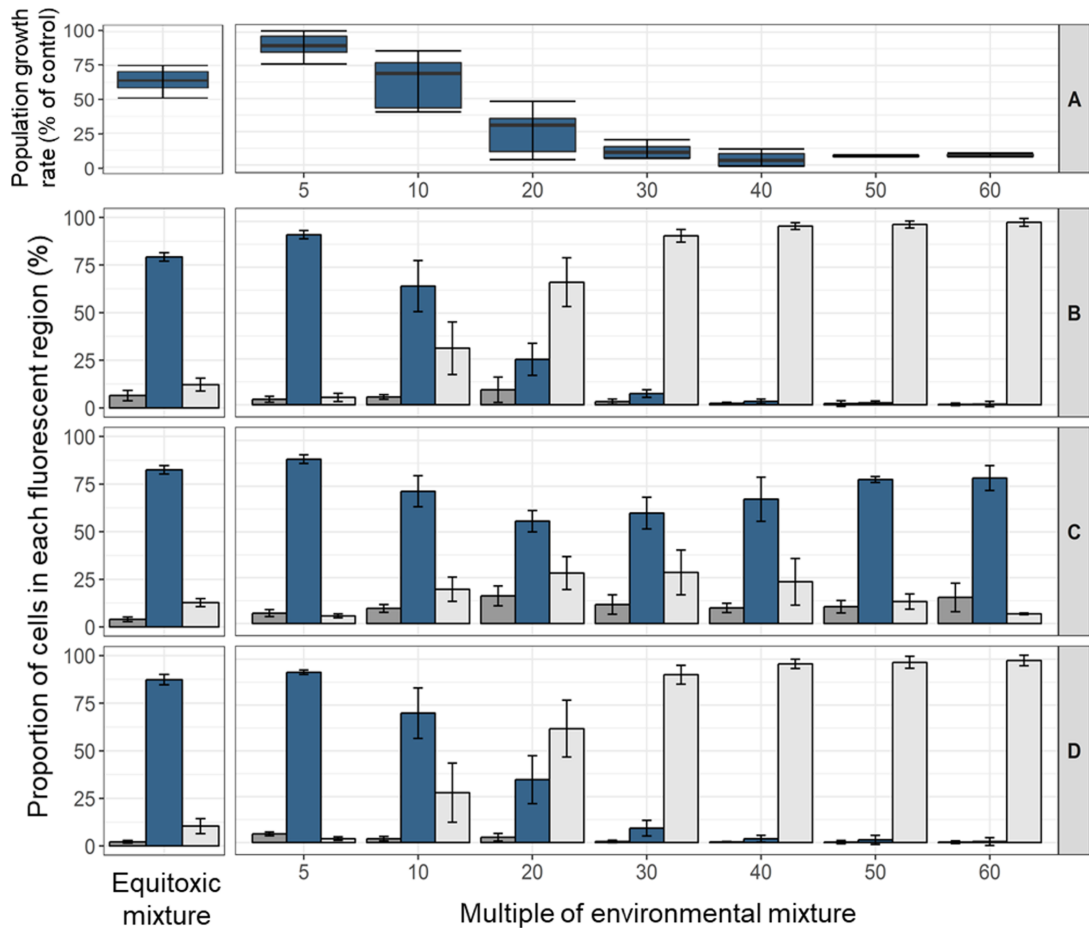


Figure 4.2: The toxicity of the equitoxic and environmentally relevant metal mixtures to the Antarctic microalgae *Phaeocystis antarctica*. Measures of toxicity are: (A) population growth rate inhibition, (B) cellular chlorophyll *a* fluorescence intensity, (C) cellular size, and (D) cellular complexity, all expressed as percent of control cell populations. Values represent the proportion of the cell population in each of three fluorescent regions: from R1 (dark grey), R2 (blue), to R3 (light grey) going left to right for each treatment as described in Section 2.2.3. Note that the R2 region was positioned to capture >95% of the healthy control cell population. Error bars represent the 95% confidence intervals. Boxplots indicate mean (central line), upper and lower quartiles (upper and lower shaded box, respectively).

Table 4.2: Metal mixture toxicity test exposure concentrations and test results for the Antarctic microalgae *Phaeocystis antarctica*. Measurements are means \pm standard deviations of: (a) metal concentrations of metal mixture treatments; (b) toxicity of metal mixtures; (c) independent action and concentration addition reference models toxicity predictions.

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

^a Represents the metal's population growth rate EC10 (95% confidence interval)

^b Numbers 5 to 60 indicate nominal multiples of the environmental mixture used as the concentration gradient in experiments with *P. antarctica*.

^c Note that the observed toxicity for relative chlorophyll *a* fluorescence, cellular size, and cellular complexity represents the percent similarity to the control cell population. It does not show the direction (increase or decrease in fluorescence intensity), which can be seen in Figure 4.2.

4.3.4 Metal mixture toxicity to *C. armigera*

Growth rate inhibition

The equitoxic mixture reduced population growth rate to $54 \pm 3\%$ compared to the control growth rate. For the environmental mixture, the population growth rate was unaffected at multiples below 30, despite having a $\sum TU_{EC10}$ above 1 (Figure 4.1). At multiples >30 , there was a concentration-dependent decrease of the growth rate to $4 \pm 5\%$ at a multiple of 100 (Table 4.3, Figure 4.3 A).

Cellular chlorophyll *a* fluorescence

The equitoxic mixture showed approximately equal proportions of the cell population with increased and decreased fluorescence intensity, with a combined $26 \pm 4\%$ of the population affected. The environmental mixture increased chlorophyll *a* fluorescence in up to 30% of the population until a multiple of 50. At multiples greater than this, 62% of the cell population had decreased fluorescence at a multiple of 100 (Table 4.3, Figure 4.3 B).

In single-metal exposures, cadmium, copper, nickel, and lead increased fluorescence intensity, while zinc caused a decrease. Based on single-metal responses, the concentrations present in both mixtures suggests only a small proportion of the cell population should be affected (see chlorophyll *a* fluorescence EC10 values, Table 3.1). The response to the equitoxic mixture agrees with this expectation. However, the toxicity of the environmental mixture was unexpected. At multiples up to 50, chlorophyll *a* fluorescence was increased, suggesting copper toxicity. Above this, fluorescence decreased, suggesting zinc toxicity. Furthermore, the population shift was greater than what could be explained solely by single-metal toxicity.

Cell size and complexity

The equitoxic mixture had a small effect on cell size and complexity, with 10% of the cell population affected (Table 4.3, Figure 4.3 C and D). In exposures to the environmental mixture, size increased in 20% of the population and complexity decreased in 30% of the population (Figure 4.3 C and D).

Single-metal treatments (except cadmium) affected the size of *C. armigera*, with nickel, lead, and zinc reducing size in a maximum of 10% of the population, while copper increased cell size in 70% of the population (Figure 3.2). However, only cadmium reduced cell complexity, with an EC10 of $269 \mu\text{gL}^{-1}$ (Table 3.1). The equitoxic mixture had concentrations of cadmium, copper, and nickel that were expected to reduce cell size, and a concentration of cadmium expected to reduce cell complexity based on single-metal exposures. However, this was not observed in the equitoxic mixture. Exposure to the environmental mixture increased cell size in a population roughly equivalent to the population with decreased complexity. These changes could be related as an increase in size without intracellular changes would lead to decreased intracellular complexity, as measured by flow cytometry. The mechanism behind the change in size is unknown but could be due to differences in osmotic pressure resulting from changes to membrane permeability.

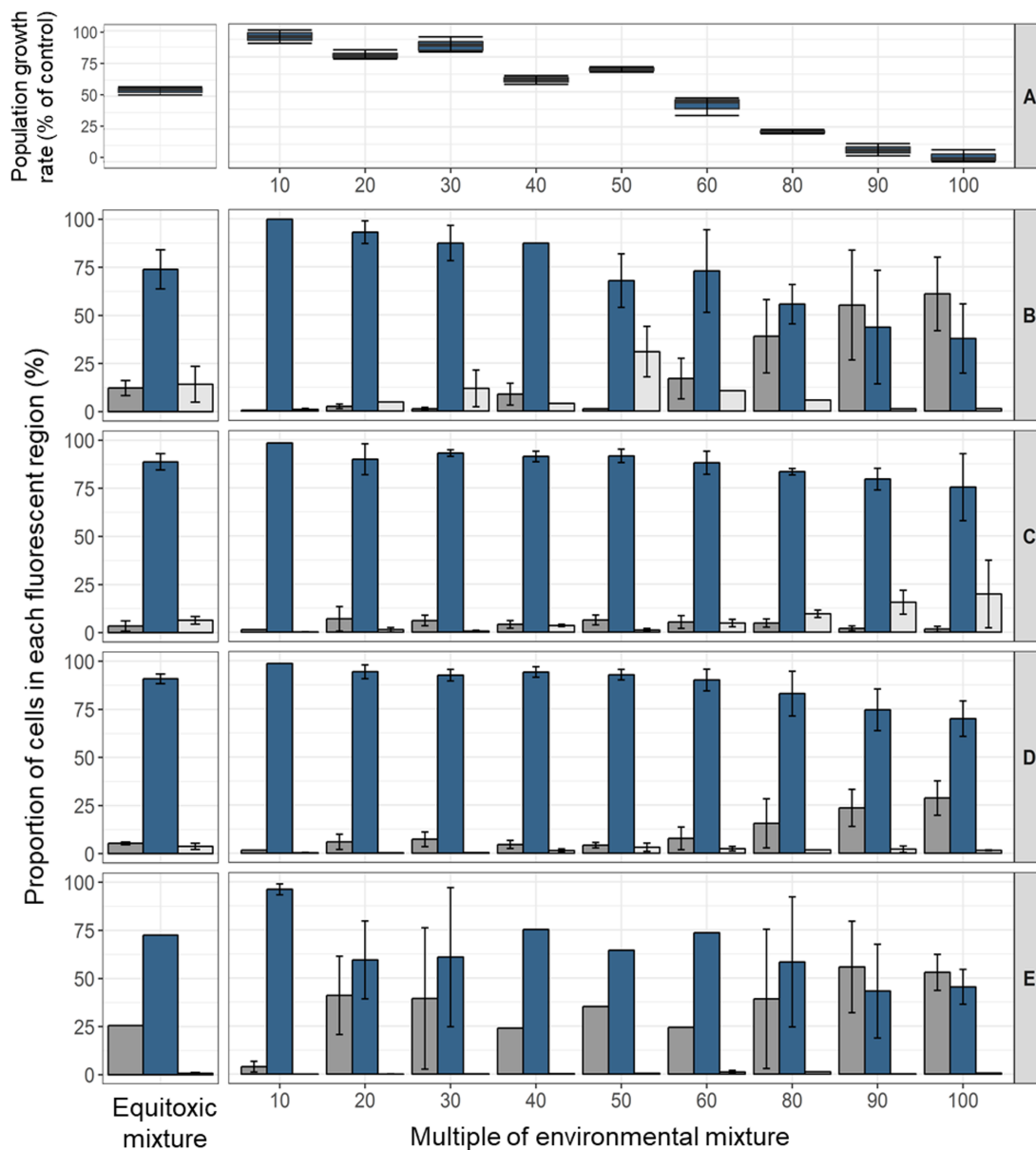


Figure 4.3: The toxicity of the equitoxic and environmental metal mixtures to the Antarctic microalgae *Cryothecomonas armigera*. Measures of toxicity are: (A) population growth rate inhibition, (B) cellular chlorophyll *a* fluorescence intensity, (C) cellular size, (D) cellular complexity, and (E) relative intracellular lipid concentrations, all expressed as percent of control cell populations. Values represent the proportion of the cell population in each of three fluorescent regions: from R1 (dark grey), R2 (blue), to R3 (light grey) going left to right for each treatment as described in Section 2.2.3. Note that the R2 region was positioned to capture >95% of the healthy control cell population. Error bars represent the 95% confidence intervals. Boxplots indicate mean (central line), upper and lower quartiles (upper and lower shaded box, respectively).

Table 4.3: Metal mixture toxicity exposures and results for the Antarctic microalgae *Cryothecomonas armigera*. Measurements are means \pm standard deviation. (a) Metal concentrations of metal mixture treatments; (b) Toxicity of metal mixtures; (c) independent action and concentration addition reference models toxicity predictions.

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

^a Represents the metal's population growth rate EC10 (95% confidence interval)

^b Numbers 5 to 60 indicate nominal multiples of the environmental mixture used as the concentration gradient in experiments with *P. antarctica*.

^c Note that the observed toxicity for relative chlorophyll *a* fluorescence, cellular size, and cellular complexity represents the percent similarity to the control cell population. It does not show the direction (increase or decrease in fluorescence intensity), which can be seen in Figure 4.3.

Intracellular lipid concentrations

The equitoxic mixture reduced the intracellular lipid concentrations in $27 \pm 11\%$ of the cell population compared to the control (Figure 4.3 E). The environmental mixture resulted in a greater reduction of lipid concentrations; however, this was not concentration dependent with 40–60% of the cell population having reduced lipids at multiples >10 .

In single-metal exposures, $\text{Pb} > \text{Cu} > \text{Cd}$ elicited the greatest effect on lipids, with EC_{10} s of 11, 33, and $89 \mu\text{g L}^{-1}$, respectively. Changes to lipid concentrations were a more sensitive measure of toxicity than changes in growth rate for lead and cadmium. The response to the equitoxic ratio was less than expected based on the concentrations of these metals. Curiously, there were no differences to lipid concentrations in exposures of equitoxic mixtures where cadmium was excluded or included at $50 \mu\text{g L}^{-1}$ or $500 \mu\text{g L}^{-1}$ (Appendix A4.6).

Based on the single-metal effects to cellular lipid concentrations, no effect was expected at treatments of the environmental mixture that had a multiple less than 50. However, there were significant reductions in the population of cells with a healthy lipid content at multiples ≥ 20 (Figure 4.3 E). This may be a result of energetically expensive cellular detoxification mechanisms depleting lipid reserves (Farese and Walther 2009; Lavoie et al. 2016).

4.3.5 Mixture interactivity

Equitoxic mixture

The equitoxic mixture had metals at concentrations that summed to $\sum \text{TU}_{\text{EC}_{10}}$ of 4.5 ± 0.8 for *P. antarctica* and 6.63 ± 0.04 for *C. armigera* (Figure 4.1), which resulted in population growth rates of $67 \pm 8\%$ for *P. antarctica* and $54 \pm 3\%$ for *C. armigera*, Figure 4.4. Both microalgae had similar responses to the equitoxic mixture; observed toxicity was predicted well by IA while CA overestimated toxicity, i.e. was antagonistic. The antagonism as measured by CA was significant for both algae, but the power of the Tukey's HSD test for *C. armigera* was low due to the low number of replicates, $n=3$. IA calculates mixture toxicity from individual contaminants under the assumption of independent modes of action, whereas CA assumes contaminants to have the same mode of action. The equitoxic mixture specifically investigated how metals may interact at concentrations where they are known to exert equivalent toxicity. The observation that IA better predicted toxicity than CA suggests that the metals have a dissimilar mode of action, rather than joint modes of toxicity (Berenbaum 1985).

Environmental mixture

Observed and predicted toxicities from the environmental mixture to the population growth rates of *P. antarctica* and *C. armigera* were concentration dependent and described well by a log-logistic model (Figure 4.5). Both models gave similar predictions to both algae; however, each alga had a different response. Antagonism to *P. antarctica* and concentration-dependent mixture interactivity to *C. armigera*, where there was antagonism at low and synergism at high effect concentrations (Figure 4.5 A and B, respectively).

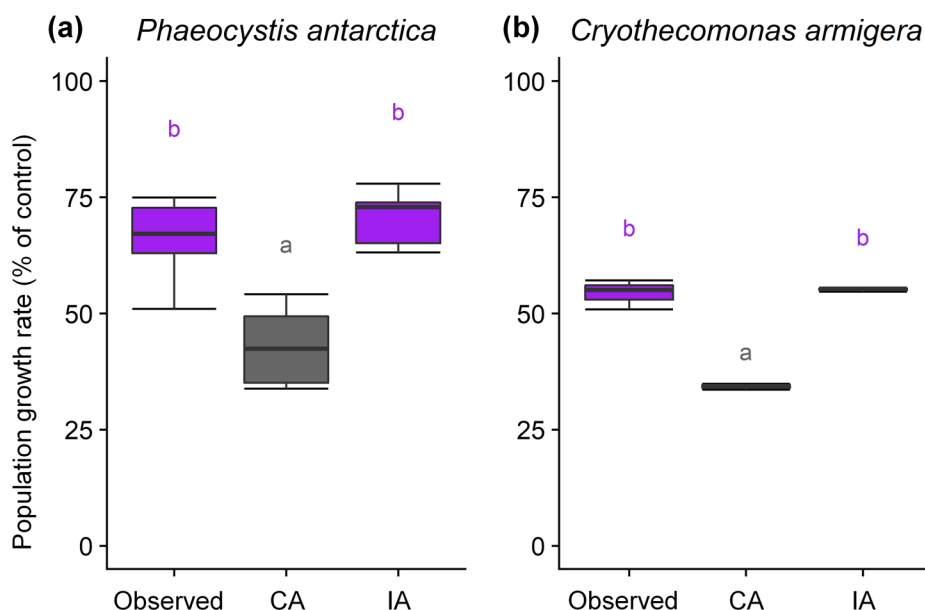


Figure 4.4: Toxicity of the equitoxic metal mixture to Antarctic microalgae: (a) *Phaeocystis antarctica*, and (b) *Cryothecomonas armigera*. Population growth rate presented as observed toxicity and predicted toxicity based on the concentration addition (CA) and independent action (IA) model. The observations or model prediction were compared to each other by Tukey's Honest Significant Difference. Different letters represent significant differences between the measurements for each microalga ($p < 0.05$), .

The only significant mixture interaction ($p < 0.05$) for the environment metal mixture ratio was antagonism measured by IA to *P. antarctica* ($p = 3.37 \times 10^{-5}$ Appendix A4.4). No other interaction was significant for either model or algae. Antagonism by CA to *P. antarctica* was not significant despite its predictions being very similar to IA.

The response to *C. armigera* was not significant, possibly because the mixture interactivity was concentration dependent. That is, significant mixture interactivity at high or low concentrations is masked when the response across the whole concentration range is assessed (Figure 4.5 B). In such cases, a full factorial experimental design (rather than individual ratios) or a different statistical approach assessing concentration-dependent mixture interactivity may be needed (Deruytter et al. 2017; Nys et al. 2015). While the synergism by IA and CA to *C. armigera* at high toxicities was not determined to be significant, similar responses are well documented with freshwater organisms (Cedergreen 2014; Nys et al. 2015, 2017c). However, this is not always the case, with nickel recently found to induce synergism at low concentrations and antagonism at high concentrations when in a binary mixture with copper (Deruytter et al. 2017).

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

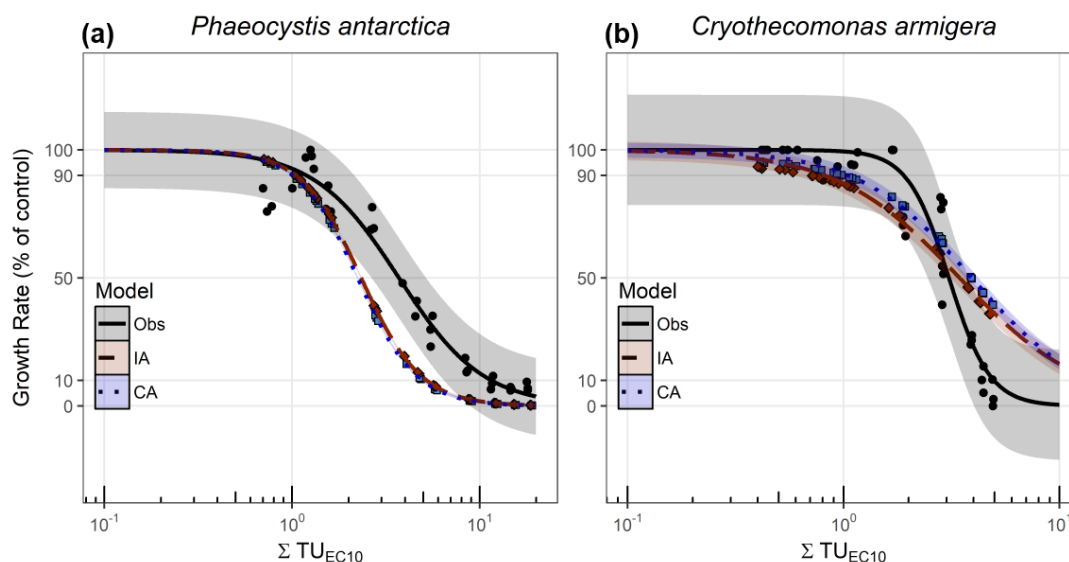


Figure 4.5: The observed and modelled toxicity of increasing multiples of the environmental mixture to the population growth rate of Antarctic microalgae (a) *Phaeocystis antarctica* and (b) *Cryothecomonas armigera*. Observed toxicity is represented by black circles, independent action (IA) predictions by red diamonds, and concentration addition (CA) by blue squares. A log-logistic model was fitted to observed toxicities (solid black line) and IA (red dashed line) and CA (dotted blue line) predictions. Ribbons represent the 95% confidence interval of the models.

Cellular biomarkers give clues to the mechanisms behind these results. The changes to chlorophyll *a* fluorescence in *C. armigera* were coincident with the mixture interactivity shift from antagonism to synergism in population growth rate. The fluorescence shifts suggest toxicity from copper at multiples <50 and zinc at multiples >50, thus zinc may be antagonistic at low concentrations but synergistic at high concentrations. However, changes to cell size and complexity suggest cadmium toxicity, possibly affecting membrane permeability. So, although copper is the most toxic and zinc the most abundant, the influence of cadmium, nickel, and lead cannot be discounted. Especially considering in the equitoxic mixture they were non-interactive by IA and antagonistic by CA.

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

A large proportion of *P. antarctica* had increased cellular complexity at low concentrations of the environmental mixture, compared to *C. armigera* (Figure 4.2 D compared to Figure 4.3 D). This could indicate detoxification of metals by intracellular sequestration in *P. antarctica*. Previous research has shown copper can be sequestered by polyphosphate bodies or phytochelatins to intracellular bodies, which would be observed as an increase to cell complexity (Adams et al. 2016; Levy et al. 2008). Such a response was not observed in *C. armigera*, suggesting different detoxification mechanisms (Figure 4.3 D).

Different chlorophyll *a* fluorescence changes in the microalgae suggest different metals are causing toxicity. *P. antarctica* had a greater proportion of cells affected than *C. armigera*, and the fluorescence shift was different. *P. antarctica* had a consistent increase, while *C. armigera* had an increase up to multiples of 50 followed by a decrease in fluorescence intensity. The increase in fluorescence intensity suggests *P. antarctica* and *C. armigera* had an impaired electron transport chain in the photosynthesis pathway (Guo and Tan 2015), possibly a result of copper toxicity. The decrease in fluorescence intensity in *C. armigera* suggests zinc toxicity, possibly impairing the light harvesting apparatus of chlorophyll moieties (Küpper and Andresen 2016). These changes in biomarkers indicate different physiological responses to metal mixtures and give insight into mechanisms behind these differences.

4.3.6 Modelling metal mixture toxicity for environmental management

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

Most metal-mixture studies only investigate binary metal mixtures. For example, in freshwater studies to green microalga zinc has shown to protect against cadmium to *Chlamydomonas reinhardtii* (Lavoie et al. 2016) and lead to *Micrasterias denticulate* (Volland et al. 2014). While cadmium has been shown to be synergistic in the presence of copper to *Chlorella sp.* (Franklin et al. 2002a). This study found that the equitoxic mixture was non-interactive by IA and antagonistic by CA and the environmental mixture elicited more complicated interactions which were microalgae dependent. More research is needed to investigate more metal mixtures, which may better represent environmental contamination.

Previous studies consistently find that CA tends to overestimate toxicity (Nys et al. 2018). This has been observed in metal mixture studies (Nagai and De Schamphelaere 2016; Nys et al. 2015, 2017a), studies with organic contaminants (Cedergreen et al. 2008), and possibly with metal and organic mixtures (Filimonova et al. 2018). As a result, CA is recommended as a conservative first-tier screening model for the environmental management of contaminant mixtures (Cedergreen 2014; Nys et al. 2018). This trend was observed in the equitoxic mixture, but not in the environmental mixture, where both models gave largely equivalent predictions to the environmental mixture (see SSEs given in Appendix A4.4). At low concentrations ($<2 \sum TU_{EC10}$), the environmental mixture has a more realistic ratio and metal concentrations than the equitoxic mixture. Thus, either IA or CA should be suitable to predict toxicity to *P. antarctica* or *C. armigera* in environmentally realistic conditions.

4.4 Conclusion

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

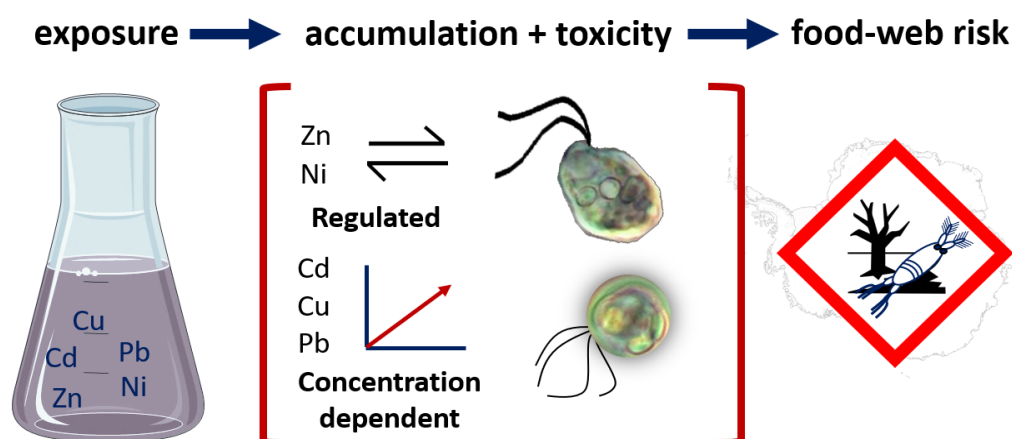
The equitoxic mixture was non-interactive by IA and antagonistic by CA to both *P. antarctica* and *C. armigera*. The environmental mixture was antagonistic by IA and CA to *P. antarctica*, with IA being a significant interaction. To *C. armigera*, the environmental mixture was concentration-dependent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

Frameworks designed to manage the environmental risk of metal mixtures typically recommend a ‘first-tier’ screening using a CA model (Backhaus and Faust 2012; Farley et al. 2015). This study showed both models are suitable at environmentally realistic ratios and concentrations of metal mixtures, where there were equivalent predictions from both models to both microalgae.

The mechanism behind the observed mixture interactivity was not determined in this chapter. Antagonism could be a result of metal competition for cellular binding sites, while synergism could be related to a breakdown of cellular metal regulation or detoxification processes. These could be investigated by understanding the cellular accumulation and partitioning of metals from metal mixture exposures. This would also inform the potential dietary risk of metal-laden microalgae to the Antarctic nearshore marine food web.

CHAPTER 5

Cellular metal accumulation in *Phaeocystis antarctica* and *Cryptothecomonas armigera* exposed to metals



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This chapter is under review for publication in *Environmental Pollution*

Koppel, D. J., Adams, M. S., King, C.K., Jolley, D.F. Preliminary study of cellular metal accumulation in two Antarctic marine microalgae – implications for mixture interactivity and dietary risk.

Author Contributions: DJK designed and ran the experiments, analysed the data, prepared all figures and wrote the manuscript. All authors contributed to the experimental design and editing of the manuscript before submission.

This is a make it work moment.
Tim Gunn

5.1 Introduction

The Antarctic microalgae *P. antarctica* and *C. armigera* have been shown to exhibit antagonistic effects from metal mixtures at an environmentally realistic ratio of five metals at low effect concentrations (Chapter 4, Figure 4.5), but the mechanisms behind these interactions are unexplained. The toxicity of metals is dependent on their ability to interact with cellular processes of an organism. This may be observed as changes to metal accumulation and fractionation (Franklin et al. 2002a; Nugroho et al. 2017; Saibu et al. 2018). Understanding the extra- and intra-cellular metal partitioning of contaminants gives insight to their cellular regulation (Duval et al. 2015), toxicity and detoxification (Lavoie et al. 2014; Perales-Vela et al. 2005; Pochodylo and Aristilde 2017; Zeng et al. 2009) and the dietary risk they pose within the food web (Luoma and Rainbow 2005).

Microalgae 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 (possibly including *C. armigera*) as juveniles (Kohlbach et al. 2017) and *P. antarctica* as adults (Haberman et al. 2003). Dietary exposure to metals is a significant source of contamination for many secondary and tertiary consumers (DeForest and Meyer 2015; Luoma and Rainbow 2005). However, the capacity of Antarctic microalgae to accumulate metals from sub-lethal exposures is not well studied. Nor is the risk they pose to the nearshore marine food web by dietary exposure.

This chapter investigated the cellular accumulation and partitioning of five 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 intra-cellular partitioning of metals in the context of understanding how cellular metal concentrations relate to previously reported toxicity endpoints for these microalgae. Based on the accumulated metals, the dietary risk posed to secondary consumers in the Antarctic food web is assessed. All metal concentrations for this chapter are presented in moles to facilitate direct comparison of metal ions accumulated by the microalgae.

5.2 Methods

5.2.1 Cellular accumulation test protocol

Microalgae were cultured and tested using the standard test protocol described in Section 2.2. The metal mixtures tested were based on an equitoxic (metals at their population growth rate EC10) and environmental ratio (a ratio of 8 Cu : 2 Cd : 5 Ni : 1 Pb : 69 Zn, multiple of 1, in nmol L⁻¹), as described in Section 4.3.2. These ratios were prepared at different multiples. The equitoxic mixture was exposed to *P. antarctica* at 0.5, 1, and 2 times the ratio and to *C. armigera* at 1 times the ratio. The environmental mixture was exposed to *P. antarctica* at multiples of 5, 10, and 20 and *C. armigera* at 10, 20, 30, 40, 60, and 80.

5.2.2 Determination of extra- and intra-cellular metal concentrations

Extra- and intra-cellular metal concentrations were determined at the end of the toxicity test (Table 2.1) using methods modified from Levy et al. (2008). Cellular metal fractions are operationally defined as: (i) dissolved, the mean of the start and end 0.45 µm-filterable concentration in the exposure; (ii) extracellular, the fraction of metal bound to the cell liberated by an ethylenediaminetetraacetic acid (EDTA) solution, and (iii) intracellular, the remaining metal accumulated in the cell following acid digestion.

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 test end 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 cells were retained and the rinse was discarded. The filter paper with algal cells was transferred into Teflon tubes with 15 mL of 0.01 M EDTA for 20 minutes, and stored on ice. The filter paper with algae cells was then transferred to a new glass filtration unit, which was used to filter the EDTA rinse solution to recapture any cells lost. The EDTA rinse solution was retained for metal analysis as the extracellular metal fraction. The rest of the EDTA solution was discarded and 30 mL of cold clean seawater was 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% HNO₃ (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 (Appendix A5.1). The average blank EDTA metal concentration was subtracted from the measured concentrations in the EDTA solutions from metal treatments. Process blanks ($n=3$) were analysed in each experiment using diluent seawater instead of an exposure solution. The measured intracellular concentrations in process blanks were subtracted from the intracellular concentrations from treatments. The efficacy of the acid digest was confirmed by analysis of certified reference material (DOLT-3, NRC Ottawa, Canada), which had recoveries of 93% Cd, 98% Cu, 96% Ni, and 105% Zn, with Pb below detection limit.

5.2.3 Metal analysis

Subsamples of the exposure solutions (dissolved fraction), EDTA rinse solution (extracellular fraction), or acid-digested cells (intracellular fraction) were analysed by ICP-AES or ICP-MS depending on the limits of detection required, 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.

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% HNO₃ 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.

A total metal recovery budget was attempted but not possible due to instrument constraints and experimental design. Specifically, the high concentrations of the exposure concentrations meant that the standard variability of ICP-AES measurements contributed too much uncertainty to detect minor changes resulting from uptake from low algal cell numbers. A metal budget approach would be possible were we using much higher cell numbers which would cause a greater depletion of metals in solution; however, this would be at the expense of environmental relevance in the bioassay.

5.2.4 Calculations and statistical analysis

Extra- and intra-cellular metal concentrations on a per cell basis were calculated using the number of cells filtered from each treatment and the metal content of the wash solution or acid digest, respectively (all given in the accompanying dataset by Koppel et al. (2018)). 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, as outlined in Chapter 2.2.3. 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 intra-cellular metal measurements was observed in 8 of 189 exposures. One experiment with *P. antarctica* had cadmium contamination in two of the three control replicates in the extra- and intra-cellular 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. Control replicates that were contaminated were removed from the dataset. All data, including contaminated replicates are presented in the dataset provided by Koppel et al. (2018).

Extracellular concentrations were normalised to cell surface area and intracellular concentrations were normalised to the volume of each microalgae. For these calculations, microalgae were measured under a phase-contrast microscope: *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 µm² and 523 µm³ for *P. antarctica* and 1484 µm² and 4691 µm³ for *C. armigera*, respectively.

Multiple linear regression was used to investigate how cellular metal fractions contribute to observed population growth rate inhibition. This was conducted in R using the base stats package (R Core Team 2016) and interpreted based on the advice given by Morrissey and Ruxton (2018). All metals of a cellular fraction were initially included. The contribution of each metal to the multiple linear regression fit was assessed by a step-wise optimisation of Akaike information criterion (AIC), and those not improving fit were removed from the analysis. Simple correlations of cellular concentrations and population growth rate were determined by Spearman's correlation. The fit of simple correlations are reported by an R^2 value adjusted for the number of model parameters (herein referred as R^2). The population growth rates and exposure concentrations are the same as those in Chapter 4, Tables 4.2 and 4.3.

5.3 Results

5.3.1 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 $2.1 \pm 1.1 \times 10^{-16}$ mol cell⁻¹ (mean \pm standard deviation of the sum of five cellular metal concentrations), respectively (Figure 5.1). Both microalgae accumulated greater total-cellular concentrations in the metal mixture treatments compared to controls (Figure 5.1). The total-, extra-cellular, and intra-cellular concentrations for *P. antarctica* and *C. armigera* from select single and mixture exposures are given in Tables 5.1 and 5.2, respectively and in Appendix A5.2.

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 5.1). 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 8.4×10^{-16} mol cell⁻¹, approximately 30% more than the sum of metal accumulation from individual exposures (6.5×10^{-16} mol cell⁻¹). Zinc had the highest total cellular concentration, followed by lead, copper, nickel, and cadmium. The difference between total cellular concentrations from single EC10 exposures and the equitoxic mixture is explained by an increase in extracellularly bound metals of 2.2×10^{-16} mol cell⁻¹, the majority of which is zinc, which was offset by a reduction in intracellular zinc (Appendix A5.2).

Cryothecomonas armigera

Single metal exposure at each metal's EC10 concentration resulted in 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 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 $85 \times 10^{-16} \text{ mol cell}^{-1}$. This is approximately a 20% reduction in accumulated metal when compared to the sum of metals from single-metal EC10 exposures ($104 \times 10^{-16} \text{ mol cell}^{-1}$). This difference is mostly due to a $23 \times 10^{-16} \text{ mol cell}^{-1}$ reduction in extracellularly bound metals, of which the majority was a decrease in zinc of $36 \times 10^{-16} \text{ mol cell}^{-1}$ with increases in other metals offsetting the difference (Table 5.2, Appendix A5.2).

Algal comparison

Greater metal concentrations were accumulated by *C. armigera* than *P. antarctica* on a moles per cell basis (Figure 5.1). However, when extra- and intra-cellular concentrations were normalised to cell surface area and volume, respectively, their concentrations were similar (Figures 5.2 and 5.3). Extracellular concentrations ranged from $10^{-20} - 10^{-17} \text{ mol } \mu\text{m}^{-2}$ and were approximately 10x higher than intracellular concentrations, which ranged from 10^{-21} to $10^{-18} \text{ mol } \mu\text{m}^{-3}$.

Two treatments allow for direct comparison of microalgal metal adsorption and uptake, the environmental mixture at a ratio of 10 (i.e. the treatments for each microalgae had equal exposure concentrations) and the equitoxic mixture (i.e. both treatments had equal theoretical toxicity): (i) the environmental mixture at a multiple of 10 led to similar extra and intracellular concentrations for both algae (Table 5.1 and 5.2), but *C. armigera* had a higher molar total of $18 \times 10^{-16} \text{ mol cell}^{-1}$ compared to *P. antarctica* at $7.3 \times 10^{-16} \text{ mol cell}^{-1}$. When normalised to cell surface area and volume, the *P. antarctica* had significantly higher extracellular cadmium and lead, and intracellular nickel concentrations. No other extra- or intra-cellular metal fraction had significant differences; (ii) the equitoxic mixture resulted in population growth rates of $64 \pm 8\%$ and $76 \pm 17\%$ for *P. antarctica* and *C. armigera*, respectively, which were not significantly different. When extra and intracellular metal fractions were normalised to surface area and volume, respectively, *C. armigera* had significantly greater extracellular nickel and zinc while *P. antarctica* had significantly greater extracellular lead concentration. Of the intracellular fraction, *P. antarctica* had greater intracellular copper and zinc concentrations.

For *C. armigera*, cadmium and lead were the only metals to have clear differences in cellular concentrations as a result of the different mixtures (see metal concentrations in the panels of Figure 5.3). For *P. antarctica*, exposure to either mixture resulted in a similar range of cellular metal concentrations (Figure 5.2), despite the different dissolved metal concentrations with the equitoxic mixture having a much higher molar total of metals than the environmental mixture.

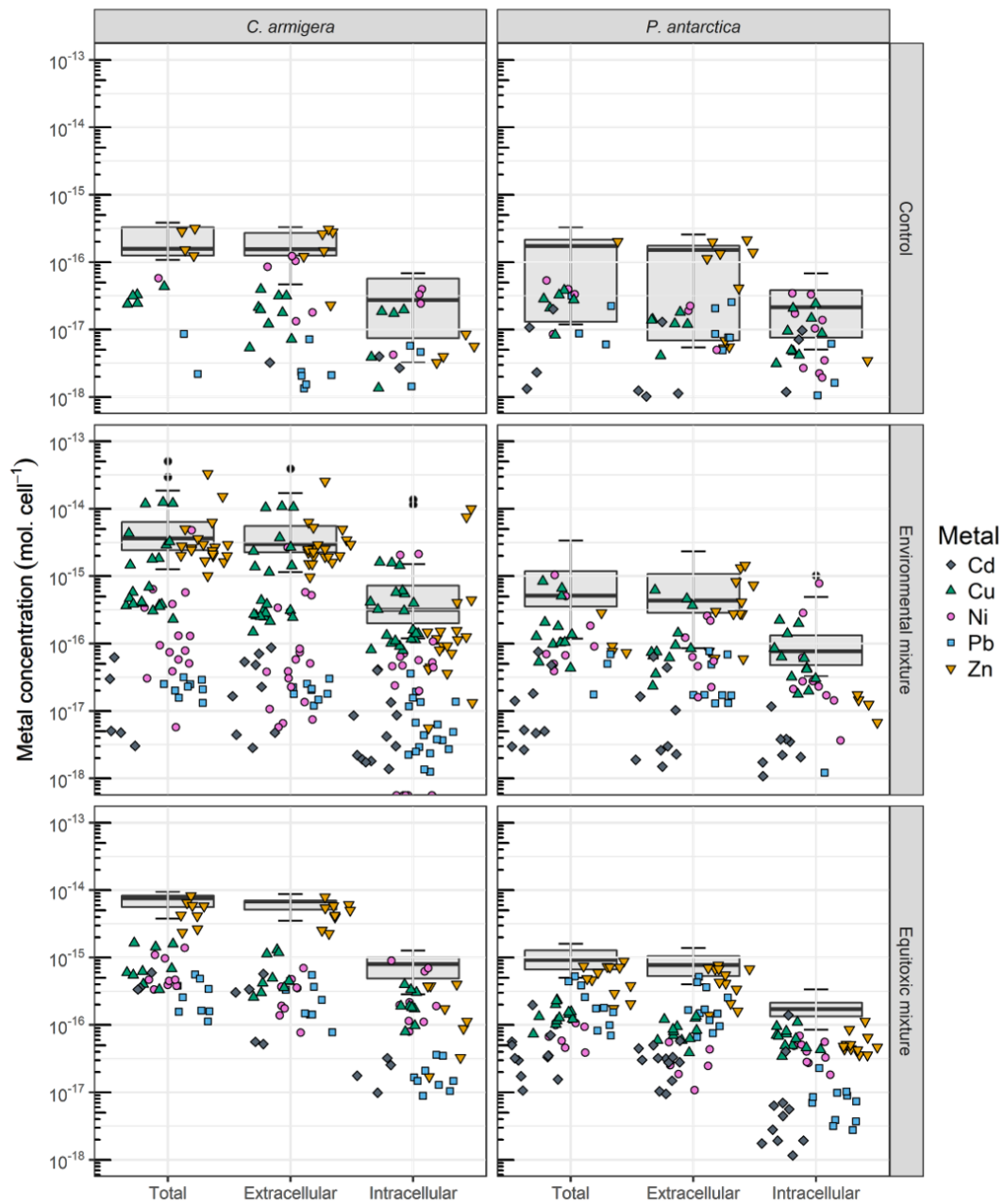


Figure 5.1: Total- extra-, and intra-cellular metal concentrations for *Phaeocystis antarctica* and *Cryptothecomonas armigera* exposed to control, environmental and equitoxic mixture treatments. Boxplots are calculated as the sum of the five metals measured, shown in the legend. Filled black circles 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 Appendix A5.2. Position of the individual metal points on the x-axis within cellular fraction group is arbitrary.

Phaeocystis antarctica

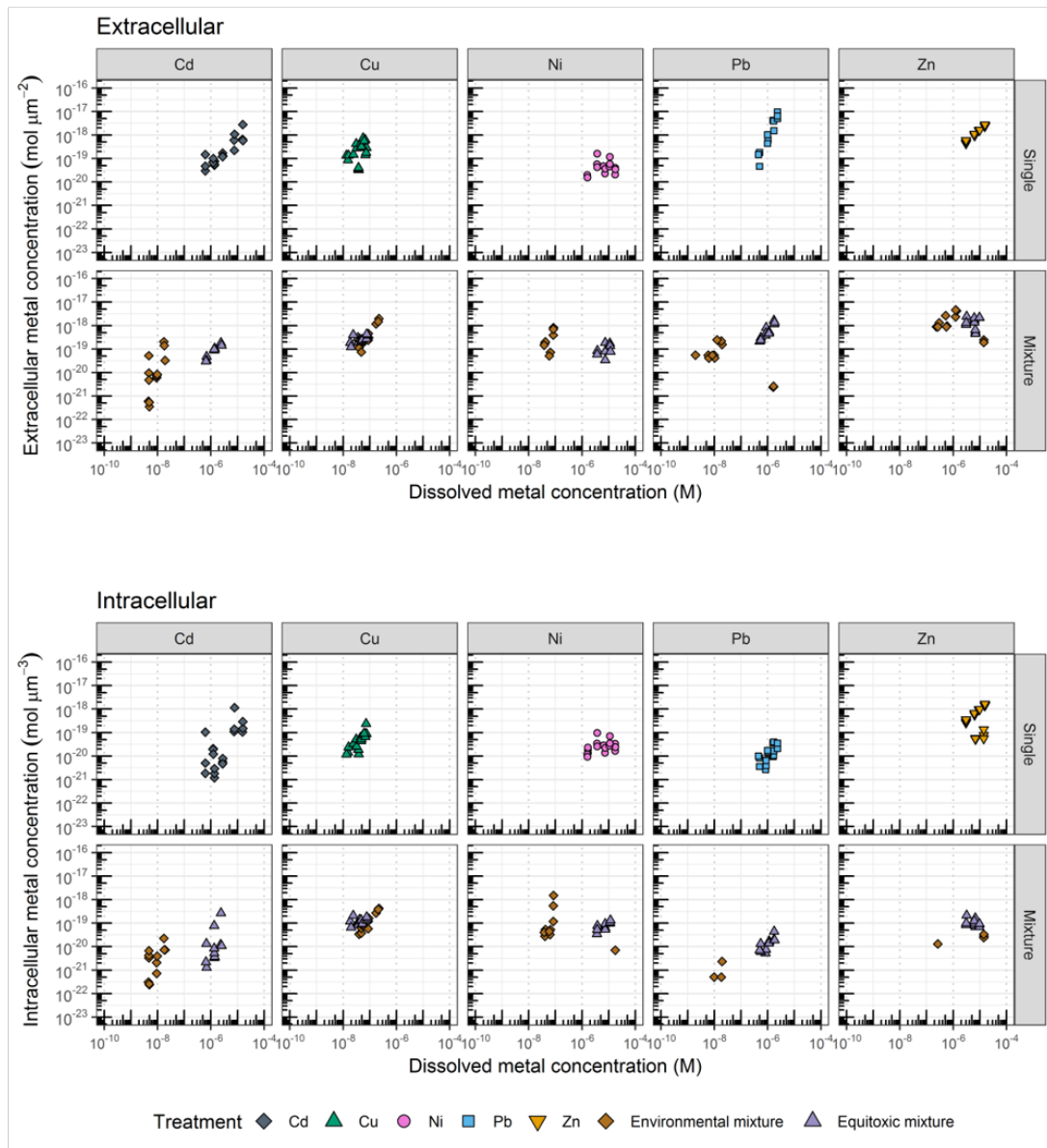


Figure 5.2: The relationship between intra- and extra-cellular metal accumulation and dissolved exposure concentrations for *Phaeocystis antarctica* in single and metal mixture exposures. Extra- and intra-cellular concentrations have been normalised to cell surface area and volume.

Cryothecomonas armigera

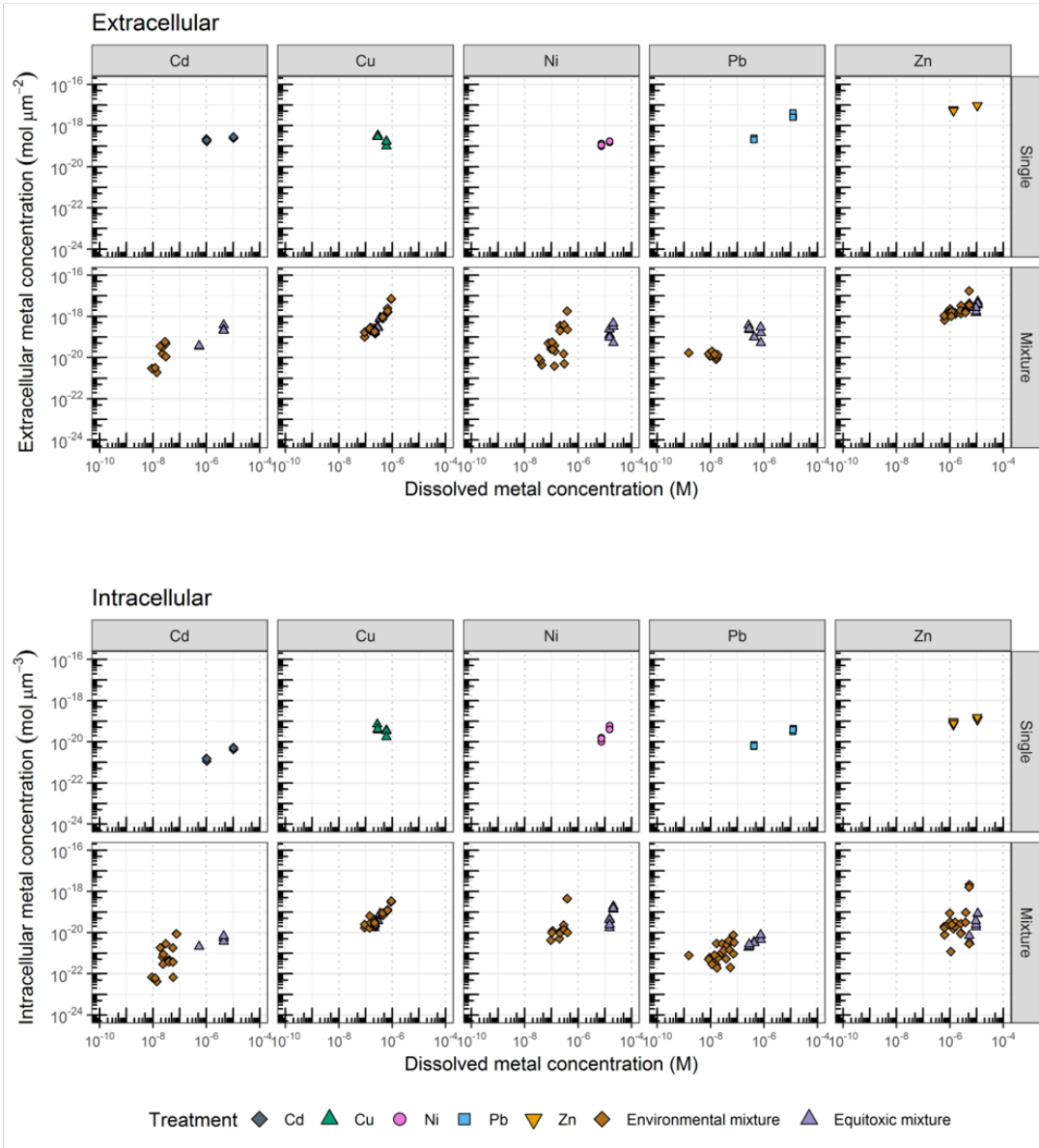


Figure 5.3: The relationship between intra- and extra-cellular metal accumulation and dissolved exposure concentrations for *Cryothecomonas armigera* in single and metal mixture exposures. Extra- and intra-cellular concentrations have been normalised to cell surface area and volume.

Table 5.1: *Phaeocystis antarctica* 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 \pm standard deviation in amol cell^{-1} ($10^{-18} \text{ mol cell}^{-1}$ total -, extra-, and intra-cellular 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 by Gissi et al. (2015).

<i>P. antarctica</i>		Cadmium	Copper	Nickel	Lead	Zinc
	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
	Exposure (μM)	1.29 \pm 0.07	0.042 \pm 0.005	17.4 \pm 0.1	0.93 \pm 0.08	3.030 \pm 0.004
Single (EC10)	Extracellular	22 \pm 5	60 \pm 60	10 \pm 3	200 \pm 100	160 \pm 30
	Intracellular	5 \pm 5	20 \pm 10	13 \pm 5	5 \pm 3	160 \pm 30
	Total	27 \pm 8	80 \pm 70	23 \pm 1	200 \pm 100	320 \pm 50
	Exposure (μM)	1.35 \pm 0.02	0.044 \pm 0.007	4 \pm 3	1.0 \pm 0.1	6.5 \pm 0.3
Equitoxic Mixture	Extracellular	31 \pm 2	75 \pm 13	32 \pm 25	170 \pm 50	360 \pm 220
	Intracellular	9 \pm 16	55 \pm 13	37 \pm 11	6 \pm 3	54 \pm 18
	Total	39 \pm 15	130 \pm 20	69 \pm 36	180 \pm 50	420 \pm 230
	Exposure (μM)	0.0094 \pm 0.0003	0.088 \pm 0.002	0.062 \pm 0.003	0.009 \pm 0.0001	0.55 \pm 0.03
Environmental mixture x10	Extracellular	2.3 \pm 0.4	100 \pm 36	19 \pm 5	16 \pm 2	500 \pm 300
	Intracellular	1.2 \pm 0.9	59 \pm 28	23 \pm 5	0.3	–
	Total	3.4 \pm 1.1	160 \pm 53	45 \pm 8	18	500 \pm 300

Table 5.2: *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 \pm standard deviation in amol cell⁻¹ (10⁻¹⁸ mol cell⁻¹ total-, extra-, and intra-cellular 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 in Chapter 3.

<i>C. armigera</i>		Cadmium	Copper	Nickel	Lead	Zinc
	EC10 (95% CI)	4.0 (2.0 - 6.1) μ M	0.35 (0.28 - 0.41) μ M	21 (19 - 23) μ M	0.73 (0.38 - 1.4) μ M	5.6 (0.63 - 11) μ M
	Exposure (μ M)	1.01 \pm 0.02	0.28 \pm 0.01	14.91 \pm 0.04	0.415 \pm 0.004	1.36 \pm 0.02
Single (EC10)	Extracellular	290 \pm 30	450 \pm 40	240 \pm 20	330 \pm 30	8300 \pm 700
	Intracellular	7 \pm 1	230 \pm 90	230 \pm 50	31 \pm 3	380 \pm 70
	Total	300 \pm 40	690 \pm 90	470 \pm 40	360 \pm 40	8600 \pm 800
	Exposure (μ M)	4.42 \pm 0.01	0.35 \pm 0.01	20.7 \pm 0.1	0.75 \pm 0.01	5.3 \pm 0.1
Equitoxic Mixture	Extracellular	400 \pm 200	1200 \pm 100	400 \pm 300	300 \pm 200	5000 \pm 800
	Intracellular	25 \pm 7	350 \pm 50	700 \pm 100	31 \pm 8	30 \pm 10
	Total	400 \pm 200	1600 \pm 100	1200 \pm 200	300 \pm 200	5000 \pm 1000
	Exposure (μ M)	0.012 \pm 0.002	0.093 \pm 0.001	0.038 \pm 0.005	0.012 \pm 0.003	0.62 \pm 0.01
Environmental mixture x10	Extracellular	4 \pm 1	220 \pm 60	10 \pm 4	16 \pm 3	1400 \pm 300
	Intracellular	0.3 \pm 0.1	100 \pm 20	–	2.1 \pm 0.8	70 \pm 30
	Total	4 \pm 1	320 \pm 80	–	18 \pm 4	1400 \pm 400

5.3.2 Drivers of mixture toxicity

Population growth rate

In single-metal exposures, cadmium and copper cellular metal fractions were strongly correlated, and lead weakly correlated, to population growth rate in *P. antarctica* (Figure 5.4 a). Following exposure to the environmental mixture, all fractions of all metals except zinc strongly correlated to *P. antarctica*. The intracellular zinc fraction had a positive while the extracellular fraction had a negative correlation to growth rate (Figure 5.4 b). The multiple linear regression incorporated all metals of a cellular fraction (i.e. total-, extra-, or intra-cellular) to examine metal interactions. Only the lead extracellular fraction was a 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 to population growth rate (Figure 5.4 c). This suggests that copper is driving toxicity proportionally to its intracellular concentration while less toxicity is observed with increasing intracellular zinc concentrations. In the environmental mixture, cellular copper fractions of *C. armigera* had the strongest correlations to population growth rate. In the multiple linear regression model all cellular copper fractions correlated to growth rate with high levels of significance (Figure 5.4 c). The partial regression of the intracellular lead fraction was negative while the extracellular lead fraction and intracellular zinc fractions were positive.

5.4 Discussion

5.4.1 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 total-, extra-, and intra-cellular concentrations than *P. antarctica* (sum of all metals as moles per cell, Tables 5.1 and 5.2, Figure 5.1). This is consistent with the theory that cellular metal binding capacity is proportional to cell size, as greater surface areas are expected to have greater concentrations of biotic ligands (Paquin et al. 2002). However, in this study, some metals had roughly equivalent cellular concentrations between the microalgae at similar exposure concentrations. 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 5.2). This is important to consider as secondary consuming organisms will have different sensitivities to metals from dietary exposure (Hook and Fisher 2002).

5.4.2 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, shown in Figure 5.4 b, where nearly all metals are negatively correlated to the growth rate of *P. antarctica* despite only copper and possibly zinc being at a high enough concentration to cause toxicity (Gissi et al. 2015). Furthermore, the correlation coefficients of metals in the environmental mixture share little similarity with the coefficients from single-metal exposures (Figure 5.4 a). Therefore, the negative correlations of cadmium, nickel, and lead could be explained by them accumulating within each cellular fraction proportionally to copper, which was likely to be driving the toxic effects. What is interesting, however, is that zinc does not follow this trend, suggesting a different uptake, regulation, or detoxification mechanism in this species.

Correlations between cellular metal fractions and population growth rates in *C. armigera* showed different patterns compared to *P. antarctica* (Figure 5.4 b). Cadmium and lead had positive correlations to population growth rate in their total- and extra-cellular fractions, but not in their intracellular fractions. This could suggest the uptake of these metals was restricted or they were excreted from their intracellular fraction, possibly by exocytosis of the phytochelatin or polyphosphate bound metals. Copper was the only metal with consistently strong negative correlations to population growth rate 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 Figure 5.4 b. Metal fractions that did not independently provide a significant improvement to the regression were removed as a parameter (Figure 5.4 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 for *P. antarctica*. The absence of a significant association between extracellular metal concentrations and growth rate most likely suggests that copper internalisation was rapid and not limited by the down-regulation of uptake pathways. In contrast, for *C. armigera*, the total-, extra-, and intra-cellular copper concentrations showed significant negative partial regressions to growth rate. This implies that copper is accumulating on and in the cell proportionally to observed toxicity. The intracellular sequestration of copper is a known detoxification strategy in marine microalgae (Adams et al. 2016; Levy et al. 2008; Smith 2014), and appears to be enhanced in *C. armigera*, as seen by the increase in intracellular copper concentrations in exposures to the equitoxic mixture compared to a single exposure of copper at similar concentrations (Table 5.2). But this is too small a sample size to determine conclusively.

Phaeocystis antarctica

Cryothecomonas armigera

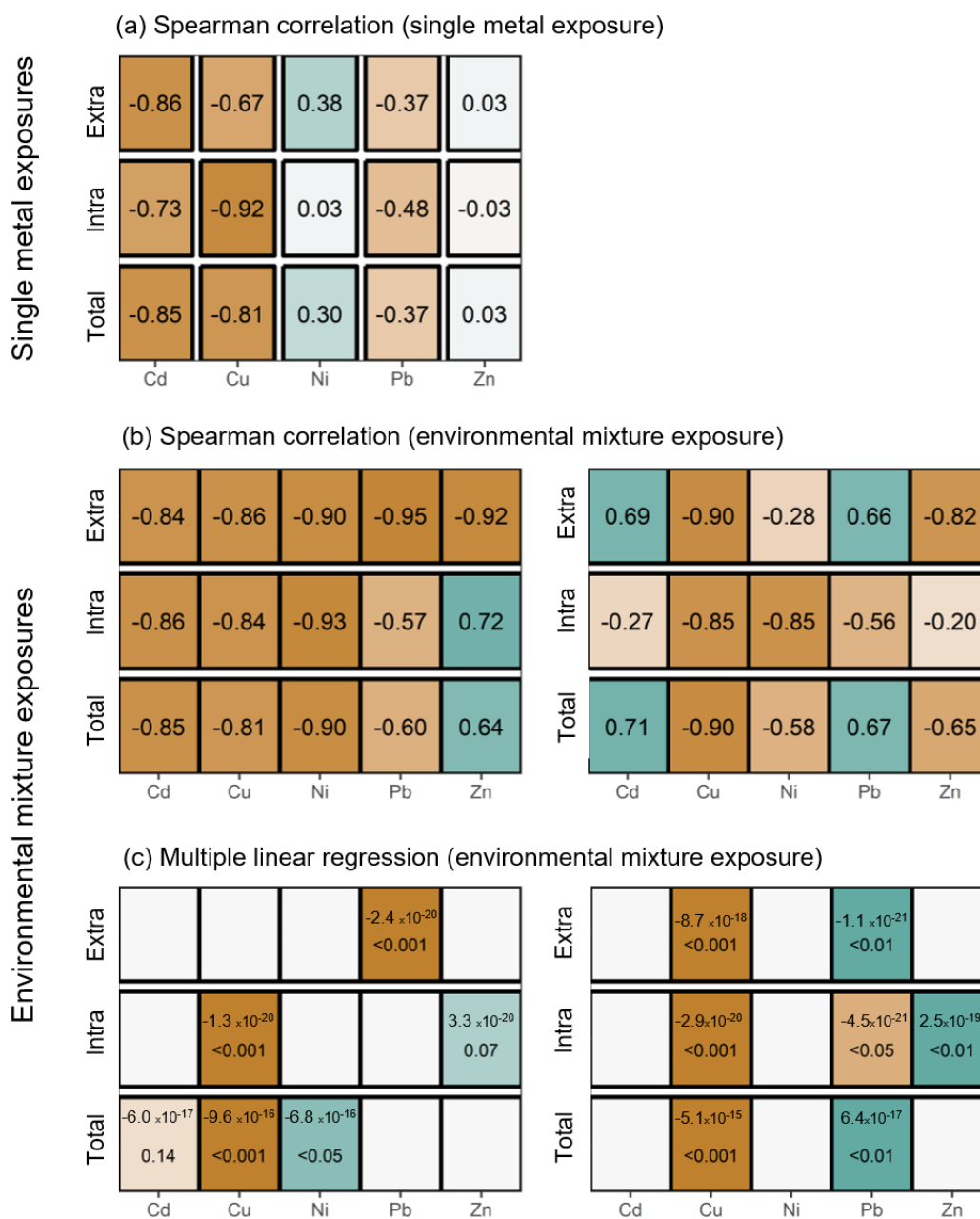


Figure 5.4: Correlation matrix of total-, extra-, and intra-cellular metal fractions as predictors of population growth rate for *Phaeocystis antarctica* and *Cryothecomonas armigera*. (a) Single metal-exposures showing Spearman correlation coefficients for individual metal fractions. (b) Exposures of the environmental mixture showing Spearman correlation coefficients. (c) the significant metal components for multiple linear regression, as determined by step-wise optimisation of Akaike information criterion, 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, indicating the strength of the correlation.

For both microalgae, intracellular zinc concentrations had a positive partial regression coefficient with growth rate. This protective effect of zinc is regularly reported in metal mixture studies (Franklin et al. 2002a; Lavoie et al. 2012; Nagai and Kamo 2014; Versieren et al. 2016). This means that, if all other metal concentrations were constant, increasing intracellular zinc concentrations increases growth rate. However, the protectiveness of zinc was different in each microalgae. When *P. antarctica* is exposed to the environmental mixture, every 1.3×10^{-20} mol Cu μm^3 decreases growth rate by 1% and every 3.3×10^{-20} mol Zn μm^3 increases growth rate by 1%. When *C. armigera* is exposed to the environmental mixture, every 2.9×10^{-20} mol Cu μm^3 reduces growth rate by 1% but every 25×10^{-20} mol Zn 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 a more toxic metal at the cell surface leading to antagonism (Lavoie et al. 2014; Volland et al. 2014). This is supported by studies showing that copper and lead share a cellular uptake transporter (Sánchez-Marín et al. 2014). Alternatively, it could indicate that intracellular lead (which is negatively correlated to growth rate) is being detoxified by removal to the cell surface (Stewart et al. 2015). 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* (Figure 4.5). The results above 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 generally inconclusive in explaining 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. 2002a; 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) and Nys et al. (2016)).

5.4.3 Predicted risk to the Southern Ocean food web

The Southern Ocean marine ecosystem has a simple food web with few species linking primary producers (i.e. marine microalgae) to tertiary consumers like fish, penguins, and whales, albeit with much spatial and seasonal variability (Hill et al. 2009). Zooplankton such as copepods and krill provide this link (Murphy et al. 2012), particularly in areas closer to the Antarctic coastline (Ward et al. 2012). Many of these zooplankton, as well as microalgae, accumulate large lipid reserves to support them through the low-productivity winters (Kohlbach et al. 2017; Mou et al. 2012), where some species migrate to deeper waters and suspend their development until the following summer (Lee et al. 1971).

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). In some studies with temperate and tropical organisms, dietary exposure to metals is a more important contributor to toxicity than is dissolved metal exposure (DeForest and Meyer 2015; Luoma and Rainbow 2005). While no studies have investigated the dietary toxicity of metal-laden microalgae to Antarctic organisms, comparison to other fresh- or sea-water organisms may inform their possible risk.

The environmental mixture is an environmentally relevant ratio of metals determined from reported concentrations in a contaminated Antarctic bay (Larner et al. 2006). At a multiple of 10 (Table 5.1), the dissolved concentrations are higher but similar to what has been reported from other contaminated Antarctic nearshore marine sites (Cabrita et al. 2017; Padeiro et al. 2016). 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. Both algae had nickel and lead concentrations under 50 amol cell⁻¹ and cadmium concentrations under 5 amol cell⁻¹ (Figure 5.1). These concentrations suggest that toxicity to secondary consumers is likely.

For example, dietary exposure of the marine diatom *Thalassiosira pseudonana* laden with copper (10 amol cell⁻¹), nickel (7.5 amol cell⁻¹), or zinc (0.24 amol cell⁻¹) to the marine copepod *Acartia tonsa* resulted in a 20% decrease to reproduction (Bielsmyer et al. 2006). Both *C. armigera* and *P. antarctica* had copper and nickel concentrations 20 times and zinc concentrations >1000 times these values when exposed to the environmental mixture (Tables 5.1 and 5.2). The freshwater microalga *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 similar to what was found on *P. antarctica* or *C. armigera* exposed to the environmental mixture at a multiple of 10 (Tables 5.1 and 5.2).

There is a paucity of data on the dietary toxicity of metals to marine organisms, but similar trends are found in dietary toxicity to freshwater organisms. Dietary cadmium (Sofyan et al. 2006), copper (De Schamphelaere et al. 2007; Rocha et al. 2016), nickel (Evens et al. 2009), and zinc (De Schamphelaere et al. 2004) have been found to be important contributors of toxicity to the temperate freshwater cladocera *Daphnia sp.*

and *Ceriodaphnia dubia*. For example, Rocha et al. (2016) found that dietary copper toxicity from the freshwater microalga *Raphidocelis subcapitata* with cellular concentrations of approximately 60 amol Cu cell⁻¹ fed to the tropical freshwater cladoceran *Daphnia laevis* resulted in significantly reduced egg and neonate production as well as lower longevities. Both *P. antarctica* and *C. armigera* had higher total copper concentrations from the environmental mixture and single-metal exposures (Tables 5.1 and 5.2). At zinc exposure concentrations comparable to this study, 0.4 µM, the intracellular zinc concentrations of *Pseudokirchneriella subcapitata* were 75 amol Zn cell⁻¹ (De Schamphelaere et al. 2004), similar to the intracellular concentrations for *C. armigera* (70 ± 30 amol Zn cell⁻¹) in the environmental mixture at a multiple of 10. When *P. subcapitata* was fed to *Daphnia magna*, there was a decrease in total reproduction of 60%.

These comparisons suggest that even the low metal concentrations reported in contaminated sites around the nearshore Antarctic marine environment (Cabrita et al. 2017; Stark et al. 2006a) may pose a risk to the Southern Ocean food web via microalgae ingestion. Experiments to investigate this link, particularly with important copepods and krill species, are needed to enable more accurate predictions of risk.

5.5 Conclusion

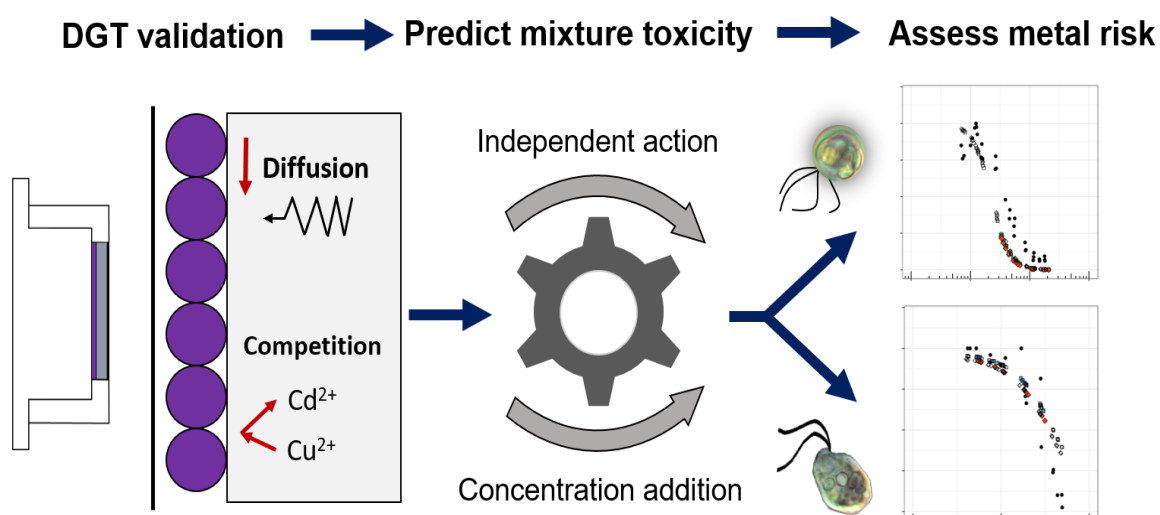
Antarctic marine microalgae *P. antarctica* and *C. armigera* employ different strategies to detoxify metal contaminants that cause toxicity; however, copper drives toxicity to both microalgae in an environmental mixture. Both *P. antarctica* and *C. armigera* accumulate metal contaminants at low, environmentally realistic, exposure concentrations that may cause toxicity to microalgae-grazing plankton in the Southern Ocean food web.

The risk of metals to the Antarctic nearshore marine ecosystem is affected by metal mixture interactions and sub-lethal metal accumulation in microalgae. *P. antarctica* and *C. 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 from sub-lethal, environmentally realistic exposure concentrations, which may in turn lead to metal bioaccumulation and or toxicity to microalgae-grazing plankton the Southern Ocean food web.

Chapters 3, 4, and 5 have furthered our understanding of contaminant impacts to the Antarctic nearshore environment by investigating the single metal toxicity to *C. armigera*, the toxicity of metal mixtures to *P. antarctica* and *C. armigera*, and by investigating potential risk to the Antarctic nearshore marine food web through dietary exposure of these microalgae at contaminated sites. To assess the risk of contaminants in the Antarctic nearshore marine environment, a robust process to measure metal contaminants that accounts for the temporal variability of contaminants and factors that modify their toxicity is needed.

CHAPTER 6

Validation of diffusive gradients in thin-films for polar marine conditions



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*What I love most about rivers is
You can't step in the same river twice
The water's always changing, always flowing*
Pocahontas

6.1 Introduction

The Protocol on Environmental Protection to the Antarctic Treaty has been in force since 1998, yet very few sites have been remediated (ATCM 2006; Errington et al. 2018; Roura 2004; Stark et al. 2006b). This is likely due to the challenges around access to sites, difficulties in contaminant monitoring, and the lack of Antarctic-specific ecotoxicology data and remediation guidelines. Simple contaminant monitoring devices are needed to overcome challenges of expensive logistics, limited personnel, and limited access to contaminated sites in Antarctica (Hodgson-Johnston et al. 2017; Raymond and Snape 2017).

Monitoring strategies in Antarctic marine environments typically investigate the presence of metals by directly measuring concentrations in water (e.g. total or dissolved) or field collected organisms (Palmer et al. 2006). These techniques provide useful information but do not necessarily inform environmental risk from metals. For example, the Antarctic nearshore marine environment is characterised by strong seasonality with ice melt, iceberg scour, and algal blooms in summer that can lead to temporal variability in contaminant concentrations (Fryirs et al. 2013). Thus, a water sample taken at a specific timepoint may not account for potential pulses of contamination (e.g. due to terrestrial ice melt or sea-ice scour of sediments), or factors known to modify metal toxicity such as metal speciation or DOC (discussed in Section 1.4.3). Measuring metal concentrations in field collected organisms will demonstrate the presence of a contaminant, but not necessarily if there is a toxic effect (e.g. metals can be stored in non-metabolically available forms Rainbow et al. (2015), Section 1.6.2). This approach also requires the collection and destructive analysis of organisms, which is not desirable for Antarctic organisms because of their long developmental times and limited geographic distribution (Chapman and Riddle 2005; Griffiths 2010).

The diffusive gradients in thin-films (DGT) technique has been used as an alternative, simple, and inexpensive contaminant monitoring tool, which measures the fraction of metals most likely to be biologically available to organisms (Amato et al. 2015, 2014; Vannuci-Silva et al. 2017). DGT has good potential to overcome the challenges associated with environmental management in the Antarctic marine environment because: (i) DGT preconcentrates analytes to a binding layer allowing for very low detection limits, which are required for the typically low contaminant concentrations present in Antarctic marine systems; (ii) DGT with a Chelex binding resin can simultaneously accumulate mixtures of cationic metals; (iii) it measures a time-averaged concentration over the deployment period that can capture pulse events (Frache et al. 2001; Stark et al. 2006b), and; (iv) studies are increasingly showing that DGT-labile metal concentrations is the fraction that best predicts toxicity to biota (Amato et al. 2015, 2016; Degryse et al. 2009; Degryse et al. 2003; Simpson et al. 2012). Thus, DGT measurements have the potential to be coupled to existing ecotoxicological data to predict metal-toxicity in Antarctic marine environments.

Coupling DGT-labile metal concentrations and single-metal toxicity data (from Chapter 3) in mixture modelling (Chapter 4), may provide a simple method to predict the risk of contaminants in the Antarctic marine environment. This process is meaningful only under the assumption that the metal fraction measured by DGT samplers (i.e. free

metal ions and metal complexes that dissociate in the DGT) is a close approximation of the biologically available fraction of metals measured in toxicity tests used to generate toxicity data. Recent research has generated single-contaminant toxicity data for a range of Antarctic organisms (Gissi et al. 2015; Hill et al. 2009; Holan et al. 2016; King and Riddle 2001; Marcus Zamora et al. 2015; Runcie and Riddle 2007). However, it is not yet clear how DGT-labile metal concentrations relate to dissolved metal concentrations in exposure solutions typical of Antarctic ecotoxicological studies, or if they can be coupled with ecotoxicology data to predict toxicity from metal mixtures.

This chapter aims to: (i) assess the performance of DGT in Antarctic cold marine environments by validating their performance in laboratory exposures to the five common metals cadmium, copper, nickel, lead, and zinc. This included determining metal diffusion coefficients, limits of detection (based on extractions of DGT binding resins from unexposed devices), minimum deployment times required to reach limits of detection at environmentally relevant exposure concentrations, method detection limits (i.e. minimum concentration in the exposure solution necessary to exceed limits of detection as a function of deployment time), and metal competition interactions for five common metals; (ii) investigate the effects of the mucogenic Antarctic microalgae, *P. antarctica*, on the uptake of metals by DGT devices co-deployed with microalgae ; and (iii) evaluate the suitability of using DGT-labile metal concentrations to predict metal-mixture toxicity to the Antarctic marine algae *P. antarctica* and *C. armigera* using previously determined single-metal toxicity data (Chapter 3).

Metal concentrations in this chapter are presented in moles because of the need to compare absolute numbers of metal ions when competition effects to the DGT binding resin were being assessed.

6.2 Method

6.2.1 Preparation of DGT devices and binding resin extraction

DGT pistons with a Chelex-100 binding layer were prepared following the procedures recommended by DGT Research (Lancaster, UK) (Davison 2016). The binding resin was a 0.4 mm thick Chelex-100 (Bio-Rad, mesh 200-400) impregnated polyacrylamide gel. During the binding resin synthesis, gravitational settling resulted in the Chelex-100 beads concentrating at the bottom of the gel. This concentrated side was placed towards the window of the DGT, in contact with the 0.8 mm thick polyacrylamide diffusive layer. A 0.13 mm thick, 0.45 μm pore size polyethersulfone filter paper was placed on top of the diffusive layer. The three layers were sandwiched on the piston base by a housing with a 2 cm diameter window. Prior to deployment, assembled pistons were conditioned for 24 h in a 0.12 M NaCl solution (Suprapur, Merck Millipore). Prepared DGT were stored moist in low-density polyethylene bags at 4 °C.

Following deployment, DGT devices were disassembled and the binding resin placed in 1 mL of 1 M HNO₃ (Suprapur grade, Merck Millipore) for ≥ 12 h on an orbital shaker. The eluants were diluted to a final concentration of 0.2% HNO₃ before metal analysis.

6.2.2 DGT performance in cold marine waters

DGT performance, aim (i), was assessed by deploying DGT to three different metal mixtures in cold marine conditions and retrieving them periodically over 36 d. Two of the mixtures used a ratio of five metals, based on measured concentrations at a historically contaminated bay in East Antarctica: 2 Cd : 8 Cu : 5 Ni : 1 Pb : 69 Zn (multiple of 1, in nM) (Larner et al. 2006). The ratio was multiplied by 10 and 500 to give two treatments termed the low and high environmental mixture, respectively. The third treatment was an equimolar mixture of five metals each at a nominal concentration of 10 μM . The average of all dissolved metal concentrations from each treatment are given in Table 6.1 (a–c).

DGT ($n = 48$ per treatment) were deployed in 20 L of 0.45 μm filtered seawater supplemented with metal solutions described above (Table 6.1 a–c). Treatments were equilibrated at 1 ± 1 $^{\circ}\text{C}$ in a temperature controlled environmental chamber for ≥ 72 h before DGT were deployed. A temperature of 1 $^{\circ}\text{C}$ was used as a compromise between the temperature of the nearshore Antarctic marine environment, which ranges from -2 to 1 $^{\circ}\text{C}$, and to ensure that the seawater would not freeze during the experiment.

A poly(methyl methacrylate) (Perspex) cube with 2.5 cm diameter holes was used to hold DGT pistons for the duration of their deployments and the solutions were agitated by magnetic stirrers or by an orbital shaker (rotating at approximately 60 RPM with an orbital diameter of 2.5 cm) for the duration of the exposure. DGT devices were retrieved periodically over 36 d, rinsed with ultrapure water and stored moist at 4 $^{\circ}\text{C}$ in clean plastic bags until they were disassembled and eluted.

Diffusion coefficients

DGT binding resins were eluted and the mass of metal accumulated (M_i , nmol) was calculated using Equation 6.1.

$$M_i = \frac{C_e(V_e + V_{gel})}{f_e} \quad (6.1)$$

Where C_e is the concentration of the eluent, V_e and V_{gel} are the volumes of the eluent and gel, respectively, and f_e is the elution factor, which was 0.8 for all metals (Zhang and Davison 1995). The diffusion coefficients for each metal was calculated for 1 ± 1 $^{\circ}\text{C}$ using Equation 6.2.

$$D_i = \frac{M_i \Delta g}{t C_b A} \quad (6.2)$$

Where D_i is the diffusion coefficient of metal i (in units of 10^{-6} $\text{cm}^2 \text{s}^{-1}$ at 1 $^{\circ}\text{C}$), M is mass (nmol) accumulated over time t (s) over the linear uptake range, Δg is the thickness of the overall diffusion layer (0.093 cm, the combined thickness of the diffusive gel and filter membrane, assuming negligible water diffusion layer thickness), C_b (μM) is the time averaged dissolved metal concentration for the linear uptake range, and A (3.14 cm^2) is the surface area of the exposure window of the DGT piston (Zhang and Davison 1995).

The linear range of metal accumulation was determined by the R^2 values of a linear model fit to each metal for each mixture against time. The slope of the longest deployment time that gave $R^2 \geq 0.95$ was selected to calculate the diffusion coefficient.

The DGT-labile metal concentration (C_{DGT} , μM) was then determined by Equation 6.3 (Zhang and Davison 1995), assuming a steady-state flux of metals to the binding resin over the deployment time.

$$C_{DGT} = \frac{M_i \Delta g}{D_i t A} \quad (6.3)$$

Limit of detection and time to limit of detection

The LOD (nmol) was determined as three times the standard deviation of M_i from blank DGT devices (≥ 3 DGT per experiment). The time to LOD was calculated as the minimum DGT deployment time needed to reach the LOD in cold marine water for a given cold marine metal concentration. Using Equation 6.3, C_{DGT} was fixed at theoretical metal concentrations (C_b , Table 6.1), which included treatments from aim (i) as well as example concentrations to help guide minimum field deployment times, including: the equivalent of the Australian and New Zealand marine water quality guideline value for 99% and 95% species protection levels (ANZG 2018), the environmental ratio (Section 5.2.1), and nearshore concentrations from Marion Cove, King George Island on the West Antarctic Peninsula (Kim et al. 2015).

Method detection limits

Method detection limits (MDL, μM) were calculated as the minimum concentrations water required for a DGT to accumulate metal to its LOD for a given deployment time. The MDL was calculated using Equation 6.3 at various time points (1 to 30 d) where M_i was the average LOD from all DGT blanks.

Competition effects

Competition effects were defined as a deviation from a C_{DGT}/C_b ratio of 1.0 ± 0.2 for a metal, occurring where $\sum M_i$ showed linear accumulation. This was also observed for some metals in the residuals of a linear model fit of the M_i over time (Appendix A6.1).

6.2.3 Investigating the influence of *Phaeocystis antarctica* to DGT metal uptake

DGT were co-deployed with *P. antarctica* to investigate how a mucogenic microalgae affects the uptake of metals by DGT, aim (ii). DGT were deployed with and without the addition of *P. antarctica* ($1-3 \times 10^3$ cells mL^{-1}) in treatments of the environmental ratio at multiples of 10, 20, 40, and 60 (equal to a molar total of 1, 2, 3 and 5 μM , respectively) and an equitoxic mixture (equal to 15 μM), where each metal was present the population growth rate inhibition EC10 for *P. antarctica* (Gissi et al. 2015). See Table 6.4 for dissolved metal concentrations.

P. antarctica was cultured as described in Section 2.2. Test solutions were 160 mL of 0.45 μm filtered seawater in 400 mL tall glass silanised beakers at 1 ± 1 °C supplemented with nutrients and metal stocks to give concentrations equivalent to their nominated mixture. Three replicates of each treatment were tested. DGT were deployed to beakers 3 d after test commencement, maintained on an orbital shaker, and retrieved after 1 to 7 d, depending on the metal solution.

The ratio of C_{DGT} / C_b was determined for each solution and significant differences between deployments with and without *P. antarctica* for each metal were determined by an analysis of variance (ANOVA) assuming normality and homogeneity of variance of the ratios.

6.2.4 Using DGT-labile metal concentrations to predict metal mixture toxicity

C_{DGT} measurements from aim (ii) were used in IA (Equation 4.5) and CA (Equation 4.7) reference mixture models, parametrised with previously determined EC10 and slope parameters from single-metal 3-parameter log-logistic concentration-response models given in Gissi et al. (2015) for *P. antarctica* and Chapter 3 for *C. armigera*. IA and CA mixture toxicity predictions from the multiples of the environmental ratio were compared to previously determined observed and predicted toxicity, which used dissolved metal concentrations in Figure 4.5.

6.3 Results

6.3.1 DGT performance in cold marine waters

Diffusion coefficients were determined to be ($\times 10^{-6}$ $\text{cm}^2 \text{s}^{-1}$ at 1 °C): Cd 2.4 ± 0.4 , Cu 2.1 ± 0.5 , Ni 2.2 ± 0.1 , Pb 2.6 ± 0.2 , and Zn 2.1 ± 0.2 as mean \pm standard deviation from deployments in the high environmental, low environmental, and equimolar solutions (Table 6.2). Only the linear portion of the gradient of metal uptake over time was used in the calculations (see deviations from uptake in Figure 6.1 and residuals of linear model fits in Appendix A6.1). The R^2 values of the fitted linear models at the different time points are given in Appendix A6.2. The parameters used to calculate diffusion coefficients (Equation 6.2) for each metal mixture are given in Appendix A6.3.

The LOD and time to LOD for DGT at 1 °C in marine waters are given in Table 6.1. The dissolved metal concentrations in the high environmental and equimolar solutions remained stable throughout the experiment (Appendix A6.4); however, metal concentrations in the low environmental treatment decreased by 20-50% over the duration of DGT deployments (Appendix A6.4), possibly due to adsorption to the container wall or metal precipitation. This decrease was accounted for by using the time-average dissolved metal concentration for each DGT's deployment period.

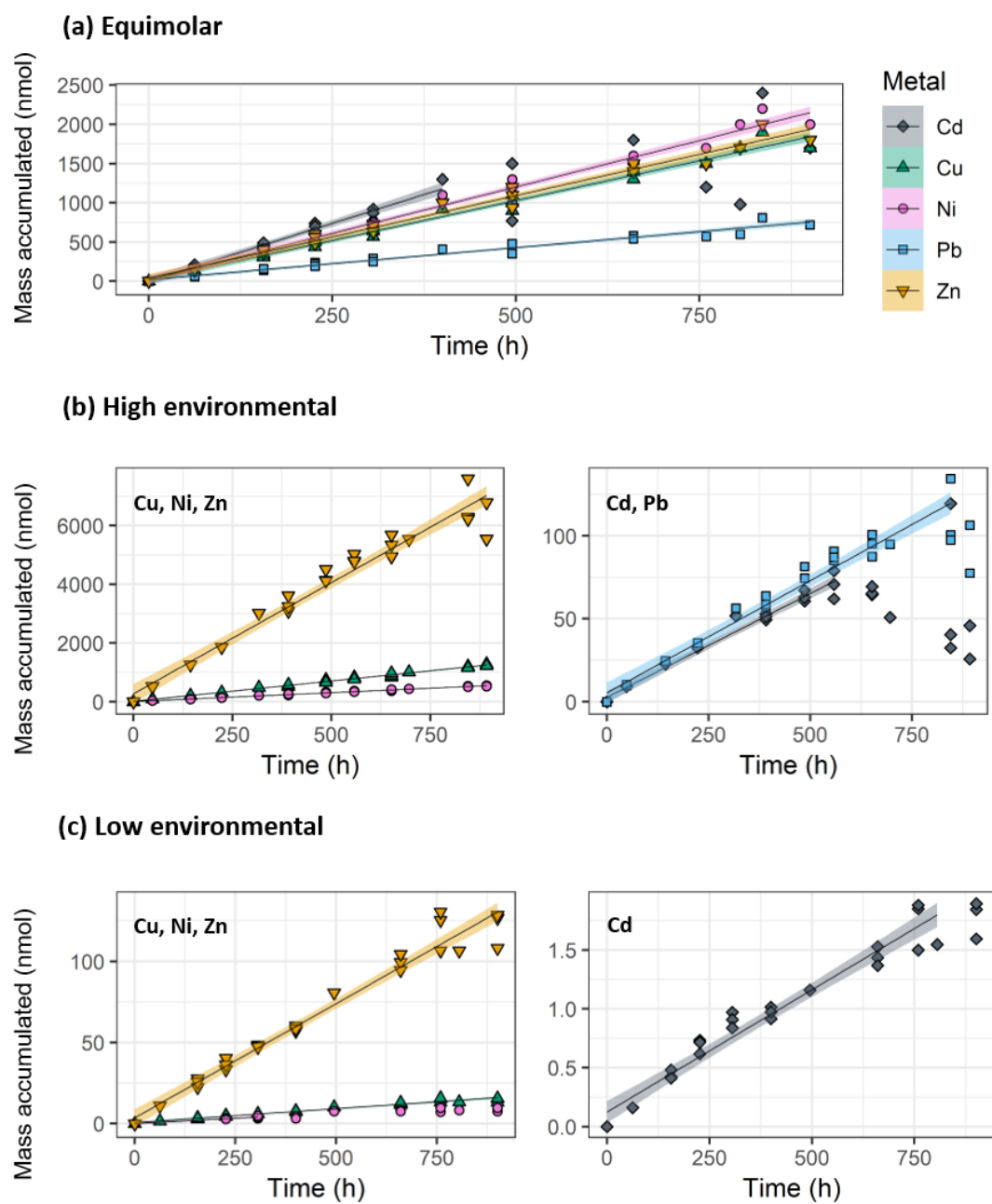


Figure 6.1: Mass of analyte (nmol) accumulated over time (h) during uptake experiments in the (a) equimolar, (b) high environmental, and (c) low environmental mixtures. Note the different y-axis scales for (b) and (c) where copper, nickel, and zinc (left) were present at higher concentrations than cadmium and lead (right). Concentrations of lead were below detection limits in the low environment mixture. Linear regressions (with 95% confidence intervals) are fitted for the interval which was used to calculate D_i , Equation 6.2. This corresponded to the longest deployment period that gave an $R^2 \geq 0.95$.

Table 6.1: Average dissolved metal concentrations for the DGT deployment period (C_b , μM), limit of detection (LOD, nmol), and time to limit of detection (h) for DGT deployments in 1 ± 1 °C seawater for (a) high environmental, (b) low environmental, (c) equimolar mixtures, (d) metals in seawater at water quality guideline values for 95% protection (ANZG 2018), (e) seawater at water quality guideline values for 99% protection (ANZG 2018), (f) environmental ratio (Larner et al. 2006), and (g) reported concentrations from the Antarctic nearshore environment (Kim et al. 2015)

(a) High environmental	Cd	Cu	Ni	Pb	Zn
C_b	0.51 ± 0.02	5.1 ± 0.2	2.4 ± 0.1	0.48 ± 0.02	33.1 ± 0.9
LOD	0.01	0.6	0.7	0.1	0.6
Time to LOD	0.4	0.3	1.7	1.3	0.05
(b) Low environmental					
C_b	0.007 ± 0.002	0.1 ± 0.01	0.05 ± 0.02	0.0015 ± 0.0003	0.57 ± 0.07
LOD	0.1	0.3	2	0.1	0.4
Time to LOD	29	16	80	395	3
(c) Equimolar					
C_b	10.4 ± 0.7	7.8 ± 0.4	9.7 ± 0.8	3.0 ± 0.2	8.9 ± 0.6
LOD	0.1	0.3	2	0.1	0.4
Time to LOD	0.02	0.2	0.4	0.2	0.2
(d) Water quality guideline values for 95% species protection					
C_b^a	0.05	0.02	1.2	0.02	0.2
LOD ^b	0.1	0.4	1.1	0.2	0.4
Time to LOD	4	79	3	29	8
(e) Water quality guideline values for 99% species protection					
C_b^a	0.006	0.005	0.12	0.01	0.1
LOD ^b	0.1	0.3	2	0.1	0.4
Time to LOD	31	341	33	57	16
(f) Environmental ratio					
C_b^a	0.009	0.002	0.005	0.001	0.07
LOD ^b	0.1	0.4	1.1	0.2	0.4
Time to LOD	22	1024	774	419	25
(g) Reported concentrations from the uncontaminated Antarctic nearshore marine environment					
C_b^a	0.00003	0.0006	0.0006	0.0001	0.002
LOD ^b	0.1	0.4	1.1	0.2	0.4
Time to LOD	6975	2647	7002	7963	1107

^a Concentration from literature used to calculate time to LOD.

^b Calculated as the average of DGT blanks from all experiments.

The minimum deployment time to exceed the LOD for all metals tested was <1 d for the high environmental and equimolar mixtures, 17 d for the low environmental mixture, 4 and 15 d for waters with concentrations equivalent to the Australian and New Zealand water quality guideline values for 95 and 99% species protection (ANZG 2018), respectively, 43 d for the environmental ratio, and 332 d for background concentrations in the Antarctic nearshore marine environment. However, times varied for individual metals (Table 6.1).

The method detection limits (MDL) for deployments of various times ranged from 165 nM for nickel in a 1-d deployment to 0.8 pM for cadmium in a 30-d deployment (Table 6.3). There is a linear relationship between the MDL and 1/t (Equation 6.3), which can be used to calculate the MDL for other deployment times.

6.3.2 Metal uptake and competition effects

The ratio of DGT-labile (C_{DGT}) to dissolved metal (C_b) concentrations for all metals in all exposures was generally 1.0 ± 0.2 (Figure 6.2). The low environmental mixture was the most variable of the mixtures, with a C_{DGT}/C_b ratio for copper and nickel of approximately 0.8, and for cadmium between 1.2 and 1.5, particularly at early time points (i.e. <400 h, Figure 6.2). Given the low metal concentrations in this treatment, this is most likely due to the increased variability in ICP-AES measurements near the detection limits for those metals.

Metal competition was observed in the equimolar and high environmental mixtures, where cadmium lost quantitative uptake (Figure 6.1 b, c). Cadmium lost linearity ($R^2 < 0.95$ of the linear model fit) after 16 d in the equimolar solution and 23 d in the high environmental mixture, where the resin had accumulated $\Sigma 5.4 \pm 0.3$ μmol of metals (at 486 h). Cadmium displacement from the resin was observed in the high environmental mixture where a maximum of 71 ± 8 nmol at 23 d decreased to 35 ± 10 nmol at 30 d. This suggests active substitution of cadmium by other metals that retained linear uptake. This was less clear for cadmium in the equimolar mixture, which had greater variability after the resin had accumulated $\Sigma 4.8 \pm 0.7$ μmol of metals at 486 h (Figure 6.1). Zinc appeared to reach saturation in both the equimolar and high environmental mixtures at around 30 d (Figure 6.1), but the experiment ended before competition affected the linearity of zinc uptake (Appendix A6.1).

Table 6.2: Comparison of diffusion coefficients (D_i) from seawater (this study), estuarine water (Larner et al. 2006), and theoretical calculations (Buffle et al. 2007). Empirically determined diffusion coefficients from this study are the mean and standard deviation of the three treatments.

Media	Temp. ^a	Sal. ^b	pH	Diffusion coefficients (10^{-6} $\text{cm}^2 \text{s}^{-1}$)				
				Cd	Cu	Ni	Pb	Zn
Seawater	1 ± 1	34	7.9 ± 0.1	2.4 ± 0.4	2.1 ± 0.5	2.2 ± 0.1	2.6 ± 0.2	2.1 ± 0.2
Estuarine	-1	20	6.7	3.0	2.6	–	3.6	2.9
Theoretical	1	–	–	2.8	2.9	2.7	3.8	2.8

^a Temperature, in °C

^b Salinity, in practical salinity units

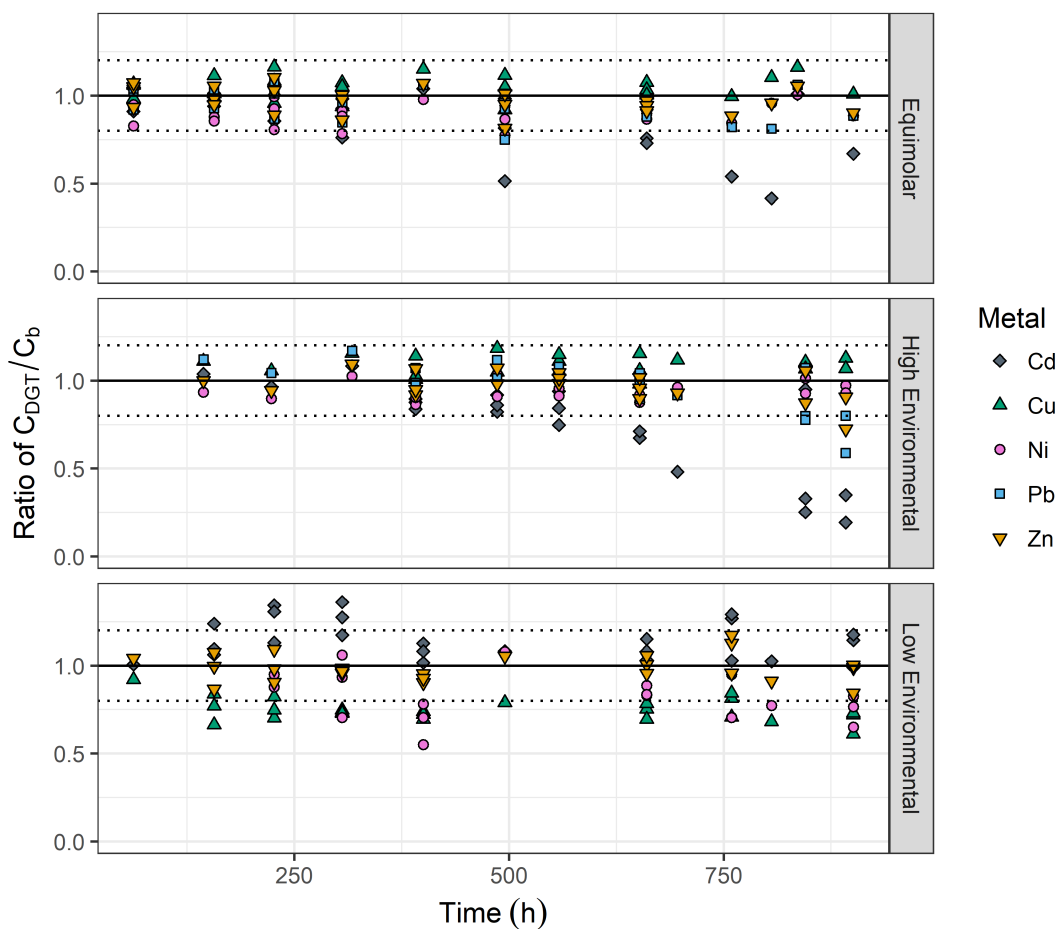


Figure 6.2: The ratio of DGT-labile to dissolved ($0.45 \mu\text{m}$ filterable) metal concentration (C_{DGT}/C_b) for the equimolar, high environmental, and low environmental metal mixtures. A ratio of 1, solid horizontal line, indicates equivalent concentrations measured. The dotted line above and below the solid line represent a 20% deviation from 1.

Table 6.3: Method detection limits (MDL, nM) at various deployment times.

Metal	LOD (nmol)	MDL (nM) for deployment times (d)				
		1	2	7	15	30
Cd	0.06	8.0	4.0	1.1	0.5	0.3
Cu	0.40	67	34	10	4.5	2.2
Ni	1.08	165	82	24	11	5.5
Pb	0.19	25	13	3.6	1.7	0.8
Zn	0.44	72	36	10	4.8	2.4

6.3.3 Investigating the influence of *Phaeocystis antarctica* to DGT metal uptake

No difference was observed between the C_{DGT}/C_b ratio in deployments in the presence and absence of *P. antarctica* for Cd ($1.0 \pm 0.2 / 1.0 \pm 0.2$), Cu ($1.0 \pm 0.4 / 0.9 \pm 0.2$), Pb ($0.9 \pm 0.1 / 1.0 \pm 0.2$), and Zn ($0.94 \pm 0.04 / 0.9 \pm 0.1$) (Figure 6.3), across all treatments. However, the C_{DGT}/C_b ratio for nickel was significantly lower in deployments in the presence of *P. antarctica* than in the absence ($0.75 \pm 0.11 / 0.86 \pm 0.14$, $p = 0.032$). The ratios from individual treatments and metals are given in Appendix A6.5.

6.3.4 Using DGT-labile metal concentrations to predict metal mixture toxicity

Both independent action and concentration addition mixture models gave similar toxicity predictions to *P. antarctica* and *C. armigera* microalgae. The models were good predictors of metal mixture toxicity to *C. armigera* within the concentration range tested, but overestimated toxicity to *P. antarctica* (Figure 6.4), suggesting antagonism.

6.4 Discussion

6.4.1 DGT performance in cold marine waters

DGT was shown to be a sensitive measure of metals in seawater, with LODs generally in the low nmol to pmol range (Table 6.1). These results suggest that DGT is suitable for the environmental monitoring of metal contamination in cold marine waters (Table 6.1). For example, a deployment period (i.e. time to LOD) of >4 or 15 d would be needed to measure seawater metal concentrations equivalent to the Australian and New Zealand marine water quality guidelines values for 95 and 99% species protection, respectively (ANZG 2018). With the detection limits of an ICP-AES, DGT would not be suitable for the measurement of trace metals in cold seawater because of the long deployment times needed (i.e. >332 d). However, DGT could be practical depending on the metal of interest if using ultra-trace metal clean laboratory techniques and sensitive analytical instrumentation, such as ICP-MS.

Table 6.4: Measured dissolved metal concentrations (C_b , μM) for the equitoxic mixture and multiples of the environmental ratio for DGT deployments in the presence + or absence - of *Phaeocystis antarctica*. No 60^- treatment was tested.

Metal	Equitoxic ⁻	Equitoxic ⁺	Multiples of the environmental ratio						
			10 ⁻	10 ⁺	20 ⁻	20 ⁺	40 ⁻	40 ⁺	60 ⁺
Cd	1.7	1.7	0.01	0.01	0.02	0.02	0.04	0.04	0.06
Cu	0.11	0.08	0.20	0.16	0.31	0.29	0.50	0.52	0.77
Ni	8.7	8.9	0.06	0.07	0.11	0.11	0.20	0.23	0.33
Pb ^a	0.54	0.44	-	-	-	-	-	-	-
Zn	3.4	3.4	0.72	0.69	1.4	1.4	2.7	2.7	4.3

^a Values missing are below detection limits, <0.009 μM

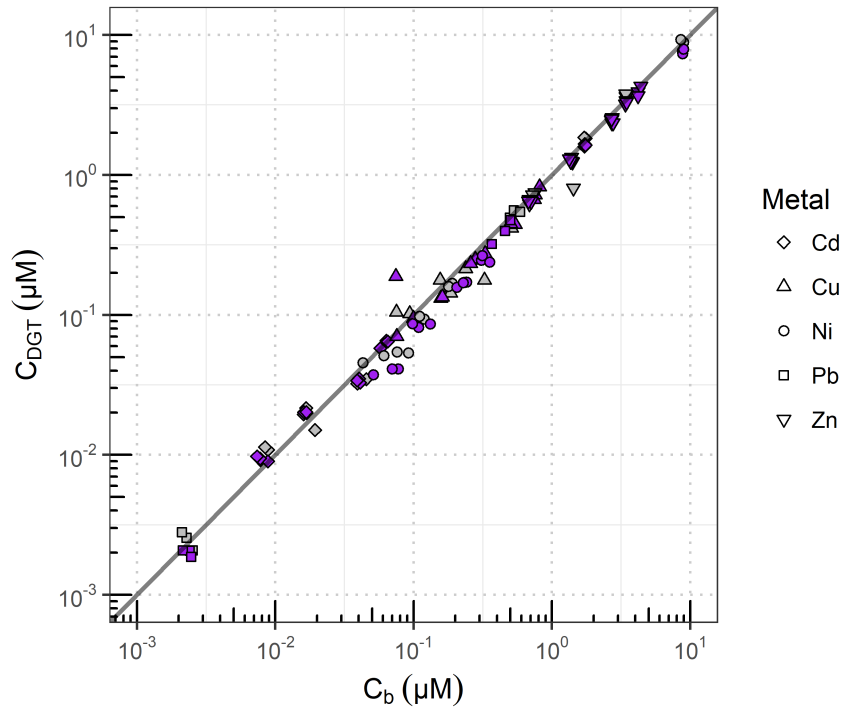


Figure 6.3: The relationship between DGT-labile (C_{DGT}) and total dissolved metal concentrations (C_b) for deployments in the equitoxic and multiples of the environmental ratio treatments in the presence (purple) and absence (grey) of *Phaeocystis antarctica*. Points falling on the 1:1 line suggest that the C_{DGT} fraction is representative of C_b in cold marine conditions.

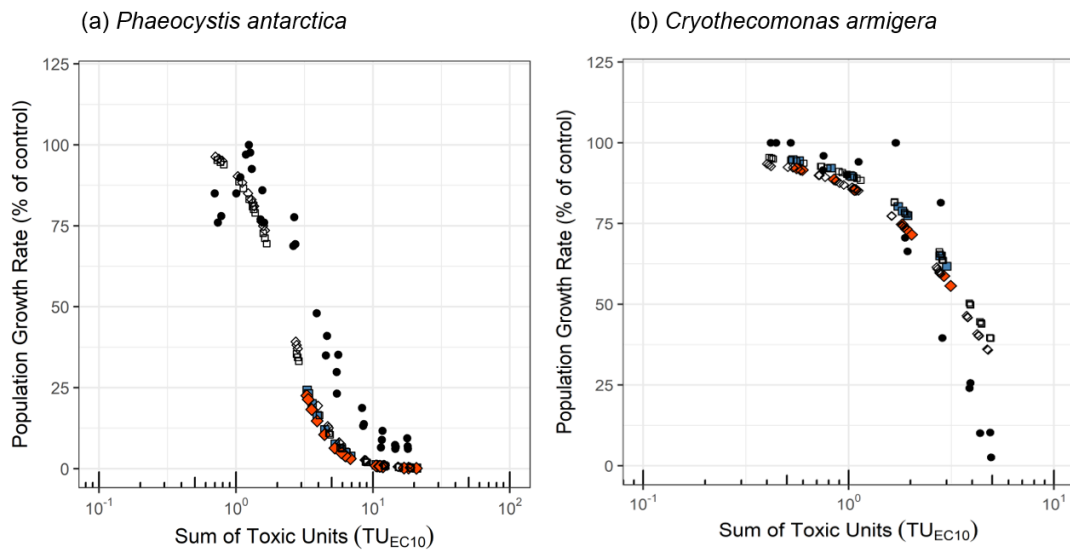


Figure 6.4: Predicted toxicity using DGT-labile metal concentrations (C_{DGT}) to *Phaeocystis antarctica* (a) and *Cryothencomonas armigera* (b). Predictions from C_{DGT} by concentration addition (blue squares) and independent action (orange diamonds) models are overlaid against previously determined toxicity predictions using dissolved metal concentrations (clear squares and diamonds, respectively) and observed toxicity (black circles) from multiples of the environmental ratio, Chapter 4. Toxicity as population growth rate inhibition is plotted against the sum of toxic units based on EC10 concentrations (ΣTU_{EC10}).

The diffusion coefficients derived in this study are the first to be determined in conditions (i.e. temperature, salinity, and pH) reflective of the nearshore Antarctic marine environment. They are up to 32% lower than those previously determined at similar temperatures (Table 6.2) (Buffle et al. 2007; DGT Research 2018; Larner et al. 2006). These reported diffusion coefficients decrease in increasingly complicated media (e.g. theoretical > estuarine > seawater), possibly due to an increased likelihood of collisions, electrostatic repulsion, changes in solution viscosity, or the presence of metal complexes (Buffle et al. 2007). Larger metal complexes have lower rates of diffusion across the DGT diffusive layer (Zhang and Davison 2000). This can be influenced by different pHs, with a lower pH increasing the likelihood of a metal existing as a free ion (Byrne et al. 1988), subsequently increasing the diffusion rate. Based on the results of this study, using previously reported diffusion coefficients could underestimate C_{DGT} calculations by as much as 32%.

The Chelex-100 resin does not act as a perfect metal sink at sub-saturation concentrations. This is an under reported finding, but in agreement with previous studies (Degryse et al. 2003; Jiménez-Piedrahita et al. 2017). A Chelex-100 resin has a capacity for divalent cations of 10-15 μmol per DGT piston (Bio-Rad laboratories 2013; Tankéré-Muller et al. 2012). In our study, competition effects were observed after approximately 20 d in the equimolar and high environmental mixtures where the mass of the five metals on the binding resin were 4.8 ± 0.7 and 5.4 ± 0.3 μmol , respectively. This highlights significant competition effects at a total molar concentration between 30-50% of the theoretical resin capacity. The competition observed agreed with the metal's selectivity for the Chelex-100 resin (normalised to zinc, at a circumneutral pH): Cu^{2+} (126) \gg Ni^{2+} (4.4) $>$ Pb^{2+} (3.9) $>$ Zn^{2+} (1) $>$ Co^{2+} (0.6) $>$ Cd^{2+} (0.4) $>$ Fe^{2+} (0.1) $>$ Mn^{2+} (0.02) (Bio-Rad laboratories 2013), i.e. of the metal analytes in the mixture, cadmium and zinc have the lowest selectivity for the Chelex-100 resin and were the first to lose linearity of their uptake (Figure 6.1). Cadmium was clearly outcompeted in the high environmental mixture, but in the equimolar mixture, this was less clear with some DGT showing expected cadmium accumulation while others showed lower accumulation after 20 d (Figure 6.1).

The relative metal concentrations, as well as their selectivity, can influence competition effects. Tankéré-Muller et al. (2012) found that Mg^{2+} and Ca^{2+} saturate a Chelex-100 binding resin when deployed in seawater for longer than 2 h, suggesting that metal binding occurs by active exchange of metals with differing selectivities on a saturated resin. However, when Fe^{2+} was present in high concentrations, both Mn^{2+} and Cd^{2+} were displaced, despite Cd^{2+} having a greater selectivity than Fe^{2+} . This may be caused by a reduction in binding affinity for metals to the Chelex-100 resin from electrostatic screening of the functional groups in high ionic strengths (Jiménez-Piedrahita et al. 2017). These confounding factors may explain the variability in the cadmium measurements in the equimolar mixture which had broadly equivalent concentrations of metals, compared to the high environment mixture where cadmium was at a much lower concentration compared to copper and zinc.

6.4.2 The influence of *Phaeocystis antarctica* on DGT metal uptake

All metals had C_{DGT}/C_b ratios >0.9 , with the exception of nickel which was 0.75 in the presence of *P. antarctica* and 0.86 in its absence (average of all treatments, Figure 6.3, Appendix A6.5). Ratios <1 are commonly observed in studies with complex matrices. For example, DGT measurements of seawater overlying sediments had C_{DGT}/C_b ratios of 0.8 for zinc and 0.25 for copper were previously reported, possibly from DOC or particle adsorption (Amato et al. 2015). For this reason, the addition of *P. antarctica* was expected to reduce the C_{DGT}/C_b ratio. *P. antarctica* is known to produce DOC as a mechanism to chelate micronutrients like iron and zinc in marine waters (Alderkamp et al. 2012; Thuróczy et al. 2012). Other micronutrients may also be bound in this process, such as nickel which is an essential metal for urease - an enzyme most marine phytoplankton use to hydrolyse urea to NH_3 and CO_2 (Dupont et al. 2010). The concentration of $1\text{-}3 \times 10^3$ cells mL^{-1} was chosen as a compromise between the keeping metals in solution (metals will adsorb to the microalga, removing them from the dissolved phase) and environmental realism (microalgal blooms in coastal waters can reach 10^5 cells mL^{-1} Deppeler and Davidson (2017)). Thus, these data show that metal binding to *P. antarctica* DOC is generally DGT-labile.

While a significant reduction in the C_{DGT}/C_b ratio was observed for nickel, it was not for other micronutrients like zinc or copper. The reduction in nickel DGT concentrations could be a Type I error, given the sample sizes of 15 and 12 for deployments in the presence and absence of *P. antarctica*, respectively. The ratio could also be affected by uncertainty in the measurement of nickel (C_b) which was present at low concentrations in multiples of the environmental ratio and has a relatively high instrument detection limit. This is most likely because of the metals tested. For example, copper has a higher binding affinity to DOC and should have a lower ratio in the presence of *P. antarctica*, compared to nickel (Smith et al. 2002).

Overall, the high ratios >0.9 for most metals suggest that dissolved metals in $0.45 \mu\text{m}$ filtered, low DOC ($1.4 \pm 0.3 \text{ mg C L}^{-1}$), seawater mediums are DGT-labile. This gives confidence in relating C_{DGT} to previously determined ecotoxicology data, which is typically derived from dissolved metal concentrations from laboratory exposures.

6.4.3 Using DGT-labile metal concentrations to predict metal mixture toxicity

DGT-labile metal concentrations and dissolved metal concentrations provided similar predictions of metal-mixture toxicity to *P. antarctica* and *C. armigera* and also highlighted metal-mixture interactivity (Figure 6.4). *P. antarctica* is known to exhibit significant antagonistic mixture interactions in response to increasing multiples of the environmental ratio, as shown in Chapter 4. The cause of the antagonism is not known but may be due to extracellular mucous production reducing the bioavailability of metals like copper and cadmium (Oleinikova et al. 2018). *C. armigera* may also exhibit synergistic interactions at high effect concentrations. This study did not test a concentration range where this was expected to have been observed.

6.4.4 Practical considerations of using DGT in cold marine environments

The Antarctic nearshore marine environment has variable metal fluxes, DOC concentrations, and concentrations of inorganic adsorbents like iron or aluminium hydroxides. In such a complex environment, DGT allows for the simultaneous uptake of common metal contaminants, with ultra-trace detection limits, time-averaged over the deployment period (Jolley et al. 2016).

Water quality guideline values specific for the Antarctic marine ecosystem have not yet been developed, so minimum deployment times to reach DGT LODs are suggested based on seawater concentrations equivalent to the Australian and New Zealand marine water quality guideline values for 99 and 95% species protection (ANZG 2018). At these concentrations a recommended minimum deployment time of at least 15 d is needed (Table 6.1). Alternatively, MDLs at various deployment times are given in Table 6.3. As per Equation 6.3, increasing deployment time will decrease MDL. It should be noted that these values will depend on the instrument sensitivity, cleanliness, and LODs achieved by each laboratory.

The risk of metal competition on the Chelex-100 resin is low for Antarctic marine deployments, given the low metal concentrations in contaminated marine areas (Cabrita et al. 2017; Larner et al. 2006; Padeiro et al. 2016; Stark et al. 2006a), and low diffusion coefficients (Table 6.2). However, where the molar total of metal-analytes with different selectivities exceeds approximately $\sum 4 \mu\text{mol}$, care should be taken in the interpretation of the results.

The application of DGT outlined in this study differs from the limited capacity in which DGT has already been used for environmental monitoring in the Antarctic environment (Cabrita et al. 2017; Larner et al. 2006; Padeiro et al. 2016; Stark et al. 2006a). These studies assessed the presence of contaminants rather than concentrations relating to the risk they pose to marine organisms. DGT measures a biologically available fraction of metals in solution, which is the fraction most likely to cause toxicity to organisms (Simpson et al. 2012). This study has shown that DGT-labile metal fractions in seawater can be used to predict the toxicity of metal contaminants to two Antarctic marine algae, where single-metal toxicity exists – at least as well as dissolved metal concentrations.

This study validates the use of DGT in polar marine environments and provides guidance around their deployment. These results should be confirmed in an *in situ* field study where factors including biofouling or interference from native fauna can be investigated.

6.5 Conclusion

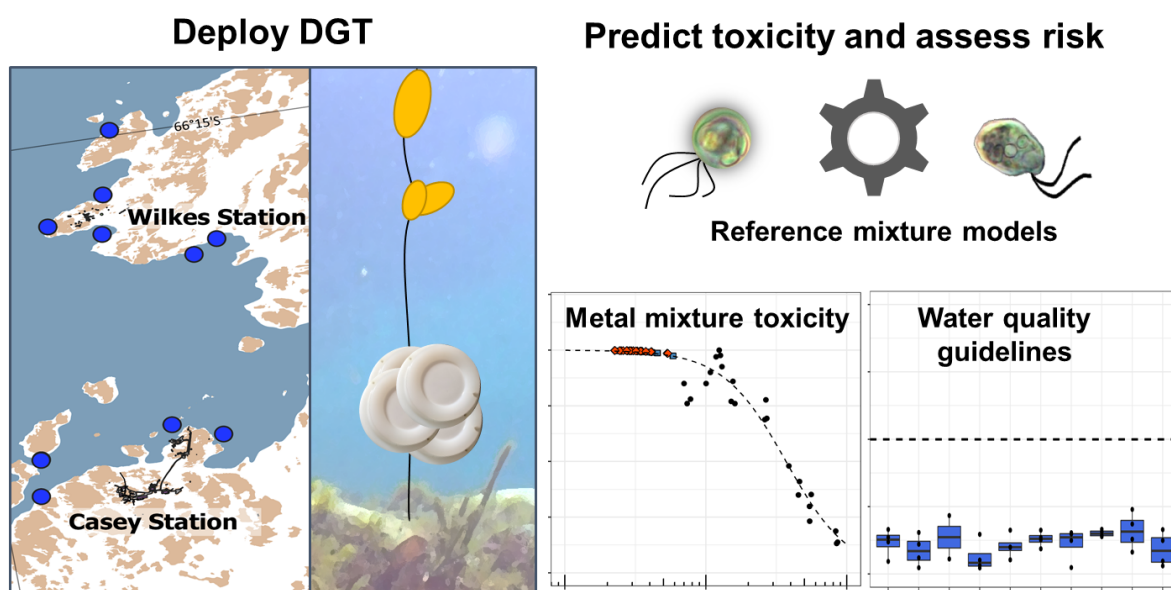
DGT were shown to be a sensitive tool to measure metals in cold marine waters, where the dynamic environment, limited accessibility, and costly logistics necessitates new monitoring techniques. This study provides empirical diffusion coefficients for five common metal contaminants, method detection limits, minimum deployment times for environmental monitoring, and highlights that metal competition at sub-saturation concentrations needs to be considered where the molar total of metals accumulated to the Chelex-100 resin exceeds approximately $\Sigma 4 \mu\text{mol}$.

DGT-labile concentrations are suitable to use in mixture toxicity modelling because they are likely to measure metal concentrations in complex environments that are bioavailable to organisms through a dissolved exposure. However, DGT-labile concentrations are equivalent to dissolved metal concentrations at predicting biological mixture toxicity interactions (i.e. synergism or antagonism). DGT is well positioned to be used in the environmental risk assessment of metal contaminants in cold marine environments, particularly with the ongoing generation of ecotoxicological data towards the derivation of Antarctic environmental quality guidelines.

This laboratory study validated DGT to measure and predict toxicity from metal mixtures in Antarctic marine conditions. However, how practical they are to use and how they perform in the Antarctic environment is unknown. Field deployments are needed to investigate their practical application to measure metal contaminants and assess the risk those metals pose in the Antarctic nearshore marine environment.

CHAPTER 7

Using diffusive gradients in thin-films assess the risk of metals and their mixtures in the Antarctic nearshore marine environment



This chapter has been submitted as a manuscript to the journal *Environmental Science and Technology*

*Be bold. Step out.
And never walk on tiptoes,
because anyone who walks on tiptoes
can never leave footprints for people to walk in.*
Leymah Gbowee

7.1 Introduction

DGT is an *in situ* passive sampling technique that has seen preliminary use in Antarctica to assess labile metal concentrations in marine waters (Cabrita et al. 2017; Larner et al. 2006; Padeiro et al. 2016; Stark et al. 2006a). The ease of use, ability to provide a time-averaged measure of the potentially bioavailable metal fraction, relatively low cost, tailorable binding resin for target analytes, preconcentration of analytes, elimination of complicated natural matrices (i.e. salts from seawater) for instrumental analysis, and simultaneous detection of contaminants, makes DGT samplers an attractive option for environmental monitoring, especially in remote and harsh environments. However, their usefulness and the practicality of their application to the Antarctic marine environment is unclear to environmental managers (Stark et al. 2006a).

Chapter 6 determined diffusion coefficients, limits of detection, method detection limits, and recommended deployment times for DGT in polar marine conditions. While DGT is predominately used to measure the bioavailable fraction of metal contaminants in the environment (Cabrita et al. 2017; Larner et al. 2006; Padeiro et al. 2016; Stark et al. 2006a), it has recently been used to predict the toxicity of contaminants to organisms (Amato et al. 2018; Remaili et al. 2016). This application was explored in laboratory experiments in Chapter 6 using single and metal mixture ecotoxicological data for *P. antarctica* (Gissi et al. 2015) and *C. armigera* (Chapters 3 and 4). DGT-labile metal concentrations can also inform on the risk of metal bioaccumulation to marine microalgae and their subsequent risk to the nearshore marine foodweb (as discussed in Section 5.4.3).

This chapter aims to validate the performance of the DGT technique for environmental monitoring and risk assessment in Antarctic marine waters, confirming the results presented in the laboratory study in Chapter 6. This will be achieved in a case study deploying DGT samplers in the nearshore marine environment at sites possibly impacted by the currently operating Casey Station and the abandoned Wilkes Station. The risk of mixtures of metal contaminants from anthropogenic activity to the nearshore marine environment will be assessed using DGT-labile concentrations from *in-situ* deployments with existing toxicological data for Antarctic organisms (such as provided in Chapter 3 and 4) and national environmental quality standards.

7.2 Methods

7.2.1 Sampling locations

Eleven sites in Newcomb Bay, in the Windmill Islands region in East Antarctica, were selected for sampling and DGT deployment (Figure 7.1). Their proximity to areas of anthropogenic disturbance, including the abandoned Wilkes station and the currently operational Casey station, the surrounding environment type, and meltwater flows based on personal observation and reports from Fryirs et al. (2015), were considerations in identifying deployment sites to give a range of potentially impacted and control sites.

7.2.2 DGT synthesis and deployments

In Antarctica, four DGT samplers were attached to acid-washed polypropylene baskets with nylon thread. The prepared DGT baskets were stored moist in low-density polyethylene bags at 4 °C until deployment. Moorings were created by connecting a mesh-bag filled with locally collected rocks (devoid of moss or lichens) to plastic buoys (2 large and 1 small) with synthetic rope. The moorings were approximately 5 m in length and designed to ensure the two large buoys remained 2 m under the water, with only the small buoy rising to the surface. This design was used with a goal to prevent significant snagging of the moorings on ice floes or bergs.

Prior to deployment, DGT baskets were attached to the mooring by plastic cable ties. Moorings were deployed to a site after the depth was confirmed to be between 3 and 5 m. Irrespective of the final deployment depth, DGT samplers were positioned 1 m above the rock bag. They were deployed for between 22 and 37 d (Figure 7.1), between December 27, 2017 and February 11, 2018. Dates for deployment were limited by access to the station wharf for inflatable rubber boat launch during the available expedition 2017–2018 summer season at Casey Station.

Following deployment, DGT devices were disassembled and the binding resin placed in 1 mL of 1 M HNO₃ (Suprapur grade, Merck Millipore) for ≥12 h on an orbital shaker. The eluents were diluted to a final concentration of 0.2% HNO₃ before metal analysis in Australia.

7.2.3 Sampling and measurements

Water sampling occurred from the side of an inflatable rubber boat at each marine site. All water samples and measurements were taken at a depth of approximately 30 to 50 cm below the surface to avoid the layer of less saline seawater that develops on the sea surface from sea-ice melt and were returned to the station laboratories for physicochemical analysis and processing for subsequent metal analysis on return to Australia.

Physicochemical parameters of seawater samples including salinity (PSU), dissolved oxygen (mg L⁻¹), and pH were measured using a Professional Plus Multiparameter Instrument (YSI, USA), calibrated as per manufacturer's instruction. Seawater for metal analysis was sampled and filtered (0.45 µm, Sartorius) directly to plastic vials. Physical weather observations including hours of sunlight and maximum and minimum air temperatures were taken from recordings published by the Australian Bureau of Meteorology's weather station at Casey Station (weather station 300017).

Following deployment, moorings were retrieved and DGT samplers recovered from the cages, rinsed with ultrapure water, and returned to the Casey Station laboratories. DGT devices were disassembled and the binding resin eluted as outlined in Chapter 6.2.1. Binding resin eluents were returned to Australia for metal analysis as outlined in Chapter 2.1.3. Two certified reference materials of natural freshwaters were analysed to validate instrument measurements, TM-24.4 (lot 0916) and TMDA-64.3 (lot 0317) (National Research Council, Canada). Metal detection limits of the ICP-AES were 0.1 µg Cd L⁻¹, 0.6 µg Cu L⁻¹, 0.5 µg Ni L⁻¹, 1.4 µg Pb L⁻¹, and 0.1 µg Zn L⁻¹.

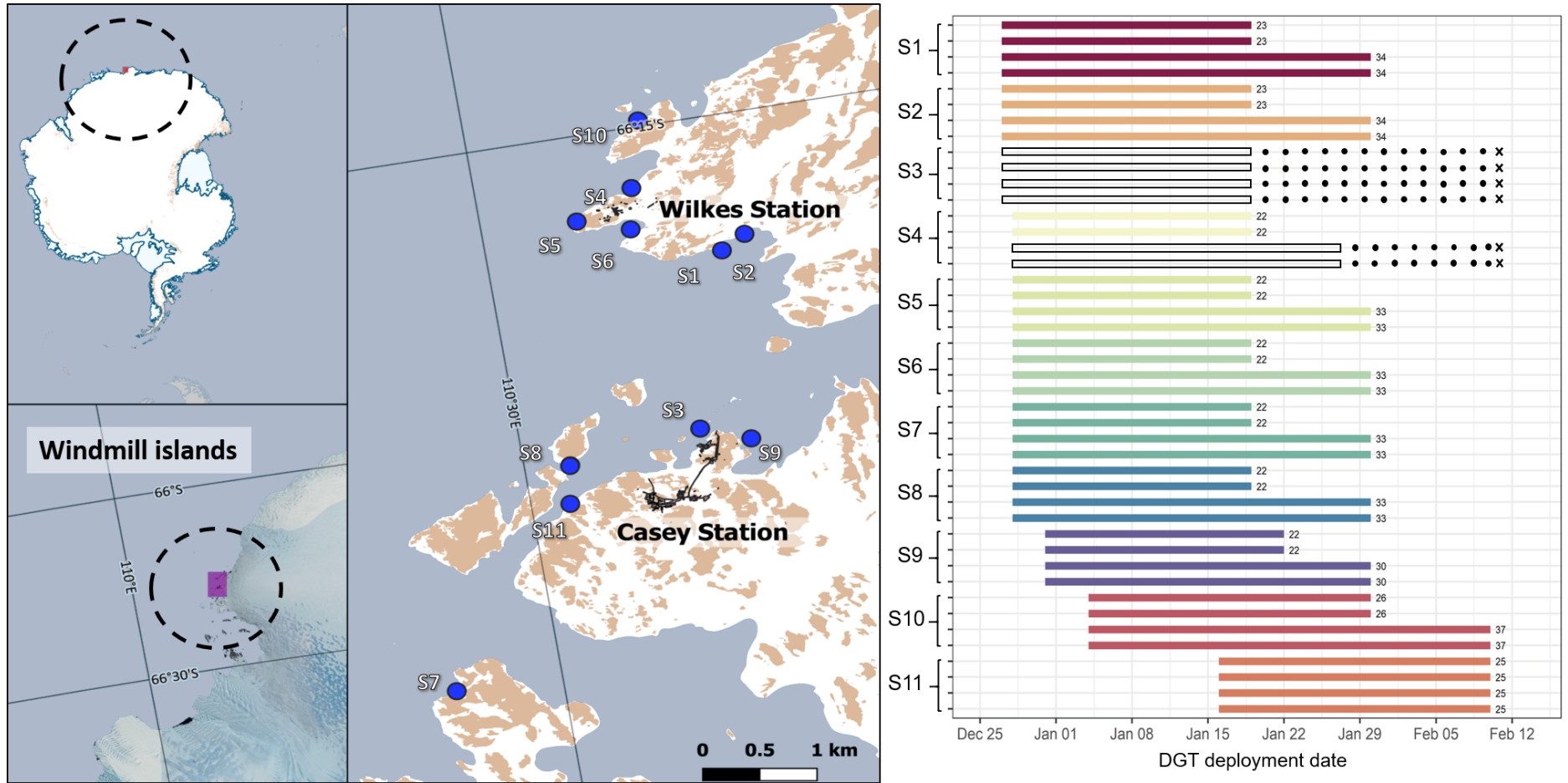


Figure 7.1: Locations and deployment durations of diffusive gradients in thin-films around Casey and Wilkes stations in the Newcomb Bay area, Windmill Islands, East Antarctica. Each bar represents one DGT deployed by mooring at a site. The number at the end of the bar indicates total days of deployment. Lost moorings are represented by an unfilled bar (indicating the dates its location was known), followed by dots indicating the period it was missing. The search for the lost moorings was terminated on the date indicated by the x.

7.2.4 Data analysis

The measured metal concentration from DGT eluants were converted to a mass of metal (M_i) accumulated to the binding resin by Equation 6.1, and converted to DGT-labile concentrations by Equation 6.3.

DGT-labile concentrations were used in mixture models to determine DGT-predicted toxicities as outlined in the laboratory validation study in Section 6.2.4 using independent action (IA, Equation 4.5) and concentration addition (CA, Equation 4.7) reference mixture models. To assist in interpreting the risk of DGT-labile concentrations, a risk quotient approach was adopted using the Australian and New Zealand marine water quality guidelines values for 99 and 95% species protection (ANZG 2018). The risk quotient was defined for each site by Equation 7.1:

$$\sum_{i=1}^n = \frac{C_{DGT}}{WQGV_i} \quad (7.1)$$

where $WQGV_i$ represents the water quality guideline value for 99 or 95% species protection for metal i , which are 0.7 and 5.5 $\mu\text{g Cd L}^{-1}$, 0.3 and 1.3 $\mu\text{g Cu L}^{-1}$, 7 and 70 $\mu\text{g Ni L}^{-1}$, 2.2 and 4.4 $\mu\text{g Pb L}^{-1}$, and 7 and 15 $\mu\text{g Zn L}^{-1}$, respectively.

7.3 Results

7.3.1 Seawater physicochemical properties

The climate for the deployment period was typical of summer at Casey Station. This was characterised by: rising sea temperature from $-1.2\text{ }^{\circ}\text{C}$ in December to $-0.2\text{ }^{\circ}\text{C}$ in the middle of January, decreasing to $-1.2\text{ }^{\circ}\text{C}$ again by February; decreasing dissolved oxygen (DO) concentration from 14 mg L^{-1} to 12 mg L^{-1} ; pH's of around 7.8 to 7.9; and increasing salinity from 29 to 35. The physicochemical properties of the nearshore marine environment, averaged across all sites, are given in Figure 7.2. All measurements were within the expected range for the nearshore marine environment, based on previous studies.

7.3.2 Metal concentrations at seawater deployment sites

Seawater dissolved metal concentrations taken at deployment sites were typically below instrument detection limits throughout the deployment period (see Section 7.2.3). Some sites had nickel and zinc concentrations around $1\text{ }\mu\text{g L}^{-1}$, and cadmium concentrations around $0.1\text{ }\mu\text{g L}^{-1}$; however, there were no clear temporal or spatial trends explaining these concentrations (Appendix A7.2).

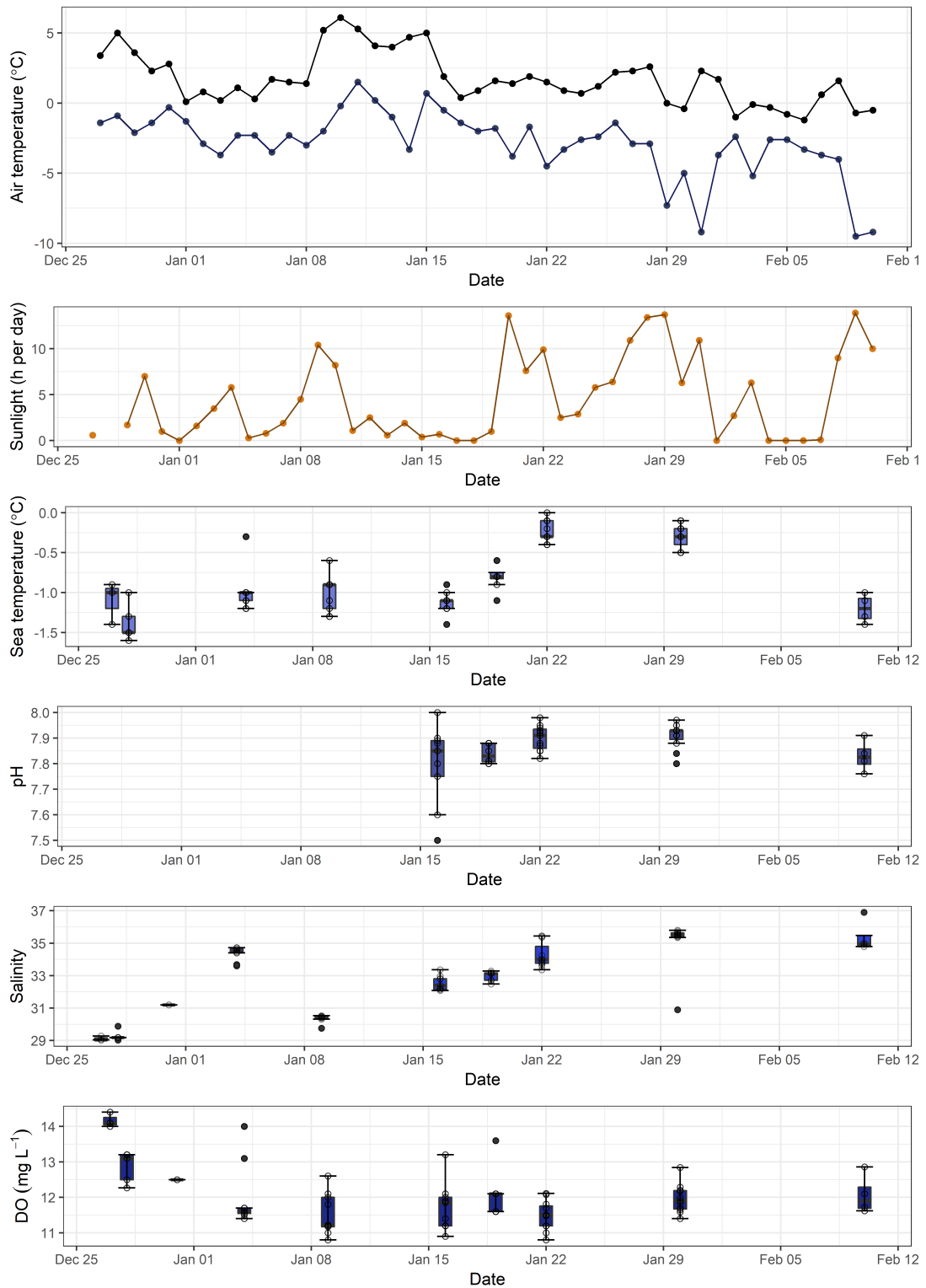


Figure 7.2: Physicochemical conditions throughout the deployment period of diffusive gradients in thin-film. Sunlight photo period and air temperature (day minimum and maximum) data taken from the Australian Bureau of Meteorology’s automatic weather station for Casey Station (Bureau of Meteorology 2018). Error bars represent 1.5 times the interquartile range with black dots outside those points outliers. An issue with the calibration of the pH probe prevented reliable measurements before January 15 2018.

DGT-labile concentrations from deployment sites were low and typical of clean nearshore marine environments (Cabrita et al. 2017; Kim et al. 2015). Lead was the only metal below detection limits (seawater concentration $<0.2 \mu\text{g L}^{-1}$, the method detection limit for DGT deployments in these conditions, times, and instrument limits of detection). The average DGT-labile concentrations (mean \pm standard deviation, with range of measurements in brackets) for all sites were: cadmium, $0.07 \pm 0.01 \mu\text{g L}^{-1}$ ($0.04 - 0.09 \mu\text{g L}^{-1}$), copper $0.28 \pm 0.08 \mu\text{g L}^{-1}$ ($0.14 - 0.47 \mu\text{g L}^{-1}$), nickel $0.39 \pm 0.07 \mu\text{g L}^{-1}$ ($0.29 - 0.62 \mu\text{g L}^{-1}$), zinc $1.3 \pm 0.5 \mu\text{g L}^{-1}$ ($0.6 - 2.3 \mu\text{g L}^{-1}$) (Figure 7.3 and Table 7.1).

7.3.3 Predicted risk to Antarctic organisms

DGT-labile cadmium, copper, nickel, and zinc concentrations were used to predict toxicity to two microalgae, *P. antarctica* and *C. armigera* using EC10 estimates and slope parameters for the individual metals, cadmium, copper, nickel and zinc. Toxicity mixture modelling predicted low toxicities to both species, with $<5\%$ growth rate inhibition (Figure 7.4).

No single DGT-labile metal concentration exceeded the EC10 or EC50 values for metal exposure to any Antarctica species that have been assessed in previous studies (Gissi et al. 2015; Koppel et al. 2017; Marcus Zamora et al. 2015; Sfiligoj 2013; Sfiligoj et al. 2015). However, DGT-labile copper concentrations (which ranged from $0.14 - 0.47 \mu\text{g L}^{-1}$, Figure 7.3) were close to the EC50 of $1.4 \mu\text{g L}^{-1}$ reported to inhibit larval development in the sea urchin *Sterechinus neumayeri* (King and Riddle 2001).

The risk quotients for 99 and 95% species protection at each site were calculated with DGT-labile cadmium, copper, nickel, lead, and zinc metal concentrations (Figure 7.5). Quotient values >1 imply a risk of toxicity from the metals to 5% or 1% of species, when applying the respective water quality guideline values (ANZG 2018). No site had metal concentrations that exceeded the quotient based on 95% guideline values; however, all sites had at least one DGT sampler which accumulated enough metal to exceed the quotient based on the 99% guideline values (Figure 7.5).

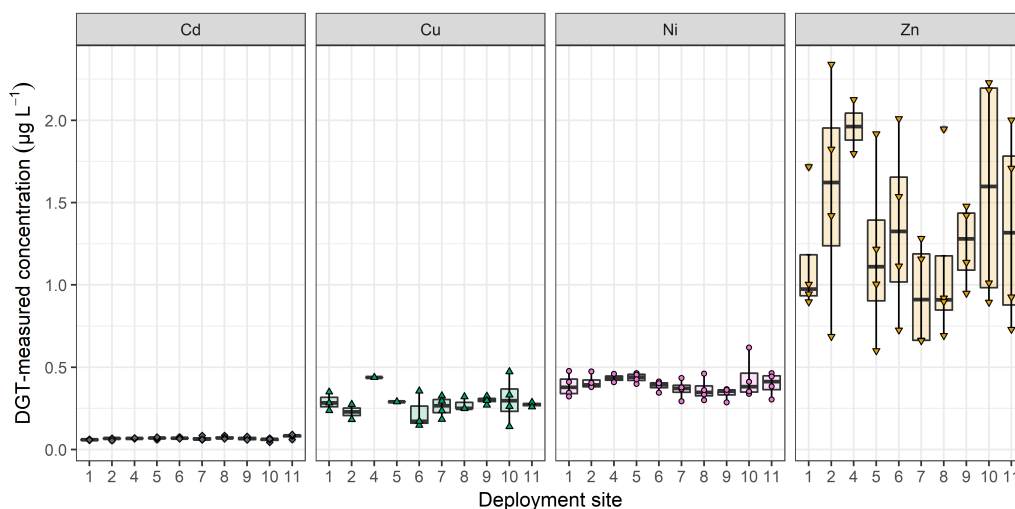


Figure 7.3: DGT-labile cadmium, copper, nickel, and zinc concentrations from 20 to 37-d deployments in the nearshore marine environment of Casey and Wilkes stations, Antarctica.

7.4 Discussion

7.4.1 Metal concentrations

Concentrations of all metals in this study were elevated compared to previous measurements in Antarctica in pristine environments reflective of coastal shelf waters (Sañudo-Wilhelmy et al. 2002) and nearshore environments with inputs from glacial melt (Kim et al. 2015), but were lower than nearshore marine waters immediately adjacent to a research station on King George Island (Cabrita et al. 2017). There were no significant differences in DGT-labile cadmium, copper, nickel, lead, or zinc concentrations between deployment sites. This suggests that the source of the metals is diffuse and geogenic in origin. In Antarctica, this could be from sediment resuspension (Husmann et al. 2012), sea ice melting (Frache et al. 2001), volcanic or hydrothermal inputs (Mão de Ferro et al. 2013), or deposition from terrestrial ice melt and the weathering of rocks (Szopińska et al. 2018).

Interestingly, no elevation in metal contaminants were observed from sites near penguin colonies, which have been proposed to be a major source of metal contaminants on the West Antarctic Fildes Peninsula and Ardley Island (Chu et al. 2019). In this study, Sites 4, 8, 10, and 11 were in close proximity to large Adelie penguin colonies, and had significant volumes of melt stream runoff from the colonies to the marine sites (personal observation). This would imply that the metal contaminants were insoluble, retained in guano, or were bound to organic carbon rendering them less bioavailable.

Table 7.1: DGT-labile concentrations from deployments to nearshore marine sites around Casey and Wilkes station, East Antarctica, compared to previously reported nearshore seawater concentrations. Values are mean \pm standard deviation (μgL^{-1}). Measurements from literature are averaged where multiple sites were tested.

	This study	Cabrita et al. (2017)	Kim et al. (2015)	Sañudo-Wilhelmy et al. (2002)
Location	Newcomb Bay	Ardley Cove	Marion Cove	Weddell Sea
Method	DGT	DGT	Chelex-100 ^a	Solvent extraction ^b
Cd	0.07 \pm 0.01	0.20 \pm 0.04	0.0031 \pm 0.0005	0.05 \pm 0.02
Cu	0.28 \pm 0.08	1.7 ^c	0.04 \pm 0.03	0.012 \pm 0.02
Ni	0.39 \pm 0.07	1.6 ^c	0.033 \pm 0.004	0.034 \pm 0.03
Pb	<0.2 ^d	0.43 \pm 0.05	0.016 \pm 0.007	0.003 \pm 0.002
Zn	1.3 \pm 0.5	2.0 \pm 0.41	0.10 \pm 0.06	0.26 \pm 0.09

^a Preconcentration of collected seawater to Chelex-100

^b Using ammonium pyrrolidine dithiocarbamate and diethylammonium

^c Approximated from figure 3 of Cabrita et al. (2017)

^d The Method Detection Limit for that element for a 37-d deployment

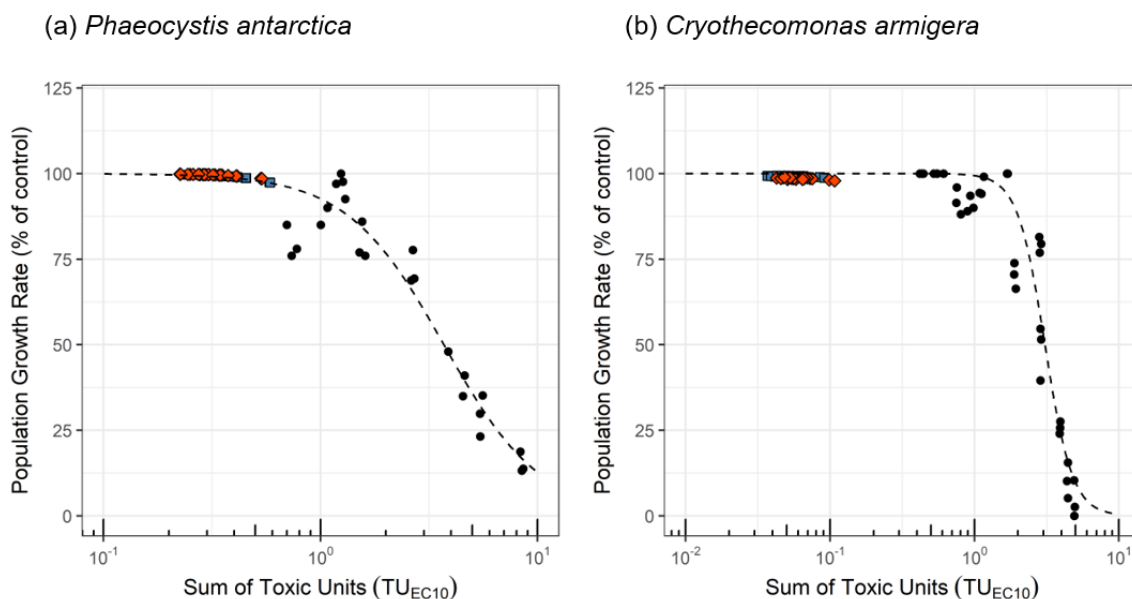


Figure 7.4: Predicted metal-mixture toxicity from DGT-labile metals to the Antarctic microalgae (a) *Phaeocystis antarctica*, (b) *Cryothencomonas armigera*. Blue squares and orange diamonds represent independent action and concentration addition reference mixture model predictions from DGT-labile metal concentrations from each DGT. All sites are plotted together as there were no significant difference between metal concentrations at each site. Black circles are observed toxicity from laboratory exposures of a mixture of 5 metals overlaid with a log-logistic model (black dotted line) from Chapter 4.

7.4.2 Predicted toxicity

For most Antarctic marine organisms, including the microalgae examined in this study, copper is the most toxic metal investigated. Copper was the only metal found to possibly be a risk to marine organisms, with concentrations near the EC50 value for the sea urchin *S. neumayeri* (EC50 of $1.4 \mu\text{g L}^{-1}$, King and Riddle (2001)). There was no difference in copper concentrations between sites (Figure 7.3), suggesting a geogenic rather than anthropogenic source. This is consistent with previous findings of bioavailable copper concentrations in Antarctic nearshore marine waters, both near research stations such as at Ardley Cove, King George Island (Cabrita et al. 2017) or Brown Bay, East Antarctica (Larner et al. 2006) and away from research stations, such as O'Brien Bay, East Antarctica (Larner et al. 2006) or Marion Cove, King George Island (up to $0.1 \mu\text{g Cu L}^{-1}$, Kim et al. (2015)), which is reflective of background surface Southern Ocean copper concentrations (Löscher 1999).

The approach to predicting metal-mixture toxicity, based on independent action or concentration addition reference models, was applied to *P. antarctica* and *C. armigera*. The response of other Antarctic species could be predicted using these same methods if the parameters needed for the mixture models are reported, and toxicity data for the range of individual metals within mixtures are available. Future studies should report the model parameters used to derive effects concentrations to facilitate their use in toxicity reference mixture models in ecological risk assessments (Nys et al. 2018).

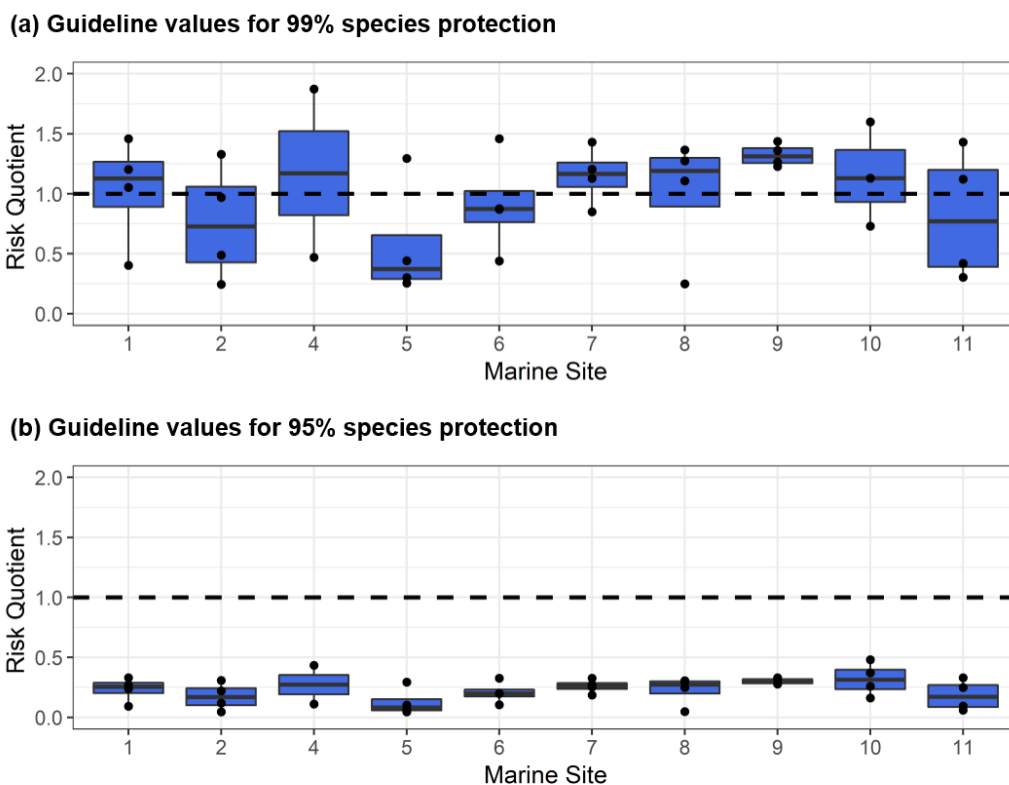


Figure 7.5: Predicted risk to the marine ecosystem based on the Australian and New Zealand domestic water quality guideline values for (a) 99% and (b) 95% species protection (ANZG 2018). Dotted line indicates a risk quotient of 1, values above which are equivalent to exceeding water quality guideline values.

7.4.3 Practicalities of using DGT samplers in the Antarctic marine environment

DGT sampler deployment in the nearshore marine environment in Antarctica was manageable, but not without its challenges. Biofouling of the DGT windows was apparent after approximately 21 d (Appendix A7.3). This was unexpected as previous studies in Antarctica using DGT have not reported biofouling (Cabrita et al. 2017; Padeiro et al. 2016; Stark et al. 2006a), even at deployment times of 29 d (Larner et al. 2006). Biofouling is dependent on the productivity of the receiving environment, which in the nearshore ecosystem is subject to intense microalgae blooms.

Biofouling is known to inhibit the diffusion of some metals to the binding resin in DGT, particularly cadmium, nickel, and zinc (Uher et al. 2012). In field deployments, this impact is difficult to quantify or assess, and so measurements from DGT with significant biofouling must be interpreted with caution (Österlund et al. 2016). In this study, DGT samplers were retrieved at two time points from Sites 1, 2, 5, 6, 7, 8, 9, and 10 (Figure 7.1). A comparison of metals concentrations in DGTs between these time points did not suggest that biofouling reduced measured metal concentrations. However, this comparison assumes comparable seawater conditions and labile metal concentrations, which we believe likely given the large overlap (i.e. >21 d) in deployment durations.

A minimum deployment period of 14 d was recommended from the results of the laboratory study in Chapter 6 for the purposes of achieving minimum detection limits for environmental management. The results of this study suggest that metal-uptake to DGT is unlikely to be affected by biofouling for deployment times <21 d. Therefore, a deployment period of 14 to 21 d is recommended for contaminant monitoring in the nearshore marine environment in Antarctica as a compromise between improving detection limits and minimising interferences from biofouling. There were initial concerns around the introduction of plastic waste and non-native microbes to the Antarctic marine environment from the moorings. These were addressed through the use of rocks sourced from the Casey quarry, which historically had been crushed for use as road and building base. The mooring setup with subsurface buoys survived significant ice floes, with only two moorings lost (presumably by iceberg ensnarement). However, some moorings were dragged around the nearshore environment during their deployment and were found 10–50 m from their original deployed locations.

7.4.4 Considerations when using DGT to predict contaminant risk

Without the ability to link the presence of contaminants to environmental harm, assessing environmental “impact” is difficult. This study demonstrates how DGT samplers are useful tools to assess metal concentrations and consider the risk they pose to the marine environment. Linking DGT-labile concentrations with toxicity thresholds for Antarctic marine organisms (including in the use of reference mixture models) or national environmental quality standards provides a benchmark against which contaminant impact or risk can be assessed.

Care should be taken when applying national environmental quality standards as they are based on the response of endemic organisms, are developed for different purposes, and may have specific caveats about environmental conditions that need to be considered. However, long-standing environmental quality standards (such as those from Europe, Canada, the United States, and Australia and New Zealand) are generally robust, as evidenced by the push for global harmonisation (Merrington et al. 2013) and their history of application in Antarctic contamination assessments (Chapman and Riddle 2005; Crockett 1997; Fryirs et al. 2015; Hill et al. 2009; Snape et al. 2001; Stark et al. 2006a). Furthermore, as the sensitivities of all tested Antarctic marine organisms to metals has been within the range for similar temperate organisms, the comparison of metal concentrations to Australian and New Zealand environmental quality standards is justified until the development of Antarctic-specific guidelines.

Using this approach will help environmental managers assess their impact to the Antarctic environment and their obligations under the Protocol, for example in environmental impact assessments (Hemmings and Kriwoken 2010), triaging the need to remediate contaminated sites (Raymond and Snape 2017), or making decisions about the protection of cultural heritage that may be causing environmental harm (Camenzuli et al. 2015).

DGT samplers deployed in marine waters measure the dissolved, labile fraction of metal contaminants. This is believed to represent the bioavailable metal fraction (Puy et al. 2016). However, dietary exposure of metals via particulate matter (Hill et al. 2009), phytoplankton (Cabrita et al. 2017), or higher-order organisms (Trevizani et al. 2018) may be important routes of exposure that also need to be investigated. For example, Stark et al. (2006b) reported that approximately 4–8 m³ of solid material was deposited into Brown Bay, adjacent to Casey Station, by melt streams each year. DGT samplers have been used in temperate and tropical sediments to assess the bioavailable metal flux from sediments to overlying waters, which has shown good agreement with toxicity and bioaccumulation to benthic organisms (Amato et al. 2018, 2015, 2014, 2016; Remaili et al. 2016). As metal concentrations in Antarctic sediments and benthic organisms are commonly reported (Ianni et al. 2010; Lenihan et al. 1990; Palmer et al. 2010; Stark et al. 2014; Trevizani et al. 2016; Vodopivec et al. 2015), DGT shows promise to assess the risk of contaminated Antarctic sediments.

So far, only DGT samplers with Chelex-100 binding resins have been used in Antarctica to assess metal contamination. However, other binding resins are commercially available which can examine the lability of other contaminants, including organic and micropollutants which are seen as emerging threats to the Antarctic nearshore marine environment (Emnet 2018). Future research should validate these binding resins for use in Antarctic marine environments.

7.5 Conclusion

This study demonstrates how DGT samplers deployed to Antarctic marine waters can assess the risk of metal contaminants. DGT were shown to give time-averaged metal concentrations with low variability, were easily deployed and recovered, and had low method detection limits. A deployment time of between of 14–21 d is recommended for Antarctic waters as a compromise between biofouling, method detection limits, and the temporal variability of contamination. Based on the results of this field study, DGT are considered be a more simple metal contaminant measurement technique than alternatives like repeat direct measurements coupled to analyte pre-concentration steps.

The DGT-labile concentrations measured in this field study were similar to previous reports for nearshore marine environments near research stations, highlighting that DGT as a robust chemical monitoring technique. The toxicity of the DGT-labile metals was assessed *in silico* using previously determined ecotoxicology data for the Antarctic microalgae *P. antarctica* and *C. armigera*. This shows that DGT can be a reliable surrogate for biological testing.

The process outlined in this study should be included in the Committee for Environmental Protection's Clean-up Manual and Guidelines for Environmental Impact Assessment in Antarctica to strengthen metal contaminant risk assessment in Antarctic marine waters.

CHAPTER 8

General discussion and
conclusions

*I did then what I knew how to do.
Now that I know better, I do better.*
Maya Angelou

THIS thesis provides information and tools that can be used by environmental managers to better assess contaminant risk in the Antarctic nearshore marine environment. There is a growing recognition from national Antarctic programs that more needs to be done to limit our impact in the Antarctic environment. This is particularly important given the growing presence from new stations, expanding research programs, and tourism.

Managing contaminants in the Antarctic environment is challenging due to the harsh conditions, isolation, and operational costs. But these are challenges that every activity in Antarctica faces. Nations operating in Antarctica cannot be complacent about their environmental impact while expanding their presence. The Antarctic Treaty System and the Protocol provide a strong governance framework to achieve the vision of maintaining Antarctica as a "natural reserve". This vision can only be achieved when the Protocol is supported by guidelines and processes that enable national programs to make informed decisions to minimise their impacts.

8.1 Assessing contaminant impacts in the Antarctic nearshore environment

This study improved our capacity to measure and assess contaminant impacts to the Antarctic nearshore marine environment by contributing ecotoxicological data for five metals to the microalga *C. armigera* (Chapter 3) and for metal mixtures to the microalgae *P. antarctica* and *C. armigera* (Chapter 4). Both microalgae were relatively tolerant to all metals, except for copper. This is fairly consistent with microalgal responses to chronic metal toxicity; however, there is wide range of sensitivities between species.

8.1.1 The toxicity of five metals to *Cryothecomonas armigera*

C. armigera was found to be a moderately tolerant species to metals (Chapter 3). It occupies a different ecological niche to *P. antarctica*, the only other Antarctic microalgae for which ecotoxicological data exists. The long developmental times of Antarctic organisms make deriving toxicity data and environmental quality standards challenging. Each organism tested requires the development of its own protocol to account for its lifecycle. *C. armigera* required 24 d to increase its cell density 16-fold, compared to the 2 to 3 d for most temperate or tropical microalgae (OECD 2011). The population growth rate EC10s for copper, lead, zinc, cadmium, and nickel were 22, 150, 370, 450, and 1200 μgL^{-1} , respectively. At the concentrations tested, only copper and nickel were sufficiently toxic to enable the derivation of EC50 values of 63 and 1570 μgL^{-1} , respectively. All metals affected the cellular physiology of *C. armigera*, including cellular chlorophyll *a* fluorescence, cell complexity and size, and lipid concentrations. However, no changes to cellular membrane permeability were observed. The reduction in cellular lipid concentrations was a more sensitive indicator of toxicity for cadmium, nickel, and lead than growth rate inhibition with EC10 values of 89, 894, and 11 μgL^{-1} , respectively, highlighting its potential as a sensitive measure of metal toxicity.

More ecotoxicological data is needed to derive Antarctic-specific environmental quality standards because there is still uncertainty about the relative toxicity of comparative species in polar, temperate, or tropical environments, and there is still a paucity of toxicological data for some classes of Antarctic organisms, particularly for microalgae (Chu et al. 2018), krill, and fish. The sensitivity of *P. antarctica* and *C. armigera* to the metals tested were broadly within the range of those of temperate microalgal species 8.1, but there is not enough data to draw ecosystem-wide conclusions. More ecotoxicology data is needed, particularly of microalga and krill. For example, no toxicity data exists for diatoms (Bacillariophyceae), which are the dominant class of microalgae in the nearshore marine environment and in sea-ice epontic communities (Alderkamp et al. 2012). Furthermore, the Antarctic krill *Euphasia superba* is considered a keystone species of the Southern Ocean food web and is cultured by the Australian Antarctic Division. However, only their sensitivity to persistent organic pollutants and microplastics have been tested (Dawson et al. 2018a,b). This should be expanded to include metals, particularly given the risk they pose from dietary exposures, shown in Chapter 5.

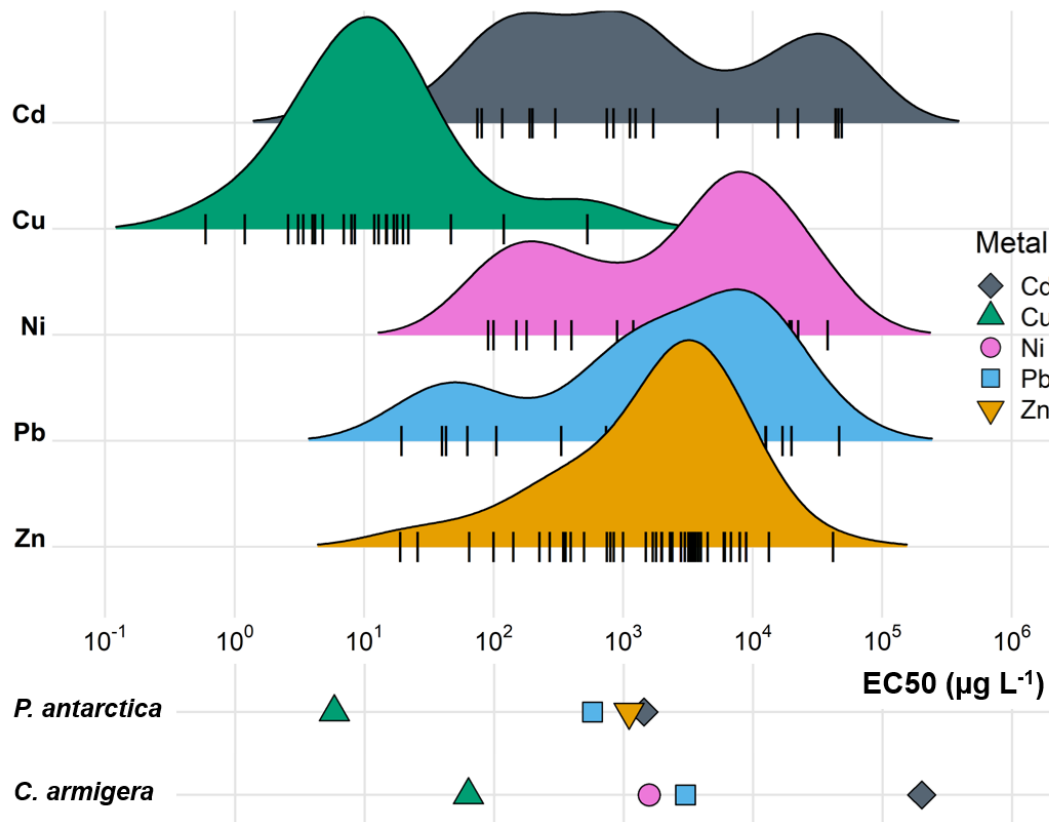


Figure 8.1: Comparison of the sensitivity of temperate and polar microalgae to cadmium, copper, nickel, lead, and zinc. Histograms of marine microalgae chronic EC50 values for population-related endpoints were taken from the USEPA ECOTOX database. These data are for comparative purposes only and do not represent a comprehensive review of temperate microalgal toxicity. Note that the EC50 values for *P. antarctica* and *C. armigera* are largely within the range of other temperate microalgal sensitivities.

Accurate toxicity estimates for Antarctic organisms can be difficult to derive because of the challenges associated with acquiring and culturing Antarctic organisms. Adopting the statistical approaches outlined in this study, as recommended by Gerhard et al. (2014) and Ritz et al. (2015), will give more robust effect concentration thresholds and reduce the error of their prediction. This is important because error does not carry through into species sensitivity distributions, the main tool used to derive ecosystem-level contaminant guideline values (Wheeler et al. 2002), nor does it carry through to some approaches in toxicity mixture modelling (e.g. a toxic unit approach like that used by Franklin et al. (2002b)). Both of these challenges can be overcome through the incorporation of an optimisation step, as outlined in Chapter 4 and developed by Hochmuth et al. (2014) and Jonker et al. (2005). However, this requires the error of model parameters to be published. This study generated the toxicity estimates using these best practice statistical approaches (Chapter 3). For example, by using parametric modelling (Ritz et al. 2015), including both inter- and intra-experimental variability into estimate derivation (Gerhard et al. 2014), and fitting toxicity models to individual responses rather than summary values, i.e. the mean of replicates (Warne and Dam 2008), lower-error toxicity estimates will be generated.

The toxicity of metals to microalgae is modified by environmental factors including the presence of DOC and should be understood in the unique Antarctic marine context. The characteristics of DOC are known to vary depending on the source (Holland et al. 2018), which can alter its metal-binding capacity (Luider et al. 2004; Schwartz et al. 2004). The Antarctic nearshore marine environment does not have the same terrestrial inputs of DOC as other marine environments (i.e. degradation of plant material). Therefore the endemic DOC is mostly microbially derived. While some characterisation has occurred, particularly for the exudates of *P. antarctica* (Alderkamp et al. 2007; Solomon et al. 2003), it is unknown to what extent this DOC will modify the toxicity of metals. This is particularly important in the Antarctic nearshore marine environment because the greatest flux of contaminants to the nearshore marine environment will occur in summer, concurrent to the bloom of marine microalgae (Schoemann et al. 2005). Furthermore, microalgae including *P. antarctica* use exudates to make trace metals more bioavailable in the Southern Ocean (Thuróczy et al. 2012). However, metal-DOC speciation changes were not observed to affect DGT-lability in the exposures outlined in Chapter 6. Thus, DOC characterisation and its impacts on metal speciation and toxicity warrants further investigation.

Salinity can affect metal speciation and toxicity. The salinity of the Antarctic nearshore marine environment is particularly dynamic due to salt exclusion from sea-ice formation and freshening from the melt of sea and terrestrial ice melt. Increasing salinities can promote greater mobility from sediments to the dissolved phase through increased competition for sediment binding sites by salt ions (Zhao et al. 2013). In addition, increased salinity promotes the formation of metal carbonate and sulphate complexes, which are considered less toxic and less soluble than the free ion of the metal (Niyogi and Wood 2004).

Decreasing salinity can decrease pH, leading to changes in metal speciation, bioavailability, and toxicity (Millero et al. 2009; Riba et al. 2004; Stumm and Morgan 1996). These processes will also be affected by the low temperatures of Antarctic seawater (-2 to 0 °C) which will reduce metal solubility and slow complexation kinetics (Byrne et al. 1988).

The effects of salinity to metal speciation and toxicity should be investigated in the context of other Antarctic environmental factors (like changing pH and the presence of DOC) to give a more holistic understanding of metal toxicity in the Antarctic marine environment.

8.1.2 The toxicity of metal mixtures to *Phaeocystis antarctica* and *Cryothecomonas armigera*

Contaminants are rarely present in the environment singly, yet environmental quality guidelines are derived from single-contaminant toxicity data. Chapter 4 found that mixture toxicity interactions can be ratio, species, and concentration dependent. Few metal mixture studies have investigated marine organisms or more than two contaminants, and many studies use unrealistically high effect concentrations. This study investigated the toxicity of five metals to the Antarctic marine microalgae *P. antarctica* and *C. armigera*. Both microalgae had similar responses to the equitoxic mixture, being non-interactive by independent action (IA) and antagonistic by concentration addition (CA) models. Toxicity from the environmental mixture was antagonistic by IA to *P. antarctica*; however, to *C. armigera* it was concentration-dependent with antagonism at low toxicities and synergism at high toxicities by both IA and CA. Given the generally low concentrations of metal contaminants (typically $<10 \mu\text{gL}^{-1}$) in nearshore marine environments of Antarctica (e.g. Figure 7.3, Cabrita et al. (2017), Larner et al. (2006), and Padeiro et al. (2016)), antagonism is the likely response to metal mixtures for these two microalgae.

During the course of this study, work has progressed to understand ways to better manage the risk of metal mixtures. The mixture toxicity modelling and significance testing approach developed by Jonker et al. (2005) and Hochmuth et al. (2014) have since been applied in other metal mixture studies (Filimonova et al. 2018; Nys et al. 2015). The use of EC10 values was also incorporated in a risk assessment framework in recent studies that highlighted that toxicity can be observed from mixtures of metals at concentrations equivalent to $<10\%$ toxic effects (Nys et al. 2018, 2017c). Chapter 4 adopted the use of EC10s in mixture toxicity modelling which allowed for the inclusion of metals for which EC50s could not be derived (i.e. cadmium, lead, and zinc, Table 3.1).

Toxicity mixture studies have largely found that the CA reference mixture model is more conservative and thus more suitable for an initial assessment of the risk of mixtures to individual organisms (Nys et al. 2017c; Versieren et al. 2016). This has since been expanded to ecosystem protections through integration with species sensitivity distributions (Nys et al. 2018). This study found either CA or IA reference mixture modelling would be suitable to assess the risk of metal mixtures, at least to these two microalgae.

More investigations are needed to assess the toxicity of contaminant mixtures to other Antarctic organisms. Organisms should be prioritised based on their single-metal sensitivities, as they are most likely to be at risk. For example, the Antarctic sea urchin *Sterechinus neumayeri* is the most sensitive Antarctic organism to copper known so far, with a larval development EC50 of $1.4 \mu\text{gL}^{-1}$ (King and Riddle 2001). Investigations should also prioritise important 'keystone' organisms for the Antarctic marine ecosystem, such as the krill *Euphasia superba*.

8.1.3 Cellular metal accumulation and partitioning

Chapter 5 determined that sub-lethal exposures of metals to *C. armigera* or *P. antarctica* led to metal accumulation that may cause dietary toxicity to higher order consumer organisms. For example, the reported DGT-measured seawater copper and zinc concentrations of up to 3.6 ± 0.6 and $14.5 \pm 0.2 \mu\text{gL}^{-1}$ in Ardley Cove, King George Island (Padeiro et al. 2016), are similar to the multiple environmental mixture exposures to *P. antarctica*, which resulted in cellular concentrations of $100 \pm 40 \text{ amol Cu cell}^{-1}$ and $210 \pm 200 \text{ amol Zn cell}^{-1}$ (Chapter 5), which is likely to cause dietary toxicity to higher order organisms (Bielmyer et al. 2006).

Cellular metal partitioning to these microalgae also explained some of the toxicity observed from exposure to mixtures of contaminants in Chapter 4 (Figures 4.2, 4.3). For both microalgae, copper was the driver of toxicity from the environmental mixture, and the cellular metal partitioning explained some, but not all, toxicity and observed mixture interactivity. For example, zinc was likely protective against copper in the environmental mixtures to both microalgae. However, the cellular metal fractions could not be used to conclusively determine the cause of the mixture interactivity or which metals in the mixtures contributed to it. A full factorial experimental design, starting with binary mixtures stepping up to the quinary mixture would be best to elucidate specific metal-metal interactions. However, this is unrealistic given the number of possible combinations that would be required. For the purposes of environmental protection, testing environmental mixtures to rule out synergism at environmentally realistic concentrations would provide confidence around the use of reference mixture models like IA and CA in risk assessment.

Dietary toxicity in the Antarctic marine ecosystem is understudied, with only a couple studies linking dietary exposure with toxicity (Dawson et al. 2018a; Hill et al. 2009). Most research in the field has focused on the bioconcentration of mercury to Antarctic birds (Calle et al. 2015; Carravieri et al. 2018) or the bioaccumulation of metals as part of contamination assessments, without links to risk or impact (Majer et al. 2014; Mauri et al. 1990; Trevizani et al. 2016). No study has investigated whether microalgae from contaminated sites cause toxicity by trophic transfer of metals, despite studies finding that cadmium, lead, and zinc accumulates in some Antarctic marine species, and has the potential to bioconcentrate (Majer et al. 2014; Santos et al. 2006; Trevizani et al. 2018).

8.2 Assessing the risk of metal contaminants to Antarctic marine organisms

The Antarctic nearshore marine environment is highly variable. Simple chemical monitoring tools are needed to properly measure and interpret the risk contaminants pose in Antarctica. This study validated the performance of DGT under polar marine conditions in the laboratory (Chapter 6) and in a pilot study in the nearshore Antarctic marine environment (Chapter 7).

DGT were identified as a preferred monitoring device because of their benefits compared to other sampling methods because:

- DGT-labile concentrations are time-averaged for their deployment period, thereby incorporating pulse releases of contaminants
- DGT are commercially available, with a variety of binding resins for different analytes
- Analytes are preconcentrated on the DGT binding resin, which allows for low method detection limits
- Preconcentration of analytes excludes the environmental matrix (i.e. the high salt content of seawater), reducing the need for further extractions or dilutions
- A labile fraction of metals are accumulated to DGT, which represents the fraction most likely to be bioavailable
- DGT are deployed *in situ* in the matrix of the ecosystem being assessed
- DGT can simultaneously accumulate contaminants, depending on the binding resin used

Chapters 6 and 7 provide guidance around the use of DGT as a chemical monitoring tool in Antarctic marine conditions. This is the first study to provide diffusion coefficients derived empirically from Antarctic marine conditions, to identify minimum deployment times, and to assess concentrations above which metal competition on the binding resin is likely. DGT were found to be useful monitoring devices because the time-integrated measurement accounts for the high temporal variability of contaminants in the nearshore marine environment (see Section 1.6). Field deployments of ≥ 21 d were believed to capture this variability (i.e. see the range of temperatures and sunlight hours in Figure 7.2) This deployment period also covered the period during the summer peak population at Casey Station (up to 120 people), which leads to greater waste water outflow. However, biofouling of the devices were observed after approximately 21 d, which is known to restrict the uptake of metals to the binding resin. This was not expected because of the cold waters, slow microalgae growth rates, and because no previous study using DGT in Antarctic marine waters have reported biofouling (Cabrita et al. 2017; Larner et al. 2006; Padeiro et al. 2016; Stark et al. 2006a), even in deployments up to 28 d (Stark et al. 2006a). Therefore, deployment times of 14 to 21 d are recommended as a compromise between detection limits, capturing contamination pulse events, and biofouling.

For DGT-labile concentrations to be used to assess metal risk, environmental managers require clear guidance about what the DGT technique actually measure. DGT samplers with a Chelex-100 resin measures the dissolved phase of contaminants ($<0.45 \mu\text{m}$) that are freely available, or easily dissociable, to the iminodiacetic acid functional group (Davison 2016). This excludes metals that are strongly bound to ligands (such as organic carbon), and is the fraction of metals in an environment shown to be most bioavailable to organisms (Amato et al. 2018, 2015, 2014; Jordan et al. 2008). Other routes of exposure, such as dietary toxicity (Chapter 5), can contribute to toxicity and should be investigated as additional lines of evidence in contaminant risk assessments.

Chapters 6 and 7 also outlines a process by which DGT-labile metal concentrations can be used to assess the risk posed by metals in the Antarctic marine environment using previously determined ecotoxicological data for Antarctic organisms. This process has been summarised in Figure 8.2. Chapter 6 showed that DGT-labile concentrations in laboratory deployments were generally equivalent to the dissolved metal concentrations used to derive metal toxicity estimates for organisms in toxicity tests (Figure 6.3). The equivalence of DGT-labile and dissolved metal concentrations in seawater matrices allowed DGT-labile metal concentrations to be incorporated into toxicity modelling using previously determined ecotoxicological data. Chapter 6 applied this process for metal mixtures in a laboratory study. DGT-labile concentrations were used in the reference mixture models outlined in Chapter 4 for *P. antarctica* and *C. armigera* to predict population growth rate toxicity. These toxicity predictions were compared to the known response of both microalgae to the metal mixture (Figure 4.5), which found good agreement (Figure 6.4). This process was then applied in a field study around Casey and Wilkes station (Chapter 7), which predicted low toxicities, <5% growth rate inhibition, to both microalgae (Figure 7.4). This process could be applied to assess the response of any Antarctic organism for which ecotoxicology data exists (Table 1.1). However, there is a risk that mixture toxicity modelling may under or over estimate toxicity depending on whether the organisms in the environment being assessed experiences antagonistic or synergistic mixture interactions.

DGT-labile metal concentrations were also compared to national environmental quality standards in Chapter 7. The water quality guideline values for each metal were derived using toxicity estimates from predominately temperate organisms and the guidelines make reference to factors that modify metal toxicity in predominately temperate marine environments. There are significant differences between temperate and Antarctic marine environments (discussed in Section 1.4.3), that will modify metal toxicity. Therefore, care should be taken when applying environmental quality standards like water quality guidelines. In the absence of Antarctic-specific environmental quality standards, however, the application of environmental quality standards from temperate environments are likely to lead to better environmental management than not using any standards, as discussed in Section 7.4.4.

DGT samplers are increasingly being used by Antarctic national programs, including the Chilean and Portuguese (Cabrita et al. 2017; Padeiro et al. 2016), and Australian programs (Larner et al. 2006; Stark et al. 2006a). This study provides timely guidance around their deployment and use in Antarctica. The application of DGT in Chapters 6 and 7 goes a step further than previous field deployments of DGT by combining DGT-labile concentrations with previously determined toxicological data to assess contaminant risk. This study demonstrates how useful this approach can be for environmental managers of nations operating in Antarctica to meet their obligations for environmental monitoring and impact assessment under the Protocol (Figure 8.2, ATCM 1991).

Future research can validate the use of different binding resins for other contaminants in a manner similar to that outlined in Chapter 6. Persistent organic pollutants or pharmaceuticals are two classes of contaminants for which there is minimal information about environmental concentrations in the Antarctic marine environment (Emnet 2018). DGT samplers can also be applied to soils and sediments to investigate contaminants that can flux from the solid phase to surrounding pore waters.

Sediment-DGT metal fluxes well correlate to toxicity in temperate marine organisms (Amato et al. 2018, 2016; Remaili et al. 2016), and warrants further investigation.

8.3 Application of this study for environmental management in Antarctica

Despite a Protocol based on sound values and principles (ATCM 1991), current practices of environmental monitoring and assessment are limited by the lack of environmental quality standards and data (Hughes 2010; Raymond and Snape 2017). Recently, the Committee for Environmental Protection provided guidance on environmental practices in Antarctica by the introduction of impact assessment guidelines (ATCM 2016), a clean-up manual (CEP 2013), and encouraging information sharing on the Antarctic Environment Portal (Klein et al. 2018).

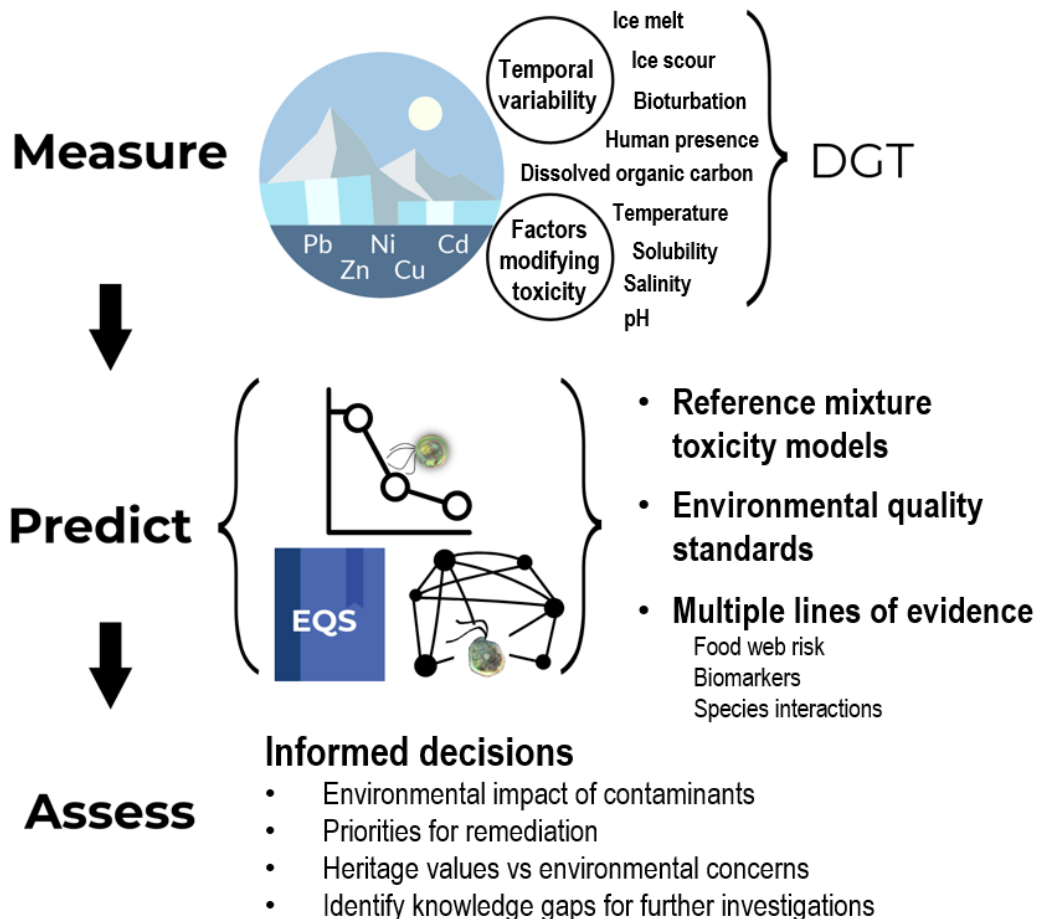


Figure 8.2: process to assess the risk of metal contaminants using diffusive gradients in thin-films and environmental toxicity data. DGT-labile concentrations account for the temporal variability of contaminant concentrations and some factors that modify toxicity. Those concentrations can be used to predict the toxicity metals singly or in mixtures to Antarctic organisms. The risk can be assessed by investigating other lines of evidence like effects to biomarkers and dietary toxicity. This process can help environmental managers make informed decisions.

However, more work is required if the standards of environmental management in Antarctic are to be raised to those of the domestic environmental quality standards of most Antarctic nations. Environmental quality standards are the link that connects the presence of a contaminant to the risk that contaminant has in the ecosystem. Without this context, the reporting of contaminant concentrations cannot be used to assess “impact” (ATCM 1991).

Annex I and III of the Protocol are directly relevant to managing contaminants in the Antarctic environment (ATCM 1991). Annex I outlines the requirements for environment impact assessments. If an activity may cause minor or transitory impact, an initial environmental evaluation is required to assess the impact and consider alternatives. These include building new structures, renovations, or scientific activities. If an initial environmental evaluation indicates that an activity will cause more than minor or transitory impact, a comprehensive environmental evaluation is required. Here, potential environmental impacts are more thoroughly investigated against the pre-impacted baseline state of the site. The comprehensive environmental evaluation is circulated and reviewed by signatory nations to the Antarctic Treaty System and are voted on by consensus at an Antarctic Treaty Consultative Meeting. Annex III requires the generator of historical waste to remove it from Antarctica unless removing waste will cause more environmental harm than leaving it, or where the waste is considered part of a structure designated a historic site or monument. Having an understanding of the impact of contaminants will improve the ability of Antarctic Treaty nations to adhere to these annexes.

The effectiveness of the environmental impact assessment process has been questioned (Pereira et al. 2017), because field studies have demonstrated inadequate protection across the continent (Cunningham et al. 2005; Kennicutt et al. 2010, 1995; Padeiro et al. 2016; Pereira et al. 2017; Snape et al. 2001) and contaminant monitoring is scarce (Hughes 2010). Similarly, very few contaminated sites have been remediated (ATCM 2006; Errington et al. 2018; Roura 2004; Stark et al. 2006b). To assist nations, the Committee for Environmental Protection developed Guidelines for Environmental Impact Assessment in Antarctica (ATCM 2016) to provide recommendations around impact identification stating “*the purpose of impact evaluation is to assign relative significance to predicted impacts associated with an activity (and the various identified alternatives)*”, where significance is defined as “... *a value judgement about the severity and importance of a change in a given environment or environmental value or resource*”. The guidelines recommend using previous assessments to assign significance on a case-by-case basis. The significance of the impact assigned being a determinant of the level of reporting and the need for monitoring.

When this process is applied to contaminants, a link between contaminant presence and impact is missing. Environmental quality standards are commonly used for this purposes across many nations (ANZG 2018; European Parliament and Council 2008; Stephen et al. 1985), but do not yet exist for Antarctica. In this way, many national programs operate to a lower standard of contaminant impact assessment in Antarctica while enforcing robust practices domestically. By developing Antarctic-specific environmental quality standards, Antarctic Treaty nations will also be able to better review each other’s environmental impact assessments (Hemmings and Kriwoken 2010).

These assessments will become more important in time in the context of worsening impacts of existing contamination from climate change related warming (McIvor 2014), and a growing anthropogenic footprint (Brooks et al. 2018; Hodgson-Johnston et al. 2017). While several nations, most notably Australia, continue to work towards the development of Antarctic-specific environmental quality standards, DGT and predictive ecotoxicological modelling can be used to assess the risk metal contaminants pose in the Antarctic nearshore marine environment.

8.4 Future research directions

This research has made significant contributions to metal contaminant measurements and toxicity assessments in Antarctic waters. It has also included a field campaign to Casey Station, East Antarctica. Studies to better understand factors that modify metal toxicity and to validate the use of the DGT technique in sediments and soils are ongoing, and are detailed below.

Development of an acute test for a terrestrial Antarctic microinvertebrate

The microinvertebrate rotifer *Adineta sp.* was isolated from the terrestrial macroalga *Prasiola crispa* in Antarctica in collaboration with Dr Kathryn Brown (Australian Antarctic Division) and Mr Gwilym Price (University of Wollongong). The acute toxicity of cadmium, copper, nickel, lead, and zinc were determined singly and in mixtures in 2 mL water exposures assessing immobility as an endpoint. Preliminary analysis suggests that *Adineta sp.* is sensitive to lead, copper, and zinc with EC50 values (and 95% confidence intervals) of 5 (0–17), 19 (6–32), and 42 (9–75) μgL^{-1} , respectively.

Factors modifying toxicity

DOC has shown to have regionally specific optical characteristics in temperate and tropical freshwaters (Holland et al. 2018), which are likely to affect how they interact with organisms (e.g. through allelopathic interactions, Long et al. (2018) and how they bind to metal contaminants). Water samples (<0.45 μm filtered) were collected from the marine sites of Chapter 7, Figure 7.1, and will be analysed for dissolved organic carbon concentration and their optical characteristics (by fluorimetric and spectroscopic methods) to understand their influence on metal toxicity, particularly through the development of the summer algal bloom (Holland et al. 2018).

Salinity is known to affect metal speciation, but its impacts in Antarctic marine conditions where low temperatures affect solubility and kinetic rates of metal-complex formation are unknown. Antarctic marine microalgae experience a wide range of salinities because of freshwater inputs from ice melt and brine rejection from sea ice formation. The toxicity and bioavailability of cadmium, copper, nickel, lead, and zinc in different salinities were assessed to *P. antarctica* using the toxicity test protocol outlined in Section 2.2. This will help understand how seasonality affects metal toxicity in the nearshore Antarctic marine environment.

Application of DGT to assess contaminants in Antarctic marine sediments and terrestrial soils

The DGT technique has been demonstrated to predict the bioavailability of metals fluxing from sediments to benthic organisms (Amato et al. 2018). This will be investigated in Antarctic conditions using field collected sediments from the nearshore marine environment. Sediments were collected using a grab sampler and equilibrated in laboratory mesocosms in Antarctica for two weeks. DGT probes were deployed to the sediments for 1 to 2 weeks and the flux of metal contaminants from above, below, and at the sediment water interface (Simpson et al. 2012) will be compared to sediment metal concentrations from a range of chemical extractions. DGT-metal flux concentrations will also be compared to metal concentrations bioaccumulated to invertebrates collected *in situ* from the sediments.

The DGT technique was also applied to Antarctic soils, collected from sites around Casey Station, Old Casey Station, and Wilkes Station. Soils were wet with ultrapure water and DGT were deployed for 9 d at 5 °C DGT-labile metal concentrations will be compared to the physicochemical properties of the soils to investigate metal risk to terrestrial organisms.

Factors driving the spatial diversity of Antarctic benthic communities

The use of environmental DNA to assess the health of complex ecosystems is a relatively recent approach that is becoming established in temperate and tropical systems to assess the environmental impact of different stressors (Chariton et al. 2016). DNA was extracted from the field collected Antarctic sediments to investigate the spatial distribution of prokaryotic and eukaryotic communities at the Antarctic nearshore marine sites (Chariton et al. 2015). Physicochemical parameters of the sediments, including DGT-labile metal fluxes, will be compared to the presence and diversity of benthic organisms to assess whether contaminant fluxes are driving changes in benthic communities.

8.5 Suggestions for further research

The derivation of water quality guidelines values for environmental quality standards specific to Antarctic requires more ecotoxicological data. More toxicity data should be collected for a range of Antarctic marine taxa including fish, invertebrates, macroalgae, and microalgae. This process is time consuming and limited by challenges around collecting, culturing, and testing Antarctic organisms. Until Antarctic-specific standards are developed, the use of national environmental quality standards should be considered for environmental management in Antarctica (e.g. Australian and New Zealand water quality guidelines (ANZG 2018), the United States of America water quality criteria (Stephen et al. 1985), the European Union environmental quality standards (European Parliament and Council 2008), etc.). As ratifying the Protocol requires the passing of domestic legislation supporting its goals, national environmental quality standards could be used as the benchmark by which nations assess the risk or impacts of contaminants.

This thesis highlighted the importance of investigating metal contaminants as they exist in contaminated sites, as mixtures. Future metal-mixture studies should be conducted with other Antarctic organisms, particularly sensitive species like the sea urchin *Sterechinus neumayeri*. Full-factorial mixture toxicity studies should be conducted to assess which, if any one, metal is particularly responsible for mixture interactivity. Very few studies in any environment have investigated mixtures of organic and inorganic contaminants should also be conducted (e.g. Filimonova et al. (2018)). Hydrocarbons (such as from diesel spills) are a major source of contamination to the Antarctic environment (Bargagli 2008), but their toxicity in mixtures with metals to Antarctic organisms is currently unknown.

Metals were shown to accumulate in *P. antarctica* and *C. armigera* at sub-lethal concentrations, and were compared to literature to assess their potential dietary risk to grazing organisms. However, with the microalgae intracellular metals can be sequestered in polyphosphate or phytochelatin-bound granules, which may not be bioavailable to grazing organisms (Pochodylo and Aristilde 2017). Dietary exposures of metal-laden Antarctic algae should be investigated, particularly to Antarctic copepods and krill, the taxa that represent the critical link between primary producers and tertiary consumers including penguins and whales (Murphy et al. 2012).

Climate change impacts to contaminant speciation and mobility in the Antarctic nearshore marine environment should be assessed. Changes to metal speciation in seawaters have been assessed for likely future changes in pH (Millero et al. 2009). However, the influence of Antarctic marine conditions to these changes is unknown, but could be modelled using a combination of speciation calculations (Tipping 1994) and climate change projections (Schofield et al. 2010).

8.6 General conclusions

THIS thesis aimed to further our understanding of metal toxicity to the Antarctic nearshore marine environment and develop a process to measure metal contaminants and assess their risks in this dynamic environment. This was achieved by successfully meeting the research objectives:

- i Single-metal toxicity data was derived for five metals to the Antarctic marine microalgae *C. armigera* (Chapter 3). The microalga was most sensitive to copper by population growth rate inhibition, but intracellular lipid concentrations were sensitive to lead and cadmium.
- ii The toxicity of a mixture of five metals to two Antarctic marine microalgae was quantified (Chapter 4). CA and IA gave generally equivalent predictions, meaning either could be used as a first-tier contaminant mixture assessment. Antagonism was observed in both microalgae at low effect concentrations from the environmental mixture. The cause of the antagonism was likely due to zinc competition with copper, as suggested by cellular metal accumulation and partitioning (Chapter 5). Both microalgae accumulated elevated concentrations of metals at sub-lethal exposure concentrations reflective of contaminated sites in the Antarctic nearshore marine environment. Literature extrapolations of metal accumulation in microalgae suggest that the concentrations accumulated by *P. antarctica* and *C. armigera* may cause dietary toxicity to higher order organisms.
- iii The applicability of diffusive gradients in thin-films (DGT) to polar marine conditions for metal contaminant measurements was confirmed. Diffusion coefficients, deployment times, method detection limits, and competition effects were determined in polar marine conditions to provide guidance around their use (Chapter 6). This was validated in a field study in Antarctica, which assessed metal concentrations in the nearshore marine environment around an operating research station and an abandoned research station (Chapter 7).
- iv A process was successfully developed to use DGT-labile concentrations to assess metal risk in the Antarctic marine environment. DGT-labile concentrations were generally equivalent to dissolved metal concentrations in a laboratory study, and able to predict metal mixture toxicity to *P. antarctica* and *C. armigera* (Chapter 6). This was extended to a field study in Antarctica, which combined DGT-labile concentrations with existing ecotoxicological data for the two microalgae and the Australian and New Zealand water quality guideline values to assess the potential risk of an operating and abandoned research station to the nearshore marine environment. The risk of metals in these areas to Antarctic marine organisms was found to be low (Chapter 7).

Environmental managers are continually seeking guidance and tools to make more informed decisions about managing our anthropogenic impact to the Antarctic environment. This thesis provides such tools and guidances, and will help protect the Antarctic nearshore marine ecosystem.

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APPENDIX A1

Appendix to Chapter 1

A1.1 Adelie penguin colony in Antarctica

Penguins inhabit coastal areas of Antarctica. They produce a large amount of organic carbon which fertilises the growth terrestrial macroalgae around the colonies (see the green colouration on the penguins). Personal photo, December 2017.

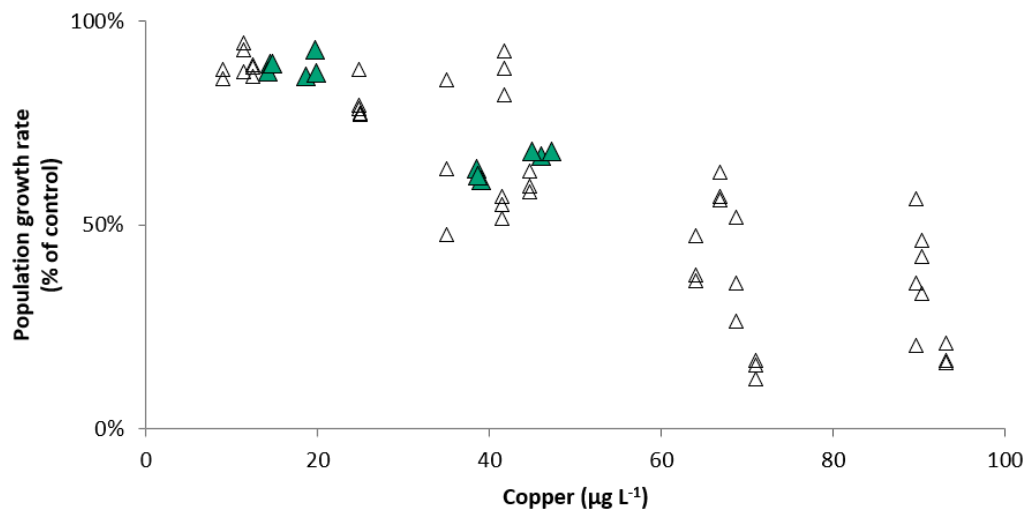


APPENDIX A2

Appendix to Chapter 2

A2.1 Comparison of *Cryothecomonas armigera*'s response to copper under different light types

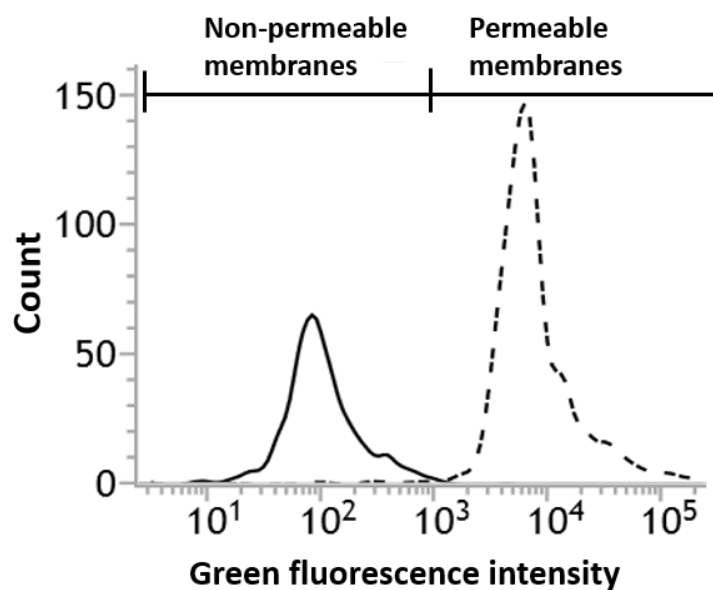
Response of *Cryothecomonas armigera* to copper (as % of control) when tested under fluorescent (grey triangles) and LED (green triangles) lights.



Appendix to Chapter 3

A3.1 Optimisation of a membrane permeability assay with *Cryothecomonas armigera*

The molecular stain SYTOX Green was used in conjunction with flow cytometry to measure membrane permeability. A procedure of 10 μM SYTOX final concentration, incubated for 5 minutes was used. A positive control was created by heat-treating control cells at 60 $^{\circ}\text{C}$ for 5 minutes. The positive control was combined with healthy control cells at a 1:1 ratio and analysed using this method to confirm peak separation. Solid line peak represents healthy control cells treated with SYTOX (non-permeable membranes). Dashed line peak represents heat-treated cells treated with SYTOX (permeable membranes). There were no observable changes in cellular membrane permeability by this method to either *P. antarctica* or *C. armigera* in any metal exposure.



A3.2 Model parameters for the toxicity endpoints of *Cryothecomonas armigera* exposed to cadmium, copper, nickel, lead, and zinc

Parameter estimates generated from models applied to single-toxicity data for *Cryothecomonas armigera* for the endpoints population growth rate inhibition, chlorophyll *a* fluorescence, cellular complexity and size, and cellular lipid concentration. Values are model parameters \pm standard error.

Endpoint	Metal	n	Model type	Inflection point (e)	Slope (β)	Upper limit (c)	Lower limit (d)*
Population growth rate	Cd	36	Mixed effect 4-parameter log-logistic [#]	200000 \pm 300000	0.4 \pm 0.1	100 \pm 1	0
	Cu	75	Mixed effect 4-parameter log-logistic	63 \pm 2	2.1 \pm 0.2	99 \pm 3	0
	Ni	45	Mixed effect 4-parameter log-logistic	1560 \pm 30	9 \pm 1	96 \pm 2	0
	Pb	45	4-parameter log-logistic	3000 \pm 1000	0.7 \pm 0.1	100*	0
	Zn	39	Mixed effect 4-parameter log-logistic	30000 \pm 20000	0.5 \pm 0.1	101 \pm 3	0
Chlorophyll <i>a</i> fluorescence	Cd	36	4-parameter log-logistic	90000 \pm 60000	0.42 \pm 0.07	100*	0
	Cu	75	–	–	–	–	–
	Ni	45	4-parameter log-logistic	2000 \pm 30	6.1 \pm 0.8	100*	0
	Pb	45	4-parameter log-logistic	2200 \pm 200	1.5 \pm 0.3	100*	0
	Zn	39	4-parameter log-logistic	50000 \pm 30000	0.7 \pm 0.1	97.8 \pm 0.7	0
Cellular complexity	Cd	36	4-parameter log-logistic	1110 \pm 40	1.5 \pm 0.1	100*	0
	Cu	75	–	–	–	–	–
	Ni	45	–	–	–	–	–
	Pb	45	–	–	–	–	–
	Zn	39	–	–	–	–	–
Cellular size	Cd	36	–	–	–	–	–
	Cu	75	4-parameter log-logistic	200 \pm 100	1 \pm 1	97 \pm 5	0
	Ni	45	4-parameter log-logistic	4000 \pm 1000	1.3 \pm 0.4	100*	0
	Pb	45	–	–	–	–	–
	Zn	39	–	–	–	–	–
Relative cellular lipid concentration	Cd	36	4-parameter log-logistic	280 \pm 20	1.9 \pm 0.2	100*	0
	Cu	75	4-parameter log-logistic	1000 \pm 800	0.7 \pm 0.2	98 \pm 2	0
	Ni	45	4-parameter log-logistic	3100 \pm 600	1.8 \pm 0.4	100*	0
	Pb	45	4-parameter log-logistic	160 \pm 50	0.8 \pm 0.2	100*	0
	Zn	39	4-parameter log-logistic [#]	30000 \pm 30000	0.8 \pm 0.3	98 \pm 1	0

* parameter was fixed at this value

[#] poor model fit with model parameters not significantly different to 0

APPENDIX A4

Appendix to Chapter 4

A4.1 Model parameters for the single-metal toxicity endpoints of *Phaeocystis antarctica*

Parameter estimates generated from models applied to single-toxicity data for *Phaeocystis antarctica* for the endpoints population growth rate inhibition, chlorophyll *a* fluorescence, cellular complexity and size. Values are model parameters \pm standard error.

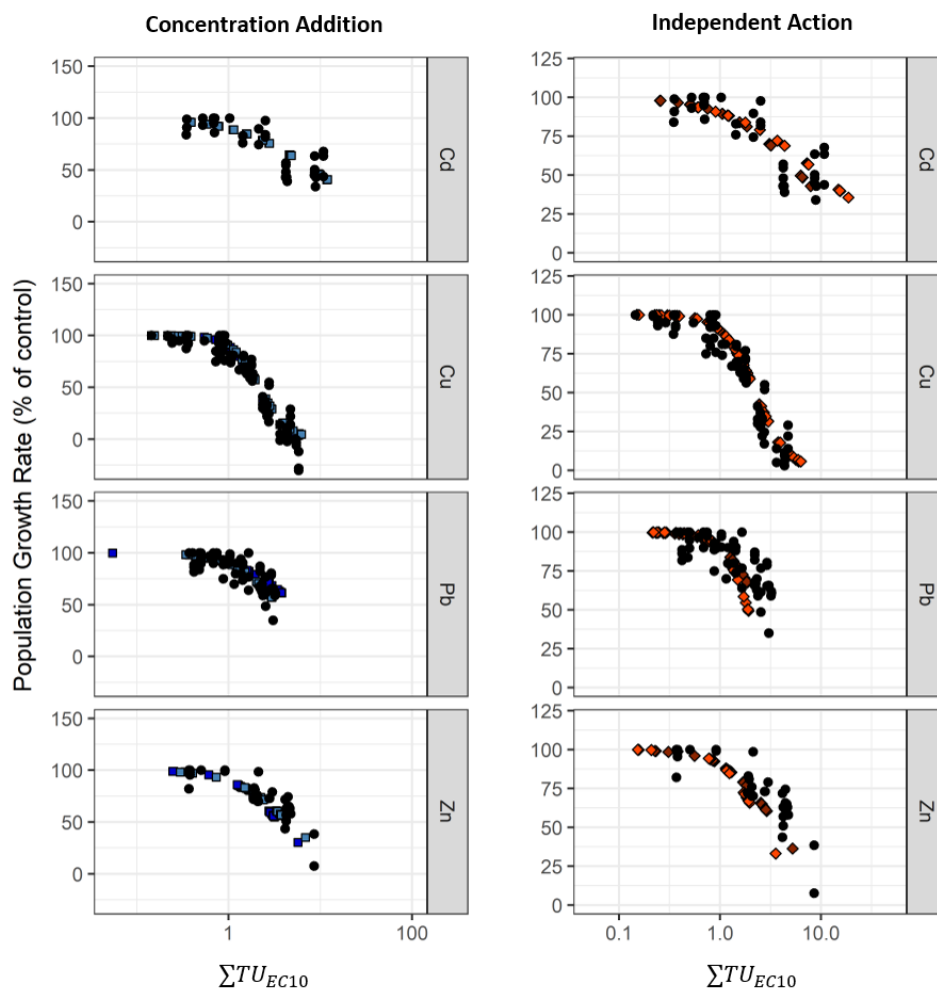
Endpoint	Metal	n	Model type	Inflection point (e)	Slope (b)	Upper limit (c)	Lower limit (d)*
Population growth rate	Cd	48	4-parameter log-logistic	1700 \pm 200	1.1 \pm 0.1	100*	0
	Cu	93	Mixed effect 4-parameter log-logistic	5.6 \pm 0.2	3.0 \pm 0.2	100	0
	Ni	39	–	–	–	–	–
	Pb	63	Mixed effect 4-parameter log-logistic	570 \pm 60	1.7 \pm 0.4	101 \pm 4	0
	Zn	39	Mixed effect 4-parameter log-logistic	1200 \pm 100	1.7 \pm 0.3	100*	0
Chlorophyll <i>a</i> fluorescence	Cd	48	4-parameter log-logistic	2400 \pm 400	0.8 \pm 0.1	100*	0
	Cu	93	Mixed effect 4-parameter log-logistic	12 \pm 2	1.6 \pm 0.1	100*	0
	Ni	39	4-parameter log-logistic [#]	130000 \pm 200000	0.3 \pm 0.2	101 \pm 10	0
	Pb	63	4-parameter log-logistic [#]	40000 \pm 100000	0.3 \pm 0.2	100*	0
	Zn	39	Mixed effect 4-parameter log-logistic [#]	20000 \pm 20000	0.5 \pm 0.2	97 \pm 5	0
Cellular complexity	Cd	48	4-parameter log-logistic	2100 \pm 300	1.0 \pm 0.2	100*	0
	Cu	93	Mixed effect 4-parameter log-logistic	12 \pm 2	1.9 \pm 0.2	100*	0
	Ni	39	–	–	–	–	–
	Pb	63	4-parameter log-logistic [#]	4000 \pm 5000	0.5 \pm 0.2	100*	0
	Zn	39	–	–	–	–	–
Cellular size	Cd	48	4-parameter log-logistic [#]	10000 \pm 40000	0.2 \pm 0.2	100*	0
	Cu	93	4-parameter log-logistic [#]	140 \pm 130	0.6 \pm 0.2	100*	0
	Ni	39	–	–	–	–	–
	Pb	63	–	–	–	–	–
	Zn	39	4-parameter log-logistic [#]	2000 \pm 2000	0.5 \pm 0.3	100*	0

* parameter was fixed at this value

[#] poor model fit with model parameters not significantly different to 0

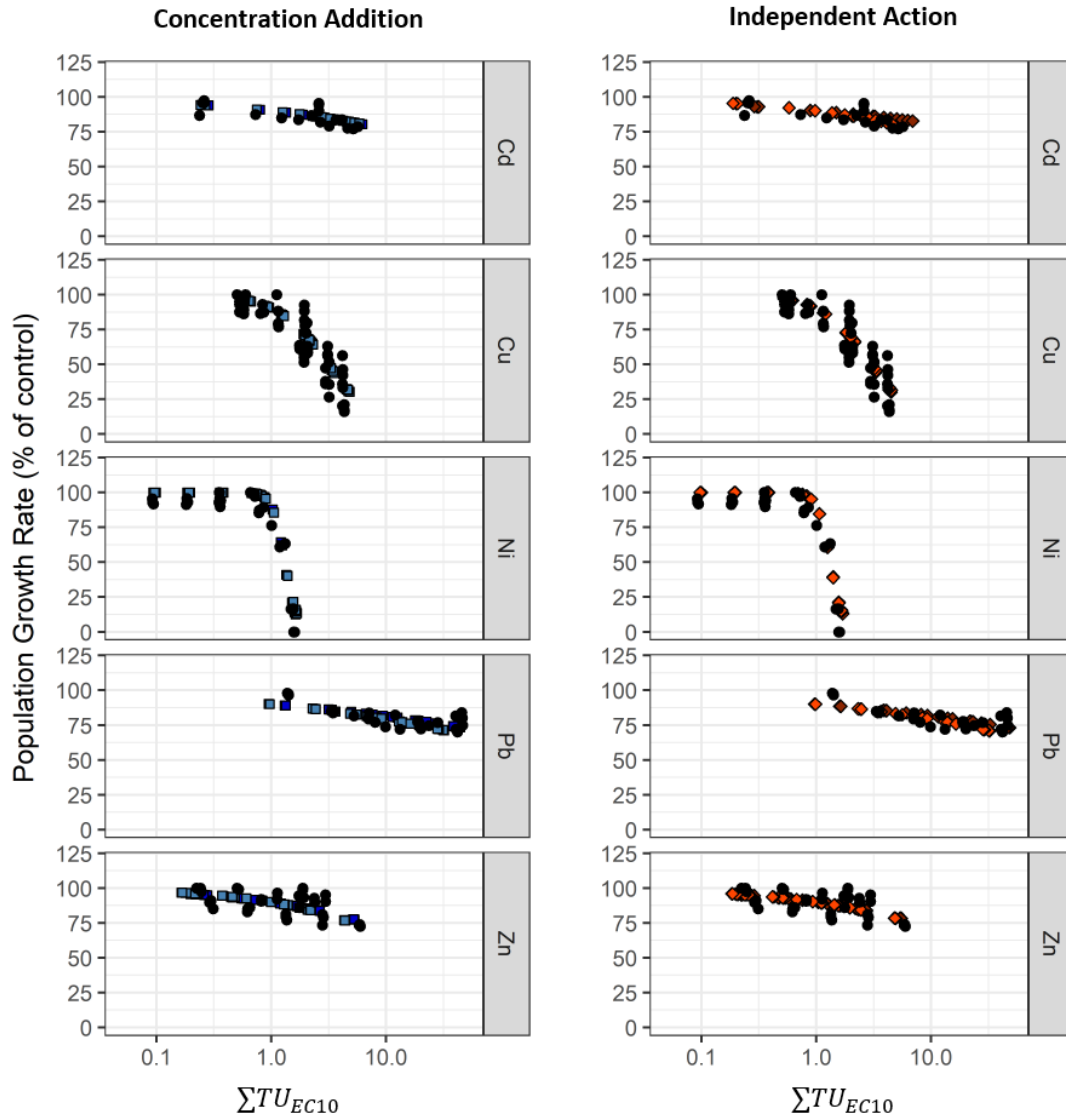
A4.2 Single metal toxicity predictions to *Phaeocystis antarctica*

Toxicity and model predictions of cadmium, copper, lead, and zinc to *Phaeocystis antarctica*. Black circles represents observed toxicity. Concentration Addition and its extension are given in the left column (dark and light blue squares, respectively) Independent Action and its extension are given in the right column (dark and light red diamonds, respectively). Observed toxicity data taken from Gissi et al. (2015). ΣTU_{EC10} = sum of toxic units in the exposure.



A4.3 Single metal toxicity predictions to *Cryothecomonas armigera*

Toxicity and model predictions of cadmium, copper, nickel, lead, and zinc to *Cryothecomonas armigera*. Black circles represents observed toxicity. Concentration Addition and its extension are given in the left column (dark and light blue squares, respectively) Independent Action and its extension are given in the right column (dark and light red diamonds, respectively). Observed toxicity data taken from Chapter 3. ΣTU_{EC10} = sum of toxic units in the exposure.



A4.4 Model parameters used in reference toxicity mixture models

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

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

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

β = slope parameter of the log-logistic model fitted to the single-metal toxicity data

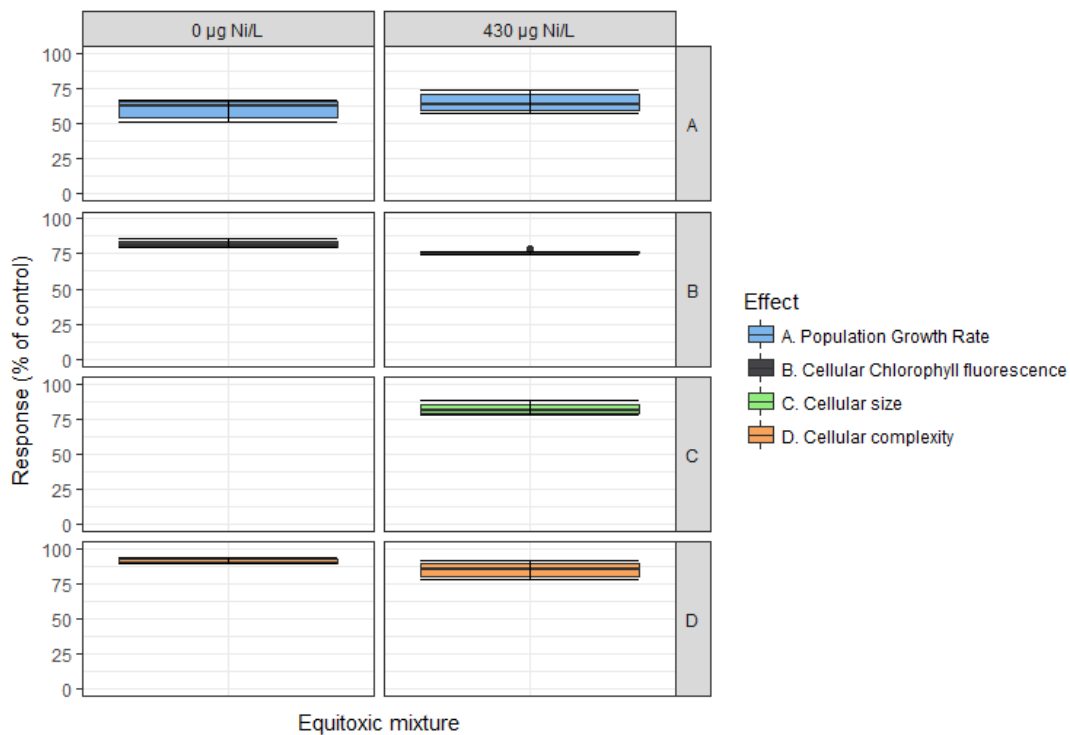
E = EC10 parameter used in lieu of the EC50 parameter of the log-logistic model fitted to the single-metal toxicity data

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

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

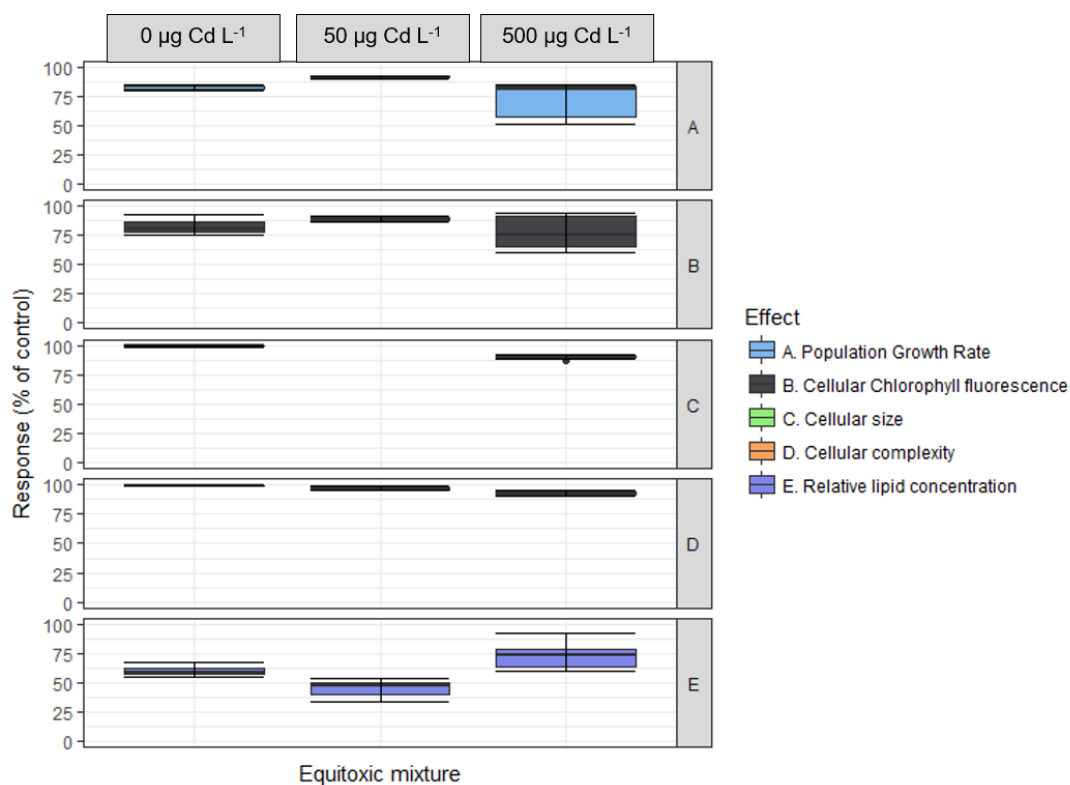
A4.5 Effect of nickel on the toxicity of the equitoxic mixture to *Phaeocystis antarctica*

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



A4.6 Effect of cadmium on the toxicity of the equitoxic mixture to *Cryothecomonas armigera*

Comparison of the toxicity of the equitoxic mixture of metals (cadmium, copper, nickel, lead, and zinc) to *Cryothecomonas armigera* with cadmium concentrations of 0, 50, and 500 $\mu\text{g L}^{-1}$. The concentration of cadmium was adjusted to investigate how cadmium influences cell size, complexity, and lipid concentration. In single metal exposures, cadmium reduced the intracellular lipid concentrations of 50% of the cell population at a concentration of 280 (229 – 330) $\mu\text{g L}^{-1}$. It also caused a coincident reduction in cellular complexity. Interestingly, there was no clear trend in intracellular lipid concentrations at increasing cadmium concentrations, nor was there a change in cellular complexity which was expected from single-metal toxicity (see Chapter 3).



APPENDIX A5

Appendix to Chapter 5

A5.1 Quality control and assurance

Quality assurance and quality control: (a) metal concentrations from process blank acid digestions; (b) metal concentrations from blank EDTA wash solution; (c) measured extra- and intra-cellular metal concentrations from control (seawater only) exposures for *Phaeocystis antarctica* and *Cryothecomonas armigera*. Blank cells indicate measurements below detection limit.

(a) Metal concentrations from process blank acid digestions

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

(b) Metal concentrations from blank EDTA wash solution

Metal	Cd	Cu	Ni	Pb	Zn
Average (μgL^{-1})	0.003	0.6	2.6	0.2	2.0
Standard deviation (μgL^{-1})	0.002	0.4	0.8	0.01	1.0
Count (n)	3	9	6	3	16

(c) Measured extra- and intra-cellular metal concentrations (mean \pm standard deviation, mol cell⁻¹) from control (seawater only) exposures for *P. antarctica* and *C. armigera*.

Metal	<i>Phaeocystis antarctica</i>		<i>Cryothecomonas armigera</i>	
	Intracellular	Extracellular	Intracellular	Extracellular
Cd	$5 \pm 5 \times 10^{-18}$	$2 \pm 5 \times 10^{-18}$	$2 \pm 2 \times 10^{-18}$	$1 \pm 1 \times 10^{-18}$
Cu	$1.1 \pm 0.8 \times 10^{-18}$	$1.2 \pm 0.5 \times 10^{-17}$	$1.2 \pm 0.9 \times 10^{-17}$	$2 \pm 1 \times 10^{-17}$
Ni	$1 \pm 1 \times 10^{-17}$	$1 \pm 1 \times 10^{-17}$	$3 \pm 2 \times 10^{-17}$	$7 \pm 5 \times 10^{-17}$
Pb	$2 \pm 3 \times 10^{-18}$	$1.3 \pm 0.8 \times 10^{-17}$	$3 \pm 2 \times 10^{-18}$	$2 \pm 2 \times 10^{-18}$
Zn	3×10^{-18}	$1.0 \pm 0.8 \times 10^{-16}$	$5 \pm 2 \times 10^{-18}$	$2 \pm 1 \times 10^{-16}$

A5.2 Cellular metal concentrations for metal mixture exposures

Cellular metal concentrations (mean \pm standard deviation) of *Phaeocystis antarctica* and *Cryptothecomonas armigera* following exposure to metal mixtures: (a) dissolved, (b) extracellular, and (c) intracellular fractions. 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 mol cell⁻¹, accounting for the different number of cells at the end of each treatment).

(a) Dissolved metal concentrations from mixture exposures

Microalga	Mixture	Dissolved metal exposure concentrations (nM)				
		Cd	Cu	Ni	Pb	Zn
<i>P. antarctica</i>	EM x5	5.0 \pm 0.2	48 \pm 6	33 \pm 8	5 \pm 2	280 \pm 20
	EM x10	10 \pm 3	88 \pm 2	62 \pm 3	9 \pm 1	550 \pm 30
	EM x20	20 \pm 1	200 \pm 20	83.5 \pm 0.8	17 \pm 4	1.27 \pm 0.07
	EC x0.5	670 \pm 20	21 \pm 3	3680 \pm 60	530 \pm 30	3100 \pm 700
	EC x1	1350 \pm 20	44 \pm 7	4000 \pm 4000	1000 \pm 100	6500 \pm 400
	EC x2	2400 \pm 100	79 \pm 5	11300 \pm 300	1800 \pm 500	9580 \pm 80
<i>C. armigera</i>	EM x10	9 \pm 1	108 \pm 2	48.9 \pm 0.4	15 \pm 4	589 \pm 1
	EM x20	21 \pm 3	190 \pm 30	96 \pm 9	19 \pm 6	1000 \pm 100
	EM x30	27 \pm 2	228 \pm 7	129 \pm 7	22.1 \pm 0.3	1500 \pm 200
	EM x40	41 \pm 2	441 \pm 5	205 \pm 6	37 \pm 5	2560 \pm 50
	EM x60	56 \pm 1	669 \pm 2	294 \pm 8	52 \pm 3	3910 \pm 70
	EM x80	40 \pm 4	911 \pm 6	389 \pm 4	73 \pm 3	5280 \pm 60
	EC x1	2000 \pm 2000	280 \pm 60	17000 \pm 3000	500 \pm 200	9000 \pm 3000

(b) Extracellular metal concentrations from mixture exposures

Microalgae	Mixture	Extracellular metal concentration (mol cell ⁻¹)				
		Cd	Cu	Ni	Pb	Zn
<i>P. antarctica</i>	Env x5	4 ± 6 x10 ⁻¹⁸	6 ± 2 x10 ⁻¹⁷	5.5 ± 0.9 x10 ⁻¹⁷	8 ± 8 x10 ⁻¹⁸	2 ± 2 x10 ⁻¹⁶
	Env x10	2.2 ± 0.4 x10 ⁻¹⁸	1.0 ± 0.4 x10 ⁻¹⁶	1.9 ± 0.5 x10 ⁻¹⁷	1.6 ± 0.2 x10 ⁻¹⁷	5 ± 3 x10 ⁻¹⁶
	Env x20	4 ± 3 x10 ⁻¹⁷	5 ± 1 x10 ⁻¹⁶	2.0 ± 0.7 x10 ⁻¹⁶	7 ± 1 x10 ⁻¹⁷	1.2 ± 0.4 x10 ⁻¹⁵
	EC x0.5	1.2 ± 0.3 x10 ⁻¹⁸	7 ± 5 x10 ⁻¹⁷	2.3 ± 0.6 x10 ⁻¹⁷	8 ± 2 x10 ⁻¹⁷	5 ± 2 x10 ⁻¹⁶
	EC x1	3.1 ± 0.2 x10 ⁻¹⁷	7 ± 1 x10 ⁻¹⁷	3 ± 2 x10 ⁻¹⁷	1.7 ± 0.5 x10 ⁻¹⁶	4 ± 2 x10 ⁻¹⁶
	EC x2	5.1 ± 0.7 x10 ⁻¹⁷	1.1 ± 0.2 x10 ⁻¹⁶	4 ± 1 x10 ⁻¹⁷	4.4 ± 0.8 x10 ⁻¹⁶	7.9 ± 0.1 x10 ⁻¹⁶
<i>C. armigera</i>	Env x10	4 ± 1 x10 ⁻¹⁸	2.2 ± 0.6 x10 ⁻¹⁶	1.0 ± 0.4 x10 ⁻¹⁷	1.6 ± 0.3 x10 ⁻¹⁷	1.4 ± 0.3 x10 ⁻¹⁵
	Env x20	4 ± 2 x10 ⁻¹⁷	3.2 ± 0.8 x10 ⁻¹⁶	6 ± 2 x10 ⁻¹⁷	2.6 ± 0.4 x10 ⁻¹⁷	2.4 ± 0.6 x10 ⁻¹⁵
	Env x30	6 ± 4 x10 ⁻¹⁷	2.7 ± 0.5 x10 ⁻¹⁶	3 ± 3 x10 ⁻¹⁷	1.9 ± 0.4 x10 ⁻¹⁷	2.0 ± 0.2 x10 ⁻¹⁵
	Env x40	<1.8 x10 ⁻¹⁷	1.3 ± 0.1 x10 ⁻¹⁵	4 ± 2 x10 ⁻¹⁶	<2.5 x10 ⁻¹⁶	3 ± 2 x10 ⁻¹⁵
	Env x60	<3.6 x10 ⁻¹⁷	2.9 ± 0.7 x10 ⁻¹⁵	2 ± 3 x10 ⁻¹⁶	<4.9 x10 ⁻¹⁶	2.6 ± 0.3 x10 ⁻¹⁵
	Env x80	<9.1 x10 ⁻¹⁷	1.06 ± 0.02 x10 ⁻¹⁴	2 ± 2 x10 ⁻¹⁵	<1.2 x10 ⁻¹⁵	1 ± 1 x10 ⁻¹⁴
	EC x1	3 ± 2 x10 ⁻¹⁶	7 ± 4 x10 ⁻¹⁶	3 ± 2 x10 ⁻¹⁶	3 ± 2 x10 ⁻¹⁶	5 ± 2 x10 ⁻¹⁵

(c) Intracellular metal concentrations from mixture exposures

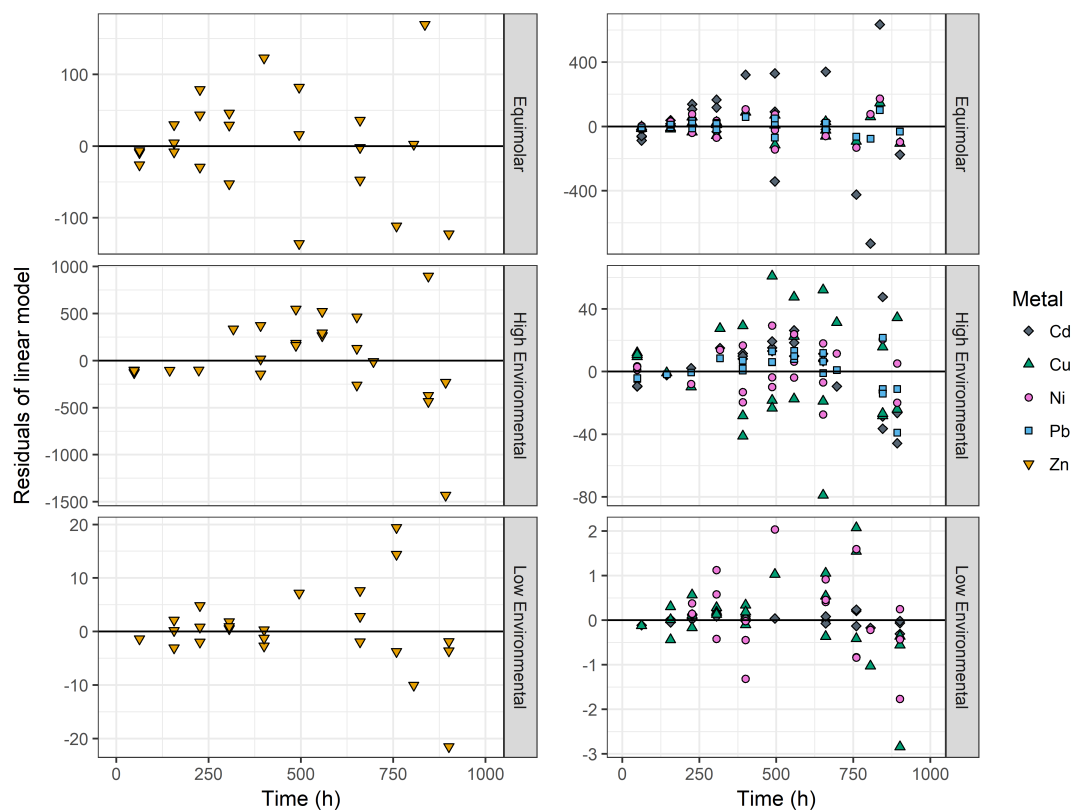
Microalgae	Mixture	Intracellular metal concentration (mol cell ⁻¹)				
		Cd	Cu	Ni	Pb	Zn
<i>P. antarctica</i>	Env x5	1 ± 1 x10 ⁻¹⁸	4 ± 2 x10 ⁻¹⁷	2 ± 1 x10 ⁻¹⁷	<3 x10 ⁻¹⁸	1.3 ± 0.4 x10 ⁻¹⁷
	Env x10	1.2 ± 0.9 x10 ⁻¹⁸	6 ± 3 x10 ⁻¹⁷	2.3 ± 0.5 x10 ⁻¹⁷	2.7 x10 ⁻¹⁹	<7 x10 ⁻¹⁷
	Env x20	6 ± 5 x10 ⁻¹⁸	1.9 ± 0.4 x10 ⁻¹⁶	4 ± 4 x10 ⁻¹⁶	7 ± 7 x10 ⁻¹⁹	<3 x10 ⁻¹⁶
	EC x0.5	3 ± 4 x10 ⁻¹⁸	7 ± 4 x10 ⁻¹⁷	3 ± 1 x10 ⁻¹⁷	5 ± 2 x10 ⁻¹⁸	7 ± 4 x10 ⁻¹⁷
	EC x1	1 ± 2 x10 ⁻¹⁷	6 ± 1 x10 ⁻¹⁷	4 ± 1 x10 ⁻¹⁷	6 ± 3 x10 ⁻¹⁸	5 ± 2 x10 ⁻¹⁷
	EC x2	5 ± 8 x10 ⁻¹⁷	8 ± 1 x10 ⁻¹⁷	5.9 ± 0.9 x10 ⁻¹⁷	1.4 ± 0.7 x10 ⁻¹⁷	4.4 ± 0.7 x10 ⁻¹⁷
<i>C. armigera</i>	Env x10	2.7 ± 0.6 x10 ⁻¹⁹	1.0 ± 0.2 x10 ⁻¹⁶	<5 x10 ⁻¹⁸	2.1 ± 0.8 x10 ⁻¹⁸	7 ± 3 x10 ⁻¹⁷
	Env x20	4 ± 3 x10 ⁻¹⁸	1.5 ± 0.8 x10 ⁻¹⁶	2 ± 3 x10 ⁻¹⁷	7 ± 6 x10 ⁻¹⁸	2 ± 1 x10 ⁻¹⁶
	Env x30	1.3 x10 ⁻¹⁷	1.2 ± 0.4 x10 ⁻¹⁶	<3 x10 ⁻¹⁷	2 ± 1 x10 ⁻¹⁸	1.1 ± 0.4 x10 ⁻¹⁶
	Env x40	2.0 ± 0.2 x10 ⁻¹⁸	3.8 ± 0.5 x10 ⁻¹⁶	4 ± 1 x10 ⁻¹⁷	7 ± 5 x10 ⁻¹⁸	7 ± 5 x10 ⁻¹⁷
	Env x60	4 ± 4 x10 ⁻¹⁸	5.8 ± 0.3 x10 ⁻¹⁶	8 ± 2 x10 ⁻¹⁷	9 ± 9 x10 ⁻¹⁸	3 ± 2 x10 ⁻¹⁶
	Env x80	4.07 ± 0.09 x10 ⁻¹⁷	1.54 ± 0.09 x10 ⁻¹⁵	1 ± 1 x10 ⁻¹⁵	2 ± 2 x10 ⁻¹⁷	6 ± 5 x10 ⁻¹⁵
	EC x1	2 ± 1 x10 ⁻¹⁷	2 ± 1 x10 ⁻¹⁶	3 ± 3 x10 ⁻¹⁶	2 ± 4 x10 ⁻¹⁷	2 ± 2 x10 ⁻¹⁶

APPENDIX A6

Appendix to Chapter 6

A6.1 Residuals from the linear model of metal uptake to DGT over time

Residuals of the linear model of metal mass over time accumulated by DGT for the equimolar, high environmental, and low environmental mixtures. (a) shows residuals for zinc, (b) shows residuals for all other metals. Note the different y-axes. Lead measurements in the low environmental treatment were below detection limit.



A6.2 R^2 values from the linear model of metal uptake to DGT over time

R^2 values of linear model fits of metal mass accumulated at different time points by DGT in the (a) equimolar mixture, (b) high environmental mixture, and (c) low environmental mixture. Lead measurements in the low environmental mixture were below detection limit.

(a) Equimolar								
Metal	Length of deployment (time, h)							
	305	400	495	660	759	805	835	900
Cd	0.97	0.97	0.88	0.92	0.87	0.78	0.80	0.81
Cu	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.99
Ni	0.98	0.98	0.98	0.99	0.99	0.99	0.99	0.99
Pb	0.98	0.98	0.96	0.98	0.97	0.97	0.97	0.97
Zn	0.98	0.98	0.98	0.99	0.99	0.99	0.99	0.99
(b) High Environmental								
Metal	Length of deployment (time, h)							
	317	390	486	557	651	696	845	892
Cd	0.99	0.98	0.99	0.98	0.94	0.89	0.63	0.45
Cu	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Ni	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Pb	0.99	0.99	0.99	0.99	0.99	0.98	0.95	0.90
Zn	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.96
(c) Low Environmental								
Metal	Length of deployment (time, h)							
	226	305	400	495	660	759	805	900
Cd	0.98	0.99	0.95	0.95	0.96	0.96	0.95	0.95
Cu	0.98	0.99	0.99	0.99	0.99	0.99	0.98	0.97
Ni	1.00	0.95	0.86	0.86	0.92	0.91	0.91	0.91
Pb	–	–	–	–	–	–	–	–
Zn	0.98	0.99	0.99	0.99	0.99	0.98	0.98	0.97

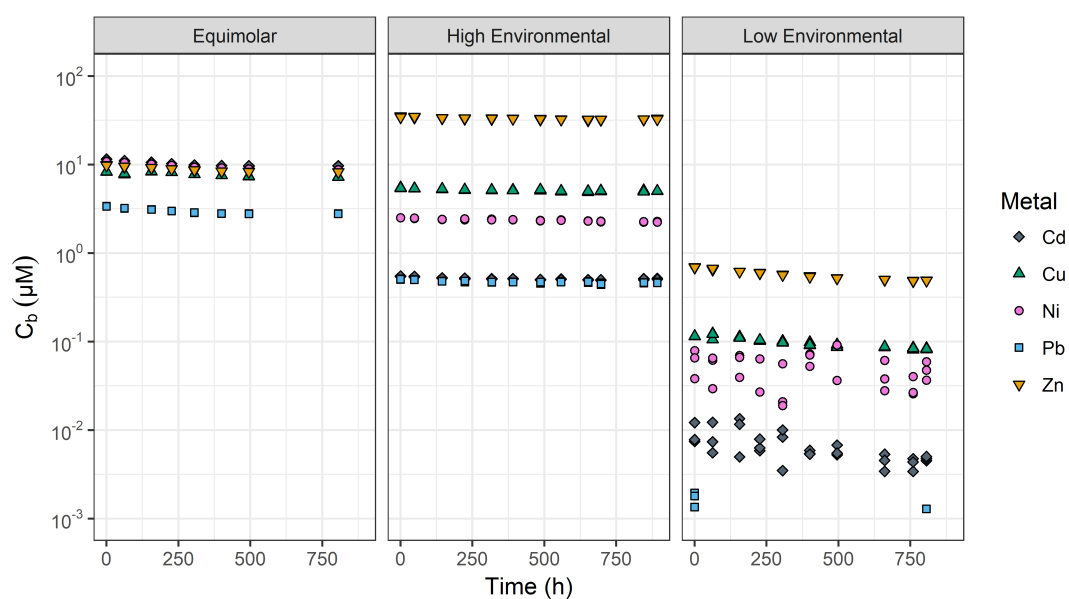
A6.3 Parameters used to calculate diffusion coefficients

Diffusion coefficients (D , $10^{-6} \text{ cm}^2 \text{ s}^{-1}$) at $1 \pm 1 \text{ }^\circ\text{C}$ for each metal in seawater were calculated using time (t , seconds), the time-averaged metal concentration of the solution for the period of linear-uptake (C_b , μM), and the mass of metal analyte accumulated in the DGT at time t (M , nmol). Lead measurements in the low environmental treatment were below detection limit.

Metal	Equimolar				High environmental				Low environmental			
	t	C_b	M	D	t	C_b	M	D	t	C_b	M	D
Cd	1440000	11	1169	2.260	2007300	0.5	72	2.056	2899800	0.007	2	2.765
Cu	3243000	7	1841	2.392	3211500	5.1	1252	2.243	3243000	0.095	16	1.539
Ni	3243000	8	2129	2.390	3211500	2.4	548	2.135	1100100	0.050	4	2.185
Pb	3243000	3	756	2.675	3042300	0.5	120	2.447	–	–	–	–
Zn	3243000	8	1931	2.280	3211500	33.1	7028	1.957	3243000	0.568	130	2.094

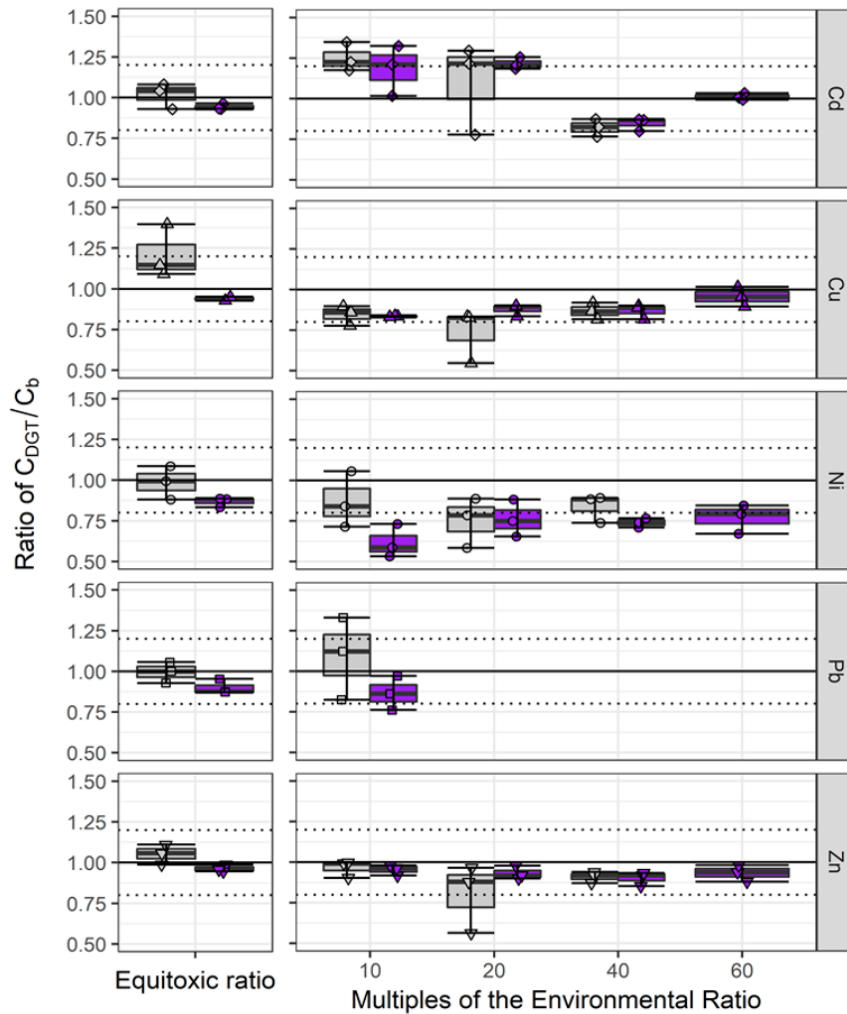
A6.4 Metal exposure concentrations over time in test to calculate diffusion coefficients

Dissolved metal concentrations for the equimolar, high environmental, and low environmental metal mixtures for the deployment period. Three subsamples were taken at each time point.



A6.5 Ratio of DGT-labile to dissolved metal concentrations in laboratory deployments with and without *Phaeocystis antarctica*

The ratio of DGT-labile to dissolved metal concentration (C_{DGT}/C_b) in the presence (purple) or absence (grey) of *Phaeocystis antarctica* at environmentally realistic concentrations of $1-3 \times 10^3$ cells mL^{-1} . The C_{DGT}/C_b ratio shows that up to 20% of the dissolved copper, nickel, and zinc metal fraction was not DGT-labile, possibly due to the formation of metal-DOC complexes. C_b for lead was generally below instrument detection limits.



APPENDIX A7

Appendix to Chapter 7

A7.1 Quality control and assurance

Quality control and assurance measurements from DGT field deployments. Including (a) instrument limit of detection and certified reference material values, (b) blank DGT concentrations and corresponding method limits of detection, and (c) method detection limits based on the blank DGT concentrations and method limit of detection.

(a) Instrument limits of detection and certified reference material (CRM) analysis using TMDA-64.4 and TM 24.4 (NRC, Canada). All values are in $\mu\text{g L}^{-1}$ unless otherwise stated.

Metal	Cadmium	Copper	Nickel	Lead	Zinc
Wavelength ^a	214.439	324.754	231.604	220.353	213.857
TMDA-64.4 measured	255	258	252	280	326
TMDA-64.4 reported	258	261	252	280	320
% of CRM	99	99	100	100	102
TM24.4 measured	4	7	5	5	30
TM24.4 reported	3.96	6.31	5.03	5.6	–
% of CRM	105	107	98	91	–

^a ICP-AES wavelength used for measurements (nm)

(b) DGT blank concentrations and LOD. All values are in $\mu\text{g L}^{-1}$ unless otherwise stated.

Metal	Cd	Cu	Ni	Pb	Zn
Instrument LOD	0.1	1	0.3	1	0.1
DGT Blank 1	0	0.8	-0.2	-1.2	3.9
DGT Blank 2	-0.1	0.5	-0.2	-0.8	8.6
DGT Blank 3	0.2	9.7	0	-0.6	20.1
DGT Blank 4	0	0.8	-0.1	0.2	4
DGT Blank 5	0	0.1	-0.1	-1	-0.1
DGT Blank 6	-0.1	-0.1	-0.2	-1	-0.1
DGT Blank 7	-0.1	0.5	-0.1	-0.5	-0.2
DGT Blank 8	0	0.2	-0.4	0.1	-0.1
DGT Blank 9	0	0.2	-0.4	-0.4	-0.1
Average blank (#5-9)	-0.05	0.2	-0.2	-0.6	-0.1
Standard deviation (#5-9)	0.04	0.2	0.2	0.5	0.006
LOD calculations ^a					
3x Standard deviation	0.1	0.6	0.5	1.4	0.1
Theoretical elution concentration ^b	4	20	14	44	4
Theoretical mass on resin (μg) ^c	0.006	0.027	0.02	0.062	0.006

^a DGT Blank 1-4 measurements were excluded from the LOD calculations due to contamination. The authors are unsure whether the contamination is from processes related to this study or another study which involved DGT deployed to soils following a deoxygenation process in nitrogen bubbled water.

^b Based on a dilution of 6.37% HNO_3 (i.e. 1 M) to 4 mL of 0.2% acid content

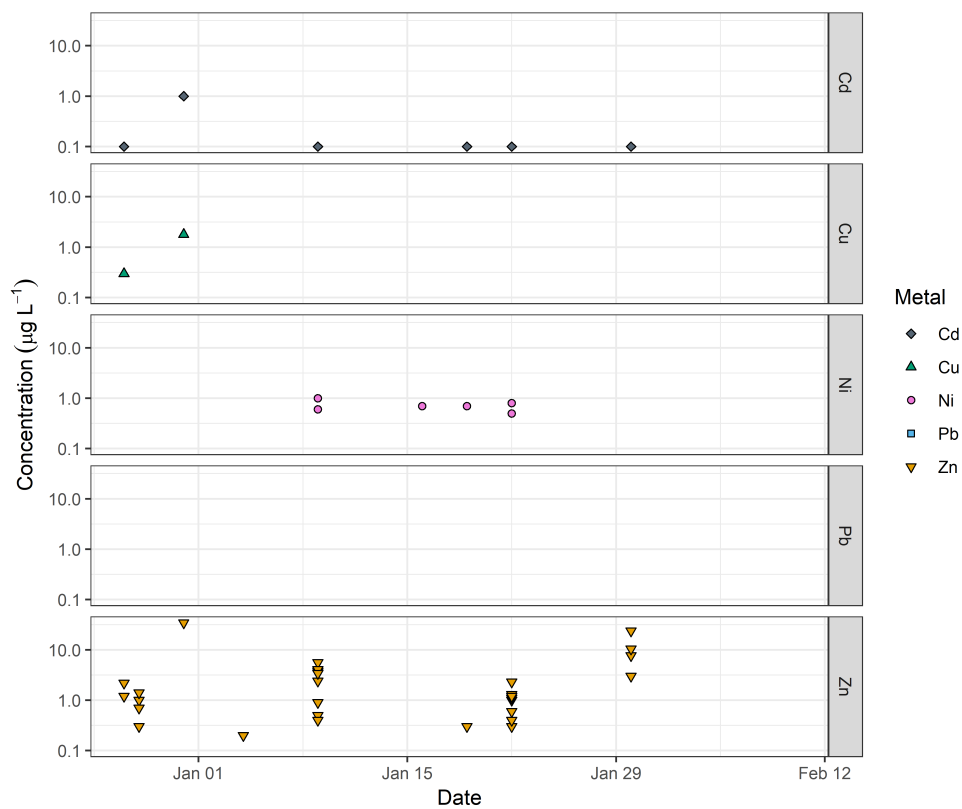
^c Calculated using Equation 6.1

(c) Method detection limits (MDL). D_i is the diffusion coefficient for each metal from Chapter 6, LOD is the limit of detection calculated as 3 times the standard deviation of undeployed DGT measured concentrations, and MDL is the method detection limit for the devices based on the LOD at a deployment time of 22 and 37 d.

Metal	D_i ($\times 10^{-6} \text{ s}^{-1} \text{ cm}^2$)	LOD (ng)	MDL (ng L^{-1}):	
			22 d	37 d
Cadmium	2.4	5.9	39	23
Copper	2.1	27	207	123
Nickel	2.2	20	140	83
Lead	2.6	62	375	223
Zinc	2.1	5.7	42	25

A7.2 Dissolved metal concentrations in seawater at DGT deployment sites

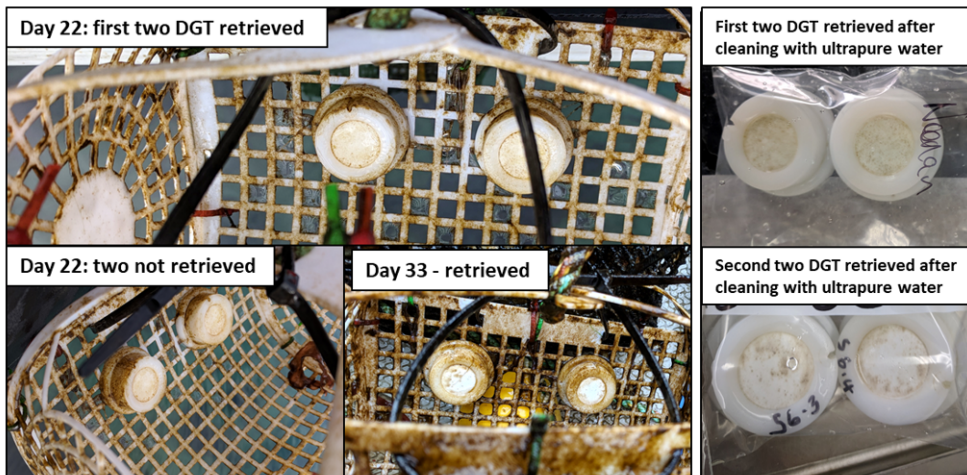
Measured dissolved metal concentrations in seawater at DGT deployment sites (all site data combined for each date and metal). The absence of points indicates measurements below detection limits (see Appendix A7.1). Note the high variability in zinc measurements.



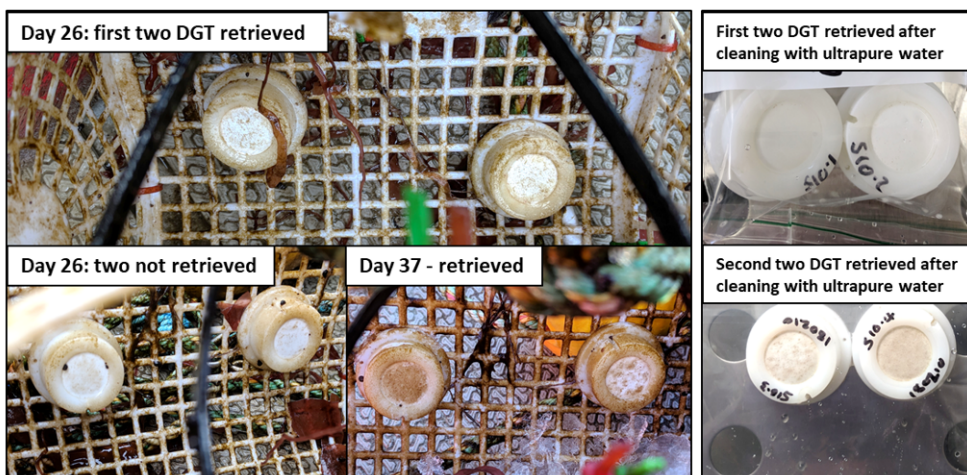
A7.3 Examples of biofouling on DGT samplers following deployment

Representative examples of DGT sampler biofouling after seawater deployments. For most sites, two DGT were retrieved after deployment for 22 and 30 d (see Figure 7.1).

Site 6



Site 10



Site 1

Site 2

Site 8

