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Assessing the chronic toxicity of nickel to a tropical marine gastropod and two crustaceans

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Abstract

The mining and processing of nickel ores from tropical regions contributes 40% of the global supply. The potential impact of these activities on tropical marine ecosystems is poorly understood. Due to the lack of ecotoxicity data for tropical marine species, there is currently no available water quality guideline value for nickel that is specific to tropical species. In this study, we investigated the toxicity of nickel to three tropical marine invertebrates, the gastropod Nassarius dorsatus, the barnacle Amphibalanus amphitrite, and the copepod Acartia sinjiensis. All toxicity tests used chronic endpoints, namely larval growth, metamorphosis (transition from nauplii to cyprid larvae) and larval development for the snail, barnacle and copepod respectively. Toxicity tests were carried out under environmentally relevant conditions (i.e. 27-30°C, salinity 34-36‰ pH 8.1-8.4). Copper was also tested for quality assurance purposes and to allow for comparisons with previous studies. The copepod was the most sensitive species to nickel, with development inhibited by 10% (EC10) at 5.5 (5.0-6.0) μg Ni/L (95% confidence limits (CL)). Based on EC10 values, the gastropod and barnacle showed similar sensitivities to nickel with growth and metamorphosis inhibited by 10% at 64 (37–91) μg Ni/L and 67 (53–80) μg Ni/L, respectively. Based on existing data available in the literature, the copepod A. sinjiensis is so far the most sensitive tropical marine species to nickel. This study has provided high quality data which will contribute to the development of a water quality guideline value for nickel in tropical marine waters. A species sensitivity distribution of chronic nickel toxicity used the data generated in this paper supplemented by available literature data, comprising 12 species representing 6 taxonomic groups. A 5% hazard concentration (HC5) was determined as 8.2 μg/L Ni.

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Medicine and Health Sciences | Social and Behavioral Sciences

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Assessing the chronic toxicity of nickel to a tropical marine gastropod and two crustaceans.

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Abstract

The mining and processing of nickel ores from tropical regions contributes 40% of the global supply. The potential impact of these activities on tropical marine ecosystems is poorly understood. Due to the lack of ecotoxicity data for tropical marine species, there is currently no available water quality guideline value for nickel that is specific to tropical species.

In this study, we investigated the toxicity of nickel to three tropical marine invertebrates, the gastropod *Nassarius dorsatus*, barnacle *Amphibalanus amphitrite,* and copepod *Acartia sinjiensis*. All toxicity tests used chronic endpoints, namely larval growth, metamorphosis (transition from nauplii to cyprid larvae) and larval development for the snail, barnacle and copepod respectively. Toxicity tests were carried out under environmentally relevant conditions (i.e. 27-30°C, salinity 34-36‰, pH 8.1-8.4). Copper was also tested for quality assurance purposes and to allow for comparisons with previous studies.

The copepod was the most sensitive species to nickel, with development inhibited by 10% (EC10) at 5.5 (5.0-6.0) µg Ni/L (95% confidence limits (CL)). Based on EC10 values, the gastropod and barnacle showed similar sensitivities to nickel with growth and metamorphosis inhibited by 10% at 64 (37-91) µg Ni/L and 67 (53-80) µg Ni/L, respectively. Based on existing data available in the literature, the copepod *A. sinjiensis* is so far the most sensitive tropical marine species to nickel. This study has provided high quality data which will contribute to the development of a water quality guideline value for nickel in tropical marine waters. A species sensitivity distribution of chronic toxicity used the data generated in this paper supplemented by available literature data, comprising 12 species representing 6 taxonomic groups. A 5% hazard concentration (HC5) was determined as 8.2 µg/L Ni.

Keywords

Tropical ecotoxicology, metals, copepod, invertebrate, bioassay

1. *Introduction*

Within the tropics, unique ecosystems harbouring rich biodiversity are juxtaposed with highly dense coastal urban populations which have the potential to impact the valuable coastal marine environment. Many countries within the tropics, particularly within the Asia-Pacific region, are extremely dependent on their coastal ecosystems for food, income (through tourism and fishing), and spiritual and cultural values. Most countries within the Asia-Pacific region are economically classified as developing, and, as a consequence, their limited environmental regulatory frameworks may hinder the implementation of environmental protection measures (Reichelt-Brushett, 2012).

The mining and production of nickel has recently intensified in the Asia-Pacific region (USGS, 2016). Approximately 70% of the world's nickel reserves are found in the tropics, contributing about 40% of the global supply (Van der Ent et al., 2013). Currently, there is limited research into the potential impacts of these activities on the coastal marine environment and also a lack of ecologically relevant risk assessment tools. These tools include bioavailability-based toxicity tests and water quality guideline values (WQGVs, i.e. a numerical threshold below which risks to aquatic ecosystems are not expected) that can be used by government and industry to contribute to the continued management of contaminants in aquatic environments and protection of local aquatic biota (Wang et al., 2014). In general, GVs for metals in tropical regions have been based on temperate data due to the limited availability of ecotoxicity data for tropical species (Howe et al., 2014; Van Dam et al., 2008). In areas of intensified nickel mining activity, it would be beneficial to have a regionally-specific GV for nickel.

Water quality GVs are increasingly being derived using species sensitivity distributions (SSDs), which estimate a protective concentration (PC), usually for 95% of species (known as the PC95 value), which corresponds to the 5% hazardous concentration (HC5). The guidance around SSDs and WQGV derivation is jurisdiction-dependent. In Europe, 10-15 species are recommended for input into SSDs (ECHA, 2008; OECD, 2011); in North America the recommendation is at least 15 species (USEPA, 2005). In Australia and New Zealand, at least eight species representing four taxonomic groups is preferred (ANZECC/ARMCANZ, 2000; Warne et al., 2015). The underlying principle in all jurisdictions is that the greater the number of species (data) used in an SSD, the more likely the PC95 is to be protective of a broad range of species in the ecosystem.

A recent review by Gissi et al. (2016) compiled and quality-checked available nickel toxicity data for tropical marine species. Using only high quality (e.g. reported measured metal concentrations in chronic toxicity tests), a total of six data points representing four taxonomic groups was found and this was insufficient to derive a GV (Gissi et al., 2016). Key data gaps identified in the review included cnidarians, molluscs, crustaceans, echinoderms, macroalgae and fish. To address this data gap, nickel toxicity data have recently been published for three different species of corals (cnidarians), based on fertilisation success (Gissi et al., 2017). In the present study, we aimed to further contribute to the body of chronic nickel toxicity data for tropical marine species, specifically for molluscs (gastropods) and crustaceans. These taxonomic groups have high ecological importance in tropical regions because of the important role they play in the food web as well as species richness and biodiversity which is highest in the tropics (Bouchet et al., 2002; Humes, 1994; Roberts et al., 2002). Additionally crustaceans and molluscs are among the most sensitive species to nickel exposure in temperate systems (Bielmyer et al., 2006; Deforest and Schlekat, 2012; Niyogi et al., 2014).

We have investigated the effects of nickel on a gastropod, the channelled dog whelk *Nassarius dorsatus,* and two crustaceans, the purple-acorn barnacle *Amphibalanus amphitrite* and the copepod *Acartia sinjiensis.* All three species tested in this study are ecologically relevant, and are found in tropical marine environments of the Indo-Pacific region. *Nassarius dorsatus* is most common in tropical North Australia, and has also been reported in coastal waters off Malaysia, Indonesia, Papua New Guinea, Fiji and the Philippines (Trenfield et al., 2016). *Amphibalanus*

amphitrite is a common biofouling organism, widely distributed across tropical to temperate waters, in the mid to low intertidal zone (Desai et al., 2006; van Dam et al., 2016). This species has been widely used as a model species in biofouling and ecotoxicity tests (Rittschof et al., 1992; van Dam et al., 2016). *Acartia sinjiensis* is found in tropical and sub-tropical brackish to marine waters in Australia and some other locations within the Asia-Pacific (Gissi et al., 2013). This species of copepod is a common food source for many higher trophic organisms (Camus and Zeng, 2008).

The objectives of this study were to assess larval growth rate for the snail, metamorphosis (successful transition from nauplii to cyprid larvae) for the barnacle and larval development for the copepod, to determine the chronic toxicity of nickel to each of the three species. In addition, copper, a commonly-used reference toxicant, was tested for quality assurance purposes, enabling comparisons with previous studies and also because copper is a widespread anthropogenic contaminant in marine environments (Levy et al., 2007). The toxicity data presented here will contribute to the further development of reliable WQGVs for nickel in tropical marine waters.

2. Materials and Methods

2.1. General laboratory techniques and reagents

Snail and barnacle toxicity tests were conducted at the Australian Institute of Marine Science (AIMS, Darwin, Northern Territory, Australia). One rangefinding and four definitive tests were conducted each for the snail and barnacle. Three definitive copepod tests were carried out at the CSIRO Land and Water laboratories in Sydney, NSW, Australia.

All glassware and plastic containers used in the tests were acid-washed in 10% (v/v) nitric acid (Merck) and thoroughly rinsed with demineralised water, followed by Milli-Q® water (MQ, 18.2MΩ/cm; Merck). Glass funnels used in barnacle tests were silanized (Coatasil, Ajax, Finechem), approximately 2 weeks prior to testing and thoroughly rinsed with demineralised water, soaked in 10% nitric acid for 24 h, and finally rinsed again with demineralised water, followed by MQ water. All metal stock solutions were made volumetrically using MQ water. Copper stock solutions of 5 and 100 mg Cu/L were prepared using copper (II) sulfate salt (A.R. grade, AJAX Chemicals, Australia). Nickel stock solutions of 10 mg/L and 100 mg/L were made using nickel (II) chloride hexahydrate salt (A.R. grade, Chem Supply, Australia). All metal stocks were acidified to 0.1% HCl (Tracepur; Merck) to ensure metals did not precipitate.

For the snail and barnacle tests, water quality parameters including dissolved oxygen (DO), pH, conductivity and salinity were measured using a multi-probe (Hach multiprobe HQ40d), which was calibrated each day according to the manufacturer's instructions. For snail tests, measurements were taken on Day 0 and every 24 h. For barnacle tests, measurements were taken on Days 0 and 4. In all copepod tests, salinity measurements were taken using a YSI salinity and conductivity meter (model30/10FT, YSI, Ohio, USA). The pH was measured using a Thermo Orion pH meter with an epoxy body probe (meter model 420, probe model ROSS815600, Thermo Fisher Scientific, USA) which was calibrated daily. Dissolved oxygen (%) was measured using an Oximeter (Oxi330WTW, Weilheim, Germany), which was calibrated immediately prior to use. Water quality parameters were measured on Day 0, Day 2 before and after renewal and on Day 3. Temperature was recorded in all toxicity tests throughout the exposure.

2.2. Toxicity tests with the snail *Nassarius dorsatus*

Culturing, larval hatching and toxicity testing with *N. dorsatus* followed the methods described in Trenfield et al. (2016), and utilised the same broodstock of snails. In brief, egg batches were laid on polystyrene tubes positioned in the broodstock tank. Prior to the eggs hatching, tubes were transferred to a clean container with filtered seawater. The egg batches were maintained at 29°C and after ~5 days larvae hatched, then were fed once per day with a mixture of the microalgae *Chaetoceros muelleri* and *Rhodomonas salina* for 2 days.

Seawater for snail tests was collected in 20-L polyethylene containers from Nightcliff Jetty, Northern Territory, Australia (12°22'59"S, 130°50'56"E)), at high tide and filtered (0.45-µm filter, Quickfilter groundwater cartridge; Thermofisher Scientific) immediately upon return to the laboratory. Filtered seawater was stored at 4°C in the dark.

Treatment solutions were prepared using seawater in 2-L high density polyethylene (HDPE) bottles 24 h prior to test commencement. Across four individual toxicity tests, nominal nickel concentrations were in the range of 50 – 1500 µg Ni/L. In the single copper test, nominal concentrations were 2, 4, 8, 12 and 20 µg Cu/L. On Day 0, unwashed axenic cultures of the microalgae *C. muelleri* and *R. salina* were added to each test container to give a final concentration of 1 $x10^4$ cells/mL of each species, then 100 mL of each treatment solution was dispensed into the relevant test container, and 10 larvae (2-day old) were gently added using a wide-mouthed glass pipette. Test containers were placed in a temperature controlled cabinet set to 28°C (as per Trenfield et al., 2016). The volume of microalgae added to the test containers each day was <4% of the total test volume. Five to six metal treatments were tested alongside a control, with three replicates per treatment. New treatment solutions were made every 24 h and prior to the addition of new food and solutions, each test container was observed and the number of living larvae were counted and checked under a stereomicroscope. Larvae without a heartbeat were classified as dead and were removed from the test containers. Sub-samples were taken from the bulk treatment solutions on Day 0, Day 2 and from one replicate container of each treatment on Day 4 to measure total and dissolved metals. Throughout the exposure, test vessels were randomized twice a day.

On Day 0, 20 larvae from the batch of larvae used in the test were selected and photographed to determine the average starting length as described by Trenfield et al. (2016). Images were captured using a Leica DFC320 camera attached to a Leica DM4000B microscope at a magnification of 250×. On Day 4, after observing larval survival, five larvae from each test container were selected at random and photographed for length measurements. Growth rate (µm/d) was calculated as (Day 4 length – Day 0 length)/4. These values were converted to percent of control for statistical analysis.

2.3. Toxicity tests with the barnacle *Amphibalanus amphitrite*

Culturing, broodstock spawning, and toxicity testing methods followed those established in van Dam et al. (2016) and utilised the same broodstock.

Seawater used in barnacle tests came from the aquaria in the AIMS laboratory, which was UV-sterilized and filtered through a 0.5-µm polypropylene cartridge filter. Treatment solutions were prepared using seawater in 1-L HDPE bottles 24 h before test commencement to ensure equilibration. Treatment solutions were made 1.5 times more concentrated than nominal values to allow for further dilution upon addition of nauplii in seawater to the test containers. Across four individual toxicity tests, five nominal concentrations of nickel were tested ranging from 50 – 500 µg Ni/L. In the single copper test, the nominal concentrations tested were 20, 40, 60, 80 and 100 µg Cu/L. Sub-samples were taken from the bulk solutions for total and dissolved metals. In each toxicity test, five metal treatments were tested alongside a control, with four replicates per treatment.

On Day 0, the microalga *C. muelleri* was filtered through a 48-µm mesh, centrifuged and washed with seawater to remove culture media. Cell densities were determined using a haemocytometer, and 1 $x10^5$ cells/mL were added to each test container. This process was followed each day using the same culture stock of algae to provide food to the nauplii throughout the exposure. The volume of algae added to the test containers each day was low (<0.5%).

Glass funnels were set up on aeration lines and 100 mL of the treatment solutions were added to the relevant funnels, as well as 10^7 cells of concentrated *C. muelleri*. Funnels were gently aerated throughout the exposure. Following induction of spawning, nauplii ≤2-h old were collected from the broodstock tank. One polystyrene jar, containing 40 mL of seawater and 5 x 10^6 cells of *C. muelleri*

was prepared for each funnel. Nauplii were transferred to a Petri dish and using a stereomicroscope, 75 nauplii were gently pipetted from the Petri dish into each polystyrene jar. One jar was then transferred into each funnel and the jar was rinsed with 10 mL of seawater that was also added to the funnel to ensure all nauplii were transferred. Funnels were capped with plastic Petri dish bases. Funnels were kept in the aquaria at 29°C, under the same conditions described in van Dam et al. (2016). Randomizing test vessels during the exposure was not possible (van Dam et al. 2016).

At 96 h, funnels were disconnected from the aeration line and the contents drained onto a 150-µm nitrile mesh. Using a stereomicroscope the number of cyprid larvae (the larval stage prior to settlement) were recorded and the success of metamorphosis was calculated as the number of cyprid larvae at 96 h/ number of nauplii at 0 h. These values were converted to percent of control for statistical analysis. One sample was taken from one replicate of each treatment to measure physico-chemical parameters and to sub-sample for total and dissolved metals.

2.4. Toxicity tests with the copepod *Acartia sinjiensis*

Copepods were cultured according to Gissi et al. (2013). Toxicity test methods followed the protocol established by Binet et al. (unpublished). Mass cultures of *A. sinjiensis* were obtained from Queensland Department of Primary Industries Northern Fisheries Centre, originally isolated from plankton collections offshore from Townsville, Queensland (Gissi et al., 2013). Seawater used in tests was collected from Cronulla, NSW, Australia, in HDPE containers, filtered through an acid-washed (10% v/v, nitric acid, Merck KGaA, Darmstadt, Germany) 0.45-µm cartridge filter (Sartobran P sterile midicap, Sartorius Stedium Biotech, Germany), and stored in polyethylene containers at 4°C in the dark.

Copepod larval development tests were semi-static with a partial renewal on Day 2. Physico-chemical parameters were recorded, and sub-samples of each treatment were collected for total and dissolved metal analyses (see Section 2.6) at test commencement (0 h), before and after renewal (48 h), and again at test completion. Three individual nickel toxicity tests were performed encompassing the nominal concentration range of 4 – 16 µg Ni/L. For the three individual copper tests, nominal concentrations ranged from 1 – 12 µg Cu/L. In each toxicity test five metal treatments were tested alongside a control with four replicates per treatment. The control treatment had eight replicates to allow for additional replicates to be sacrificed when checking the larval development ratio (LDR) on Day 3.

Bulk solutions of nickel and copper treatments were prepared in clean acid-washed polycarbonate containers in filtered seawater with added microalgal food on Day 0 (8×10^4 cells/mL *Tisochrysis lutea/*mL and 0.63×10^4 cells/mL *Tetraselmis chuii*) and Day 2 (4×10^4 cells/mL *Tisochrysis lutea/*mL and 0.31×10^4 cells/mL *Tetraselmis chuii*). Microalgae were centrifuged prior to addition to treatments to remove algal growth medium. The total volume of algae added to each test container was <0.5%. On Day 0, 60 mL of each test treatment was dispensed into clean acid-washed 250-mL polycarbonate containers and allowed to equilibrate to 30 °C for 1 h in the test cabinet.

Adult copepods were isolated 24 h prior to test commencement. The isolated adults were fed a mixture of microalgae, *Tisochrysis lutea* and *Proteomonas sulcata* (1:8). On the day of test commencement (Day 0), eggs were isolated from adult cultures, rinsed with seawater into a 100-mL beaker and returned to the culture cabinet until required. Prior to inoculation of eggs, any nauplii that hatched were removed by rinsing with MQ and the eggs concentrated again in seawater (Binet et al. unpublished). This egg concentrate was counted on the microscope (Olympus SZX10, Japan, 10x magnification) to determine the egg density and volume of inoculum such that approximately 40-60 eggs were added to each test container with 60 mL of treatment solution. The inoculated test was placed in the test cabinet and the time recorded. Throughout the exposure, test containers were randomized in the cabinet twice a day.

On Day 2, a partial renewal was completed by replenishing each test container with 120 mL of freshly prepared test treatment solution with algae. The test was terminated after approximately 80 h, or when a minimum of 50% of animals in the controls had developed into copepodites (LDR ≥ 50%) (ISO, 2015; OECD, 2007). Following measurement of physico-chemical parameters and sub-sampling for metals, all containers were fixed with formalin and stained with Rose Bengal (Binet et al., unpublished). These containers were stored in the dark at 4 °C for a minimum of 24 h before LDR assessments were performed.

Fixed samples were filtered through a 54-µm sieve, rinsed with MQ water and counted under a dissecting microscope (Olympus SZX10, Japan, x10 magnification) to determine number of eggs, nauplii and copepods. The larval development ratio was calculated (LDR = ∑copepodite/∑(copepodite + nauplii)). These values were converted to percent of control for statistical analysis.

The samples were also analysed using a zooplankton analyser (Zooscan 3, Hydroptic Model #ZSCA03, France). The scanned data were processed and analysed using Zooprocess 7.22 (http://www.zooscan.obs-vlfr.fr/rubrique.php3?id_rubrique=49?lang=en) and Plankton Identifier v1.3.4 (http://www.obs-vlfr.fr/~gaspari/Plankton_Identifier/index.php) as per Binet et al. (unpublished).

Use of the Zooscan to count and identify the larval stage of each animal detected by the software as either eggs, naupliar stage 1 and 2 (N1N2), naupliar stage 3 and 4 (N3N4), naupliar stage 5 and 6 (N5N6), copepodite stages 1, 2 and 3 (C1C2C3) and copepodite stages 4, 5 and 6 (C4C5C6) has been validated in our laboratories (Binet et al. unpublished). These groupings were required to achieve the lowest error rate in the software's ability to accurately categorise animals. In this study, toxicity estimates were based on the larval development ratio determined by microscope counts. The Zooscan data was used to investigate the number of animals at each stage of development.

2.5. Chemical analyses

All plastic ware used for metal sub-sampling was acid washed (10% v/v, Tracepur; Merck) and rinsed with MQ water in a semi-clean room. All dissolved metal sub-samples were filtered through acid-washed syringes and 0.45-µm sterile filters (Sartorius Ministart® Syringe Filter, Germany), collected in acid-washed 5-mL polypropylene vials and acidified to either 0.2% (dissolved metals) or 2% (total metals) with Tracepur nitric acid (Tracepur; Merck). Samples were stored at 4°C in the dark until analysis. Most samples were analysed using inductively coupled plasma-atomic emission spectroscopy (ICP-AES 730ES), except for copper samples which were below, or close to the detection limit of the ICP-AES (~1 µg Cu/L). Instead these samples were analysed by ICP–mass spectrometry (ICP-MS; Agilent 7500CE), with a detection limit of ~0.1 µg Cu/L. Quality assurance procedures included matrix-matched calibration standards, drift standards and seawater blanks. Background concentrations of metals in seawaters used in tests and the limit of detection (LOD) for each metal analysed are presented in the supplementary information (Tables S2-4).

Sub-samples were taken from the seawater used in all tests to measure dissolved organic carbon (DOC). Samples were filtered through a 0.45-µm filter and collected in a glass vial with 2 mL of concentrated H_2SO_4. Analysis of DOC was done by the National Measurement Institute (NMI), Sydney, Australia.

2.6. Statistical analyses

For snail and copepod tests, the measured dissolved metal values were used to calculate the time weighted average concentration (TWA). For barnacle tests where only two measurements were taken, at Days 0 and 4, the mean was calculated. The TWA or mean dissolved metal concentrations were used in the statistical analyses. For each species, data from all four tests (or three tests for the copepod) were combined and analysed using the free software R (version 3.3.2, 2016-10-31) (R Core

Team, 2016) in the drc package (version 3.0-1, (Ritz et al., 2015)). For each dataset, 3 different models including the log-logistic models with 3 and 4 parameters, LL.3 and LL.4, and the Weibull models with 3 parameters (W1.3) were fitted to the concentration-response curve. The model of best fit was selected using the Akaike Information Criterion (AIC, model of best fit had the lowest AIC value, Table S1) and by visual assessment of the model. The chosen model was then used to determine toxicity estimates (EC10, EC20, EC50). The GGplot package was used to generate a graphical representation of the data and the model used to calculate toxicity estimates.

3. Results

3.1. Quality control

The background concentrations of metals in the seawater used in all tests were generally below the limit of detection (LOD) (Supplemental material Table S2-4). Additionally metals in seawater used for culturing were also below LOD (data not shown). For all three species, in all toxicity tests, physico-chemical parameters remained within the expected ranges. For the snail, pH ranged from 8.1 - 8.4, salinity ranged from 35-37‰; for the barnacle, pH ranged from 8.2-8.4, salinity was 33-34‰; and for the copepod, pH ranged from 8.1-8.2, salinity ranged from 34-36‰. In all tests, DO was maintained above 90%. In all toxicity tests, the temperature was maintained within 0.5°C of the required temperature; 28°C for the snail, 29°C for the barnacle and 30°C for the copepod. The average concentration of DOC in the filtered seawater used in snail tests was 0.7 ± 0.1 (SE) mg/L; in barnacle and copepod tests it was 0.8 ± 0.1 mg/L. This is within the range found for tropical coastal waters; concentrations of DOC in South East Asia and Melanesia have been reported around 0.5 – 4 mg/L (Ross Smith, pers. comm.).

In all tests, the measured dissolved nickel concentrations were within 12% of the nominal values, and in most cases dissolved nickel made up 95-100% of the total fraction. The exception was in the copepod test, where the difference between nominal and measured nickel concentrations (based on a time-weighted average, TWA) was up to 21% and the dissolved nickel was between 88-100% of the total fraction. In all copper tests, up to 67% of dissolved metal was lost during the exposure. Dissolved measured TWA values for copper were within 64% of nominal values. The fraction of dissolved copper in all tests ranged from 58-100% (Table S5-7).

All snail, barnacle and copepod tests in this study met the criteria for control treatments (Trenfield et al.,2016; van Dam et al., 2016; Binet et al. unpublished). In this study the mean growth rate of snail larvae in control treatments, across four individual tests was 62 ± 3.7 µm/d (±SE). Across four individual toxicity tests with the barnacle, the mean % of successfully transitioned larvae in control treatments was 86 ± 2.6 % (±SE). Across three individual copepod tests the average LDR in the control treatments was 68 ± 4% (±SE).

3.2. Toxicity of nickel to the snail, barnacle and copepod

Based on EC10, EC20 and EC50 (± 95% CLs) values of 5.5 (5.0 - 6.0) µg Ni/L, 6.6 (6.1-7.0) and 8.6 (8.3 - 8.9) µg Ni/L respectively, the copepod was the most sensitive species to nickel following an 80-h exposure. The snail and barnacle were of similar sensitivity to nickel at low concentrations, i.e., EC10 values were 64 (37-91) µg Ni/L and 67 (53-80) µg Ni/L, respectively, following a 96-h exposure. However, the EC50 values for these two species were substantially different: 478 (420-536) µg Ni/L and 171 (160-182) µg Ni/L for the snail and barnacle, respectively (Table 1). The concentration-response curves for nickel toxicity to snail, barnacle and copepod are shown in Figure 1A-C.

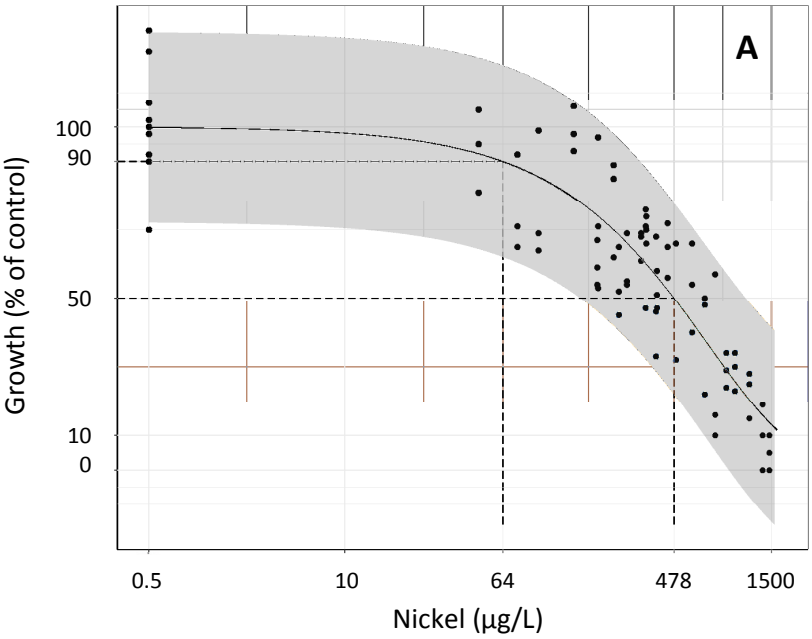
3.3. Toxicity of copper to the snail, barnacle and copepod

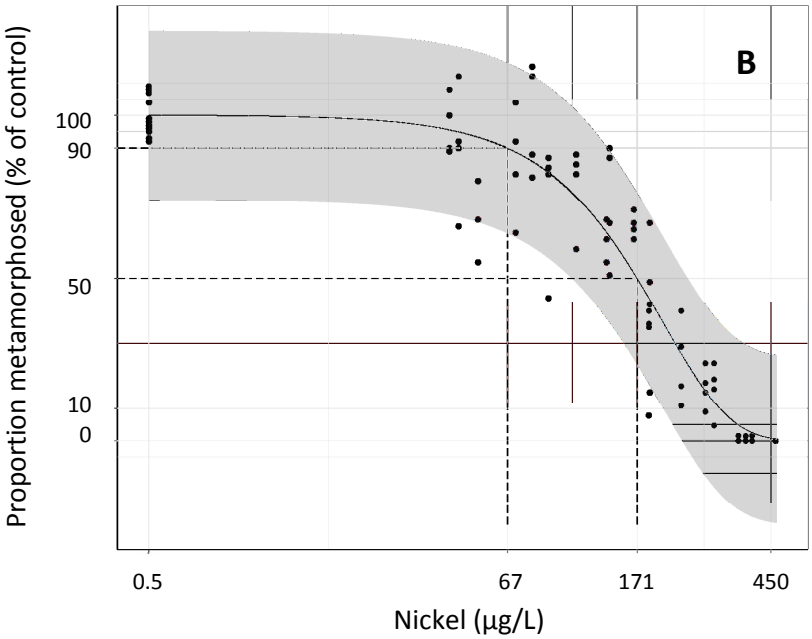
Copper was more toxic than nickel to all species tested (Table 1, Figure 2). The copepod was the most sensitive species with an EC10 of 1.4 (1.2-1.8) µg Cu/L, followed by the snail (EC10 of 3.7 (2.7-4.7) µg Cu/L) and the barnacle (EC10 of 10 (5-15) µg Cu/L) (Table 1). The toxicity estimates in this

study are similar to those in previous studies with the snail (Trenfield et al., 2016), the barnacle (van Dam et al., 2016) and the copepod (Binet et al unpublished), showing that these test protocols are robust and reproducible.

Table 1. Toxicity of nickel and copper to invertebrates, using dissolved (0.45-µm filtered) measured concentrations. Values in parentheses are 95% confidence intervals. All toxicity estimates were calculated using the Weibull model 1.3 in the drc package in R.

	Toxicity estimates, µg/L (95% confidence limits)					
	Nickel			**Copper**		
	EC10	**EC20**	**EC50**	**EC10**	**EC20**	**EC50**
Gastropod						
Nassarius dorsatus	64 (37-91)	143 (103-182)	478 (420-536)	3.7 (2.7-4.7)	4.8 (3.9-5.7)	7.1 (6.4-7.8)
Crustaceans						
Amphibalanus amphitrite	67 (53-80)	97 (84-110)	171 (160-182)	10 (5-15)	15 (9.4-20)	26 (22-30)
Acartia sinjiensis	5.5 (5.0-6.0)	6.6 (6.1-7.0)	8.6 (8.3-8.9)	1.4 (1.2-1.8)	1.9 (1.6-2.2)	2.8 (2.6-2.9)


A
Growth (% of control)
100
90
50
10
0
0.5
10
64
478
1500
Nickel (µg/L)


B
Proportion metamorphosed (% of control)
100
90
50
10
0
0.5
67
171
450
Nickel (µg/L)

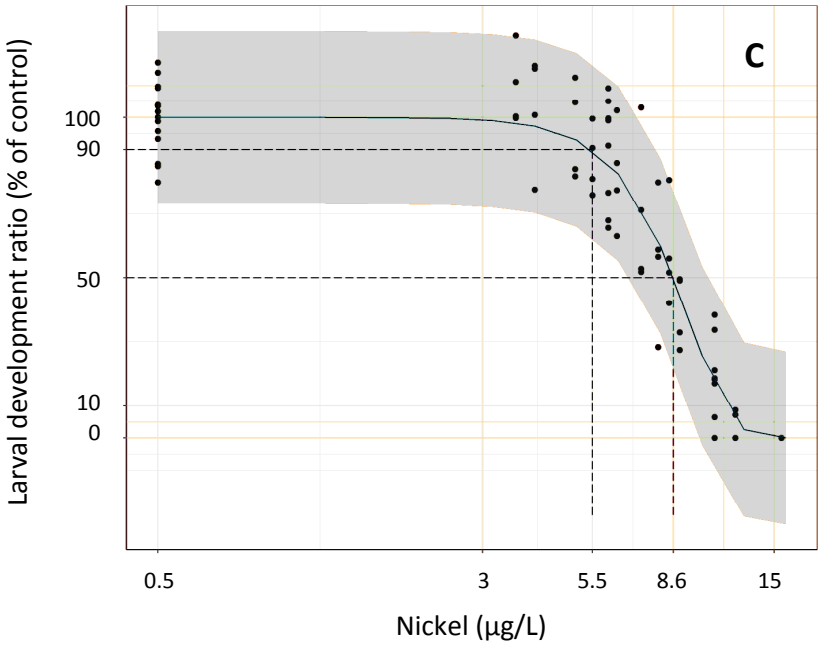
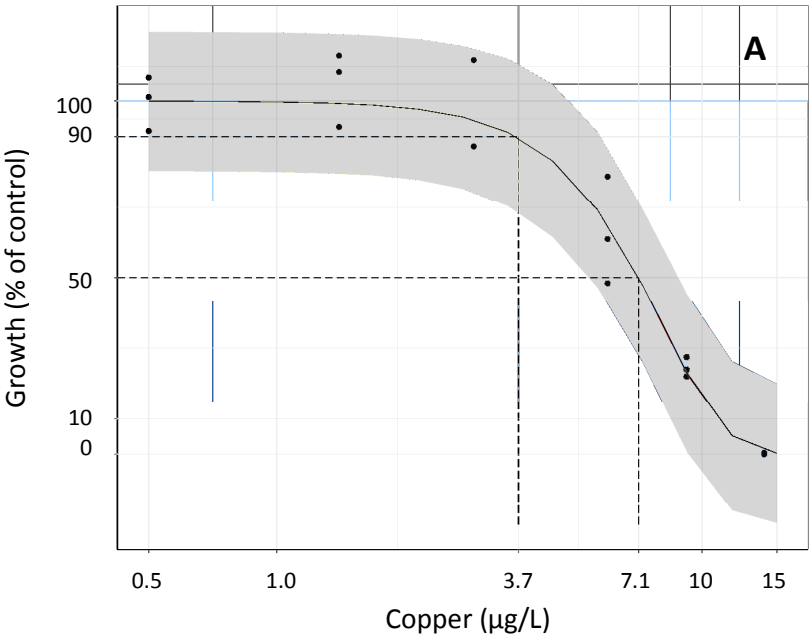
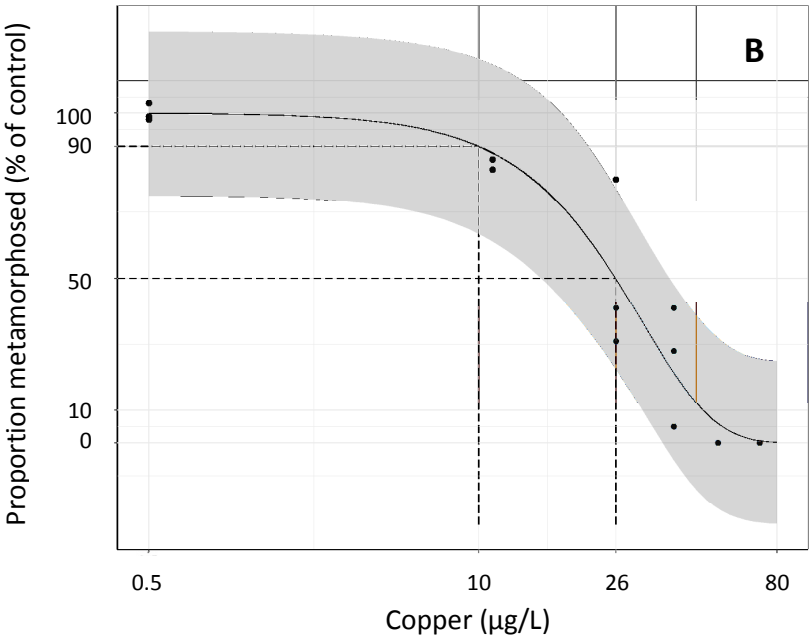


Figure 1. Toxicity of nickel to A) the snail *Nassarius dorsatus*, B) the barnacle *Amphibalanus amphitrite* and C) the copepod *Acartia sinjiensis*. For Figures A and B, each point represents 1 replicate from 4 individual toxicity tests; for Figure C, each point represents 1 replicate from 3 individual toxicity tests. The black line indicates the Weibull 1.3 model, fitted to the data to calculate toxicity estimates. The grey ribbon indicates the 95% prediction interval of the model, and the dashed lines point to the 10% and 50% effect concentrations, calculated from the model. Control concentration was set to 0.5 µg Ni/L, which is approximately half the limit of detection for nickel on the ICP-AES. Note different scales on x-axis.


A
Growth (% of control)
100
90
50
10
0
0.5
1.0
3.7
7.1
10
15
Copper (µg/L)


B
Proportion metamorphosed (% of control)
100
90
50
10
0
0.5
10
26
80
Copper (µg/L)

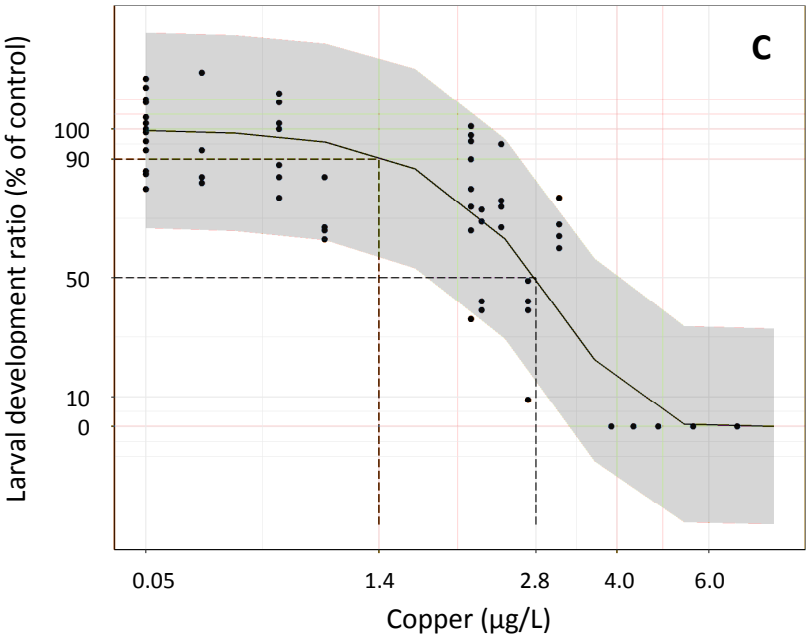


Figure 2. Toxicity of copper to A) the snail *Nassarius dorsatus*, B) the barnacle *Amphibalanus amphitrite* and C) the copepod *Acartia sinjiensis*. For Figures A and B, each point represents 1 replicate from 1 individual toxicity tests; for Figure C, each point represents 1 replicate from 3 individual toxicity tests. The black line indicates the Weibull 1.3 model, fitted to the data to calculate toxicity estimates. The grey ribbon indicates the 95% prediction interval of the model, and the dashed lines point to the 10% and 50% effect concentrations, calculated from the model. Control concentration was set to 0.5 µg Cu/L, which is approximately half the limit of detection for copper on the ICP-AES. For Figure C, the value is set to 0.05 µg Cu/L, approximately half the limit of detection on the ICP-MS. Note different scales on x-axis.

3.4. The effect of nickel and copper on the different stages of copepod development

Following 80-h exposure, 58-72% of copepods in control treatments had developed to the first stages of copepodite (C1C2C3), 22-33% were at nauplii stages 5 and 6 (N5N6), 5-7% were at nauplii stages 3 and 4 (N3N4) and 2-3% were at nauplii stages 1 and 2 (N1N2) (Figure 3). Nickel inhibited the development of nauplii into copepodites. As the concentration of nickel increased, the proportion of copepods at stages C1C2C3 decreased, and at the highest concentration tested (16 µg Ni/L), there were no copepodites (Figure 3) and only 3% of nauplii were at stages N5N6. There was no linear decrease in the proportion of N5N6 nauplii with increasing nickel concentrations. The proportion of nauplii at N3N4 increased with increasing nickel concentration from 5% at 3.7 µg Ni/L to 86% at 16 µg Ni/L. On average, the proportion of N1N2 nauplii slightly increased with increasing nickel concentration.

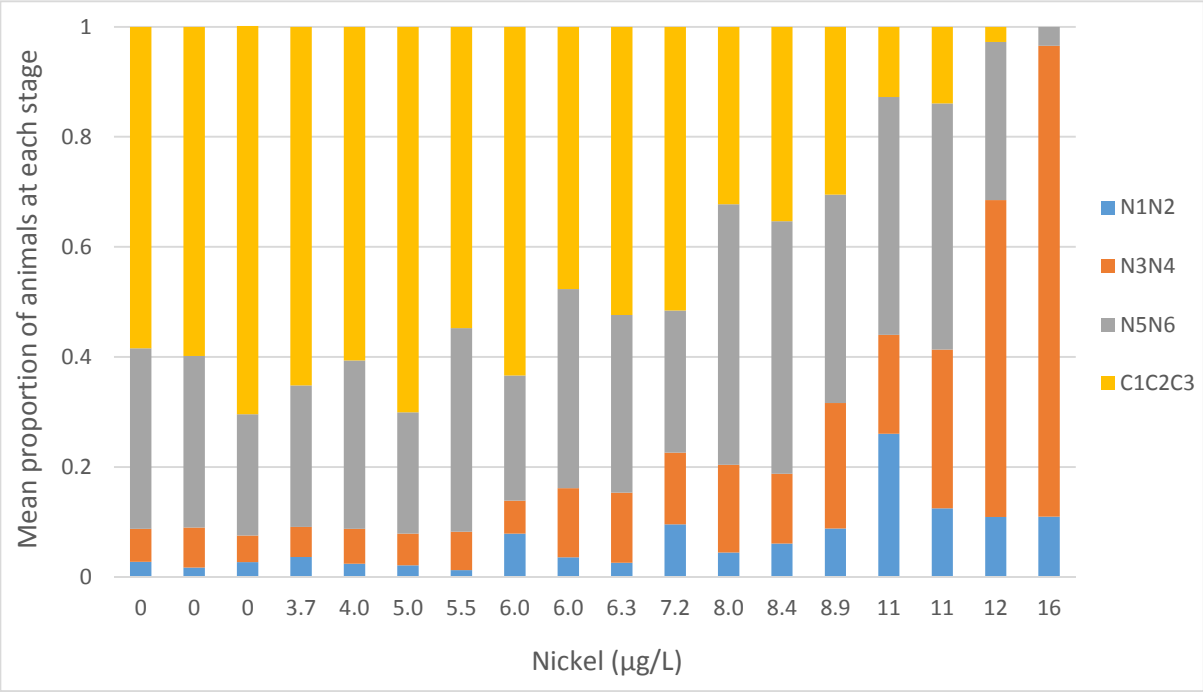


Figure 3. The effect of nickel on the different stages of copepod development over ~ 80 hours, expressed as the proportion of animals at each stage of development as determined by the Zooscan. Values are the means of 4 replicates per treatment compiled from 3 individual toxicity tests.

4. Discussion

4.1. Toxicity of nickel to gastropods

When comparing the EC10 and EC20 values, the snail and barnacle demonstrated similar sensitivity to nickel. However, based on the EC50 values, the snail *Nassarius dorsatus* was the least sensitive to nickel out of the three species tested. Two previous studies have reported acute toxicity for the tropical marine snail *Babylonia areolata*. Survival of adult snails after 96-h exposure was inhibited by 50% (LC50, 95% confidence limits) at 36000 (35000-28000) µg Ni/L and for juvenile snails at 200 (110-340) µg Ni/L, respectively (Hajimad and Vedamanikam, 2013; Vedamanikam and Hayimad, 2013). No chronic nickel toxicity data are available for marine gastropods (temperate or tropical), but, for temperate marine bivalves, EC10 values range from 61 – 431 µg Ni/L (Deforest and Schlekat, 2012). *Nassarius dorsatus* is therefore the most sensitive marine gastropod to nickel so far reported.

4.2. Toxicity of nickel to crustaceans

This is the first report on the toxicity of nickel to a tropical marine barnacle. Previous acute studies reported EC50 values for nickel to tropical marine crustaceans ranging from 7.2 – 18000 µg Ni/L (Gissi et al., 2016), yet chronic toxicity data are far more limited. One study showed that after an 11-d exposure, growth in a tropical marine shrimp, *Artemia franciscana* was reduced (by approximately 50%) at 3 µg Ni/L, but toxicity estimates were not reported (Asadpour et al., 2013). Chronic nickel toxicity data are available for several temperate marine and estuarine crustaceans. Reproductive output in three species of euryhaline copepods was reduced at 10 µg Ni/L (Mohammed et al., 2010). Reproduction in the temperate copepod *Acartia tonsa* was reduced by 20% at 2.4 µg Ni/L after a 7-d dietary exposure to nickel (Bielmyer et al., 2006). Survival of *A. tonsa* nauplii after 7-d exposure to nickel was reduced by 50% at 39 µg Ni/L, and egg hatching success after 4-d exposure to 25 – 100 µg Ni/L was reduced by 14 - 20% (Zhou et al., 2016). Gorbi et al. (2012) found similar results for the same species showing that nauplii survival was reduced by 50% between 29 – 76 µg Ni/L after 7-d exposure. The temperate marine mysid shrimp, *Mysidopsis intii*, demonstrated similar sensitivity to nickel; 28-d growth was reduced by 10% at 45 µg Ni/L (Deforest and Schlekat, 2012). Based on the current chronic toxicity data available for nickel, crustaceans, compared to all other marine organisms, show the highest sensitivity to nickel. *Acartia sinjiensis,* tested in this study, is the most sensitive tropical marine organism to nickel (Table 2).

In this study, the individual nauplii and copepodite stages of *A. sinjiensis* were assessed and showed that concentrations ≥ 8 µg Ni/L reduced the number of copepodites. This important developmental stage (from nauplii to copepodite) requires extensive physiological change and is often the most sensitive developmental window for *Acartia* species (OECD, 2007).This test is terminated at approximately 80 h, when >50% of animals in controls are, at a minimum, at the 7^{th} stage of development out of 13 stages within the copepod life-cycle. Like other larval development tests, it is not known whether longer exposure would reveal whether copepods are experiencing a delay in larval development, or an inability to develop any further. Regardless, observed inhibition in larval development will ultimately result in reduced population growth rate overall.

Compared to other marine copepods, *A. sinjiensis* is one of the most sensitive species to nickel exposure. This could be due to differences in the test method used in the current study compared with methods used for other copepod species, including factors such as test endpoint, diet and the type of algae and its concentration. In the present study, algae were centrifuged and resuspended in natural filtered seawater to remove nutrient rich culture media, while, in other studies, algae were not rinsed of culture media prior to addition to test solutions (Bielmyer et al., 2006; Gorbi et al., 2012; Mohammed et al., 2010; Tlili et al., 2015; Zhou et al., 2016). This can reduce the bioavailability and subsequent toxicity of nickel because algal culture media contains ligands such as

ethylenediaminetetraacetic acid (EDTA) which can bind metals. The algal cell densities used in our tests were also between 1-3 orders of magnitude lower than previous studies (Bielmyer et al., 2006; Mohammed et al., 2010; Zhou et al. 2016). In those studies, where a higher algal cell density was used, a greater depletion of dissolved metal would occur (Franklin et al., 2002), which may lead to a decrease in metal availability, reduced metal uptake, and lower toxicity if the metal then becomes less available to the copepod. Lower cell densities are more ecologically relevant and it is evident that the nutritional status of the copepods was not reduced because of the successful development observed in the control treatments (60-80% LDR in controls, in all tests).

In comparison to the snail also tested in this study, the copepod was 12 times more sensitive (based on EC10 values). Algae provided as a food source to the snails during exposure were not washed prior to addition to the test solutions. It is possible, as discussed above, that ligands in the culture media could reduce the bioavailability and toxicity of nickel to the snails. However, the concentrations of total and dissolved metals were measured throughout, and for the snail tests, the proportion of dissolved nickel was >95% (data not shown). Therefore, it is unlikely that the culture media from the algae altered the bioavailability and toxicity of nickel to snails.

4.3. Nickel in the environment

Concentrations of nickel in surface marine waters are typically < 5 µg Ni/L (Apte et al., 2006, DeForest and Schlekat 2012). However, at more polluted sites, nickel concentrations have been reported in the range of 15 - 2000 µg Ni/L (Denkhaus and Salnikow, 2002; Eisler, 1998; Pyle and Couture, 2012). The nickel concentrations used in this study are environmentally relevant, and significant effects were observed at 5 – 67 µg Ni/L for the copepod, snail and barnacle. If these organisms were exposed to elevated concentrations of nickel in the environment, adverse effects could occur. This suggests that nickel could pose a risk to tropical biota in coastal waters.

In natural systems, metals may be directly absorbed by organisms from the water column or assimilated via dietary exposure routes. Toxicity will depend on whether the metal is biologically available, its cellular mode of action and the rate with which it gets eliminated from the body (Luoma, 1983). In this study, algae were added to all three tests as a food source throughout exposure. In a recent study by Tlili et al. (2015), the adult copepods *Pseudodiaptomus marinus* were exposed for 7 days to nickel by water exposure only and through dietary exposure via an alga *Isochrysis galbana* which was dosed with sub-lethal concentrations of nickel. It was shown that *I. galbana* had a higher uptake rate than the copepod (0.51 µg/L/d compared to 0.17 µg/L/d), and that the copepod accumulated more nickel through dietary exposure (0.17 µg/L/d), than a water-only exposure (0.15 µg/L/d) (Tlili et al., 2015). According to Tlili et al. (2015), the majority of nickel accumulated by microalgae is distributed as soluble substances which may be easily assimilated by higher trophic organisms which feed on algae. In the snail, barnacle and copepod tests, >95% of total nickel was in the dissolved phase, therefore it can be assumed that exposure was predominately via the water route.

4.4. Compilation of tropical marine nickel toxicity data

As discussed above (Sections 4.1 and 4.2), it is clear that the sensitivity of marine species to nickel varies greatly. There is no clear pattern in sensitivity based on the geographical location of species, i.e., temperate versus tropical. In Australia, because of the limited test data on endemic species, for the derivation of WQGVs greater emphasis is placed on maximising the number of species and taxa for which toxicity data are available, rather than prioritise data for endemic species (Warne et al., 2014, 2015). In contrast, the USEPA stipulate that all toxicity data used to derive GVs should be for species that live and breed in North America (USEPA, 2007). Canada requires that non-indigenous species can be used to derive GVs if it can be demonstrated that they are an appropriate surrogate species and if the exposure conditions, under which the toxicity data was derived, were relevant to Canadian waters (CCME, 2007).

Prior to our research, only six chronic reliable toxicity data had been reported for nickel exposure to tropical marine species, half of which represented microalgae and cyanobacteria, the group least sensitive to nickel (Gissi et al., 2016). With the addition of the toxicity data for *Acroproa aspera, Acropora digitifera* and *Platygyra daedalea* (Gissi et al., 2017) and the data in this paper for the copepod, snail and barnacle, there are now sufficient tropical data to derive an interim marine GV that is specific for tropical waters. This is frequently not the case and in these instances supplementation with the abundant temperate data is frequently practised to fill data gaps (Merrington et al., 2014), but in these cases the derived GVs are not truly region specific.

Table 2. Toxicity estimates for the biological effect of nickel on a range of tropical marine organisms, summarised from Gissi et al. (2016). Values compiled for input into species sensitivity distribution (SSD).[a]

Phylum	Common name used in SSD	Scientific Name	Endpoint	Temperature (°C)	Toxicity measure	Reported toxicity value (µg/L)	Toxicity value used in SSD (µg/L)	Reference
Cyanobacteria	Cyanobacteria	*Cyanobacteria Cyanobium* sp.	inhibition in growth rate	25	IC10	3700	3700	Alqueza and Anastasi (2013)
Bacillariophyta	Microalgae	*Ceratoneis closterium*[b]	inhibition in growth rate	27	IC10	4330	4330	CSIRO. Gissi et al. Pers comm.
Haptophyta	Microalgae	*Isochrysis* sp.	inhibition in growth rate	27	IC10	337	340	CSIRO. Gissi et al. Pers comm.
Echinodermata	Sea urchin	*Diadema savignyi*	fertilisation and development	25	NOEC	23.5	24	Rosen et al. (2015)
Annelida	Polychaete	*Hydriodes elegans*	larval settlement	28	NOEC	162[c]	32	Gopalakrishnan et al. (2008)
Cnidaria	Anemone	*Aiptasia pulchella*	reproduction-total number juveniles	25	EC10	66	66	Howe et al. (2014)
Cnidaria	Coral	*Platygyra daedalea*	fertilisation	25	NOEC	920	920	Gissi et al. 2017
Cnidaria	Coral	*Acropora digitifera*	fertilisation	25	EC10	2000	2000	Gissi et al. 2017
Cnidaria	Coral	*Acropora aspera*	fertilisation	25	NOEC	<280	NA	Gissi et al. 2017
Mollusca	Snail	*Nassarius dorsatus*	growth	29	EC10	64	64	This study
Crustaceae	Barnacle	*Amphibalanus amphritrite*	metamorphosis	29	EC10	67	67	This study
Crustaceae	Copepod	*Acartia sinjiensis*	development	30	EC10	5.5	5.5	This study

EC10 = 10% effect concentration. IC10 = 10% inhibition concentration. NOEC = No observable effect concentration.

[a] This table only includes chronic, measured nickel data which passed the quality assurance criteria established in Gissi et al. (2016), ANZECC/ARMCANZ (2000), and Warne et al. (2015).

[b] Previously known as *Nitzcshia closterium*

[c] Chronic EC50 converted to NOEC value, divide by 5 (Warne et al., 2015) NA = not applicable, toxicity value not used in SSD

The full tropical dataset of 12 values is shown in Table 2. For coral, *Acropora aspera*, the endpoint was a NOEC value of <280 µg/L and there were insufficient points on the concentration response curve to derive an EC10 (Gissi et al. 2017) so this test was omitted from the SSD. The NOEC value for the polychaete, *Hydriodes elegans* was derived by applying a factor of 5 to a chronic EC50 value (ANZECC/ARMCANZ, 2000; Warne et al., 2015). Greater reliability GVs can be derived without the inclusion of converted data (Warne et al., 2015). However in this instance, there was no difference in the derived PC95 value and so these data were included. The SSD shown in Figure 4 was derived using BurrliOz Version 2 software (Barry, 2014). The type of distribution that is fitted to the data is automatically determined by the software; in this study, data were fitted to a Burr Type II distribution.

The derived tropical marine PC values for different levels of ecosystem protection are shown in Table 3 (derived based on ANZECC/ARMCANZ, 2000 and Warne et al., 2015). It is the PC95 value of 8.2 µg Ni/L that would be mostly applied in slightly-to-moderately disturbed systems. This value is above the level typically reported for background concentrations of nickel in seawater (<5 µg Ni/L, Apte et al., 2006, DeForest and Schlekat 2012). Warne et al. (2015) provide guidance for assessing the reliability of PC values derived from SSD methods. This is based on the sample size (number of species for which toxicity data are available), the type of data (chronic, chronic and acute, or converted values), and by visual assessment of the fit of the SSD to the toxicity data (i.e. good or poor) (Warne et al., 2015). In the SSD shown in Figure 4, there are a total of 11 chronic data points (one of which was converted) and the fit of the SSD to the toxicity data is poor, placing the classification of this PC95 value as moderate reliability. It should be noted that this is not a regulatory value, but a reflection of the continued and ongoing research to produce high quality nickel toxicity data for tropical marine waters.

Table 3. Protection Concentration (PC) values derived using the species sensitivity distribution shown in Figure 4, for different levels of protection based on Warne et al. (2015).

Level of protection	PC (±95% CL)
PC90 – 90% species protection for disturbed systems	14 (8.1-52)
PC95 – 95% species protection for slightly-to-moderately disturbed systems	8.2 (2.6-29)
PC99 – 99% species protection for pristine systems	3.3 (0.13-12)

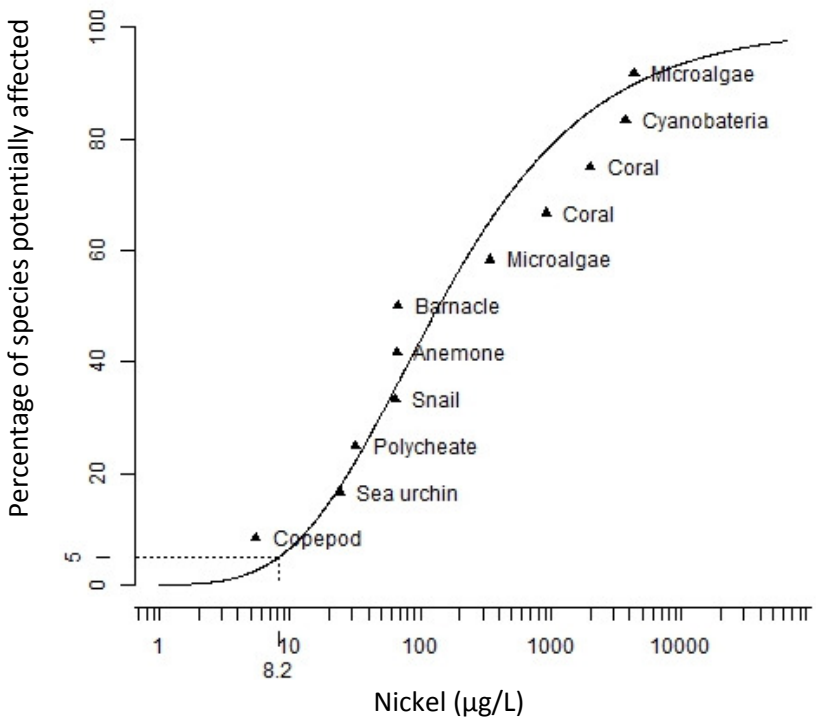


Figure 4. Species sensitivity distribution using chronic, measured nickel toxicity data, for data that passed the quality assurance criteria established in Gissi et al. (2016). The value indicated on the x-axis by the dotted line represents the Protective Concentration for 95% of species (PC(95)).

There are inherent uncertainties that should be considered when applying GVs, for example, the difference between laboratory waters and natural waters, and the absence of insensitive taxa such as fish from the SSD. In this instance, it is also important to note that the SSD and derived PC95 value for nickel is largely driven by the copepod data, the most sensitive species; it should be considered that this is a relatively new endpoint when evaluating ecological relevance. Guideline values ultimately provide guidance and where uncertainties arise, additional lines of evidence should be sought.

5. Conclusion

The development of ecologically relevant risk assessment tools for nickel in tropical marine environments is hindered due to the paucity of data on the effects of nickel on key tropical species. In this study we have reported the toxicity of nickel to one gastropod and two crustaceans, relevant to tropical Asia-Pacific. These test organisms were ideal species to include in the SSD for nickel due to their sensitivity to the metal, in particular the copepod. With the inclusion of ecologically relevant species, and high quality chronic toxicity data, greater reliability can be placed on the PC95 value reported in this study. It is anticipated that the data presented here will contribute to the development of an ecologically relevant water quality guideline for nickel in tropical marine waters. Given the potential concentrations of nickel in the environment in close surrounds to nickel mines and facilities, there is potential risk of nickel toxicity to tropical marine organisms in these locations.

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Supplementary material.

Table S1. Models and the corresponding Akaike Information Criterion (AIC) values used in the drc package in R. Three different models were fitted to each data set (species and metal). The model of best fit was chosen based on the lowest AIC value, and by visual assessment of the curve. The selected model was then used to determine toxicity estimates for each species and metal tested.

Species	*Nassarius dorsatus*		*Amphibalanus amphitrite*		*Acartia sinjiensis*	
Metal	Nickel	Copper	Nickel	Copper	Nickel	Copper
Model	AIC values					
Weibull 1.3	678	128	738	145	632	653
Log Logistic 3	682	129	742	148	633	649
Log Logistic 4	678	192	741	146	832	845

Table S2. Background concentrations of metals in seawater used in snail tests. LOD = Limit of detection. Values in bold exceeded the LOD.

Metal (µg/L)	Al	Ba	Cd	Co	Cr	Cu	Mn	Ni	Pb	Se	V	Zn
LOD	0.30	0.07	0.20	1.0	0.77	0.50	0.06	2.0	2.1	2.6	0.20	0.18
Ni test 1	<0.30	**2.0**	<0.20	<1.0	<0.77	<0.50	**0.70**	<2.0	<2.1	**4.3**	<0.20	**5.5**
Ni test 2	<0.30	**1.8**	<0.20	<1.0	<0.77	<0.50	**1.0**	<2.0	<2.1	<2.5	<0.20	<0.18
Ni test 3	<0.30	**1.8**	<0.20	<1.0	<0.77	<0.50	**0.93**	<2.0	<2.1	<2.5	<0.20	**1.1**
Ni test 4	<0.30	**1.9**	<0.20	<1.0	<0.77	<0.50	**0.82**	<2.0	<2.1	<2.5	<0.20	**5.2**
Cu test 1	<0.30	**1.9**	<0.20	<1.0	<0.77	<0.50	**1.1**	<2.0	<2.1	**3.5**	<0.20	<0.18

Table S3. Background concentrations of metals in sea water used in barnacle tests. LOD = Limit of detection. Values in bold exceeded the LOD.

Metal (µg/L)	Al	As	Ba	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Se	V	Zn
LOD	0.23	2.0	2.1	0.14	0.29	0.46	0.45	0.78	0.18	1.2	2.7	2.5	1.1	0.68

Ni test 1	<0.23	**3.8**	**2.2**	<0.14	<0.29	<0.46	<0.45	<0.78	<0.18	<1.2	<2.7	<2.5	**2.7**	<0.68
Ni test 2	<0.23	**4.7**	**2.8**	<0.14	<0.29	<0.46	<0.45	<0.78	<0.18	<1.2	<2.7	<2.5	<1.1	<0.68
Ni test 3	<0.23	**4.8**	<2.1	<0.14	<0.29	<0.46	<0.45	<0.78	<0.18	<1.2	<2.7	**2.6**	<1.1	<0.68
Ni test 4	**4.5**	<2.0	**3.8**	<0.14	<0.29	<0.46	<0.45	<0.78	<0.18	<1.2	<2.7	<2.5	<1.1	<0.68
Cu test 1	<0.23	**4.0**	<2.1	<0.14	<0.29	<0.46	<0.45	<0.78	<0.18	<1.2	<2.7	<2.5	<1.1	<0.68

Table S4. Background concentrations of metals in sea water used in copepod tests. LOD = Limit of detection. Values in bold exceeded the LOD. Each test included Ni and Cu treatments.

Metal (µg/L)	Ag	Al	As	Ba	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Se	V	Zn
LOD	2.0	0.36	3.5	0.13	0.13	0.74	0.79	1.1	0.35	0.12	0.92	3.2	4.6	0.59	0.15
Test 1	<2.0	<0.36	<3.5	**0.75**	<0.13	<0.74	<0.79	<1.1	<0.35	<1.2	<0.92	<3.2	<4.6	<0.59	**6.6**
Test 2	<2.0	<0.36	<3.5	<0.13	<0.13	<0.74	<0.79	<1.1	<0.35	**0.22**	<0.92	<3.2	<4.6	<0.59	<0.15
Test 3	<2.0	<0.36	<3.5	<0.13	<0.13	<0.74	<0.79	<1.1	<0.35	<1.2	<0.92	<3.2	<4.6	<0.59	<0.15

Table S5. Comparison of nominal and measured dissolved nickel and copper concentrations in 4-day toxicity tests with the snail *Nassarius dorsatus*. TWA = Time weighted average.

Test	Nominal, µg/L	Measured dissolved, µg/L			
		Day 0	Day 2	Day 4	TWA
Ni test 1	200	193	193	194	194
	240	234	233	238	235
	280	274	273	280	275
	320	313	316	318	324
	360	349	352	351	342
	400	388	392	391	390
Ni test 2	150	145	148	146	147
	250	247	252	248	250
	350	344	347	343	345
	450	442	441	444	442
	600	587	591	593	590
	900	882	882	886	883
Ni test 3	50	48	49	47	48
	100	100	97	96	97
	500	493	489	482	488
	700	689	685	685	686
	1000	982	974	975	976
	1500	1476	1448	1477	1462
Ni test 4	80	75	78	75	76
	200	198	195	195	196
	400	392	386	386	387
	800	786	768	770	773
	1200	1169	1151	1158	1157
	1400	1370	1340	1340	1350
Cu test 1	2	2.1	<0.5	0.7	1.4
	4	3.6	0.2	2.1	2.9
	8	7.3	3.5	4.8	6.0
	12	11	7.5	7.9	9.2

	20	17	13	11	14

Table S6. Comparison of nominal and measured nickel and copper concentrations in 4-day toxicity tests with the barnacle *Amphibalanus amphitrite*.

Test	Nominal, µg/L	Measured dissolved, µg/L		
		Day 0	Day 4	Mean
Ni test 1	50	44	44	44
	100	92	89	90
	150	137	137	137
	200	189	183	186
	500	461	466	463
Ni test 2	50	49	45	47
	75	74	68	71
	150	144	136	140
	300	305	290	298
	400	402	383	392
Ni test 3	60	55	53	54
	120	112	109	110
	200	189	185	187
	280	283	276	280
	380	379	372	375
Ni test 4	90	84	76	80
	180	173	161	167
	250	241	228	235
	350	368	344	356
Cu test 1	20	15	7	11
	40	33	18	26
	60	50	28	39
	80	65	40	53
	100	87	55	71

Table S7. Comparison of nominal and measured dissolved nickel and copper concentrations in 3-day toxicity tests with the copepod *Acartia sinjiensis*. TWA = Time weighted average. Day 2B = samples taken on Day 2 before renewal of test solutions. Day 2A = samples taken on Day 2 after renewal of test solutions. NA = not analysed.

Test	Nominal, µg/L	Measured dissolved, µg/L				
		Day 0	Day 2B	Day 2A	Day 3	TWA
NICKEL						
Test 1	4	3.5	4.2	3.3	2.9	4.0
	6	6.3	5.6	5.4	5.9	6.0
	8	7.2	7.2	7.4	8.5	7.9
	12	13	12	12	11	12
	16	16	16	15	15	16
Test 2	4	3.6	3.5	4.1	3.4	3.6
	6	4.9	5.6	5.2	4.7	5.5
	8	6.5	6.3	6.6	6.0	6.3
	10	8.7	7.8	8.7	8.5	8.4
	12	10	10	9	11	11
Test 3	5	5.0	4.5	4.3	5.0	5.0
	7	6.1	5.4	6.9	5.7	6.0
	9	7.0	6.4	7.2	7.8	7.2
	11	8.3	8.5	9.6	8.9	8.9
	13	11	10	11	11	11
COPPER						
Test 1	1	1.2	0.7	1.1	0.8	0.9
	3	2.8	1.3	2.2	2.2	2.1
	6	3.4	1.8	2.9	2.8	2.7
	9	5.9	2.8	5.1	4.1	4.5
	12	8.4	4.7	7.7	6.3	6.8
Test 2	1	0.9	NA	1.0	0.8	0.9
	3	3.0	1.3	2.2	2.0	2.1
	6	2.9	1.1	2.6	2.3	2.2
	9	5.0	2.9	4.3	3.6	3.9
	12	6.8	3.7	6.8	5.5	5.6
Test 3	1	0.8	0.4	0.9	0.5	0.6
	2	1.5	0.6	1.5	1.1	1.1
	4	3.3	1.6	2.7	2.2	2.4

6	3.8	2.2	3.7	2.6	3.1
8	5.8	3.1	4.8	3.4	4.3