Sensitivity of Antarctic marine invertebrates and microalgae to metal exposure

by

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Abstract

Water quality guidelines contribute to the successful management of anthropogenic contaminant impacts in many temperate, tropical and some Arctic marine environments. Despite the remoteness and restricted colonisation of the Antarctic continent, a number of coastal marine sites near established research stations have been impacted and contaminated by human activities. Sources of contaminants include leachates from legacy waste dumping sites and abandoned research stations as well as ongoing discharge of untreated effluent and accidental fuel spills. Even though the extent of contamination is relatively localised, provisions of Annex III to the Protocol on Environmental Protection to the Antarctic Treaty stipulate that these sites must be cleaned up and remediated unless the remediation process would cause further negative impact. Identifying contaminated sites, planning remediation activities and monitoring the success of the clean-up process as well as managing ongoing impacts from current human activities in Antarctic ecosystems are significantly challenging due to the absence of Antarctic specific, environmental guidelines. Developing region specific guidelines requires understanding the relative sensitivities of local biota to contaminant exposure, data for which is commonly obtained through toxicity tests. Few toxicological studies have been conducted with Antarctic marine species, and only a couple have addressed the unique biochemical and physiological adaptations of biota to their cold, stable environment by incorporating more appropriate, long exposure durations. The aims of this research were to develop longer-term contaminant bioassay methodologies with a number of Antarctic marine invertebrates and microalgae to determine point estimates for the future development of Antarctic specific water quality guidelines. The microalgal growth inhibition bioassay using flow cytometry was successfully developed for use with an endemic, ubiquitous Antarctic phytoplankton species, *Phaeocystis antarctica* with results indicating this species to be more sensitive to copper exposure than other temperate and

tropical species. A longer (21 d) bioassay using a sensitive behavioural endpoint was successfully developed for adults and juveniles of the littoral amphipod, *Paramoera walkeri*. Lethal and behavioural response rates varied following exposure to individual metals including copper, cadmium, zinc, lead and nickel, with the behavioural response of active behaviour being more sensitive than lethality. Juvenile *P. walkeri* were more sensitive to copper than adults. Investigating effects of climate change variables in association with contamination showed that slight increases in water temperature increased the toxicity of cadmium and copper exposure to *P. walkeri* adults. A 30 d bioassay was successfully developed for the benthic amphipod, *Orchomenella pinguides* and the lethal responses to copper, cadmium, zinc, lead and nickel exposure were determined. Point estimates for exposure to the metals were determined by two analytical methods; the traditional Probit Maximum Likelihood method and a newly developed statistical model, the generalised additive mixed model (GAMM). The newly developed GAMM improves on point estimation by traditional Probit analyses by including all the observations taken throughout the longer bioassay duration and incorporating the time-varying metal concentrations into the model. By exploiting all the time-series data simultaneously, GAMM provides a better estimation of the toxicity response through time.

The point estimates determined for these Antarctic marine species provide essential toxicological data needed for the future development of Antarctic specific water quality guidelines. Bioassay methodologies developed are crucial components of environmental risk assessments and provide a means of planning, monitoring and evaluating remediation activities. Through the increased understanding of species contaminant sensitivities, current and future human activities in Antarctic ecosystems can be better managed to reduce the longevity and severity of anthropogenic contaminant impact.

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Chapter 1

General Introduction

Antarctic marine ecosystems and biota

Antarctica is the most remote continent in the southern hemisphere. Physical geography and climatic conditions restricted exploration of the continent until the early 1900's. Since that time, human visitation and habitation has increased from earlier fishing, sealing and whaling ventures to today's scientific research expeditions and recreational tourism (Bargagli 2005). Despite being designated as "a natural reserve, devoted to peace and science," Antarctica is no longer a pristine wilderness, and is affected both by local and by global anthropogenic activities (Bargagli 2005). In order to conserve and protect Antarctic ecosystems from anthropogenic contaminants, it is essential that we understand the unique characteristics of the endemic species and ecosystems in which they live.

There is a high level of endemism for species in the Southern Ocean and around the Antarctic continent, as the extreme temperature gradient of the Polar Front (Antarctic circumpolar current at 55°S) has acted as an oceanic barrier to spatial dispersion of marine organisms (Bargagli 2008). This has strongly influenced the evolution of Antarctic marine species, resulting in lower diversity relative to temperate and tropical ecosystems and highly specialised adaptations to living in the narrow thermal limits of waters in this region (Pörtner 2006).

Antarctic coastal water temperatures are low and stable with a mean annual temperature of -1.8 °C (Clarke 1988) and a winter low of -2 °C to summer high of +1 °C (McClintock et al. 2008). With water temperature as the exception, much of the physical and biological environment of Antarctica is characterised by seasonal fluctuations. Variations in solar radiation affect the extent and stability of coastal sea ice. The presence of sea ice influences transmission of solar radiation to the marine ecosystems, reducing wind-driving mixing of the water

column and promoting water column stratification and increased physical disturbance of benthos through ice scour (Chapman and Riddle 2005a). The combined influence of sea ice and solar radiation affects the seasonal primary production in Antarctic coastal waters. In spring and summer, trapped phytoplankton is released from melting sea ice and quickly develops into dense blooms in response to increased solar radiation and high nutrient availability (Clarke 1988; McClintock et al. 2008). This seasonal primary production provides the basis for the annual food web, sustaining the rich pelagic and benthic communities of Antarctic marine ecosystems.

Due to the stable, cold temperature, Antarctic marine species are largely stenothermal, having a much narrower thermal tolerance range than species from temperate or tropical regions (Peck et al. 2004). Adaptations to the cold temperature have increased the longevity of marine organisms which also have lower metabolic rates (Pörtner et al. 2007), a tendency towards gigantism and high lipid content for energy storage (Goerke et al. 2004). Reproductive strategies among the benthos are dominated by brooding with larvae taking longer to develop with fewer individuals being released compared with analogous temperate and tropical species (Dell 1972).

Impacts of human activities in Antarctica

Ice-free coastal regions are the most likely to be impacted by human activities as they provide the best access points from ship to land for conducting scientific research and constructing permanent stations. There are currently 90 active research stations in Antarctica, 40 of which are operated all year round with a total peak capacity of almost 4,500 people during the summer (The Council of Managers of Antarctic Programs, COMNAP 2012). Australia has four research stations operated all year round; Casey, Davis and Mawson are located within the Australian Treaty area of East Antarctica on the continent and the fourth is on the subantarctic Macquarie Island (Figure 1).

Figure 1: Map of Antarctica depicting the locations of Australia's Research Stations (Australian Antarctic Data Centre 2012)

Over 20 years ago, the potential for increased human activities and the associated human derived impacts to the Antarctic environment was recognised and the Protocol on Environmental Protection to the Antarctic Treaty (more commonly known as The Madrid Protocol) was developed in 1991 and ratified in 1998. The Madrid Protocol details five Annexes for environmental protection in Antarctic Treaty areas with Annex III: Waste Disposal and Waste Management, providing direction for safe waste management planning and practices, prohibited products and obligations for waste removal and contaminated site remediation. Despite the ratification of Annex III to The Madrid Protocol, previous waste management practices have resulted in a legacy of

contamination at several current and abandoned research station sites (Snape et al. 2001; Stark et al. 2006a). Disposal of waste to tip sites, open air burning, discharge of untreated sewage and depositing of waste onto sea ice before the summer melt were common practice prior to The Madrid Protocol. Contamination from these sources as well as abandoned research stations typically extends for hundreds of meters (Stark et al. 2003a) but accidental fuel spills have had further reaching contaminant effects as they have occurred in both terrestrial and coastal marine locations and are readily dispersed (Kennicutt et al. 2010). To improve protection of marine ecosystems from contamination and to meet obligations of the Madrid Protocol it is essential that contaminated sites be effectively identified, managed and remediated.

Some studies have identified contaminant impacts in nearshore and terrestrial sites in Antarctica, such as the old waste dumping site of Winter Quarters Bay near McMurdo Station. High levels of hydrocarbon, polychlorinated biphenyls (PCB) and metal contaminants in the sediments of Winters Quarters Bay are comparable to the most polluted ports in the world and have dramatically affected the benthic community structure along the pollution gradient (Lenihan 1992; Lenihan and Oliver 1995a). Background levels of trace metals in Antarctic waters are relatively stable compared with concentration ranges from other oceans (Honda et al 1987). Analysis of samples taken from 12 locations in East Antarctica showed metal concentration ranges of 0.21-0.49 µg/L of lead, 0.03 – 0.10 µg/L of cadmium, 0.18-1.17 µg/L of copper, 0.41 – 1.16 µg/L of zinc and 0.30-0.54 µg/L of nickel (Honda et al 1987). An extensive assessment of contamination at Casey Station, East Antarctic identified numerous sites surrounding the station that had been impacted by metal and organic chemical contaminants (Deprez et al. 1999). The old waste tip site in Thala Valley was of particular concern, with high concentrations of petroleum hydrocarbons and metals such as copper, lead, zinc, nickel, cadmium, chromium and mercury found in leachates, melt pools and soils (Deprez et al. 1999). Due to the topography of Thala Valley, contaminants from the tip site were mobilised by

meltwaters and by tidal inundation, leading to accumulation of copper, lead and zinc in the nearshore sediments of the adjacent Brown Bay (Deprez et al. 1999; Snape et al. 2001). Dissolved concentrations of metals were measured in the water column by DGT samplers (diffusive gradients in thin films) with background levels below detection limits at the reference site of McGrady Cove Those measured in Brown Bay were generally below Australian Water Quality Guideline levels (Stark et al 2006b). Significant differences however were detected in the benthic community structure between the impacted site of Brown Bay and the reference sites (Stark 2000). Correlating biological patterns with sediment contamination indicated lower species diversity and the dominance of opportunistic species such as polychaetes in Brown Bay, both of which were attributed to anthropogenic impacts of cadmium, copper, lead and zinc; making these metals a primary concern for local contamination (Stark et al. 2003b). Identified as the highest environmental risk site in the Australian Antarctic Territory at the time (Snape et al. 2001) and under obligations to Annex III of The Madrid Protocol, waste was removed from the tip site of Thala Valley and a carefully designed, pioneer remediation program was conducted to limit any further environmental impact being caused (Stark et al. 2006b). The clean-up process was a unique undertaking, the first of its kind in the Australian Antarctic Territory, and not only utilised dig and haul methods for removing the contaminated materials, but also incorporated biological monitoring of the ecological impact of the remediation activities (Stark et al. 2006a).

Although chemical contamination from human activities is the most widespread environmental impact in Antarctica (Bargagli 2005), there is the potential for these local impacts to be enhanced by additional human induced-stressors associated with climate change (Tin et al. 2009). Climate change has already been detected in the Antarctic region with the rate of warming on the Antarctic Peninsula being one of the highest seen globally in recent times (Anisimov et al. 2007). The atmospheric temperature of the Western Antarctic Peninsula has risen by 3 °C, which has in turn caused nearshore sea temperatures to rise above

1 °C during the summer months (Meredith and King 2005). As previously mentioned, Antarctic species are stenothermal and are therefore particularly susceptible to the effects of small temperature increases (Meredith and King 2005; Peck et al. 2004, 2006). If current climate change trends continue, a rise of 1-2 °C would impact Antarctic species competitiveness with potential immigrant species and threaten the survival of populations (Anisimov et al. 2007; Meredith and King 2005; Peck et al. 2004). Increased temperatures will also affect the continental ice and permafrost areas of Antarctica with the retreat of many glacier termini (Cook et al. 2005) and decreased snow cover (Fox and Cooper 1998) having already been measured on the Antarctic Peninsula. Changes in sea ice extent have even identified from whaling logs taken during the 1950-70's (de la Mare 1997) with further reductions in duration and extent of sea ice formation seen in the Bellingshausen Sea (Meredith and King 2005). Surface meltwater runoff and ground water discharges from permafrost to the nearshore marine environments have also increased (Vaughan 2006) and will continue to increase with further warming (Anisimov et al. 2007). Changes in primary production have also been affected by sea ice retreat and fresh water influxes, which is predicted to impact the balance between the main phytoplankton grazers: krill and salps (McClintock et al. 2008). The intertidal and nearshore marine species which are most vulnerable to impacts from human derived chemical contamination are also at greatest risk to climate change temperature effects with the impacts on Antarctic marine species of the combined stressors completely unknown. With Antarctic marine food webs already considered the most fragile ecosystems susceptible to environmental change (Peck et al. 2004), additional exposure to human contamination could have unprecedented negative effects on ecosystem structure and function.

Development of water quality guidelines for key contaminants of concern in Antarctica

Environmental Risk Assessments and contaminated site remediation and management in Antarctica is challenging as specific guidelines and monitoring protocols for the Antarctic environment have not yet been developed (Chapman and Riddle 2003; Stark et al. 2006b; Snape et al. 2003). Development of water quality guidelines are necessary for managing the negative effects of contaminants on aquatic ecosystems, and as ecosystem types vary globally due to structure, physiochemical properties and inhabiting biota, universal guidelines cannot be applied cross-regionally. Specific guidelines that have been developed for use in tropical, temperate and some Arctic regions may be able to be adapted for use in other areas provided sufficient information on local conditions and biotic responses is available (ANZECC/ARMCANZ 2000). Currently, there is insufficient ecotoxicological information for Antarctic marine species responses to contaminant exposure, and no water quality guidelines have been developed.

Trigger values form the basis of water quality guidelines and represent concentrations of specific chemicals that if exceeded, have the potential to cause a negative effect in the ecosystem (ANZECC/ARMCANZ 2000). The level of effect that the trigger value is defined for is predetermined, and is generally set to protect 95% or 90% of species within that ecosystem. Deriving trigger values not only incorporates regionally specific abiotic factors of aquatic ecosystems but most importantly, it involves assessing the conservation value, level of pre-existing disturbance and the sensitivity of the local biota to contaminant exposure. In the case of Antarctica, the protection value of 99% would be more appropriate given that this level of protection under the ANZECC/ARMCANZ guidelines (2000) is prescribed for aquatic ecosystems with high conservation value. The most common method of determining species sensitivity to a contaminant is through acute or chronic toxicity tests. These bioassays expose

individuals to a range of concentrations of a given contaminant for a defined exposure period after which observations are made based on a certain endpoint such as mortality, growth or behavioural change. Statistical analysis is then performed to determine point estimates. For a mortality endpoint, the point estimate is defined as the lethal concentration (LC) of the contaminant that causes X % mortality in the test species. The concentration that causes 50% mortality of the test species is termed the median lethal concentration (LC_{50}). Sub-lethal endpoints such as growth or behaviour are termed effective concentrations (EC) and like lethal concentrations, are contaminant concentrations estimated to cause the particular effect in X % of the test species. Few toxicity tests have been undertaken with Antarctic marine species to date, and their sensitivity to contaminants is largely unknown. Without further toxicity testing to generate more point estimates, trigger values for contaminants and subsequent water quality guidelines cannot be developed for Antarctic marine ecosystems.

Development of appropriate methods for toxicity tests with Antarctic biota

Only a small number of toxicity tests with Antarctic marine biota have been completed to date (Chapman and Riddle 2005a, Duquesne et al. 2000; Hill et al 2009; King and Riddle 2001; Liess et al. 2001). Some of these studies employed short-term testing durations taken from standard methods developed for temperate and tropical test species and only reported the LC_{50} values and do not investigate more subtle sub-lethal impacts (Duquesne et al. 2000). Studies that used longer exposure durations showed that Antarctic organisms have longer acute response times and when comparing results with temperate and tropical species, they were more sensitive to contaminant effects (Chapman and Riddle 2005a, King and Riddle 2001). Using the limited data available from short-term tests (1 and 4 day LC_{50}), Chapman and Riddle (2005a) found that applying the

acute criteria of the United States Environmental Protection Agency (US EPA) would protect Antarctic marine invertebrates from the metal contaminants tested. However, applying the combined temperate guidelines and criteria for chronic exposure to the longer-term test results showed that trigger values were not protective for some metals (Chapman and Riddle 2005a). Using more relevant, longer exposure durations was an important factor in King and Riddle's (2001) study that determined metal impacts on developmental stages of the Antarctic sea urchin *Sterechinus neumayeri*. Sensitivity of larval stages of the Antarctic urchin to metals were greater than related species from temperate and tropical regions, when actual larval stages as opposed to exposure durations were compared (King and Riddle 2001). The longer acute response times and longer development times of Antarctic marine biota highlight the need for developing Antarctic specific toxicity methods with longer test durations to appropriately address their unique life history, ecophysiology and environmental characteristics (Chapman and Riddle 2005a, 2005b; King and Riddle 2001).

In addition to exposure duration, the endpoint evaluated in toxicity tests affects the contaminant sensitivity determined for a species. Sub-lethal endpoints such as molecular, physiological or behavioural changes are early warning indicators of contaminant effects and have been used in environmental monitoring programs worldwide (Galloway et al. 2002). An ideal endpoint selected for use in routine toxicity testing would be robust yet sensitive, and simple to evaluate as well as rapid and cheap to perform (Mills et al. 2006). Behavioural endpoints are more sensitive than mortality, integrating the contaminant impacts on complex biochemical and physiological processes that enable an individual to function normally within its ecosystem (Mills et al. 2006). The majority of toxicity tests with Antarctic marine species have focused on mortality as the endpoint, with the exception of Hill et al (2009) who investigated the effects of metal contaminated sediments on the behavioural and survival of an Antarctic spirorbid, *Spirorbis nordenskjoldi*. They found the behavioural endpoint to be an order of magnitude more sensitive to metal exposure than the survival endpoint. As Antarctic marine species show longer acute time responses to contaminant

exposure (Chapman and Riddle 2005a), evaluating alternative sub-lethal endpoints would improve sensitivity and decrease the exposure duration required to observe a response. Only one study to date with Antarctic biota has investigated behavioural changes (Lenihan et al. 1995b). In this study, the behaviour of a benthic amphipod, tanaid, cumacean and heart urchin exposed to contaminated sediments was assessed to explain the difference in species diversity at contaminated and reference sites near McMurdo Station, however point estimate values were not derived (Lenihan et al. 1995b). Ecologically relevant behaviours associated with movement and locomotion are ideal for endpoint development as they affect an organisms ability to find prey, avoid predation and engage in reproductive events (Mills et al. 2006).

While developing toxicity tests that incorporate longer exposure durations is essential for improved determination of contaminant sensitivity of Antarctic marine species (Chapman and Riddle 2005a, 2005b; King and Riddle 2001), this will present analytical difficulties concerning the longer-term, time-series data that is generated. Binomial concentration-response data from toxicity tests is generally analysed using the Probit model combined with Maximum Likelihood to fit a regression. This regression is used to determine point estimates of effective or lethal concentrations of the contaminant that would cause a response in X % of a population (EC_x / LC_x). The exposure duration of the tests and the endpoint that is subsequently analysed is generally standardised to species or taxa, and chosen irrespective of the contaminant being tested (Baas et al. 2010). Despite routine gathering of data from intermediate time points, traditional practice in toxicological analyses determines point estimates from response data for only one endpoint (e.g. mortality / behaviour / biomarker change) which has been observed at a single, specified time during a bioassay (e.g. 24 h, 48 h). This practice disregards the cumulative effect of contaminant exposure through time, limiting understanding of temporal contaminant effect and the ability to extrapolate effects across toxicants, species and ecosystems (Heckmann et al. 2010). This approach has also been widely criticised (Álvarez et al. 2006; Baas et al. 2010; Crane and Grosso 2002; Heckmann et al. 2010; Jager

et al. 2006) and developing statistical methods which incorporate time-series data would greatly improve the interpretation of longer-term bioassay results and risk assessment outcomes for contaminant impact management in Antarctica.

Selected test species

A number of Antarctic marine microalgae and invertebrates were selected for development of toxicity test and metal contaminant sensitivity assessment. Selection was based on satisfying a number of factors; ecological important, inhabiting coastal waters, reliably accessible and abundant, and amenable to laboratory and handling conditions. Of the six phytoplankton species assessed for growth inhibition bioassay development, *Phaeocystis antarctica* was tested for sensitivity to copper exposure. *Phaeocystis antarctica* has a complex life cycle, having many free-living cell forms only a few micron in diameter as well as forming mucilaginous colonies a few millimetres in diameter in the open ocean and coastal waters of Antarctica (Marchant et al. 2005; Schoemann et al. 2005). It is a dominant algae inhabiting the sea ice during winter and readily disperses into the ice-edge water column where it is the first to bloom during the summer months, representing ~ 90 % of the phytoplankton cells at the peak of the bloom (Davidson and Marchant 1992). Grazed by copepods, krill and other zooplankton, *P. antarctica* is an ecologically significant phytoplankton species, contributing to more than 65 % of the annual primary production of the southern ocean (Schoemann et al. 2005).

A common and ecologically important gammarid amphipod, *Paramoera walkeri* inhabits the littoral benthos and sub-fast ice of Antarctic coastlines. An omnivorous species which feeds on phytoplankton and zooplankton as well as detritus, *P. walkeri* has been used successfully in previous toxicity studies (Clason et al. 2003; Duquesne et al. 2000; Duquesne and Riddle 2002; Duquesne and Liess 2003; Liess et al. 2001; Stark et al. 2006). It is reliably collected from the

under surface of fast ice as well as the benthic sand, mud and rocky substrates and amongst macrophytes between 0-5 m in depth (Sagar 1980). The life cycle of the species is longer than 2 years with females maturing sexually 19 months after hatching. Female *P. walkeri* develop their embryos for 4.5 months from May - October, where after the eggs hatch, they continue to brood their young for a further 40-50 days (Sagar 1980).

The deposit feeding and macrophage (Dauby et al 2001), benthic amphipod *Orchomenella pinguides* (Lysianassidae) was selected as a test species due to its different habitat niche and feeding strategy to *P. walkeri*. Further ecological information on this species is limited to personal observations (Bianca Sfiligoj). Adults measure approximately 5-9mm in length, having an orange carapace and red eyes. They can live in high densities on mostly sand / mud substrates between 10 - 20m in depth in the Antarctic near shore environment.

Skenella paludionoides is an epiphytic microgastropod and was chosen as a test species as it is commonly found on macrophytes in littoral habitats of Antarctic coastlines. It has been identified as the dominant epiphytic invertebrate on the macrophyte, *Desmarestia chordalis* from samples taken near the Japanese Antarctic research base of Syowa Station (Takeuchi and Watanabe 2002). Information for this species is mostly limited to personal observations noted during collections and experiments. *Skenella paludionoides*, of the family Cingulopsidae, has a spiral shell that is often black but can also be found as a dark grey/brown colour. The adults are approximately 2mm in length and lay masses of yellow eggs onto firm substrates such as the base of macrophyte fronds and rocks (personal observations, Bianca Sfiligoj).

Juveniles of the heart urchin *Abatus shackletoni* were selected as a test species to investigate sensitivity of an early life history stage and as a representative from the Echinoidea class. *Abatus shackletoni* burrow 5 – 10 cm into soft-sediment (David et al 2005) and loose gravel substrates of Antarctic coastal habitats and have been found at depths between 3 – 18 m (Kirkwood and Burton 1988). Adults measure between 40 and 67 mm in diameter and the females

brood an average of 80 eggs and juveniles in the brood pouches of the petals (David et al 2005). The juveniles are a red-brown colouration, turning brown as adults (personal observation, Bianca Sfiligoj).

Another Lysianassid amphipod species *Tryphosella murrayi* was used as a test species during this study. Information for this species is mostly limited to personal observations during collections and experiments however, there are documented collections from coastal and continental shelf sites around the Antarctic coastline between 5 and 920m of depth (De Broyer et al 2007). Adults collected for experiments were between 12 and 15 mm in length, having a yellow carapace and red eyes (personal observations, Bianca Sfiligoj).

Selected test metals

Metals can be categorised as essential or non-essential depending on their requirements for biological functions and processes within organisms. Both categories of metals are taken up by organisms from their surrounding environment from water, sediment and dietary sources. Five metals that were detected at high concentrations in tip leachates from Thala Valley were selected for toxicity test application in this research; copper, cadmium, zinc, lead and nickel (Deprez et al 1999).

Phytoplankton take up trace metals in two stages; firstly, the metal ion binds to proteins on the cell membrane surface and secondly, the metal is transported internally mostly through protein channels and by carrier-proteins (Rainbow 1997; Stoiber et al 2010). Marine invertebrates such as amphipods take up trace metals through absorption through permeable surfaces such as the gills and the gut (Rainbow 2007). Metals can become toxic to an organism if present at concentrations greater than required for normal biological function. Toxicity will occur if the rate of metal uptake exceeds the rate at which the organism can detoxify (bind and store) or excrete the metabolically available form of metal (Marsden and Rainbow 2004; Rainbow 2002; Rainbow 2007). Importantly, metal

accumulation rates vary between organisms, with toxicity being metal and species specific and reliant upon a number of biological and physiological characteristics (Rainbow 2002; Rainbow 2007).

Abiotic factors such as temperature, salinity and pH also influence metal uptake by affecting metal speciation and therefore, bioavailability of metal ions (Rainbow et al 1990). Metal ions in aquatic invertebrates can be regulated by metallothioneins. Metallothioneins are non-enzymatic proteins that contain thiol groups, which bind to particular metals (Amiard et al 2006). Metallothionein proteins can contain several metal atoms of copper, zinc, cadmium, mercury and silver when present and are believed to control the homeostatic cycling and detoxification of these metals (Amiard et al 2006). The following section summarises the role and toxicity potential of each of the major metals of interest.

Copper

Copper is an essential metal and in trace concentrations, is necessary for the proper biological function of a variety of structural and enzymatic processes that result in growth, development and maintenance in an organism (Gaetke 2003; Stauber and Davies 2000). Both the oxidised (cupric Cu^{2+}) and reduced (cuprous Cu^{+}) forms of copper can participate in intracellular redox reactions. The most powerful reactive oxygen species (ROS) in biological systems, hydroxyl, is produced when cupric is reduced to cuprous through the Haber-Weiss reaction. Hydroxyl can interact with almost every type of biological molecule and cause oxidative damage to lipids, proteins and DNA (Buettner 1993; Kawanishi 1989, Stoiber et al 2010). Within phytoplankton cells, copper oxidises thiol groups, inhibiting spindle formation and cell division and therefore restricting formation of new daughter cells (Stauber and Florence 1987). Amphipods readily take up copper from the surrounding environment where it is metabolically available for biological processes such as producing the respiratory protein, haemocyanin (Rainbow 2002), and being detoxified and stored by metallothionein (Amiard et al 2006).Copper can be neurotoxic to some invertebrates through inhibiting the

production of the neural transmission enzyme acetylcholinesterase (Brown et al 2004). Many research studies have investigated the accumulation and toxicity of copper exposure on marine phytoplankton and amphipod species from a variety of geographical regions (e.g. Ahsanullah and Florence 1984; Bat et al 1998; Brown et al 2004; Clason and Zauke 2000; Debelius et al 2009; Duquesne et al 2000; Duquesne and Riddle 2002; Duquesne and Liess 2003; Florence and Stauber 1986; Franklin et al 2001; Gopalakrishna Bhat and Vamsee 1993; Johnson et al 2007; King et al 2005, 2006; Levy et al 2007, 2008; Stauber and Florence 1987).

Cadmium

Cadmium is a non-essential metal yet has similar chemistry to the essential metal, zinc, and potentially shares similar uptake pathways in aquatic invertebrates (Rainbow 1997). Cadmium is redox inactive and does not directly exert oxidative stress on cells through the formation of ROS as copper does, however it is toxic in low concentrations by binding to thiol groups and reducing antioxidant capacity and enzyme function (Ercal et al 2001; Stoiber et al 2010). As cadmium is non-essential and has no biological role with an organism, toxic effects will only be avoided if the elimination rate (through detoxification or excretion) matches the uptake rate (Rainbow 2002). Crustacea accumulate cadmium through detoxification, typically as metallothionein (Rainbow 2007). Cadmium has been shown to decrease oxygen consumption, metabolic activity and ventilation rates in the dogwhelk *Nucella lapillus* (Leung et al 2000) A number of studies have investigated the effects of cadmium exposure and accumulation on marine phytoplankton and amphipods (e.g. Bargagli et al 1996; Bat et al 1998; Felten et al 2008; Jamers et al 2009; Payne and Price 1999; Rainbow and White 1989; Stoiber et al 2010; Vellinger et al 2012; Wang and Wang 2009; Yu et al 2005).

Zinc

Zinc is an essential metal and is a key component of many enzymes including carbonic anhydrase (Rainbow 2002). Zinc is involved in a large number of

metabolic processes such as the synthesis and decomposition of carbohydrates, proteins and lipids and also involved in transcription and translation of genetic codes (Vallee 1993). The copper and zinc containing superoxide dismutase (Cu,Zn-SOD) is an extremely important antioxidant (Yim 1993). Zinc also stimulates the synthesis of metallothionein which can affect the metabolic availability of copper. Copper has a higher binding affinity to metallothionein than zinc, so with increased production of metallothionein stimulated by higher levels of zinc, more copper is sequestered and bound to the thiol groups of the metallothionein protein (Fosmire 1990). Toxic effects and accumulation of zinc have been investigated through a number of studies on algae and invertebrates (Ahsanullah and Arnott 1978; Bat et al 1998; Chapman and McPherson 1993; Chong and Wang 2001; Ciutat and Boudou 2003; Johnson et al 2007; King et al 2006; Rainbow and Luoma 2011).

Lead

Lead, like cadmium, is redox inactive and exerts oxidative stress in organisms indirectly by depleting antioxidants in cells leading to an increase in ROS (Ercal et al 2001). Lead can adversely affect reproduction in invertebrates and affect the growth of algae (U.S.EPA 2011). Lead toxicity in natural waters can be reduced by low solubility through complexation with dissolved organic matter and adsorption to suspended humic and clay materials (ANZECC/ARMCANZ 2000). Toxic effects and accumulation of lead have been studied on a number of algae and invertebrate species (Chapman and McPherson 1993; Debelius et al 2009; Fichet et al 1998; Satoh et al 2005; Schubauer-Berigan et al 1993).

Nickel

Nickel is an essential metal for some organisms, primarily plants and algae that use nickel to produce the enzyme urease (Rees and Bekheet 1982). Nickel toxicity can produce oxidative stress which can impact on DNA, increase lipid perioxidation, deplete glutathione and alter calcium and sulphhydryl homeostasis (Das 2008). Effects of nickel toxicity and accumulation have been

studied in a number of algae and invertebrates (Ahsanullah 1982; Doig and Liber 2006; Ferreira et al 2010; Hunt et al 2002; Keithly et al 2004).

Summary and thesis objectives

Further toxicity tests using species across a range of taxonomic groups and trophic levels (microalgae, invertebrates and vertebrates) are needed in order to derive Antarctic specific water quality guidelines and remediation targets (Chapman and Riddle 2005b, 2003; Snape et al. 2001). By addressing the ecophysiological characteristics of Antarctic marine biota through the development of tests with longer exposure durations and more appropriate and sensitive endpoints, whilst applying more appropriate statistical methods that integrate responses through time, we will be better able to estimate sensitivities of Antarctic marine biota to chemical contaminant exposure as well as to develop biological contaminant monitoring techniques. This will facilitate the development of water quality guidelines and remediation targets for Antarctic marine ecosystems, provide risk assessment methods and information for improved identification of contaminated sites, and improve our ability to manage anthropogenic contaminants in the Antarctic environment.

The overall aim of the thesis is to investigate the sensitivity of Antarctic marine invertebrate and microalgae to metal contaminant exposure, to provide point estimates to incorporate into species sensitivity distributions in order to develop site-specific Antarctic water quality guidelines. Specific objectives were to: 1) develop bioassay methodologies for contaminant testing with coastal Antarctic marine invertebrate and microalgae species; 2) assess their sensitivity to known anthropogenic metal contaminants over longer exposure periods than those used in standard toxicity tests with temperate and tropical species; and 3) to develop new statistical methods to analyse time-series mortality data generated by longer-term bioassay exposures.

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Chapter 2

Screening of Antarctic microalgae for suitability in toxicity tests using flow cytometry analysis and the effects of copper on *Phaeocystis antarctica* (Karsten)

This chapter is presented as a manuscript prepared for publication

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Abstract

Water quality guidelines contribute to the successful management of anthropogenic contaminant impacts in many temperate, tropical and some Arctic marine environments. Despite the existence of legacy sites of contamination and the ongoing discharge of sewage wastewaters to the marine environments near Antarctic research stations, no water quality guidelines have been established for this region. Developing guidelines requires an understanding of the sensitivities of a range of Antarctic marine biota including microalgae, invertebrates and vertebrates, to contaminants. This study evaluated the suitability of six, ecologically important Antarctic marine microalgae as potential toxicity test species using flow cytometry. *Phaeocystis antarctica* was found to be the most suitable species for use with the flow cytometry technique as it had the fastest growth rate out of the six species screened. *Phaeocystis antarctica'*s sensitivity to copper was assessed using standard microalgal growth rate inhibition bioassay techniques modified for use with the Antarctic species. *Phaeocystis antarctica* was highly sensitive to copper exposure, with 100% growth rate inhibition at only 8.86 µg/L and mean 50% and 10% growth rate inhibition concentrations of 3.78 µg/L (± 0.5 SD) and 1.92 µg/L (± 0.46 SD) respectively. Due to analytical detection limits, we could not accurately quantify concentrations in the copper treatments below 2 µg/L and therefore these values are estimated from linear regression analysis of the nominal and measured copper concentrations. As *P. antarctica* shows a high sensitivity to copper it is recommended that these tests be repeated to qualify the sensitive point estimates which have been determined for copper. The results of this work supports the further development of this species as a biological monitoring tool for determining contaminant point estimates of effect to contribute to the development of Antarctic specific water quality guidelines.

Key words

phytoplankton, water quality guideline, toxicity, growth inhibition bioassay, metal, contamination

Introduction

Human activity in Antarctica has caused significant ecological impact to terrestrial and marine environments through chemical contamination and physical disturbance at localised scales (Tin et al. 2009). Past practices and on-going activities associated with logistics, research station habitation, waste disposal, tourism and fisheries operations have resulted in impacts to the environment (Tin et al. 2009). Chemical contamination of nearshore marine habitats has been identified as one of the most widespread and long-lived impacts on Antarctic environments (Bargagli 2005). Sites affected by contamination require remediation under Annex III - Waste disposal and Waste Management, of the Protocol on Environmental Protection to the Antarctic Treaty (the Madrid Protocol), with the provision that no further environmental damage is caused by the clean-up process. However, determining sites that require remediation, monitoring remediation activities, setting appropriate remediation targets, as well as protecting against future impacts, requires knowledge of contaminant levels that can negatively affect Antarctic biota (Snape et al. 2003).

Contaminant concentration thresholds (trigger values) listed in water quality guidelines (eg. ANZECC/ARMCANZ 2000) have been developed for many temperate and tropical regions. Due to a paucity of ecotoxicological data, guideline values for polar marine environment protection have not yet been developed (Chapman and Riddle 2003, 2005a). Few toxicological studies with Antarctic marine species have been conducted with most focusing on the effects of metals and UV-B exposure effects on the survival of the amphipod,

Paramoera walkeri (Duquesne et al. 2000; Duquesne and Riddle 2002; Duquesne and Liess 2003; Stark et al. 2006). One study has investigated the effect of metal exposure on the embryonic and larval development of the sea urchin, *Sterechinus neumayeri* (King and Riddle 2001), and a single study has investigated the effect of cadmium on a sea ice alga *Chlamydomonas* sp. (Yu et al. 2005). This lack of sensitivity data also affects the possibility of applying water quality guidelines from other regions for use in Antarctica, as it is not known whether Antarctic species have similar sensitivities compared with analogous temperate and tropical species (Chapman and Riddle 2005a). Further toxicity tests using species across a range of taxonomic groups and trophic levels (microalgae, invertebrates and vertebrates) are needed in order to determine threshold concentrations to be used in the development of Antarctic specific water quality guidelines and remediation targets (Chapman and Riddle 2003, 2005b; Snape et al. 2001a; Stark et al. 2006; Tin et al. 2009).

Microalgae form the foundation of energy transfer in aquatic ecosystems (Debelius et al. 2009). Able to quickly respond to bioavailable contaminants, microalgae are sensitive indicators of environmental change, and as such have been used extensively as both biomonitors and as test species in water quality guideline development in temperate and tropical regions (Debelius et al. 2009; Franklin et al. 2005; Johnson et al. 2007; Stauber and Davies 2000). Growth rate inhibition is commonly used as one of the most sensitive endpoints in microalgal toxicity testing. A reliable, fast and effective technique to measure growth rate inhibition is the flow cytometry method that can accurately determine live algal cell counts (Franklin et al. 2001, 2005; Hadjoudja et al. 2009; Jamers et al. 2009; Stauber et al. 2002). Population-based endpoints such as growth rate inhibition are highly relevant, representing a significant level of ecological impact by affecting species competition, succession and community structure and function (Stauber and Davies 2000). Combining the potential sensitivity and ecological importance of microalgae in the Antarctic food web with the effective flow cytometry technique, the growth rate inhibition bioassay is an ideal test to

develop for assessing the sensitivity of Antarctic microalgae to known anthropogenic contaminants.

Antarctic microalgae are diverse, composed of hundreds of species from more than 20 different classes within three different phyla (Marchant and Scott 2005). Due to the dynamic seasonal fluctuations in the Southern Ocean, Antarctic microalgae have evolved to inhabit the open water column, form dense benthic mats (Cunningham et al. 2005), colonise sea ice brine channels and grow on the underside of sea ice (Marchant and Scott 2005). Growth rates of micro algae are influenced by temperature, light irradiance and nutrient availability. Due to stable low temperatures and high nutrient availability of Antarctic waters, variations in growth rates of Antarctic micro algae is controlled by the seasonal light regime (Sakshaug and Holm-Hansen 1986; Wright et al 2010). When light is the major controlling factor for phytoplankton growth rates, photoadaptations such as variations in chlorophyll content and photosynthetic responses to irradiance, influence the way that algal cells respond to varying light conditions (Sakshaug and Holm-Hansen 1986). Compared with temperate or tropical species, Antarctic micro algae generally have lower growth rates, with rates measured for 15 species ranging between 0.32 -0.72 doublings per day (Sommer 1989). During the Antarctic summer, reduced ice cover and longer hours of daylight stimulate the rapid formation of dense microalgal blooms with colony forming and large celled diatom species leading the initial bloom (Perrin and Marchant 1987; Clarke and Leakey 1996). The initial size of the phytoplankton stock is an important factor in determining the exponential progress of the bloom and particular areas like the marginal ice zone which have a large seeding stock of phytoplankton from the sea ice will have a positive impact on the rapid development rate of the bloom (Sakshaug et al 1991; Wright et al 2010).

In respect to the diverse characteristics and life strategies of Antarctic phytoplankton, selection of candidate species for bioassay development was based on a number of criteria. Consideration was given to species at most risk of impact to a range of contaminants, which narrowed potential candidates to

coastal species. Species must also have significant ecological importance (i.e. abundant / common / key food source) so that extrapolation to ecosystem impacts can be better inferred from single species test results. Candidate species should also have characteristics that make them suitable for laboratory culture as well as manipulation and analysis using flow cytometry with the most important of these being the ability to culture solitary cells. The species should be sufficiently sensitive to contaminant impacts to provide the most sensitive point estimates for protective guideline development.

Six common and ecologically important microalgae fulfilled these criteria and were selected for screening to identify species most suitable test development with flow cytometry. The micro algae were the prymnesiophyte *Phaeocystis antarctica,* three bacilliariophytes; *Chaetoceros* sp.1, *Cylindrotheca closterium,* and *Nitzschia* sp., the cryptophyte *Geminigera cryophila* and the dinophyte *Polarella glacialis*. Suitability was determined based on their solitary cell culture, growth rate and amenability to measuring cell density using flow cytometry.

Diatoms such as *Chaetoceros* sp, *C. closterium* and *Nitzschia* sp are generally unicellular, composed of silicate cell walls and reproduce vegetatively through binary fission (Scott and Thompson 2005). Twenty-five diatom species represent the *Chaetoceros* genus in Antarctic waters that are generally characterised by rectangular valves with long setae that are solitary and or, chain forming depending on the specific species (Scott and Thompson 2005). *Cylindrotheca closterium* is a solitary diatom that can be needle like, straight, sigmoidal or sickle shaped and while it can be confused with *Nitzschia longissima* it is generally broader and a more delicate cell (Scott and Thompson 2005). *Nitzschia* sp. are solitary cells or can be attached end to end and generally are lanceolate in shape (Scott and Thompson 2005). Cryptophytes such as *G. cryophila* are delicate flagellates that are widespread in Antarctic marine waters (Scott and van den Hoff 2005). Dinoflagellates such as *P. glacialis* are motile cells with two flagella and have few representative species in Antarctic waters compared with

temperate and tropical regions (McMinn and Scott 2005). *Polarella glacialis* has a dormant cyst and motile flagellate cell forms (McMinn and Scott 2005).

Of the six species, *P. antarctica* showed the greatest potential through the screening trial. *Phaeocystis antarctica* is the only *Phaeocystis* species found in Antarctic waters and is particularly common in coastal areas in the Ross Sea and Prydz Bay (Marchant et al. 2005; Schoemann et al. 2005). It has a complex life cycle, existing either as small flagellated and non-flagellated solitary cells (~ 4 µm diameter), or in dense blooms of spherical, mucilaginous colonies (~ 10^7 cells/L) (Marchant et al. 2005; Schoemann et al. 2005). Contributing to approximately 65% of the total annual primary production in the Southern Ocean, *P. antarctica* plays an important role in nutrient transfer to higher trophic levels, and in cycling carbon dioxide and sulfur between the ocean and the atmosphere (Schoemann et al. 2005). *Phaeocystis antarctica,* like many marine micro and macroalgae, produces significant amounts of dimethylsulfide (DMS) through the enzymatic cleavage of dimethyl-sulfonio-proprionate (DMSP), in response to light and temperature stresses (Baumman 1994; Cantoni and Anderson 1956). DMSP is an osmolyte and is produced by algae to aid in osmotic regulation (Yoch 2002). DMSP is synthesized through the pathway: methionine → 4-methylthio-2-oxobutyrate → 4-methylthio-2-hydroxybutyrate → 4-dimethylsulfonio-2-hydroxybutyrate → DMSP (Gage et al 1997). The release of DMS to the atmosphere by *P. antarctica* has a significant influence on regional cloud formation and ultimately on global climate (Davidson and Marchant 1992; Gaebler-Schwarz et al. 2010). Copepods, krill and other zooplankton graze extensively on *P. antarctica* (Arndt and Swadling 2006; Davidson and Marchant 1992; Haberman et al. 2003; Smith 2003).

Given the ecological importance of this species and its suitability to flow cytometry analysis, *P. antarctica's* sensitivity to the metal exposure was assessed with the adapted growth rate inhibition bioassay. Having a high surface-to-volume ratio and multiple membrane metal-ion binding sites, microalgae are particularly sensitive to metal exposure (Johnson et al. 2007). Cellular

membranes are generally impermeable to metal ions and binding of a metal ion to a protein (ligand) on the cell's membrane surface is required for transportation into the cell (Sunda and Huntsman 1998). These transportation proteins are however, not specific to particular metals and will bind and facilitate the transport of both essential and non-essential metals through the cell's membrane (Sunda and Huntsman 1998).Copper was chosen as the reference metal for its use as a standard toxicant in algal bioassay testing (Stauber and Florence 1987) and it is a known anthropogenic contaminant in Antarctic marine coastal waters and sediments (Deprez et al. 1999). As an essential trace element, copper is actively taken up by algal cells but can be toxic at concentrations as low as 1 µg/L (Levy et al. 2007); not much higher than concentrations that stimulate algal growth (ANZECC/ARMCANZ 2000; Florence and Stauber 1986). *Phaeocystis antarctica's* sensitivity to copper exposure is compared with other temperate and tropical species and due to this high level of sensitivity observed, this species is recommended for further development as a biomonitor for Antarctic coastal waters.

Materials and Methods

General analytical

Glassware (Erlenmeyer and volumetric flasks, beakers and Schott bottles) were soaked overnight in 10% (v/v) nitric acid (HNO_3) and rinsed thoroughly with reverse osmosis (Milli-RO) water. All test glassware was capped with double folded aluminium foil lids and sterilised by autoclaving. Seawater to be filtered for use in algal culturing and growth inhibition bioassays was collected from the shoreline of Trumpeter Bay, Bruny Island, Tasmania (43˚9'28"S, 147˚23'52"E) at a depth of 1-1.5 m and filtered to 0.22 µm then stored in new, black, 20 L carboys at 2 ˚C. All reagents were analytical grade. Nutrient stock solutions of sodium nitrate (26 mM $NaNO_3$ UNIVAR) and potassium dihydrogen orthophosphate (1.3 mM KH_2PO_4 UNIVAR) were prepared in accordance with Franklin et al. (2005) and stored at 4 ˚C. Minimal nutrient seawater was prepared

for each growth rate inhibition bioassay by adding 10 mL of each $NaNO_3$ (15 mg NO_3^-/L) and KH_2PO_4 (1.5 mg PO_4^{3-}/L) stocks per litre of filtered seawater (FSW)(Franklin et al. 2005). Background levels of NO_3^- and PO_4^{3-} were not measured in the FSW. A 250 mL copper stock solution of 500 mg/L was prepared by dissolving copper sulfate ($CuSO_4.5H_2O$) in highly purified (Milli-Q) water in acid-washed, high-density polyethylene bottles. Working stock solutions of copper at 1 mg/L and 25 mg/L were made by diluting the original stock with Milli-Q water. No pH adjustments were made in stock, media or exposure solutions.

Microalgae culturing

Isolates of *P. antarctica* (Prymnesiophyceae, strain number AAD 113, isolated by Andrew Davidson from a seawater sample collected from Davis Station, East Antarctic in 1989, solitary cell phase), *G. cryophila* (Cryptophyceae), *P. glacialis* (Dinophyceae), *Chaetoceros* sp.1 (Bacilliariophyceae)*, C. closterium* (Bacilliariophyceae) and *Nitzschia* sp. (Bacilliariophyceae) from the Australian Antarctic Divisions' algal culture collection (Kingston, Tasmania, Australia) were selected for the screening test. *Phaeocystis antarctica* was maintained in GSe/5 medium (Blackburn et al. 2001), with the remaining five species maintained in f/2 medium (Jeffrey and LeRoi 1997). Aseptic transfers for culture maintenance occurred monthly for all species into new, sterile 70 mL plastic sample containers. Cultures were kept in a temperature-controlled room at 2 ± 2 ˚C with a 12 h light/dark cycle and light source intensity ~ 100 µmol. photons/m^2/s.

Flow cytometric analysis

In addition to being a rapid, accurate and effective technique for determining cell densities, flow cytometry has additional advantages over other techniques such as microscopy. Using lasers to excite fluorescence in algal cells, flow cytometry can quickly differentiate between live and dead cell counts. The laser optics also determine shape and size of cells, and together with the fluorescence characteristics of algae, flow cytometry can differentiate between different species in a heterogeneous sample. It is also sufficiently sensitive to count cell

densities below 10^2 cells / mL (Franklin et al 2000). In this study, a BD-FACSCalibur flow cytometer and CellQuest software were used to count cells in test solutions by identifying individual cells/particles based on their fluorescent and light scattering properties after excitation at 488 nm (laser). Cell density was calculated using the number of cells counted in a sample and the differences in the weight of the sample before and after flow cytometric analyses. PeakFlow™ flow cytometry 2.5 µm green reference bead solution diluted with Milli-Q was added to each sample as an internal standard and was accounted for in the cell density determination (bead volume constituted ~ 5% of sample volume). Fluorescent detector settings of the flow cytometer were optimized for each species, being adapted from Franklin et al. (2005).

Microalgal screening test

The initial screening test to identify which of six Antarctic microalgae exhibited growth rates suitable for flow cytometry analysis was conducted using four-week-old parent cultures from the AAD culture library. Aseptic technique was used to inoculate daughter working cultures. Approximately 5 mL from each parent culture was transferred to new, sterile 70 mL containers, each holding either f/2 or GSe/5 high nutrient media. Cell densities were analysed by flow cytometry every 24 h for 13 d and densities are shown in Table 1. Cell densities for *Nitzschia* sp. and *C. closterium* were determined by flow cytometry after using the adapted solution homogenisation method (to separate clumped cells) as described by Stauber et al. (1994), utilising a 45 mL volume homogeniser instead of a 15 mL homogeniser.

Table 1: Morphological characteristics and cell density increases over 13 days for the six Antarctic microalgal species in the screening test at 2 ˚C.

	Cell				
Species	**Size**	**Shape**	**Density (0 d) (× 10^4 cells/mL)**	**Density (13 d) (× 10^4 cells/mL)**	**Density increase factor (0 – 13 d)**
***Nitzschia* sp.**[1]	10-120 µm apical axis	lanceolate	0.56	6.55	11
Cylindrotheca closterium [1]	30-400 µm apical axis	elongated cylinder	0.56	1.72	3
Geminigera cryophila [2]	15-17 µm	ovate and flattened	0.95	10.72	11
Phaeocystis antarctica [3]	Solitary = 5-8 µm Colony = 10 µm – 2 cm diameter	spherical	12	302.8	25
Polarella glacialis [4]	Vegetative cell = 10-15 µm Cyst = 12-17 µm	prolate - spherical	0.25	1.36	6
***Chaetoceros* sp.1** [1]	10 - 50 µm	rectangular with long spiny setae	0.02	0.02	0

[1] (Scott and Thomas 2005)
[2] (Scott and van den Hoff 2005)
[3] (Marchant et al. 2005)
[4] (McMinn and Scott 2005)

Growth rate inhibition bioassays

Growth rate inhibition bioassays were conducted with the two most suitable species from the screening test; *P. antarctica* and *G. cryophila*. Two growth rate inhibition bioassays were attempted with *G. cryophila.* Following a range finder pilot test with control and four copper treatments (a gradient of 0, 8, 47, 88 and 401 µg/L), two growth rate inhibition bioassays were conducted with *P. antarctica*. Test 1 comprised two replicates for each of the seven copper treatments and three control replicates, and Test 2 comprised three replicates for six copper treatments and control replicates. Nominal and measured copper concentrations are presented in Table 2.

High density daughter cultures were grown for *P. antarctica* and *G. cryophila* in 250 mL glass Erlenmeyer flasks for use in the growth rate inhibition bioassays and then placed into a controlled temperature cabinet (CTC) for 4-6 days for pre-test acclimation and to reach the exponential growth phase. The temperature of the CTC was set to 2 ± 1 ˚C with a 16:8 h high-light (90 ± 9 µmol. photons/m^2/s): low-light (6-7 µmol. photons/m^2/s) intensity cycle. The light regime was designed to mimic the ambient daily light cycle of an Antarctic summer; the time that metal contaminated meltwater is most likely to enter the nearshore marine environment (Snape et al. 2001b). Algal inoculum was prepared as described by Franklin et al. (2005), with daughter cultures being concentrated and rinsed of the f/2 media through four spins at 2500rpm for 7 min in the centrifuge. The method was modified as we used new plastic, 50 mL centrifuge tubes and the centrifuge was pre-cooled to 2 ˚C.

Test solutions were prepared within 24 h of the start of bioassays by dispensing 50 mL of test water (low nutrient seawater ± copper spike) to 125 ml glass Erlenmeyer flasks with aluminium foil lids. Test flasks were placed in the CTC to reach test temperature prior to algal inoculation.

Algal inoculums were added to the test flasks within 2 h of inoculum preparation at a cell density of 1-3 x 10^4 cells/mL for both *P. antarctica* and *G. cryophila* tests. Test flasks were positioned randomly inside the CTC. Cell densities were

analysed every 24 h by flow cytometry until a 16-fold density increase was measured in the controls (Franklin et al. 2005).

Copper analysis

Nominal concentrations of copper are defined as the target concentration of copper designed for each exposure treatment. These nominal concentrations are required in order to measure specific volumes of the copper stock solution for spiking the filtered seawater to create the test exposure solutions. The measured concentrations of copper are those analytically determined in the exposure solutions and include the copper concentration delivered through the measured aliquot of highly concentrated copper stock solution, the contribution from the natural seawater and any other trace contamination. Estimated copper concentrations are defined as those which have been mathematically determined from a linear regression analysis of the nominal and measured concentrations as they were below detectable limits of the analytical technique.

Exposure solutions for each treatment in all tests were sampled for measuring dissolved copper. Samples were taken at the beginning of each bioassay prior to adding the algal inoculum through a 0.45 µm syringe filter and were acidified to 1% (v/v) with ultra pure HNO_3 and stored at room temperature until analysed (Johnson et al. 2007; Levy et al. 2007; Stauber et al. 2005, 2008) by inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian 720-ES). Standard calibrating procedures using single element primary standards and seawater blanks were performed prior to metal analyses of the original undiluted samples with ICP-OES with repeat analysis of individual samples being performed during the analytical run to verify precision of concentrations measured.

The analytical detection limit for copper was 2 µg/L. Concentrations in the copper exposure solutions which measured less than this value were estimated through linear regression analysis. A linear regression was performed for Test 1 and 2 individually, plotting the nominal values (x) against the measured values (y). The equation for each regression was used to calculate the copper

concentrations in the lower exposure solutions. Test 1 had a linear regression of $y = 0.9053x$ with a highly significant fit to the data having an R^2 value of 0.99. Test 2 had a linear regression of $y = 0.9893x$ with a highly significant fit to the data having an R^2 value of 0.98. Nominal, measured and estimated copper concentrations are presented in Table 2. Salinity and pH were measured at the beginning and end of each bioassay using a Mettler Toledo SG78 metre calibrated with buffers for measuring water at 2 °C. The total sample volume removed from test flasks throughout the experiment did not exceed 10% of the initial total test volume.

Table 2: Nominal, measured and estimated concentrations of copper (µg/L) used in growth rate inhibition bioassays with *Phaeocystis antarctica.*

	Test 1		Test 2	
Treatment	**Nominal**	**Measured/Estimated**	**Nominal**	**Measured/Estimate**
Control	0	0.00 [a]	0	0.00 [a]
Copper 1	0.13	0.12 [b]	0.25	0.25 [b]
Copper 2	0.25	0.23 [b]	0.5	0.49 [b]
Copper 3	0.5	0.45 [b]	1	0.99 [b]
Copper 4	1	0.91 [b]	2.5	2.00
Copper 5	2.5	2.53	5	4.51
Copper 6	5	4.78	10	10.23
Copper 7	10	8.86	-	-

[a] below detection limit of 2 µg Cu/L
[b] below detection limit of 2 µg Cu/L; concentration estimated as described in Materials and Methods section

Statistical analyses

Statistical analyses of *P. antarctica* test data followed methods described by Franklin et al. (2005) with modification of the growth rate being calculated over 7 and 8 d instead of the standard 72 h. Cell division rates per hour (µ) for each replicate were calculated as the slope value from a linear regression of $\log_{10}$ cell density over time (h). For each replicate, the slope (specific growth rate) was converted to a daily growth rate using the following formula (Franklin et al. 2005): Growth rate (doublings per day) = µ × 24 × 3.32 (constant)

The calculated specific growth rates and measured / calculated copper concentrations were used to produce concentration-response curves in

ToxCalc™ (Tidepool Software). No observable effect concentrations (NOEC) and the Lowest observable effect concentrations (LOEC) were determined using Dunnett's test for test 2. Unfortunately due to the variability in the growth response in test 1 for the 4.78 µg/L treatment no NOEC or LOEC could be determined Concentrations causing 10% growth rate inhibition (IC_{10}) and 50% growth rate inhibition (IC_{50}) were determined for each test using linear interpolation (Table 3).

Results

Salinity (33 ± 0.5 ‰) and pH (8.07 to 8.43 from start to finish) remained stable throughout all of the tests. Temperature of the CTC was 2.1 ˚C ± 0.5 SD throughout the testing period.

Microalgal screening test

Cell densities for the six test species were determined over two weeks with initial cell densities and increases presented in Table 1. *Phaeocystis antarctica* grew fastest throughout the screening test showing an average of 0.40 doublings per day and was chosen as the most suitable species for the growth rate inhibition bioassay. *Geminigera cryophila* also showed potential as a test species with an average of 0.29 doublings per day, however, the inoculum preparation and growth media rinsing method proved too vigorous with the centrifuge speed of 2500 rpm producing a tangled thread of lysed cells. Subsequent trials reducing the centrifuge speed to 1500 rpm and spinning only twice produced an intact *G. cryophila* inoculum. The proceeding *G. cryophila* growth rate inhibition bioassay was however not successful as cell density in the control treatments only increased three fold by day 12. This is significantly different to the 11-fold increase in cell density measured on day 13 of the screening test (Table 1). The flow cytometer analyses for light scatter (indicating cell size and shape) and chlorophyll fluorescence (indicating species type and health) for *Nitzschia* sp*., C. closterium* and *Chaetoceros* sp. 1 showed no clear pattern, with detection points

broadly distributed over the individual cytometric histograms. As a result, it was impossible to distinguish live from dead cells and therefore accurate densities of live cells could not be determined. These species were subsequently deemed unsuitable for further testing in this study.

Polarella glacialis was also unsuitable for use in the growth rate inhibition bioassay as two distinct, live cell populations appeared on day 3 of the screening test making it difficult to determine if the populations were the same species or different morphologies of the same species.

Growth rate inhibition bioassays with *Phaeocystis antarctica*

The end of each test was determined when cell density had increased 16-fold in the controls that resulted in Test 1 having duration of 8 d and Test 2 being 7 d (Franklin et al 2005). Average growth rates for the controls in all 3 tests (pilot range-finder and two definitive bioassays) were 0.59 to 0.60 cell doublings per day over a 7 or 8 d period with a coefficient of variation of 1 - 2%, well below the 10% standard variability criteria for control treatments (Franklin et al. 2005). These results also show the cells were in a consistent, exponential growth phase for the duration of the tests with an example presented for control replicate number 2, from Test 2 (Figure 1). All copper treatments tested (total dissolved copper: 8 - 400 μg/L) in the range finder pilot test caused cell densities to decrease from day 1 of the experiment, indicating that all copper concentrations tested resulted in cell death, not just growth rate inhibition.

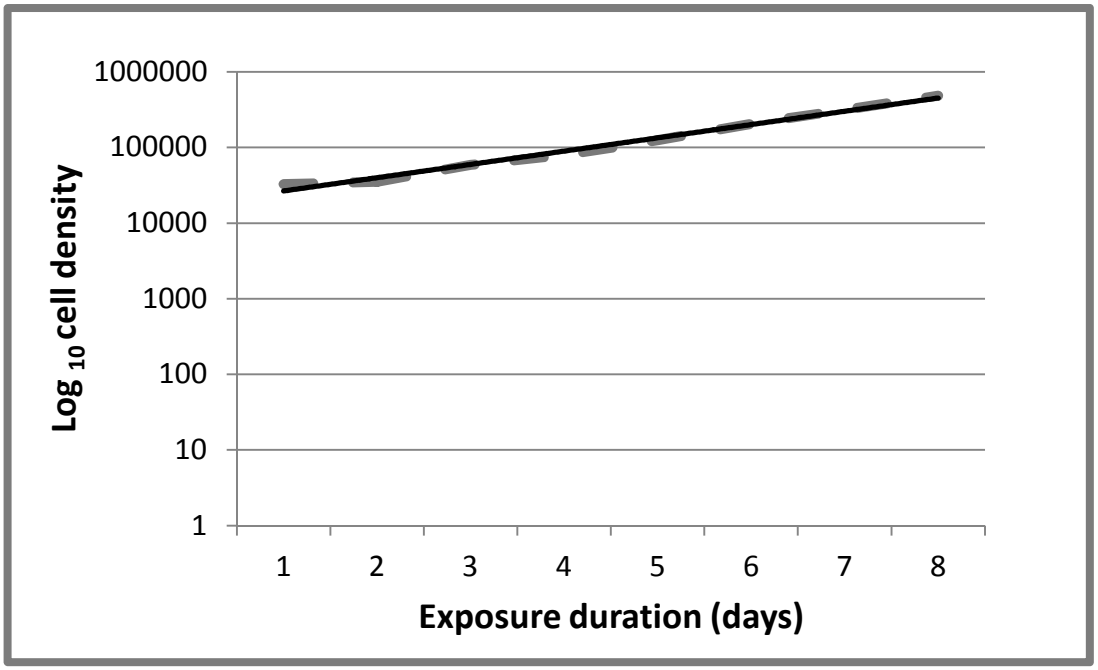

Figure 1: Log_{10} cell density of *Phaeocystis antarctica* growth (dashed blue line) with an exponential trend line (solid black line) fitted for the control replicate number 2 from Test 2.

Lowering the copper concentration in the two replicate tests to a nominal range of 0.13 to 10 µg/L resulted in a range of responses from no growth rate inhibition (100% growth compared to the growth rate in the control) to complete inhibition in algal growth rate (0% growth compared to control) observed in the 8.86 µg/L treatment (Figure 2). The growth inhibition (%) for each treatment as a percentage of the controls for both tests are presented in Figure 2.

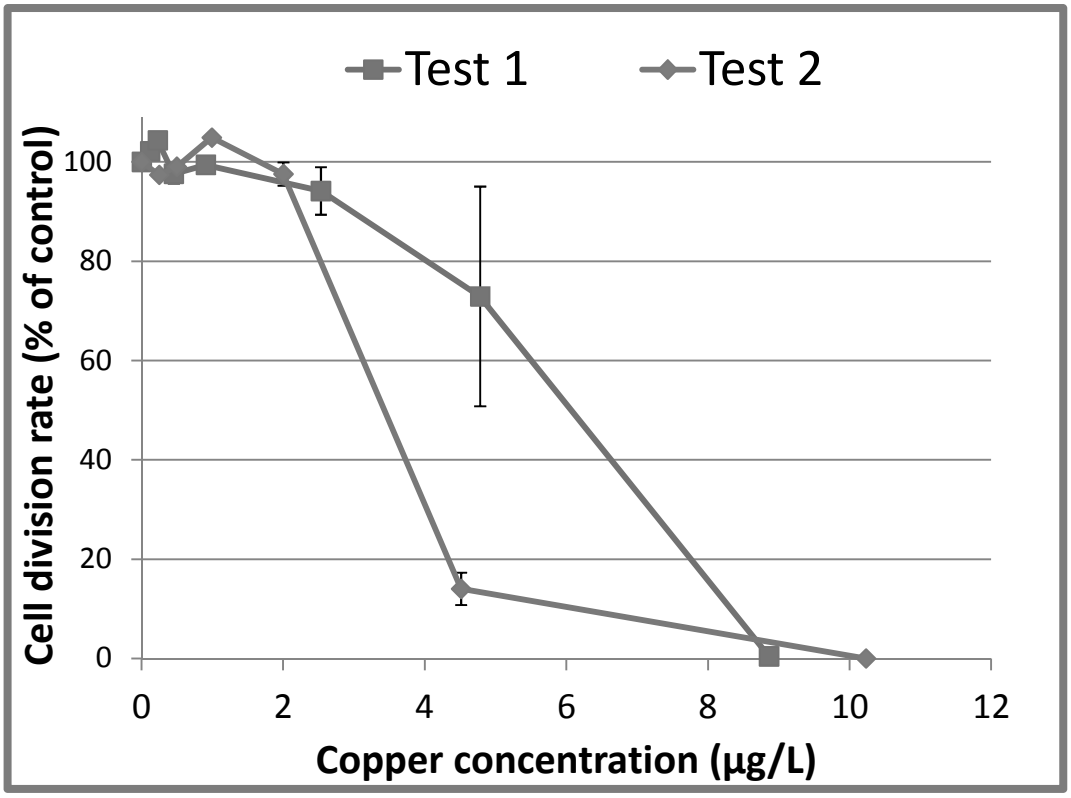

Figure 2: Average growth rate inhibition of *Phaeocystis antarctica* (± 1 SE) as a percentage of the control growth rate, to copper on day 8 and 7 of the exposure in Tests 1 and 2 respectively.

In Test 1, the 10 µg/L treatment had a total dissolved copper concentration of 8.86 µg/L and test 2 had 7.8 µg/L with the nominal, measured and estimated values presented in Table 2. The average IC_{50} for copper in Tests 1 and 2 was 3.78µg/L (see Table 3 for IC_{10} and IC_{50} values for individual tests). There was high variability in growth inhibition at the 3.78 µg/L copper treatment between the two replicates of Test 1 compared with the three replicates in the analogous (4.51 µg/L) treatment in Test 2 (Figure 2).

Table 3: Copper concentrations (µg/L) causing no observable effect (NOEC), the lowest observable effect (LOEC), 10% growth rate inhibition (IC_{10}) and 50% growth rate inhibition (IC_{50}) ± Standard Deviation (SD) for *Phaeocystis antarctica.*

Test	NOEC	LOEC	IC_{10} ± SD	IC_{50} ± SD
1			1.6± 0.25	4.14 ± 0.07
2	2	4.51	2.25 ± 0.03	3.43 ± 0.04
Average			1.92 ±0.46	3.78 ± 0.5

Discussion

The effects of anthropogenic contaminants on Antarctic species are not well understood yet this information is essential for protecting the marine ecosystems and for deriving water quality guidelines. Developing toxicity tests with Antarctic species offers unique challenges due to the cold conditions in which these species live, and as a result, standard test protocols need to be adapted to account for their slower growth rates, longer development times, and slower acute responses (Chapman and Riddle 2003, 2005b; King and Riddle 2001). As shown in this study, traditional microalgal growth rate inhibition endpoints of 48 and 72 h are inappropriate for higher latitude species, which have slower growth rates and hence, slow to respond to exposure to contaminants. Therefore, it is important that standard toxicological methods are developed to reflect the different characteristics of Antarctic species and of their environment.

Selection of a suitable Antarctic microalgal test species

The initial screening of microalgae in this study found five of the six species were unsuitable for flow cytometric analysis at this time. Irregular patterns of light

scatter and chlorophyll fluorescence intensity measured for *Nitzschia* sp., *C. closterium* and *Chaetoceros* sp. 1 may have been caused by natural variation in cell size and chlorophyll content for these species and/or may have been due to cell clumping. *Nitzschia* sp. and *C. closterium* are prone to sticking together and to surfaces, preventing accurate analysis of individual cells. Stauber et al. (1994) developed a homogenisation method using a glass, hand homogeniser with Teflon pestle for the temperate *C. closterium* species (also commonly called *Nitzschia closterium*), which provided accurate cell density measurements by separating clumps of cells into single cells. We attempted to use this method during this study, but the substitution of a 45 mL homogeniser for the prescribed 15 mL size may have decreased the effectiveness of the homogenisation method and therefore affected the density determination of these cultures.

Cell density for *Chaetoceros sp.1* was also difficult to determine with flow cytometry. The orientation of the *Chaetoceros* sp. cells within the flow cytometer sheath may not have been uniform due to their shape and the long setae which project from both ends of the rectangular frustules. This could have influenced the laser light scatter and inaccurately determined the cell density. The initial cell density was also very low, which may have affected the growth rate of the culture. *Chaetoceros* sp.1 was not investigated further due to the difficulty in determining the cell density with flow cytometry. This species could however be amenable to growth tests that utilise microscopy for cell count determination.

Polarella glacialis was unsuitable for use in the growth rate inhibition bioassay as two distinct cell populations appeared on day 3 of the screening test, making identification of the cells and subsequent cell density measurements difficult with flow cytometry. These populations may represent the two different life cycle stages of *Polarella:* a motile dinoflagellate and a resting, spiny cyst (Montresor et al. 2003).

Geminigera cryophila cell density increased 11-fold over 13 days in the screening test, but only three fold over 12 days in the growth rate inhibition trial. During

inoculum preparation, the algal cells are concentrated through repeat rinses in filtered seawater in a centrifuge to remove the high nutrient culture media that has been shown to interfere with metal sensitivity assessment (Stauber and Florence 1989). *Geminigera cryophila* has been described as a "delicate flagellate" (Scott and van den Hoff 2005), indicating that it is possible that the additional stress of the centrifuge rinse method, despite modification including reduced speed and fewer rinsing steps (Franklin et al. 2005) may have suppressed this species' growth rate. As *G. cryophila* is from a different subkingdom to *P. antarctica*, and is also widely distributed in nearshore Antarctic marine environments (Scott and van den Hoff 2005), further test development would provide useful additional microalgae sensitivity data to be incorporated in the development of Antarctic water quality guidelines.

Phaeocystis antarctica proved to be the most suitable bioassay species as it was amenable to flow cytometric determination of cell density and demonstrated exponential growth rates through the test period, increasing at least 16-fold over 8 days. Based on these characteristics the growth rate inhibition bioassay was trialled using the solitary cell culture of *P. antarctica* to determine its sensitivity to copper.

Growth rate inhibition bioassay with *Phaeocystis antarctica*

The growth rate inhibition bioassay was successfully applied to the Antarctic microalgae, *P. antarctica,* with all test acceptability criteria being met with low variability in control growth rates (≤ 2%) and in the environmental parameters (pH < 0.5 ; salinity < 1 ‰; temperature = 2.1 ˚C ± 0.5 SD). The variation in pH is within allowable limits, however, it did increase marginally in alkalinity by the end of the testing period. This increase can be attributed to the increased production of oxygen by the increasing number of algal cells which affected the carbonate chemistry of the test water.

There were difficulties in accurately measuring the lower copper concentrations in the exposure solutions. The working detection limit of 2 µg/L limited the accurate calculation of toxicity indices in this study. ICP-OES have been used in

many other bioassays to determine total copper concentrations for calculating toxicological indices (Levy et al. 2007; Stauber et al. 2005, 2008) and have had copper detection limits in saline water of ~1 µg/L. Accuracy of metal analyses could be improved by adopting trace metal methodologies (Milne et al. 2010), silanzing the borosilicate glassware (Franklin et al. 2005) and pre-soaking in exposure solutions to reduce metals adhering to test vessels, as well as acidifying the copper stock solution to pH < 2 to maintain dissolved ionic copper and storing the stock in a refrigerator for preservation prior to its use (Franklin et al. 2005). Once metal analyses have been improved, the growth rate bioassay should be repeated with these modifications to precisely determine copper toxicity indices for *P. antarctica*.

Free ions and labile complexes are the most bioavailable forms of copper with uptake into algal cells controlled by either thermodynamics or kinetic transport mechanisms (Stauber and Davies 2000; Stauber and Florence 1987). Once transported into the algal cell, bioavailable copper directly contributes to the production of reactive oxygen species (ROS) through the oxidisation of thiol groups (Stauber and Florence 1987). The production and action of the free radical ROS can reduce the cell's antioxidant capacity, interfere with photosynthesis and inhibit cell division (Stauber and Florence 1987; Stoiber et al 2010) and therefore, bioavailable copper can significantly impair the overall survival and existence of algal species in contaminated waters. While dissolved (0.45 µm filterable) copper was measured in this study, the amount of copper which is actually bioavailable may be anywhere from 0-100% of the total concentration (Stauber and Davies 2000). Due to the filtration preparation of the seawater, it is expected that the majority of the dissolved fraction of the copper would be bioavailable due to the reduced capacity for binding to particulate matter. Despite copper treatment concentrations being below detectable levels, and speciation of copper not identified, there were clear toxic impacts inhibiting growth of *P. antarctica* indicating copper was present in bioavailable forms and at concentrations above the threshold level for the organism.

Antarctic marine environments have a high conservation value and should be afforded the highest species protection level of 99% under the ANZECC/ARMCANZ (2000) guidelines. Trigger values for copper for temperate fresh water and marine ecosystems as derived from statistical distribution methods, are listed as1 and 0.3 µg/L of total copper respectively under the 99% species protection level in the ANZECC/ARMCANZ (2000) guidelines. Determining sensitivity of *P. antarctica* to copper exposure based on the bioavailable concentration of copper will provide more sensitive calculations of point estimates and contribute to the development of more sensitive trigger values for Antarctic marine ecosystems.

Colony-forming microalgal species are generally unsuitable for toxicity tests due to difficulties in accurately determining cell densities. As *P. antarctica* has both solitary and colonial cell phases, using this species as a monitoring tool is restricted to testing solitary cell cultures irrespective of the counting method used. The solitary cell phase of *P. antarctica* is an ecologically important stage in this species life cycle, being an important food source for zooplankton, and a dominant microalga in coastal areas during the Antarctic winter and spring (Smith 2003). At the end of spring, *P. antarctica* begins to form colonies of cells enveloped in a polysaccharide matrix in response to nutrient availability, increased light intensity and grazing pressure (Schoemann et al. 2005). Transitioning from spring through summer, *P. antarctica* can be found in both colonial and solitary cell forms with the dominant form fluctuating temporally (Smith 2003). Although the mechanisms controlling the switching of *P. antarctica* between solitary and colonial cell phase have yet to be fully understood (Smith 2003) both forms would be at risk of contaminant impact in coastal Antarctic marine waters.

A unique feature of the *P. antarctica* growth rate inhibition bioassay is the longer test duration compared to the standard 48 and 72 h microalgal tests applied with temperate and tropical species (Stauber et al. 1994). The prolonged exposure was necessary due to test acceptability criteria stipulated in Franklin et

al (2005) requiring a 16 fold increase in cell density in the controls when conducting growth inhibition bioassays with flow cytometry. In Franklin et al (2005), this equates to a 72hr exposure at a specific growth rate of 0.9 doublings per day. With the control growth rates being 0.59 - 0.60 doublings per day for *P. antarctica* in exposure bioassays of this study, the duration of the tests needed to be extended to 7 and 8 d to comply with test acceptability recommendations. Levy et al (2007) have investigated the effects of copper on a number of different temperate and tropical marine phytoplankton species. During the 72 h exposures, the temperate strain of the baccillariophyte, *Nitzschia closterium* showed a growth rate of 1.53 cell doublings per day, while *Phaeodactylum tricornutum* showed a growth rate 1.78 cell doublings per day. Higher growth rates of 1.85 cell doublings per day were seen for the tropical prymnesiophyte, *Isochrysis* sp., which is three times faster than the Antarctic prymnesiophyte, *P. antarctica*. The tropical cryptophyte *Proteomonas sulcata* has a significantly higher growth rate than the Antarctic cryptophyte, *G. cryophila* having 1.54 and 0.29 cell doublings per day respectively. Due to the slower growth rates seen for the Antarctic phytoplankton species compared with temperate and tropical species, growth inhibition bioassays need to be run for longer than 72 h to meet test acceptability criteria and allow for comparable and biologically relevant endpoints for determining Antarctic species sensitivity in relation to species from other regions.

Whilst longer exposures may be more representative of field disturbance durations where contaminated meltwaters enter the Antarctic coastal environment intermittently during the seasonal melt period (Snape et al. 2001a), the increased exposure test duration may affect estimates of sensitivity of this species due to changes in exposure concentration and water chemistry throughout the test. Bioavailable copper ion concentration would have decreased in the test solution by initial adsorption to the glass container and then continued to decrease through the test duration via uptake and ligand binding by the increasing number of algal cells (Stauber et al. 2005). Future testing should incorporate a pre-soaking treatment with the test solution water

to reduce metal adsorption to the glass container as well as regular water sampling throughout the test exposure duration to monitor concentration fluxes, thereby improving metal sensitivity assessment.

Whilst *P. antarctica* was found to be sensitive to copper exposure in this study, generalisations as to the sensitivity of Antarctic microalgae cannot be made as no comparable data exists from other Antarctic species. One study investigated the effects of cadmium on the Antarctic sea ice microalga *Chlamydomonas* sp.*,* in which it was reported that a reduction in growth occurred after 13 days of exposure at 8.9 mg/L, from which an IC_{25} of 17.9 mg/L was estimated (Yu et al. 2005). Whilst Yu et al*.* (2005) also used a longer exposure duration for the Antarctic microalga than those used for temperate or tropical microalgal bioassays, they used nutrient rich f/2 culture media throughout their experiments and did not report the initial cell density for the bioassays. The use of f/2 media and high initial cell densities (>10^4 cells / mL) have been shown to significantly reduce the toxicity of metals to marine and freshwater microalgae due to increased removal of free bioavailable ions from the solution through chelation (Stauber and Davies 2000; Stauber and Florence 1989). Using the f/2 media therefore would have significantly reduced the toxic action of cadmium and therefore the true sensitivity of *Chlamydomonas* has been underestimated in this study.

One study has investigated the effects of copper on the temperate species *Phaeocystis cordata,* using two different experimental culturing methods with artificial seawater medium, (AQUIL) during 72 h bioassays (Lombardi and Maldonado 2011). Compared to the control growth rates, *P. cordata* exhibited a 53% reduction in growth when exposed to total copper concentration of 3.17 mg/L and a 40% reduction at 5.08 mg/L for the semicontinuous and batch culture methods respectively. Despite the variability between growth reduction rates for the two culturing methods, this temperate species of *Phaeocystis* is approximately 1000 times less sensitive to copper than *P. antarctica*.

Copper concentrations shown to inhibit growth in the temperate Prymnesiophyte, *Isochrysis galbana* (Debelius et al. 2009; Satoh et al. 2005; Wilson and Freeburg 1980), are considerably higher than those shown for *P. antarctica* in this study (Table 4). *Phaeocystis antarctica's* sensitivity to copper is more similar to the tropical strain of *I. galbana* from Tahiti, which has a 72 hr IC_{50} of 4 µg/L for copper when tested with a non-copper complexing media and low test cell densities of 10^3 cells/mL (Levy et al. 2007)(Table 4). *Phaeocystis antarctica* is more sensitive to copper than the temperate strain of the diatom *Cylindrotheca closterium* (Table 4). Among the most sensitive marine species to copper exposure, *C. closterium* is used as a standard test species for monitoring contamination in temperate marine environments (Stauber et al. 1994) and growth inhibition data for *C. closterium* was included in the development of the ANZECC/ARMCANZ (2000) temperate marine trigger value of 1.3 µg/L for total copper. *Phaeocystis antarctica* is also more sensitive than the temperate marine species, *Phaeodactylum tricornutum* (Franklin et al. 2001) and tropical *Nitzschia closterium* (Johnson et al. 2007), which have both been used as environmental monitoring tools.

Table 4: Growth inhibition concentrations (IC_{50}) for temperate and tropical phytoplankton species exposed to copper for 72 hr.

Region	Species	IC_{50} (µg/L)	Study
Temperate	*Isochrysis galbana*	58	Debelius et al. (2009)
		110 – 1000	Wilson and Freeburg (1980)
		4200	Satoh et al. (2005)
	Cylindrotheca closterium	16	Stauber and Florence (1989)
	Phaeodactylum tricornutum	9	Franklin et al. (2001)
	Nitzschia closterium	40	Johnson et al. (2007)
Tropical	*Isochrysis galbana*	4	Levy et al. (2007)

With refinement of the contaminant analytical technique and silianzing of test glassware, further sensitivity testing with copper and additional contaminants should be undertaken with *P. antarctica*. High variability in algal growth at the 4 µg/L concentration of copper in Test 1, indicates that future tests should increase the number of replicates per treatment and the range of copper treatments above and below 4 µg/L. This would decrease variability in the average response, enabling a more accurate determination of copper concentration effecting growth inhibition of *P. antarctica*. Future studies should also use low cell densities (10^3 cells/mL) as research has shown that high cell densities ($10^4 – 10^6$ cells/mL) increase cell binding sites for copper and subsequently limit the bioavailable free copper in solution, thereby potentially underestimating toxicity (Franklin et al. 2005). The high cell density of 10^4 cells/mL used in *P. antarctica* tests may have led to an underestimation of species sensitivity in this study. *Nitzschia* sp. and *C. closterium* should be investigated further since their suitability as test species could be improved by homogenisation prior to measuring cell density (Stauber et al. 1994). If successful, both species could be tested for sensitivity to contaminants using the growth rate inhibition bioassay. *Cylindrotheca closterium* comprises temperate and tropical strains that have been successfully used as toxicity test species in contaminant assessments of marine waters and in the development of water quality guidelines (ANZECC/ARMCANZ 2000).

This study has shown *P. antarctica* to be not only sensitive to copper exposure, but also exhibits a greater sensitivity to copper than temperate and tropical microalgal species tested to date, yet further work is required in improving analytical techniques for copper determination in order to calculate toxicity indices (Levy et al. 2008). It is important that future ecotoxicological tests be developed with endpoints and exposure durations that are relevant to the slower metabolism, growth rates and longer response times already identified for Antarctic species (Chapman and Riddle 2005b). Through this process, Antarctic specific toxicity testing protocols and water quality guidelines can be developed to ensure the protection of the Antarctic marine environment

through impact assessment of human activities and through appropriate management of site remediation efforts.

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Chapter 3

Interactive effects of elevated temperature and metal exposure on the behaviour and survival of adult and juvenile Antarctic amphipods, *Paramoera walkeri*

This chapter is presented as a manuscript prepared for publication

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Abstract

Managing contaminant impacts on nearshore marine ecosystems in Antarctica requires an understanding of the sensitivities of local biota to contaminant exposure. Few ecotoxicological studies on Antarctic biota have been undertaken to date with most studies having utilised standard bioassay procedures with short durations and mortality endpoints such as those used for temperate and tropical species. These standard methods however, do not adequately address the ecophysiological adaptations of Antarctic biota that have evolved to survive the low stable temperatures and fluctuating levels of sunlight, sea ice and primary production, as well as the unique physical characteristics of the Antarctic marine environment. This study aimed to develop longer duration bioassays and a sensitive behavioural endpoint with the littoral Antarctic amphipod, *Paramoera walkeri*. Bioassays of 21 d in length were developed and a more sensitive behavioural endpoint of normal activity was determined for the adult *P. walkeri*. The impact of metals on survival of both juvenile and adult *P. walkeri* was investigated to determine the relative sensitivity of different life history stages. Juveniles were found to be more sensitive to copper than adults. Evidence of global warming, indicated by increased Antarctic coastal sea surface temperatures prompted the investigation of elevated temperature as an additional stressor to metals affecting survival in adults. Elevated temperatures were found to increase the toxicity of copper and cadmium, decreasing the survival rate of *P. walkeri* adults. The successful development of the longer-term adult and juvenile bioassays as well as the development of the sensitive behavioural endpoint has provided essential methodologies and contaminant information for the future development of Antarctic specific water quality guidelines.

Key words

Toxicity test, mortality, global warming, water quality guideline development, *Paramoera walkeri*

Introduction

Many coastal regions near research stations in Antarctica have been impacted by anthropogenic metal and organic contaminants (Deprez et al. 1999; Lenihan 1992; Stark et al. 2003, 2004). Waste disposal activities such as sea icing, disposal to tip sites, sewage discharge as well as fuel spills, have been the main sources of chemical contamination to nearshore marine ecosystems in Antarctica (Snape et al. 2001). Whilst most of these activities have ceased since the establishment of the Protocol on Environmental Protection to the Antarctic Treaty (the Madrid Protocol, 1991), annual summer meltwater migration through contaminated sites on land continue to mobilise and transport contaminants to nearshore ecosystems (Snape et al. 2003). Under the Madrid Protocol, contaminant sources are to be removed and contaminated sites remediated provided the remediation activities do not cause further impacts. To better manage environmental contamination and target remediation efforts, appropriate risk assessment methods including standard toxicity tests and environmental guidelines specific to Antarctica need to be developed. This information will aid the development of appropriate remediation criteria by providing accurate environmental monitoring tools and setting acceptable endpoints for the clean-up process.

Developing guidelines for managing contaminant impacts on aquatic ecosystems requires an assessment of the sensitivity of local representative species to contaminants, incorporation of the abiotic factors of the local environment that may modify biotic response, and the establishment of a suitable level of protection (e.g. 95% or 99%) (ANZECC/ARMCANZ 2000). Toxicity tests are used

to determine sensitivities of individual species to contaminant exposure by providing concentration-response data for generating point estimates. Few toxicity tests have been conducted on Antarctic marine species with most applying standardised methods developed for temperate and tropical marine species (Chapman and Riddle 2005). Studies have found that Antarctic marine invertebrates have longer acute contaminant response times than species from warmer regions (Duquesne et al. 2000; King and Riddle 2001). A small number of tests that used longer exposure durations found Antarctic marine invertebrates to be equally or more sensitive to contaminant exposure than species from lower latitudes (King and Riddle 2001; Chapter 2; Sfiligoj et al. in prep-a). Standardised, bioassay methods developed for temperate and tropical marine species which typically include relatively short exposure durations, may not be appropriate for Antarctic species which have adapted to the unique characteristics of the Antarctic environment (Chapman and Riddle 2005; King and Riddle 2001; Chapter 2; Sfiligoj et al. in prep-a;).

The Antarctic marine environment is characterised by stable, low seawater temperatures and highly dynamic seasonal fluctuations in solar radiation, primary production and sea ice cover. Predictable variations in these parameters have greatly influenced the unique ecophysiological adaptations of local nearshore Antarctic species that in turn, influence their response to anthropogenic contaminant exposure (Chapman and Riddle 2005). Larval developmental times are slower for many Antarctic marine species (Peck 2002) and King and Riddle (2001) found the larvae of the sea urchin *Sterechinus neumayeri* to be more sensitive to metal contaminant exposure than analogous species from temperate and tropical regions. Antarctic marine invertebrates are generally long lived (Sagar 1980), have reduced metabolic rates (Peck 2002), lower surface area to volume-ratios related to gigantism (Chapelle and Peck 1995), all of which could influence their longer acute response times to contaminant exposure as a result of slower uptake rates (Kahle and Zauke 2003). Despite the potentially slower uptake rates for contaminants by Antarctic marine invertebrates, the lower metabolic rates and lower energy usage of these

organisms indicate a reduced ability to respond to increasing contaminant concentrations through slower rates of detoxification and excretion (Chapman and Riddle 2005). This would mean that the metabolically active forms of contaminants such as copper and cadmium, could have a longer resident time in the organism's cells and potentially cause more oxidative stress through increased free radical production (Rainbow 2002). The longer life span and longer life cycles of the organisms also increase the length of time they are exposed to, and can accumulate metal contaminants. Physiological impairment or even death may occur prior to engaging in sexual reproductive events and securing the future of the species' population. Therefore, polar species could be more sensitive overall to longer-term contaminant exposures than temperate or tropical organisms (Chapman and Riddle 2005).

Antarctic marine species are also stenothermal, having narrow thermal tolerance ranges making them vulnerable to even slight temperature increases (Peck et al. 2004). Temperature increases associated with climate change have already been detected in the terrestrial and nearshore marine ecosystems of the Western Antarctic Peninsula (Meredith and King 2005). Species inhabiting these regions are also at risk of anthropogenic contaminant impacts due to extensive human activities in the area. Given the sensitivity of early life stages to contaminants, the potential for synergistic effects of temperature rise with contaminant exposure and the longer acute response times of Antarctic marine species, toxicity tests that address these factors need to be undertaken. By incorporating different life history stages, elevated temperatures and longer exposure durations into the development of appropriate Antarctic bioassay methods we will be better able to assess Antarctic marine species sensitivity to contaminants.

The Antarctic gammarid marine amphipod, *Paramoera walkeri*, is an ideal test species for developing appropriate bioassay methods. This species has been used in a small number of previous studies investigating effects of exposure to metal contaminants and UV-B radiation on survival (Clason et al. 2003; Duquesne and Liess 2003; Duquesne et al. 2000; Liess et al. 2001), and as a

biomonitor during the remediation of a contaminated site at Casey Station, East Antarctica (Duquesne and Riddle 2002; Stark et al. 2006). *Paramoera walkeri* is omnivorous, feeding on phytoplankton, zooplankton and detritus in sub-fast ice and shallow benthic littoral, marine ecosystems (0-15 m depth). The life cycle of *P. walkeri* is greater than 2 years with females taking >19 months to reach sexual maturity (Sagar 1980). *Paramoera walkeri* have a circumpolar distribution (Sagar 1980) although Duquesne and Liess (2003) reported that these amphipods were absent from suitable marine habitats which were adjacent to contaminated sites near Casey Station, East Antarctica.

Despite the promising work already conducted with *P. walkeri*, the only bioassay endpoint evaluated to date is survival. Sub-lethal, behavioural endpoints are more sensitive than lethal endpoints for measuring contaminant effects as they integrate the impacts on complex biochemical and physiological processes in a defined characteristic that can be visually observed and quantified (Mills et al. 2006). Behavioural traits such as movement and locomotion are highly ecologically relevant as they can impact on an organism's ability to function successfully in their ecosystem. Numerous studies have investigated contaminant impacts on swimming speed, sediment burrowing and crawling and attachment behaviours with temperate and tropical marine invertebrate species (Burlinson and Lawrence 2007; Busdosh 1981; Faimali et al. 2006; Mills et al. 2006; Sheffrin et al. 1984). Lenihan et al. (1995) included behavioural endpoints of burrowing and avoidance in their laboratory tests and field study in which they exposed Antarctic amphipod, tanaid, cumacean and heart urchin species to contaminated sediments from Winter Quarters Bay near McMurdo Station, Antarctica. They found these endpoints to be highly relevant for understanding the difference in species diversity observed at contaminated and uncontaminated sites within this area. Hill et al (2009) investigated effects on the behaviour and survival of an Antarctic spirorbid *Spirorbis nordenskjoldi,* from exposure to sediments contaminated with single metals (copper, cadmium, zinc) and metal mixtures and concluded that the behavioural sub-lethal endpoint was an order of magnitude more sensitive than the lethal endpoint. *Paramoera*

walkeri is a highly mobile species (Rakusa-Suszczewski 1972), and like other gammarids, movement is essential for acquiring food, avoiding predators and for mating (Mills et al. 2006). Given the increased sensitivity of sub-lethal endpoints relative to survival, alterations in normal behavioural characteristics represent an ideal endpoint with which to better assess the effects of contaminants on *P. walkeri*.

This study aimed to: 1) determine sensitive behavioural endpoints for *P. walkeri* for evaluating the effects of exposure to metal contaminants, 2) derive point estimate concentrations for lethal and sub-lethal effects for *P. walkeri* adults exposed to five metals, 3) investigate the interactive effects of elevated temperature with copper and cadmium exposure on *P. walkeri* adults, and 4) investigate the relative sensitivity of juvenile and adult *P. walkeri* to copper.

Materials and Methods

Animal and seawater collection

Paramoera walkeri adults including brooding females were collected using dip nets from nearshore, rocky substrate habitats in >1 m depths near Davis Station (68˚35'S 77˚58'E) and newly released juveniles were collected near Shirley Island, Casey Station (66˚17'S 110˚31'E), East Antarctica. Test seawater was collected approximately 500m – 2km up current and away from coastal contaminant inputs from station and sewage outfalls .Seawater was filtered to 1 µm and had a salinity of 33 - 34 ppt, and pH of 7.99 - 8.05 as measured by a Mettler Toledo SG78 metre calibrated with buffers for measuring water at 0 °C. Dissolved oxygen (DO) of the filtered seawater was > 90%.

General analytical

Equipment preparation

All plastic ware and glassware was soaked in 10% (v/v) analytical grade nitric acid (HNO_3) for a minimum of 8 h prior to use in tests.

Test solution preparation

Stock solutions (500 mg/L) of copper, cadmium, zinc, lead, and nickel were prepared in high-density 250 mL polyethylene NALGENE screw cap bottles using metal salts and highly purified (Milli Q) water. Metal stock solutions were prepared using 0.4911 g of $CuSO_4.5H_2O$, 0.2853 g of $3CdSO_4.8H_2O$, 0.5062 g of $NiCl_2.6H_2O$, 0.2606 g of $ZnCl_2$ and 0.1678 g of $PbCl_2$. Test exposure solutions were prepared by diluting metal stock solutions with 1 µm filtered seawater (FSW).

Test parameters

Samples of test solutions were taken for metal analysis at the start and end of tests, and before and after each water renewal during tests. Ten mL was taken from a single replicate vial for each exposure treatment and for the control, and was filtered using a 0.45 µm syringe filter tip then acidified to 1% (v/v) using ultra pure HNO_3. Metal analysis was conducted using an inductively coupled plasma optical emission spectrometer (ICP-OES, Varian 720-OES). Salinity and pH were measured in the control treatments only. All tests were conducted in controlled temperature cabinets, which were monitored using data loggers that recorded the temperature at 10 min intervals. A 16:8 h light/dark photoperiod using cool white fluorescent lamps was used to mimic ambient light intensity and the longer day length during Antarctic spring/summer months.

Bioassays

Bioassays conducted

Pilot tests were conducted to establish appropriate test concentrations and methods for bioassays including test duration and feeding and water change regimes. Two replicate tests (Adult tests 1 and 2) were conducted with adult *P. walkeri* (8-13 mm in length) exposed to copper, cadmium, nickel, zinc and lead as single contaminants at ambient Antarctic seawater temperatures of -1 ± 0.5 ˚C over 21 d. An additional elevated temperature of 2.4 ± 0.2 ˚C was tested in Adult test 2 for copper and cadmium treatments. Both behavioural and lethal endpoints were evaluated in the adult tests. Three tests were conducted with juvenile *P. walkeri* in which juveniles were exposed to copper at ambient

Antarctic seawater temperature (-0.9 ± 0.5 ˚C). The first two tests (Juvenile tests 1 and 2) used the smallest individuals caught by hand-held nets in the field, the lengths of which matched those of juveniles newly released from brood pouches (1.75 – 2.3 mm) (Rakusa-Suszczewski 1972). Juvenile tests 1 and 2 evaluated mortality after 10 d exposure to copper. Test 3 used juveniles of 1.8 - 2.2 mm in length that were collected immediately after release from brooding females in the laboratory and evaluated mortality and behaviour after 14 d exposure to copper and cadmium.

Bioassay procedure

Adult *P. walkeri* were acclimated to laboratory conditions for < 10 d prior to beginning tests 1 and 2. Juveniles collected from the field for tests 1 and 2 were acclimated for 24 hrs prior to test use. Brooding females collected from the field were isolated and juveniles to be used in test 3 were separated upon release from the marsupium. Observations of mortality and behaviour were made at 4, 7, 14 and 21 d for the adult tests. Mortality observations for juvenile tests 1 and 2 were made at 4, 7 and 10 d and mortality and behavioural observations were made at 4, 7 and 14 d for juvenile test 3. The exposure solution in tests was renewed and water samples taken at 7, 14 and 21 d for adult tests and at 7 and 14 d for juvenile test 3. Juvenile tests 1 and 2 were static tests with no exposure solution renewal. Approximately 18 h prior to the start of tests and prior to water renewals, adult *P. walkeri* were fed granulated fish pellets (Granumarin, Sera) and ~ 5 mL of concentrated algae mixture of E-Z larvae (250-600 µm) *Thalassiosira weissflogii* 1200 (CCMP: 1051 7-20 µm), *Pavlova* 1800 (CCMP:359 4-7 µm) and *Isochrysis* 1800 (CCMP:1324 5-7 µm) and juveniles were fed ~ 5 mL of the concentrated algae mixture. Each test had four replicates per treatment. Each replicate contained either 10 adult *P. walkeri* or 15, 10 or 6 juveniles (juvenile test 1, 2 and 3 respectively). Amphipods were exposed to 200 mL of test solution in 250 mL polypropylene test containers with screw cap lids. A small square of acid washed, plastic mesh (6 x 4 cm in size for adults and 2 x 2 cm for juveniles) was added to each container to provide a surface habitat for amphipods to cling to.

Behavioural endpoint categories

At each observation, amphipods surviving were assigned to one of four behavioural categories that were identified and described in the pilot tests and are detailed below:

1. "Active" behaviour is defined as normal activity characterised by swimming / active clinging to the plastic mesh or walking on the bottom of the container,
2. "Side-fast" behaviour represents the first departure from normal behaviour where the amphipod is observed laying on its side with open or curled posture, fanning pleopods (hyperventilation) and moving pereopods / antennae. Amphipods displaying this behaviour did not start swimming when they were stimulated by touch,
3. "Side-slow" behaviour characterised by amphipods lying on their side or back with open posture, pleopods moving slowly or irregularly with little or no pereopod / antennae movement and no response to stimulus, and
4. "Side twitching" behaviour is characterised by amphipods lying on their side or back with open posture, pleopods twitching (hypoventilation), no other detectable movement of pereopods / antennae and no response to stimulus.

Whilst there appeared to be a pattern of increased severity in response to metal exposure through these behavioural categories, preliminary data analyses indicated that some amphipods switched between displaying different affected behaviour categories and did not exhibit a progressive response from category 1 through to category 4. However, once an amphipod displayed any deviation from active behaviour (i.e. categories 2 - 4), there was no evidence of reversal. Therefore, categories 2 to 4 describing affected behaviour were grouped together as the "inactive" category, and behavioural analysis was based on comparing the active category to the dead and inactive categories.

Statistical analyses

Point estimates including LC_x and EC_x values were determined by Maximum Likelihood Probit analysis using the EPA recommended methods in the ToxCalc™ program (v5.0.26; Tidepool Scientific Software). Data was arcsin square root transformed as necessary for analyses. Metal exposure concentrations used for data analysis were actual measured concentrations rather than nominal values. Mean concentrations were determined for each exposure period from water samples taken at the beginning and end of each period.

Results

Salinity, pH and temperature remained relatively constant throughout all tests. Mean salinity and pH were stable at 33.8 ± 1.3 ppt and 7.8± 0.3 SD respectively. Average control survival was 93% in adult test 1 and 75% in adult test 2 at 21 d. Average control survival was 80% in juvenile tests 1 and 2 at 10 d. In juvenile test 3 average control survival was 91% at 4 d, 62% at 7d, but only 29% at 14 d.

Behavioural and survival responses in adult tests at ambient temperature

Behavioural and mortality response times for *P. walkeri* were different for each metal. Copper was the most toxic, producing significant behavioural changes and mortality after 4 d exposure in both tests. Cadmium was the second most toxic metal, with significant mortality observed at 7 d in test 1. Zinc was the next most toxic metal with significant responses occurring between 7 and 14 days. Lead and nickel produced different responses in the two replicate tests. In test 1, lead was highly toxic and as toxic as copper. Interestingly in this test the second lowest exposure concentration caused greater mortality than the highest concentration. In test 2 both nickel and lead produced more atypical dose-response relationships with higher rates of mortality observed at the lower concentrations and higher survival in metal treatments than in the controls. In the case of nickel, behavioural and lethal effects were observed in test 1,

allowing for point estimates to be calculated (Table 1). No point estimates for nickel could be calculated from test 2. Random variability in individual sensitivity could account for the differences seen between the two tests in regards to the responses to lead and nickel exposure.

Deviation from normal, active behaviour represented a more sensitive endpoint than survival for *P. walkeri* adults exposed to metals. The three categories (2, 3 and 4) which described the metal effected behaviours of side-fast, side-slow and side-twitching were combined together as the inactive category as no clear degenerative behavioural pattern was observed for *P. walkeri* during the exposures. The progression from normal, active behaviour, through to inactivity and dead categories is illustrated clearly in test 1 (Figure 1). The degree of sensitivity difference between behavioural and lethal endpoints varied for each metal and each observational time point. On days 7 and 14 the average LC_{10} values for copper were 677 µg/L and 462 µg/L respectively, considerably higher than the behavioural EC_{10} values of 244 µg/L and 71 µg/L (Table 1). Sensitivity differences were less pronounced for cadmium with average LC_{10} values on days 14 and 21 of 299 µg/L and 147 µg/L respectively and EC_{10} values of 205 µg/L and 108 µg/L (Table 1).

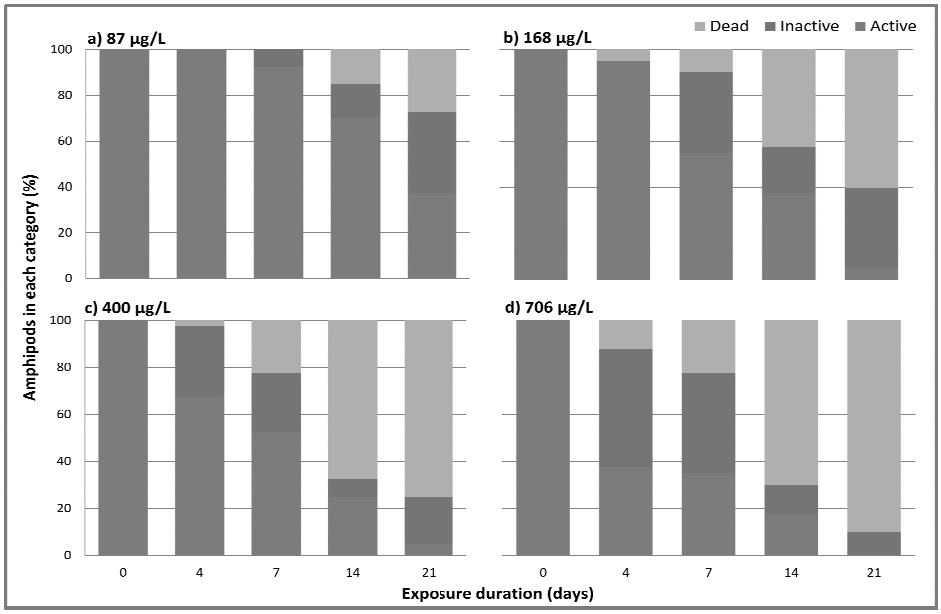

Figure 1: Behavioural changes and mortality of adult *Paramoera walkeri* exposed to copper at concentrations of a) 87 µg/L, b) 168 µg/L, c) 400 µg/L and d) 706 µg/L over 21 days in Test 1. Note the progression of effects observed from active, to inactive, to dead for each copper treatment.

Table 1: Average point estimates and standard error concentrations (µg/L (SE)) for behavioural and mortality endpoints of two adult *P. walkeri* tests (EC_x / LC_x) with copper, cadmium, zinc and lead for observation days 4, 7, 14 and 21 of the bioassays. Point estimates reported for nickel are from test 1 only.

	Copper				**Cadmium**		**Zinc**		**Lead**	**Nickel**		
LC / EC	**4**	**7**	**14**	**21**	**14**	**21**	**14**	**21**	**21**	**7**	**14**	**21**
LC_{10}	677 (21)	462 (209)	33 (26)	21 (20)	299 (120)	147 (64)	-	185 (7)	229 (201)	705	337	232
EC_{10}	244 (170)	71 (1)	16 (12)	22 (4)	205 (65)	108 (8)	162 (73)	290 (98)	30 (29)	347	321	268
LC_{50}	-	-	288 (15)	106 (70)	519 (168)	320 (170)	-	1005 (150)	-	-	-	-
EC_{50}	669 (7)	360 (41)	112 (58)	61 (1)	389 (129)	247 (61)	1937 (978)	761 (61)	-	-	-	864

Relative sensitivity of juvenile and adult survival

Juveniles were more sensitive to exposure to copper than were adults. Control survival in juvenile tests 1 and 2 was > 80% over the 10 d test duration. Control survival in juvenile test 3 with newly released juveniles however decreased throughout the test with 91% survival at 4 d, 62% at 7 d and only 29% at 14 d. Average LC_{10} and LC_{50} values for juveniles exposed to copper for 4 d were 295 µg/L and 337 µg/L, and for 7 d were 166 µg/L and 325 µg/L respectively. The longer time required for the adults to respond prevented the calculation of LC_{50} point estimates for 4 and 7 d. The LC_{10} values for copper for adults (677 µg/L on 4 d and 462 µg/L on 7 d) are more than twice those determined for the juveniles.

Influence of elevated temperature on response to metals

Amphipod survival in control treatments was not significantly different between the different temperature treatments of 2.4 ± 0.2 °C with 75 % (± 6.5 % SE) surviving in the ambient Antarctic seawater temperature of -0.9 ± 0.5 °C and 64 % (± 5 % SE) surviving in the 2.4 ± 0.2 °C temperature until day 21 (Figure 2a). Amphipods exposed to copper and cadmium at this elevated temperature however, demonstrated increased sensitivity to metals compared those in ambient Antarctic seawater temperature (Figures 3a and b). As illustrated for one copper treatment in Figure 2b, survival decreased more rapidly for amphipods exposed to copper at the elevated temperature than those exposed to copper at ambient Antarctic seawater temperature.

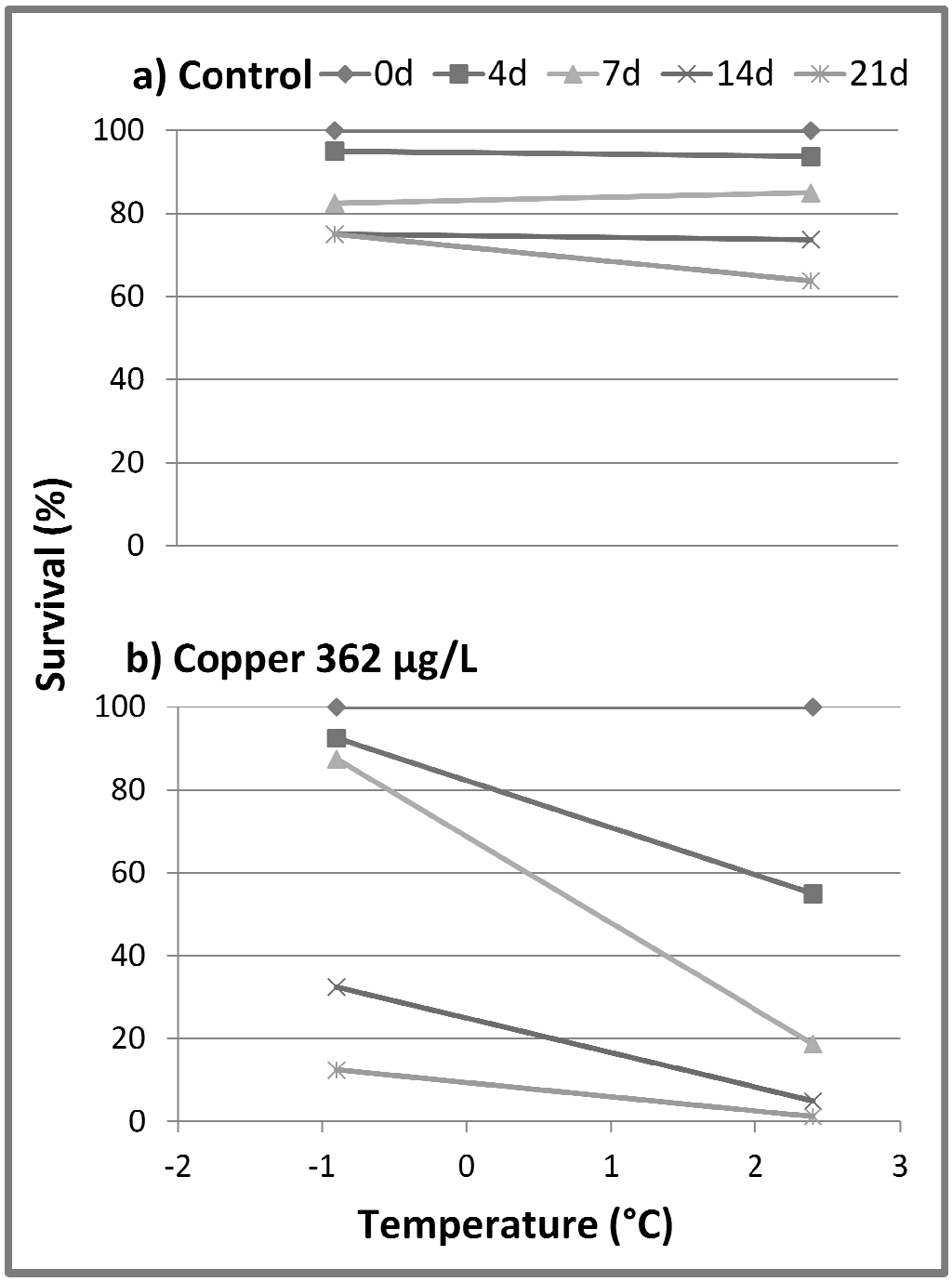

Figure 2: Effect of temperature on survival of *Paramoera walkeri* adults over 21 days in a) control treatment and b) copper 362 µg/L treatment from Test 2 for days 4, 7, 14 and 21 at - 0.9 and 2.4 °C

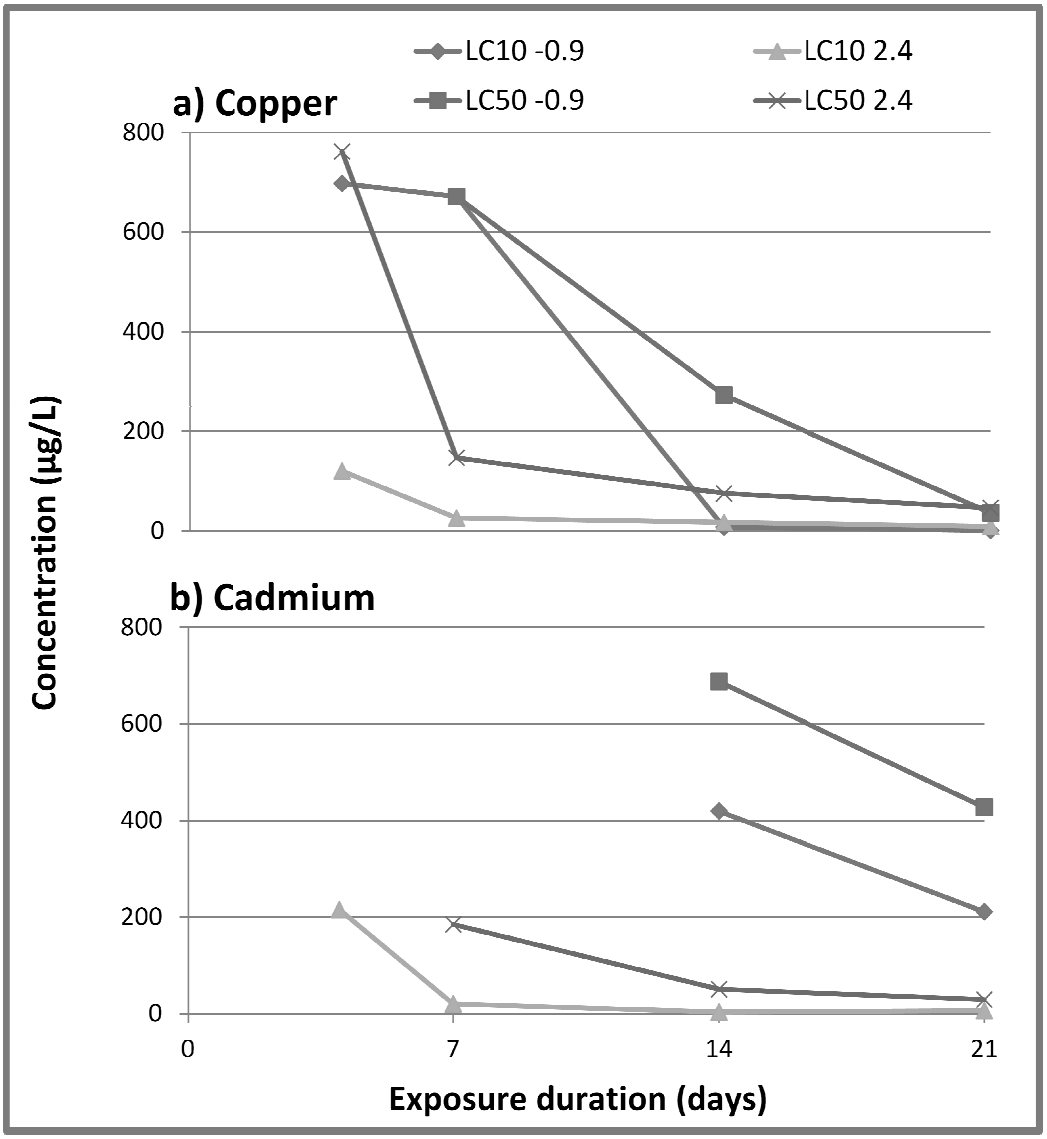

Figure 3: Effect of exposure to a) copper and b) cadmium on *Paramoera walkeri* adults over 21 days, LC_{10} and LC_{50} values are shown for - 0.9 and 2.4 °C temperature treatments in Test 2.

Discussion

Managing anthropogenic contaminant impacts on Antarctic ecosystems requires the development of specific environmental guidelines that integrate the unique environmental and biological conditions of the region. This study has shown the importance of using longer exposure durations when assessing contaminant impacts on Antarctic marine biota which have specific ecophysiological traits such as lower metabolic rates, longer life spans and longer developmental times that impact their response rates to contaminants. *Paramoera walkeri* potentially have a slower metabolic rate, however, Duquesne et al (2000) showed that accumulation rates of metals by *P. walkeri* were comparable to those of temperate and tropical species. The life span (>2yr) of *P. walkeri* and developmental times are considerably longer than those of temperate or tropical species which live between 4months and 1yr in duration (Highsmith and Coyle 1991; Sagar 1980). Toxicological tests with Antarctic ectotherms need to be run for longer durations to assess sensitivities that can be comparable to the effects observed on temperate and tropical species during shorter tests. King and Riddle (2001) have explicitly shown the importance of assessing biologically relevant exposure durations when comparing species sensitivity between latitudes with their research on metal effects on developmental endpoints of an Antarctic echinoid species.

Juvenile amphipods were more sensitive than adults, and sub-lethal behavioural endpoints such as departures from normal activity, were more sensitive than mortality and are recommended as sensitive indicators of contaminant-induced stress in *P. walkeri* exposed to metals. Climate change effects are already present in Antarctica (Meredith and King 2005), and this study has demonstrated the importance of incorporating climate change variables into contaminant impact studies as increased temperature increased the severity of impact and sensitivity of *P. walkeri* to metals.

Biological response times and sensitivity to metals

Longer acute response times to all metals tested were observed for lethal endpoints for *P. walkeri* adults and delayed behavioural responses occurred with zinc, lead, and nickel exposures. This delay in response prevented the determination of average EC and LC point estimates for most of the 4 d observation points for each metal during the 21 d tests. Aqueous toxicity tests with temperate and tropical amphipod species often use a 4 or 10 d exposure period. These durations are clearly not long enough to elucidate a response in the Antarctic amphipod, and it is therefore difficult to make latitudinal comparisons of the sensitivities of species by comparing point estimate values for a given exposure period.

In a previous study, LC_{50} values for copper and cadmium were determined for *P. walkeri* adults following 4 and 8 d exposures (Duquesne et al. 2000). While the 4 d LC_{50} value could not be calculated in the present study, the average 4 d LC_{10} value determined for copper exposure on *P. walkeri* is 677 µg/L, and the 4 d EC_{50} value using the behavioural change endpoint is 669 µg/L. In comparison, the 4 d LC_{50} determined by Duquesne et al. (2000) for *P. walkeri* was 970 µg/L. Mortality and behavioural response times for *P. walkeri* adults varied between metals with exposure effects first elicited by copper, then cadmium, zinc, lead and nickel. The relative toxicity of these metals to *P. walkeri* adults through comparisons of point estimates shows the same pattern. A similar pattern in metal toxicity has also been observed with the benthic Antarctic marine amphipod, *Orchomenella pinguides* (Chapter 4; Sfiligoj et al. in prep-b) and for the temperate marine amphipod, *Melita plumulosa* (King et al. 2006a). Sensitivity of *P. walkeri* adults also varied considerably between replicate tests as indicated by the large standard errors for the average point estimates generated (Table 1).

Paramoera walkeri did not respond in a typical concentration-response manner when exposed to nickel and lead. In adult test 1, slightly higher mortality was observed in the lower exposure concentrations of nickel and lead compared with the highest concentration. In test 2, a higher survival rate was observed in

individuals exposed to nickel and lead relative to the control, with those exposed to the highest concentrations of each metal, showing the highest survival. Nickel has been shown to be one of the least toxic metals to invertebrates with a 96 hr LC_{50} of 34.7 mg/L reported for the Australian marine amphipod *Allorchestes compressa* (Ahsanullah 1982). King et al. (2006a) also found *M. plumulosa* adults and juveniles to be relatively insensitive to nickel exposure. Arctic amphipods have shown high tolerance to zinc and lead (Chapman and McPherson 1993), showing little to no response to exposures over 4 days even at the highest concentrations tested (Pb 3.5 mg/L and Zn 11.8 mg/L). It is difficult to explain why effects on *P. walkeri* behaviour and morality were observed at the lower exposure concentrations of nickel and lead as opposed to the higher concentrations. In consideration of the results from previous studies, it could be expected that *P. walkeri* would respond in a normal pattern to increasing concentrations of nickel and lead but could possibly have a significant tolerance to these metals. Alternatively, solubility of the metals in the exposure solutions may have been affected by an unidentified factor and therefore affected the responses observed for *P. walkeri* to these metals. A number of steps were incorporated into the test methodology to minimise impacts on metal solubility. All stock solutions of metal salts were prepared using highly purified, Milli-Q water to reduce metal complexation. Particulate matter was removed through 1 µm filtration of the seawater prior to making exposure solutions to further reduce complexation of the metals. Exposure solutions were also well mixed to ensure the metals were dissolved prior to dispensing to the replicate containers which had been pre-soaked in nitric acid baths. The dissolved metal concentrations were then quantified by measuring 0.45µm filtered samples wit ICP-OES instrumentation that had been acidified with 1% ultra pure nitric acid.

Variability in response rates of both the juvenile and adult *P. walkeri* to the individual metals are indicative of their different uptake rates and pathways that depend on the organisms' biological requirements of that metal and the ability of the organism to bind and metabolically remove or excrete the excess metal. Toxicity occurs when uptake exceeds detoxification capabilities and production

of free radical oxygen species increases, causing damage to lipids, proteins and DNA (Buettner 1993; Kawanishi 1989, Stoiber et al 2010).

Metals such as copper and zinc are considered essential and are readily taken up by organisms, as they are required for normal biological function and physiological processes such as growth and development. The difference in the rate of toxic action of these two metals on *P. walkeri* could be attributed to the neurotoxicity of copper, where in excess concentrations, copper can inhibit the production of the neural transmission enzyme acetylcholinesterase in some invertebrates (Brown et al 2004). Inhibition of acetylcholinesterase would directly affect activities such as movement and respiration, which could explain the greater sensitivity of the behavioural category as well as the higher sensitivity of *P. walkeri* to copper toxicity.

Nickel is an essential metal for some organisms, primarily plants and algae which use nickel to produce the enzyme urease (Rees and Bekheet 1982). Nickel toxicity can produce oxidative stress that can impact on DNA, increase lipid perioxidation, deplete glutathione and alter calcium and sulphhydryl homeostasis (Das 2008). Despite the apparent toxicity of nickel, little effect was seen on *P. walkeri*, which is most likely due to the exposure concentrations not being high enough to elicit a typical concentration-response.

Although considered non-essential, it has been suggested that cadmium could share similar up take pathways to zinc and is therefore readily taken up from the environment (Rainbow 1997). Invertebrates, particularly crustacea accumulate cadmium typically by the protein metallothionein rather than excrete it directly (Rainbow 2007). Lead is not an essential metal and therefore any that is accumulated must be metabolically removed through detoxification to prevent toxicity (Rainbow 2002). Increased pressure on detoxification processes increases energetic costs that can impair biological function and activities such as movement. Despite cadmium and lead both being non-essential metals, the faster rate of cadmium toxicity in comparison to lead implies that the uptake rate for cadmium by *P. walkeri* was higher.

Organisms have different strategies for regulating body concentrations of toxic metals through detoxification by binding to proteins and storage as granules to directly excreting the excess metals (Rainbow 2002). Under increased copper concentrations, the caridean shrimp, *Palaemon elegans* stores detoxified copper in granules in the hepatopancreas which then have the potential to be excreted through the gut (Rainbow 2002) until the detoxifying threshold is exceeded and toxicity occurs through free radical oxidative stress (Rainbow and White 1989; Rainbow et al 1990). In comparison, when exposed to zinc, toxicity occurs in *P. elegans* when the rate of uptake exceeds excretion as zinc remains in a metabolically active form and is not regulated through detoxification in this species (Rainbow and White 1989). Another crustacean, the amphipod *Echinogammarus pirloti* shows different detoxification strategies to the *P. elegans* and accumulates zinc through detoxification but does not readily regulate copper, with the majority being metabolically active due to biological requirements of haemocyanin production (Rainbow and White 1989; Rainbow 2002). Unfortunately no specific information on detoxification strategies exists for *P. walkeri* however, Duquesne et al (2000) found that *P. walkeri* accumulated copper at a higher rate to cadmium and that neither metal was regulated in terms of maintaining constant body concentrations. They therefore proposed that both copper and cadmium were being detoxified through metal-binding proteins or through the formation of insoluble granules.

Comparison of the sensitivity of juveniles and adults

Early life history stages of numerous marine invertebrate species have been shown to be more sensitive to contaminant exposure than are adults (Ahsanullah and Florence 1984; Sheffrin et al. 1984). Using sensitive early life history stages in toxicity tests will produce more ecologically relevant data for the development of trigger values and water quality guidelines that better predict the worst-case contaminant effects in ecosystems (Green et al. 1986).

Comparing average LC_{10} values at 4 d endpoint, the new cohort of juveniles were more sensitive than the *P. walkeri* adults to copper exposure (LC_{10} values of 295 µg/L and 677µg/L respectively). King et al (2006b) found similar results with newly released (<7 d from marsupium) juvenile amphipods of *Hyale longicornis*, *Melita awa*, *Melita matilda* and *Melita plumulosa* being more sensitive to copper and zinc after 4 d exposures than adults exposed for 10 d. The life history stage chosen however is important, as studies have shown mixed results in terms of the relative sensitivity of different stages. Green et al. (1986) found juveniles of a fresh water isopod, *Asellus aquaticus* that had just been released from the marsupium, were the most sensitive life history stage to cadmium exposure, being more than three times more sensitive than embryos and ten times more sensitive than adults. Juvenile *P. walkeri* hatch in October and are released from the brooding marsupia in early-mid November (Rakusa-Suszczewski 1972). The timing of juvenile release coincides with the spring/summer melt period (October – December) and therefore, the peak time for contaminant influx to the nearshore marine environments. As a result, juveniles may have an increased susceptibility to metal and other contaminant exposure. After hatching, female juvenile *P. walkeri* require 19 months of development to reach sexual maturity. Sagar (1980) observed numerous females with degenerated oostegites (flattened plates forming the marsupium), gills and coxae (appendage segment) after releasing juveniles from the marsupium. This suggests that female *P. walkeri* die soon after releasing the juveniles (Sagar 1980). Tip leachates from Thala Valley near Casey Station had high copper concentrations ranging from 6.3 – 19.0 mg/kg which are significantly higher than the point estimates determined for juvenile *P. walkeri* survival in this study. Upon entering the near shore marine waters it would be expected that the tip leachates would rapidly dilute and juveniles would have to be within close proximity to the point source to be immediately affected by contaminant toxicity. Metal contamination however is persistent in aquatic environments due to cycling of bioavailability through binding, accumulation and re-suspension (Ciutat and Boudou 2003; Lenihan 1992). Long-term accumulation of metals in near shore environments

combined with the long development and maturation of juvenile *P. walkeri,* could increase the potential for losses of juveniles from exposure to metal contaminants. This in turn could have significant, long-term effects on coastal populations of *P. walkeri*.

Behavioural endpoints

Behavioural endpoints have been shown to be sensitive indicators of sub-lethal contaminant effects as they integrate the complex responses of an organism's biochemical and physiological processes to toxicant exposure (Mills et al. 2006). As observed in this study, departures from normal behavioural activity of *P. walkeri* represent a more sensitive endpoint than mortality to metal exposure and being a precursor to mortality were also quicker to identify. A number of behaviours were categorised in an attempt to identify a progressive pattern of increasing toxic effect, however, some switching was observed between the behavioural categories. Burlinson and Lawrence (2007) indicate similar difficulties with their behavioural study of the worm, *Hediste diversicolor,* where no defined sequence of behaviours were categorised from low to high concentrations of copper, but responses became more pronounced as concentrations increased. Ventilation rate of pleopods was also investigated as a behavioural characteristic for *P. walkeri* and similar to findings by Vellinger et al. (2012), the pattern of hyper or hypoventilation was too complex to successfully describe due to the irregular switching that was observed between the pleopod fanning rates throughout the exposure periods. This irregularity may have occurred because of the different toxic actions that the different metals were exerting on the amphipod. Cadmium for example, has been shown to reduce oxygen consumption, metabolic and ventilation rates in dogwhelks and potentially similar toxicity effects depressed the ventilation rate of *P. walkeri* amphipods exposed to cadmium in this study (Leung et al 2000). Copper is neurotoxic to some invertebrates as it inhibits the production of acetylcholinesterase, an enzyme involved with neural transmission. By interrupting nervous system transmission, normal physiological function,

movement and therefore the ability to control ventilation rates could have been affected in *P. walkeri* exposed to copper (Brown et al 2004).

Whilst recovery from behavioural changes was not investigated for *P. walkeri* in this study, the departure from normal activity progressed to mortality under the sustained exposure conditions of the bioassays. The impact of metal exposure on behaviour was most noticeably an effect on mobility. Stressors that reduce an organism's capacity for movement can significantly impact their survival fitness. Movement can be directly affected by metal toxicity through the suppression of acetylcholinesterase and therefore neural transmission activity (Brown et al 2004) and indirectly through reduced metabolic rate (Leung et al 2000). Increased demand on detoxifying and storage of toxic metals increases energetic costs and can limit available energy for biological functions such as movement (Sherwood et al 2001). For gammarid amphipods, normal movement and activity is of high ecological importance, being necessary for foraging, predator avoidance and reproductive behaviours (Mills et al. 2006). As the first behavioural change categorised described *P. walkeri's* inability to swim, any departure from normal activity has the potential to impact survival even if there is scope for recovery once the exposure is removed. Combining the ecological significance of mobility for *P. walkeri*, the higher sensitivity of the behavioural endpoint and shorter time required observing a contaminant exposure response; behavioural change is a more ecologically significant endpoint for ecotoxicological evaluation with this species and should be incorporated in future contaminant sensitivity tests.

Effects of increased temperature on toxicity of metals

Variations in natural environmental conditions such as temperature, dissolved oxygen (Ferreira et al. 2010) and UV radiation (Duquesne and Liess 2003; Liess, et al. 2001) may act as additional stressors, and have been shown to increase the sensitivity of organisms to contaminants. Due the temperature stability of Antarctic waters, most marine invertebrates such as *P. walkeri* have evolved to live within a narrow 6-7 °C temperature window compared with temperate

species that have a much larger tolerance of 15-25 °C (Peck 2002). Biological functions of some Antarctic marine invertebrates are significantly impaired at temperatures only a few degrees above the average (-1.8 °C) (Clarke 1988; Peck et al 2004). Surface waters of littoral zones will be among the first to experience increased temperatures from effects of climate change (Sokolova and Lannig 2008), and changes have already been measured for the Western Antarctic Peninsula (Meredith and King 2005). Combining the stenothermal nature of *P. walkeri* with their littoral habitat, this amphipod is among the most susceptible to increases in temperature from climate change effects as well as impacts from metal inputs (Peck et al 2004; Rakusa-Suszczewski 1972).

In this study, increased temperature both reduced the response time of amphipods and increased metal toxicity. The combination of the stress of metal exposure and slightly elevated temperature resulted in increased mortality in *P. walkeri* adults (see Figure 2). Point estimates for copper and cadmium were significantly lower for the amphipods exposed at the higher temperature of 2.4 °C in test 2. Control mortality was similar between the temperature treatments (Figure 3). Previous studies have shown that small increases in temperature (2-3 °C) can cause significant negative physiological effects in Antarctic marine species (Peck et al. 2004). Increased temperature causes an increase in oxygen demand and heart rate that leads to a decline in blood oxygen content (Peck et al 2004). As a result, the maintenance metabolic rate increases and a threshold can then be reached where oxygen demand outweighs supply, negatively affecting essential biological functions such as feeding, movement, and reproduction as well as reducing the organism's capacity for detoxifying and excreting excess metals. Increased temperature can increase metal accumulation rates through higher metal exposure due to increased ventilation rates driven by higher oxygen demand (Sokolova and Lannig 2008). Higher ventilation, metal accumulation and oxygen consumption rates have been seen in the oyster, *Crassostrea virginica* exposed to a combination of cadmium and higher temperature compared with those exposed only to cadmium (Lannig 2006). Ion pumps and facilitated diffusion are the main uptake pathways for metals into

cells of aquatic ectotherms. These processes are temperature dependent where a slight increase in temperature can significantly increase the uptake of metals (Sokolova and Lannig 2008).Increasing metal uptake rates through increasing temperature has also been shown to increase metallothionein production in the dog whelk, *Nucella lapillus* when exposed to both high temperature and cadmium (Leung et al 2000).

As seen in the results of the present study, the added stress of increased temperature compounds the effect of metal exposure on the behaviour and mortality of *P. walkeri*. Despite annual temperature cycles around continental Antarctica being relatively stable within the narrow range of -1.8 - 1 °C (Chapman and Riddle 2005), rapid warming of the ocean west of the Antarctic Peninsula to an average summer temperature above 1 °C has been recorded over the last half century (Meredith and King 2005). Increased pressure on coastal marine ecosystems from anthropogenic impacts occurs during the summer months when human habitation of scientific bases increases and meltwaters mobilise toxicants from contaminated sites. If climate change continues to influence sea surface temperature rises around the Antarctic continent, littoral marine invertebrate species, like *P. walkeri,* could be even more susceptible to impacts from localised metal contamination. Like other Antarctic marine invertebrates, developmental and sexual maturation times of *P. walkeri* are significantly longer than temperate or tropical species. If a significant contaminant impact is sustained to a *P. walkeri* population, their ability to recover and the survival of the population could be significantly impaired.

Optimisation of parameter and toxicity test development

Control mortality

P. walkeri adults and juveniles were sensitive to the laboratory test conditions, indicated by decreased survival in the control treatments in some of the bioassays. For standard toxicity tests with amphipods, 30% mortality in control treatments is generally considered acceptable (Environment Canada 2005). On this basis, the adult tests (75% survival by 21 d) and juvenile tests 1 and 2 (80%

survival by 10 d) are within acceptable limits. The exception to this was juvenile test 3 in which control survival had decreased to 29% at 14 d. As we have shown the juveniles to be a more sensitive life history stage of *P. walkeri* amphipods to metal contaminant impact, it is recommended that further testing be conducted with this age class to determine more optimal bioassay test conditions methods including handling, solution renewal, and feeding regimes. Longer duration exposures similar in length to the adult bioassay would then be able to be conducted and better comparisons made between the sensitivities of juvenile and adult *P. walkeri*.

Variability of responses between tests

There was high variability in the responses of adults for each metal between replicate tests. This result was not entirely unexpected as, despite using identical methodologies, tests were conducted a year apart with amphipods sourced from different locations (although none of these sites were impacted by local contaminant inputs). The two consecutive experimental summer seasons were characteristically different, with fast ice breaking out in late December in the first season, allowing for 24 hr light penetration to the littoral zone and subsequent microalgal blooms to be longer in duration than the second season where fast ice remained until late January, periodically reforming until March. Liess et al. (2001) investigated the combined effects of UV-B radiation on copper exposure on *P. walkeri* and found, despite UV-B not affecting the chemical structure of copper, the amphipods exposed to both UV and copper had significantly reduced survival (40%) after 12 d. In the present study, it is possible that adults tested in the first season (test 1) had been impacted from UV-B stress in the field prior to testing, affecting their sensitivity to metal exposure in the bioassay. It is difficult to control for pre-existing environmental conditions when using animals collected directly from the field, hence laboratory cultures are often used and this is being explored further for *P. walkeri*.

Test duration and endpoint

The variability in responses of *P. walkeri* between tests makes it difficult to suggest a single exposure duration for a standardised bioassay method which is shorter than the 21 d testing period used. Exposure durations for bioassays with temperate and tropical species are standardised irrespective of the contaminant being tested (Baas et al. 2010). However, the significant difference in *P. walkeri's* response times to different metal contaminants suggests this is not feasible for this species. Behavioural change was a more sensitive endpoint than mortality and was reliably affected by copper, cadmium, and zinc at a faster rate. It would be beneficial therefore to develop a standardised test duration based on behavioural change that would have the advantage of being a more sensitive endpoint as well as being able to be completed in a shorter time period. This could positively affect the control survival rate and increase test acceptability, as well as increase the number of tests conducted during Antarctic field seasons. Therefore, for future sensitivity testing with *P. walkeri*, it is recommended that the endpoint of active behaviour be evaluated for effects of contaminant exposure.

Number of individuals per treatment

The highest variability in control survival occurred in the juvenile test 3 where only six individuals were used in each replicate. The number of test individuals was limited to 6 due to organism availability which can be a problem when relying on field collections, especially under logistically difficult conditions in Antarctica. By increasing the number of juveniles to at least 10 to 15 individuals, the test acceptability with bioassays using newly released juveniles would potentially improve and allow better statistical comparisons to be made between juvenile and adult responses to metal contaminant exposure.

Evaluation of test species and recommendations

To develop trigger values for water quality guidelines it is important that species that are sensitive to contaminant exposure are assessed for concentration point estimates. Despite seeing variability in responses between tests in this study, *P.*

walkeri has been a successful experimental species in previous studies having been used in toxicity testing, bioaccumulation studies and biological monitoring programs. When developing point estimates it is important that the response of the species, and not simply of one population at one point in time, are represented in the toxicity data. By incorporating spatial and temporal variability, our study has captured a broader range of responses for *P. walkeri* and produced point estimates that reflect the sensitivity variability within the species rather than for a single population. Accuracy of the point estimates generated could be improved with further testing. Ideally, toxicity tests with *P. walkeri* would be conducted with individuals sampled at different geographic locations to incorporate differences in population genetics, and conducted during the same research season. The tests would then need to be repeated across separate research seasons to incorporate temporal variability into the response data. Alternatively, individuals could be collected from different geographic locations and grouped together for toxicity testing under the same laboratory conditions or possibly established in an aquarium facility for culturing. Experimental designs like these, while practically feasible in many temperate and tropical locations, are rarely realistic under Antarctic circumstances. Conducting research in Antarctica is significantly constrained by numerous logistical issues such as seasonal access to the continent, restrictions on sampling locations due to permits and regulations as well as weather conditions and sea ice cover, and financial limitations that can restrict research programs being conducted over successive seasons. Collecting and transporting organisms to establish cultures in aquarium facilities is also logistically difficult logistically with the stress experienced from the air or sea transport quite often resulting in mortality of the more sensitive individuals from the population which can then bias results determined for contaminant sensitivities. Therefore, where possible, extensive handling and transportation of tests species should be minimised for accurate evaluation of species sensitivity to contaminant exposure.

Conclusion

This study successfully developed a longer-term bioassay method and more sensitive behavioural endpoints for evaluating contaminant effects on adult *P. walkeri*. The relative toxicity varied between metals, in the following order from most to least toxic: copper > cadmium > zinc > lead > nickel. This is a similar pattern as observed for another Antarctic marine amphipod, *O. pinguides* (Chapter 4; Sfiligoj et al. in prep-b) and for a temperate amphipod *M. plumulosa* (King et al. 2006). Juveniles were successfully evaluated for sensitivity to copper exposure, and, as they were more sensitive than adult *P. walkeri,* further bioassay method development should be done to improve the control mortality in the juvenile tests. The additional stress of slightly elevated temperature compounded the negative effect of metal exposure on *P. walkeri* with the adults responding more quickly in the elevated temperature than those exposed to only copper or cadmium. Establishing a standardised exposure duration for bioassays with *P. walkeri* has proven to be difficult due to the difference in response times to the different metals. For toxicity tests evaluating lethal endpoints with *P. walkeri*, an exposure period of 21 days should be the minimum time employed. Using the more sensitive endpoint of active behaviour, the exposure duration could be reduced to 14 days. Based on results from this study and evidence from another study with the Antarctic amphipod, *O. pinguides* (Chapter 4; Sfiligoj et al. in prep-b), it is likely that a minimum of 14 days is required for toxicity testing with *P. walkeri.*

The point estimates generated from this study will contribute to the development of species sensitivity distributions for determining water quality guidelines and trigger values that are specific to Antarctic marine ecosystems. With this information, contamination from past human activities can be better managed by providing essential guidance information for remediation activities. Future human activities can also be better managed through improved contaminant mitigation planning to ensure the long-term protection of these unique ecosystems.

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Chapter 4

Improved methods for determining the toxicity of metals to an Antarctic amphipod by joint modelling of survival response to exposure concentration and duration

This chapter is presented as a manuscript prepared for publication

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Abstract

Developing water quality guidelines for Antarctic marine environments requires an understanding of the sensitivity of local biota to contaminant exposure. Experiments with Antarctic invertebrates have shown that responses to contaminants are delayed compared to temperate and tropical species in standard toxicity tests. Test methods which take into account the biological characteristics of these cold climate species and of their environment therefore need to be developed. This study investigated the effects of five metals on survival of an Antarctic amphipod, *Orchomenella pinguides*, over a 30 day exposure period. Multiple observations assessing mortality rate response to metal exposure were made. As traditional toxicity tests with quantal data sets use methods such as Maximum Likelihood Regression (Probit analysis) and Spearman- Kärber which treat individual time period endpoints independently, a new statistical model was developed to integrate the time-series concentration-response data. Interval-censored survival times were modelled using a generalized additive mixed model (GAMM) which incorporates all data from multiple observation times to enable better time integrated point estimates to be determined. Response times varied for different metals with amphipods responding faster to copper than to cadmium, lead or zinc. Nickel exposure (up to 1 mg/L) did not affect amphipod survival. While lethal concentrations (LC_{10} and LC_{50}) were similar when estimated by both the Probit and the GAMM analyses, incorporating all the time-series data into the GAMM model strengthens the accuracy of the point estimation. The confidence intervals for estimated lethal concentrations by time were wider for the GAMM since it accounts for variation due to lack of fit as well as binomial variation where only this last source of error is considered in Probit analyses. Using longer exposure durations and utilising the GAMM model to integrate the response of test organisms through time will provide improved methodologies for accurately assessing sensitivities of Antarctic marine invertebrates to contaminants.

Key words

contamination, *Orchomenella pinguides*, bioassay, survival through time, water quality guidelines

Introduction

Several studies have identified nearshore marine environments and biota in Antarctica that have been significantly affected by anthropogenic chemical contamination (Cunningham et al. 2005; Deprez et al. 1999; Duquesne and Riddle 2002; Lenihan 1992; Lenihan and Oliver 1995; Stark et al. 2003, 2004). This contamination is mostly associated with past and current human activities at Antarctic research stations, including waste disposal to tip sites, "sea-icing" of waste material, discharge of sewage and accidental fuel spills, all of which have resulted in organic and metal contaminant inputs to nearshore ecosystems (Snape et al. 2001a, 2003; Townsend and Snape 2008). Managing and predicting the impacts of contaminants in Antarctica relies upon an understanding of the sensitivity and response of local marine biota to anthropogenic contaminant exposure.

Currently there is little toxicological data on the sensitivity of Antarctic biota to metals (Chapman and Riddle 2005a; Duquesne et al. 2000; King and Riddle 2001; Liess et al. 2001). This paucity in data is primarily due to logistical constraints (i.e. transport, sea ice coverage, weather conditions), which restrict routine access to Antarctic marine biota for toxicity testing, and to the slow response times of Antarctic biota such that toxicity tests must often run for several weeks to months to elicit a response. Standard bioassay replication is therefore rarely achieved and often, only small data sets are generated for contaminant effects on individual species. To maximise the use of this data and to better understand the sensitivity of Antarctic marine biota to contamination it is important that all data generated in a toxicity test be incorporated in statistical analyses. Further

testing with species from a range of trophic and taxonomic levels using appropriate test methodologies should also be undertaken.

Accurate determination of metal sensitivity requires the development of appropriate toxicity test durations that address the unique evolutionary adaptations of Antarctic marine biota (Chapman and Riddle 2005a). Many species exhibit ecophysiological traits such as lowered metabolic rates (Peck 2002), longer life-spans, longer larval development (King and Riddle 2001), reduced surface area to volume ratios, higher lipid content and therefore lipophilic contaminant retention (Goerke et al. 2004), all of which could affect response times to contaminant exposure. The few studies conducted with Antarctic marine biota using longer exposures found that extended test durations were required to elicit contaminant-induced responses that were similar to temperate and tropical species exposed to contaminants over short-term test durations (Chapter 2; Sfiligoj et al. in prep-a; King and Riddle 2001). Additionally, some studies found that Antarctic species were more sensitive to contaminants than analogous temperate and tropical species (Chapter 2; Sfiligoj et al. in prep-a; King and Riddle 2001). These research findings highlight the importance of developing appropriate, longer exposure duration bioassays for assessing the impacts of contaminants on Antarctic marine biota and for deriving accurate point estimate concentrations.

While employing longer exposure durations is necessary for bioassays with slow responding Antarctic species (Chapman and Riddle 2005a, 2005b; King and Riddle 2001), they present issues relating to appropriate statistical analyses. The exposure duration is generally standardised to species or taxa and is chosen irrespective of the contaminant being tested (Baas et al. 2010).Traditionally, one endpoint (e.g. mortality / behaviour/ biomarker change) is observed at a single specified time (exposure duration) and is analysed to derive a lethal or effective concentration for a given contaminant. In many tests however, multiple observations are made throughout the bioassay, yet this time-series data is rarely incorporated into the derivation of point estimates. This practice of

disregarding all the observational data gathered through a bioassay and only using the information taken from the one endpoint at the end of the exposure has been widely criticised (Baas et al. 2010; Crane and Grosso 2002). Risk assessment outcomes could therefore be affected by failing to incorporate the cumulative effects of contaminant exposure through time (Baas et al. 2010; Crane and Grosso 2002; Heckmann et al. 2010; Jager et al. 2006).

Current methods such as Maximum Likelihood Regression using the Probit transform (Probit analysis) and the Spearman-Kärber Method are unable to measure cumulative effects of contaminant exposure through time (Bedaux and Kooijman 1994; Jager et al. 2006). A toxicity model using Dynamic Energy Budget theory or DEBtox, was developed to incorporate time into toxicity testing (Kooijman and Bedaux 1996). DEBtox is a process based analysis of physiological parameters and links an organism's essential processes (i.e. feeding, digestion, respiration, maintenance, growth, development, reproduction, survival) through mechanistic assumptions of first order kinetics to the exposure time and exposure concentration of tested toxicants (Kooijman et al. 1998; Jager et al. 2006). As this model is process-based, it requires choosing DEB parameters of that process which the contaminant will impact, having good starting values for those parameters (which are not easily obtained (Bedaux and Kooijman 1994)) and then fitting an appropriate model to the data. Unfortunately, these steps require significant experience on behalf of the researcher and prior parameter knowledge for the test species (Kooijman and Bedaux 1996). These issues impact the application of the DEBtox model with Antarctic marine species at this time.

To better understand the sensitivity of Antarctic marine biota to a range of contaminants, a new model was developed by Candy et al (in prep; Appendix 1) which can incorporate all time point responses and time varying concentrations to model contaminant effects through time. Using a generalized additive mixed model (GAMM) the bioassay component has been combined with survival analysis (i.e. using interval-censored survival times). The bioassay component assumes a Weibull tolerance distribution on the concentration whereas, the

commonly used Probit analysis uses log of concentration, and assumes this distribution is lognormal. The log-hazard function (instantaneous mortality rate function) is modelled by the GAMM using a flexible function that does not correspond to a particular parametric family of survival-time distributions, instead, GAMM chooses the best model and reports diagnostics on the fit of that model to the data (Appendix 1; Candy et al. in prep). This means that log-concentration as applied in a Probit linear regression is used but mortality is modelled through time using the actual interval-censored mortality data. As a result, GAMM can produce point estimates for LC_x/EC_x for any day within the test period. Candy et al. (in prep; Appendix 1) demonstrate how separate bioassays can be jointly modelled and differences between the individual bioassays can be explored and accounted for as random effects. In addition, a simple empirical approach is described where time varying concentrations are accounted for using a Calibrated Cumulative Exposure (CCE) variable to replace terms in the standard GAMM. The model using CCE can also be applied when concentrations are assumed time-invariant. Control mortality can also be incorporated into the model predictions using Abbott's correction (Finney 1971). Applying this newly developed GAMM will allow for a greater understanding of the influence of time on contaminant exposure response, increased sensitivity assessment accuracy, and add strength to lethal and effective concentration values as all data collected contributes to the derived point estimates.

This study had 3 main objectives: 1) develop appropriate methods for a longer duration bioassay using the benthic amphipod *Orchomenella pinguides* (Walker, 1903) (Lysianassidae) as a representative, common coastal Antarctic marine invertebrate in East Antarctica; 2) examine the sensitivity and response time of *O. pinguides* to five metals (copper, cadmium, zinc, lead and nickel) which are common at contaminated sites in Antarctica (Cunningham et al. 2005; Deprez et al. 1999; Snape et al. 2001b); 3) develop a statistical model to analyse mortality data from multiple observations through time to more accurately assess the sensitivity of this species and to produce more robust point estimate concentrations in comparison to a traditional statistical approach using the

Probit analysis method. This new model is described in detail in Candy et al. (in prep; Appendix 1).

Materials and methods

Species description and sample collection

The benthic amphipod *Orchomenella pinguides* (Lysianassidae) was selected as a test species due to its potential for contaminant impact. Being a deposit feeding macrophage, *Orchomenella pinguides* lives in close association with sand and mud sediments in near shore habitats of Antarctic coastal waters (Dauby et al 2001). *Orchomenella pinguides* sized between 6 – 8 mm in length were collected using baited invertebrate traps deployed in nearshore, soft sediment and rocky substrate habitats at approximately 10-20 m depth at various locations near Davis Station (68˚35'S 77˚58'E) in East Antarctica.

Seawater used in tests was collected approximately 40 m offshore from Davis Station, from 2-2.5 m depth, 500m north and up current from the sewage outfall and away from any obvious anthropogenic contaminant inputs. Seawater was filtered to 1 µm. The filtered seawater (FSW) had a salinity range of 33 – 34 ppt, and pH of 7.99 – 8.05 as measured by a Mettler Toledo SG78 metre calibrated with buffers for measuring water at 0 °C. Dissolved oxygen (DO) of the filtered seawater was > 90%.

General analytical

Equipment preparation

All plastic ware and glassware used for test preparation and in tests were soaked in 10% (v/v) analytical grade nitric acid (HNO_3) for a minimum of 8 h prior to use.

Test solution preparation

Stock solutions (500 mg/L) of copper, cadmium, zinc, lead and nickel were prepared in high-density 250 mL polyethylene NALGENE screw cap bottles using highly purified (Milli Q) water and the following metal salts: 0.4911 g of $CuSO_4.5H_2O$, 0.2853 g of $3CdSO_4.8H_2O$, 0.5062 g of $NiCl_2.6H_2O$, 0.2606 g of $ZnCl_2$

and 0.1678 g of $PbCl_2$. Test exposure solutions were prepared by diluting metal stock solutions with FSW. The pH of stock and exposure solutions were not adjusted.

Test procedure

Pilot tests were conducted to establish appropriate bioassay methodology, specifically exposure concentrations, test duration, and feeding and water change regimes. Amphipods used in bioassays were acclimated in the laboratory for ~ 5 days prior to the start of tests. Two replicate bioassays were conducted at Davis Station, Antarctica, during the austral summer of 2010-11 in which adult *O. pinguides* were exposed to copper, cadmium, nickel, zinc and lead as single contaminants.

Bioassays were conducted over 30 d. Mortality observations coincided with water changes and water sampling on days 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30. Granulated fish pellets (Granumarin, Sera) were dispensed to each replicate ~ 18 h before each water change. Bioassay 1 consisted of four test concentrations for each of the five metals and one control treatment. Bioassay 2 included an additional concentration for cadmium, nickel and lead. In both bioassays, four replicates per treatment were used. Each replicate contained 10 adult *O. pinguides* (approximately 5 – 7 mm in length) exposed to 200 mL of test solution in capped 250 mL polypropylene containers. A small square of acid washed, plastic mesh (6 x 4 cm) was added to each container to provide a surface habitat for amphipods to cling to. As exposure solutions were made for all replicates in a treatment in one batch, 10 mL water samples from both old and new replacement solutions were taken from a single replicate vial per treatment for metal analysis. Water samples were filtered through a 0.45 µm syringe filter tip then acidified with 1% (v/v) ultra pure nitric acid (HNO_3) and analysed using an inductively coupled plasma optical emission spectrometer (ICP-OES, Varian 720-ES). Average exposure concentrations for all treatments and their standard error (SE) are presented in Table 1. Salinity and pH were measured in the control treatments only. All bioassays were conducted in controlled temperature

cabinets that were set to -1.0 °C and monitored using data loggers, which recorded internal air temperature at 10 min intervals. A 16:8 h light/dark cycle using cool white fluorescent lamps was used to mimic ambient light intensity and daylight hours during Antarctic spring/summer months.

Table 1: Average exposure concentrations (µg/L) for metal treatments used in bioassays with *Orchomenella pinguides*. Concentrations with standard errors (SE) are averaged from samples taken from each water renewal and measured using ICP-OES. Concentrations of all five metals in the control were below detection limits (DL) which are listed for each metal.

Metal	Detection limit (µg/L)	Nominal metal concentrations (µg/L)	Average measured metal concentration and SE (µg/L) Bioassay 1	Bioassay 2
Copper	2.7	100	85 (2.4)	85 (1.3)
		200	177 (4.2)	170 (4.4)
		400	377 (14.9)	347 (7.8)
		800	766 (38)	714 (14.4)
Cadmium	0.7	100	108 (1)	106 (1.2)
		200	223 (4.6)	215 (1.8)
		400	439 (5.2)	433 (3.0)
		800	871 (12.5)	878 (8.7)
		1200	-	1306 (6.2)
Zinc	1.1	250	237 (2.8)	227 (1.9)
		500	471 (3.8)	434 (16.3)
		1000	918 (8.1)	993 (72.8)
		2000	1800 (96)	1816 (21.5)
Nickel	2.6	100	93 (1)	94 (1.6)
		200	188 (1.4)	183 (2.2)
		400	381 (3.8)	366 (3.4)
		800	764 7.2)	732 (7.8)
		1200	-	1123 (43.9)
Lead	8.7	100	90 (2.1)	85 (1.7)
		200	178 (3.7)	181 (1.4)
		400	363 (5.2)	343 (3.1)
		800	710(12.5)	674 (8.9)
		1200	-	1020 (13.6)

Data analyses

Mean exposure concentrations for each metal treatment were calculated using the measured concentrations from the water samples taken at the beginning and end of each 3 d exposure period. These measured values as opposed to nominal values were used in analyses to derive point estimates.

Statistical methods

Mortality data was parametrically analysed using traditional Probit Maximum Likelihood Regression to derive LC_{10} and LC_{50} values for copper, cadmium, zinc and lead for each observational time endpoint (day 3, 6, 9, 12, 15, 18, 21, 24, 27, 30). This was done using mortality data from the individual bioassays and with the data for both bioassays combined. A new statistical model was applied using a generalised additive mixed model (GAMM) which is described in detail in Candy et al. (in prep; Appendix 1). The GAMM was run for the two bioassays individually and was modified to run the data from both tests pooled together. Both the individual endpoint Probit analyses and GAMM analyses were carried out using the R software package. Mortality responses were not analysed for nickel due to the lack of response to the concentrations used in the exposure

Results

Salinity, pH and temperature remained constant throughout both bioassays. Mean salinity was 34.6 ppt ± 0.4 SD, mean pH was 7.81 ± 0.2 SD and temperature -0.9 ˚C ± 0.5 SD.

Response of *Orchomenella pinguides* to metal exposure

No mortalities were observed in the control treatment in Bioassay 1, and an average of 8% mortality at 30 d was observed in Bioassay 2. Response times of *O. pinguides* to metal exposure were similar for both bioassays, however varied between metals (Figure 1 and 2). *Orchomenella pinguides* responded slowly to metals with almost all individuals surviving exposure to copper up to day 3, day 6 for cadmium, day 12 for zinc and day 15 for lead in both bioassays (Figure 1 and 2). No significant mortalities were observed following exposure to nickel up to

day 30, even at the highest test concentration (1.12 mg/L). *S*ensitivity to copper, cadmium, zinc and lead exposure increased with increasing exposure time (Figure 1 and 2) as indicated by a decrease in LC_{50} values (Table 2). As shown in Figures 1, 2 and Table 2, copper was the most toxic metal, followed by cadmium, lead and zinc. Nickel was not toxic to *O. pinguides* at any of the concentrations tested therefore no results for nickel are presented.

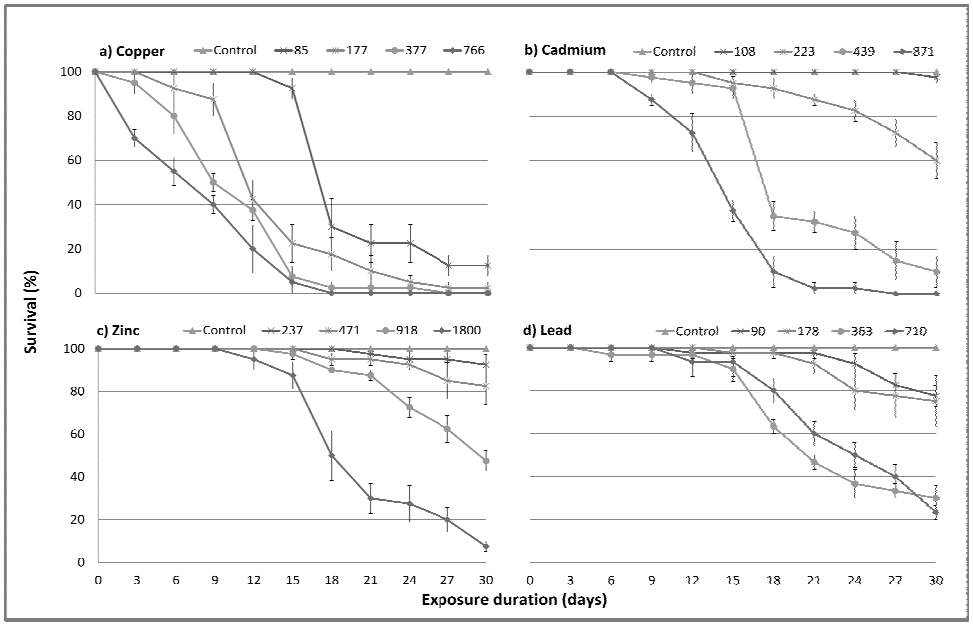

Figure 1: Survival of *Orchomenella pinguides* exposed to a) copper, b) cadmium, c) zinc and d) lead in Bioassay 1; values are average ± 1 SE; all concentrations are in µg/L).

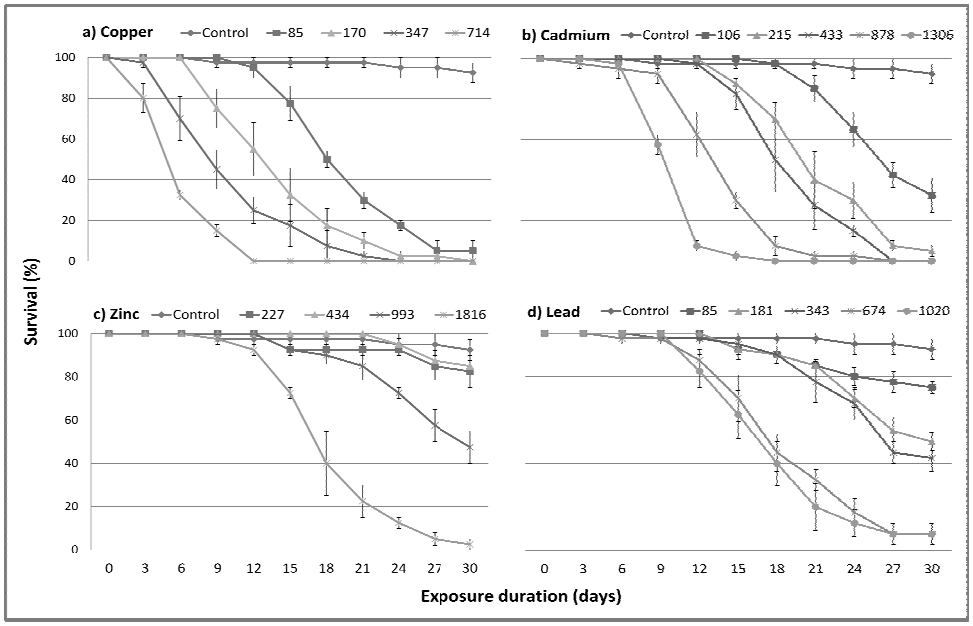

Figure 2: Survival of *Orchomenella pinguides* exposed to a) copper, b) cadmium, c) zinc and d) lead in Bioassay 2 (values are average ± 1 SE; all concentrations are in µg/L).

Table 2: LC_{10} and LC_{50} estimates and 95% Confidence Limits (CL) in brackets for *Orchomenella pinguides* exposed to metals for up to 30 days (estimates are from GAMM[a] with data combined across two replicate bioassays; estimates are not reported (-) if outside the range of concentrations tested).

Exposure duration (days)	Copper		Cadmium		Zinc		Lead	
	LC_{10} (CL)	LC_{50} (CL)	LC_{10} (CL)	LC_{50} (CL)	LC_{10} (CL)	LC_{50} (CL)	LC_{10} (CL)	LC_{50} (CL)
7	416 (330, 487)	-	1005 (635, 1623)	-	-	-	-	-
10	197 (156, 231)	441 (365, 545)	582 (388, 813)	-	-	-	-	-
14	98 (78, 115)	218 (181, 271)	305 (200, 408)	896 (675, 1254)	1461 (980, 2079)	-	414 (256, 621)	
21	42 (33, 50)	94 (78, 117)	123 (79, 171)	362 (277, 463)	533 (369, 687)	1517 (1201, 1930)	97 (60, 144)	596 (440, 818)
28	24 (19, 28)	52 (43, 64)	76 (56, 98)	191 (134, 252)	324 (212, 435)	923 (728, 1141)	54 (37, 76)	286 (205, 382)
30	20 (16, 24)	45 (37, 56)	71 (51, 90)	164 (110, 225)	288 (188, 397)	821 (628, 1035)	51 (35, 71)	256 (177, 349)

[a] Based on model (4) of Candy et al. (in prep; Appendix 1) with CCE fitted using actual time-varying concentrations

Statistical results

As low control mortality (0-8%) was observed in both bioassays, the Abbots correction extension to the GAMM was not applied in this study. Slow mortality responses affected ability for point estimates to be derived by both GAMM and Probit analyses. LC_{10} and LC_{50} estimates for the combined bioassay data sets determined by GAMM are presented in Table 2. Using the methods described in Candy et al. (in prep; Appendix 1), only cadmium exhibited significant differences ($P<0.05$) between individual bioassays. LC_{50} estimates for cadmium in Bioassay 1 also differed between the statistical methods used, with estimates from Probit analysis being consistently lower than those from GAMM, although still within the confidence intervals of GAMM (Figure 3). The difference between the two methods in LC_{50} estimates for cadmium in Bioassay 1 particularly on day 18, relate to a sharp decrease in survival between day 15 and 18 for the 439 µg/L treatment (Figure 1). Mortality of *O. pinguides* exposed to zinc was consistent through the exposure periods, and this is reflected in the similarities of the Probit and GAMM estimates (Figure 4).

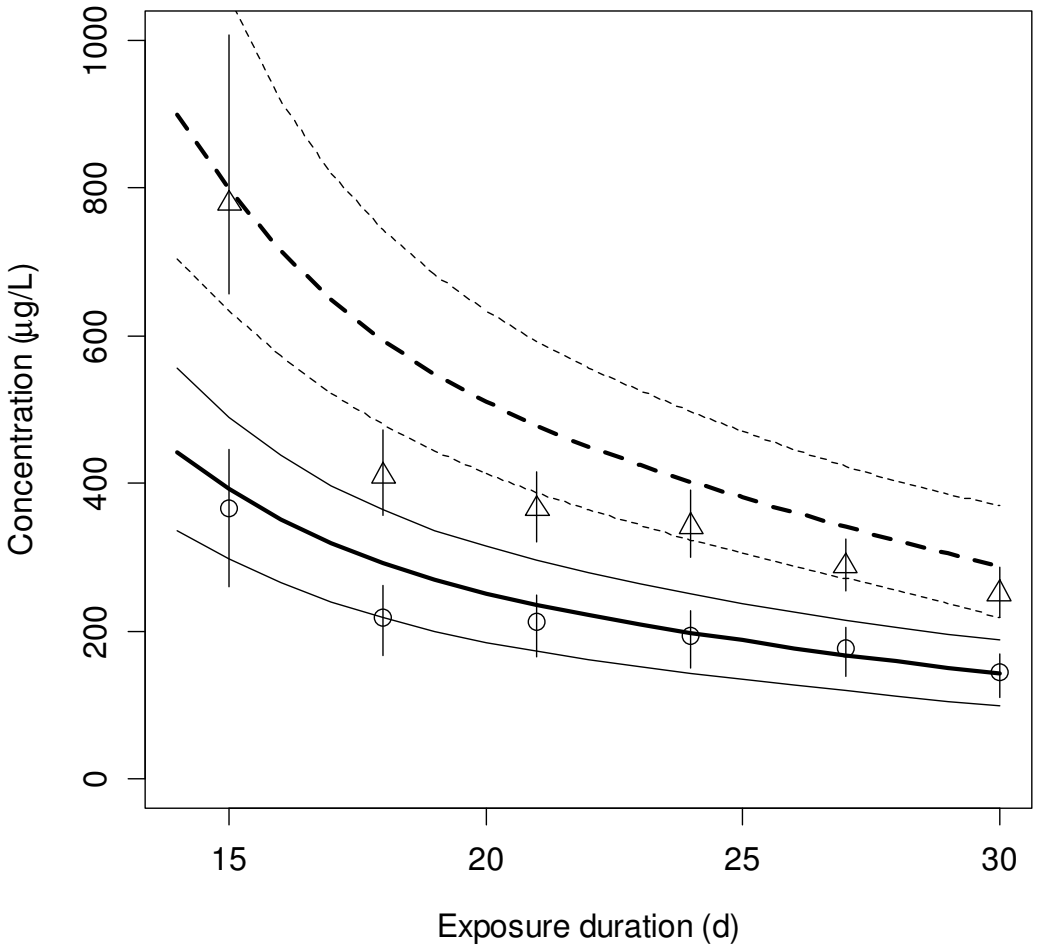

Figure 3: Lethal concentration (LC) values for *Orchomenella pinguides* estimated from GAMM (lines) and Probit analysis (points) for cadmium in Bioassay 1. LC_{10} (GAMM = solid line ____, Probit = circles) and LC_{50} (GAMM = dashed lines _ _ _ _, Probit = triangles) with 95% confidence bounds shown for the GAMM (thin lines) and probits (bars).

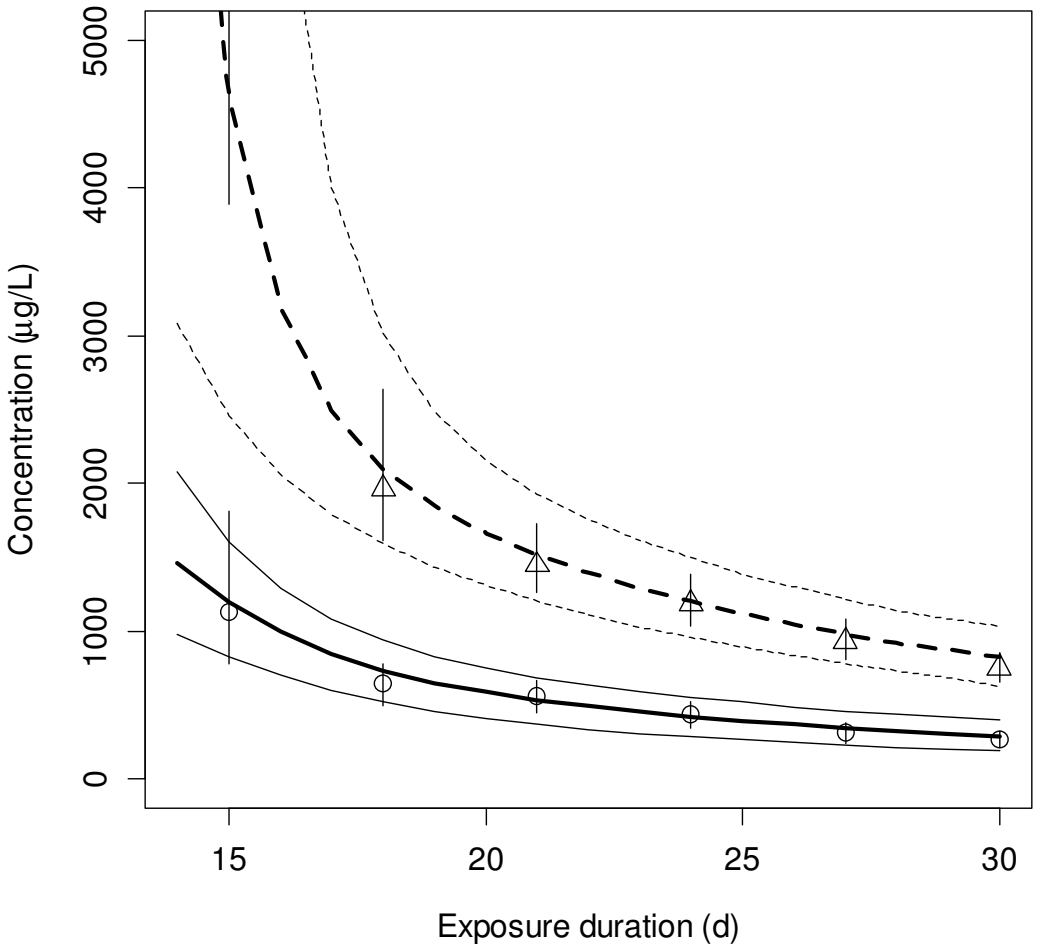

Figure 4: Lethal concentration (LC) values for *Orchomenella pinguides* estimated from GAMM (lines) and Probit analyses (points) for zinc using the combined data set from Bioassay 1 and 2. LC_{10} (GAMM = solid line ____, Probit = circles) and LC_{50} (GAMM = dashed lines _ _ _ _, Probit = triangles) with 95% confidence bounds shown for the GAMM (thin lines) and probits (bars)..

Discussion

Sensitivity of *Orchomenella pinguides* and response time to metal exposure

The 30 d exposure period used in this study allowed sufficient time for mortality to occur in *O. pinguides,* and enabled concentration-response data to be generated for each metal with the exception of nickel. The mortality response differed between metals with toxicity decreasing from copper > cadmium > lead > zinc > nickel over the 30 day exposure period. A similar pattern of metal toxicity has also been recorded for *Paramoera walkeri* (Chapter 3; Sfiligoj et al. in prep -b) the temperate amphipod *Melita plumulosa* (King et al. 2006a) over a 4 d exposure period. King et al (2006b) found a similar toxicity pattern for six Australian and New Zealand amphipod species, with *Corophium colo, Grandidierella japonica, Hyale longicornis, Melita awa, Melita matilda* and *Melita plumulosa* all being more sensitive to copper than zinc exposure in 10 d water-only tests. However, Bat et al (1998) found the burrowing marine amphipod *Corophium volutator* to be less sensitive to copper than zinc or cadmium in water only exposures. Conversely, the North American marine amphipods, *Corophium insidiosum* and *Elasmopus bampo* were found to be more sensitive to copper and cadmium than zinc or lead in Reish's (1993) research. Anderson et al (2008) found differing patterns of toxicity for the North American estuarine amphipods *Ampelisca abdita* and *Eohaustorius estuarius* during sediment exposures with *A. abdita* showing higher sensitivity to copper and *E. estuaries* being more sensitive to pesticides than metals. Despite specific modes of action differing for each metal such as varying rates of free radical generation or neurotoxicity, there does not appear to be a clear pattern of increasing metal toxicity across amphipod species. Nickel did not affect the survival of *O. pinguides* up to the highest concentration tested. King et al (2006) found *M. plumulosa* to exhibit a 10 d Ni LC_{50} of 2590 µg/L, which is more than double the highest average exposure concentration of 1120 µg/L used in this study with *O. pinguides*. Despite the longer exposure duration used, the

response time of *O. pinguides* to metal exposure is significantly longer than temperate and tropical species and it is most likely that the nickel concentrations used during the bioassays were too low to elicit any harmful effect.

In addition to the longer response time of *O. pinguides* to metal exposure compared with temperate and tropical amphipods, *O. pinguides* sensitivity increased through time. Only a few ecotoxicology studies have investigated and identified longer response times coupled with increasing sensitivity of Antarctic marine invertebrates to metal exposure. Another Antarctic marine amphipod, *Paramoera walkeri*, has a longer response time to metal contaminant effects with sensitivity to exposure also increasing through time from 4 d to 8 d exposures (Duquesne et al. 2000) and this pattern was also observed over the longer exposure durations in Chapter 3, Sfiligoj et al (in prep-b). King and Riddle (2001) found longer exposure durations to be important when comparing metal contaminant sensitivities of specific developmental stages of Antarctic echinoid larvae with temperate and tropical species. In addition to highly seasonal primary productivity, low stable temperatures of Antarctic marine environments are a significant driver of physiological adaptations in endemic marine invertebrates (Clarke 1988). Adaptations include slow development and growth, reduced metabolic rate (Peck 2002), longer life spans, increased body size (Chapelle and Peck 1999) and higher lipid content (Goerke et al. 2004). Despite these characteristics, Duquesne et al (2000) showed that accumulation rates of metals by the Antarctic amphipod *P. walkeri* were comparable to those of temperate and tropical species, yet, they did not regulate body concentrations of copper or cadmium during exposure. Although metal ion uptake rates may be similar for *O. pinguides,* accumulation of toxicants and the associated effects on toxicity level are not easily linked (Simpson and King 2005) and therefore the long acute time responses of *O. pinguides* would be influenced by a combination of biochemical processes such as lowered metabolic rates and temperature-dependent toxicokinetics. Using longer exposure durations in bioassays is therefore essential when accurately determining Antarctic marine biota sensitivity to contaminant effects.

Chapman and Riddle (2005) reviewed the available polar toxicity data with comparisons to the short-term tests (acute) and long-term test (chronic) values from temperate water quality guidelines for Australia, New Zealand, Canada and the United States. They concluded that based on short-term tests, polar species were relatively insensitive to metal contaminants and the corresponding U.S. EPA acute guidelines would be protective for copper, chromium, lead and zinc exposure. However, when comparing the polar species data to the chronic values from long-term temperate tests, the guidelines would not be protective for copper, but would be for cadmium and zinc (Chapman and Riddle 2005). Lethal concentration estimates (LC_{50}) and associated endpoint times (day) for the Antarctic amphipods *O. pinguides and P. walkeri* (Chapter 3: Sfiligoj et al in prep b) and temperate amphipod species are presented in Table 2 for sensitivity comparison. *Paramoera walkeri* and *O. pinguides* have similar sensitivities to copper, cadmium and zinc (Table 2). There are a number of instances where long-term test endpoints (14 and 21 d) for the Antarctic species have similar point estimate values compared with the 4 and 10 d values for the temperate species (Table 2). These results suggest that over biologically relevant exposure durations, the two Antarctic amphipods are similar in sensitivity to temperate species and as suggested by Hill et al (2009) temperate water quality guidelines could be protective of Antarctic species with the additional consideration of Antarctic appropriate exposure durations.

Table 2: Lethal concentration estimates (LC_{50} µg/L ± standard error) determined for *Paramoera walkeri* for each metal. LC_{50} estimates (with 95 % confidence limits) for *Orchomenella pinguides* and amphipods from other regions are presented with endpoint durations (day).

		LC_{50} (µg/L)					
Species	**Day**	**Copper**	**Cadmium**	**Zinc**	**Lead**	**Nickel**	**Study**
Paramoera walkeri	14	288 (15)	519 (168)	-	-	-	This study
	21	106 (70)	320 (170)	1005 (150)	-	-	
Orchomenella pinguides	10	441 (365, 545)	-	-	-	-	(Chapter 4: Sfiligoj et al in prep b)
	14	218 (181, 271)	896 (675, 1254)	-	-	-	
	21	94 (78, 117)	362 (277, 463)	1517 (1201, 1930)	596 (440, 818)	-	
Melita plumulosa	10	180 (30-260)	470 (400–540)	900 (750–1020)	1270 (940–1550)	2590 (2030–3050)	(King et al 2006a)
Corophium colo	4	>950		>4500			(King et al 2006b)
Grandidierella japonica	4	250 (120-410)		1560 (1090–2010)			(King et al 2006b)
Hyale longicornis	10	> 190		> 1940			(King et al 2006b)
Melita awa	10	150 (120-190)		710 (570 – 830)			(King et al 2006b)
Melita matilda	10	220 (200-240)		730 (560-890)			(King et al 2006b)

It is also important to consider the environmentally realistic duration of contaminant exposure when determining sensitivity of marine biota to contaminant effects. Persistence and bioavailability of metals in aquatic systems varies with respect to the input source, chelating properties of the receiving water, sediment storage capacity, and rate of dilution / dispersion of the contaminant based on residence time (Bartram and Ballance 1996). It could be argued that the longer exposure durations employed in this study (30 d) do not accurately reflect the natural exposure duration expected in nearshore Antarctic marine ecosystems. However, metals are persistent contaminants and are mobilised from terrestrial contaminant sources such as tip sites during summer melt periods and delivered to nearshore Antarctic marine areas (Snape et al. 2001b). Metals not removed by biota or dispersed by water movement during the summer may become bound to the sediment where they are potentially bioavailable at a later stage through physical disturbance (such as ice scour, shipping activities or turbulent water mixing) (Lenihan 1992) and bioturbation (Ciutat and Boudou 2003). In the case of Brown Bay at Casey Station, East Antarctica, metal contaminants known to be highly toxic at elevated levels have been found in the marine sediments, indicating that dilution and dispersion of contaminated meltwaters from Thala Valley tip was not rapid enough to prevent accumulation in nearshore sediments (Snape et al. 2001b). The persistence of elevated metals in the benthic zone can also be enhanced by the presence of sea ice cover that significantly reduces wind driven mixing of the coastal waters for most of the year (Chapman and Riddle 2005a; King and Riddle 2001). With the combined influence of these dynamic processes, it is probable that biota within Brown Bay experience longer periods of exposure to these metal contaminants.

While longer exposure durations will allow accurate assessment of biological response to determine increasing sensitivities of Antarctic marine biota to contaminant impact, increasing acute bioassay times from the current "standard" 4 day bioassays to greater than 28 days could reduce the number of replicate bioassays which can be run during a short term, Antarctic field season. Field seasons are conducted during the spring / summer months from November

through to February when access to nearshore ecosystems for collecting test species is less restricted by weather and sea ice conditions. Combining extended bioassay exposure durations, short experimental seasons and reduced access to resources from logistical constraints, standard bioassay replication is rarely achieved. It is therefore extremely important that all data collected from one bioassay can be successfully exploited to derive better point estimates from more appropriate, Antarctic relevant, time endpoints. In order to address this, the new statistical GAMM model which incorporates the multiple time point observations into the point estimate determination was successfully developed (Appendix 1; Candy et al. in prep).

Comparison of Statistical Methods

Point estimates generated by the GAMM converged to similar values towards the end of the exposure period to those generated by Probit analysis for the two bioassays. This was not unexpected as GAMM uses a similar log-concentration approach to relate mortality response to contaminant exposure. However, GAMM models this as a trend in time and exploits all the interval-censored mortality data simultaneously rather than considering them as discrete and unrelated events across time. As a result, GAMM gains "strength" in point estimate generation by using all the time-series data obtained in the bioassays. Therefore, differences in point estimates for LC_x values particularly at earlier and midrange observation times are due to smoothing the time trends in mortality as a function of concentration using a single, cubic spline model over time. Diagnostic plots for lack of fit random effects for cadmium and copper showed no obvious systematic departures over time as a results of this simple, parsimonious model of time trends. There was an indication of a difference between the two bioassays for the case of cadmium and further bioassays would be needed to determine if this is due to unavoidable experimental variability or if one of these bioassays gave exceptional results.

In some instances such as cadmium in Bioassay 1, the GAMM generated LC_{50} estimates with 95% confidence limits that were wider than those from the Probit

analysis (Figure 3). This is due to the additional variance estimated for lack-of-fit random errors. Also, the time trend obtained by the GAMM is more robust to "noise" in the interval-censored mortality data; smoothing out these effects when estimating trend and attributing this noise to lack-of-fit random error. By smoothing through the interval data, GAMM can give more general and interpretable estimates of average responses of a population to contaminant exposure.

Even though the point estimates for the two methods were similar, GAMM has significant advantages over Probit analyses that merit its use. Probit analyses can only generate point estimates at times when observations physically took place unless a further analysis to fit a smooth trend through the endpoint-specific point estimates is undertaken. As part of a single, comprehensive analysis, GAMM allows generation of point estimates for any day within a bioassay regardless of when the physical observations were made.

Conclusion

Longer duration exposures resulted in increasing sensitivity in *O. pinguides* to metal contamination. The GAMM model produced similar LC_x estimates to Probit analysis but was able to model mortality trends more accurately through time by exploiting all the time-series data simultaneously. Increased accuracy in metals sensitivity analyses can therefore be achieved by using longer duration exposures in contaminant bioassays and estimates generated from GAMM.

Using longer exposure durations for contaminant bioassays with Antarctic species, and analysing the time-series data with GAMM, will provide increased generality of results and increased accuracy of uncertainty in estimates for metal sensitivity analyses. It is envisaged that this new bioassay procedure and analytical approach, including the ability to model survival when concentrations

vary over time using the CCE-based GAMM, will allow for more accurate development of Antarctic specific water quality guidelines.

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Chapter 5

General Discussion

The purpose of this study was to assess the sensitivity of a range of Antarctic marine species to anthropogenic metal contaminants. This was done both to provide robust routine procedures for toxicity tests using appropriate statistical methods to be used as monitoring components of risk assessments in the future, and to provide data to inform the development of site specific Environmental Guidelines for Antarctica. Tests with longer exposure durations than those used in routine toxicity tests with analogous temperate species were developed for three Antarctic marine species; the prymnesiophyte phytoplankton *Phaeocystis antarctica,* the littoral gammarid amphipod, *Paramoera walkeri*, and the benthic lysianassid amphipod *Orchomenella pinguides*. A new statistical model (Appendix 1; Candy et al. in prep) which incorporates time into the survival analysis was developed in collaboration with Steven Candy (Australian Antarctic Division) and applied successfully to the lethal response data of *O. pinguides*. Contaminant sensitivities to key metals of concern in Antarctic marine ecosystems were determined for each species, and comparisons with temperate and tropical species sensitivity data were made where possible.

Establishing water quality guidelines requires determining contaminant sensitivities of local biota through toxicity testing. The need for site specific environmental guidelines for Antarctica has been well documented (Chapman and Riddle 2003, 2005a, 2005b; King and Riddle 2001; Snape et al. 2001; Snape et al. 2003; Stark et al. 2003, 2006). Such guidelines are required for the development, implementation and monitoring of remediation activities of contaminated sites and to inform future planning of human activities in Antarctica.

Test methodology development

Longer exposure duration

Few contaminant bioassay studies have been conducted using Antarctic marine species, with most applying standardised methods developed for temperate and tropical organisms (Chapman and Riddle 2005b). Importantly, longer-term exposures are needed to assess the delayed responses of Antarctic biota to metal contaminant exposure (Chapman and Riddle 2005a,b; King and Riddle 2001). The low stable temperatures of Antarctic waters have produced ecophysiological traits such as low metabolic rates that can impact the response time of these organisms to metal contamination (Peck 2002). Metal toxicity occurs in an organism when metabolically active forms are taken up in concentrations exceeding those which can be removed through detoxification or excretion (Marsden and Rainbow 2004), allowing the enhanced production of free oxidative radicals to damage lipids (Buettner 1993), proteins and DNA (Kawanishi et al 1989; Stoiber et al 2010) as well as directly affect neurological function (Brown et al 2004). Due to the reduced metabolic rates of Antarctic ectotherms, their uptake rate kinetics would also be reduced, meaning metal accumulation would happen at a slower rate than biota from temperate or tropical regions (Chapman and Riddle 2005b). However, when exposed to high concentrations of metals in contaminated environments, their capacity to effectively and rapidly detoxify metabolically active metals could also be reduced, and therefore their sensitivity to metal toxicity could be much greater than species from non-polar areas (Chapman and Riddle 2005a,b).

The work presented in this thesis extends beyond previous limited research by developing new longer-term bioassays with three Antarctic marine species. Routine toxicity tests were successfully developed for the amphipods *O. pinguides* and *P. walkeri* and for the phytoplankton *P. antarctica* to assess sensitivity to specific metals. Toxicity tests were also developed for the marine microgastropod *Skenella paludionoides,* the benthic amphipod *Tryphosella*

murrayi and juveniles of the heart urchin *Abatus shackletoni*. Due to limited access to individuals of these three species for testing, sufficient test replication was not achieved but the results of the tests are presented in Appendices 2 - 4. All six species were found to have delayed responses to metal exposure compared with species from warmer regions at lower latitudes, which reinforces the importance of using longer exposure durations for Antarctic toxicity tests. These results also corroborate findings from previous toxicity studies where Antarctic marine species displayed longer acute response times to contaminant exposure (Duquesne et al. 2000; King and Riddle 2001; Liess et al. 2001). Average point estimates determined for metal contaminant exposure for all species tested are presented in Table 1.

Table 1: Average point estimates (µg/L) for growth inhibition concentrations (IC_x) and lethal concentrations (LCx) of metal contaminant exposure determined for all six species tested in this study. Estimates are given for the minimum time endpoints where concentrations could be accurately determined by regression analysis and Maximum Likelihood Probit analysis.

			µg/L	
Species	**Metal**	**Day**	**IC / LC_{10}**	**IC / LC_{50}**
Phaeocystis antarctica	Copper	7.5	1.92	3.78
Paramoera walkeri juvenile	Copper	4	295	166
		7	337	325
Paramoera walkeri adult	Copper	4	551	-
		7	414	-
		14	98	343
	Cadmium	14	200	346
	Zinc	21	185	1005
	Lead	21	229	-
	Nickel	21	232	-
Orchomenella pinguides	Copper	10	197	441
		14	98	218
	Cadmium	14	305	896
		21	123	362
	Lead	21	97	596
Skenella paludionoides	Copper	21	93	231
	Zinc	21	824	1217
	Cadmium	28	982	1091
Abatus shackeltoni	Copper	14	601	170
Tryphosella murrayi	Copper	14	243	432
		21	162	238
		28	96	160

Microalgal growth inhibition bioassay

Microalgae have been successfully developed as sensitive biomonitors of water quality in temperate and tropical regions with contaminant point estimate information used to develop trigger values for water quality guidelines (Franklin et al. 2005; Johnson et al. 2007; Stauber and Davies 2000). This study was the first to successfully develop a longer-term microalgal growth inhibition bioassay using flow cytometry with an Antarctic phytoplankton species. The *P. antarctica* growth inhibition bioassay provides a sensitive endpoint for determining contaminant point estimates and evaluating anthropogenic contaminant impacts in Antarctic marine ecosystems. *Phaeocystis antarctica* showed such high sensitivity to copper exposure that metal concentration analytical detection limits were surpassed. Improving analytical detection limits will improve point estimate determination for copper exposure to *P. antarctica* and provide sensitive toxicity indices for contaminant trigger value derivation. With slight modifications to solution homogenisation methods and inoculum preparation, other species screened during the experiment (*Nitzschia* sp*., C. closterium* and *G. cryophila*) could also be developed for determining contaminant point estimates. Further development of *Nitzschia sp,* and *C. closterium* would be beneficial as temperate forms of this species have already been developed and used in routine water quality monitoring programs (Stauber et al*.* 1994). This would provide a basis for sensitivity comparison between polar and temperate species and aid in adapting trigger values from temperate marine ecosystems to Antarctic specific guidelines.

Sensitive behavioural endpoints

While mortality is commonly used as an endpoint in toxicity assessments, disturbances to the environment and inhabiting biota occur at far lower concentrations than those required to cause death (Mills et al. 2006). Behavioural endpoints are more sensitive than mortality as they integrate the

contaminant impacts on complex biochemical and physiological processes that enable an individual to function normally within its ecosystem (Mills et al. 2006).

This study is the first to report behavioural endpoints with Antarctic marine amphipod species for toxicity testing. Behavioural changes in the amphipod *P. walkeri* occurred at lower concentrations and could be evaluated over a shorter period of time relative to mortality. Behavioural endpoints were investigated with *O. pinguides*, however, they were not reported in the manuscript due to the focus of the paper and have been included into the Appendix 5. Behavioural endpoints were also developed and assessed for the black microgastropod *Skenella paludionoides* (Appendix 2), the juvenile heart urchin *A. shackletoni* (Appendix 3), and the large yellow amphipod, *T. murrayi* (Appendix 4). With each species, behaviour was a more sensitive indicator of metal contaminant impact than mortality.

Numerous behavioural categories were assigned to *P. walkeri* individuals describing specific characteristics related to movement, activity, and ventilation. However, the most robust category observed in this study was normal, active behaviour as departures from this active state to inactivity indicated the first physiological effects of contaminant exposure. Changes in behaviours relating to mobility and activity have also been assessed as sensitive sub-lethal endpoints with species from lower latitudes. Sediment burrowing time and re-emergence (Scarlett et al. 2007), food foraging and spatial mobility (Busdosh 1981), as well as ventilation rate of pleopods (Vellinger et al. 2012), have been identified as more sensitive indicators of contaminant impacts on amphipods compared to lethality. Ventilation rate in *P. walkeri* was investigated in this study however, as in previous amphipod ventilation rate investigations using *Gammarus duebeni* (Lawrence and Poulter 1998) the hyper or hypoventilation response pattern of *P. walkeri*, partly as a function of extended duration of exposure, was too complex to quantify. Behavioural endpoints relating to changes in mobility, such as the change from active behaviour to inactivity described for *P. walkeri* are important in ecotoxicology studies as they are more sensitive, they are observed earlier,

and are as simple to evaluate as lethality (Vellinger et al. 2012). The behavioural endpoint of normal activity established for *P. walkeri* is an ecologically relevant endpoint as this behaviour impacts the amphipods' ability to evade predators, to forage for food and to engage in reproduction (Mills et al. 2006).

Use of sensitive early life history stages

Early life history stages of marine invertebrates are highly sensitive to contaminant exposure (Ahsanullah and Florence 1984; Gopalakrishnan et al. 2008). Due to their higher sensitivity, early developmental and juvenile stages of biota can reveal effects of contaminant exposure more quickly than adults. As early life history stages are also physically smaller than adult stages, the space and test solution volume required for tests is reduced. One of many benefits of using early life history and juvenile stages is increasing the number of replicate invertebrate tests that can be conducted over the short summer Antarctic season when test individuals are accessible in the field. Toxicity tests conducted in this study show juveniles of *P. walkeri* to be more sensitive to copper than adult amphipods, as has been shown in several amphipod test species from lower latitudes (King et al. 2006; Ahsanullah and Florence 1984). A previous study by King and Riddle (2001) investigated metal toxicity to sea urchin larvae and found the developing *Sterechinus neumayeri* larvae to be highly sensitive to copper and cadmium exposure. The behavioural and lethal responses to copper exposure on juvenile heart urchins, *A. shackletoni*, were also investigated in this study, with a significant behavioural response occurring within two days indicating a high sensitivity to copper (Appendix 3). Finally, the degree of deleterious response to contaminant exposure may not necessarily follow a gradation correlated to life stage. Green et al. (1986) found that embryo and adult isopods of *Asellus aquaticus* were significantly less sensitive to cadmium exposure than newly released juveniles.

Based on these findings, further toxicity testing with early life history stages should be undertaken with Antarctic marine species to increase the suite of

more sensitive point estimates for water quality guideline derivation. In particular, the sensitivity of various life stages should also be compared for each individual species to ensure the most sensitive stage is utilised for testing.

Interactive effects of climate change on contaminant impacts

Environmental parameters outside those normally experiences, can result in additional stress to an organism exposed to a contaminant, which can increase the sensitivity of an organism's response (Vellinger et al. 2012). With climate change resulting in measurable changes to environmental parameters in the Antarctic such as increased seawater temperature, reduced sea ice cover / increased solar radiation (Meredith and King 2005) and increased freshwater run-off (Vaughan 2006), it is important to evaluate the possible interactive effects of these stressors with contaminant exposure on Antarctic marine species. The combined effects of UV-B radiation and metal contaminant exposure were investigated in a previous study and shown to produce a more sensitive response in *P. walkeri* exposed to copper and cadmium (Liess et al. 2001). As Antarctic marine invertebrates have been shown to be sensitive to even slight increases in temperature (1-2 °C) (Peck et al. 2004) and temperature influences metal solubility and therefore bioavailability (Vellinger et al. 2012), the effects of a slightly increased temperature to cadmium and copper toxicity was explored in this study and was shown to increase the sensitivity of mortality responses in *P. walkeri*. As this is the first reported study to evaluate temperature and contaminant effects on Antarctic marine species, no direct comparisons can be made. However, *P. walkeri* observations agree with findings from similar studies on species from lower latitudes exposed to contaminants and higher than normal temperatures (Vellinger et al. 2012). In addition to mortality, strong correlations have been identified between the physiological and biochemical response to contaminants and higher temperature exposure. Reduced oxygen consumption rate and glycogen concentrations, increased

metallothionein induction, and higher bioaccumulation rates were observed in the marine dogwhelk, *Nucella lapillus* exposed to cadmium at a 10 °C treatment compared to 5 °C treatment (Leung et al. 2000). Behaviour and physiological changes of the freshwater gammarid amphipod *Gammarus pulex* were also exacerbated when high temperatures were combined with cadmium exposure (Vellinger et al. 2012). High temperature and cadmium exposure increased mortality and standard metabolic rates in the eastern oyster *Crassostrea virginica* compared to no significant changes in condition or survival rate observed for oysters exposed to higher temperature only (Lannig et al. 2006).

If Antarctic marine waters continue to increase in temperature, marine invertebrate species like *P. walkeri* could be more susceptible to long-term impacts from localised metal contamination. Development and sexual maturation times for *P. walkeri*, like other Antarctic species, are significantly longer than temperate and tropical species (Sagar 1980). The fitness of a population could therefore be significantly affected by exposure to contaminants as their ability to recover has already been impaired by the elevated temperature. Understanding the increased sensitivity levels of Antarctic marine biota to contaminants in the presence of additional environmental stressors such as elevated temperatures is therefore essential to provide future protection of species in the climate changing environment.

Improved statistical methods for small data sets incorporating responses through time

The development of the new statistical approach using a Generalized Additive Mixed Model (GAMM) has improved our ability to accurately estimate the toxicity of contaminants on Antarctic species and in addition, allowed more accurate quantification of the uncertainty in these estimates. Current methods such as Maximum Likelihood Regression using the Probit transform (Probit analysis) have been criticised for their inability to measure cumulative effects of

contaminant exposure through time (Bedaux and Kooijman 1994; Jager et al. 2006). As longer exposure durations (e.g. > 21 d) have been used in this research, it is essential that the pattern of toxicity response to cumulative exposure can be examined. The GAMM can take the response data from multiple time points and incorporate either time-constant or time-varying concentrations for the contaminants to determine point estimates for any binomial endpoint (e.g. effective or lethal concentrations). These estimates can be obtained not only for the days of observation, but also be calculated for each day of the bioassay. Through the simultaneous exploitation of all the time point response data the GAMM can fit a data-driven function and smooth through potentially noisy time interval response data to provide more robust predictions of general contaminant responses for that species. Due to the general modelling framework that the GAMM provides, it can analyse combined data sets from both multiple and individual bioassays and, at the same time, investigate the effect of the individual bioassays as a fixed or random factor. These features maximise the information from the response data generated which is particularly important with Antarctic species as standard bioassay replication is rarely achieved due to logistical constraints that frequently results in small data sets.

As shown in this study, GAMM can predict similar point estimates to Probit analyses as they share a similar concentration-response relationship, but overall, GAMM is a sophisticated improvement to Probit. This statistical model not only has future application potential within Antarctic ecotoxicology studies but could also be applied to Arctic studies and to current tropical and temperate standard methods to improve point estimation by assessing the cumulative effects of exposure to a contaminant through time.

Conclusions

This study has shown that longer test durations are required for determining lethal and sub-lethal impacts of metal contaminant exposure on Antarctic marine macroinvertebrates and microalgae. Current testing methods standardised for temperate and tropical species are therefore not applicable to polar species, which have lower metabolic rates, slower development, are longer lived, and respond more slowly to contaminant exposure. Longer exposure durations were required for the microalgal growth inhibition bioassay exposing *P. antarctica* to copper than bioassay durations (48 – 72 hr) normally applied to temperate and tropical species. *Phaeocystis antarctica* is highly sensitive to copper exposure and more sensitive than lower latitude species that have previously been developed as contaminant biomonitors. This encourages the continued development of *P. antarctica* as a contaminant assessment tool for the Antarctic marine environment. Response times to different metals varied for each invertebrate species and between species. *Paramoera walkeri*, *O. pinguides* and *Skenella* sp. all responded faster to copper exposure than to nickel or lead. This suggests that toxicity test durations may not be able to be standardised across all contaminants or even particular orders of organisms, and long exposures (i.e. 14 - 21 d) incorporating observations at regular intervals will need to be considered for future experiments with Antarctic biota.

The behavioural activity endpoint developed in this study has shown to be a quicker and more sensitive endpoint for *P. walkeri* adults than survival when evaluating impacts of metal contaminants. As longer bioassay exposure periods have been established as necessary for determining lethal point estimates with Antarctic marine biota, being able to utilise a quicker and more sensitive behavioural endpoint would shorten the test duration and increase the number of tests that could be conducted within a short summer field season.

This study found juvenile *P. walkeri* amphipods were more sensitive to copper exposure than the adults. This finding is similar to that exhibited in species from

lower latitudes and provides a faster and more sensitive life history stage for evaluating contaminant impacts in Antarctic marine environments. This developmental stage is also potentially more vulnerable to contaminant exposure from terrestrial sources, as juvenile *P. walkeri* are released from the marsupium at the beginning of summer, coinciding with summer meltwater inputs of contaminants into the nearshore zone.

A slight elevation in water temperature increased the toxicity impact of copper and cadmium to *P. walkeri* adults. This finding indicates a deleterious synergistic effect of temperature and metal exposure with Antarctic marine biota. If climate change effects continue to warm the nearshore marine environments, Antarctic species inhabiting these ecosystems are potentially more vulnerable to contamination impacts through temperature enhanced, metal toxicity.

The new statistical method developed by Steven Candy has provided a more sophisticated method for analysing the binary toxicological data collected during this study. Applying the GAMM to the *O. pinguides* data set showed similar results to the Probit model and maximum likelihood estimates. The GAMM model has a number of benefits for point estimation over the typical Probit model approach as it can incorporate all the time interval-censored data into one integrated analysis as well as utilising time-varying concentrations in the analysis whilst performing internal checks to assess the fit of the chosen model. GAMM is also able to determine point estimates for every day within the exposure period, which is particularly useful with the long exposure durations required for tests with Antarctic species and for the future development of biological monitoring tools.

Recommendations for future research

The successful development of the longer-term bioassays with *P. walkeri, O. pinguides* and *P. antarctica* have provided a basis on which to continue

contaminant sensitivity testing with additional toxicants and climate change variables. Incorporating the faster, more sensitive behavioural activity endpoint developed for *P. walkeri* will allow more tests to be conducted within the short summer field season. A similar behavioural endpoint developed for *O. pinguides* (Appendix 5) also provided a more sensitive and faster characteristic for evaluating contaminant responses and should be incorporated into future testing to increase the number of test species and tests able to be completed during a short summer field season. Initial results from the pilot studies for *S. paludionoides, T. murrayi* and *A. shackletoni* indicate the methodologies developed are sound and these tests could be repeated to provide more comprehensive information for determining point estimates with these species. Behavioural endpoints were also investigated with these species, showing more sensitive responses to the metal contaminants. Repeat testing will validate their selection as an appropriate endpoint for observation.

Applying the new GAMM statistical model to the new binary data generated by future testing using the developed bioassay methodologies developed in this study will allow for further validation of the benefits of GAMM over traditional statistical methods. GAMM can also be applied to binary toxicological data from other regions such as the Arctic where species also display a slower response rate to contaminants exposure than lower latitude species that also produce data sets with multiple time point observations and time-varying concentrations.

Point estimates generated in this study contribute to the data necessary for producing species sensitivity distributions that will aid in the development of contaminant trigger values and subsequent water quality guidelines for the Antarctic marine environment. These water quality guidelines will be used in risk assessments for planning activities in Antarctic environments, identifying sites that are already contaminated and setting remediation targets for the clean-up of contaminated sites. With some minor modifications to the methodologies developed to improve control mortality with *P. walkeri* juveniles and sensitivity of metal concentration analyses for microalgal testing, these species investigated

could be utilised as biological tools for monitoring the remediation activities during environmental clean-up programs and determining the progress and success of the remediation effort. This research will help provide for the improved management of human activities in the Antarctic region and increase protection of the unique Antarctic marine species and their ecosystems.

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Glossary

Acronyms

AAD	Australian Antarctic Division
ANZECC	Australian and New Zealand Environment and Conservation Council
ARMCANZ	Agriculture and Resource Management Council of Australia and New Zealand
DO	Dissolved oxygen
ICP-OES	Inductively coupled plasma optical emission spectrometry
LOEC	Lowest observed effect concentration
NEC	No effect concentration
NOEC	No observable effect concentration
ROS	Reactive Oxygen Species
USEPA	United States Environmental Protection Agency
WQG	Water Quality Guideline

Measurement units

M	Molarity = number of moles divided by volume
mL	millilitre
µL	micro litre
mg /L	milligram per litre

µg /L	microgram per litre
µm	micron / micrometer
ppt	parts per thousand
v/v	volume per volume
µmol. photons/m^2/s	a measurement for light called the Photosynthetic Photon Flux

Terminology

Abiotic	The non-living components of a system
Acclimation	Short-term adaptation of individual organisms to specific environmental conditions
Acute toxicity	Rapid adverse effect (e.g. death) caused by a substance in a living organism. Can be used to define either the exposure or the response to and exposure (effect)
Adsorption	The taking up of one substance at the surface of another
Anthropogenic	Produced or caused by humans
Benthic	Referring to organisms living in or on the sediments of aquatic habitats (lakes, rivers, ponds, etc.)
Benthos	The sum total of organisms living in, or on, the sediments of aquatic habitats
Binding sites	Sites on a substrate where chemical interaction with an indicator (qv) may occur. The interaction may be electrostatic, polar, hydrogen bonding or covalent bonding.

Bioaccumulation	General term describing a process by which chemical substances are accumulated by aquatic organisms from water, either directly or through consumption of food containing the chemicals
Bioassay	A test that exposes living organisms to several levels of a substance that is under investigation, and evaluates the organisms' responses
Bioavailable	The fraction of the total of a chemical in the surrounding environment that can be taken up by organisms. The environment may include water, sediment, soil, suspended particles, and food items.
Biota	The sum total of the living organisms of any designated area
Bioturbation	The physical disturbance of sediments by burrowing and other activities of organisms
°C	Degrees Celsius
Chelate	The type of co-ordination compound in which a central metal ion is attached by co-ordinate links to two or more non-metal atoms in the same molecule, called ligands
Chronic	Lingering or continuing for a long time; often for periods from several weeks to years. Can be used to define either the exposure of an aquatic species or its response to an exposure (effect). Chronic exposure typically includes a biological response of relatively slow progress and long continuance, often affecting a life stage.

Complexation	The formation of a compound by the union of a metal ion with a non-metallic ion or molecule called a ligand or complexing agent
Concentration	The quantifiable amount of chemical in, say, water, food or sediment
Contaminant	Biological (e.g. bacterial and viral pathogens) and chemical (see Toxicants) introductions capable of producing an adverse response (effect) in a biological system, seriously injuring structure or function or producing death
Control	That part of an experimental procedure which is like the treated part in every respect except that it is not subjected to the test conditions. The control is used as a standard of comparison, to check that the outcome of the experiment is a reflection of the test conditions and not of some unknown factor
Cumulative	Resulting from successive additions at different times or in different ways
Depuration	Process that uses a controlled aquatic environment to reduce the level of pathogenic organisms that may be present in live shellfish
Detection limit	The smallest concentration or amount of a substance that can be reported as present with a specified degree of certainty by definite complete analytical procedures
Detoxify	To remove a metabolically active, toxic substance through binding to a metabolic protein or sequestered in a solid granule form

Dinoflagellates	Major class of marine algae that move by flagella. They are often red in colour, and can produce strong toxins that can kill many fish and other marine organisms
Dose	The quantifiable amount of a material introduced into an animal
Early life-stage test	28-day to 32-day exposures (60-day post-hatch for salmonids) of the early life stages of a species of fish from shortly after fertilisation through embryonic, larval and early juvenile development. Data are obtained on survival and growth.
EC_{50}	Median effective concentration - The concentration of material in water that is estimated to be effective in producing some lethal response in 50% of the test organisms. The LC50 is usually expressed as a time-dependent value (e.g. 24-hour or 96-hour LC50).
End-points	Measured attainment response, typically applied to ecotoxicity or management goals
Endemic, endemism	Confined in occurrence to a local region
Guideline trigger values	These are the concentrations (or loads) of the key performance indicators measured for the ecosystem, below which there exists a low risk that adverse biological (ecological) effects will occur. They indicate a risk of impact if exceeded and should 'trigger' some action, either further ecosystem specific investigations or implementation of management/remedial actions.
Guideline	Numerical concentration limit or narrative statement

(water quality)	recommended to support and maintain a designated water use
Hepatopancreas	Digestive gland or midgut gland of arthropods, molluscs and fish
LC50	Median lethal concentration - The concentration of material in water that is estimated to be lethal to 50% of the test organisms. The LC50 is usually expressed as a time-dependent value, e.g. 24-hour or 96-hour LC50, the concentration estimated to be lethal to 50% of the test organisms after 24 or 96 hours of exposure.
Leachate	Water that has passed through a soil and that contains soluble material removed from that soil
Ligand	A molecule, ion or atom that is attached to the central atom of a co-ordination compound, a chelate or other complex. May also be called complexing agent.
LOEC	Lowest observed effect concentration - The lowest concentration of a material used in a toxicity test that has a statistically significant adverse effect on the exposed population of test organisms as compared with the controls.
Metallothionein	Metallothionein (MTs) are non-enzymatic proteins with a low molecular weight, high cysteine content, no aromatic amino acids and heat stability
Median	Middle value in a sequence of numbers
Neurotoxin	Toxic substances which adversely affect the nervous system

NOEC	No observed effect concentration - The highest concentration of a toxicant at which no statistically significant effect is observable, compared to the controls; the statistical significance is measured at the 95% confidence level
Osmolyte	Compounds affecting osmosis which are soluble in the solution within a cell, or in the surrounding fluid and aid in cell volume maintenance
pH	Value that represents the acidity or alkalinity of an aqueous solution. It is defined as the negative logarithm of the hydrogen ion concentration of the solution.
Pollution	The introduction of unwanted components into waters, air or soil, usually as result of human activity; e.g. hot water in rivers, sewage in the sea, oil on land
Reactive Oxygen Species	Chemically reactive molecules containing oxygen.
Speciation	The intimate chemical environment of the indicator (qv), that is the compound or ion of which it forms a part
Standard	In regards to water quality - an objective that is recognised in enforceable environmental control laws of a level of government
Tolerance	The ability of an organism to withstand adverse or other environmental conditions for an indefinitely long exposure without dying
Toxicant	A chemical capable of producing an adverse response (effect) in a biological system at concentrations that might be encountered in the environment, seriously injuring

	structure or function or producing death. Examples include pesticides, heavy metals and biotoxins (i.e. domoic acid, ciguatoxin and saxitoxins).
Toxicity test	The means by which the toxicity of a chemical or other test material is determined. A toxicity test is used to measure the degree of response produced by exposure to a specific level of stimulus (or concentration of chemical).
Trigger values	These are the concentrations (or loads) of the key performance indicators measured for the ecosystem, below which there exists a low risk that adverse biological (ecological) effects will occur. They indicate a risk of impact if exceeded and should 'trigger' some action, either further ecosystem specific investigations or implementation of management/remedial actions.
Uptake	A process by which materials are absorbed and incorporated into a living organism

Appendix 1

Modelling interval-censored survival times and estimation of lethal concentrations in bioassays using Generalized Additive Mixed Models

This manuscript is presented as prepared for publication

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Abstract

In bioassays where survival is measured for a range of concentrations of toxicants such as metals, emphasis is on a fixed exposure period and typically Probit analysis is carried out using an assumed normal tolerance distribution on the log-concentration scale. In survival studies involving interval-censored survival times, the log of the hazard function is typically modelled as a parametric or non-parametric function of time interval mid-points or start-points, respectively. For bioassay studies which observe cumulative mortality over consecutive periods of exposure, separate Probit analyses and calculation of lethal concentrations (LC) can be carried out for survival at the end of each exposure period (i.e. time interval end-point). This method is compared to combining survival time and bioassay approaches where the log-hazard function is modelled as a linear function of log-concentration to which is added a parametric function of time that is modelled by a smoothing spline. Assuming periodic mortality as binomially distributed conditional on the number of individuals surviving at the start of each interval, this model is fitted as a Generalized Additive Mixed Model (GAMM) using an approximation of the log of the integral of the hazard function. Random effects are defined by the interaction of concentration level with time intervals by defining each as discrete-level factors. Generalisation of this model to multiple bioassays is given and diagnostics for testing the validity of assuming the effects of bioassay and its interaction with concentration level and time interval are random are given. Numerical methods are used to obtain LC values from the fitted GAMM since

analytical expressions are not easily available. A simple empirical approach to allowing for time-varying concentrations using a Calibrated Cumulative Exposure scale is described which can also be used as an alternative model for mortality response to a range of concentrations.

The GAMM approach is demonstrated and its advantages over multiple Probit analyses are examined using data from two replicate bioassays of the effect of each of copper and cadmium exposure on survival of an Antarctic amphipod, *Orchomenella pinguides*, observed in 3-d intervals up to 30 d.

Keywords

metal toxicity - survival time - periodic mortality - generalized additive mixed model - Antarctic amphipod *Orchomenella pinguides*

Introduction

In ecotoxicology experiments (i.e. bioassays) where survival is measured for a range of concentrations of toxicants such as metals, classical techniques place emphasis on a fixed exposure period and typically Probit analysis is carried out using an assumed normal tolerance distribution on the log-concentration scale (Finney, 1971). This approach treats each time point independently, and therefore does not attempt to model the underlying processes in time. However, Heckman et al. (2010) emphasise the importance of time as a factor in stress ecology and ecotoxicology given that organisms are dynamic systems with life history characteristics that express underlying processes in time.

Bedaux and Kooijman (1994) and Kooijman and Bedaux (1996) developed the DEBtox model of survival in bioassays that combine dose-response and time-response using a proportional hazards function (Cox and Oakes 1984) where this function models the instantaneous mortality rate as proportional to the concentration of a chemical in the animal as far as it exceeds a, so-called, no-effect concentration. DEBtox uses a simple one-compartment kinetics model to parameterise the model in terms of environmental concentration since the concentration of the chemical within the animal is not directly observed. The log-hazard is nonlinear in the parameters defined as the no-effect concentration (NEC) and the elimination rate, while additive for the log of the killing rate parameter with dimension $(\text{concentration} \times \text{rate})^{-1}$. Although these process rate parameters are interpretable from theory (Jager et al. 2006), Bedaux and Kooijman (1994) note that the nonlinear form of the model can be difficult to estimate being sensitive to the starting values of parameters with good starting values not easily determined. Even though this model is relatively sophisticated in terms of dynamic processes for concentration levels within the animal compared to the approach introduced below, the shape of the time component of the hazard function is a simple logistic model with single parameter, the elimination rate, and has an upper limit with time of one. Bedaux and Kooijman (1994) used maximum likelihood defined from a product multinomial distribution for the interval-censored survival times quantified as counts of deaths for each consecutive observation period.

Our approach also uses the proportion hazards model, does not use process parameters that have to appear nonlinearly in the log-hazard function, and models the shape with time using a flexible cubic smoothing spline model. Assuming periodic mortality as binomially distributed conditional on the number surviving at the start of each interval, this model is fitted as a Generalized Additive Model (GAM) (Wood, 2006) using an approximation of the integral of the hazard function using the mid-point rule which implies a complementary log-log link function. No starting values for parameters are required to carry out estimation using standard, freely-available software that can fit GAMs.

This approach of approximating the integral of the log-linear model of the hazard function, which is a parametric function of time, using the mid-point rule has been used previously for fish exposed to a range of zinc concentrations (Candy, 1986), for tree survival (Candy, 1989, 1997a) , and for leaf survival (Egli and Schmid, 2001). The parametric function of time was either linear or quadratic in the above applications but the method used in these applications of fitting a Generalized Linear Model (GLM) (McCullagh and Nelder, 1989) extends to higher degree polynomials or any parametric function that can be expressed as additive terms where parameters appear linearly (i.e. the linear predictor in a GLM). The Generalized Additive Model (GAM) (Wood, 2006) extension to the GLM introduced here extends this parametric function of time to an empirical smooth for the shape of the log-hazard function over time. Further, the extension to a mixed model (i.e. random effects) version of the GAM to give a Generalised

Additive Mixed Model (GAMM) (Wood, 2006) is investigated as a means of providing model fit diagnostics. Given assumptions about the randomness of lack-of-fit deviations (i.e. concentration x day random effects), it can provide more accurate estimates of the precision of parameter estimates than the GAM. The estimates of precision of model parameter estimates are then reflected in the precision of estimates of lethal concentration (LC). Generalisation of this model to multiple bioassays is given and diagnostics for testing the validity of assuming the effects of bioassay and its interaction with concentration level and time interval are random are given. Numerical methods are used to obtain LC values from the fitted GAMM since analytical expressions are not easily available. Given an initial fit of the model the shape of the log-hazard function over time it is shown how the accuracy of the mid-point rule approximation to the integral of the log-hazard function can be evaluated.

As in DEBtox we assume the concentration in the environment is kept constant over time for each concentration level in the experiment. However, a simple empirical approach to allowing for time-varying concentrations is given as an extension to the GAMM that assumes concentrations are fixed values over time. Péry et al. (2001) developed three alternative approaches to extending DEBtox to allow for elimination of the toxicant from the experimental environment. Two of the approaches involve process models which are not investigated here. Their third approach is more empirical and involves using the time-varying concentrations in DEBtox after smoothing their values using a cubic smoothing

spline but note that, in contrast to approach used here, this spline model does not “smooth” the dose-response relationship between survival and concentration.

The GAMM approach is demonstrated and advantages over multiple Probit analyses examined using data from two replicate bioassays of 40 animals each investigating the survival of an Antarctic amphipod, *Orchomenella pinguides*, for each of the metal contaminants of copper (Cu) and cadmium (Cd) observed in 3-d intervals up to 30 d. Details of the experimental setup and the complete set of bioassays with application of the methods described in this paper are given in Sfiligoj et al. (in prep).

Modelling dose-survivorship response

In Bedaux and Kooijman (1994) the key assumption is that the hazard function is proportional to concentration of the compound in the animal over time where the function form of the time decay is a simple exponential function of time and the elimination rate parameter. Here physiological parameters such as this rate parameter are not specified. Instead, the log hazard function is specified using an empirical smooth of the interval-censored survival times (i.e. specified as periodic mortality) using a Generalized Additive Model (GAM) (Wood, 2006). To fit this model the periodic mortalities are modelled as a set of continuation ratios (Fienberg, 1980) as follows.

The response variable is the number dying in period i for concentration level j (i.e. C_j) of n_{ij} given the number surviving at the start of period i, N_{ij} where period i is defined as starting at time t_{ij} and ending at time $t_{i+1,j}$ where $i = 1,\ldots,I_j$ and $j = 1,\ldots,J$. The length of time period i is $t_{i+1,j} - t_{ij}$ which in most bioassays will be a constant time period across observed (i.e. censoring) times and concentration levels but the method described here allows these periods to vary. The variable period length, $\Delta t_{ij} = t_{i+1,j} - t_{ij}$ and period mid-point $t'_{ij} = t_{ij} + \Delta t_{ij}/2$, are required to define the model. Given the number at the start of the bioassay is M_j then $N_{ij} = M_j - \sum_{i'=1}^{i-1} n_{i'j}$ for $i = 2,\ldots,I_j$ and $N_{1j} = M_j$.

Observed values of n_{ij} that are zero are included in the fit as long as the corresponding value of N_{ij} is greater than zero. Values of n_{ij} and N_{ij} where N_{ij} is zero are therefore excluded from the fit since they are not informative since the number of animals at risk is zero. As a result the number of observed periodic mortalities may be less for higher concentrations (i.e. I_j depends on j).

The probability, p_{ij}, defined as the expected value of n_{ij}/N_{ij} is defined by the model as

$$p_{ij} = 1 - exp\left[-exp\left\{ln\left(\Delta t_{ij}\right) + \beta_0 + \beta_1 ln\left(C_j\right) + s\left(t'_{ij}, k\right)\right\}\right] \quad (1)$$

where β_0 and β_1 are regression parameters to be estimated and $s\left(t'_{ij},k\right)$ is a smooth function of time with parameter k the dimension of the basis used to represent the smooth term [Wood, 2006, see also `choose.k` in `gam{mgcv}` help for the `mgcv` package in the R-software (R Development Core Team, 2006)]. Model (1) can be fitted as a Generalized Additive Model (GAM) (Wood, 2006) with a binomial distribution for n_{ij} conditional on the total N_{ij}, complementary log-log link function and "offset" of $ln\left(\Delta t_{ij}\right)$. If $s\left(t'_{ij},k\right)$ is replaced by a parametric function of time such as a quadratic, $s\left(t'_{ij}\right)=\beta_2 t'_{ij}+\beta_3 t'^2_{ij}$ or a simple monotonically increasing function of time such as $s\left(t'_{ij}\right)=\beta_2 ln\left(t'_{ij}\right)$, then Model (1) can be fitted as a Generalized Linear Model (GLM) (McCullagh and Nelder, 1989). Given the case where the same time points are observed for each concentration level, $t_{ij}=t_i$ then by specifying these time points as a discrete categorical variable (i.e. factor) and replacing the term $s\left(t'_{ij},k\right)$ by the set of dummy variables representing the time factor combined with corresponding parameters, excluding the first time point which is specified by β_0, this defines $s\left(t'_{ij}\right)$ as a step-function. This model corresponds to the time interval-censored form (Bartlett, 1978) of Cox's semi-parametric proportional hazards model (Cox and Oakes, 1984).

The relationship to the hazard function to model (1) (i.e. instantaneous mortality rate) function, $h(t)$ is defined as $h(t)=f(t)/S(t)$, where $f(t)$. is the

probability density function for the exact survival times, and $S(t)=1-F(t)$ where $F(t)=\int_0^t f(\tau)d\tau$ is the cumulative density function. Assuming a log-linear (i.e. proportional hazards) model for the hazard function then

$$h(t)=\lambda(t)exp\left(e^{\beta_0}C_j^{\beta_1}\right).$$

Since

$$S(t)=exp\left\{-e^{\beta_0}C_j^{\beta_1}\int_{-\infty}^{t}\lambda(\tau)d\tau\right\}$$

if

$$P_{ij}=1-\frac{S\left(t_{i+1,j}\right)}{S\left(t_{ij}\right)}=1-exp\left\{-e^{\beta_0}C_j^{\beta_1}\int_{t_{ij}}^{t_{i+1,j}}\lambda(\tau)d\tau\right\}$$

and $\lambda(t)=exp\{s(t)\}$, then p_{ij} from Equation (1) is an approximation to P_{ij} for general function $s(\tau)$ via the mid-point rule since

$\int_{t_{ij}}^{t_{i+1,j}} exp\{s(\tau)\}d\tau \cong \Delta t_{ij} exp\left\{s\left(t'_{ij}\right)\right\}$. Note that if $s(t)=\beta_2 ln(t)$ then exact survival times have a Weibull distribution with shape parameter β_2+1 (Aitken and Clayton, 1980). Also if $\beta_2=1$ then $\lambda(t)$ is a linear function in time and as a result the mid-point rule gives the exact value of the integral and p_{ij} is therefore exactly equal to P_{ij}.

Fixing the exposure period to a constant, then the cumulative mortality across increasing values of concentration, with values given by general C, is $1-S(C)=1-exp\{-e^{\beta_0}C_j^{\beta_1}\}$ which corresponds to a Weibull distribution with shape parameter β_1 for the tolerance distribution on the scale of C. The classical Probit analysis for log-concentration assumes a lognormal tolerance distribution on the C scale. Note that the lognormal and Weibull distributions can take very similar shapes for their probability density function and in fact share the same variance-to-mean relationship. Therefore, for a fixed exposure period the complementary log-log and probit links corresponding to Weibull and lognormal tolerance distributions should give very similar predictions for survival and LCs.

Returning consideration to the multiple-time points model, the smoothing spline term $s(t'_{ij},k)$ in Equation (1) allows a flexible shape for $log\{\lambda(t)\}$ within a given period. The approximation of P_{ij} by p_{ij} will be accurate enough for practical purposes as long as $\lambda(t)$ is approximately linear over the time range of each period with widths of Δt_{ij}, either due to $\lambda(t)$ being well approximated by $s(t)=ln(t)$ over the whole time range or, for general $\lambda(t)$, departure from linearity is relatively small within each period. Note that this flexible function $s(t)$ for the log of the hazard function means that calculation of the proportion surviving to a given time is not carried out using any standard parametric family

of survival-time distributions. However, the advantage of applying this approximation is that standard, freely available software can be used to fit model (1) such as the `gam` function in the `mgcv` package for the R-software whereas analytical or numerical integration of $\lambda(t)$ (other than by the mid-point rule) cannot be implemented using GAM or GLM fits using standard software. The estimation of parameters in the GAM is carried out using a penalised iteratively re-weighted least squares (P-IRLS) algorithm that maximises the penalised likelihood given the number of (k), and values of knot points, and the estimate of the smoothing parameter for each spline term. The estimate of the smoothing parameter for a GAM uses generalised cross validation (GCV) if the scale parameter of the dispersion model (McCullagh and Nelder, 1989) is unknown and a rescaled Akaike Information Criterion (AIC) when the scale parameter is known. In the case of binomial data the scale parameter is known and takes a value of 1. The theory is described in Wood (2006) and specific details for the use of the `mgcv` package are given in the package documentation so these will not be repeated here (see `gam` and `gamm` functions in R-help for `mgcv`). In most cases specification of knot points can be left to the `gam` and `gamm` functions to be automatically determined. Once an initial fit of model (1) is carried out the adequacy of the mid-point rule approximation can be evaluated as described later using the example datasets.

The use of standard software also allows model (1) to be extended to mixed effect versions described as Generalized Additive Mixed Models (GAMMs). The

mgcv library allows such models to be fitted as described below. The extension to model (1) given below allows model lack of fit to be evaluated and if appropriate incorporated as random effects to account for additional uncertainty compared to a binomial sampling distribution. Also the extension of combining multiple bioassays is described.

Creating a factor version of concentration level, $C_j^{(F)}$, and a factor version of time start-points, $t_i^{(F)}$, assuming $t_{ij} = t_i$ then the interaction of $C_j^{(F)}$ and $t_i^{(F)}$, specified as $C_j^{(F)} : t_i^{(F)}$ can be specified as a random effect term and added to the linear predictor of model (1) to give model (2)

$$p_{ij} = 1 - exp\left[-exp\left\{ ln\left(\Delta t_{ij}\right) + \beta_0 + \beta_1 ln\left(C_j\right) + s\left(t'_{ij}, k\right) + \nu_{ij} C_j^{(F)} : t_i^{(F)} \right\} \right] \quad (2)$$

where $\{\nu_{ij}\}$ is a set of random effects which are assumed independent, normally distributed with mean zero and common variance σ_ν^2. If the time points are not the same across concentration levels then a factor defining the unique combinations of $C_j^{(F)}$ and t_{ij} should replace $C_j^{(F)} : t_i^{(F)}$ in model (2). Model (2) can be fitted using the gamm function from mgcv.

The gamm function estimates model (2) by iterating between a gam fit and a linear mixed model (LMM) fit using the gammPQL function, where gam supplies a set of working regressor variables, that express the model as a LMM (i.e. linear

function of parameters and random effects), to `gamm` and `gammPQL` (which fits the LMM using `lme`) and returns the set of updated parameter estimates, random effects estimates, and an estimate of σ_ν. The linear and nonlinear components of the spline, $s\left(t'_{ij},k\right)$, are estimated as fixed and random effects, respectively (Wood, 2006). The iterative cycle is repeated until convergence. This approach has been described for Generalized Linear Mixed Models (GLMMs) as penalized quasi-likelihood (PQL) (Breslow and Clayton, 1993) and has good estimation properties in estimating σ_ν^2 as long as the binomial sample sizes, N_{ij} here, are reasonable, with PQL failing badly in the case of matched case control studies where the binomial sample size per treatment/covariate combination is always 1 (Breslow and Clayton, 1993). One advantage of PQL over other approaches such as marginal maximum likelihood that integrates over the distribution of the random effects, is that estimates of the $\{\nu_{ij}\}$ are obtained and these can be examined using data diagnostic methods to determine if the lack of fit they represent is random and of similar magnitude across concentration levels and time points or if certain combinations of these two factors give very different estimates of lack of fit compared to other combinations. This can give insight into potential problems with experimental techniques or alternatively that the model is itself inadequate. The property of the model that the $\{\nu_{ij}\}$ operate on the linear predictor scale means that the reduction in N_{ij} with time as survival decreases does not strongly influence the scale of $\{\nu_{ij}\}$.

If a number of separate bioassays have been carried out then model (2) can be extended in a number of alternative ways to model the combined dataset while examining the effect of Bioassay. In the example given later there were two bioassays so the factor $E_l^{(F)}$, $l = 1,\ldots,L,$ is defined as a two-level factor so that $L = 2$. One way to model the effect of Bioassay is to treat $E_l^{(F)}$ as a fixed-effect factor to give

$$p_{ijl} = 1 - exp\left[-exp\left\{ \begin{array}{l} ln\left(\Delta t_{ijl}\right) + \beta_0 + \alpha_{0l} E_l^{(F)} + \beta_1 ln\left(C_{jl}\right) + \\ \alpha_{1l} E_l^{(F)} ln\left(C_{jl}\right) + s\left(t'_{ijl} : E_l^{(F)}, k\right) + v_{ijl} C_{jl}^{(F)} : t_{il}^{(F)} \end{array} \right\} \right]. \quad (3)$$

Note that the random effects, $\{v_{ijl}\}$, are extended to include lack of fit across as well as within bioassays and that concentration levels and time points do not have to be the same across bioassays. Also the $\alpha_{0l}, l = 2,\ldots,L$, and $\alpha_{1l}, l = 2,\ldots,L$ are fixed parameters to be estimated, and the smoothing spline has a separate shape for each bioassay as specified by $s\left(t'_{ijl} : E_l^{(F)}, k\right)$. The dataset might not allow this level of complexity to be successfully estimated and some model reduction may be required starting from dropping the separate shape terms and thereby simplifying to the common shape spline term of $s\left(t'_{ijl}, k\right)$. A simple way to obtain lethal concentration estimates (as described later) by averaging across bioassays is to fit the $\alpha_{0l}, l = 1,\ldots,L$, and $\alpha_{1l}, l = 1,\ldots,L$ as two sets of random effect estimates with zero mean and

variances of σ_0^2 and σ_1^2, respectively, and covariance σ_{01}. Note that `gamm` does not have the facility to fit the term $s\left(t'_{ijl} : E_l^{(F)}, k\right)$ with $E_l^{(F)}$ specified as a random factor term. For prediction the random effects can either be integrated out of Equation (3), to give the posterior mean in a Bayesian interpretation, or alternatively set to zero, to give the posterior mode. The latter approach is adopted here since predictions in this case can be obtained using the `predict` function in R. Note that the additional uncertainty in fixed effect parameter estimates (including those involved in the spline term) due to the estimation of random effects is not removed by this approach.

If there are a sufficient number of different levels of concentration then a further generalisation is to replace the term $\beta_1 ln\left(C_{jl}\right)$ with a spline term, $s\left\{ln\left(C_{jl}\right), k'\right\}$, so that the assumption of linearity of response (i.e. on the linear predictor scale) with log of concentration level can be relaxed.

Péry et al. (2001) considered the case of time varying concentrations as an extension to the DEBtox model where they investigated three different approaches to account for the progressive elimination, or lowering of concentration, of a toxicant in the environment (i.e. in the experimental setup). Here a simple empirical approach is applied. If the time varying concentration, assuming a single bioassay for simplicity, is given by C_{ij}, then the cumulative exposure is given by $C_{ij}^{*}\left(\beta_1\right) = \sum_{i'=1}^{i} \Delta t_{i'j} C_{i'j}^{\beta_1}$, where β_1 is a parameter to be

estimated. Therefore the terms $\beta_1 ln\left(C_j\right)+s\left(t'_{ij},k\right)$ in Model (2) are replaced by $s\left(C'_{ij}\ (\beta_1),k\right)$ and the offset $ln\left(\Delta t_{ij}\right)$ is replaced by an offset of $ln\left\{\Delta C^*_{ij}(\beta_1)\right\}$ where $C'_{ij}\ (\beta_1)$ is the mid-point value of $C^*_{ij}(\beta_1)$ between values for time points indexed by i and $i+1$. This in effect replaces the mid-point approximation to the log of the integral of the hazard for a time scale of days to a time scale of cumulative exposure. Note that even if $C_{ij}=C_j$ (i.e. concentrations do not vary over time) $s\left(C'_{ij}\ (\beta_1),k\right)$ is not equivalent to $\beta_1 ln\left(C_j\right)+s\left(t'_{ij},k\right)$ since although there is no variation through time there is variation across concentration levels as indexed by j so that $s\left(C'_{ij}\ (\beta_1),k\right)$, for a given value of β_1, smooths across the product of $C_j^{\beta_1}$ and $t'_{ij}=\sum_{i'=1}^{i}\Delta t_{i'j}-\Delta t_{ij}/2$ and not just across t'_{ij}. So as well as accounting for time-varying concentrations this model based on a Calibrated Cumulative Exposure (CCE), $C'_{ij}\ \left(\hat{\beta}_1\right)$ is an alternative model to Model (2). The random effect terms are those described for Model (2) based on the C_j expressed as a factor even when actual concentrations vary across time so that $C_j=\sum_{i=1}^{I_j}C_{ij}/I_j$ (i.e. the average concentrations). Note also that estimation of β_1 no long fits neatly within the specification of the GAMM fitting algorithm. However, a simple profile estimate can be obtained by repeatedly fitting this model for a grid of fixed values for β_1 and using the "Proportion of Deviance Explained" (PDE) (i.e. the generalisation to non-Gaussian distributions in the general family of exponential distributions of the R^2 or "proportion of variance

explained") (McCullagh and Nelder, 1989) as the objective function to be maximised across the profiled values of β_1. Other potential objective functions are discussed later. Another issue that should be considered for this approach, apart from determining an appropriate objective function, is that the uncertainty in predictions of periodic mortality, and therefore survival and LCs, cannot take into account uncertainty due to the covariance between the profile estimate of β_1 and the other estimated parameters.

Returning to fixed concentrations over time and replicate bioassays, a reduced form of model (3) was found to be a useful model where Bioassay was treated as random given that there were only two bioassays in the study dataset with this model used for prediction of LCs

$$p_{ijl} = 1 - exp\left[-exp\left\{ln\left(\Delta t_{ijl}\right) + \beta_0 + \beta_1 ln\left(C_{jl}\right) + s\left(t'_{ij}, k\right) + \nu_{ijl} C_{jl}^{(F)} : t_{il}^{(F)}\right\}\right]. \quad (4)$$

For prediction purposes the term $\nu_{ijl} C_{jl}^{(F)} : t_{il}^{(F)}$ is set to zero so it only affects the $\hat{p}_{ijl}$ indirectly via the effect of this random term on the estimates of the parameters in the fixed effect terms and, as mentioned previously, this random effect term directly affects the predicted uncertainty in the estimates of $\hat{p}_{ijl}$.

Calculation of Lethal Concentrations

Since $\hat{p}_{ijl}$ only predicts periodic mortality, two steps are required to estimate LC values using the values of proportion surviving, $S_i(t)$, at any time point, t, that are required (i.e. the LCs will be a smooth function of time). A corresponding analytical expression to that used to obtain LC values from probit analysis carried out separately for each time period end-point cannot be obtained from models (1) to (4) for two reasons. Firstly, it is not possible to express the spline term in a mathematical expression that can be easily inverted to obtain LC values and, secondly, obtaining $S_i(t)$ requires recursive use of the equation for $\hat{p}_{ijl}$ as described next.

To estimate $S_{j'}(t_{i'})$ from a set of predicted values of $\hat{p}_{j'}(t_{i'})$ from for example model (2), as a general function of time and concentration level [i.e. replacing t'_{ij} with any set of sequential concentration levels, $C_{j'}$, indexed by $j' = 1,\ldots,J'$ combined with any set of sequential time points, $t_{i'}$, indexed by $i' = 1,\ldots,I'$ in the model for p_{ij}], where $\hat{p}_{j'}(t_{i'})$ is obtained from the `predict` function in the `mgcv` library, then $S_{j'}(t_{i'})$ is given by

$$S_{j'}(t_{i'}) = \hat{p}_{j'}(t_{i'}), \qquad i' = 1$$

$$= \left\{1 - \sum_{h=1}^{i'-1} S_{j'}(t_h)\right\} \hat{p}_{j'}(t_{i'}), \qquad i' = 2,\ldots,I'-1$$

$$= \left\{ 1 - \sum_{h=1}^{I'-1} S_{j'}(t_h) \right\}, \qquad i' = I'.$$

(5)

Therefore Equation (5) can be used to predict survival proportion, or surviving numbers as $M_j S_{j'}(t_{i'})$, for the concentration levels and time points used in the bioassay or for any other set of values within the corresponding time range (i.e. GAMMs are not recommended for predicting outside the range of the variable over which the smoothing is carried out).

To determine LC values, for example for survival of 0.9 (i.e. cumulative mortality of 0.1) giving the LC10 value, predicted values of $S_{j'}(t_{i'})$ can be obtained for a fine grid of values for log concentration between the minimum and maximum values used combined with a set of time points (e.g. daily) between some sensible minimum and the maximum exposure time observed in the experiment. The LC10 can be found by searching the corresponding grid of values of $S_{j'}(t_{i'})$ and locating the value of log concentration that gives $S_{j'}(t_{i'})$ closest to 0.9. The function `predict` also allows approximate standard errors on the linear predictor scale to be obtained. Therefore, approximate 95% confidence limits can be obtained for LC values by repeating the above procedure while replacing $\hat{p}_{j'}(t_{i'})$ with $1 - \exp\left\{-\exp\left[\hat{\eta}_{j'}(t_{i'}) + 2SE_{\hat{\eta}}\right]\right\}$ for the upper and $1 - \exp\left\{-\exp\left[\hat{\eta}_{j'}(t_{i'}) - 2SE_{\hat{\eta}}\right]\right\}$ for the lower limit, respectively, where $SE_{\hat{\eta}}$ is the

standard error on the linear predictor scale and the linear predictor is given by $\hat{\eta}_{j'}(t_{i'}) = log\left\{-log\left[1-\hat{p}_{j'}(t_{i'})\right]\right\}$. This procedure can be applied for any required LC value.

Example of survival of an Antarctic amphipod under Cadmium and Copper exposure of 30 d

The GAMM approach is demonstrated using data from two replicate bioassays (i.e. Bioassays 1 and 2) of 40 animals each on the effect of each of cadmium and copper exposure on survival of a common Antarctic amphipod, *Orchomenella pinguides*, observed in 3-d intervals up to 30 d (Sfiligoj et al. in prep).

Figure 1 shows the number of surviving animals from a starting number of 40 for each of 4 concentrations of cadmium (Cd) over a total period of 30 d with observations made every 3 d for the first bioassay. The points are observed number surviving, the solid lines are predictions from Equation (5) using the GAMM model (2) fitted to the periodic mortality data (not shown), and the dashed lines join the estimated survival for the probit model estimated for each period end-point and fitted directly to the survival numbers using a GLM with probit link function and regressor variable of log of concentration. Figure 2 is the estimated function $s\left(t'_{ij}, k\right)$ within model (2) with k taking the default value in

`gamm` (see see R help file for the `s` function in the `mgcv` library) along with approximate 95% confidence bounds.

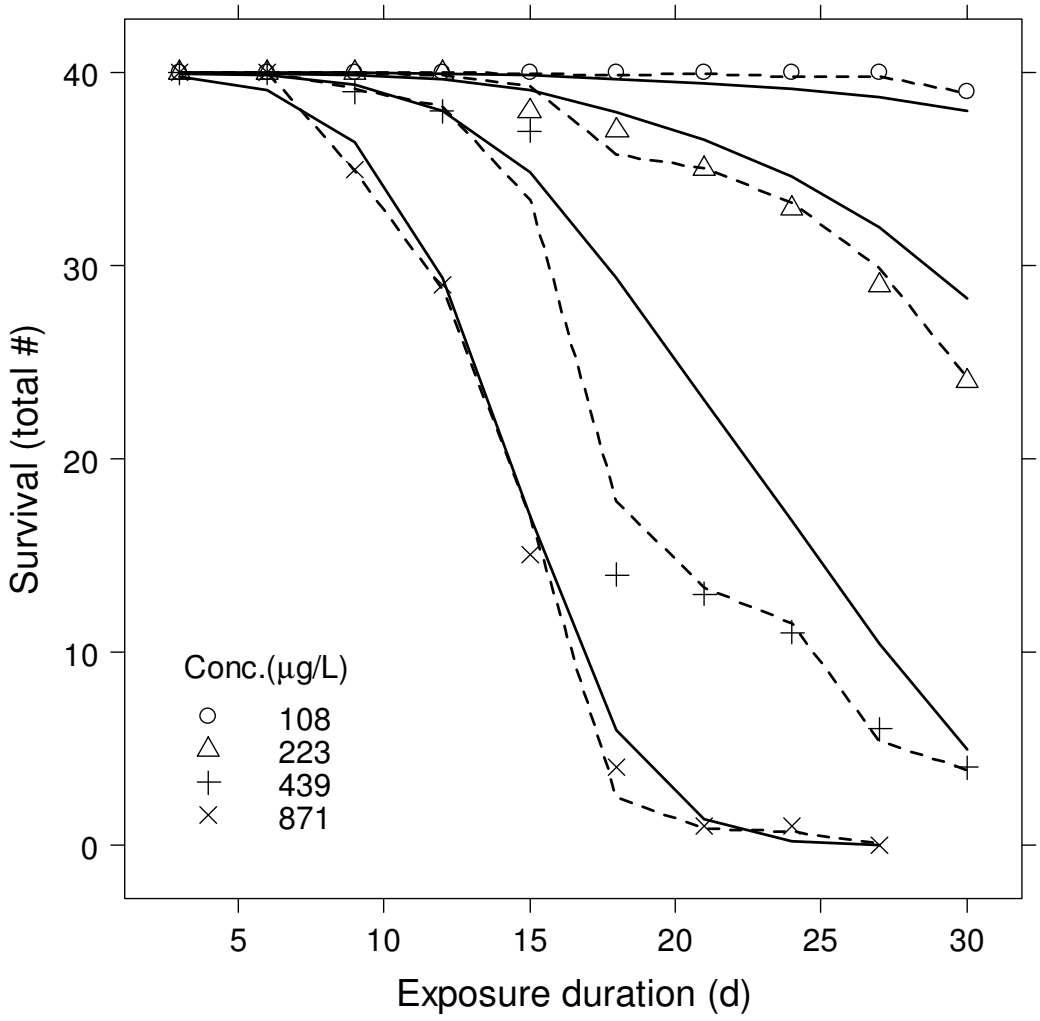

Fig. 1 Survival curves for the amphipod *Orchomenella pinguides* including both observed and predicted from both the GAMM (solid lines) and the day-specific probit analyses (joined dashed line segments) for cadmium Bioassay 1 data.

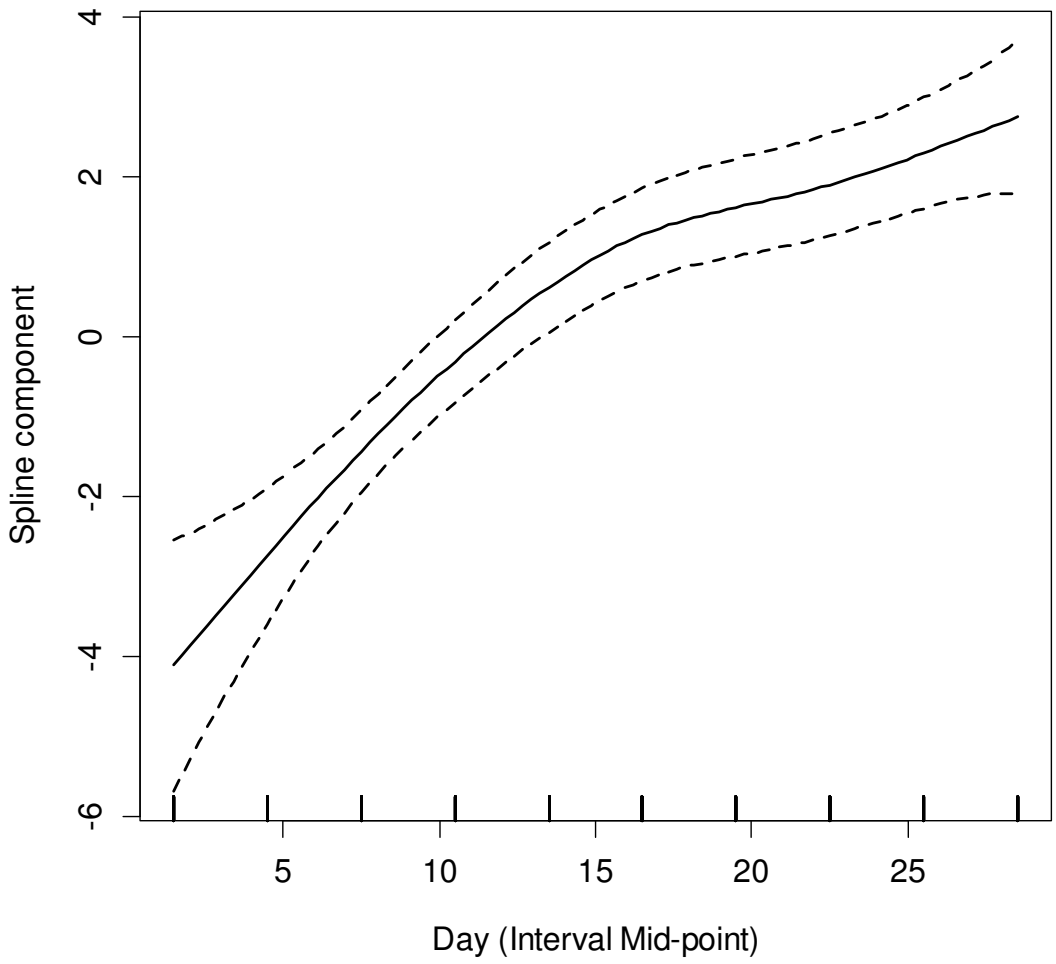

Fig. 2 GAMM spline, $s\left(t'_{ij},k\right)$, for Days for cadmium Bioassay 1 with approximate 95% confidence bands for the spline component.

Data from both bioassays for Cd exposure were combined and Model (4) was fitted. Plots of the estimated random effects $\left\{\tilde{v}_{ijl}\right\}$ indicated that there were systematic differences between bioassays. Model (4) does not include the factor `Bioassay_f` as a fixed effect so to investigate this effect, an intermediate model between the maximal fixed effect model (3) and model (4) was fitted. This model

adds terms of $\alpha_{0l}B_l^{(F)}$ and $\alpha_{1l}B_l^{(F)}ln\left(C_{jl}\right)$ to model (4) which involves addition of 2 parameters $(\alpha_{02},\alpha_{12})$. Since α_{01} and α_{11} are 'aliased' with $\beta_{01}=\beta_0$ and $\beta_{11}=\beta_1$, respectively, the parameters $(\alpha_{02},\alpha_{12})$ represent differences from the bioassay-specific parameters (i.e. $\alpha_{02}=\beta_{02}-\beta_{01},\alpha_{12}=\beta_{12}-\beta_{11}$). The `gamm` syntax for this model is

```
gamm.01 <- gamm(formula = BinY ~ offset(Day.inc.off) + LogConc +
     Bioassay_f + Bioassay_f:LogConc + s(Day.mid),
     family=binomial(link="cloglog"),
     weights=data.NoContr$Nbin,random=list(Concxday.f=~1),
     data=data.NoContr,
     niterPQL=150,control=lmeControl(maxIter=150, msMaxIter=150))
```

where

`BinY=` n_{ijl}/N_{ijl} , `Day.inc.off=` $ln\left(\Delta t_{ij}\right)$, `LogConc=` $ln\left(C_{jl}\right)$, `Bioassay_f=` $B_l^{(F)}$ `Day.mid=` t'_{ij} , `Concxday.f=` $C_{jl}^{(F)}:t_{il}^{(F)}$, `Nbin=` N_{ij} .

The estimates of parameters for this model were $\beta_0=-24.45(\mathrm{SE}=1.51,\ \mathrm{P}<0.001)$, $\alpha_{02}=9.24(\mathrm{SE}=1.99,\ \mathrm{P}<0.001)$, $\beta_1=3.23(\mathrm{SE}=0.26,\ \mathrm{P}<0.001)$, $\alpha_{12}=-1.29(\mathrm{SE}=0.34,\ \mathrm{P}<0.001)$, and $\sigma_\nu=1.23$. Therefore there is a significant difference between bioassays in periodic mortality over concentrations and time. Note that the maximal fixed effect model (3) which includes the additional term $s\left(t'_{ijl}:B_l^{(F)},k\right)$ specified by

`s(Day.mid, by=Bioassay_1) + s(Day.mid, by=Bioassay_2)`, where `Bioassay_1` and `Bioassay_2` are the appropriate (0,1) dummy variables, failed to converge.

Figure 3 shows the estimated values of the random effects $\{\tilde{\nu}_{ijl}\}$ from the extension of model (4) when data from both Bioassays were combined and the terms involving `Bioassay_f` given above were dropped. Note that the concentrations of Cd were planned to closely match across Bioassays: the very low (107), low (219), medium (436), and high values (874) while there was an extra very high (1306) concentration also tested in Bioassay 2. So the concentration levels in Figure 3 are the above integerised average across the two bioassays for each of the above general levels so they differ very slightly from those given in Figure 1. The sign and scale of the $\{\tilde{\nu}_{ijl}\}$ are informative. Higher positive values of $\{\tilde{\nu}_{ijl}\}$ indicate that model (4) including only fixed effects (i.e. setting the $\{\nu_{ijl}\}$ to zero) under-estimates the level of periodic mortality. Larger negative values indicate the reverse. However, the difference between observed and predicted survival is not a simple cumulative summation of the random effects for those estimated for periodic mortality shown in Figure 3 since Equation (5) must be applied to obtain estimates of survival.

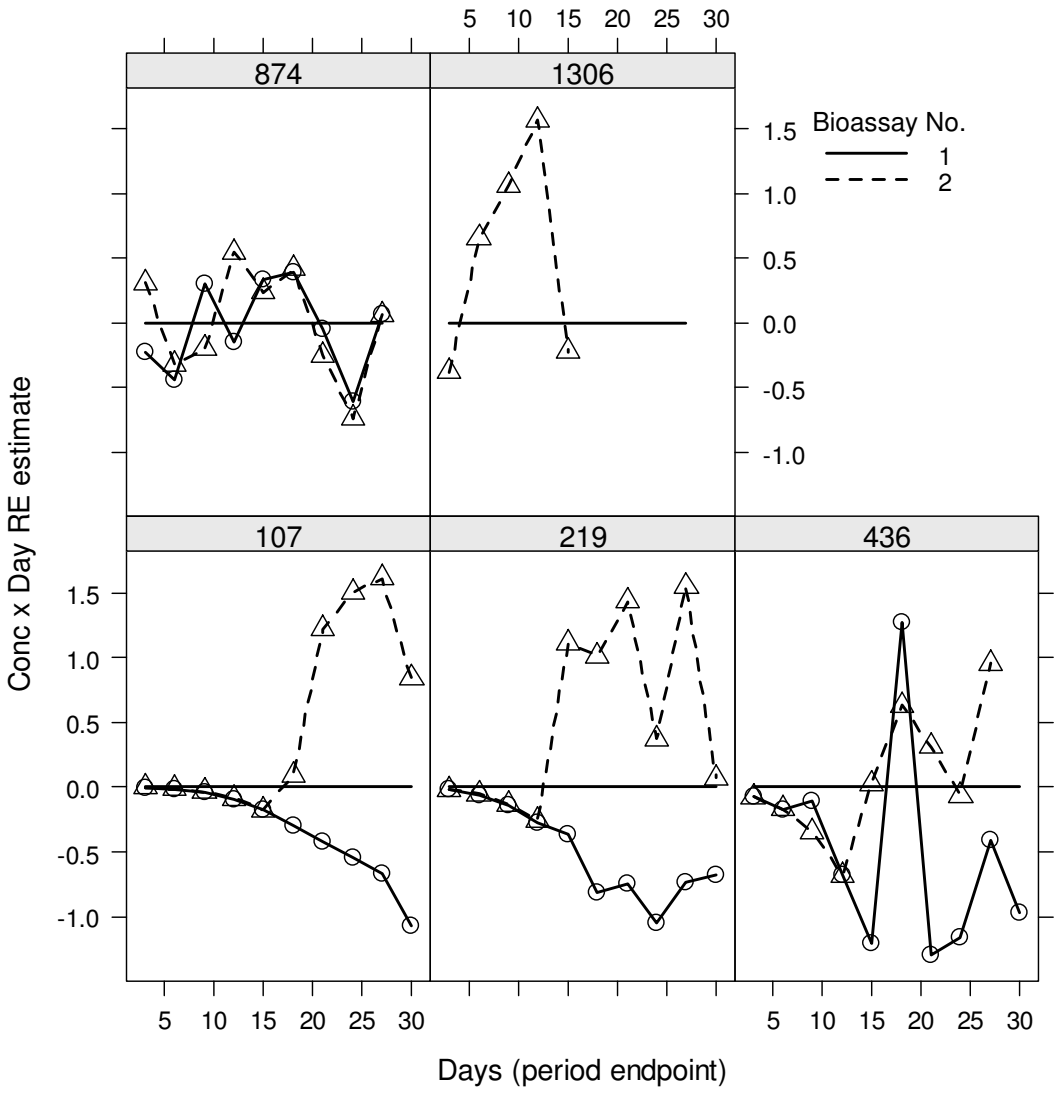

Fig. 3 Concentration by day factor random effect estimates versus Days for cadmium

and Model (4) fitted to the combined bioassay data.

Figure 4 shows the equivalent figure to Figure 3 but for Cu and for the fit of model (4). In this case there does not appear to be any consistent and substantial difference between the two Bioassays and this was confirmed by

estimates of both α_{02} and α_{12} , when model (4) was extended (AIC=196.4) as was carried out for Cd, that were found to be not statistically different from zero (*P*>0.05) with the Model (4) AIC of 195.5.

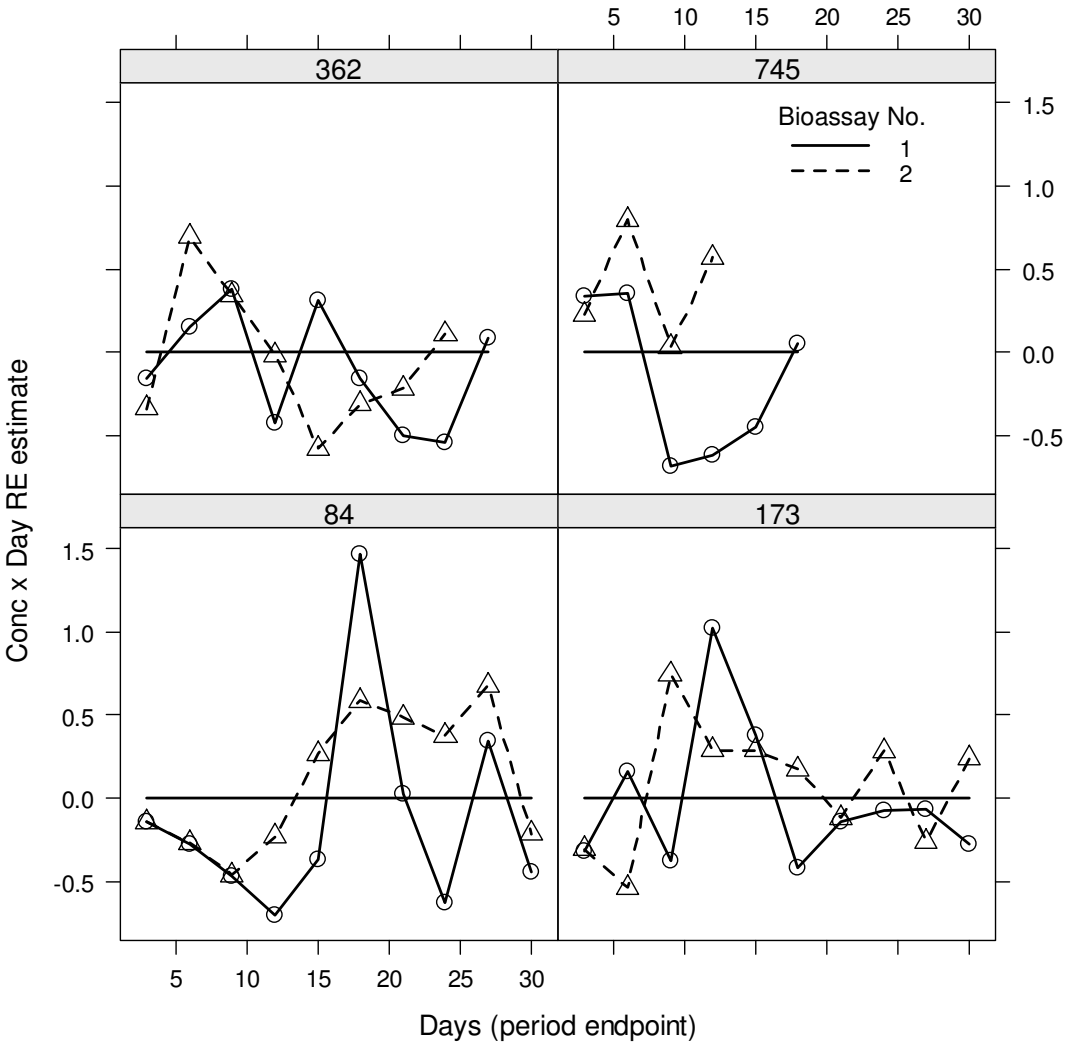

Fig. 4 Concentration by day factor random effect estimates versus Days for copper and Model (4) fitted to the combined bioassay data.

Figure 5 shows the average concentrations of Cu (i.e. C_{jl}) and period-specific concentrations (i.e. C_{ijl}) for both Bioassay 1 and 2. The extension of Model (4) to

account for time-varying concentration using CCE, $C'_{ij}\left(\hat{\beta}_1\right)$, gave a maximum profile PDE estimate of β_1 of 0.58 with corresponding maximum PDE of 0.647. This PDE is greater than the Model (4) value of 0.592 and therefore demonstrates an improved fit to the periodic mortality data. However, this was not due to accounting for time-varying concentrations but due to the use of a time scale of CCE since when $C'_{ij}\left(\hat{\beta}_1\right)$ was calculated using average values of concentration applied to all time intervals the profile estimate of β_1 was also 0.56 with corresponding maximum PDE of 0.646.

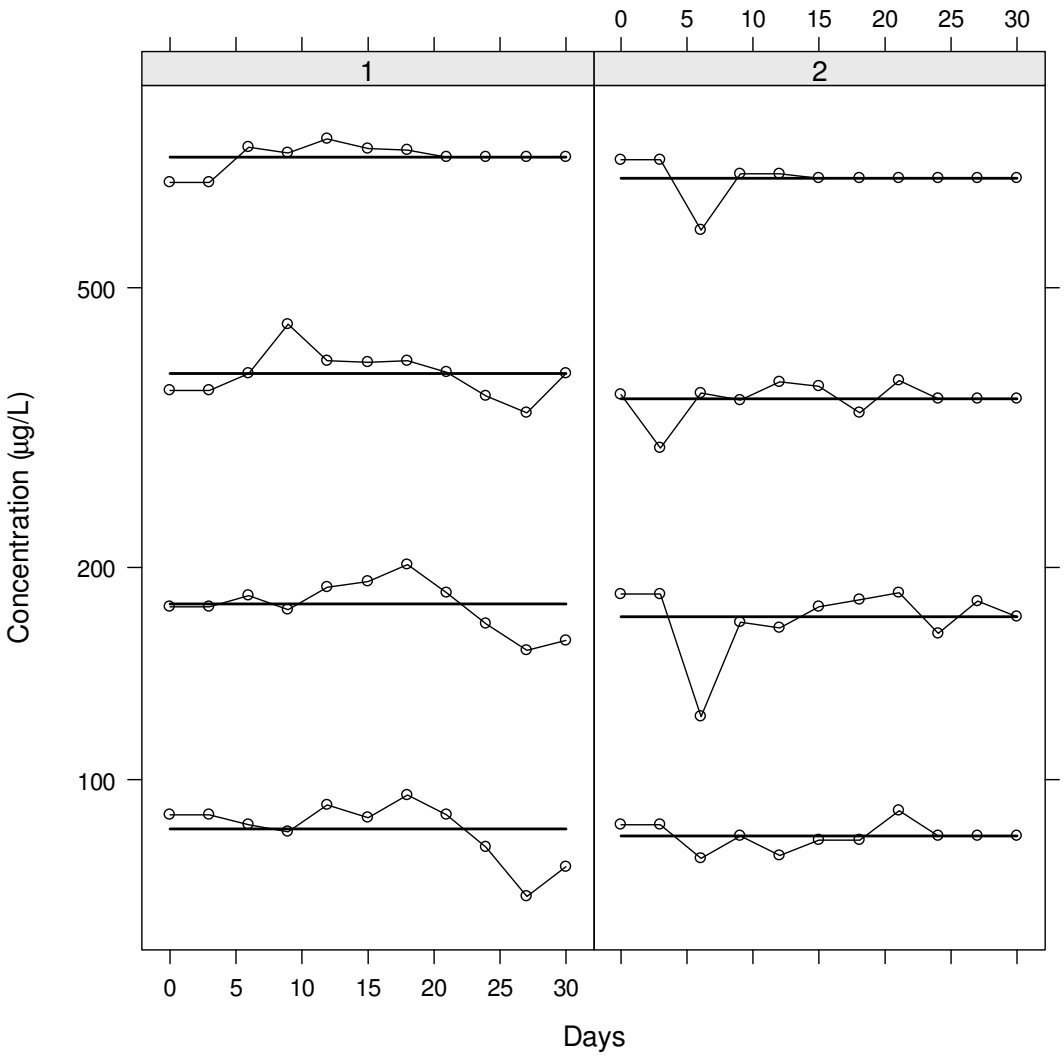

Fig. 5 Average concentrations (log scale) for copper (heavy solid line) and measured period-specific concentrations (lines with circles) for Bioassays 1 and 2.

Figure 6 gives the estimated function $s\left\{C'_{ij}\left(\hat{\beta}_1\right), k\right\}$ for the model using actual time-varying concentrations.

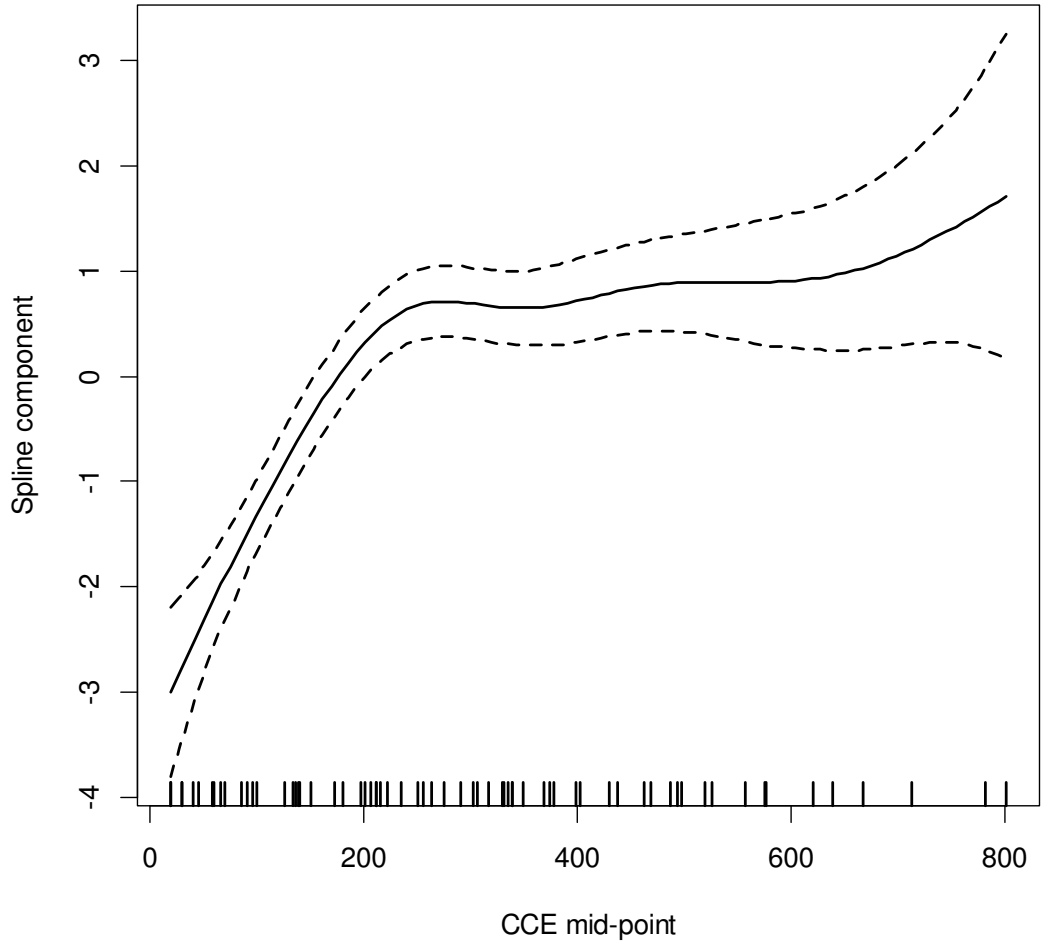

Fig. 6 GAMM spline, $s\left\{C'_{ij}\left(\hat{\beta}_1\right),k\right\}$, for Calibrated Cumulative Exposure (CCE) with $\hat{\beta}_1 = 0.50$ using time-varying concentrations for copper and Bioassays 1 and 2 combined with approximate 95% confidence bands for the spline component.

Figure 7 shows the log of the integral of the fitted hazard function of the smooth $s\left(t'_{ij},k\right)$ shown in Figure 2 using daily time steps (i.e. midpoint rule with $\Delta t_{ij} = 1$) compared to that of the actual observation time periods Δt_{ij} of 3 d, and for

comparison using time periods of 10 d (i.e. t'_{ij} interval midpoints of 5, 15, and 25 d). Therefore the most accurate integral is the sum of the midpoint rectangles using daily time steps for a given interval of either 3 d or 10 d (lines in Figure 7). For example, for the 3 d comparison this approximation is $log\left\{\sum_{j=j'}^{j'+3} s\left(t'_{ij},k\right)\right\}$ for $t'_{ij'} = t_{ij'} + 0.5, +1.5, +2.5$ compared to $log\left\{3s\left(t'_{ij},k\right)\right\}$ for $t'_{ij'} = t_{ij'} + 1.5$.

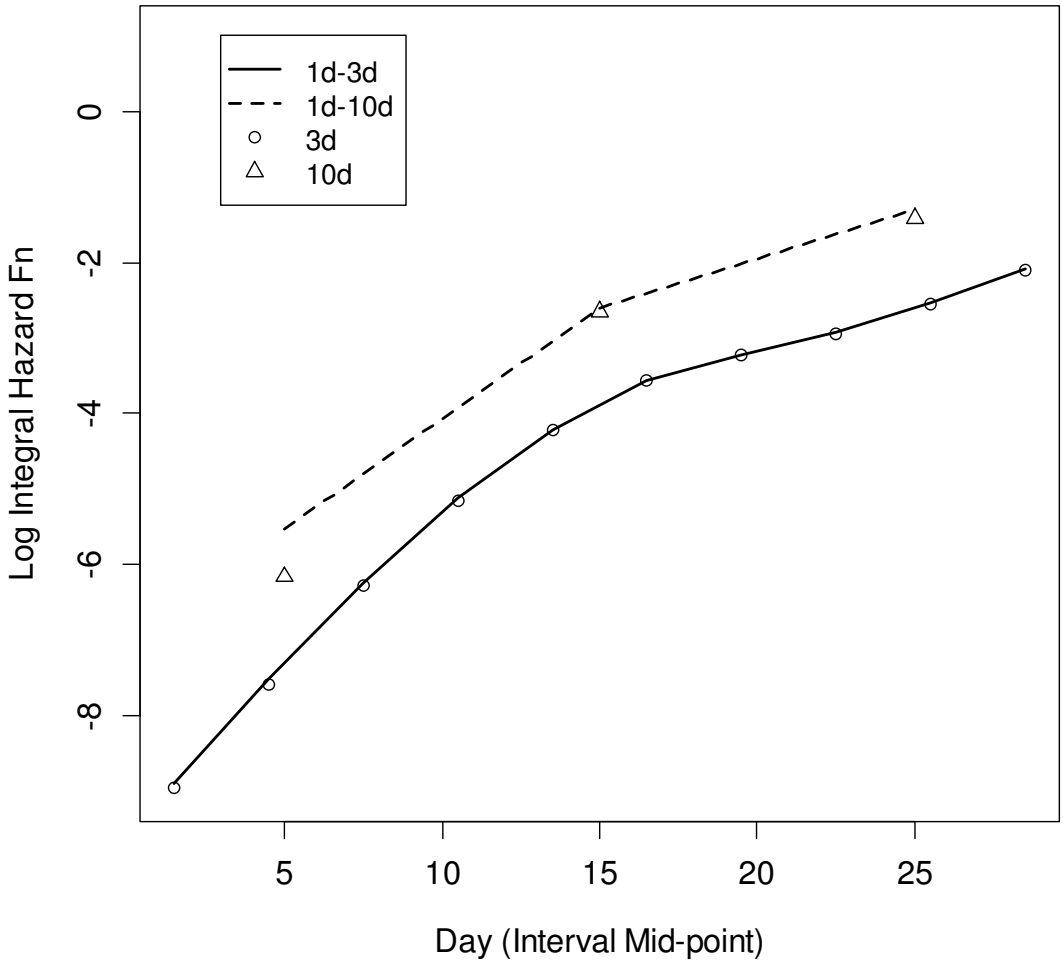

Fig. 7 Log of the integral of the fitted hazard function for cadmium and Bioassay 1 using 1 d, 3 d, and 10 d intervals where the 1 d midpoint rule approximation is

summed over 3 d (solid line) and 10 d (dashed line) and compared to 3 d (circles) and 10 d (triangles) midpoint rule approximations.

Figure 8 shows the LC values for survival probabilities 0.5 (i.e. fraction mortality of 0.5, LC50) for Cu obtained from model (4). Approximate 95% confidence are bounds shown along with the corresponding values from the probit analysis (points) at each time period end-point with approximate 95% confidence bounds also shown (bars). The confidence bounds for the LCs derived from the probit analyses were obtained using Fieller's theorem (Finney, 1971). Figure 8 also shows these LC values for the version of Model (4) that replaces terms involving the log of concentration and days with the spline in the CCE variable with prediction based on actual time-varying concentrations and the estimate of β_1 of 0.58.

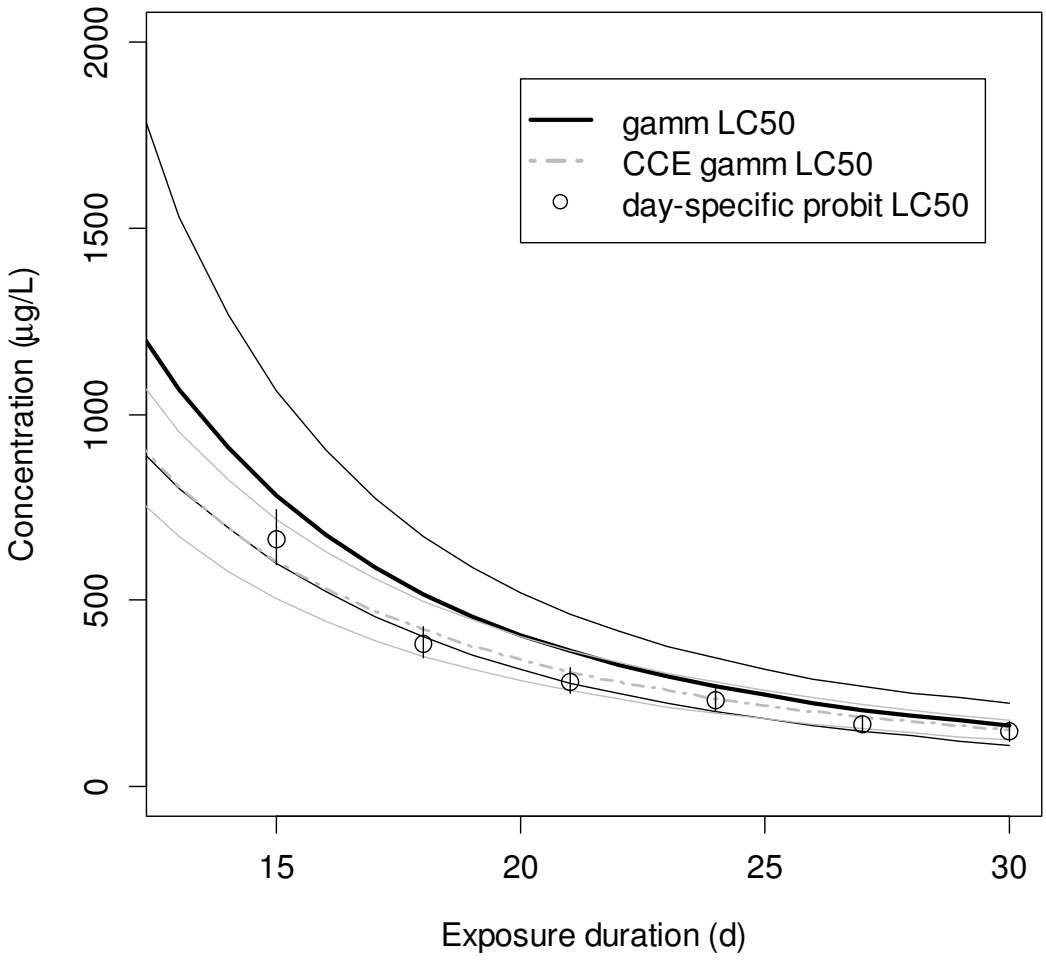

Fig. 8 LC curves by Days for Model (4) versus those for endpoint-specific probits for combined bioassays data for Cu and LC of 0.5 (black solid) with corresponding LC curves for time scale of Calibrated Cumulative Exposure (grey dash-dot line). Approximate 95% confidence bounds shown for LCs for the GAMMs (thin lines) and probits (bars).

Discussion

Using the midpoint rule to approximate the log of the integral of the hazard function is seen to be very accurate for 3 d time intervals given the shape of the hazard function shown in Figure 2. If this function was piecewise linear for each time interval then the midpoint approximation would be exact. The reason the approximation is very good is that the fitted function would be very well approximated by piecewise linear components for the observed 3 d intervals. If 10 d intervals had been used, Figure 7 shows that the midpoint approximation is still quite accurate, however, if the mortality was only observed every 10 d then the ability to model the shape of the hazard function would be limited to a linear or possible quadratic trend without being able to detect more subtle shapes such as that shown in Figure 2. Given the fitted shape of the log-hazard function over time it is shown in Figure 7 that the approximation to its integral is sufficiently accurate for practical application. Therefore observed mortality in 3 d intervals over the 30 d Bioassay was a prudent approach. Such recommendations need to be specific to the particular taxa and experimental conditions applied so that the observation period is adequate to allow accurate modelling of the hazard function which will depend on the rate at which the hazard function changes with time.

Given the midpoint approximation is sufficiently accurate, which can be evaluated as described above using an initial fit of the GAMM, the approach

described is quite flexible in modelling bioassay/survival data in studies of this type. Multiple bioassays can be combined and the proportional hazards assumption can be tested using the diagnostics described, including plotting estimates of Concentration x Day random effect estimates for each bioassay. The approach of combining bioassays can be investigated using bioassay as a fixed effect, and also allows one to average across bioassays, if this is appropriate, simply by fitting bioassay and its interaction with concentration level and day factors as random effects. More than two bioassays would be required to model the main effect of bioassay as a random effect but this is easily accommodated in the GAMM. Using the log of observation period intervals, Δt_{ij} , also allows bioassays where this period is not constant to be accommodated in the GAMM. The log-linear assumption of concentration level on mortality can be tested by fitting a spline to log concentration level if there are a sufficient number of levels tested to allow more flexibility in modelling the shape of the dose-response relationship.

The issue of time-varying concentrations studied by Péry et al. (2001) in extending the DEBtox model was considered and a simple approach to accounting for this using a GAMM was investigated. The GAMM applying the CCE variable as a replacement for time in days could be considered for bioassays where progressive elimination of the toxicant is more substantial than that seen in the example on Cu exposure given here. Even in this example replacing the combined log-linear dose-response and hazard function based on day with a combination of these as quantified by the CCE variable not only has the potential

to fit a model of mortality response to time-varying concentrations but also can improve the fit for the case where concentrations remain over time at, or close to, their average value. The profiling of the parameter β_1 in calculating the CCE and fitting it within a spline term in the GAMM is an extension of the GAMM fitting procedure. Other objective functions besides PDE could be used such as the AIC obtained from the `lme` component of the GAMM which needs to be minimised to find the minimum profile estimate. However, a limitation of this measure is that the `lme` fit cannot constrain the residual variance to 1, with empirical values obtained in this study ranging from approximately 0.4 to 1.2. A residual variance of 1 corresponds to a binomial distribution conditional on the random effects. While profiling using AIC it was observed that the proportion of variance explained by the residual term compared to that for the random effect term could vary substantially making the profile estimation problematic. The PDE is a marginal goodness-of-fit statistic since the random effect estimates must be set to zero to calculate it. Alternatively, other marginal statistics could be used such as -2 times the marginal log-likelihood with random effects integrated out of the penalised log-likelihood. Further research on the best method of profiling using simulation studies is worth pursuing given that the GAMM based on CCE has potential to provide a general empirical framework for joint modelling of response-time and response-dose relationships for time-constant and time-varying concentrations in ecotoxicological studies. Ideally, process models could be applied (Péry et al., 2001) if there were sufficient understanding of the causes of the variation over time in concentration either with or without intervention by

the experimenter to maintain concentration levels. However, even given this understanding the data required to successfully estimate process model parameters may not be feasible to obtain.

Sanchez-Bayo and Goka (2007) recommend hyperbolic relationships between each of time and response and dose and response where response is cumulative mortality over time periods as a percentage of the number of animals at the start of the experiment. They use ordinary least squares and their model lacks generality since they do not combine parametric models for dose-response and time-response into a single model. Further the origins for their family of hyperbolic curves for time response requires data-specific values of the "closest date for which no mortality was recorded", or in other words, the time just prior to first response in terms of mortality must be specified, however, they give no model to predict these values. The survival-time analysis under a range of fixed concentrations (with extension to variable concentrations) assumes that when there are animals at risk (N_{ij} > 0) under some exposure to toxicants then given sufficient sample size a positive number of animals are expected to suffer mortality, whereas under smaller sample sizes, zero periodic mortality is also likely as part of stochastic conditional binomial variation with probability determined by p_{ij} . Therefore zero periodic mortality is informative as long as N_{ij} is greater than zero and should not be excluded from model fit as in Sanchez-Bayo and Goka (2007). Further, a binomial distribution has a greater justification

both in theory and practice than an equal-variance, Gaussian error distribution implied by their ordinary least squares approach.

The GAMM provides a flexible way of smoothing through noisy bioassay/survival data by assuming a smooth trend in the survival over time exists compared to modelling using classical probit/logit models at each interval end-point. Using random effect terms also allows this noise to be reflected in the uncertainty of LC values (Figure 8) both within and across replicate bioassays.

Defining a single random effect variance as in Model (4) for random effects corresponding to combinations of bioassay, concentration level, and time factor is simplistic but in the example datasets with only two replicated bioassays for each metal there was little scope for fitting more complex random effect models. With a larger number of replicate bioassays then Bioassay could form a random effect term with the concentration level by time factor term nested within Bioassay. The concentration level by time factor interaction term clearly does not represent nested sampling but is strictly a lack-of-fit error term which corresponds to the lack-of-fit error component in the case of linear regression with replicate observations at design points for which Draper and Smith (1998) partition the error variance into a lack-of-fit error component and a pure error component (i.e. binomial variation in our case). Additional replication would allow the examination of these random effect estimates that make up the interaction as to whether they can validly be characterised as random or whether they represent systematic variation. Data diagnostics for the random

effect estimates as displayed in Figures 3 and 4 would be more useful in investigating these issues when there are a larger number of replicate bioassays. Systematic departures from the underlying model of a smooth nonlinear time-response and linear or smooth nonlinear dose-response relationships can indicate if these smooth relationships are an adequate approximation to reality when there are possibly discrete change-points in response caused by thresholds being exceeded (i.e. the tolerance distribution may not be well described by a continuous probability density function). The NEC is an example of such a discrete change-point in response for the lower range of concentration. In this case, lack of fit may be evident as systematic effects and even in this case describing such systematic effects using random effect estimates on the complementary log-log link scale may be more useful than using model departures for periodic mortality because of scale restrictions in this last case. Examination of systematic effects as random effects can thus to contribute to understanding toxicity as a process in time (Baas et al. 2010).

More accurate methods of estimating random effect estimates, their variance, and simultaneously estimated fixed effects exist in the case of Generalised Linear Mixed Models (GLMMs) and GAMMs such as marginal likelihood approaches (McCulloch, 1997) and Markov Chain Monte Carlo sampling methods (Gilks et al. 1996; Vaida and Xu, 2000). The `lme` methods that `gamm` uses, applies Residual Maximum Likelihood (REML) to a first order linear approximation to the GAMM as described for GLMMs by Schall (1991) (see also Candy, 1997b) and identified as a Penalised Quasi-Likelihood (PQL) approach by Breslow and Clayton (1993).

This approach has been demonstrated (Breslow and Clayton, 1993) to perform poorly in terms of estimation of random effect variances for binary data (e.g. matched case-control studies) that assume a binomial distribution with sample size of one. However, this situation improves rapidly as the binomial sample size increases, so in this study with $M_j = 40$ animals per bioassay the PQL approximate estimates compared to those obtained using the exact marginal likelihood are expected to be quite good. This simple assumption needs to be qualified since the binomial sample size decreases as animals die off so that some values of N_{ij} can be as low as 1. Further research involving simulation studies is require to investigate the effect of the presence of such small conditional sample sizes used in fitting the GAMM using continuation ratios combined with PQL. Therefore the `gamm/lme` approach applied here should not be considered unless the initial number of animals used for each concentration level in bioassays is an adequately sized sample.

The approach described does not explicitly incorporate control mortality. The animals in the control treatments did not suffer natural mortality to any significant extent (Sfiligoj et al. in prep), however, if control mortality is of sufficient practical importance given attempts to eliminate it by improving the experimental setup are not entirely successful then the output from the GAMM can be adjusted for control mortality using Abbott's correction (Finney, 1971). A smooth of the control mortality over the experimental observation periods can be fitted using a GAM or GAMM similar to those described above. If control

mortality for the set of (toxicant-induced) stressed animals is assumed to act independently to the effect of the toxicant (i.e. the mortalities can be attributed to either natural stress or toxicant-induced stress and not a combination of both) then the predicted survival (i.e. 1 minus cumulative mortality) for a given concentration and exposure period, $S_{j'}(t_{i'})$ from equation (5), is replaced by $S_{j'}(t_{i'}) / S_0(t_{i'})$ where $S_0(t_{i'})$ is predicted survival probability for controls to time $t_{i'}$.

The GAMM approach using the approximation to the log of the integral of the hazard function provides a convenient framework to fit flexible proportional hazards models including fixed and random effects. The cubic smoothing splines within the general GAMM structure allow flexible, empirical modelling of the hazard function over time, and can also model the shape of the dose-response curve if a sufficient number of concentration levels have been used and a linear term in log of concentration is inadequate. Extensions to non-proportional hazard models, including accounting for time-varying concentrations can also be investigated.

The R-software and `mgcv` library are both freely available and the R-code used in this study can be provided on request.

Acknowledgements

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Appendix 2

Toxicity tests with Antarctic microgastropods, *Skenella paludionoides* (E. A. Smith, 1902)

Description:

Information for this species is limited to personal observations during collections and experiments. *Skenella paludiniudes* are a microgastropod of the family Cingulopsidae with spiral shell that is often black but also a dark grey/brown in colour. The adults are approximately 2mm in length and lay masses of yellow eggs onto hard substrates. They are often found in Antarctic coastal waters on macrophyte fronds attached to rocky substrates (personal observations, Bianca Sfiligoj).

Pilot Test

Materials and Methods

Adult *Skenella paludionoides* were collected using dip nets from nearshore, rocky substrate habitats in >1 m depth near Casey Station, East Antarctica (66˚17'S 110˚31'E). Bioassay treatments of copper, cadmium and zinc consisted of 4 replicate 70 mL acid washed plastic vials, each containing 10 individuals in 50 mL of test solution. Test seawater was collected approximately 40 m offshore at 2-2.5 m depth away from any obvious anthropogenic contaminant inputs. Seawater was filtered to 0.22 µm and had a salinity of 33 - 34 ppt, dissolved oxygen (DO) content > 90%, and pH of 7.99 - 8.05. Vials were kept in a constant temperature chamber at 0.5 (± 0.4) °C and a 12:12 hour photoperiod, and no food was added during the test. At each observation, individuals were assigned into 1 of 4 behavioural categories based on activity or appearance as follows:

- **Active:** gastropod is attached to container, moving and active with foot protruding from the shell and with tentacles erect

- **Not attached:** gastropod is not attached to the container, is not moving and with foot retracted but operculum open allowing water exchange
- **Closed:** gastropod is not attached with operculum closed tight
- **Dead:** gastropod is not attached, not moving and foot is hanging limply from shell

Behaviour and mortality was evaluated over a 35 d exposure period with observations and removal of dead individuals on day 4, 7, 14, 21, 28 and 35 of the test. Renewal of test solutions was also conducted on these days, with 10 mL water samples taken both before and after renewals for metal analysis. Samples of test solutions taken at the start of the tests and throughout the tests were filtered through 0.45 µm syringe tip and preserved with 1% (v/v) of ultra pure nitric acid (HNO_3) then analysed using an inductively coupled plasma optical emission spectrometer (ICP-OES). Mean exposure concentrations were determined for each exposure period from water samples taken at the beginning and end of each period, and these average measured metal concentrations were used for determination of point estimates. Sub-lethal (based on active category; EC_{10} and EC_{50}) and lethal (LC_{10} and LC_{50}) point estimate values were determined by Maximum Likelihood Probit analysis in Toxcalc™ (v5.0.26; Tidepool Scientific software).

Results

Behaviour was a more sensitive endpoint than mortality with a pattern of progression through categories from Active, to Not attached, to Closed and then to Dead. There was a small amount of switching between the categories however, so the Active category endpoint was chosen to be analysed for sub-lethal behavioural effects, as departure from this category indicated evidence of negative effects from the metal exposure. Point estimates for active behaviour (EC_{10} and EC_{50}) and Lethal (LC_{10} and LC_{50}) endpoints are presented in Table 1. Responses to metal exposure in terms of survival and active behaviour are presented in Figures 1 and 2 respectively.

Table 1: Point estimates (µg /L) for copper, zinc and cadmium based on active behaviour (EC_{10} and EC_{50}) and survival (LC_{10} and LC_{50}) of the microgastropod *Skenella paludionoides* over 35 days. (95% confidence limits in brackets where possible for calculation).

		Exposure duration (days)					
		4	**7**	**14**	**21**	**28**	**35**
Copper	**LC_{10}**				89 (72 – 101)		
	LC_{50}				124 (111 – 138)		
	EC_{10}	62 (39 - 81)	134 (75 - 184)				
	EC_{50}	142 (117 - 167)	382 (304 - 481)				
Zinc	**LC_{10}**				490 (405 - 556)		461
	LC_{50}				647 (572 - 731)		514
	EC_{10}		792	501	465	442	445
	EC_{50}		989	550	552	534	499
Cadmium	**LC_{10}**				1090 (740 - 1307)	982	
	LC_{50}				1467 (1188 - 1679)	1091	
	EC_{10}		1390		457	319 (163 - 431)	196 (114 - 254)
	EC_{50}				902	589 (438 - 709)	339 (264 - 403)

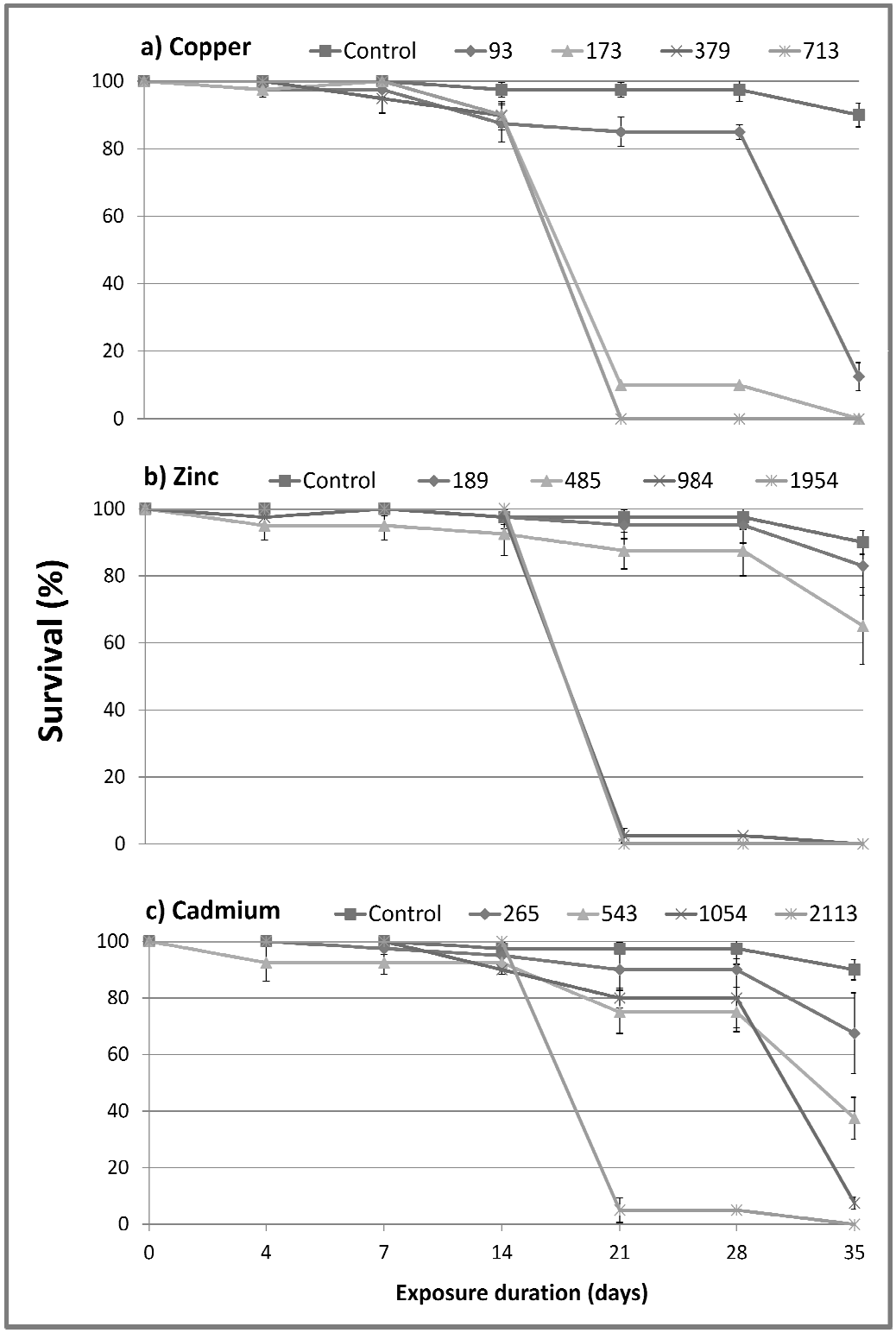

Figure 1: Survival of the microgastropod *Skenella paludionoides* exposed to a) copper, b) zinc and c) cadmium (µg/L) over 35 d. (Error bars shown are ± 1 SE).

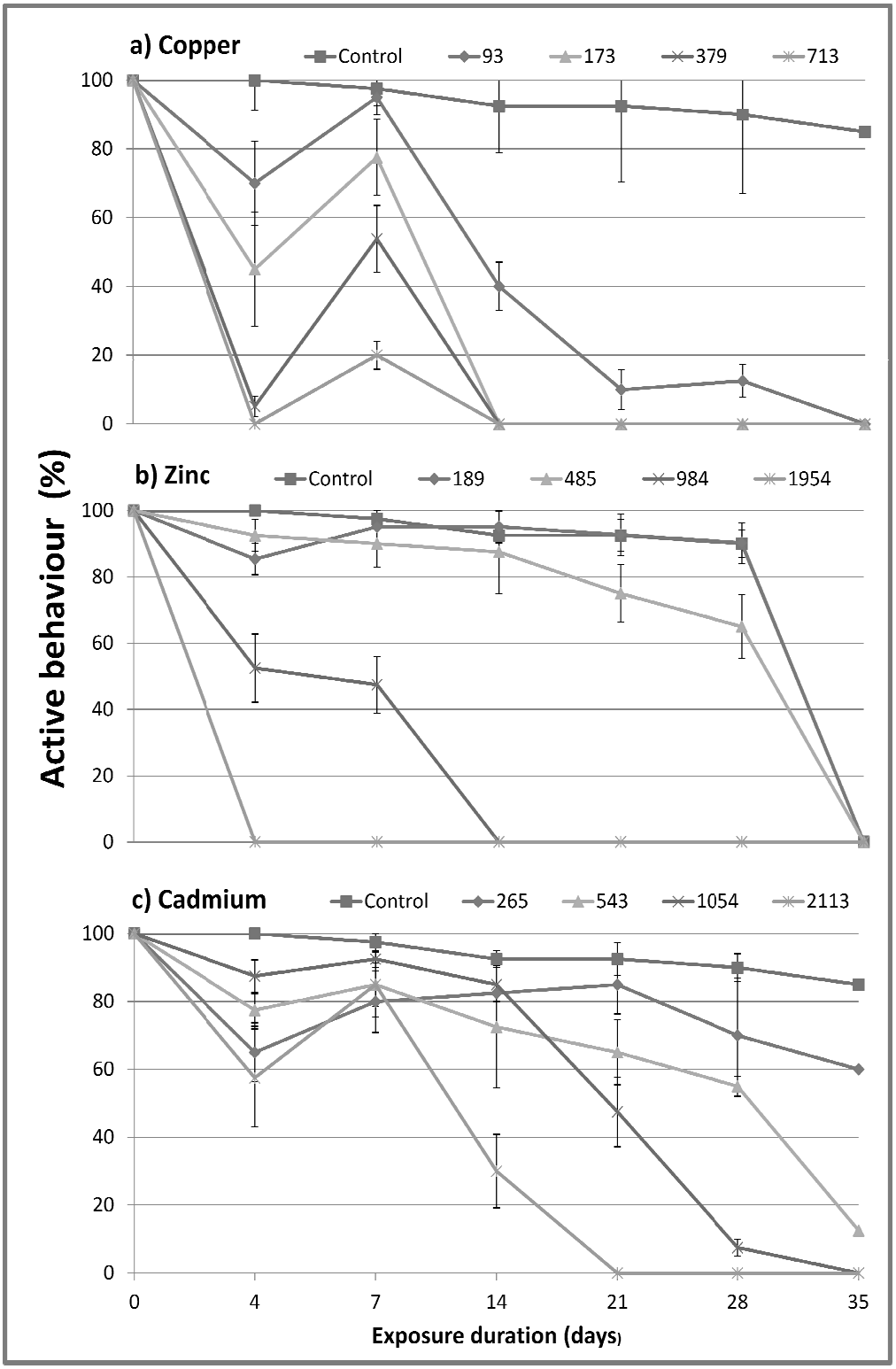

Figure 2: Active behaviour of the microgastropod *Skenella paludionoides* exposed to a) copper, b) zinc and c) cadmium (µg/L) over 35 d. (Error bars shown are ± 1 SE).

Test 2

Materials and Methods

Adult *Skenella paludionoides* were collected using dip nets from nearshore, rocky substrate habitats in >1 m depth near Davis Station, East Antarctica (68°35' S 77°58' E). Bioassay treatments of copper, cadmium, zinc lead and nickel consisted of 4 replicate 70 mL acid washed plastic vials, each containing 10 individuals in 50 mL of test solution. Test seawater was collected approximately 40 m offshore at 2-2.5 m depth away from any obvious anthropogenic contaminant inputs. Seawater was filtered to 1 µm and had a salinity of 33 - 34 ppt, dissolved oxygen (DO) content > 90%, and pH of 7.99 - 8.05. Vials were kept in a constant temperature chamber at 0.5 (± 0.4) °C and a 12:12 hour photoperiod, and no food was added during the test. At each observation, individuals were assigned into 1 of 4 behavioural categories based on activity or appearance as described in the pilot study.

Behaviour and mortality was evaluated over a 21 d exposure period with observations and removal of dead individuals on day 7, 14 and 21 of the test. Renewal of test solutions was also conducted on these days, with 10 mL water samples taken both before and after renewals for metal analysis. Samples of test solutions taken at the start of the tests and throughout the tests were filtered through 0.45 µm syringe tip and preserved with 1% (v/v) of ultra pure nitric acid (HNO_3) then analysed using an inductively coupled plasma optical emission spectrometer (ICP-OES). Mean exposure concentrations were determined for each exposure period from water samples taken at the beginning and end of each period, and these average measured metal concentrations were used for determination of point estimates. Sub-lethal (based on active category; EC_{10} and EC_{50}) and lethal (LC_{10} and LC_{50}) point estimate values were determined by Maximum Likelihood Probit analysis in Toxcalc™ (v5.0.26; Tidepool Scientific software).

Results

As with the pilot test, behaviour was a more sensitive endpoint than mortality. However, a small amount of switching between the behavioural classification categories resulted in Active category being chosen as the behavioural endpoint. Responses were slower compared with the pilot test, with cadmium, nickel and lead failing to elicit any significant response. Point estimates for active behaviour (EC_{10} and EC_{50}) and lethal (LC_{10} and LC_{50}) endpoints are presented in Table 1. Lethal responses are presented in Figures 1 and active responses are presented in Figure 2.

Table 1: Point estimates for Active (EC_{10} and EC_{50}) and Lethal (LC_{10} and LC_{50}) endpoints with 95% confidence limits for *Skenella paludionoides* exposed to copper and zinc for 21 d

		Exposure duration (days)		
		7	**14**	**21**
Copper (µg /L)	**LC_{10}**			96
	LC_{50}			338
	EC_{10}		22 (1 – 40)	43 (1 – 57)
	EC_{50}	41	49 (13 – 69)	74 (53 – 88)
Zinc (µg /L)	**LC_{10}**			1157 (850 – 1352)
	LC_{50}			1787 (1575 – 2056)
	EC_{10}	811		530 (379 – 642)
	EC_{50}	1193		898 (768 – 1034)

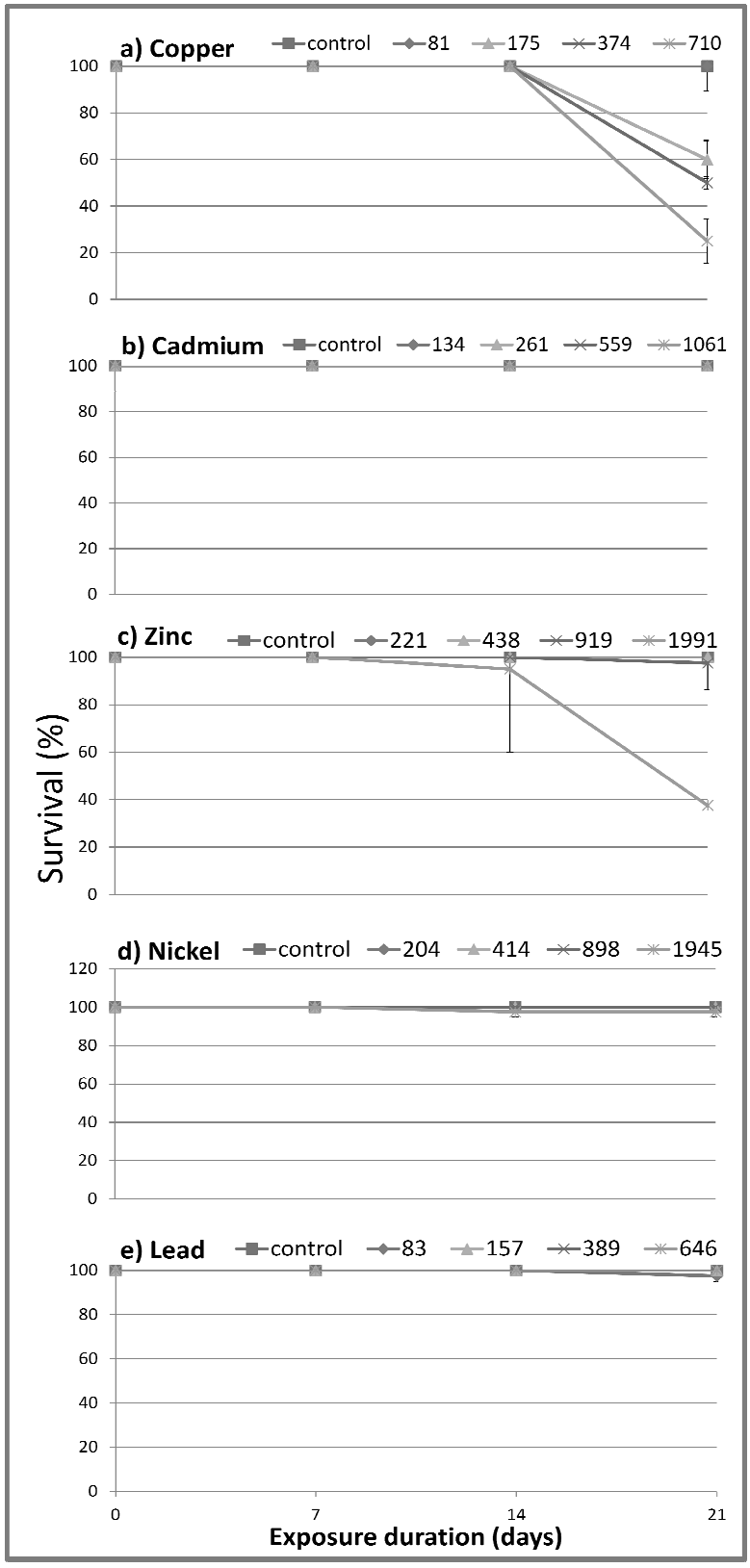

Figure 1: Survival of the microgastropod *Skenella paludionoides* exposed to a) copper, b) cadmium, c) zinc, d) nickel and e) lead (µg/L) over 21 d. (Error bars shown are ± 1 SE).

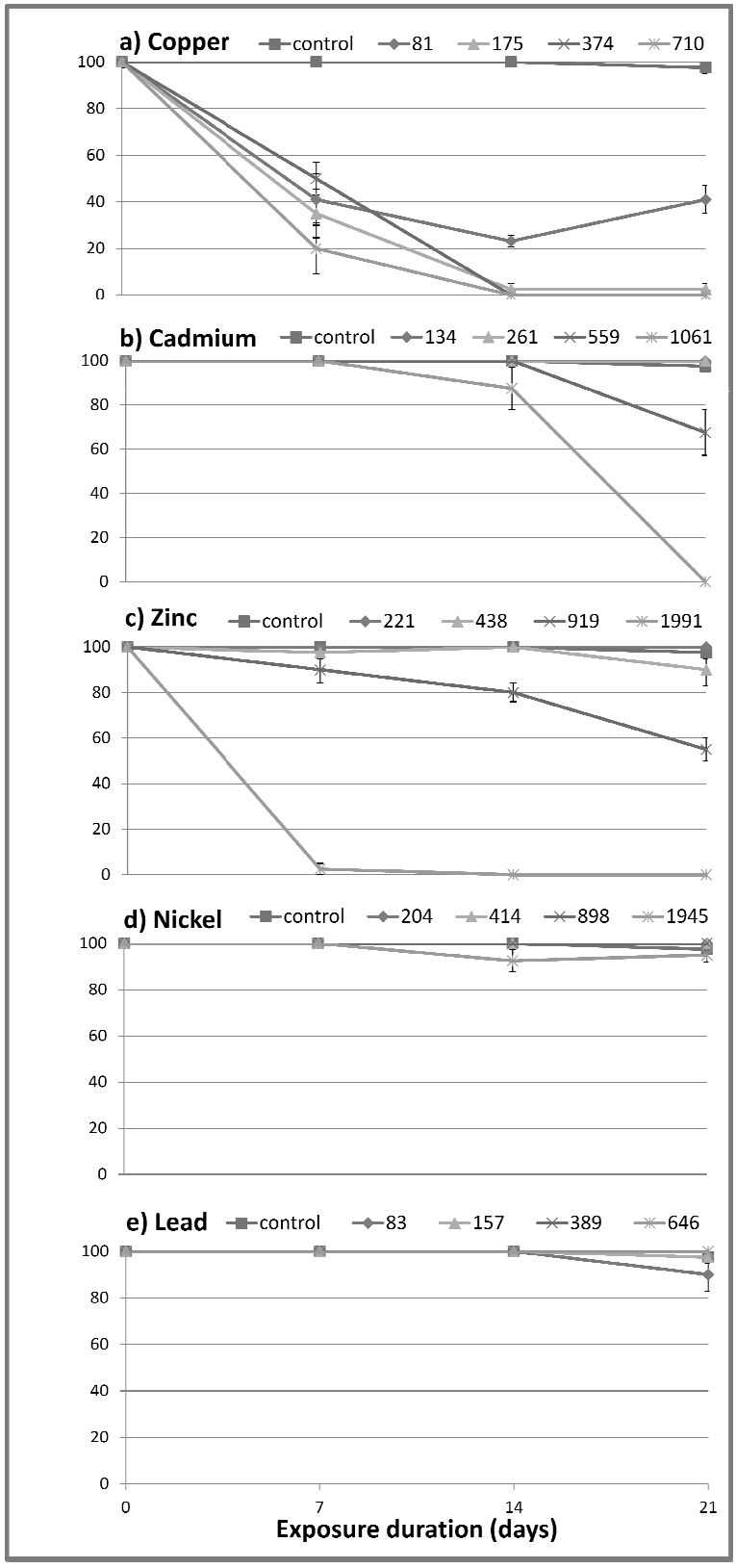

Figure 2: Active behaviour of the microgastropod *Skenella paludionoides* exposed to a) copper, b) cadmium, c) zinc, d) nickel and e) lead (µg/L) over 21 d. (Error bars shown are ± 1 SE).

Appendix 3

Toxicity tests with Antarctic juvenile heart urchins, *Abatus shackletoni* (Koehler, 1911)

Description:

Abatus shackletoni are heart urchins which inhabit Antarctic coastal benthic areas of soft-sediment and loose gravel substrates at depths between 3 – 18 m (Kirkwood and Burton 1988). Adults measure between 40 and 67 mm and the females carry an average of 80 eggs and juveniles in the brood pouches of the petals (David et al 2005). The juveniles are a red-brown and turn brown as adults (personal observation, B. Sfiligoj).

Pilot Test

Materials and methods

Adult *Abatus shackletoni* were collected using a sieve from nearshore sandy sediments near Davis Station (68˚35'S 77˚58'E), East Antarctica at ~3m depth. Fifteen females were dissected and juvenile urchins were gently removed from brood pouches and allowed one day to acclimate to -0.9 °C ± 0.4 and no light conditions of control temperature cabinet conditions. Six juveniles were placed into 200 mL, acid washed plastic containers for each of 4 replicates for control and copper treatments. Test seawater was collected approximately 40 m offshore at 2-2.5 m depth away from any obvious anthropogenic contaminant inputs. Seawater was filtered to 1 µm and had a salinity of 33 - 34 ppt, dissolved oxygen (DO) content > 90%, and pH of 7.99 - 8.05.

At each observation, individuals were assigned into 1 of 5 behavioural categories based on activity or appearance as follows:

- **Spines moving:** urchin spines moving freely and continuously

- **Spines slow:** urchin spines moving infrequently and slower than previous category
- **Not moving:** urchin spines not moving at all
- **Spine colour:** urchin spines undergoing colour change from yellow/orange/red to black, spines not moving/moving slowly
- **Dead:** urchin spines and test are discoloured and decaying, covered with small parasitic crustacean and spines are slack

Behaviour and mortality was evaluated over a 21 d exposure period with observations and removal of dead individuals on day 1, 2, 4, 7, 10, 14 and 21 the test. Water sampling and water renewal occurred on days 0, 7, 14 and 21 with 10 mL water samples taken both before and after renewals for metal analysis. Samples of test solutions taken at the start of the tests and throughout the tests were filtered through 0.45 µm syringe tip and preserved with 1% (v/v) of ultra pure nitric acid (HNO_3) then analysed using an inductively coupled plasma optical emission spectrometer (ICP-OES). Mean exposure concentrations were determined for each exposure period from water samples taken at the beginning and end of each period, and these average measured metal concentrations were used for determination of point estimates. Sub-lethal (based on active category; EC_{10} and EC_{50}) and lethal (LC_{10} and LC_{50}) point estimate values were determined by Maximum Likelihood Probit analysis in Toxcalc™ (v5.0.26; Tidepool Scientific software).

Results

Behaviour was a more sensitive endpoint than mortality with a pattern of progression from Spines moving, through to Spines slow, Not moving and Dead categories. Spine colour change occurred when the spines were still moving or had stopped, so this category was harder to fit into a pattern. As with the *Skenella paludionoides* microgastropod, the initial departure from the normal active behaviour of continuously moving spines was used as the analytical behavioural endpoint, now termed “active”. Point estimates for active behaviour

(EC_{10} and EC_{50}) and Lethal (LC_{10} and LC_{50}) endpoints are presented in Table 1. Lethal and active behaviour responses to the metal exposure are presented in Figures 1 and 2.

Table 1: Point estimates calculated for active behaviour (EC_{10} and EC_{50}) and Lethal (LC_{10} and LC_{50}) endpoints with 95% confidence limits in brackets for *A. shackletoni* juveniles exposed to copper for 21 d

		Exposure duration (days)			
		2	**4**	**7**	**14**
Copper (µg /L)	**LC_{10}**				76 (29 – 121)
	LC_{50}				262 (182 – 360)
	EC_{10}	43 (0 – 120)	13 (2 – 27)	10 (0 – 21)	
	EC_{50}	480 (216 – 1722)	74 (40 – 110)	27 (6 – 41)	

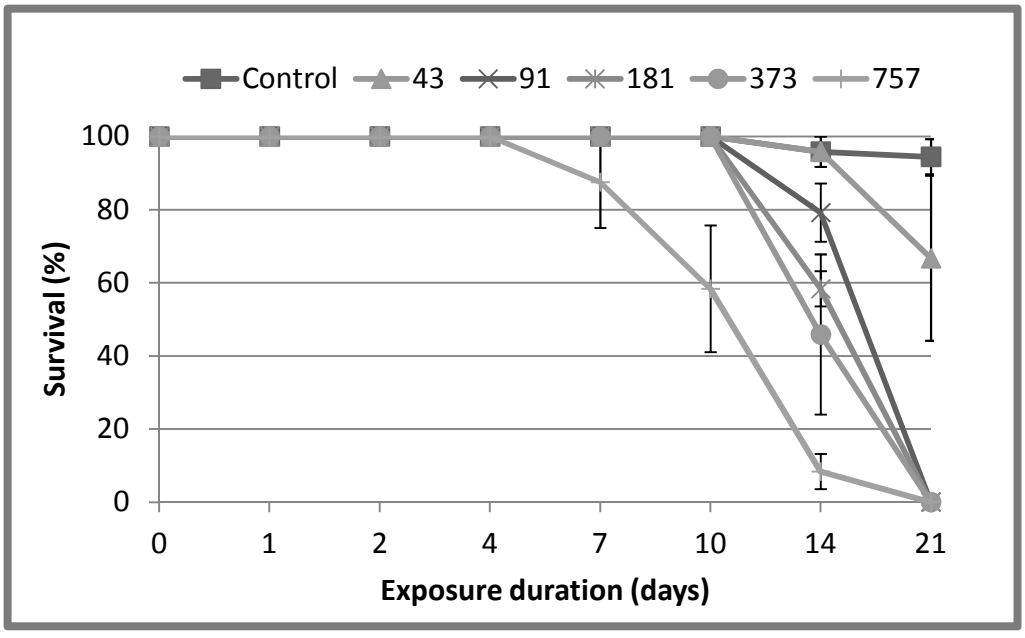

Figure 1: Survival (%, with error bars shown as ± 1 standard error) of *A. shackletoni* juveniles exposed to copper (µg /L) for 21 d

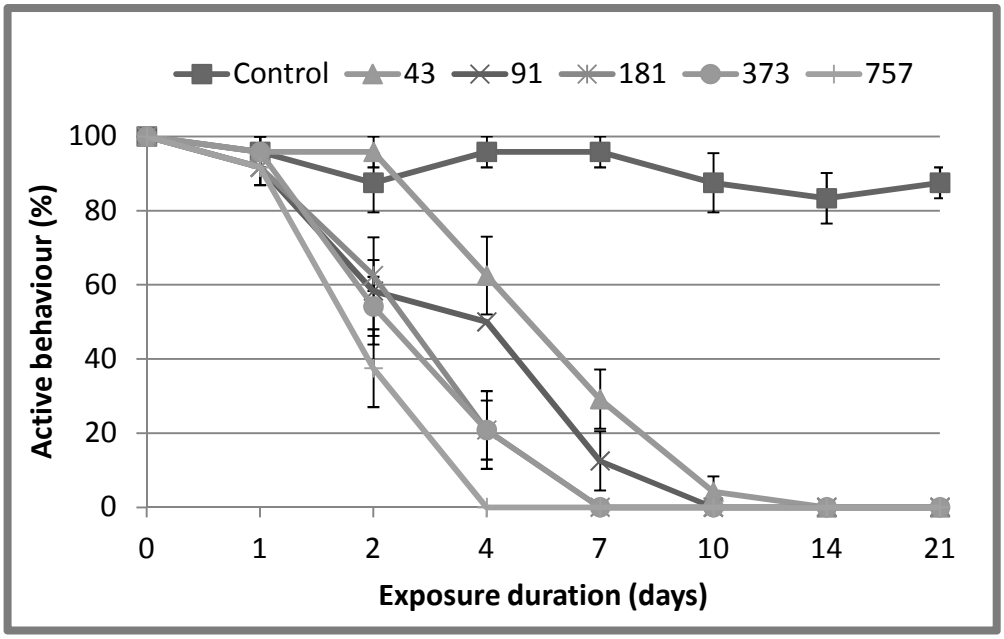

Figure 2: Active behaviour (%, with error bars shown as ± 1 standard error) of *A. shackletoni* juveniles exposed to copper (µg /L) for 21 d

Test 2

Materials and methods

Adult *Abatus shackletoni* were collected using a sieve from nearshore sandy sediments near Davis Station (68˚35'S 77˚58'E), East Antarctica at ~3m depth. Fifteen females were dissected and juvenile urchins were gently removed from brood pouches and allowed one day to acclimate to -0.9 °C ± 0.4 and no light conditions of control temperature cabinet conditions. Six juveniles were placed into 200 mL, acid washed plastic containers for each of 4 replicates for control and copper treatments. Test seawater was collected approximately 40 m offshore at 2-2.5 m depth away from any obvious anthropogenic contaminant inputs. Seawater was filtered to 1 µm and had a salinity of 33 - 34 ppt, dissolved oxygen (DO) content > 90%, and pH of 7.99 - 8.05.

At each observation, individuals were assigned into 1 of 5 behavioural categories based on activity or appearance as described in the pilot study.

Behaviour and mortality was evaluated over a 21 d exposure period with observations and removal of dead individuals on days 2, 4, 7, 10 and 14 of the test. Water sampling and water renewal occurred on days 0, 7 and 14 with 10 mL water samples taken both before and after renewals for metal analysis. Samples of test solutions taken at the start of the tests and throughout the tests were filtered through 0.45 µm syringe tip and preserved with 1% (v/v) of ultra pure nitric acid (HNO_3) then analysed using an inductively coupled plasma optical emission spectrometer (ICP-OES). Mean exposure concentrations were determined for each exposure period from water samples taken at the beginning and end of each period, and these average measured metal concentrations were used for determination of point estimates. Sub-lethal (based on active category; EC_{10} and EC_{50}) and lethal (LC_{10} and LC_{50}) point estimate values were determined by Maximum Likelihood Probit analysis in Toxcalc™ (v5.0.26; Tidepool Scientific software).

Results

As with the Pilot test, behaviour was a more sensitive endpoint than mortality with a clear progression through the behavioural categories from Spines moving, through to Spines slow, Not moving and Dead categories. Spine colour change occurred when the spines were still moving or had stopped, so this category was harder to fit into a pattern. The initial departure from the normal active behaviour of continuously moving spines was used as the analytical behavioural endpoint, termed "active". Point estimates for active behaviour (EC_{10} and EC_{50}) and Lethal (LC_{10} and LC_{50}) endpoints are presented in Table 1. Lethal and active behaviour responses to the metal exposure are presented in Figures 1 and 2.

Table 1: Point estimates calculated for active behaviour (EC_{10} and EC_{50}) and Lethal (LC_{10} and LC_{50}) endpoints with 95% confidence limits for *A. shackletoni* juveniles exposed to copper for 14 d

		Exposure duration (days)			
		2	**4**	**7**	**14**
Copper (µg /L)	**LC_{10}**			312	45 (26 – 58)
	LC_{50}			1291	78 (61 – 99)
	EC_{10}	23 (7 – 39)	16 (3 – 30)	2 (0 – 12)	
	EC_{50}	75 (48 – 106)	55 (29 – 80)	13 (0-34)	

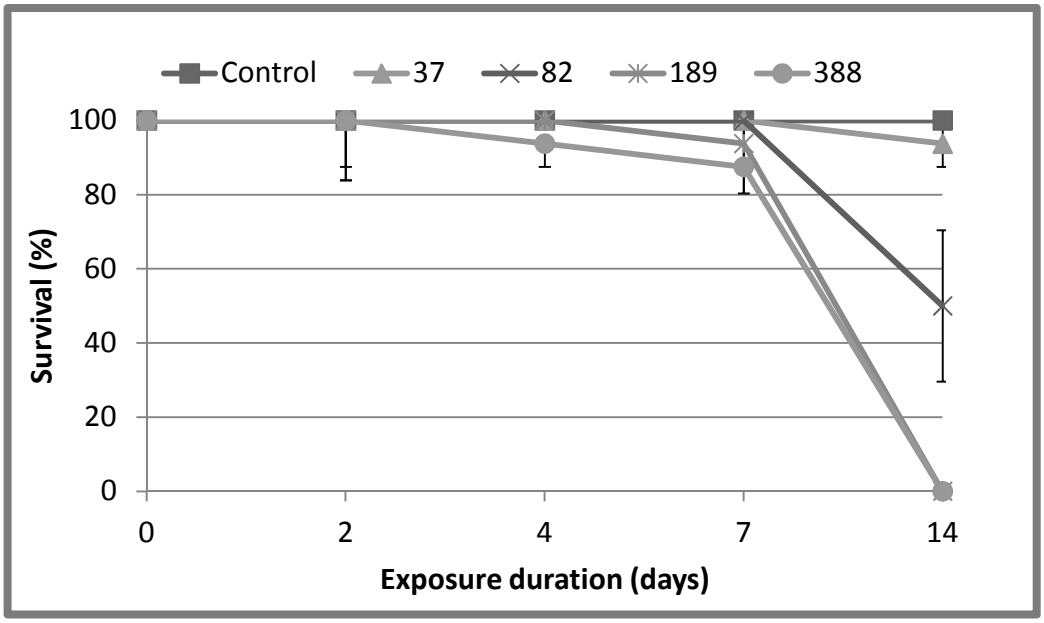

Figure 1: Survival (%, with error bars shown as ± 1 standard error) of *A. shackletoni* juveniles exposed to copper (µg /L) for 14 d

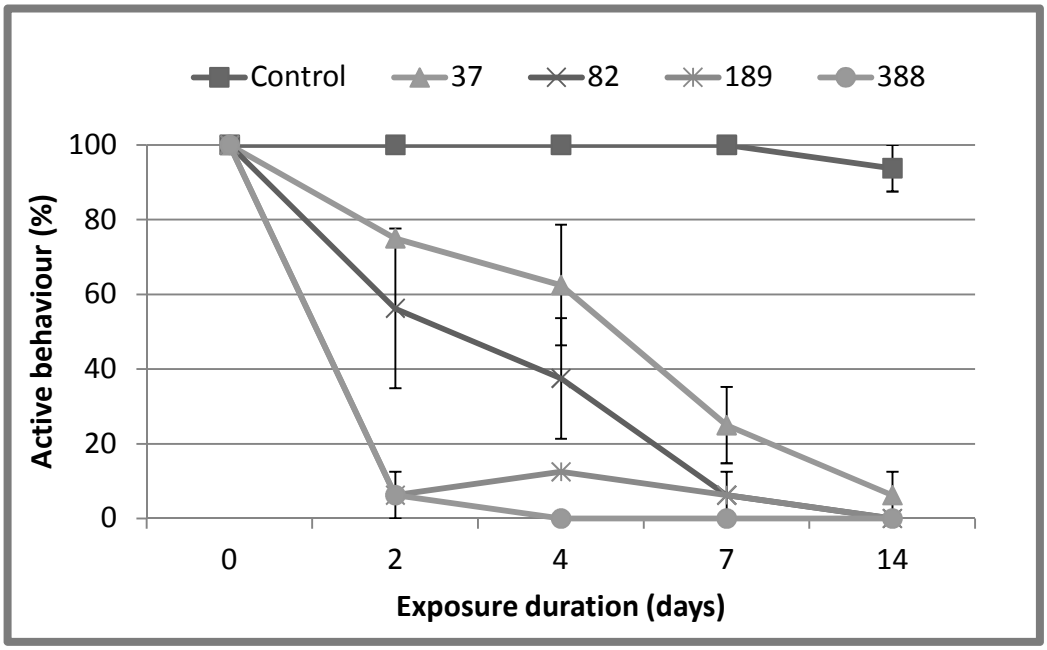

Figure 2: Active behaviour (%, with error bars shown as ± 1 standard error) of *A. shackletoni* juveniles exposed to copper (µg /L) for 14 d

References

David B, Choné T, Mooi R, De Ridder C (2005) Antarctica Echinoidea. Volume 10 Synopses of Antarctic Benthos, Volume 35 These Zoologicae. A.R.G Ganter Verlag K.G.Czech Republic. p 204

Kirkwood JM, Burton HR (1988) Macrobenthic species assemblages in Ellis Fjord, Vestfold Hills, Antarctica. Marine Biology 97 (3):445-457. doi:10.1007/bf00397776

Appendix 4

Toxicity test with the Antarctic amphipod, *Tryphosella murrayi* (Walker, 1903a)

Description:

Information for this species is mostly limited to personal observations during collections and experiments. *Tryphosella murrayi* of the Lysianassidae family, has been collected from many coastal and continental shelf sites around the Antarctic coastline between 5 and 920m of depth (De Broyer et al 2007). Adults collected for experiments were between 12 and 15 mm in length having a yellow carapace and red eyes (personal observations, Bianca Sfiligoj).

Pilot Test

Materials and methods

Adult *Tryphosella murrayi* were collected using dip nets from nearshore, rocky substrate habitats in >1 m depth near Casey Station, East Antarctica (66˚17'S 110˚31'E). Bioassay treatments of copper consisted of 4 replicate 500 mL acid washed plastic containers each containing 5 individuals. Plastic mesh was placed within the test containers to provide a clinging surface.

Test seawater was collected approximately 40 m offshore at 2-2.5 m depth away from any obvious anthropogenic contaminant inputs. Seawater was filtered to 0.22 µm and had a salinity of 33 - 34 ppt, dissolved oxygen (DO) content > 90%, and pH of 7.99 - 8.05. Vials were kept in a constant temperature chamber at -1 (± 0.7) °C over a 12:12 hour photoperiod, with no food added during the test.

At each observation, individuals were assigned into 1 of 6 behavioural categories based on activity or appearance as follows:

- **Active:** amphipod actively clinging to submerged mesh / swimming / walking / feeding
- **Curled:** amphipod in a curled posture on their side on the bottom of the container
- **Out of water:** amphipod actively clinging to mesh but are position above the water line
- **Open posture:** amphipod on their side with open posture fanning their pleopods quickly
- **Negligible movement:** amphipod on their side with open posture and little to no movement of appendages including pleopods
- **Dead:** amphipod not moving and not responding to stimulus

Behaviour and mortality was evaluated over a 46 d exposure period with observations and removal of dead individuals on days 2, 4, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, 30, 32, 35, 37, 39, 42 and 46 of the test. Renewal of test solutions was also conducted on these days, with 10 mL water samples taken both before and after renewals for metal analysis. Samples of test solutions taken at the start of the tests and throughout the tests were filtered through 0.45 µm syringe tip and preserved with 1% (v/v) of ultra pure nitric acid (HNO_3) then analysed using an inductively coupled plasma optical emission spectrometer (ICP-OES). Mean exposure concentrations were determined for each exposure period from water samples taken at the beginning and end of each period, and these average measured metal concentrations were used for determination of point estimates. Sub-lethal (based on active category; EC_{10} and EC_{50}) and lethal (LC_{10} and LC_{50}) point estimate values were determined by Maximum Likelihood Probit analysis in Toxcalc™ (v5.0.26; Tidepool Scientific software) with Abbott's correction being applied when required prior to modelling point estimates to correct for control mortality.

Results

Point estimates for LCx and ECx are presented in Table 1. Control survival was > 80% for the duration of the exposure period with average responses to copper exposure presented in Figure 1. Behaviour was a more sensitive endpoint than mortality yet there was some switching between the categories, particularly between the active and curled behaviours. This lead to a noisy set of response data for the active category and as such, does not display a typical concentration response curve (Figure 2) compared with the lethal endpoint (Figure 1). Amphipods in the copper treatments also climbed out of the exposure water more often than in the controls. This behaviour potentially indicates a drive to escape the copper contaminated water. However, as the amphipods removed themselves from the exposure solution, they have not all sustained the same level of exposure. Future tests should ensure clinging surfaces do not extend above the water line.

Table 1: Point estimates for copper (µg /L) calculated for active behaviour (EC_{10} and EC_{50}) and Lethal (LC_{10} and LC_{50}) endpoints which could be calculated with 95% confidence limits in brackets for *T. murrayi* exposed to copper for up to 37d

	Exposure duration (days)											
	9	**11**	**14**	**16**	**18**	**21**	**23**	**25**	**28**	**31**	**35**	**37**
LC_{10}	472 (201-600)	402 (204-514)	243 (149-296)	217 (139-276)	180 (102-233)	162 (106-196)	162 (106-196)		96 (40-130)	83 (40-112)	81 (47-103)	77 (47-95)
LC_{50}	887 (698-2099)	779 (620-1302)	432 (352-535)	405 (329-501)	313 246-384)	238 (197-285)	238 (197-285		160 (112-198)	146 107-183)	125 (978-152)	114 (91-140)
EC_{10}		342 (93-462)	145 (8-251)	165 (22-117)	167	158		173	61 (10-98)	72 (27 – 98)		
EC_{50}		505 (290-648)	312 (102-449)	264 (117-348)	206	196		199	123 (58-171)	112 (73-144)		

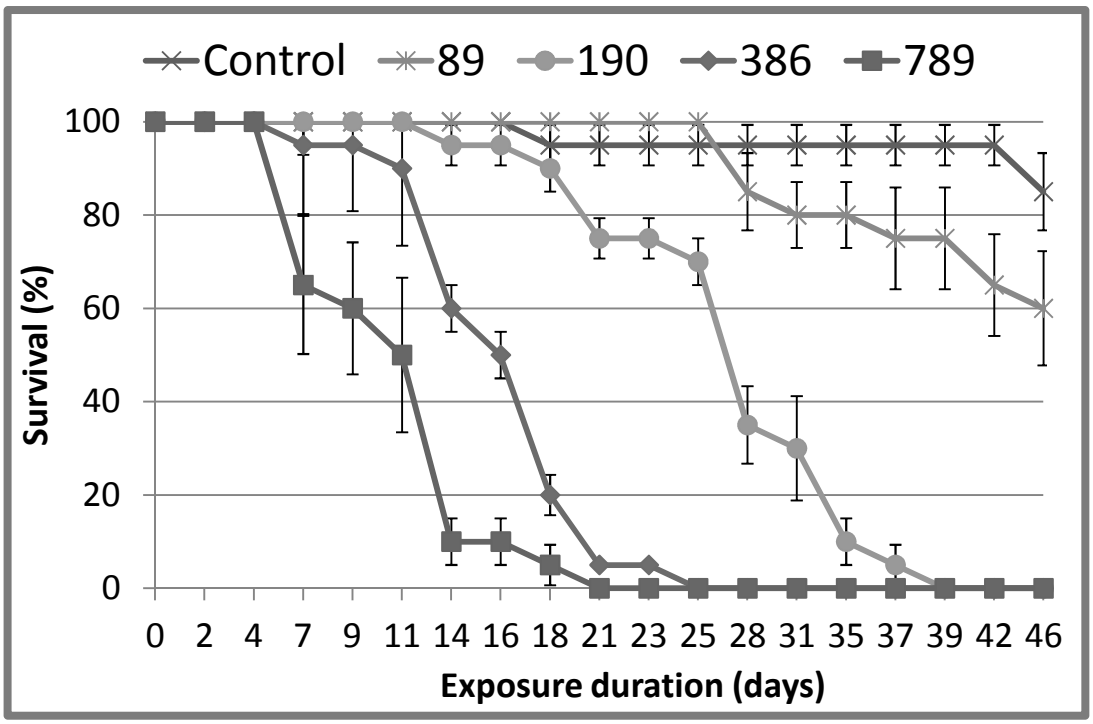

Figure 1: Survival (%, with error bars shown as ± 1 standard error) of *T. murrayi* exposed to copper (µg /L) for 46 d

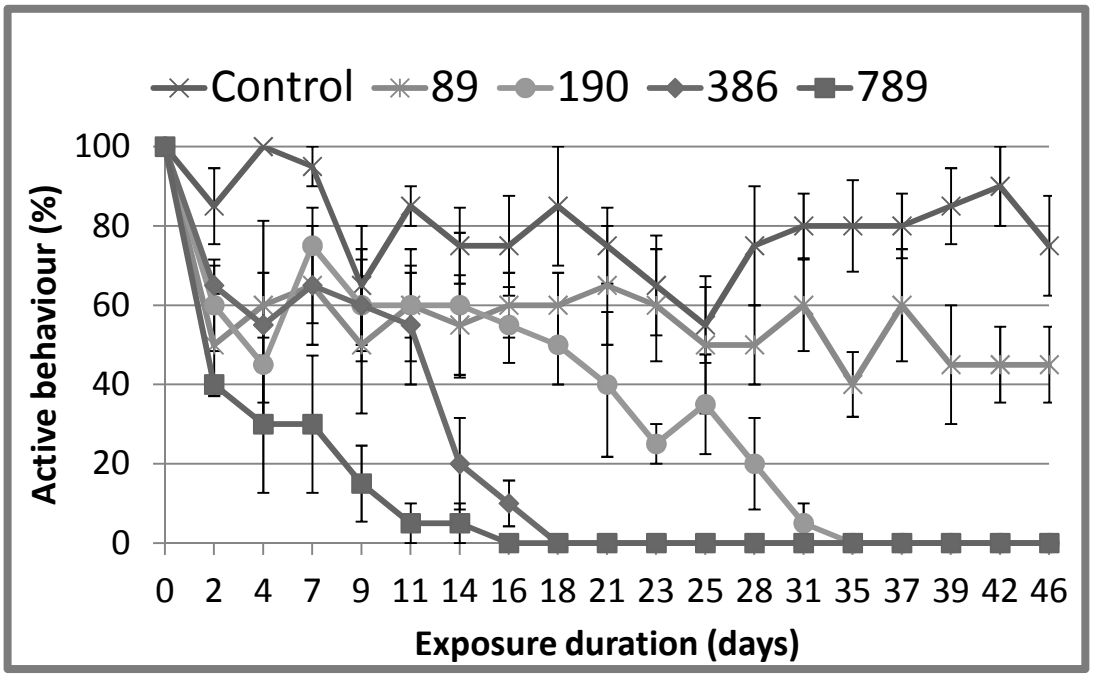

Figure 2: Active behaviour (%, with error bars shown as ± 1 standard error) of *T. murrayi* exposed to copper (µg /L) for 46 d

References

De Broyer C, Lowry J, Jazdzewski K, Robert H (2007) Catalogue of the gammaridean and corophiidean Amphipoda (Crustacea) of the Southern Ocean with distribution and ecological data. In: De Broyer C (ed) Census of Antarctic Marine Life. Synopsis of the Amphipoda of the Southern Ocean vol 1. Bulletin de l'Institut royal des Sciences naturelles de Belgique Biologie 77 pp 1-325

Appendix 5

Behavioural endpoint and point estimate results for the amphipod *Orchomenella pinguides* (Walker, 1903)

Description

The benthic amphipod *Orchomenella pinguides* (Lysianassidae) is a deposit feeding and macrophage (Dauby et al 2001). Adults measure approximately 5-9mm in length, having an orange carapace and red eyes. They can live in high densities on mostly sand / mud substrates between 10 - 20m in depth in the Antarctic near shore environment (personal observations, Bianca Sfiligoj).

Materials and methods

Behavioural observations were conducted during the same two bioassays with *Orchomenella pinguides* that have been reported in detail in Chapter 4: Improved methods for determining the toxicity of metals to an Antarctic amphipod by joint modelling of survival response to exposure concentration and duration. Animal collection, equipment and test solution preparation and toxicity test procedures are described in the Materials and methods section of Chapter 4.

At each observation, *O. pinguides* individuals were assigned into 1 of 4 behavioural categories based on activity or appearance as follows:

- **Active:** behaviour is defined as normal activity characterised by swimming / active clinging to the plastic mesh / walking on the bottom of the container /resting in a curled posture but activated to swimming if stimulated but touch,

- **Side-fast:** behaviour represents the first departure from normal behaviour where the amphipod is observed laying on its side in an open posture or repeatedly stretching from open posture to a curled posture. Pleopods are fanning (hyperventilation) and pereopods / antennae are moving. Amphipods displaying this behaviour did not start swimming when they were stimulated by touch,

- **Slow-twitching:** amphipod is laying on its side with pleopods fanning slowly or twitching. No movement of pereopods or antennae and no movement in response to a touch stimulus

- **Dead:** amphipod not moving and not responding to stimulus

Behavioural (based on active category; EC_{10} and EC_{50}) point estimate values were determined by Maximum Likelihood Probit analysis in Toxcalc™ (v5.0.26; Tidepool Scientific software).

Results

Behaviour was a more sensitive endpoint than mortality yet there was some switching between the categories, particularly between the Side fast and Slow twitching categories. The active behaviour category was statistically analysed as the sub lethal behavioural endpoint using Probit analyses in the ToxCalc software. Point estimates for the active behavioural category (EC) are presented in Table 1. Active behavioural responses to metal exposure for *O. pinguides* in bioassay 1 and bioassay 2 are presented in Figure 1 and 2 respectively.

Table 1: Average point estimates for copper, cadmium, zinc and lead based on active behaviour (EC_{10} and EC_{50}) of *Orchomenella pinguides* over 30 d in bioassay 1 and 2. (Numbers in brackets are 95% confidence limits for point estimates. Where no data is shown, point estimates were unable to be calculated.) Point estimates for survival of *O. pinguides* can be found in Chapter 4, p 97.

		Exposure duration (days)									
		3	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
Copper (µg/L)	EC_{10}	155	12	4	11		3	10	9		
	EC_{50}	397	99	44	69	1	25	34	27		
Cadmium (µg/L)	EC_{10}		1444	633	479	275	159	91	96	63	52
	EC_{50}		1241	737	502	360	215	174	158	128	89
Zinc (µg/L)	EC_{10}				1028	1170	843	697	638	558	517
	EC_{50}				2104	1731	1311	1143	1065	935	872
Lead (µg/L)	EC_{10}		730	589	320	304	245	202	114	59	61
	EC_{50}		3481	2664	1043	801	839	477	334	258	227

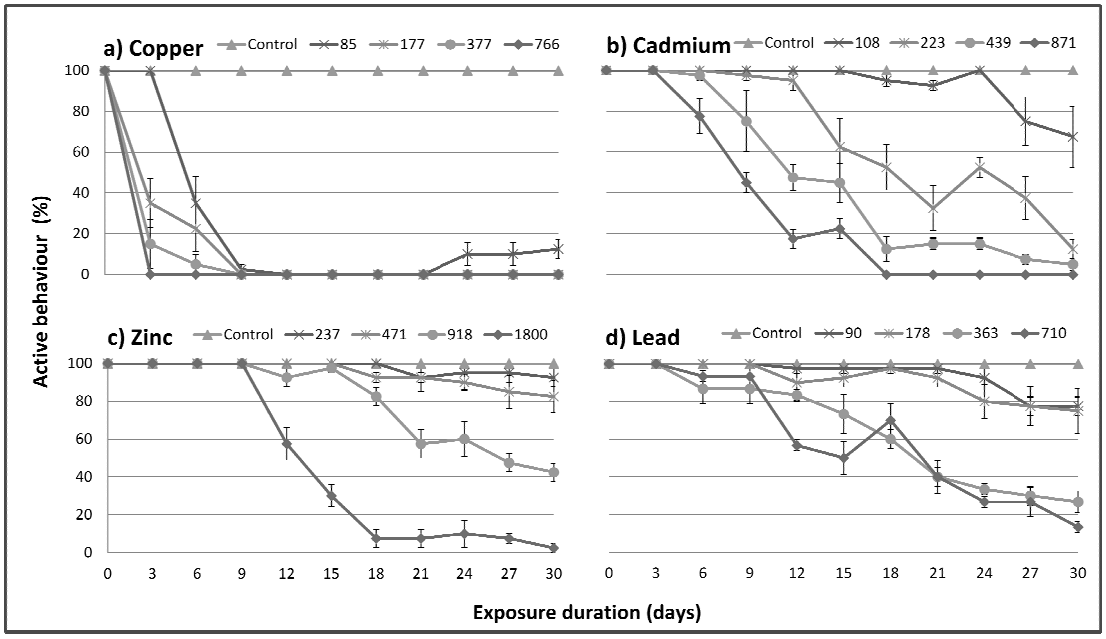

Figure 1: Active behaviour of *Orchomenella pinguides* exposed to a) copper, b) cadmium, c) zinc and d) lead (µg/L) over 30 d in bioassay 1. (Error bars shown are ± 1 SE).

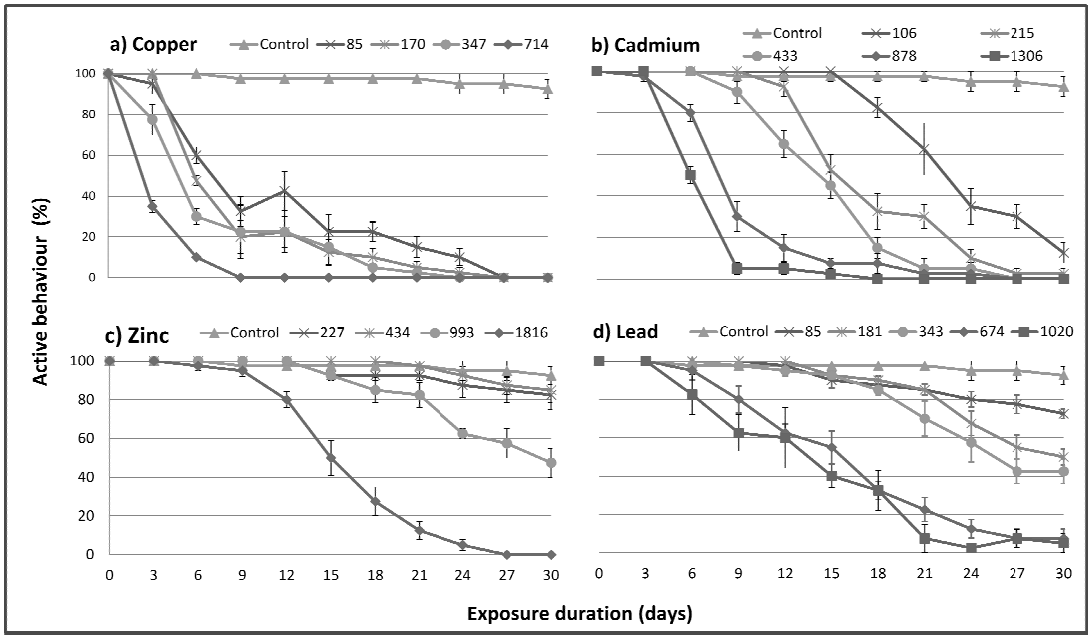

Figure 2: Active behaviour of *Orchomenella pinguides* exposed to a) copper, b) cadmium, c) zinc and d) lead (µg/L) over 30 d in bioassay 2. (Error bars shown are ± 1 SE).

References

Dauby P, Scailteur Y, Chapelle G, De Broyer C (2001) Potential impact of the main benthic amphipods on the eastern Weddell Sea shelf ecosystem (Antarctica). Polar Biology 24 (9):657-662. doi:10.1007/s003000100265