Preliminary Investigation of Effects of Copper on a Terrestrial Population of the Antarctic Rotifer *Philodina sp.*

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ABSTRACT

Terrestrial microinvertebrates in Antarctica are potentially exposed to contaminants due to the concentration of human activity on ice-free areas of the continent. As such, knowledge of the response of Antarctic microinvertebrates to contaminants is important to determine the extent of anthropogenic impacts. Endemic Antarctic *Philodina sp.* were extracted from soils and mosses at Casey station, East Antarctica and exposed to aqueous Cu for 96 h. The *Philodina sp.* was sensitive to excess Cu, with concentrations of 36 µg L^{-1} Cu (48 h) and 24 µg L^{-1} Cu (96 h) inhibiting activity by 50%. This is the first study to be published describing the ecotoxicologically derived sensitivity of a rotifer from a terrestrial population to metals, and an Antarctic rotifer to contaminants. It is also the first study to utilise bdelloid rotifer cryptobiosis (chemobiosis) as a sublethal ecotoxicological endpoint. This preliminary investigation highlights the need for further research into the responses of terrestrial Antarctic microinvertebrates to contaminants.

Keywords:
Antarctica, rotifer, toxicity, terrestrial, copper.

Abbreviations:
EC, Effective concentration; LC, Lethal Concentration; LOEC, Lowest Observable Effect Concentration; NOEC, No Observable Effect Concentration.

INTRODUCTION

Ice-free areas in Antarctica make up less than 0.5% of the total landmass and include areas of exposed soil and rock (Brooks et al., 2019). Antarctica's soils have low productivity and the dominant plants are non-vascular consisting of bryophytes (mosses and liverworts) and lichens yet support the majority of terrestrial diversity on the continent (Lee et al., 2017). Biotic activity is highly cyclical with above zero temperatures, increased sunlight, and free water from melting ice and snow during the summer season creating favourable conditions for adapted biota in an environment that is cold, dark, and dry for much of the year (Pugh and Convey, 2008).

The relatively short history of human activity in Antarctica has resulted in localised areas of contamination, which has been largely concentrated in ice-free areas (Aislabie et al., 2004; Brooks et al., 2019). Elevated concentrations of metals and hydrocarbons attributable to anthropogenic sources have been found in soils around Antarctic and subantarctic research stations compared to nearby sites with minimal historic human activity; for example, near a Robert Island field hut (de Lima Neto et al., 2017) and at New Zealand's Scott Base (Sheppard et al., 2000). Snape et al., (2001) Substantial differences have been found in metal concentrations in contaminated vs. non-impacted control sites at Australia's Casey station with notably elevated concentrations of total arsenic (12 mg kg^{-1}), cadmium (24 mg kg^{-1}), copper (2199 mg kg^{-1}), and nickel (94 mg kg^{-1}) at a tip site, vs. non-impacted control sites (1.0 mg kg^{-1}As; 0.2 mg kg^{-1}Cd; 33 mg kg^{-1}Cu; 38 mg kg^{-1}Ni) among others (Snape et al., 2001).

The impacts of soil contamination on biota is typically assessed using ecotoxicological testing, the results of which have been used to derive soil quality guidelines and assessment frameworks (e.g. National Environment Protection (Assessment of Site Contamination) Measure in Australia (National Environmental Protection Council, 2013). There are, however, currently no soil quality guidelines developed for Antarctica to inform risk assessments to support environmental management of contaminated sites. Using endemic organisms is critical when assessing local impacts, and particularly so in Antarctica due to its unique soil and environmental conditions, which differ substantially even from the most comparable areas of the Arctic (Goryachkin et al., 2004).

Terrestrial environments in Antarctica consist of soils and mosses that provide habitat for the simple ecosystems These ecosystems lack macroinvertebrates such as earthworms and insects, with microinvertebrates such as nematodes, tardigrades and rotifers comprising the dominant terrestrial fauna (Hogg et al., 2006; Yergeau and Kowalchuk, 2008; Nielsen and Wall, 2013). However, studies that assess the effect of contaminants on soil dwelling microinvertebrates are limited, and there are no established standard toxicity testing protocols for Antarctic terrestrial microinvertebrate species. The first paper assessing ecotoxicological effects of Cu on an Antarctic terrestrial nematode (*Plectus murrayi*) was published recently (Brown et al., 2020). The limited research in this area is partially due to difficulty in accessing Antarctic field sites, and in culturing the indigenous biota due to lack of knowledge of basic life history traits. Impacts from contamination of Antarctic soils has previously focussed on microbial communities, e.g. Yergeau and Kowalchuk (2008), Flocco et al. (2019), and Pudasaini et al. (2019).

To improve our understanding of the impact of metal contaminants on terrestrial Antarctic microinvertebrates, this study investigated the toxicity of aqueous Cu to a terrestrial population of the Antarctic rotifer *Philodina sp*. Copper was chosen as the toxicant as it commonly found at elevated concentrations in human-impacted sites in Antarctica.

MATERIALS AND METHODS

Sample collection and invertebrate isolation

Soil and moss samples for the isolation of microinvertebrates were collected from the vicinity of Casey station in the Windmill Islands region of East Antarctica in January and February 2019. Sample collection, isolation and toxicity testing were completed at Casey station, East Antarctica and chemical analysis completed upon return to Australia.

Samples were collected in sterile polypropylene centrifuge tubes, and polyethylene zip lock bags. For soil collection, the surface of the ground was scraped to remove large stones and debris, then loosened to a depth of ~5 cm with mattocks and steel probes and scooped into UV stabilised polyethylene zip-lock bags (Prospectors Pro Earth). Mosses were chosen for sampling based on visual assessment of good health (deep green colour, high density tufts and moderate-high moistness) and collected by cutting small sections (2-3 cm^2) with a steel spatula and tweezers, thus minimising disruption of moss beds.

Invertebrates were extracted from samples by hydrating a small portion of moss or soil (0.1-2 g) with deionised ultrapure water (Millipore MilliQ, 18.2 MΩ cm) in a petri dish, agitating the sample for a few seconds, then allowing the sample to settle for 20 minutes before observing for invertebrate movement under a stereomicroscope (Leica Wild M8). Observed invertebrates were isolated using a micropipette and transferred into petri dishes containing ultrapure water.

Morphologically consistent rotifers were segregated and identified as *Philodina sp.* via the key provided by Ricci and Melone (2000). *Philodina sp.* were the dominant morphospecies and were collected in petri dishes and stored in a temperature-controlled cabinet at 10 °C with a 12/12 hour light/dark cycle until testing commenced. Petri dishes initially contained only ultrapure water but as rotifers were added, clumps of green unicellular algae from samples were also transferred to provide additional food while organisms were accumulated for testing.

Test solution preparation

A Cu stock solution (3 mg/L) was prepared by dissolving 4 mg $CuCl_2.2H_2O$ (>99% purity, Ajax Finechem UNIVAR) in 500 mL of ultrapure water. Nominal Cu concentrations at the test start were 0, 10, 130, 200, 400 and 600 µg L^{-1}. Treatment concentrations were based on the known sensitivity of another Antarctic terrestrial microinvertebrates(Brown et al., 2020). These concentrations are also environmentally relevant, being comparable to water-extractable Cu concentrations reported at a former tip site at Casey station (Snape et al., 2001).

Test setup

Testing was conducted in a 48 microwell plate and consisted of five Cu treatments plus a control of ultrapure water, with 4 replicates per treatment. Each replicate well contained ten individual *Philodina sp.*, allocated randomly from a pool of 250 individuals. Individuals were transferred from isolation dishes into a 'rinse dish' containing only ultrapure water the day prior to test commencement and algae clumps were avoided during this step. Rotifers were transferred individually from the rinse dishes into the test wells using a micropipette, along with 80 µL water from the isolation dish. This was repeated until 10 individuals and 800 µL of 'transfer water' was present in each well. Food was not added to the test wells; however, the transfer water likely contained microbes suitable for consumption.

After all rotifers were added to wells, ultrapure water and Cu stock solution (total of 400 µL) were added sequentially to produce the nominal treatment concentrations in a final volume of 1200 µL/well. Well plates were kept in a temperature-controlled cabinet at 10 °C with a 12/12 hour photoperiod for the duration of the test.

Observations

The test duration was 96 h, with rotifers assessed at 48 h and 96 h. Assessments were conducted using a Leica Wild M8 stereo microscope at 18-50x magnification. Individuals were scored as active (visibly moving, feeding or swimming) or immobile (no movement of the individual for 10 seconds, no response to gentle prodding with metal probe). Immobile individuals were further classified as alive or dead. Immobile individuals classified as alive were observed in a contractedstate and despite no external movement or activity, otherwise showed signs of life (still actively anchored to the test vessel or internal movement visible). Dead individuals were observed fully extended and relaxed with no anchor to the vessel or internal movement observed.

Chemical analysis

After the test, solutions from each well were acidified to 3% nitric acid (AR grade 69.0-70.0% w/w, Rowe Scientific) and refrigerated at 4°C until returned to Australia for analysis. Copper concentrations in test solutions were measured using inductively coupled plasma - mass spectrometry (ICP-MS; Agilent 7000 series, Agilent Technologies Australia, Mulgrave, Victoria). Samples were measured against a calibration curve of ICP-MS standards (Agilent Multi-element calibration standard-2A; 5-100 µg L^{-1} Cu), with nitric acid blanks measured after every 8 samples. Samples were diluted to fit within the calibration curve based on nominal concentrations. The limit of detection (LOD) for analysis for Cu was 0.093 µg L^{-1}. Measured concentrations for each nominal concentration (0, 10, 130, 200, 400 & 600 µg L^{-1} Cu) were (mean ± SD): 0 ± 0, 13 ± 17, 128 ± 11, 192 ± 108, 391 ± 172 and 598 ± 137 µg L^{-1} Cu respectively. Measured concentrations were used for all statistical analysis and graphics.

Statistical analyses

Response data were calculated as percent survival (active plus alive-immobile) and percentage of active individuals observed for each replicate and timepoint. The test criteria for quality control was ≥80% of organisms active in the controls.

Dose-response modelling utilised the *drc* (Ritz et al., 2015) and *tidyverse* (Wickham et al., 2019) packages in R 3.6.1 (R Core Team, 2019). Response data and Cu exposure concentrations for each treatment were fitted to six polynomial regression models (3-, 4- and 5- parameter log-logistic, 3 parameter log-logistic with fixed upper limit of 100, 4-parameter Weibell type 1 and 4 –parameter Weibell type 2) using *drc*. Model fit was determined using Lack of Fit and Akaike Information Criterion (AIC), with a good fitting model having a low AIC and non-significant lack of fit (p>0.05). The best-fitting model was chosen for determination of Effective Concentrations (ECs); in all cases, this was a Weibell type 1, four-parameter model.

Effective Concentrations (µg L^{-1} Cu) were derived from the modelled curves for active individuals using *drc* and plotted using *ggplot2* and *ggpubr* R packages (Kassambara, 2020).

Lowest Observed Effect Concentrations (LOECs) and No Observed Effect Concentrations (NOECs) were reported for both active and alive individuals. Data were first checked for homogeneity via Levene's Test, after which Two-way Analysis of Variance (ANOVA) (time x treatment) followed by Tukey's multiple comparisons test were conducted at each time point using the *rstatix* R package as appropriate (Kassambara, 2021). Differences were considered significant at $p \leq 0.05$

RESULTS AND DISCUSSION

All individuals in control replicates were alive at the end of the test, and the majority were active (89 ± 8% (mean ± SD); Figure 2); as such, testing met quality control criteria. The two endpoints (alive and active individuals; Figure 1) gave different assessments of toxicity; activity was more responsive and consistent across replicates while survival was more variable across treatments and within replicates. Copper concentrations of ≥ 192 µg L^{-1} had significantly higher mortality of rotifers than the control treatment (p=0.0115; Figure 1). Significantly lower activity was observed at Cu concentrations ≥ 128 µg L^{-1} compared to control (p<0.0001; Figure 3).

We hypothesise that using activity as a sublethal endpoint will be more sensitive and environmentally relevant as it represents the stage of exposure at which the interaction of the rotifers with their environment is first inhibited, removing them from active function in the soil ecosystem.

Cryptobiosis is currently understood to be induced by one of 5 stressors; desiccation (anhydrobiois), osmotic pressure (osmobiosis), freezing (cryobiosis), low oxygen (anoxybiosis), and environmental chemicals (chemobiosis) with the latter two poorly studied and as such lacking a body of evidence (Mobjerg and Neves, 2021). Tuns are the most well studied crytobiotic state in rotifers and have been observed in response to desiccation. We propose that the contraction and suspension of activity observed in the present study could be an example of chemobiosis. Their prevalence was observed to be dose-dependent on the concentration of dissolved copper in the treatments (Figure 1). Inactive individuals could not be distinguished from tuns of this species previously observed in response to desiccation (McCarthy pers. obs.). Similar discussions are reported in the literatureregarding tardigrade cryptobiosis, with evidence of dose-dependent chemobiosis in response to Cu exposure in *Echiniscus testudo* and *Halobiotus crispae* (Hygum et al., 2017).

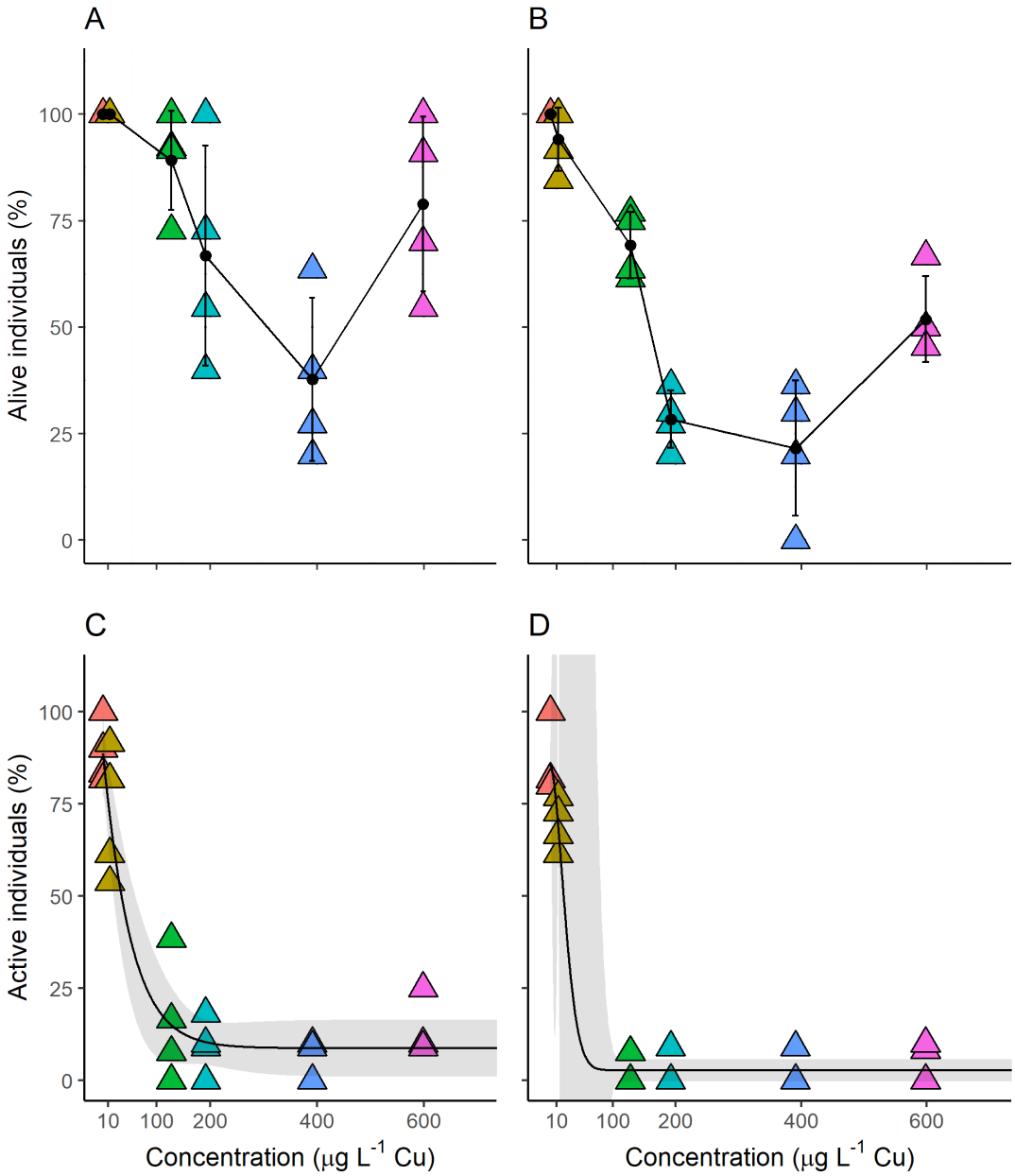

Figure 1. Response of *Philodina sp.* in terms of survival and activity to exposure to Cu at 48 h (A, C) and 96 h (B, D). Plots A and B show mean survival ± standard deviation (black points), Plots C and D show activity data with modelled dose response curve and shaded ± 95% confidence intervals Markers are colour-coded by treatment, equations for modelled data available in supplementary information S1.

None of the models fit the mortality data, with the model options all having significant Lack of Fit (p<0.05) and high AIC, so derivation of lethal concentrations (LCs) was not possible. Sublethal endpoints by their nature appear earlier in an exposure period than lethal endpoints and it is likely that the test duration was insufficient for mortality trends to become apparent in these data (Snell et al., 2017).

For the activity data, a Weibell 1.4 model was found to be the most appropriate for both time points based on an insignificant goodness of fit (p>0.05) and comparison of AIC values between trialed models. The EC_{50} values for activity had increased sensitivity to Cu with exposure time, with lower concentrations of Cu required for a given response at 96 h compared to 48 h (Table 1), data for lower sensitivity estimates were unclear. Uncertainty in EC estimates remained high as evidenced by the large confidence intervals. This uncertainty was likely in part due to using organisms isolated directly from field samples. While individuals of consistent size were targeted, they are likely to represent a wider spread of maturity and genetic variation than could be obtained using cultured test organisms. As test organisms were collected from soils away from any obvious sources of contamination, and with low background concentrations of contaminants (data not shown), previous exposure to Cu or other contaminants is unlikely to and have substantially impacted sensitivity.

Snape et al. (2001) (Mikkonen et al., 2018); (de Caritat and Grunsky, 2013)).

Table 1. Summary of effective concentrations (EC_x), no observed effect concentrations (NOECs) and lowest observed effect concentrations (LOECs) for activity and survival of *Philodina* sp. in an aqueous medium in response to exposure to copper (µg L^{-1}). The 95% confidence intervals are in parenthesis. (See Figure 1 and Supplementary Information S1 for the graphs and model equations respectively

	Activity					**Survival**	
Test duration	**NOEC**	**LOEC**	**EC_{10}**	**EC_{20}**	**EC_{50}**	**NOEC**	**LOEC**
(h)	µg L^{-1}						
48	13	128 ****	6 (0-14)	12 (0-25)	36 (11-62)	128	192 **
96	13	128 ****	9 (0-46)	13 (8-18)	24 (0-201)	13	128 *

Significant difference compared to the control: *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001

This is the first published study on the response of an Antarctic rotifer (terrestrial or aquatic) to Cu. The *Philodina sp.* used in this test were found to be highly sensitive to Cu compared to the only other Antarctic terrestrial microinvertebrate that has been tested, the nematode *P. murrayi* (Brown et al., 2020). No mortality of *P. murrayi* was observed for 3 days at 500 ug/L (the maximum exposure concentration tested; Brown et al., 2020), while mortality in *Philodina sp.* was observed at 192 and 128 µg/L following 2d and 4d exposure respectively (Table 1, LOECs). Sublethal endpoints for the two studies were not directly comparable. Differences in sensitivity between these species are likely due to differences in tolerance to Cu but may also be due to different exposure periods and endpoints used.

There are few published studies on the effects of Cu on *Philodina sp.* and only one has reported a sublethal endpoint (Table 2). Snell et al. (2017) reported both reproduction and ingestion endpoints for a *Philodina sp.* to Cu over a short time period, however differences in endpoints and timing of when responses were assessed make direct comparison with the current study difficult.

Table 2. Summary of global published studies on the toxicity of Cu to rotifers. Numbers from the present study presented in bold with 95% confidence intervals in parenthesis.

Species	Class	Endpoint	EC/LC_{50} ($\mu g\ L^{-1}$)	Exposure duration (h)	Test medium	Natural habitat	Temperature (°C)	Study
Philodina sp. (a)	**Bdelloidea**	**Activity**	**36 (11-62)**	**48**	**Ultrapure water**	**Moss & soil**	**10**	**Present study**
			24 (0-201)	**96**				
Philodina acuticornis	Bdelloidea	Mortality	1500	48	Soft artificial dilution water	Freshwater	20	(Buikema et al., 1974)
			800	96				
Philodina sp. (b)	Bdelloidea	Mortality	440	24	EPA medium	Freshwater	25	(Snell et al., 2017)
		Reproduction	350	120				
		Ingestion	140	1				
Brachionus calyciflorus	Monogononta	Mortality	17	24	Moderately hard synthetic freshwater	Freshwater	10	(Snell et al., 1991b)
Brachionus plicatilis	Monogononta	Mortality	130	24	Synthetic seawater	Seawater	25	(Snell and Persoone, 1989)
Brachionus plicatilis	Monogononta	Mortality	63	24	Synthetic seawater	Seawater	25	(Snell et al., 1991a)

Rotifers in the environment belong to two distinct classes (Bdelloidea and Monogononta) with potentially important differences in environmental stress response between the classes. *Philodina* genus (including the Antarctic species tested here) belong to the bdelloid class and can enter a dormant cryptobiotic state in response to physical stress throughout their life cycle. In comparison, *Brachionus sp.* (Table 2), belongs to the Monogononta class and responds to stress by producing 'resting eggs' (diapause) which are resistant to environmental stress, but hatched individuals lack the equivalent of a cryptobiotic state (C., 2001). This makes it difficult to directly compare sensitivities between the two groups, especially in relation to the use of activity as an endpoint. That is, a non-moving bdelloid may be alive but in a dormant state, while a non-moving monogonont is very likely dead.

Studies with Antarctic species introduce a number of challenges compared to temperate and tropical species. The natural environment is a large factor, as conditions vary substantially not only seasonally but also day to day. In many locations, air temperatures are below freezing for most of the year, and even during the summer months, temperatures fluctuate and sub-zero temperatures are common. This means organisms in the field are exposed to variable conditions

(temperature, availability of free water) throughout their active period, making accurately simulating field conditions in the lab a difficult process. For test consistency, our study was conducted at 10 °C, the upper range of temperatures reported in surface soils at Casey station (McWatters et al., 2016).

Utilising indigenous species for ecotoxicological testing is particularly important for unique or extreme environments, where using a generic model species or procedures are not appropriate. This study builds on the knowledge of the sensitivity of Antarctic terrestrial microinvertebrates to contaminants, first presented by Brown et al. (2020), providing preliminary response data for a novel test species bdelloid rotifer,. Sourcing the *Philodina sp.* from mosses and soils around Casey station provided an accessible and environmentally relevant test species for exposure to toxicants; being the dominant morphospecies in the samples taken and sourced close to the station where contamination events are most likely.

To the authors' knowledge, this is the first study to utilise entering the immobile cryptobiotic state as an end point in toxicity test with bdelloid rotiferas an endpoint in ecotoxicity testing. While similar, Snell and Persoone (2021) used emergence from a tun state as an indicator of sensitivity, not entering cryptobiosis as in the present study. Delineating active individuals from those that were alive but in an immobile cryptobiotic state enabled the derivation of effective concentration estimates for individuals based on behavioural responses to Cu exposure. Behavioural responses are generally considered to be a sensitive and accurate approach to assessing toxicants impacts (Gerhardt, 1996), and thus we suggest further use of this immobile cryptobiotic state for establishing ecotoxicity endpoints in species that utilize this response to environmental stress.

CONCLUSION

To our knowledge, this is the first published ecotoxicology study using a terrestrial rotifer population as the test organism, with few published studies on the general sensitivity of terrestrial rotifer populations to contaminants (e.g. a North American field study including rotifer responses to sulfur dioxide by Leetham et al. (1982)). In comparison, there have been multiple ecotoxicological studies on aquatic rotifer populations (e.g. Table 2). It's not currently clear how distinct aquatic and terrestrial rotifer populations are however research in Antarctica has shown that Antarctic rotifers show high levels of genetic endemism across the continent, suggesting that the inherently isolated terrestrial populations are likely to be distinct (Velasco-Castrillón et al., 2014; Iakovenko et al., 2015).

Thus, this research provides important new information on how an Antarctic bdelloid rotifer responds to excess Cu. This research has demonstrated the potential to use cryptobiosis in developing behavioural ecotoxicity endpoints. It is suggested that future research includes longer exposure durations to potentially derive lethal toxicity estimates, and development of reproductive endpoints to determine chronic toxicity for the species. In addition, as the population was terrestrial, it is suggested more soil-like test media are investigated to improve environmental relevance.

CONFLICT OF INTEREST STATEMENT:

The authors declare that they have no known conflicts of interest including but not limited to intellectual property, relationships with other entities, or financial gain that could be perceived to have influenced the work reported in this paper.

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404

SUPPLEMENTARY INFORMATION

Table S1. **Model equations for Activity data.**

Below are the model equations used to obtain the Activity EC values presented in this study. They are presented here to allow for the determination of different ECs to those presented in the paper. Weibell type 1, four-parameter model used throughout. y = active individuals (% of control), x = Cu concentration in test media (µg L^{-1})

Weibell type 1, four-parameter model - generalised	$y = c + (d - c)exp^{(-exp^{(b(\log x - \log e))})}$
Activity at 48 h	$y = 8.74 + (88.72 - 8.74)e^{(-e^{(1.03(\log(x) - \log(51.96)))})}$
Activity at 96 h	$y = 2.76 + (85.46 - 2.76)e^{(-e^{(1.78(\log(x) - \log(29.65)))})}$