Environmental Toxicology and Chemistry—Volume 43, Number 7—pp. 1583–1591, 2024
Received: 2 January 2024 | Revised: 21 February 2024 | Accepted: 7 April 2024

Environmental Toxicology

Glutathione-Mediated Metal Tolerance in an Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*)

Rebecca J. Webb,[a,*] Christopher Cuff,[b] and Lee Berger[a]

[a]Melbourne Veterinary School, University of Melbourne, Werribee, Victoria, Australia
[b]C&R Consulting, Townsville, Queensland, Australia

Abstract: The spread of the amphibian chytrid fungus *Batrachochytrium dendrobatidis*, which causes the disease chytridiomycosis, has resulted in amphibian declines and extinctions worldwide. Some susceptible amphibian species can persist in contaminated habitats, prompting the hypothesis that *B. dendrobatidis* might be sensitive to heavy metals. We tested a panel of 12 metals to rank their toxicity to *B. dendrobatidis* zoospores and zoosporangia during a 6-h exposure. To better understand the mechanism for metal detoxification, we also evaluated whether glutathione is required for metal tolerance by depleting cellular glutathione before metal exposure. In addition, we investigated whether prior exposure to low metal concentrations impacted tolerance of subsequent exposure, as well as identifying metal combinations that may act synergistically. Silver (Ag), cadmium (Cd), and copper (Cu) were particularly toxic to *B. dendrobatidis*, with zoospore minimum lethal concentration values of 0.01 mM (Ag), 0.025 mM (Cd), and 0.5 mM (Cu). These three metals along with zinc (Zn) were also inhibitory to zoosporangia, with minimum inhibitory concentration values of 0.005 mM (Ag), 0.04 mM (Cd), 0.075 mM (Cu), and 0.04 mM (Zn). The fungicidal effects of several metals was reduced when assays were conducted in nutrient medium compared with synthetic pond water, highlighting the need for careful in vitro assay design and interpretation. Glutathione depletion strongly influenced tolerance of Cd and Ag (85% and 75% less growth, respectively) and moderately influenced tolerance of Cu, Zn, and lead (37%, 18%, and 14% less growth, respectively), indicating the importance of glutathione for metal detoxification. In general, the minimum metal concentrations that inhibited growth of *B. dendrobatidis* far exceeded values detected in contaminated amphibian habitats in Australia, suggesting that metal contamination alone may not have a strong protective effect against chytridiomycosis. We discuss future research directions to futher understand the potential for dissolved metals to create chytrid refuges. *Environ Toxicol Chem* 2024;43:1583–1591. © 2024 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Amphibians; *Batrachochytrium*; Chytrid; Freshwater toxicology; Heavy metals

INTRODUCTION

The Chytridiomycota, or "chytrids," are a group of ancient, early diverging fungi found in almost every environment on earth (Longcore & Simmons, 2020). Most chytrids are saprobes; however, there are some parasitic species which infect plants, algae, and insects (Gleason et al., 2008). Only two species are known to parasitize vertebrates, *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus, and its salamander-infecting sister species *B. salamandrivorans*. Chytrids are characterized by their unwalled motile flagellated zoospores, which allow dispersal through moist environments. The short-lived zoospores of *B. dendrobatidis* are attracted to the host epidermis by chemotaxis (Thekkiniath et al., 2013) and then penetrate the skin via a germ tube (Greenspan et al., 2012), before developing into zoosporangia. Mature zoosporangia divide asexually into infective zoospores, which are released into the environment via discharge tubes. As disease burdens increase, host skin function decreases, leading to electrolyte imbalance and cardiac arrest.

Chytridiomycosis due to *B. dendrobatidis* has been named the worst disease of wildlife (Skerratt et al., 2007), having caused severe declines and extinctions in hundreds of amphibian species. Its epidemiology is strongly influenced by temperature (Bradley et al., 2019; Greenspan et al., 2017); however, the interaction between chytrid fungi and the chemical properties of aquatic environments could also drive infection dynamics.

This article includes online-only Supporting Information.

* Address correspondence to rebecca.webb@unimelb.edu.au
Published online 10 May 2024 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.5885

Batrachochytrium dendrobatidis is sensitive to salt (Stockwell et al., 2012), and elevated water salinity is associated with lower disease prevalence and transmission in both experimental (Clulow et al., 2018) and natural (Heard et al., 2014) settings, giving rise to the idea of "salt refuges" (Stockwell et al., 2015). The perplexing observations that vulnerable green and golden bell frogs (*Litoria aurea*) can thrive in highly disturbed habitats, such as disused quarries (White & Pyke, 2014), and that amphibian species richness can be higher in polluted urban areas (Lane & Burgin, 2008) prompted the suspicion that *B. dendrobatidis* might be sensitive to metal contamination (Department of Environment and Heritage, 2006; Threlfall et al., 2008). Two metals, copper (Cu) and zinc (Zn), have been shown to inhibit in vitro growth of *B. dendrobatidis* (Boisvert & Davidson, 2011; Threlfall et al., 2008); and Cu can reduce disease in infected hosts (Parris & Baud, 2004). The effect of other metals is unknown, but if they suppress *B. dendrobatidis* growth more so than amphibian health, contaminated habitats may provide "metal refuges" from chytridiomycosis.

The toxic effect of heavy metals appears largely due to the induction of oxidative stress (Wysocki & Tamás, 2010), and therefore it is not surprising that the antioxidant glutathione can be protective (Jozefczak et al., 2012). Glutathione also possesses a thiol group with high metal affinity, allowing detoxification via chelation and sequestration (Pócsi et al., 2004). Glutathione is important during cadmium (Cd) stress in *B. dendrobatidis* (Webb et al., 2024), but the role of glutathione in the tolerance of other metals is unknown. Because Cd stimulates a rapid increase in cellular glutathione (Webb et al., 2024), it is possible that pre-exposure to metal might prime cells and protect against future metal stress.

In the present study, we investigated the sensitivity of *B. dendrobatidis* zoospores and zoosporangia to a panel of 12 metals. The minimum lethal concentration (MLC) for zoospores is an indication of metal concentrations which may prevent disease transmission. In zoosporangia, which are often more resistant, we determined the minimum inhibitory concentration (MIC) as an indication of metal concentrations which may prevent or reduce disease progression. Because environmental heavy metals are unlikely to occur in isolation, we also investigated whether exposure to a mix of two metals produces a synergistic effect. Previous studies of metal susceptibility involved exposing *B. dendrobatidis* to metals in a nutrient-rich growth medium (Threlfall et al., 2008), which may not represent realistic amphibian habitats. Therefore, we tested the effect of exposure medium on metal toxicity, comparing growth medium to synthetic pond water. Finally, we established whether tolerance of each metal involves the glutathione system by depleting cellular glutathione before metal exposure and whether metal pre-exposure can prime *B. dendrobatidis* cells for increased metal tolerance.

METHODS

B. dendrobatidis culture

The three *B. dendrobatidis* cultures used in the present study were isolated from two naturally infected wild frogs collected from remote, sparsely populated locations and from an invasive newt collected from an urban site: a whistling tree frog (#46 Waste point—*Litoria verreauxii*—5-2013-LB,RW), a Victorian smooth froglet (#107 Eildon—*Geocrinia victoriana*—2022-RW), and a smooth newt (#109 Melbourne—*Lissotriton vulgaris*—2023-LB) following standard protocols (Fisher et al., 2018; Waddle et al., 2018). Cultures were maintained in tryptone–gelatin hydrolysate–lactose (TGhL) medium in 75-cm^2 culture flasks (TPP) following standard in vitro protocols (Longcore et al., 1999; Robinson et al., 2020).

Growth assays

Two assays were used to estimate growth: a methylene blue assay, which is an inexpensive method based on cell volume, and BacTitre-Glo, a commercial kit assay based on levels of adenine triphosphate (ATP). The methylene blue assay is a modified version of a cell viability assay (Webb et al., 2019) used for MIC determination. After exposure to metals, medium was removed, and cells were fixed with 70% ethanol for 1 min. The cells were stained with 0.2 mg/mL methylene blue for 1 min, followed by gentle washing with water to remove traces of dye. The dye was eluted using hydrochloric acid (HCl), with 40 µL 0.1 M HCl added per well, and incubated for 15 min; the HCl was transferred to a new 96-well plate and absorbance measured at 650 nM using an Omega Polarstar spectrophotometer (using 0.1 M HCl as a blank). The amount of growth was determined as relative to the untreated control after subtracting the value of the negative control. The BacTitre-Glo assay (Promega) is an ATP cell viability assay which can detect subtle differences in *B. dendrobatidis* growth (Webb et al., 2024). Cell growth was measured per the manufacturer's instructions. Growth was calculated from the blank-corrected relative light units as relative to the average control value.

Metals

Metal solutions were prepared by dissolving metal salts in MilliQ water, followed by filter sterilization and stored in glass vials at 4 °C. The metals used were silver as $AgNO_3$ (Sigma), aluminum as $AlCl_3 \times 6H_2O$ (BHD), barium as $BaCl_2 \times 2H_2O$ (Univar), Cd as $CdCl_2$ (Sigma), cobalt (II) as $CoCl_2 \times 6H_2O$ (Merck), Cu (II) as $CuCl_2$ (Sigma), chromium (II) as $CrCl_2$ (Sigma), iron (III) as $FeCl_3 \times 6H_2O$ (Sigma), manganese (II) as $MnCl_2 \times 4H_2O$, nickel (II) as $Ni0_4S \times 7H_2O$ (Fluka), lead (II) as $Pb(NO_3)_2$ (Sigma), and Zn as $ZnCl_2$ (Sigma). Metal concentrations for each experiment are listed in the Supporting Information. We used metal chlorides where possible because this form is commonly found in the environment.

Toxicity of individual metals to zoospores

The acute toxicity of each metal was determined by exposing zoospores to a range of metal concentrations for 30 min, adapting a method from Berger et al. (2009). Pure zoospore suspensions were obtained by removing the TGhL medium from

mature flasks and incubating the zoosporangia monolayer with sterile dilute salt (DS) solution (Cashins et al., 2013), pH 6.7, for approximately 2 h (Prostak & Fritz-Laylin, 2021; Robinson et al., 2020). The zoospore solution was syringe-filtered using a 10-μm isopore filter (Millipore) to exclude zoosporangia and concentrated by centrifugation at 2500 *g* for 5 min. Zoospores (5×10^4 in 100 μL) were added to 96-well plates along with the relevant metal, with two replicate wells per concentration. After 30-min incubation, wells were observed under an inverted microscope (CK2 Olympus), and zoospore viability was ranked based on movement compared to the control. The MLC was designated as the lowest concentration with no zoospore movement after 30 min. Zoospores were observed again after 24 h to confirm lack of growth. Zoospore MLC values were tested using Isolate #107 for all metals, and a subset of metals were also tested on Isolates #46 and #109.

Toxicity of individual metals to zoosporangia

The MIC of each metal was determined by exposing zoosporangia to metal solutions for 6 h and then monitoring growth. Zoospores were added to 96-well plates at 5×10^4 zoospores per well and incubated at 20 °C in TGhL overnight to encyst into zoosporangia. Excess TGhL medium was removed, and the adhered cells were gently washed with sterile DS. Two replicate wells were exposed to each concentration of metal in 200 μL DS and incubated at 20 °C. Concentrations for each metal are listed in Supporting Information, Figure S5. After 6 h, 20 μL of 10 × TGhL solution was added, and the plates were incubated at 20 °C. Positive (DS only) and negative (70% ethanol) controls were included. After 48 h, cells were observed under an inverted microscope, and growth was calculated relative to the control using a modified methylene blue assay (Webb et al., 2019). The MIC was determined as the lowest concentration in which growth did not differ from the negative control (Rollins-Smith et al., 2002). The MIC values for all metals were determined using Isolate #107, and a subset of metals were tested on Isolates #46 and #109. We also quantified the lowest concentration where zoosporangia started to display negative effects (e.g., stunted growth or large vacuoles), using the MIC graphs in combination with light microscopic observations. We have termed this point the "minimum effect concentration" (MEC). Exposure to the MEC will produce mild growth inhibition.

Effect of media on metal toxicity

To test whether the exposure solution influenced toxicity, one concentration (2 × MIC) was chosen for each metal and formulated in TGhL nutrient medium (pH 7.2) and DS (pH 6.7). Zoospores were added to 96-well plates at 5×10^4 zoospores per well and incubated at 20 °C overnight to encyst into zoosporangia. Excess TGhL medium was removed, cells were washed with DS, and 200 μL metal solution was added in either TGhL or DS. After 6 h, metal solutions were removed and replaced with fresh TGhL medium, and the cells were incubated at 20 °C overnight. Relative growth was measured using the BacTitre-Glo assay, calculated as the growth in TGhL divided by the growth in DS for each metal pair.

Metal interactions and toxicity to zoosporangia

Because environmental metals rarely occur in isolation, the toxicity of metal mixtures was compared to single-metal exposures on zoosporangia. A subset of seven metals was chosen to produce six pairs. For each pair combination, cells were exposed to the two metals each at 50% MEC, as well as individually at 100% MEC. Zoospores were added to 96-well plates at 5×10^4 zoospores per well and incubated at 20 °C overnight to encyst into zoosporangia. Excess TGhL medium was removed, and the adhered cells were gently washed with sterile DS. Zoosporangia were exposed to single- or combined-metal solutions in 200 μL DS and incubated at 20 °C. After 6 h, 20 μL of 10 × TGhL solution was added, and the plates were incubated at 20 °C. Growth was measured at 48 h using the BacTitre-Glo assay. Metal interactions were characterized using the "excess over Bliss" approach (Liu et al., 2018), in which synergistic interactions are defined as producing a greater effect than the additive effect of each metal individually (e.g., less growth in mix compared to alone $S < 0$). Similarly, antagonistic interactions are those in which the two metals produce a reduced effect compared to each metal individually ($S > 0$); Garza-Cervantes et al., 2017; Hegreness et al., 2008).

$$S = (\text{Metal}^{a+b} \times 2) - (\text{Metal}^{a} + \text{Metal}^{b})$$

Role of glutathione in metal tolerance

The requirement of glutathione for tolerance of each metal was determined by depleting cellular glutathione before metal exposure. Zoosporangia were prepared as previously described, before exposure to 30 mM buthionine sulfoximine (BSO; Sigma) for 6 h to deplete cellular glutathione (Webb et al., 2024). After BSO treatment, all traces of media/BSO were removed and replaced with 150 μL metal DS solution corresponding to the MEC for each metal. After 6-h incubation at 20 °C, 20 μL 10 × TGhL and 30 μL 200 mM BSO were added and the plates returned to 20 °C. Growth was quantified using the BacTitre-Glo assay after 48 h, and two-way analysis of variance (GraphPad Prism) used to determine if the interaction between metal and BSO differed from the control. Buthionine sulfoximine is only minimally effective against zoospores (Webb et al., 2024); therefore, glutathione depletion experiments can only be conducted using zoosporangia.

Adaptation of zoosporangia to metal stress

The ability of *B. dendrobatidis* to adapt to metal stress was explored by exposing cells to a high metal concentration following preexposure to a low metal concentration. A subset of six metals was chosen, representing both glutathione-dependent (Ag, Zn, Cu, Cd) and -independent (Ni, Co, Al) tolerance mechanisms, as identified in the above BSO experiments.

Zoosporangia were prepared as previously described, then exposed to 200 µL metal in DS at 50% MEC for 6 h, after which the metal solution was removed, and cells were allowed to recover in TGhL overnight. The following day preexposed and naive cells were exposed to 200 µL metal solution at 150% MEC for 6 h. Growth was quantified using the BacTitre-Glo assay after 48 h, and independent *t* tests (Prism, Ver. 9) were used to determine if pretreated cells displayed increased metal tolerance ($p < 0.05$).

RESULTS

Toxicity of individual metals to zoospores and zoosporangia

Twelve metals were tested against *B. dendrobatidis* for acute toxicity (MLC) in zoospores and inhibition of growth in zoosporangia (MEC and MIC; Table 1). The most toxic metal was Ag, causing almost instant zoospore death and a zoosporangia MIC value in the micromolar range; Cd, Cu, and Zn followed with MIC and/or MLC values of ≤0.1 mM. The metals Mn and Ba were not toxic to *B. dendrobatidis* and did not cause complete growth inhibition or zoospore death at any of the concentrations tested. A subset of metals (Cu, Zn, Pb, Ni, and Cr) was retested with two additional *B. dendrobatidis* isolates, which were mostly in agreement, except Isolate #46 was more resistant to Cu (Supporting Information, Figures S7 and S8).

Metal interactions and toxicity to zoosporangia

Experiments with six metal pairs indicated that metal toxicity did not drastically change when another metal was present (Figure 1). Mild synergistic interactions ($S \leq 0$) were observed between Ag/Cd, Cu/Zn, and Cu/Ni, whereas mild antagonistic interactions ($S \geq 0$) were observed in Ag/Pb. No interaction (additive effect, $S = 0$) was observed for Cd/Zn and Co/Ni (Supporting Information, Figure S6).

TABLE 1: Toxicity of 12 metals to *Batrachochytrium dendrobatidis*

Metal	Mass	MEC (mM)	MIC (mM)	MLC (mM)	BSO effect	Media effect
Silver (Ag)	107	0.00085	0.005	0.01	++	++
Cadmium (Cd)	112	0.0075	0.04	0.025	++	++
Copper (II) (Cu)	63	0.01	0.075	0.5	+	++
Zinc (Zn)	65	0.02	0.04	>2	+	+
Chromium (II) (Cr)	51	0.075	0.5	>2	–	–
Cobalt (II) (Co)	58	0.04	0.5	>2	–	++
Nickle (II) (Ni)	58	0.15	1	>2	–	+
Iron (III) (Fe)	55	0.4	>2	0.5	–	–
Aluminum (Al)	26	1	>2	0.4	–	–
Lead (II) (Pb)	207	1.5	>2	>2	+	++
Manganese (II) (Mn)	54	1.5	>2	>2	–	–
Barium (Ba)	137	5	>2	>2	–	–

The effect of pre-exposure to BSO (a glutathione inhibitor) on growth indicates whether glutathione is important for metal tolerance. The media effect indicates the size of the reduction in toxicity of each metal when delivered in tryptone–gelatin hydrolysate–lactose medium compared to the dilute salt solution used as our standard.
MEC = minimum effect concentration (6-h exposure causes a noticeable negative effect on zoosporangia); MIC = minimum inhibitory concentration (6-h exposure inhibits growth of zoosporangia); MLC = minimum lethal concentration (lack of zoospore motility within 30 min); BSO = buthionine sulfoximine; ++ = major effect; + = minor effect; – = little or no effect.

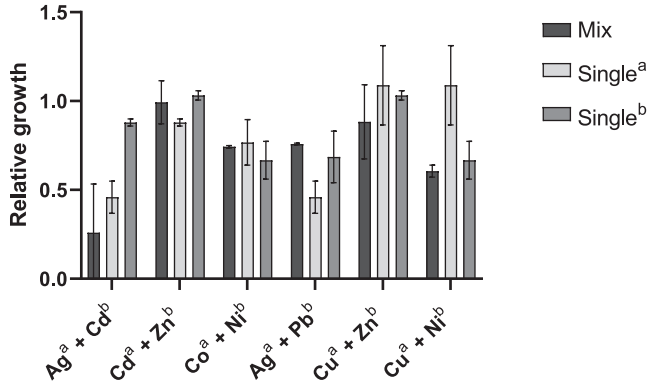

FIGURE 1: Effect of metal combinations on *Batrachochytrium dendrobatidis* zoosporangia. Zoosporangia were exposed to a mixture of two metals at 50% minimum effect concentration (MEC) and compared to exposure to each metal separately at 100% MEC. Bars represent growth relative to the untreated control of two replicate wells per condition. Mild synergistic effects were observed for Ag/Cd, Cu/Zn, and Cu/Ni, whereas mild antagonistic effects were observed for Ag/Pb. No interaction (additive effect) was observed for Cd/Zn and Co/Ni.

Effect of medium on metal toxicity

The exposure medium affected toxicity in all 12 metals tested to various degrees (Figure 2). All metals were less toxic when diluted in TGhL, but this was most apparent in Ag, Pb, Co, Cu, and Cd. The toxicity of Cr, Mn, and Al was only slightly reduced in TGhL compared to DS.

Role of glutathione in metal tolerance

Buthionine sulfoximine was used to inhibit glutathione synthesis in cells prior to metal exposure to detect changes in metal tolerance due to glutathione depletion (Figure 3). Both Ag and Cd were significantly more toxic to glutathione-depleted zoosporangia (Ag $F_{(1,7)} = 32.75$, $p = 0.0007$; Cd $F_{(1,8)} = 40.16$, $p = 0.0002$). Cells treated with BSO displayed severely stunted growth compared to cells exposed to Ag or Cd only (85% and 75% less growth, respectively). The metals Cu, Zn, and Pb were also more toxic to BSO-treated cells, although the effect was smaller (37%, 18%, and 14% less growth, respectively; Cu $F_{(1,8)} = 7.336$, $p = 0.0267$; Zn $F_{(1,8)} = 10.01$, $p = 0.0133$; Pb $F_{(1,8)} = 9.710$, $p = 0.0145$).

Adaptation of zoosporangia to metal stress

Cells were exposed to low metal concentrations to investigate whether this primes cells against future metal challenges. Seven metals were investigated, including four which were influenced by glutathione (Ag, Zn, Cu, Cd) and three which were not (Ni, Co, Al). In all cases except Co, pre-exposed cells were significantly more sensitive when challenged with high metal concentrations

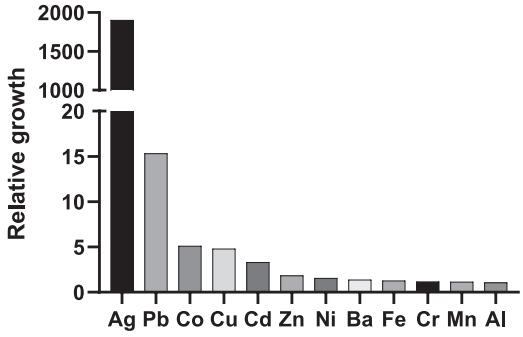

FIGURE 2: Relative growth of *Batrachochytrium dendrobatidis* exposed to metal in tryptone–gelatin hydrolysate–lactose (TGhL) liquid medium compared to the same concentration in dilute salt (DS) solution. All metals were less toxic when delivered in TGhL growth medium relative to DS solution.

(Figure 4). Cells pre-exposed to glutathione-dependent metals had an average of 90%, 36%, 51%, and 53% less growth compared to naive cells for Ag, Cd, Cu, and Zn, respectively. Pre-exposure to glutathione-independent metals resulted in 39%, 11%, and 43% less growth for Ni, Co, and Al, respectively.

DISCUSSION

Our results show that *B. dendrobatidis* varies in susceptibility to 12 metals commonly encountered in the environment. Most metals produced a typical dose response curve, indicating a lack of the hormetic effect sometimes reported for low metal concentrations (Gajewska et al., 2022; Morkunas et al., 2018). The only exception was Cu, in which a low conconcentrations resulted in slightly increased growth. Silver was the most toxic metal to *B. dendrobatidis* zoospores and zoosporangia, followed by Cd, Cu, and Zn. Silver is well known for its antimicrobial properties (Clement & Jarrett, 1994) and is especially toxic toward aquatic organisms. Our results align with previous work that established that *B. dendrobatidis* is sensitive to Cu (Boisvert & Davidson, 2011; Deknock et al., 2022), with reported inhibitory concentrations of $CuSO_4$ at 200 ppm (~1.2 mM). Interestingly, the toxicity of the four most toxic metals was influenced by

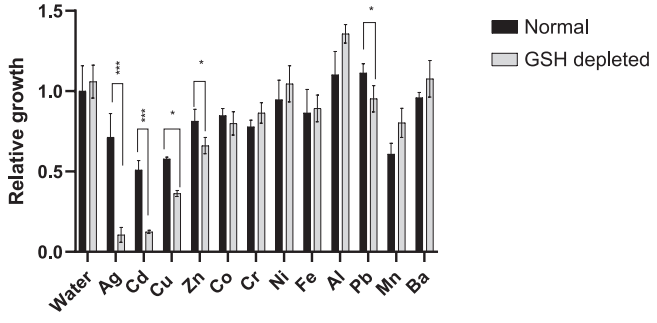

FIGURE 3: The effect of glutathione depletion on metal tolerance in *Batrachochytrium dendrobatidis*. Zoosporangia pretreated with buthionine sulfoximine (a glutathione inhibitor) were more sensitive to Ag and Cd and slightly more sensitive to Cu, Zn, and Pb. Mean growth relative to the control calculated from three replicates. Error bars represent SD: $*p \leq 0.05$; $***p \leq 0.001$. GSH = glutathione.

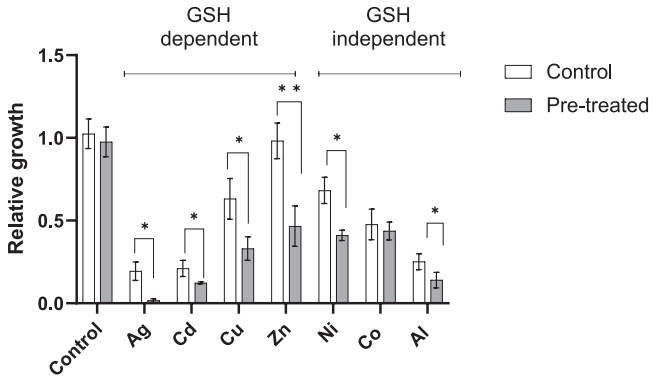

FIGURE 4: The effect of pretreatment on metal tolerance of *Batrachochytrium dendrobatidis*. Zoosporangia pretreated with low-level metal exposure did not display increased tolerance of subsequent metal exposure, regardless of whether tolerance involves glutathione. Mean growth relative to the control calculated from three replicates. Error bars represent SD: $*p \leq 0.05$; $**p \leq 0.01$. GSH = glutathione.

glutathione availability. These metals also tended to be those with the heaviest mass (Table 1).

Zoosporangia under glutathione deprivation were very susceptible to Cd and Ag, with 75% to 85% less growth in cells preexposed to BSO. Tolerance of the next two most toxic metals, Cu and Zn, also appears to involve glutathione, with 18% to 37% less growth in response to glutathione depletion. The Pb tolerance was also slightly lower in BSO-treated cells. The importance of glutathione during Cd detoxification has been well documented in many fungi. Glutathione can remove Cd by either forming a complex directly or serving as a precursor for a phytochelatin complex, before exportation to a vacuole (Cobbett & Goldsbrough, 2002; Li et al., 1996). Fungi respond to Cd exposure by upregulating glutathione production (Khullar & Reddy, 2019; Vido et al., 2001) and glutathione deficiency results in increased Cd susceptibility (Clemens et al., 1999; Glaeser et al., 1991; Gutiérrez-Escobedo et al., 2013; Khullar & Reddy, 2019; Mutoh & Hayashi, 1988; Prévéral et al., 2006). The role of glutathione in Ag, Cu, and Zn detoxification in *B. dendrobatidis* is less consistent with reports from other fungal species. Tolerance of Ag stress can involve glutathione (Fraser et al., 2002) but is more frequently associated with metallothioneins, which are another class of thiols (Osobová et al., 2011; Robinson et al., 2021). Glutathione was not required for Cu or Zn tolerance in *Saccharomyces cerevisiae* (Gharieb & Gadd, 2004), although glutathione was associated with tolerance of both Cu (Germann & Lerch, 1987) and Zn (Rama Rao et al., 1997) in *Neurospora crassa*. In *B. dendrobatidis*, the strong growth inhibition after Cd and Ag exposure indicates that glutathione might be solely responsible for the detoxification of these two metals, whereas the mild growth inhibition after Cu, Zn, and Pb suggests that *B. dendrobatidis* might employ alternative mechanisms for their clearance. In the present study we tested three *B. dendrobatidis* isolates and found some variation in metal tolerance, especially regarding Cu. This highlights the importance of expanding in vitro assays to include multiple isolates if possible. Because Cu is a common contaminant of freshwater systems (Eisler, 1998), understanding the mechanism of increased Cu tolerance warrants further investigation.

Previous research has found that Cd exposure stimulates a rapid increase in cellular glutathione concentration, with a doubling in total glutathione 24 h after Cd exposure (Webb et al., 2024). Therefore, we reasoned that metal pretreatment might prime cells for subsequent metal challenges via an increase in glutathione. However, we found that cells pre-exposed to low levels of metal were more susceptible to metal stress. This was most pronounced in the glutathione-dependent metals such as Ag, Zn, and Cu, suggesting that perhaps glutathione supplies were exhausted by the first exposure. Future work could investigate whether supplementation with glutathione precursors such as cystine or methionine (Wang et al., 1997) could alleviate the constraints of increased glutathione synthesis, and therefore increase metal tolerance via priming, or whether long-term exposure over multiple generations can produce evolution of metal tolerance.

Realistic environmental conditions are likely to contain multiple metals, so we assessed six pairs of metals to investigate whether metal interactions might change toxicity. We particularly wanted to investigate whether metal combinations would produce unpredictable synergistic effects compromising the applicability of our in vitro MIC values (Table 1). Selection of metal ions was based on their co-existence in natural systems and similarities in their hydrogeochemical and crystal chemical behaviors. The metal pairs based on common co-occurrence were Ag/Pb, Zn/Cd, and Cu/Zn. We also chose metals that are structurally similar (e.g., Co/Ni and Cd/Zn) or that might compete for glutathione (Ag/Cd and Ag/Pb). We found no indication of dramatic interactions between any of the six metal pairs tested. Instead, the effect on growth generally followed an additive pattern; for example, 50% Cd + 50% Zn provided the same inhibitory effect as 100% Cd or 100% Zn. Therefore, total metal concentration is a key parameter for prediction of *B. dendrobatidis* growth inhibition, and the concentration of all metals should be considered. The only metal pair showing signs of a noticable synergistic relationship was Ag/Cd. Because tolerance of both of these metals is glutathione-dependent, it is possible that this is due to glutathione competition. This initial investigation of possible synergistic effects could be expanded by testing multiple pairwise combinations at different concentrations or proportions (Garza-Cervantes et al., 2017).

We found that the choice of exposure medium can drastically change the results of in vitro metal sensitivity assays. There was a large discrepancy between the toxicity of Ag, Pb, Co, and Cu delivered in a pond water substitute compared to an artificial nutrient broth. Previous studies using TGhL may therefore have underestimated the effect of metals such as Cu on *B. dendrobatidis*. It must be noted that TGhL is slightly alkaline and DS slightly acidic, and although this is well within the range that *B. dendrobatidis* can tolerate (Piotrowski et al., 2004), the difference in pH may account for some of the observed difference in metal toxicity between the two exposure media. This highlights the need for careful consideration of experimental design and interpretation of results.

Comparing the metal sensitivity of *B. dendrobatidis* to other fungal species is difficult because many studies have been biased toward highly tolerant species collected from contaminated sites, often with the aim of bioremediation (Zafar et al., 2007).

TABLE 2: Inhibitory concentrations of metals for *Batrachochytrium dendrobatidis* in context of levels (1) detected in amphibian habitats, (2) listed as safe in Australian freshwater guidelines, and (3) that are lethal to amphibians

Metal	Bd MEC/MLC from the present study (μM)	Range in habitat (μM; Threlfall et al., 2008)	99%–80% ANZECC (μM)	Amphibian 96-h LC50 (μM)
Ag	0.85	0.009	0.0002	0.04 (Khangarot & Ray, 1987)
	10	0.019	0.002	0.23 (Khangarot et al., 1985)
Al	1000	0.462	1.038	9
	400	126	5.769	38 (Freda, 1991)
Cd	7.5	0.009	0.001	0.3
	25		0.007	4.5 (Brutyn et al., 2012)
Cr	75	0.020	NA	
	>2000	0.137		
Cu	10	0.032	0.016	0.3
	50	1.4	0.040	25 (Freda, 1991)
Fe	400	0.545	NA	35 (Schuytema & Nebeker, 1996)
	500	64		
Mn	150	0.130	22.222	
	>2000	20	66.667	
Ni	150	0.017	0.138	
	>2000	0.138	0.293	
Pb	1500	0.005	0.005	77 (Enuneku & Ezemonye, 2012)
	>2000	0.184	0.045	
Zn	20	0.169	0.037	20
	>2000	16	0.477	500 (Schuytema & Nebeker, 1996)

Total dissolved heavy metal concentrations detected in suspected chytrid refuge habitats in Australia, from Threlfall et al. (2008). Also included for context are the default guideline values based on Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand (2000) for 99% and 80% species protection and 96-h median lethal concentration values for various amphibian species. The concentration required to affect *B. dendrobatidis* zoosporangia (MEC) or kill zoospores (MLC) far exceeded the habitat values for all metals except Al, Cu, and Zn.
Bd = *Batrachochytrium dendrobatidis*; MEC = minimum effect concentration; MLC = minimum lethal concentration; ANZECC = Australian and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand (2000); LC50 = median lethal concentration.

In addition, our discovery that growth medium can substantially affect susceptibility complicates comparisons between studies. However, despite this, it appears that *B. dendrobatidis* is relatively sensitive to metals because the MIC values are orders of magnitude lower than those for similar species. For example, soil chytrids from New South Wales, Australia, tolerated Cu and Zn at levels 10 times higher than *B. dendrobatidis* (Henderson, 2018). Likewise, the model yeast *S. cerevisiae* can tolerate 10 times higher concentrations of Ag, Cu, and Cr (Prévéral et al., 2006).

Despite the sensitivity of *B. dendrobatidis* to metals, the levels found in contaminated Australian sites where *L. aurea* persists do not appear high enough to reduce disease. The maximum values detected in *L. aurea* habitats (Threlfall et al., 2008) were well below the MEC for most metals (Table 2), although the levels of Al, Cu, and Zn approached inhibitory levels at one heavily contaminated site. Another related chytridiomycosis-susceptible bell frog species, *Litoria raniformis*, also appears to persist in urban environments prone to metal contamination via stormwater runoff (B. Casey RMIT, personal communication, December 15, 2023). The concentrations of metals (Al, Cu, Fe, and Zn) detected at these sites (B. Casey RMIT personal communication, December 15, 2023) were also much lower than the values required to inhibit *B. dendrobatidis* in our in vitro assays. Amphibians are sensitive to heavy metal contamination (Ficken & Byrne, 2013), and only Cu and Zn have *B. dendrobatidis* MEC values lower than the highest median lethal concentration for amphibian larvae (Table 2). There is some evidence that Cu can influence *B. dendrobatidis* infection dynamics in the field; however, the results are inconsistent (Chew, in press). Our in vitro experimental design necessitated an acute 6-h metal exposure, and it is likely that chronic exposure could cause growth inhibition of *B. dendrobatidis*, or disrupt transmission, at lower concentrations. Zoosporangia within the epidermis may also experience higher than background concentrations as a result of bioaccumulation, or alternatively they may be shielded from direct contact with dissolved heavy metals by the host cells. In addition, the bioavailability and toxicity of metals in the environment will vary depending on numerous factors including the major ion composition, presence of organic matter, pH, temperature, coastal influence, and rainfall events. Therefore, the present values cannot be directly extrapolated to the field but are useful to indicate potential impacts of dissolved metals. Because water temperature and salinity are known to affect chytridiomycosis dynamics, future work could investigate the potential interplay of these factors. In addition, rather than prepare metal solutions in the laboratory, future work could test the chemical content and fungicidal effects of water collected from a range of sources for a realistic assessment (Boisvert & Davidson, 2011). Measuring skin levels in wild frogs would assess the potential for bioaccumulation to result in exposures above environmental levels (Prokić et al., 2016). We found that total metal concentration is a key parameter because of the additive inhibitory effect. However, the minimum concentrations observed to impact *B. dendrobatidis* growth during acute exposure far exceed the acceptable safe metal concentrations according to Australian guidelines, as well as the concentrations detected in habitats where susceptible amphibians persist. Further investigation of the effect of chronic metal exposure in combination with enviromental variables is needed to resolve whether dissolved metals have potential to create chytrid refuges.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5885.

Acknowledgments—The present study was funded by the Walter Fisher Grant for Mycology Research, on behalf of the Royal Society of Queensland, and the Australian Research Council (DP220101361). The authors thank B. Cuff, M. West, L. Brannelly, A. Chew, D. De Angelis, B. Casey, A. Aquilina, and L. Skerratt for their preliminary work and assistance. Open access publishing facilitated by The University of Melbourne, as part of the Wiley - The University of Melbourne agreement via the Council of Australian University Librarians.

Conflict of Interest—The authors declare no conflict of interest.

Author Contribution Statement—**Rebecca J. Webb**: Conceptualization; Formal analysis; Funding acquisition; Investigation; Writing–original draft. **Christopher Cuff**: Methodology; Writing–review & editing. **Lee Berger**: Conceptualization; Supervision; Resources; Writing–review & editing.

Data Availability Statement—All data are presented in the article.

REFERENCES

Australian and New Zealand Environment and Conservation Council & Agriculture and Resource Management Council of Australia and New Zealand. (2000). *Australian and New Zealand guidelines for fresh and marine water quality*.

Berger, L., Speare, R., Marantelli, G., & Skerratt, L. F. (2009). A zoospore inhibition technique to evaluate the activity of antifungal compounds against Batrachochytrium dendrobatidis and unsuccessful treatment of experimentally infected green tree frogs (Litoria caerulea) by fluconazole and benzalkonium chloride. *Research in Veterinary Science*, *87*, 106–110.

Boisvert, S. P., & Davidson, E. W. (2011). Growth of the amphibian pathogen, *Batrachochytrium dendrobatidis*, in response to chemical properties of the aquatic environment. *Journal of Wildlife Diseases*, *47*(3), 694–698. https://doi.org/10.7589/0090-3558-47.3.694

Bradley, P. W., Brawner, M. D., Raffel, T. R., Rohr, J. R., Olson, D. H., & Blaustein, A. R. (2019). Shifts in temperature influence how *Batrachochytrium dendrobatidis* infects amphibian larvae. *PLOS ONE*, *14*(9), Article e0222237. https://doi.org/10.1371/journal.pone.0222237

Brutyn, M., D'Herde, K., Dhaenens, M., Van Rooij, P., Verbrugghe, E., Hyatt, A. D., Croubels, S., Deforce, D., Ducatelle, R., Haesebrouck, F., Martel, A., & Pasmans, F. (2012). *Batrachochytrium dendrobatidis* zoospore secretions rapidly disturb intercellular junctions in frog skin. *Fungal Genetics and Biology*, *49*(10), 830–837. https://doi.org/10.1016/j.fgb.2012.07.002

Cashins, S. D., Grogan, L. F., McFadden, M., Hunter, D., Harlow, P. S., Berger, L., & Skerratt, L. F. (2013). Prior infection does not improve survival against the amphibian disease chytridiomycosis. *PLOS ONE*, *8*(2), Article e56747. https://doi.org/10.1371/journal.pone.0056747

Clemens, S., Kim, E. J., Neumann, D., & Schroeder, J. I. (1999). Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO Journal*, *18*(12), 3325–3333. https://doi.org/10.1093/emboj/18.12.3325

Clement, J. L., & Jarrett, P. S. (1994). Antibacterial silver. *Metal-Based Drugs*, *1*(5–6), 467–482. https://doi.org/10.1155/mbd.1994.467

Clulow, S., Gould, J., James, H., Stockwell, M., Clulow, J., & Mahony, M. (2018). Elevated salinity blocks pathogen transmission and improves host survival from the global amphibian chytrid pandemic: Implications for translocations. *Journal of Applied Ecology*, *55*(2), 830–840. https://doi.org/10.1111/1365-2664.13030

Cobbett, C., & Goldsbrough, P. (2002). Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology*, *53*, 159–182. https://doi.org/10.1146/annurev.arplant.53.100301.135154

Deknock, A., Pasmans, F., van Leeuwenberg, R., Van Praet, S., De Troyer, N., Goessens, T., Lammens, L., Bruneel, S., Lens, L., Martel, A., Croubels, S., & Goethals, P. (2022). Impact of heavy metal exposure on biological control of a deadly amphibian pathogen by zooplankton. *Science of the Total Environment*, *823*, Article 153800. https://doi.org/10.1016/j.scitotenv.2022.153800

Department of Environment and Heritage. (2006). *Background document for the Threat Abatement Plan: Infection of amphibians with chytrid fungus resulting in chytridiomycosis*.

Eisler, R. (1998). *Copper hazards to fish, wildlife, and invertebrates: A synoptic review*. US Department of the Interior, US Geological Survey.

Enuneku, A. A., & Ezemonye, L. I. (2012). Acute toxicity of cadmium and lead to adult toad *Bufo maculatus*. *Asian Journal of Biological and Life Sciences*, *1*(3), 238–241.

Ficken, K. L. G., & Byrne, P. G. (2013). Heavy metal pollution negatively correlates with anuran species richness and distribution in south-eastern Australia. *Austral Ecology*, *38*(5), 523–533. https://doi.org/10.1111/j.1442-9993.2012.02443.x

Fisher, M. C., Ghosh, P., Shelton, J. M. G., Bates, K., Brookes, L., Wierzbicki, C., Rosa, G. M., Farrer, R. A., Aanensen, D. M., Alvarado-Rybak, M., Bataille, A., Berger, L., Böll, S., Bosch, J., Clare, F. C., Courtois, E. A., Crottini, A., Cunningham, A. A., Doherty-Bone, T. M., … Garner, T. W. J. (2018). Development and worldwide use of non-lethal, and minimal population-level impact, protocols for the isolation of amphibian chytrid fungi. *Scientific Reports*, *8*(1), Article 7772. https://doi.org/10.1038/s41598-018-24472-2

Fraser, J. A., Davis, M. A., & Hynes, M. J. (2002). A gene from *Aspergillus nidulan* with similarity to *URE2* of *Saccharomyces cerevisiae* encodes a glutathione *S*-transferase which contributes to heavy metal and xenobiotic resistance. *Applied and Environmental Microbiology*, *68*(6), 2802–2808. https://doi.org/10.1128/AEM.68.6.2802-2808.2002

Freda, J. (1991). The effects of aluminum and other metals on amphibians. *Environmental Pollution*, *71*(2), 305–328. https://doi.org/10.1016/0269-7491(91)90035-U

Gajewska, J., Floryszak-Wieczorek, J., Sobieszczuk-Nowicka, E., Mattoo, A., & Arasimowicz-Jelonek, M. (2022). Fungal and oomycete pathogens and heavy metals: An inglorious couple in the environment. *IMA Fungus*, *13*(1), Article 6. https://doi.org/10.1186/s43008-022-00092-4

Garza-Cervantes, J. A., Chávez-Reyes, A., Castillo, E. C., García-Rivas, G., Ortega-Rivera, O. A., Salinas, E., Ortiz-Martínez, M., Gómez-Flores, S. L., Peña-Martínez, J. A., Pepi-Molina, A., Treviño-González, M. T., Zarate, X., Cantú-Cárdenas, M. E., Escarcega-Gonzalez, C. E., & Morones-Ramírez, J. R. (2017). Synergistic antimicrobial effects of silver/transition-metal combinatorial treatments. *Scientific Reports*, *7*(1), Article 903. https://doi.org/10.1038/s41598-017-01017-7

Germann, U. A., & Lerch, K. (1987). Copper accumulation in the cell-wall-deficient slime variant of *Neurospora crassa*. Comparison with a wild-type strain. *Biochemical Journal*, *245*(2), 479–484. https://doi.org/10.1042/bj2450479

Gharieb, M. M., & Gadd, G. M. (2004). Role of glutathione in detoxification of metal(loid)s by *Saccharomyces cerevisiae*. *BioMetals*, *17*(2), 183–188. https://doi.org/10.1023/B:BIOM.0000018402.22057.62

Glaeser, H., Coblenz, A., Kruczek, R., Ruttke, I., Ebert-Jung, A., & Wolf, K. (1991). Glutathione metabolism and heavy metal detoxification in *Schizosaccharomyces pombe*. *Current Genetics*, *19*(3), 207–213. https://doi.org/10.1007/BF00336488

Gleason, F. H., Kagami, M., Lefevre, E., & Sime-Ngando, T. (2008). The ecology of chytrids in aquatic ecosystems: Roles in food web dynamics. *Fungal Biology Reviews*, *22*(1), 17–25.

Greenspan, S. E., Bower, D. S., Webb, R. J., Roznik, E. A., Stevenson, L. A., Berger, L., Marantelli, G., Pike, D. A., Schwarzkopf, L., & Alford, R. A. (2017). Realistic heat pulses protect frogs from disease under simulated rainforest frog thermal regimes. *Functional Ecology*, *31*(12), 2274–2286. https://doi.org/10.1111/1365-2435.12944

Greenspan, S. E., Longcore, J. E., & Calhoun, A. J. K. (2012). Host invasion by *Batrachochytrium dendrobatidis*: Fungal and epidermal ultrastructure in model anurans. *Diseases of Aquatic Organisms*, *100*(3), 201–210.

Gutiérrez-Escobedo, G., Orta-Zavalza, E., Castaño, I., & De Las Peñas, A. (2013). Role of glutathione in the oxidative stress response in the fungal pathogen *Candida glabrata*. *Current Genetics*, *59*(3), 91–106. https://doi.org/10.1007/s00294-013-0390-1

Heard, G. W., Scroggie, M. P., Clemann, N., & Ramsey, D. S. L. (2014). Wetland characteristics influence disease risk for a threatened amphibian. *Ecological Applications*, *24*(4), 650–662.

Hegreness, M., Shoresh, N., Damian, D., Hartl, D., & Kishony, R. (2008). Accelerated evolution of resistance in multidrug environments. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(37), 13977–13981. https://doi.org/10.1073/pnas.0805965105

Henderson, L. E. (2018). *The effect of metals on growth, reproduction and attachment of zoosporic true fungi* (Publication No. 18611) [Doctoral dissertation, University of Sydney]. Sydney eScholarship. http://hdl.handle.net/2123/18611

Jozefczak, M., Remans, T., Vangronsveld, J., & Cuypers, A. (2012). Glutathione is a key player in metal-induced oxidative stress defenses. *International Journal of Molecular Sciences*, *13*(3), 3145–3175.

Khangarot, B. S., & Ray, P. K. (1987). Sensitivity of toad tadpoles, *Bufo melanostictus* (Schneider), to heavy metals. *Bulletin of Environmental Contamination and Toxicology*, *38*(3), 523–527. https://doi.org/10.1007/BF01606623

Khangarot, B. S., Sehgal, A., & Bhasin, M. K. (1985). "Man and biosphere"—Studies on the Sikkim Himalayas. Part 5: Acute toxicity of selected heavy metals on the tadpoles of *Rana hexadactyla*. *Acta Hydrochimica et Hydrobiologica*, *13*(2), 259–263. https://doi.org/10.1002/aheh.19850130223

Khullar, S., & Reddy, M. S. (2019). Cadmium and arsenic responses in the ectomycorrhizal fungus *Laccaria bicolor*: Glutathione metabolism and its role in metal(loid) homeostasis. *Environmental Microbiology Reports*, *11*(2), 53–61. https://doi.org/10.1111/1758-2229.12712

Lane, A., & Burgin, S. (2008). Comparison of frog assemblages between urban and non-urban habitats in the upper Blue Mountains of Australia. *Freshwater Biology*, *53*(12), 2484–2493. https://doi.org/10.1111/j.1365-2427.2008.02068.x

Li, Z. S., Szczypka, M., Lu, Y. P., Thiele, D. J., & Rea, P. A. (1996). The yeast cadmium factor protein (YCF1) is a vacuolar glutathione *S*-conjugate pump. *Journal of Biological Chemistry*, *271*(11), 6509–6517. https://doi.org/10.1074/jbc.271.11.6509

Liu, Q., Yin, X., Languino, L. R., & Altieri, D. C. (2018). Evaluation of drug combination effect using a Bliss independence dose–response surface model. *Statistics in Biopharmaceutical Research*, *10*(2), 112–122. https://doi.org/10.1080/19466315.2018.1437071

Longcore, J. E., Pessier, A. P., & Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, *91*(2), 219–227. https://doi.org/10.1080/00275514.1999.12061011

Longcore, J. E., & Simmons, D. R. (2020). Chytridiomycota. In Encyclopedia of life sciences. John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470015902.a0000349.pub4

Morkunas, I., Woźniak, A., Mai, V. C., Rucińska-Sobkowiak, R., & Jeandet, P. (2018). The role of heavy metals in plant response to biotic stress. *Molecules*, *23*(9), Article 2320.

Mutoh, N., & Hayashi, Y. (1988). Isolation of mutants of *Schizosaccharomyces pombe* unable to synthesize cadystin, small cadmium-binding peptides. *Biochemical and Biophysical Research Communications*, *151*(1), 32–39. https://doi.org/10.1016/0006-291X(88)90555-4

Osobová, M., Urban, V., Jedelský, P. L., čBorovička, J., Gryndler, M., Ruml, T., & Kotrba, P. (2011). Three metallothionein isoforms and sequestration of intracellular silver in the hyperaccumulator *Amanita strobiliformis*. *New Phytologist*, *190*(4), 916–926. https://doi.org/10.1111/j.1469-8137.2010.03634.x

Parris, M. J., & Baud, D. R. (2004). Interactive effects of a heavy metal and chytridiomycosis on gray treefrog larvae (*Hyla chrysoscelis*). *Copeia*, *2004*(2), 344–350. https://doi.org/10.1643/CE-03-116R1

Piotrowski, J. S., Annis, S. L., & Longcore, J. E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, *96*(1), 9–15.

Pócsi, I., Prade, R. A., & Penninckx, M. J. (2004). Glutathione, altruistic metabolite in fungi. *Advances in Microbial Physiology*, *49*, 1–76. https://doi.org/10.1016/s0065-2911(04)49001-8

Prévéral, S., Ansoborlo, E., Mari, S., Vavasseur, A., & Forestier, C. (2006). Metal(loid)s and radionuclides cytotoxicity in *Saccharomyces cerevisiae*. Role of YCF1, glutathione and effect of buthionine sulfoximine. *Biochimie*, *88*(11), 1651–1663. https://doi.org/10.1016/j.biochi.2006.05.016

Prism (Version 9) [Computer software]. (2020). GraphPad.

Prokić, M. D., Borković-Mitić, S. S., Krizmanić, I. I., Mutić, J. J., Trifković, J.Đ., Gavrić, J. P., Despotović, S. G., Gavrilović, B. R., Radovanović, T. B., Pavlović, S. Z., & čSaičić, Z. S. (2016). Bioaccumulation and effects of metals on oxidative stress and neurotoxicity parameters in the frogs from the *Pelophylax esculentus* complex. *Ecotoxicology*, *25*(8), 1531–1542. https://doi.org/10.1007/s10646-016-1707-x

Prostak, S. M., & Fritz-Laylin, L. K. (2021). Laboratory maintenance of the chytrid fungus *Batrachochytrium dendrobatidis*. *Current Protocols*, *1*(12), Article e309. https://doi.org/10.1002/cpz1.309

Rama Rao, V. S. K. V., Wilson, C. H., & Maruthi Mohan, P. (1997). Zinc resistance in *Neurospora crassa*. *BioMetals*, *10*(3), 147–156. https://doi.org/10.1023/A:1018339425355

Robinson, J. R., Isikhuemhen, O. S., & Anike, F. N. (2021). Fungal–metal interactions: A review of toxicity and homeostasis. *Journal of Fungi*, *7*(3), Article 225.

Robinson, K. A., Pereira, K. E., Bletz, M. C., Carter, E. D., Gray, M. J., Piovia-Scott, J., Romansic, J. M., Woodhams, D. C., & Fritz-Laylin, L. (2020). Isolation and maintenance of *Batrachochytrium salamandrivorans* cultures. *Diseases of Aquatic Organisms*, *140*, 1–11. https://doi.org/10.3354/dao03488

Rollins-Smith, L. A., Carey, C., Longcore, J., Doersam, J. K., Boutte, A., Bruzgal, J. E., & Conlon, J. M. (2002). Activity of antimicrobial skin peptides from ranid frogs against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. *Developmental & Comparative Immunology*, *26*(5), 471–479. https://doi.org/10.1016/S0145-305X(01)00088-X

Schuytema, G. S., & Nebeker, A. V. (1996). *Amphibian toxicity data for water quality criteria chemicals* (EPA/600/R-96/124). US Environmental Protection Agency.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., Hines, H. B., & Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, *4*(2), 125–134. https://doi.org/10.1007/s10393-007-0093-5

Stockwell, M. P., Clulow, J., & Mahony, M. J. (2012). Sodium chloride inhibits the growth and infective capacity of the amphibian chytrid fungus and increases host survival rates. *PLOS ONE*, *7*(5), Article e36942. https://doi.org/10.1371/journal.pone.0036942

Stockwell, M. P., Clulow, J., & Mahony, M. J. (2015). Evidence of a salt refuge: Chytrid infection loads are suppressed in hosts exposed to salt. *Oecologia*, *177*(3), 901–910. https://doi.org/10.1007/s00442-014-3157-6

Thekkiniath, J. C., Zabet-Moghaddam, M., San Francisco, S. K., & San Francisco, M. J. (2013). A novel subtilisin-like serine protease of *Batrachochytrium dendrobatidis* is induced by thyroid hormone and degrades antimicrobial peptides. *Fungal Biology*, *117*(6), 451–461. https://doi.org/10.1016/j.funbio.2013.05.002

Threlfall, C., Jolley, D., Evershed, N., Goldingay, R., & Buttemer, W. (2008). Do green and golden bell frogs *Litoria aurea* occupy habitats with fungicidal properties? *Australian Zoologist*, *34*(3), 350–360.

Vido, K., Spector, D., Lagniel, G., Lopez, S., Toledano, M. B., & Labarre, J. (2001). A proteome analysis of the cadmium response in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, *276*(11), 8469–8474. https://doi.org/10.1074/jbc.M008708200

Waddle, A. W., Sai, M., Levy, J. E., Rezaei, G., van Breukelen, F., & Jaeger, J. R. (2018). Systematic approach to isolating *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, *127*(3), 243–247. https://doi.org/10.3354/dao03203

Wang, S. T., Chen, H. W., Sheen, L. Y., & Lii, C. K. (1997). Methionine and cysteine affect glutathione level, glutathione-related enzyme activities and the expression of glutathione S-transferase isozymes in rat hepatocytes. *Journal of Nutrition*, *127*(11), 2135–2141. https://doi.org/10.1093/jn/127.11.2135

Webb, R. J., Berger, L., Skerratt, L. F., & Roberts, A. A. (2019). A rapid and inexpensive viability assay for zoospores and zoosporangia of *Batrachochytrium dendrobatidis*. *Journal of Microbiological Methods*, *165*, Article 105688. https://doi.org/10.1016/j.mimet.2019.105688

Webb, R. J., Rush, C., Berger, L., Skerratt, L., & Roberts, A. (2024). Glutathione is required for growth and cadmium tolerance in the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Biochemie*, *220*, 22–30.

White, A., & Pyke, G. (2014). Distribution and conservation status of the green and golden bell frog *Litoria aurea* in New South Wales. *Australian Zoologist*, *30*(2), 177–189. https://doi.org/10.7882/az.1996.010

Wysocki, R., & Tamás, M. J. (2010). How *Saccharomyces cerevisiae* copes with toxic metals and metalloids. *FEMS Microbiology Reviews*, *34*(6), 925–951. https://doi.org/10.1111/j.1574-6976.2010.00217.x

Zafar, S., Aqil, F., & Ahmad, I. (2007). Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. *Bioresource Technology*, *98*(13), 2557–2561. https://doi.org/10.1016/j.biortech.2006.09.051

