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Hazard/Risk Assessment

The Effects of Nickel and Copper on Tropical Marine and Freshwater Microalgae Using Single and Multispecies Tests

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Abstract: Microalgae are key components of aquatic food chains and are known to be sensitive to a range of contaminants. Much of the available data on metal toxicity to microalgae have been derived from temperate single-species tests with temperate data used to supplement tropical toxicity data sets to derive guideline values. In the present study, we used single-species and multispecies tests to investigate the toxicity of nickel and copper to tropical freshwater and marine microalgae, including the free-swimming stage of *Symbiodinium* sp., a worldwide coral endosymbiont. Based on the 10% effect concentration (EC10) for growth rate, copper was two to four times more toxic than nickel to all species tested. The temperate strain of *Ceratoneis closterium* was eight to 10 times more sensitive to nickel than the two tropical strains. Freshwater *Monoraphidium arcuatum* was less sensitive to copper and nickel in the multispecies tests compared with the single-species tests (EC10 values increasing from 0.45 to 1.4 µg Cu/L and from 62 to 330 µg Ni/L). The *Symbiodinium* sp. was sensitive to copper (EC10 of 3.1 µg Cu/L) and less sensitive to nickel (EC50 >1600 µg Ni/L). This is an important contribution of data on the chronic toxicity of nickel to *Symbiodinium* sp. A key result from the present study was that three microalgal species had EC10 values below the current copper water quality guideline value for 95% species protection in slightly to moderately disturbed systems in Australia and New Zealand, indicating that they may not be adequately protected by the current copper guideline value. By contrast, toxicity of nickel to microalgae is unlikely to occur at exposure concentrations typically found in fresh and marine waters. *Environ Toxicol Chem* 2023;42:901–913. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Metals; Multispecies test; Phytoplankton; Risk assessment; Water quality guidelines

INTRODUCTION

Microalgae are photosynthetic organisms that play a vital role in aquatic ecosystems; they are the primary link in aquatic food chains and are sensitive indicators of environmental change (Kuzminov et al., 2013; Levy et al., 2007; Rasdi & Qin, 2015). In addition, microalgae are easy to culture and are amenable to laboratory testing (Franklin et al., 2004; Stauber, 1995). Growth rate (cell division) tests are short-term and can provide high-quality chronic ecotoxicity data. Given their known sensitivity to contaminants, particularly metals (Franklin et al., 2000; Stauber & Davies, 2000), it is surprising that algal data are not generally included in species sensitivity distributions (SSDs) for deriving water quality criteria in the United States (Stephen et al., 1985, and subsequent US Environmental Protection Agency water quality criteria documents). Microalgae are, however, a key component of SSDs used to derive environmental quality standards and water quality guideline values in other jurisdictions, including the European Union, Canada, Australia, and New Zealand.

The sensitivity of microalgae to metals varies and is influenced by a wide range of biotic and abiotic factors. Taxonomic class, cell size, prior exposure to metals, water quality parameters, and

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toxicokinetic and toxicodynamic processes all influence metal bioavailability and consequently toxicity to microalgae (Johnson et al., 2007; Levy et al., 2007; Price et al., 2021; Stauber & Davies, 2000; Stauber & Florence, 1987). Much of the available international data on metal toxicity to microalgae have been derived from single-species tests using temperate species. Limited studies have been undertaken on the effects of metals on tropical microalgae in comparison with temperate microalgae (Binet et al., 2018; Gissi et al., 2016), necessitating the extrapolation of temperate data to protect tropical systems. Tropical environments are unique in their range of sensitive habitats, endemic taxa, and high biodiversity. For this reason and the difficulty in predicting the sensitivity of species from different climatic regions (Chapman et al., 2006), new ecologically relevant risk-assessment tools and guideline values are required to protect these tropical freshwater and coastal marine systems.

Tropical freshwater microalgae, including chlorophytes and chrysophytes, are dominant in many freshwater systems and have been used in both single-species and multispecies tests to assess the toxicity of copper and herbicides (Franklin et al., 2004; Stone et al., 2019; Yu et al., 2007). Multispecies tests use several species of microalgae that naturally co-occur in a microcosm, providing a more environmentally realistic approach to the assessment of contaminants than single-species tests. Algae may compete for available nutrients or produce exudates that influence the growth of other species or alter the bioavailability of contaminants in the tests. The development of multispecies tests with microalgae has been limited by our ability to distinguish and count cells in mixed populations. Flow cytometry is one technique that has been used to discriminate microalgal species based on their size and pigments' fluorescent properties when excited by light of different wavelengths. Franklin et al. (2004) developed both marine and temperate multispecies tests using flow cytometry, and further studies on algae in multispecies tests have been undertaken, including by Yu et al. (2007), de Morais et al. (2014), and Stone et al. (2019).

Marine microalgae from the classes Dinophyceae, Bacillariophyceae, and Coccolithophyceae were selected for the study because they play important roles in tropical marine ecosystems. Of the dinoflagellates, the genus *Symbiodinium* is particularly important as an endosymbiont in corals (zooxanthellae). Prior to being incorporated as endosymbionts in corals, *Symbiodinium* species are free-swimming phytoplankton in the water column and can potentially be exposed to contaminants and other stressors (Rodriguez et al., 2016). In addition, it has been found that species of *Symbiodinium* accumulate many metals in greater concentrations than coral tissues (Reichelt-Brushett & McOrist, 2003). If coral–algal symbioses become compromised by stressors such as increased temperature or changes in water quality (e.g., metal contamination), corals may expel the algal endosymbionts or "bleach" and thus lose a major energy source (Bielmyer et al., 2010; Reichelt-Brushett & McOrist, 2003). Therefore, understanding the impacts of metal contaminants on this taxon is highly relevant to its inclusion in SSDs to derive water quality guideline values for tropical systems.

Copper and nickel were selected as the metals of interest because of their widespread use in industrial and marine applications and their known toxicity to aquatic biota at low exposure concentrations (Australian and New Zealand Governments [ANZG], 2018). Copper is a ubiquitous contaminant with a range of sources including stormwater runoff and industrial inputs that enter both freshwater and marine environments (Comber et al., 2022). Nickel mining in tropical regions is an expanding industry, and with limited high-quality chronic data available for tropical species, it is important to address this data gap (Binet et al., 2018).

The aim of the present study was to provide data on the toxicity of copper and nickel to tropical marine and freshwater microalgae. We also compare the sensitivities of temperate and tropical strains of the marine diatom *Ceratoneis closterium* and the sensitivity of tropical freshwater algal species in multispecies tests compared with single-species tests.

METHODS

General laboratory techniques and reagents

Population growth rate–inhibition tests were conducted in borosilicate glass Erlenmeyer flasks which were pretreated with Coatasil (Ajax Finechem) to prevent adsorption of metals to the glass. All glassware and plasticware were acid-washed in 10% (v/v) nitric acid (reagent-grade; Merck, Darmstadt, Germany) for at least 24 h and rinsed five times with demineralized water, followed by five rinses in ultrapure water (Milli-Q®, 18 MΩ/cm; Merck).

All metal stock solutions were made volumetrically using ultrapure water. Copper stock solutions of 5 and 100 mg/L were prepared using copper (II) sulfate salt (analytical reagent–grade; AJAX Chemicals, Australia), and nickel stock solutions of 10 and 100 mg/L were made using nickel (II) chloride hexahydrate salt (analytical reagent-grade; Chem Supply, Australia). All stock solutions were acidified to 0.1% HCl (Tracepur; Merck).

Test diluent was either 0.45-µm filtered (Sartobran P Sterile Midicap; Sartorius Stedim Biotech) seawater collected from Oak Park, Cronulla, New South Wales, Australia, or 0.45-µm filtered (Merck Millipore; nitrocellulose) synthetic freshwater (moderately hard, at either 80–90 mg $CaCO_3$/L or 100–120 mg $CaCO_3$/L; Franklin et al., 2002; Stone et al., 2019).

Physicochemical parameters were measured in all test treatments at the beginning and end (0 and 72 h) of the tests using instruments that were calibrated per the manufacturer's instructions. Salinity measurements were taken using a YSI salinity and conductivity meter (model 30/10FT). The pH was measured using a Thermo Orion pH meter with an epoxy body probe (meter model 420, probe model ROSS815600; Thermo Fisher Scientific), which was calibrated daily. Temperature was maintained at 27 ± 1 °C for tropical tests and 21 ± 1 °C for temperate tests throughout the exposures.

Test species and culture conditions

The present study used three tropical marine species, a temperate strain of one marine species, and four tropical freshwater species, from five different algal taxonomic classes (Table 1). Two different strains of tropical *C. closterium* were

TABLE 1: Microalgal species investigated in the present study: Description, isolate origins, and culture conditions

Algal species	Class	Origin of isolate	Culture medium	Temperature (°C)
Marine				
Ceratoneis closterium (temperate)	Bacillariophyceae	Port Hacking, Sydney	f[a]	21 ± 2
Ceratoneis closterium (tropical)	Bacillariophyceae	Coral sea	G/2[b]	27 ± 2
Ceratoneis closterium (tropical)	Bacillariophyceae	Coral sea	f/2[c]	27 ± 2
Tisochrysis lutea	Coccolithophyceae	Tahiti	f/2	27 ± 2
Symbiodinium sp. Freud clade C	Dinophyceae	Hawaii	f/2[d]	27 ± 2
Freshwater				
Chlorella sp.	Chlorophyceae	Lake Aesake, Papua New Guinea	JM/2.5[e]	27 ± 2
Monoraphidium arcuatum	Chlorophyceae	Lake Aesake, Papua New Guinea	JM/2.5	27 ± 2
Pediastrum duplex	Chlorophyceae	Lake Aesake, Papua New Guinea	JM/2.5	27 ± 2
Nannochloropsis-like sp.	Chrysophyceae	Lake Aesake, Papua New Guinea	MBL/2[f]	27 ± 2

[a]f medium (Guillard & Ryther, 1962) with the trace metal concentrations halved.
[b]G/2 medium (Loeblich & Smith, 1968).
[c]f/2 medium—half strength f medium (above).
[d]f/2 medium with double the iron concentration of f/2 to ensure cells were in exponential growth phase for testing (Supporting Information, Table S1).
[e]JM/2.5 medium (Schlösser, 1982)—diluted.
[f]MBL/2 medium (Stein, 1973)—half strength.

used in the study to investigate any difference in the two strains, both isolated from the same geographic area and cultured in different media types. The marine species were originally obtained from the CSIRO Australian National Algae Culture Collection (Hobart, Australia). The freshwater species were isolated from Lake Aesake, Papua New Guinea, and maintained at CSIRO Land and Water (Sydney, Australia). One of the freshwater species, isolated from Papua New Guinea, was genetically characterized by Stone et al. (2019), with a positive match (>90%) for two genera. Morphological assessment resulted in *Nannochloropsis* sp. being the most likely match. Therefore, it is referred to as *Nannochloropsis*-like sp., following Stone et al. (2019). All species were maintained under axenic conditions in controlled light (70 µmol photosynthetically active radiation photons/m^2/s, on a 12: 12-h light: dark cycle) and temperature-controlled growth cabinets. Descriptions of the culture conditions are provided in Table 1.

Growth rate–inhibition tests

Single-species tests. The chronic toxicity of nickel and copper to three marine species (see Table 1, including three strains of *C. closterium*) and two freshwater species (*Chlorella* sp. and *Monoraphidium arcuatum*) was determined in single-species tests. All tests followed the methods of Stauber et al. (1994), Franklin et al. (2004), and Organisation for Economic Co-operation and Development (OECD, 2011). Test conditions are outlined in Supporting Information, Table S1. Low-nutrient test solutions of natural seawater or synthetic freshwater were supplemented with 1.5 g NO_3^-/L ($NaNO_3$; BDR analytical reagent-grade) and 0.15 g PO_4^{3-}/L (KH_2PO_4; BDR analytical reagent-grade) stocks, giving final concentrations of 1.5 mg NO_3^-/L and 0.15 mg PO_4^{3-}/L, to sustain exponential algal growth throughout the 72-h exposure. Each test included four replicate controls and two replicates per metal concentration (4–16 concentrations). Measured dissolved (<0.45 µm) copper test concentrations ranged from 0.46 to 8.8 µg/L in the synthetic freshwater tests and from 0.22 to 30 µg/L in tests with seawater. Measured dissolved (<0.45 µm) nickel concentrations ranged from 3.3 to 9400 µg Ni/L in synthetic freshwater tests and from 10 to 50 000 µg Ni/L in tests with seawater. A smaller number of treatments were tested for copper because it is routinely used in our laboratory as a reference toxicant, and we have an established data set for some of the species in the study. Because of the large experiment sizes, using both nickel and copper, we reduced the number of copper treatments tested. Three tests were carried out with each species/strain for each metal, excepting *C. closterium* and *M. arcuatum*, where only two tests were performed (Table 2).

Cultures of microalgae (in exponential growth phase, 5–6 days) were used for all toxicity tests. Microalgae were centrifuged at 280 *g* for 7 min and washed four times with test diluent to remove nutrient-rich culture media, except for *Symbiodinium* sp., which was not washed. Initial investigations found that the growth rate of *Symbiodinium* sp. was significantly reduced after washing and centrifugation. This could indicate a potential sensitivity of the flagellated motile stage of *Symbiodinium* sp. to the forces of centrifugation (Yamashita & Koike, 2016). Concentrated microalgae were vortexed or homogenized in a hand-held tissue grinder (15 ml; Wheaton) to resuspend or dislodge clumps of microalgal cells. Cell densities were calculated using flow cytometry (FACSCalibur; BD Bioscience), and each flask was inoculated with 2 to 3×10^3 cells/ml and incubated in a temperature- and light-controlled cabinet. These low initial cell densities were used to better emulate cell densities in the natural environment and to reduce any potential metal speciation changes (with pH change) over the duration of the test (Franklin et al., 2002).

Cell densities were determined at 0, 24, 48, and 72 h using flow cytometry (FACSCalibur; BD Sciences). Cells were excited at 488 nm, and densities of microalgae were measured using chlorophyll *a* autofluorescence and side-angle light scatter. Fluorescent beads (BD Trucount™ Tubes; BD Biosciences) were added to each sample as an internal counting standard (Franklin et al., 2001).

TABLE 2: Mean 72-h EC10 and 72-h EC50 values[a] for microalgae exposed to dissolved (<0.45 μm) nickel and copper in single-species tests

		Cu (μg/L)		Ni (μg/L)	
	n	72-h EC10	72-h EC50	72-h EC10	72-h EC50
Marine species					
Ceratoneis closterium (temperate)	2(Cu)3(Ni)	1.1 (0.9–1.3)	3.1 (2.7–3.5)	260 (200–320)	1000 (900–1100)
Ceratoneis closterium (tropical, G/2)	3	1.0 (0.9–1.1)	3.4 (3.2–3.7)	3100 (2800–3500)	6500 (6300–6800)
Ceratoneis closterium (tropical, f/2)	2	0.9 (0.5–1.3)	2.7 (2.1–3.3)	2400 (2000–2900)	6000 (5700–6400)
Tisochrysis lutea	3	2.3 (1.9–2.7)	5.8 (5.5–6.1)	360 (300–410)	890 (800–970)
Symbiodinium sp.	3	3.1 (2.7–3.6)	5.6 (5.3–5.9)	ND	>1600
Freshwater species					
Chlorella sp.	3	0.7 (0.5–1.0)	2.8 (2.5–3.2)	22 (18–26)	170 (150–180)
Monoraphidium arcuatum	2	0.5 (0.4–0.5)	1.1 (1.0–1.2)	62 (48–76)	480 (430–530)

[a]The 72-h EC10 and 72-h EC50 values were derived by combining data (from individual replicates) from *n* tests.
Numbers in parentheses are the 95% confidence limits.
EC10/EC50 = 10% and 50% effect concentrations, respectively; ND = not determined.

Growth rates (cell division rates, μ) were calculated as the slope of the regression line from a plot of $\log_{10}$ (cell density) versus time (hours). Growth rates for each treatment flask were converted to a percentage of control. Test acceptability criteria included <1 pH unit change in control flasks, control growth rates ≥0.92 doublings/day, and <20% variation in control growth rates (Franklin et al., 2001; Markich et al., 2005; OECD, 2011).

Multispecies tests. The chronic toxicities of copper and nickel to three tropical freshwater microalgae were determined when exposed together in a multispecies test. *Pediastrum duplex*, *M. arcuatum*, and *Nannochloropsis*-like sp. were chosen as the test species because they co-occur naturally in the environment (Stone et al., 2019). The procedure was similar to that used in the single-species tests, except that 1 ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid (HEPES) buffer (10 mM) and 0.33 mg Fe/L (as iron [III] chloride) were added to 49 ml synthetic freshwater. The HEPES buffer was added following the method of Stone et al. (2019), initially developed for an herbicide study. It has been shown that HEPES is a very weak metal complexing buffer; therefore, it has been widely used in metal ion studies and shown not to affect metal bioavailability (Ferreira et al., 2015). The addition of iron ensured that all species achieved acceptable growth rates over the 72-h test duration (Stone et al., 2019). Six control replicates and two replicates per metal concentration (5–16 concentrations per test) were tested, with two tests for each metal. Measured dissolved (<0.45 μm) copper and nickel test concentrations ranged from 0.31 to 290 μg/L and from 7.2 to 4300 μg/L, respectively.

Cultures of microalgae were centrifuged, vortexed, and washed using synthetic freshwater per the single-species method. Cell densities were calculated using flow cytometry (FACSVerse; BD Bioscience). Each flask was inoculated with the three species to achieve a total initial cell density of 3×10^3 cells/ml (Supporting Information, Table S1). The number of cells added for each species was proportional to their surface area to avoid the confounding effect of increased biomass for metal binding on toxicity. The cells were added at an approximate ratio of 5:2:23 for *M. arcuatum*, *P. duplex*, and *Nannochloropsis*-like sp., respectively (Franklin et al., 2004; Stone et al., 2019). The flasks were incubated in a temperature- and light-controlled cabinet at 27 °C.

Cell densities were determined at 0, 24, 48, and 72 h, using flow cytometry. To perform quality assurance/quality control tasks including laser alignment daily BD FACSuite Research Cytometer Setup and Tracking Beads were used. Aliquots from each flask were homogenized in a hand-held tissue grinder (2 ml; Wheaton) prior to counting to break up any cell clumps. Cell densities were determined after differentiating the three microalgal populations based on their cell size and fluorescence properties (Stone et al., 2019).

Growth rates (cell division rates, μ) were calculated as the slope of the regression line from a plot of $\log_{10}$ (cell density) versus time (hours). Growth rates for each treatment flask were converted to a percentage of control. Test acceptability criteria were based on those of Stone et al. (2019).

Metal and dissolved organic carbon analyses

Immediately after the algal inoculum was added to all flasks, subsamples were taken for metal and dissolved organic carbon (DOC) analyses.

Subsamples were taken at 0 and 72 h for dissolved (filtered, 0.45 μm, using acid-washed syringes and filters) metal analyses. All samples were acidified to 0.2% HNO_3 (Merck; Tracepur) and analyzed using inductively coupled plasma atomic emission spectrometry (ICP-AES; Spectro Flame-EOP; Spectro Analytical Instruments, Kleve, Germany). The

instrument detection limit was 2.0 and 0.41 µg/L in seawater and freshwater, respectively, for copper and 6.7 and 0.57 µg/L in seawater and freshwater, respectively, for nickel. For all test treatments with nominal concentrations less than the detection limits of the ICP-AES, samples were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7500CE), for example, copper, where the detection limit on the ICP-MS is approximately 0.1 µg/L. For ICP-AES and ICP-MS analyses, metal concentrations were calculated from a matrix-matched calibration curve (0.45 µm filtered seawater or ultrapure water, acidified with 0.2% HNO_3) and a drift standard was incorporated into the analytical procedure. The average (0 and 72 h) measured dissolved (<0.45 µm) metal concentrations were used in the statistical analyses to derive 72-h effect concentrations (ECs).

Subsamples were taken from control flasks with both seawater and synthetic freshwater, to measure DOC at the beginning of each test (0 h). Samples were filtered (0.45 µm) and collected in a glass vial with 2 ml of concentrated H_2SO_4. Analysis of DOC was carried out at the National Measurement Institute (Sydney, Australia).

Statistical analyses

Data analysis and figure generation were performed using the statistical environment RStudio (RStudio Team, 2020). All data from individual tests for each species (two or three tests; see Tables 2 and 3) were pooled before statistical testing was performed. The method to derive 72-h effect concentrations using the "Dose Response Curve" R package (R Foundation for Statistical Computing, 2021; Ritz et al., 2015) was as described by Koppel et al. (2017). Akaike's information criterion was used to determine model suitability where multiple models were tested (Pinheiro & Bates, 2000). Generally, a three-parameter Weibull (Type 1 or 2) or four-parameter log-logistic-type model (and, where applicable, upper and lower asymptotes were fixed at 100% and 0%, respectively) provided the best fit to the data. All models (and corresponding parameters) used to derive 72-h EC values are given in Supporting Information, Table S3. When 10% EC (EC10) values could not be determined, Bonferroni's *t* test was used to determine which treatments were significantly different from the control (two-tailed, $p < 0.05$) to estimate the no-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC) using ToxCalc (Ver 5.0.23).

RESULTS

Quality assurance

Toxicity tests. Results for control acceptability criteria are presented in Supporting Information, Table S2. The mean ± 1 standard deviation (SD) of DOC concentrations in control flasks of seawater and synthetic freshwater were 1.5 ± 0.25 mg/L ($n = 6$) and 0.55 ± 0.07 mg/L ($n = 2$), respectively. Mean ± SD concentrations of dissolved copper and nickel in control flasks with seawater were 0.5 ± 0.3 ($n = 14$) and 1.9 ± 1.1 µg/L ($n = 14$), respectively. For controls flasks with synthetic freshwater, mean ± SD concentrations of dissolved copper and nickel were 0.6 ± 0.5 ($n = 7$) and 1.1 ± 0.7 µg/L ($n = 7$), respectively. For control flasks with seawater, the mean ± SD salinity was 36 ± 0.6 ppt ($n = 14$).

Dissolved metal loss throughout test duration. In all tests, the dissolved nickel concentrations remained fairly constant over the 72-h test durations (<10% loss over 72 h), whereas copper concentrations decreased >10% over the 72 h, particularly in the low-copper treatments. For this reason, average (0 and 72 h) measured dissolved copper and nickel concentrations were used to calculate 72-h EC values for all species in the present study. Low cell densities were used in all algal tests to try to minimize copper depletion in solution by algal exudates; however, copper losses still occurred, likely due to absorption to glass flasks and adsorption or absorption by algal cells (Franklin et al., 2002; Levy et al., 2007). Franklin et al. (2002) found 7%–30% of copper was adsorbed in the flasks over the 72-h test duration. At 10^3 cells/ml, approximately 50%–60% of the total copper at the end of the bioassay was still present in solution (as dissolved, 0.45 µm), in line with the findings in the present study (Franklin et al., 2002).

Metal analyses. A drift standard was used to monitor sample drift within analyses. The sample drift was <20% for nickel and copper and was corrected using drift correction calculations.

TABLE 3: Mean 72-h EC10 and 72-h EC50[a] values for freshwater microalgae exposed to dissolved (<0.45 µm) nickel and copper in multispecies tests

Freshwater species tested in the multispecies bioassay	*n*	Cu (µg/L)		Ni (µg/L)	
		72-h EC10	72-h EC50	72-h EC10	72-h EC50
Monoraphidium arcuatum	2	1.4 (1.2–1.6)	3.7 (3.5–3.8)	330 (280–380)	1100 (970–1200)
Pediastrum duplex	2	0.5 (0.4–0.6)	3.7 (3.4–4.0)	190 (150–220)	480 (420–530)
Nannochloropsis-like sp.	2	13 (9.5–16)	15 (13–17)	14 (8.5–19)	94 (79–110)

[a]The 72-h EC10 and 72-h EC50 values were derived by combining data (from individual replicates) from *n* tests.
Numbers in parentheses are the 95% confidence limits.
EC10/EC50 = 10% and 50% effect concentrations, respectively.

Toxicity of nickel to marine and freshwater microalgae in single-species tests

The five marine species varied in sensitivity to nickel by over an order of magnitude, with EC10 values ranging from 260 to 3100 µg Ni/L (Table 2 and Figure 1). The most sensitive tropical marine species was the haptophyte *Tisochrysis lutea* (EC10 of 360 µg Ni/L). No EC10 value could be determined confidently for *Symbiodinium* sp., but the NOEC for nickel was 310 µg/L and the LOEC was 440 µg/L. The temperate strain of *C. closterium* was the most sensitive marine species to nickel, with an EC10 of 260 µg Ni/L. The two tropical strains of *C. closterium* were not different in sensitivity at both EC10 and EC50 (concentration–response curve plotted for tropical *C. closterium* f/2 in Figure 1 only; see Supplementary Information, Figure S4, for the concentration–response curve for *C. closterium* G/2). The tropical strains of *C. closterium* were less sensitive (EC10 values >2400 µg Ni/L) to nickel than the temperate strain (EC10 of 260 µg Ni/L). The two freshwater microalgal species were more sensitive to nickel than the marine species, with EC10 values of 22 and 62 µg Ni/L for *Chlorella* sp. and *M. arcuatum*, respectively.

Toxicity of copper to marine and freshwater microalgae in single-species tests. Copper was more toxic than nickel to all microalgae, with EC10 values of 0.5–3.1 µg/L, two to four orders of magnitude lower than nickel (Table 2 and Figure 2). Generally, the freshwater species were more sensitive than the marine species to copper at both the EC10 and EC50. *Symbiodinium* sp. and *T. lutea* were slightly less sensitive to copper

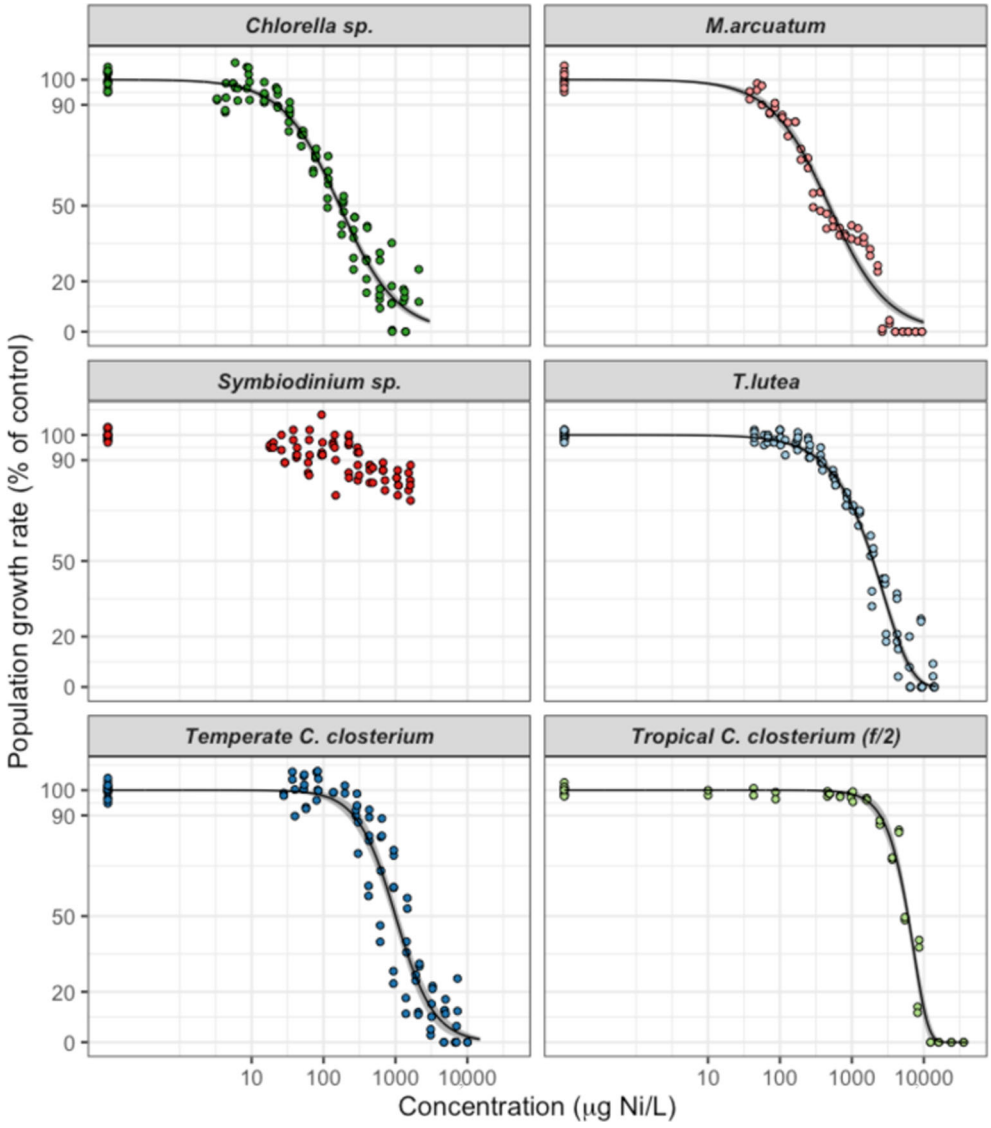

FIGURE 1: Concentration–response curves for microalgae in single-species tests exposed to concentrations of nickel. Growth rate is plotted against dissolved (0.45 µm filterable) average nickel concentration. Each point on the plot is a single test replicate, and the gray ribbons represent the 95% confidence limits of the toxicity estimates.

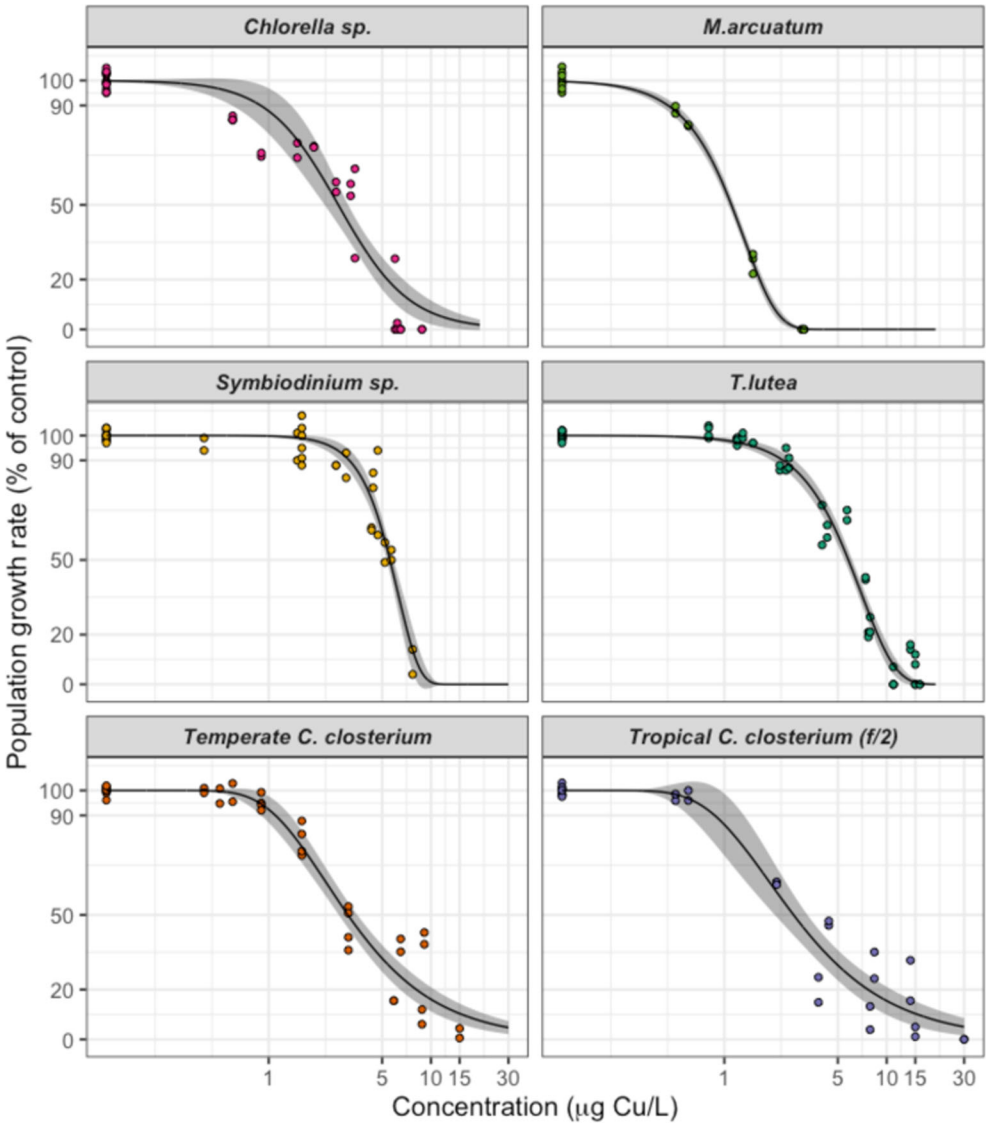

FIGURE 2: Concentration–response curves for microalgae in single-species tests exposed to concentrations of copper. Growth rate is plotted against dissolved (0.45 μm filterable) average copper concentration. Each point on the plot is a single test replicate, and the gray ribbons represent the 95% confidence limits of the toxicity estimates.

than the *C. closterium* strains. The two tropical strains and the temperate strain of *C. closterium* were all very similar in sensitivity at the EC10 and EC50 (Table 2). The concentration–response curve for one tropical strain of *C. closterium* (f/2) is presented in Figure 2 (See Supplementary Information, Figure S5 for the concentration response curve for C. closterium (G/2)).

Chlorella sp. and *M. arcuatum* were not different in sensitivity at the EC10, though *M. arcuatum* was more sensitive than *Chlorella* sp. at the EC50 and more sensitive than all marine species at both the EC10 and EC50.

Toxicity of nickel and copper to freshwater microalgae in multispecies tests. *Nannochloropsis*-like sp. had the lowest control growth rate of the three species, as found by Stone et al. (2019). When comparing the control growth rates of *M. arcuatum* in the single and multispecies tests, stimulation was observed in the multispecies tests, with control growth rates increasing from 2.0 ± 0.01 in the single-species tests to 2.6 ± 0.08 in the multispecies tests.

All three species varied in sensitivity to nickel (Table 3 and Figure 3). The order of most to least sensitive species to nickel was *Nannochloropsis*-like sp., *P. duplex*, and then *M. arcuatum*, with respective EC10 values of 14, 190, and 330 μg Ni/L (Table 3). *M. arcuatum* was between four and five times (when comparing EC10 and EC50 values, respectively) less sensitive to nickel when exposed in the multispecies compared with the single-species test exposure.

P. duplex was the most sensitive species to copper, with an EC10 of 0.5 μg Cu/L, followed by *M. arcuatum* (EC10 of 1.4 μg Cu/L) and *Nannochloropsis*-like sp. (EC10 of 13 μg Cu/L). The *Nannochloropsis*-like sp. response to copper was highly variable in the low treatments, with some stimulation also observed. *P. duplex* and *M. arcuatum* showed the

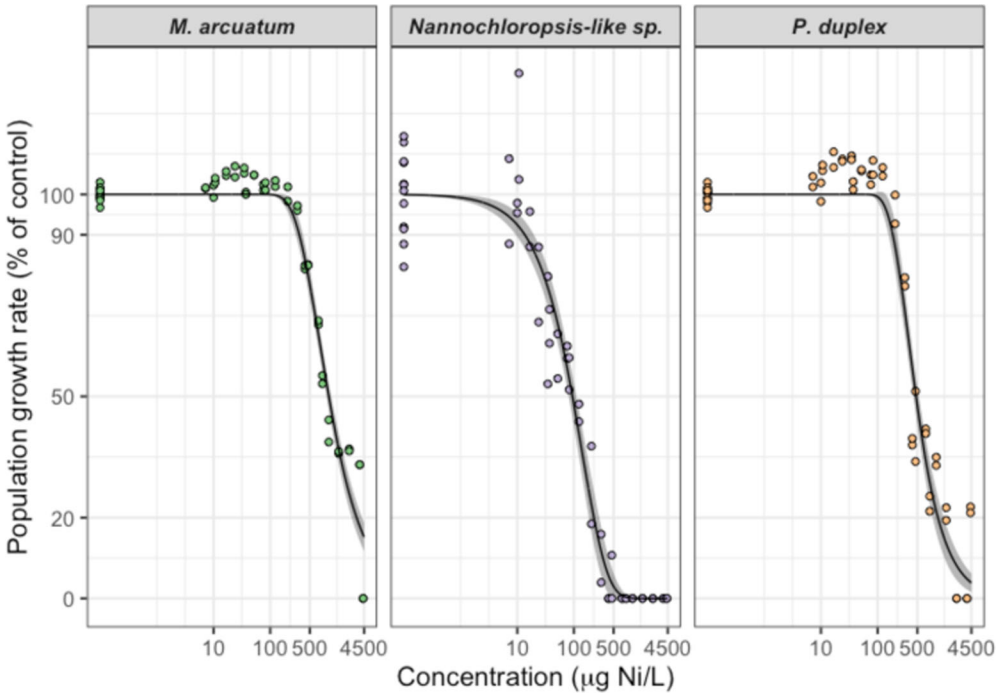

FIGURE 3: Concentration–response curves for microalgae in multispecies tests exposed to concentrations of nickel. Growth rate is plotted against dissolved (0.45 µm filterable) average nickel concentration. Each point on the plot is a single test replicate, and the gray ribbons represent the 95% confidence limits of the toxicity estimates.

same sensitivity to copper at the EC50 (Table 3 and Figure 4). *M. arcuatum* was approximately five times less sensitive to copper when exposed in the multispecies tests compared with the single-species tests. The EC10 increased from 0.5 to 1.4 µg Cu/L.

DISCUSSION

The present study has contributed high-quality data on the toxicity of copper and nickel to tropical marine and freshwater microalgae. However, it is difficult to compare our results to

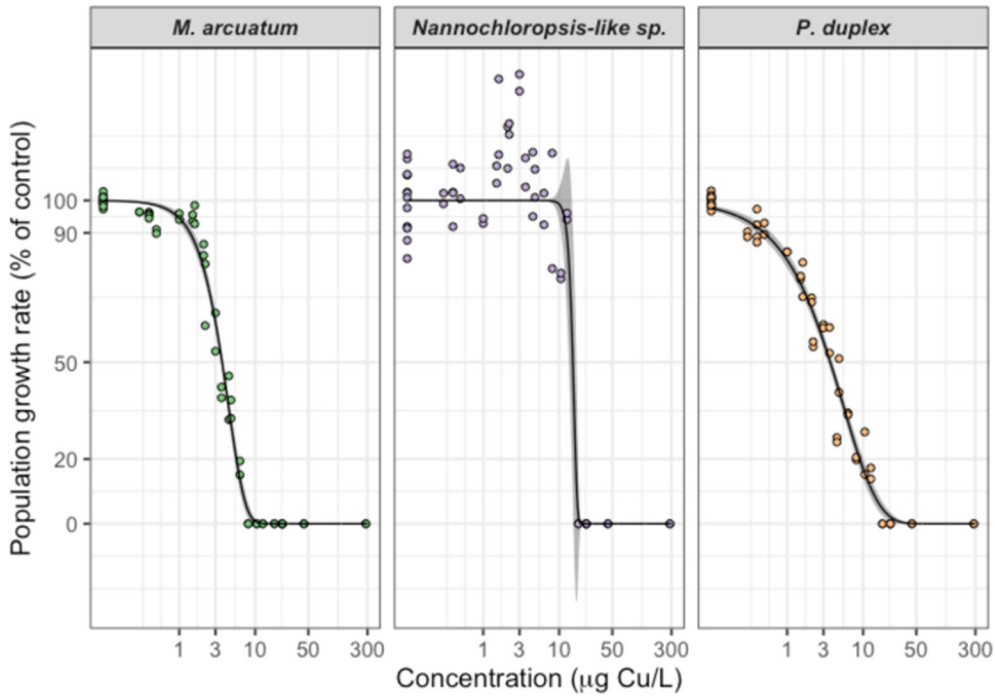

FIGURE 4: Concentration–response curves for microalgae in multispecies tests exposed to concentrations of copper. Growth rate is plotted against dissolved (0.45 µm filterable) average copper concentration. Each point on the plot is a single test replicate, and the gray ribbons represent the 95% confidence limits of the toxicity estimates.

previous studies, largely because most previous toxicity tests have been carried out using high initial cell densities and full-strength culture media, containing chelators and adsorbents that can modify metal bioavailability and consequently toxicity (Stauber & Davies, 2000).

Toxicity of nickel to tropical microalgae

Marine microalgae were relatively insensitive to nickel compared with freshwater microalgae, with EC50 values generally an order of magnitude higher than for freshwater species. In a compilation of nickel effects data for all tropical marine biota, Gissi et al. (2016) concluded that microalgae were less sensitive to nickel than invertebrates, with sea urchins being the most sensitive taxa. Importantly, the coral endosymbiont *Symbiodinium* sp. in the present study was also insensitive to nickel (EC50 > 1620 μg/L, the highest test concentration), similar to other coral life stages (Gissi et al., 2016). Given that dissolved nickel concentrations in coastal waters are typically <5 μg/L (Apte et al., 2006), this suggests that nickel is unlikely to be a risk in marine systems, except in highly contaminated waters where up to 2 mg/L nickel has been reported (Pyle & Couture, 2012).

By contrast, freshwater microalgae were found to be among the most sensitive tropical taxa to nickel, together with macrophytes and a snail (Binet et al., 2018). Reported EC50 values ranged from 41 to 2410 μg Ni/L (Binet et al., 2018; Deleebeeck, De Laender, et al., 2009) over a range of water chemistry conditions. The EC50 values for the freshwater species in the present study fell within this reported range, 170–480 and 94–1100 μg Ni/L for single-species and multispecies tests, respectively. It is well known that pH, hardness, and, to a lesser extent, DOC influence the speciation, bioavailability, and toxicity of nickel in freshwaters, so direct comparison of species sensitivities to nickel is difficult without normalization to account for water chemistry (Peters et al., 2018). Recent studies have confirmed that DOC has less influence on nickel bioavailability to microalgae in freshwaters when compared with the influence of DOC on copper bioavailability (Macoustra et al., 2021).

Toxicity of copper to tropical microalgae

Tropical marine and freshwater microalgae were similarly sensitive to copper, with EC50 values several orders of magnitude lower than EC50 values for nickel. The microalgae tested as single species in our study are among the most sensitive taxa to copper, with EC50 values <6 μg Cu/L and EC10 values <3 μg Cu/L.

Stauber and Davies (2000) compiled data on the toxicity of copper to marine and freshwater microalgae. The EC50 values ranged from 7 to 640 μg Cu/L for marine microalgae and from 1.5 to 430 μg Cu/L for freshwater species. This included three tropical *Chlorella* species, which were among the most sensitive freshwater species to copper. Stauber and Davies (2000) showed that the sensitivities of microalgae to copper were influenced by water chemistry conditions, including whether tests were conducted in nutrient-rich culture media, test endpoints, initial cell densities, exposure conditions and duration, as well as prior exposure to metals, making comparison of species' sensitivities to copper between studies problematic. Trenfield et al. (2022) found that an Australian isolated tropical *Chlorella* sp. was sensitive to copper when tested at the same low initial cell density as in the present study (EC50 9.1 [95% confidence limits 5.1–7.4]). The tests were performed in low-hardness Magela Creek water (<0.45 μm fraction), with lower pH and higher temperatures than in the present study. The tropical *Chlorella* sp. species used in our study has been tested by Johnson et al. (2007) with copper under identical water chemistry and similar test conditions, except the initial cell density was 10 times higher (2 to 4×10^4 cells/ml) than that used in our study. Johnson et al. (2007) calculated a mean ± SD 72-h EC50 of 7.9 ± 1.8 μg Cu/L, higher than the value calculated in the present study (EC50 of 3 [95% confidence limits 2.5–3.5] μg Cu/L). This is in agreement with Franklin et al. (2002), who showed that 72-h EC50 values for this species increased from 4.6 to 16 μg Cu/L as initial cell densities increased from 10^2 to 10^5 cells/ml. Using measurements of labile, extracellular, and intracellular copper, Franklin et al. (2002) found that at higher initial cell densities less copper was bound to the cells, resulting in less copper uptake and lower toxicity. In the higher copper test concentrations, an increase in complexed copper was observed, and the authors suggested that algal exudates may contribute to the complexation of copper and a reduction in copper binding to the algal cells at higher initial cell densities. A further comparison between the results of Johnson et al. (2007) and the present study shows differences in the sensitivity of the tropical marine alga *C. closterium* (previously known as *Nitzschia closterium*) to copper. With similar water chemistry and test conditions, except 10 times higher initial cell densities, the mean ± SD 72-h EC50 was 40 ± 4 μg Cu/L, much higher than our reported EC50 of 3.3 (3.1–3.6) μg Cu/L.

Toxicity of metals to *Symbiodinium* sp.

Because of the high ecological importance of the genus *Symbiodinium* in tropical marine ecosystems, understanding the impacts of metal contaminants on this taxon is highly relevant to its inclusion in SSDs to derive water quality guideline values for tropical systems. This is an important contribution of data on the chronic toxicity of nickel to the coral endosymbiont *Symbiodinium* sp. in the free-swimming phytoplankton stage because this is, to our knowledge, the first data of this kind. Data on the effects of other metals to *Symbiodinium* sp. are limited. Kuzminov et al. (2013) demonstrated that copper, zinc, cadmium, and lead disrupted photosynthesis in *Symbiodinium* sp., which could potentially reduce their growth and the supply of energy to corals. In an earlier study by Goh and Chou (1997), 40 μg Cu/L significantly reduced the growth of *Symbiodinium* sp. Their tests were conducted in a nutrient-rich medium which is known to reduce the toxicity of metals through chelation and adsorption. This is in contrast to our study, which showed that the growth rate of *Symbiodinium* sp. was inhibited by 10%

following exposure to only 3.3 μg Cu/L, concentrations that could be found in coastal waters.

Tropical versus temperate species sensitivities

Based on our tests with two tropical strains and one temperate strain of the marine diatom *C. closterium*, the tropical strains were less sensitive to nickel, but of similar sensitivity to copper, as the temperate strain. Limited studies using SSDs have shown that tropical marine biota are no more or less sensitive to contaminants than their temperate counterparts and that it is difficult to predict sensitivity between different climatic regions (Chapman et al., 2006; Gissi et al., 2020; Warne et al., 2018). Wang et al. (2014) found only small differences in acute toxicity of chemicals between tropical and temperate marine biota, including microalgae, macrophytes, invertebrates, and fish, with no general trend observed for different chemicals.

For copper, the temperate marine dinoflagellate *Heterocapsa niei* had a similar sensitivity to that of the tropical dinoflagellate *Symbiodinium* sp. tested in the present study; 72-h EC50 values for these species were 4.8 and 5.6 μg Cu/L, respectively (Levy et al., 2007). Levy et al. (2007) found that the temperate strain of *C. closterium* (previously known as *N. closterium*) was less sensitive to copper than both the temperate and tropical strains tested in the present study; the 72-h EC50 value was 18 μg Cu/L, six times higher than the 72-h EC50 values calculated in the present study. Two differences between the methods of the present study and those of Levy et al. (2007) were the nitrate concentrations used in the test exposure and different methods of analyzing dissolved copper exposure concentrations. Similarly, a temperate strain of *Isochrysis galbana* (Debelius et al., 2009) was less sensitive than a tropical strain (*T. lutea*, previously known as *I. galbana*), tested in the present study and by Levy et al. (2007). Test conditions differed in Debelius et al. (2009), including having 10-fold higher initial cell densities and the use of continuous light for test exposures. These studies highlight the difficulties of comparing species sensitivities between regions because of the different test conditions used.

Peters et al. (2019) have compared the nickel toxicity thresholds, overall SSDs, and closely related groups of species using temperate and tropical freshwater data sets, with and without bioavailability normalization. Bioavailability normalization made little difference to the derived guideline values, largely because ecotoxicity data were derived under high nickel bioavailability conditions, for example, low DOC. Although direct comparison of the temperate and tropical SSDs was confounded by the lower taxa diversity and lack of insensitive taxa such as mollusks and fish in the tropical SSD, they did show that nickel thresholds (95% protective concentration values), which ranged from 3.5 to 8.6 μg/L, were similar between tropical and temperate distributions. Stauber et al. (2021) also showed that there was little difference in the sensitivities of tropical and temperate freshwater biota to nickel.

Multispecies tests

The species in the present study were used following the study of Stone et al. (2019), who developed a multispecies test with freshwater tropical microalgae to test the effect of herbicides on microalgal growth. Species co-occurring in the environment were mixed together on the basis of equivalent surface areas to avoid the confounding effect of increased biomass for metal binding on toxicity.

In our study, *M. arcuatum* growth was stimulated in the controls (no added metal) in the multispecies tests compared with the single-species tests, and the mean ± SD growth rates were 2.6 ± 0.08 and 2.0 ± 0.01, respectively. This may have been due to different medium conditions (no additional iron or HEPES in single-species exposures) or to the presence of the other algae. In contrast, Stone et al. (2019) found that there was no difference in control growth rates of *M. arcuatum* in single-species tests and in the presence of *P. duplex* and *Nannochloropsis*-like sp. when the same media type was used for all tests. In applying the newly developed multispecies tests (developed for pesticide toxicity), methods were followed per Stone et al. (2019), and further testing would be required to match the medium conditions in the present study's single-species tests and to compare the growth rates of *M. arcuatum*. Other studies have shown that the growth of some freshwater algae does increase in the presence of other species, presumably due to allelopathic effects from exudates (de Morais et al., 2014; Vasconcelos et al., 2002).

The control growth rate of *Nannochloropsis*-like sp. in the multispecies test was more variable in the present study than the Stone et al. (2019) study. However, the control growth rate variability of *Nannochloropsis*-like sp. was still within acceptable limits (<20%). Because there are limited toxicity test data for this species under these laboratory conditions, the control growth rate variability across tests is not yet well understood.

M. arcuatum was less sensitive to copper and nickel in the multispecies tests compared with the single-species tests, with the 72-h EC50 values increasing significantly from 1.1 to 3.7 μg Cu/L and from 480 to 1100 μg Ni/L, respectively. Differences in media conditions such as pH may have led to a reduction in sensitivity, and further testing is required to investigate this. Nickel toxicity has previously been shown to decrease with a decrease in pH and to increase as hardness decreases (Deleebeeck, De Schamphelaere, & Janssen, 2009). A similar reduction in copper toxicity was found for the marine microalga *Phaeodactylum tricornutum*, with the 72-h EC50 value increasing from 13 μg Cu/L in the single-species test to 24 μg Cu/L in the multispecies test (Franklin et al., 2004). These authors found that the reduction in copper toxicity was not due to differences in copper complexing capacity in solution as a result of exudate production by the other algae. They hypothesized that this was potentially due to speciation changes. We did not test this in our study, but we can conclude that changes in the toxicity of copper were not due to differences in initial biomass because all algae in the mixture were added with the same biomass based on algal cell surface area. However, *M. arcuatum* growth was stimulated in

the controls (no added metal) in the multispecies tests compared with the single-species tests, so it is possible that less metal bound per cell resulted in a reduced metal toxicity in the multispecies test. The opposite was found for copper by Franklin et al. (2004); that is, the toxicity of copper to the temperate freshwater alga *Trachelomonas* sp. was greater in the presence of two other species (*Pseudokirchneriella subcapitata* and *Microcystis aeruginosa*), with 72-h EC50 values decreasing from 10 to 3 μg/L. It is possible that exudates produced by the other algae in response to copper had an inhibitory effect on *Trachelomonas* sp. growth, not observed in the absence of copper.

Incorporation of toxicity data in water quality guidelines

The Australian and New Zealand water quality guideline values for nickel and copper in both marine and freshwaters are currently being revised (ANZG, 2018). The proposed draft nickel guideline value for 95% species protection for slightly to moderately disturbed marine systems is 6 μg Ni/L (Gissi et al., 2020) and is based on a combination of temperate and tropical data, including the microalgal data derived from the present study. The proposed freshwater nickel guidelines will also include our data and will be normalized for water chemistry using recently developed bioavailability models (Stauber et al., 2019, 2021). The current guideline for copper is 1.3 μg Cu/L for 95% species protection in slightly to moderately disturbed marine waters. However, both temperate and tropical strains of *C. closterium* in the present study had EC10 values below the current guideline. Similarly, the current freshwater copper guideline of 1.4 μg Cu/L for 95% species protection may not be sufficiently protective of two freshwater microalgae used in the present study.

CONCLUSIONS

Single-species and multispecies tests with a range of tropical marine and freshwater microalgae have provided much needed data on the effects of nickel and copper on these tropical primary producers. These new data are now being incorporated into SSDs to derive improved water quality guideline values in Australia and New Zealand.

The results from the single and multispecies tests highlight possible differences in sensitivity of algal species when either coexposed or individually exposed to metals in toxicity tests. Multispecies tests can be considered to be more environmentally relevant than single-species tests because microalgae occur naturally as mixed communities.

Copper was found to be more toxic than nickel to all species, confirming earlier studies that showed that copper may be a risk to aquatic biota. Of most concern is that three species (one marine and two freshwater microalgae) had EC10 values below the current copper water quality guideline value for 95% species protection in slightly to moderately disturbed systems in Australia and New Zealand. In contrast, toxicity of nickel to microalgae is unlikely to occur at exposure concentrations typically found in fresh and marine waters.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://10.1002/etc.5565.

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Data Availability Statement—The data have been uploaded to the CSIRO Data Access Portal. The DOI is doi.org/10.25919/bwcn-rm71. Data, associated metadata, and calculation tools are also available from the corresponding author (kitty.mcknight@mq.edu.au).

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