FIRST DESCRIPTION OF THE ENVIRONMENTAL NICHE OF THE EPIBENTHIC DINOFLAGELLATE SPECIES *COOLIA PALMYRENSIS, C. MALAYENSIS AND C. TROPICALIS* (DINOPHYCEAE) FROM EASTERN AUSTRALIA[1]

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Running Title: Environmental niche of *Coolia* from Australia

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Abbreviations:

BHAB, Benthic Harmful Algal Bloom

CFP, Ciguatera Fish Poisoning

HAB, Harmful Algal Bloom

Abstract:

Environmental variables such as temperature, salinity and irradiance are significant drivers of microalgal growth and distribution. Therefore, understanding how these variables influence fitness of potentially toxic microalgal species is particularly important. In this study, strains of the potentially harmful epibenthic dinoflagellate species *Coolia palmyrensis*, *C. malayensis* and *C. tropicalis* were isolated from coastal shallow water habitats on the east coast of Australia and identified using the D1-D3 region of the large subunit (LSU) ribosomal DNA (rDNA). To determine the environmental niche of each taxon, growth was measured across a gradient of temperature (15-30 °C), salinity (20-38) and irradiance (10-200 µmol photons · m^{-2} · s^{-1}). Specific growth rates of *Coolia tropicalis* were highest under warm temperatures (27 °C), low salinities (ca. 23) and intermediate irradiance levels (150 µmol photons · m^{-2} · s^{-1}), while *C. malayensis* showed the highest growth at moderate temperatures (24 °C) and irradiance levels (150 µmol photons · m^{-2} · s^{-1}) and growth rates were consistent across the range of salinity levels tested (20-38). *Coolia palmyrensis* had the highest growth rate of all species tested and favoured moderate temperatures (24 °C), oceanic salinity (35) and high irradiance (>200 µmol photons · m^{-2} · s^{-1}). This is the first study to characterise the environmental niche of species from the Benthic Harmful Algal Bloom genus *Coolia* and provides important information to help define species distributions and inform risk management.

1. Introduction:

Dinoflagellates are microscopic, photosynthetic organisms that occupy a variety of niches in freshwater, estuarine and marine habitats. They have a variety of lifestyles, including being suspended in the water column (planktonic) or living in close assocation with both organic

(e.g. macrophytes, seagrass) (Shah et al. 2013; Mabrouk et al. 2014) and inorganic (e.g. coral rubble, sand) (Ballantine et al. 1985; Faust 1995) substrates (epiphytic). Some microalgal species known as Harmful Algal Bloom (HAB) species, produce potent toxins that can accumulate in the food web, and when consumed by humans (Berdalet et al. 2016) or marine life, can cause illness and death (Fire et al. 2010, Bottein et al. 2011, Phillips et al. 2011). Toxin producing dinoflagellates that occupy the benthic environment are known as Benthic HAB species (BHAB) and have been implicated in a number of human food related illnesses including Ciguatera Fish Poisoning (CFP) (Yasumoto et al. 1979, Yasumoto et al. 1977), clupeotoxism (Aligizaki et al. 2011), and respiratory (Durando et al. 2007) and dermatologic (Tubaro et al. 2011) conditions. Globally, the frequency of such BHAB outbreaks is thought to be increasing (Perini et al. 2011).

Common BHAB genera include *Gambierdiscus* Adachi & Fukuyo, *Fukuyoa* Gómez, Qui, Lopes & Lin, *Ostreopsis* Schmidt, *Amphidinium* Claparède & Lachmann, *Prorocentrum* Ehrenberg and *Coolia* Meunier, the focus of this study. Like many microalgal genera, *Coolia* has been the object of considerable taxonomic confusion. The genus was originally described by Meunier (1919) but was later grouped with the genus *Ostreopsis* by Lindemann (1928) due to morphological similarities. It was then transferred to the genus *Glenodinium* by Biecheler (1952) before being reinstated to *Coolia* after more detailed morphological examination by Balech (1956). This differentiation has since been confirmed with phylogenetic analysis (Penna et al. 2005, Dolapsakis et al. 2006). Additionally, for more than 90 years, the genus was thought to be monotypic, consisting only of *Coolia monotis*. However, since 1995, an additional six species have been described. These are all morphologically very similar but genetically distinct. The genus now also includes *Coolia tropicalis* (Faust 1995), *C. areolata* (Ten-Hage et al. 2000), *C. canariensis* (Fraga et al.

2008), *C. malayensis* (Leaw et al. 2010), *C. santacroce* and *C. palmyrensis* (Karafas et al. 2015).

Holmes *et al.* (1995) performed the original toxicological investigations on a strain of *Coolia* (then described as *C. monotis* but later confirmed to be *C. tropicalis* by Mohammad-Noor et al. (2013)) isolated from Queensland, Australia. The strain was found to produce cooliatoxin, a lipophilic, polyether toxin similar to yessotoxin. Likewise, a strain of *Coolia* sp. (species unconfirmed) isolated from Japan was also found to produce cooliatoxin (Nakajima et al. 1981). More recently, Wakeman et al. (2015) characterised five more analogs of yessotoxin, distinct from cooliatoxin, from a strain of *C. malayensis* (Okinawa, Japan). Despite proving lethal to mice (Carlson et al., 1984) and invertebrates (Rhodes and Thomas 1997, Leung et al. 2017) and showing haemolytic activity (Cruz and Okolodkov 2016), these lipophilic polyether toxins are yet to be implicated in any human illness events. Given that some isolates produce cooliatoxin and others produce different yessotoxin analogs, the toxicology of the genus is currently unresolved.

Coolia is commonly collected from the surface of macrophytes in shallow water habitats and the genus appears to have a worldwide distribution (Fig. 1), although understanding the biogeography of species has been hindered by taxonomic uncertainty of cultured isolates and field samples. Nonetheless, patterns are beginning to emerge (Fig. 1). *Coolia malayensis* is the most widely distributed species occurring across tropical and temperate locations (David et al. 2014, Momigliano et al. 2013, Jeong et al. 2012, Leaw et al. 2010, Wakeman et al. 2015, Gomez et al. 2016, Rhodes et al. 2014, Tawong et al. 2015, Mohammad-Noor et al. 2013, Leung et al. 2017, Karafas et al. 2015, Leaw et al. 2016, Fraga et al. 2008, Rhodes et al. 2000). *Coolia monotis* appears to be restricted to the northern hemisphere with most records arising from the Mediterranean Sea (Faust 1992, Aligizaki and Nikolaids 2006, Armi et al. 2010, Dolapsakis et al. 2006, Ben-Gharbia et al. 2016, David et al. 2017, David et al.

2014, Laza-Martinez et al. 2011, Fraga et al. 2008, Mohammad-Noor et al. 2013, Penna et al. 2005, Karafas et al. 2015, Leaw et al. 2016, Rhodes et al. 2000, Simoni et al. 2004, Pagliara and Caroppo 2012, Cohu and Lemee 2012, Feki-Sahnoun et al. 2014, Cruz and Okolodkov 2016, Nguyen 2014). *Coolia canariensis* occurs in the North East Atlantic Ocean but is also found in the Western Pacific Ocean (David et al. 2014, Momigliano et al. 2013, Jeong et al. 2012, Laza-Martinez et al. 2011, Fraga et al. 2008, Mohammad-Noor et al. 2013, Leung et al. 2017, Nguyen 2014). *Coolia tropicalis* is similarly found in the Western Pacific Ocean but also in the Western Atlantic and Western Indian oceans (Fraga et al. 2008, Momigliano et al. 2013, Rhodes et al. 2014, Mohammad-Noor et al. 2013, Leung et al. 2017, Leaw et al. 2016, Rhodes et al. 2014, Nguyen 2014)). The only known location of *C. areolata* is of the type species from the South Western Indian Ocean at La Réunion Island and Mozambique (Ten-Hage et al. 2000). *Coolia palmyrensis* has been collected in the West and Central Pacific Ocean and the West Atlantic Ocean (Momigliano et al. 2013, Leung et al. 2017, Karafas et al. 2015) whereas *C. santacroce* has only been found in the western Atlantic Ocean (Karafas and Tomas 2015, Karafas et al. 2015; Fig. 1).

In Australia, there have been very few studies evaluating the distribution, taxonomic identity and toxicity of BHAB species. One study which used detailed phylogenetic analyses to describe the taxonomic identity of *Coolia* isolated from the Great Barrier Reef region identified four species (*C. canariensis, C. malayensis, C. palmyrensis* and *C. tropicalis*) (Momigliano et al. 2013). Another two studies which used a metabarcoding approach, detected *Coolia* spp. (*C. monotis* and *C. canariensis*) at almost all sampling sites including Broome, Western Australia, and Wagonga inlet and Merimbula in southern New South Wales (Kohli et al. 2014a, Kohli et al. 2014b). These findings suggest the genus *Coolia* may be widespread in Australia, however many locations are yet to be surveyed.

Beyond their presence, little is known about the environmental niche of *Coolia* species, although their wide geographic distribution (Fig. 1) suggests this genus can occupy a large range of conditions. Early work on an unidentified *Coolia* isolate which assessed the influence of temperature on growth showed cells have a doubling rate of approximately three to four days at a moderate temperature of 23 °C (Faust 1992), while faster growth can be achieved at a warmer temperature of 29 °C (Morton et al. 1992). Since then, Cruz and Okolodkov (2016) measured the growth of *C. monotis* (identified based only on morphological characteristics) across a gradient of temperature from 5-30 °C, finding the optimal range to be between 15 and 25 °C. Another recent study measured growth across a gradient of irradiance (0-300 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}) finding strains of *C. monotis* with no morphological or molecular differences, isolated from three locations on the Iberian peninsula respond differently, each having vastly different irradiance optima (David et al. 2017). The combined effects of temperature and salinity of a strain of *C. malayensis* and *C. tropicalis* (confirmed species identity) from Malaysia, showed that high temperatures (30 °C) at salinity levels close to that of oceanic seawater (35) yielded greatest growth for both species (Mohammad-Noor et al. 2013). Although these studies provide important insight into how environmental conditions influence the growth of *Coolia* species, considerable work remains to be done.

Like other microalgal species, BHABs require irradiance, nutrients and inorganic carbon (CO_2) in combination with specific physicochemical variables such as temperature, pH and salinity to photosynthesise and grow. In shallow marine habitats, temperature, salinity and irradiance levels are the most likely physicochemical variables influencing species distributions and relative cell abundance under nutrient replete conditions. In this study, we investigated how environmental variables influence the growth of *Coolia* strains isolated from the east coast of Australia. Specifically, we measured growth across a gradient of

temperature (15-30 °C), salinity (20-38) and irradiance (10-200 µmol photons · m^{-2} · s^{-1}). To the best of our knowledge, this is the first study to comprehensively characterise the environmental niche of *Coolia* and examine its fitness landscape.

2. Methods:

2.1 Culture establishment

Epiphytic microalgae were removed from one individual of the macroalgal species *Padina* sp. and *Chnoospora* sp. collected from 1 m depth at Heron Island lagoon, Queensland, Australia (23.4423°S, 151.9148°E) on the 27th July 2014 (austral winter). One sample of a seagrass (*Zostera* sp.) was collected at a depth of 1 m from Merimbula inlet, New South Wales, Australia 36.8979°S, 149.9044°E) on the 7th April 2014 (austral autumn) (Fig. 1). Single cells of *Coolia* were isolated using the micropipette technique (Andersen and Kawachi 2005), established as monoclonal cultures and maintained in modified K medium (Litaker et al. 2009) made from sterile aged natural seawater at a salinity of 32. Established cultures (Table 1) were maintained at 24 °C under ~100 µmol photons · m^{-2} · s^{-1} on a 12:12 Light:Dark cycle in 25 cm^2 (70 mL) or 75 cm^2 (250 mL) sterile vented polystyrene tissue culture flasks (Falcon, Corning, New York, USA), oriented horizontally.

2.2 DNA extraction and phylogenetic analyses

Approximately 100 mL of each established *Coolia* isolate was centrifuged at 1000 g for 10 minutes to pellet the cell material. DNA was extracted using a MoBio Power Soil DNA Extraction Kit (Qiagen, Carlsbad, CA, USA) following the manufacturer's instructions and sent to a commercial service (Australian Genomic Research Facility (AGRF), Queensland, Australia) where the D1-D3 region of the large subunit ribosomal DNA (LSU rDNA) was

amplified using the primers D1R-F (Scholin et al. 1994) and D3-R (Nunn et al. 1996) under conditions described in Rhodes et al. (2014). Amplification products (~ 950 bp) were purified and sequenced in both directions using the Sanger sequencing platform. Phylogenetic analyses were conducted in Geneious v9.1.5 (Kearse et al. 2012). Forward and reverse sequences from this study were joined and aligned with publicly available sequences of *Coolia* and *Ostreopsis* downloaded from GenBank (www.ncbi.nlm.nih.gov), using the MUSCLE algorithm (maximum number of iterations 8) (Edgar 2004), with *Ostreopsis* species sequences used as out-groups (*Ostreopsis* cf. *siamensis* HQ414222; *Ostreopsis* cf. *ovata* JX065571). Alignments were truncated to 776 bp and a Maximum Likelihood phylogenetic tree was generated using PHYML with 1,000 bootstraps (Guindon and Gascuel, 2003) using a GTR substitution model and an estimated gamma distribution. Bayesian analyses was performed using MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) by means of the GTR+G (general-time reversible with gamma-shaped among-site variation) model and was carried out in four simultaneous runs with four chains each for 3.1 x 10^6 generations, sampling every 1,000 trees and 1,000 trees were discarded as burn in.

2.3 Growth estimates

In vivo chlorophyll *a* fluorescence was used as a proxy for cell abundance to measure growth of *Coolia* spp. during experiments. To confirm *in vivo* chlorophyll *a* fluorescence is correlated with *Coolia* cell abundance, all strains except *C. tropicalis* (UTSHI2D1) (Table 1) were grown in 75 cm^2 (250 mL) sterile vented polystyrene tissue culture flasks (Falcon, Corning, New York, USA) in triplicate under culture maintenance conditions. A 1 mL aliquot was removed from each flask every 3 to 4 days and the *in vivo* chlorophyll *a* fluorescence was measured for each sample using a fluorometer (Turner Designs Trilogy Laboratory Fluorometer®, USA) and then preserved with 1% Lugol's iodine solution. The cell abundance for all three replicates of each strain and species was enumerated using a

Sedgewick Rafter counting chamber (McAlice, 1971) under a Nikon Eclipse TS100 inverted light microscope for at least 3 time periods. The correlation between *in vivo* chlorophyll *a* fluorescence and cell abundance was calculated using linear regression analyses in OriginPro 8 (OriginLab Corporation, Northhampton, MA, USA). While temperature, salinity and irradiance niche estimate experiments were not carried out concurrently, growth rate estimates were conducted similarly with sampling continuing until the stationary phase of growth was reached. Stationary phase was defined as two consecutive sampling periods where cell abundance did not increase.

Regression analysis showed a linear relationship between *in vivo* chlorophyll *a* fluorescence measured in relative fluorescence units (rfu) and cell abundance (n=15), for each species of *Coolia* used in this study (Supplementary Fig. 1) (*C. tropicalis*, r^2=0.87; *C. palmyrensis*, r^2=0.86; *C. malayensis*, r^2=0.90), demonstrating this fluorometric method can provide a robust estimate of cell abundance. *In vivo* chlorophyll *a* fluorescence was therefore used as a rapid method to estimate cell abundance and calculate growth rates in all experiments in this study.

2.4 Temperature niche estimates

Each established strain of *Coolia* was inoculated into triplicate 25 cm^2 sterile vented polystyrene tissue culture flasks (Falcon, Corning, New York, USA), with 50 mL fresh standard modified K medium at a salinity of 32, at a standardised cell density of approximately 10 cells mL^{-1}. Flasks were placed horizontally under ~100 µmol photons · m^{-2} · s^{-1} measured using a 4π light sensor (WALZ, Model US-SQS/L, Germany) in air on a 12:12 Light:Dark cycle and incubated at 15, 18, 21, 24, 27 and 30 °C in controlled-temperature incubators (Climatron® Plant Growth Cabinet, Australia). Light levels were chosen to avoid both light limitation and light inhibition based on pilot studies. Flasks were acclimated to the

experimental conditions for seven days before growth measurements began. *In vivo* chlorophyll *a* fluorescence was measured on a 1 mL aliquot of each replicate culture every three to four days until the cultures reached stationary phase. The maximum growth rate was calculated using the slope of the linear portion of the natural logarithm transformed cell abundance curve.

2.5 Salinity niche estimates

Salinity was adjusted to the experimental treatments of 20, 26, 32 and 38 by diluting aged natural seawater with sterile MilliQ water or adding sterile artificial hypersaline seawater prepared as per Berges et al. (2001). Nutrients were added as per standard modified K medium and each strain of *Coolia* was inoculated into triplicate 25 cm^2 sterile vented polystyrene tissue culture flasks (Falcon, Corning, New York, USA) at a standardised cell density of approximately 10 cells mL^{-1}. Flasks were incubated in controlled-temperature incubators (Climatron® Plant Growth Cabinet, Australia) set at a constant temperature of 24 °C under ~100 µmol photons · m^{-2} · s^{-1} on a 12:12 Light:Dark cycle. Flasks were acclimated to the experimental conditions for seven days before *in vivo* chlorophyll *a* fluorescence was measured every three to four days on a 1 mL aliquot of culture, until the stationary phase of growth was reached.

2.6 Irradiance niche estimates

Strains of *Coolia* were inoculated into triplicate 25 cm^2 sterile vented polystyrene tissue culture flasks (Falcon, Corning, New York, USA), with 50 mL fresh standard modified K medium as a salinity of 32, at a standardised cell density of approximately 10 cells mL^{-1}. Flasks were placed horizontally in controlled-temperature incubators (Climatron® Plant Growth Cabinet, Australia) set at a constant temperature of 24 °C under different irradiance levels (10, 50, 100 and 200 µmol photons · m^{-2} · s^{-1}), on a 12:12 Light:Dark cycle. Flasks

were acclimated to the experimental conditions for seven days after which growth was measured every three to four days on a 1 mL aliquot of culture using i*n vivo* chlorophyll *a* fluorescence until the stationary growth phase was reached or culture death occurred.

2.7 Estimating optimum growth conditions and niche width

The calculated maximum growth rate for each strain from each experiment was plotted as a function of the environmental variable (e.g. temperature, irradiance, salinity) to form a reaction norm. The shape of the reaction norm was then fitted to estimate fitness parameters such as the conditions under which optimum growth (T_{opt}) was achieved and the limits of growth (T_{min} and T_{max}). Temperature and salinity reaction norms were both fitted in the R environment version 3.2.3 (R Core Team, 2012) with a function first applied to phytoplankton by Eppley (1972) and recently modified by Thomas et al. (2012).

This function describes the temperature or salinity dependent specific growth rate using the following equation:

$$f\ (\mathrm{E}) = ae^{bE}\left[1 - \left(\frac{E - z}{w/2}\right)\right]^2$$

where growth rate *f* is a function of either temperature or salinity (E). The shape of the reaction norm is controlled by species traits, *z* and *w*. The niche width is given by *w* and species trait *z* determines the location of the maximum of the quadratic portion of the function.

Irradiance reaction norms were fitted with a Monod function with a photoinhibition term as was used in Megard et al. (1984) and Litchman (2000) using R version 3.2.3 (R Core Team 2012).

$$\mu = \mu_{max} \frac{I}{I + k + \frac{I^2}{k_{inh}}} - r$$

where μ is the specific growth rate (day^{-1}), μ_{max} is the maximum specific growth rate (day^{-1}), I is the irradiance (µmol photons · m^{-2} · s^{-1}), k is a half-saturation constant (µmol photons · m^{-2} · s^{-1}), r is the metabolic loss rate and k_{inh} is the photoinhibition constant (µmol photons · m^{-2} · s^{-1}).

For temperature, salinity and light, the optimum growth range was calculated to be the point at which 80% of the optimum growth was achieved. The minimum and maximum point along the environmental cline where growth was less than zero was used to estimate the niche width.

3. Results:

3.1 Culture identification and observations

In this study, seven cultures of *Coolia* were established, four from Heron Island, Queensland and three from Merimbula New South Wales, Australia (Table 1). Phylogenetic analyses of 52 *Coolia* LSU rDNA (D1-D3 region) sequences representing six of the seven described species in the genus (sequences for *C. areolata* are not available in GenBank) grouped into six distinct clades (Fig. 2). Strains UTSHI1D5, UTSHI2D4 and UTSHI2D1, isolated from Heron Island, grouped with other *C. tropicalis* sequences with high support (100%/1.0). Strain UTSHI3C5 grouped with *C. palmyrensis* (91%/0.99), while all three strains isolated from Merimbula, New South Wales (UTSMER17A5, UTSMER17D2 and UTSMER17A6), grouped with *C. malayensis* (97%/1.0) (Fig. 2).

3.2 Thermal niche estimates

The maximum growth rate (μ_{max}) for each strain of *Coolia* tested in this study was between 0.21 and 0.29 day^{-1} (Table 2). The temperature at which maximum growth occurred (T μ_{max}) was similar at the intraspecific level between strains but showed substantial differences between species (Table 2, Fig. 3, and Fig. 4). *Coolia tropicalis* had the highest optimum temperature (T μ_{max}) for growth at 27.0-27.6 °C, followed by *C. palmyrensis* at 24.5 °C, then *C. malayensis* at 23.4-23.8 °C reflecting their geographic origin (Table 1). The optimum temperature range (i.e. 80% of maximum) (T_{opt}) for growth of *C. tropicalis* was much narrower (ca. 3-4 °C) than for other species (ca. 7-8 °C) suggesting some degree of temperature specialisation (Table 2 and Fig. 3). The minimum temperature at which growth ceased differed between species but was consistent at the intraspecific level. *C. palmyrensis* and *C. malayensis* were able to grow at temperatures as low as 14 °C, while the minimum temperature for growth (T_{min}) of *C. tropicalis* was 17 °C (Table 2 and Fig. 3). Interestingly, all strains had a maximum temperature tolerance (T_{max}) of approximately 30 °C except *C. palmyrensis* which was found to have an upper thermal tolerance of 32 °C (Table 2 and Fig. 3).

3.3 Salinity niche estimates

Maximum growth rates of most strains under different salinities (μ_{max}) ranged from 0.08 to 0.16 day^{-1}, except for *C. palmyrensis* which grew 0.39 day^{-1} (Table 3). The optimum salinity levels for growth (S μ_{max}) were brackish (23-29) for most strains, while *C. palmyrensis* had maximum growth under more saline oceanic conditions (35.6; Table 3). The shape of each salinity reaction norm diverged between species but was similar at the intraspecific level (Fig. 5). Growth was within 80% of the maximum (S_{opt}) between salinities 21 and 30 for *C. tropicalis* and *C. palmyrensis* but *C. malayensis* appeared to be more euryhaline, growing maximally between salinities 12 and 30 (Table 3 and Fig. 5). The salinity niche width of *C.*

tropicalis was approx. 20 (i.e. between 17 and 40) while for *C. malayensis* it exceeded 50 (<10 and >60) (Table 3 and Fig. 5).

3.4 Irradiance niche estimates

Coolia palmyrensis had the highest maximum growth rate at 0.25 day^{-1} in the irradiance experiments (μ_{max}), well above other species which ranged between 0.14 and 0.17 day^{-1} (Table 4). There were large intraspecific differences in the level of irradiance at which maximum growth was achieved (I μ_{max}), ranging from 130 to greater than 200 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (Table 4 and Fig. 6). Interestingly, the shape of the curves did not differ enormously between species or strains (Fig. 4 and Fig. 6), therefore estimates of the optimum irradiance was similar across all taxa (approximately 60-200 µmol photons $\cdot m^{-2} \cdot s^{-1}$) and the estimated niche widths were the same (0 to<200 µmol photons $\cdot m^{-2} \cdot s^{-1}$) (Table 4 and Fig. 6).

4. Discussion

Environmental variables such as temperature, salinity and irradiance influence microalgal growth and ultimately shape species distributions, but the niches of many benthic harmful algal bloom species (BHAB) are undescribed. In this study, we characterised the environmental niche of *Coolia malayensis*, *C. palmyrensis* and *C. tropicalis* isolated from tropical and temperate locations in eastern Australia for the first time. Despite living in similar shallow coastal habitats, growth optima were different amongst taxa. *C. tropicalis* growth was greatest at warmer temperatures (27 °C), lower salinities (ca. 23) and moderate irradiance (150 µmol photons $\cdot m^{-2} \cdot s^{-1}$). In comparison, optimum growth of *C. malayensis* occurred under moderate temperature (24 °C) and irradiance (150 µmol photons $\cdot m^{-2} \cdot s^{-1}$) but was seemingly unaffected by salinity; *C. palmyrensis* growth peaked at moderate

temperatures (24 °C) under oceanic salinities (35) and relatively high irradiance (>200 µmol photons · m^{-2} · s^{-1}). This is the first study to quantify how temperature, salinity and irradiance influence growth of *Coolia* spp. from eastern Australia. We found interspecific differences were greater than intraspecific variation, however, the number of strains included in our analyses was limited and therefore this pattern may not hold when further strains are compared.

4.1 Culture identification

Strains of *Coolia* in this study were isolated from two locations in eastern Australia, selected based on previous reports of BHABs (Merimbula, New South Wales, Kohli et al. (2014a); Great Barrier Reef, Queensland Australia, Momigliano et al. (2013)). This study was by no means a comprehensive survey, but our findings show there were no species common to both locations. *Coolia tropicalis* and *C. palmyrensis* were both isolated from Heron Island (tropical region) and only *C. malayensis* was isolated from Merimbula (temperate region). Very little is known about the diversity of *Coolia* spp. in Australia. Momigliano et al. (2013) completed a comprehensive morphological and molecular analysis of four strains isolated from three sites in the Central Great Barrier Reef region identifying *C. tropicalis, C. malayensis* and *C. canariensis*, along with a strain that could not be identified at the time but has since been classified as *C. palmyrensis* (Karafas et al. 2015, Gómez et al. 2016, Leaw et al. 2016). These findings, along with the results from this study suggest *C. tropicalis*, *C. palmyrensis* and *C. canariensis* are distributed throughout the Great Barrier Reef region, while *C. malayensis*, although present in tropical regions elsewhere, may have a more temperate distribution in eastern Australia. In two studies where metabarcoding was used to describe benthic dinoflagellate species diversity, a *Coolia* sp. was identified at Broome, Western Australia and both *C. monotis* and *C. canariensis* in southern New South Wales (Kohli et al. 2014a, Kohli et al. 2014b). While it is difficult to accurately identify to species

level using the metabarcoding approach, these studies indicate the *Coolia* genus may be widely distributed in Australia. The distribution of *C. canariensis* may also extend to temperate eastern Australia and other species, such as *C. monotis,* may also be present, however, considerable work remains to be done before we have a thorough understanding of the biogeography of *Coolia* spp. in Australia.

4.2 Coolia tropicalis

C. tropicalis was first described by Faust (1995) with cells collected from Belize, Japan, Puerto Rico and Reunion Island in the South West Indian Ocean. Since then, the species has been documented in many shallow water habitats (2-8 m) throughout Asia including Vietnam (Nguyen 2014), Japan (Tawong et al. 2015), Hong Kong (Leung et al. 2017), Malaysia and Indonesia (Mohammad-Noor et al. 2013). Thus, the current known distribution of *C. tropicalis* suggests the species may be restricted to tropical locations between the latitudes of 23 °S to 23 °N (Fig. 1). In this study, *C. tropicalis* was collected at Heron Island (latitude 23 °S), which may be close to its southern limit, and it was not found at the temperate sampling location (latitude 36 °S).

Thermal niche results from this study also support that *C. tropicalis* may have a distribution restricted to tropical locations. The optimum temperature for growth (T μ_{max}) was considerably higher (27 °C) and the optimum range (T_{opt}) narrower (± 1.5-2 °C) than the other species tested, suggesting *C. tropicalis* could be a high temperature specialist. Indeed, the minimum temperature threshold for growth (T_{min}) or the lower niche limit was approximately 17 °C, 3 °C higher than the other strains, providing further evidence that growth may be limited to tropical locations. It was surprising that the maximum temperature threshold (T_{max}) or the upper niche limit was only 30 °C, given that tropical ocean waters can reach temperatures substantially higher than this. However, the experiments in this study

examined growth under constant temperature, whereas in natural environments, short-term cooling due to diurnal or tidal fluctuations may allow growth at higher day-time temperatures.

The optimum salinity for growth (S μ_{max}) of *C. tropicalis* was approximately 25, much lower than seawater. The tolerable range of salinities was however very large (18 to 40, suggesting a mechanism to adapt to large fluctuations in salinity during tropical monsoonal rains. Mohammad-Noor et al. (2013) found contrasting results, with *C. tropicalis* from Indonesia growing optimally at salinity 35, suggesting there is some variability in osmoregulation amongst strains.

Coolia tropicalis strains from this study were able to grow across a very broad range of irradiance levels. Optimum growth (I μ_{max}) occurred at levels of approximately 50 to ~ 200 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. To our knowledge, the only study that has assessed the influence of irradiance on the growth rate of *Coolia* found three *C. monotis* strains isolated from 1 m depth at different locations along the Atlantic Coast of the Iberian Peninsula each responded differently (David et al. 2017). However, the range of irradiance levels that produced the highest growth rates were similar to this study (approx. 50 to 300 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}).

4.3 Coolia malayensis

In contrast to the restricted distribution of *C. tropicalis*, *C. malayensis* appears to have a widespread global distribution (Fig 1). The species was first described by Leaw et al. (2010) from type strains collected in Malaysian waters. Since the original description, the species has been documented in many locations throughout Asia (Jeong et al. 2012, Mohammad-Noor et al. 2013, Tawong et al. 2015, Wakeman et al. 2015, Leaw et al. 2016, Leung et al. 2017), the South Pacific Ocean (Rhodes *et al.* 2000, Momigliano et al. 2013, Rhodes et al. 2014, Leaw et al. 2016), North America (Fraga et al. 2008, Karafas et al. 2015, Leaw et al.

2016) and South America (Gómez et al. 2016), occurring at latitudes between 36 °S to 34 °N (Fig. 1). The *C. malayensis* isolates from this study were isolated from temperate waters at a latitude of 36 °S, around the southern limit of its documented distribution (Fig. 1).

The thermal niche of *C. malayensis* strains in this study was greater than 15 °C. Both strains tolerated temperatures as low as 14 °C and as high as 30 °C, with an optimum at approximately 24 °C. The ($T\ \mu_{max}$) for *C. malayensis* strains was lower than for *C. tropicalis*. This divergence likely represents differences in temperature regimes at the different isolation locations for the two species, with *C. malayensis* originating from a cool temperate climate while *C. tropicalis* originated from a warm tropical location.

It remains to be determined whether a *Coolia* strain isolated from a tropical location would be more like a temperate strain of the same species or a tropical strain of a different species. The optimum temperature range (80% of the maximum growth rate) (T_{opt}) extended over 7-8 °C suggesting this species could be a thermal generalist, consistent with its broad latitudinal distribution.

The growth of *C. malayensis* strains used in this study appeared unaffected by salinity, growing at similar rates across all levels measured (20-38). Mohammad-Noor et al. (2013) measured the growth rate of a strain of *C. malayensis* originally isolated from Malaysia across a similar salinity gradient (20-40) finding that it had the highest growth rate at salinity 30 when grown at 25 °C, in contrast to salinity 35 when grown at 30 °C. However, similar to our study, growth was achieved across all salinities tested. Like strains of *C. tropicalis* measured in this study and *C. monotis* strains measured by David et al. (2017), *C. malayensis* was able to grow across a range of irradiance. *Coolia malayensis* therefore appears to have the broadest fundamental niche of the three *Coolia* species isolated in this study.

4.4 Coolia palmyrensis

C. palmyrensis is one of the newly described species in the *Coolia* genus. It was first described by Karafas et al. (2015) based on a strain isolated from Palmyra Atoll in the central Pacific Ocean and another from the Dominican Republic in the Caribbean Sea. The original description also included a previously unidentified strain reported as *Coolia* sp. (strain number NQAIF103, GenBank accession number HQ897277; Momigliano et al. (2013)) from Australia. The only other report of *C. palmyrensis* in the literature is by Leung et al. (2017) who isolated and identified three strains from two locations in Hong Kong, although sequences available in GenBank (www.ncbi.nlm.nih.gov) suggest that the distribution might extend to Fiji and Spain (Fig. 2). While it has only recently been described, the distribution so far seems to be limited to tropical locations (Fig. 1).

As only one strain of *C. palmyrensis* was established in this study, it is difficult to draw conclusions about the environmental niche of the species. However, it is evident that the established isolate differs from the other taxa. *Coolia palmyrensis* is the smallest of the seven species in the genus (Karafas et al. 2015) and had the fastest growth rate (up to 0.39 day^{-1}), consistent with the general pattern observed across microalgal lineages that smaller cells grow faster (Litchman et al. 2007). Established from a tropical location, it was expected that the *C. palmyrensis* isolate would have a similar environmental niche to *C. tropicalis*. This, however, was not the case (Tables 2, 3, 4 and Figs. 3, 4, 5 and 6).

The optimum temperature for growth (T μ_{max}) was 24 °C, ca. 3 °C lower than the strains of *C. tropicalis* isolated from the same tropical location and more similar to the strains of *C. malayensis* isolated from temperate Australia (Table 2). *Coolia palmyrensis* also differed from *C. tropicalis* in that it had a much wider thermal niche, suggesting it may be more of a thermal generalist like *C. malayensis* (Fig. 4). The optimum salinity for growth (S μ_{max}) was also much higher at 35, and growth remained high at salinities as low as 20 and as high as 40.

Coolia palmyrensis was also found to grow well at relatively high irradiance levels but like all other species tested, significant growth was measured across a wide range of irradiance.

4.5 Conclusions

This is the first study to comprehensively characterise how temperature, salinity and irradiance affect growth of *C. tropicalis, C. palmyrensis* and *C. malayensis* from eastern Australia and apply this information to help define species distributions. We demonstrated that *C. tropicalis* is more of a thermal specialist, growing optimally within a narrow range of high temperatures characteristic of tropical locations, and prefers brackish salinity and moderate irradiance levels. *Coolia malayensis* prefers more temperate thermal conditions, with moderate irradiance levels and appears euryhaline. Although *C. palmyrensis* was isolated from the same tropical location as the *C. tropicalis* strains, the environmental niche of this species appears to be more like temperate *C. malayensis*. While these results show that *Coolia* spp. can grow across a broad range of environmental conditions, further research is required to examine the toxicology of the *Coolia* genus and examine how environmental variables influence toxicity. This information will provide vital information to help inform species distributions, particularly under changing climate conditions.

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Author Contributions:

M.E.L isolated, established and maintained the strains of *Coolia*, carried out all DNA extractions, experimental work, data analysis, designed the study and drafted the manuscript; K.F.S assisted with sequencing and phylogenetic analyses and helped write the manuscript; M.A.D participated in the design of the study and helped write the manuscript.

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Tables

Table 1. Identity, geographic origin and GenBank accession number for strains of *Coolia* isolated in this study.

Species Name	Strain Code	Site of Isolation	GenBank Accession No.
C. tropicalis	UTSHI1D5	Heron Island Lagoon, QLD, Australia	MH319018
C. tropicalis	UTSHI2D4	Heron Island Lagoon, QLD, Australia	MH319017
C. tropicalis	UTSHI2D1	Heron Island Lagoon, QLD, Australia	MH319016
C. palmyrensis	UTSHI3C5	Heron Island Lagoon, QLD, Australia	MH319015
C. malayensis	UTSMER17A5	Merimbula Lake, NSW, Australia	MH319014
C. malayensis	UTSMER17D2	Merimbula Lake, NSW, Australia	MH319013
C. malayensis	UTSMER17A6	Merimbula Lake, NSW, Australia	MH319012

Table 2. Maximum growth rate (μ_{max}), the temperature (°C) at which maximum growth was reached (T μ_{max}), the optimum (>80% of μ_{max}) temperature range for growth (T_{opt}) and the minimum and maximum temperatures where growth ceased (T_{min} and T_{max}), calculated from the temperature reaction norm function for each strain of *Coolia* spp.

Species	μ_{max} (d^{-1})	T μ_{max} (°C)	T_{opt} (°C)	T_{min} (°C)	T_{max} (°C)
***C. tropicalis* (UTSHI1D5)**	0.25	27.4	25.3-28.8	<10	30.1
***C. tropicalis* (UTSHI2D4)**	0.28	27.0	24.8-28.5	17.1	30.0
***C. tropicalis* (UTSHI2D1)**	0.21	27.6	25.7-28.9	17.8	30.1
***C. palmyrensis* (UTSHI3C5)**	0.29	24.5	20.5-28.1	14.7	32.2
***C. malayensis* (UTSMER17A5)**	0.21	23.4	19.9-26.6	14.9	30.1
***C. malayensis* (UTSMER17A6)**	0.25	23.8	20.1-26.9	14.5	30.2

Table 3. Maximum growth rate (μ_{max}), the salinity at which maximum growth occurred (S μ_{max}), the optimum salinity range for growth (S_{opt}) and the minimum and maximum salinity where growth ceased (S_{min} and S_{max}) calculated from the salinity reaction norm function for each strain of *Coolia* spp.

Species	μ_{max} (d^{-1})	S μ_{max}	S_{opt}	S_{min}	S_{max}
***C. tropicalis* (UTSHI1D5)**	0.15	23.6	21.3-27.0	19.2	41.6
***C. tropicalis* (UTSHI2D4)**	0.12	24.2	21.4-28.0	18.7	36.7
***C. tropicalis* (UTSHI2D1)**	0.08	26.0	21.9-30.7	17.5	37.3
***C. palmyrensis* (UTSHI3C5)**	0.39	35.6	25.9-42.5	<10	49.0
***C. malayensis* (UTSMER17A5)**	0.10	23.4	16.3-23.4	<10	>60
***C. malayensis* (UTSMER17A6)**	0.16	29.3	12.1-29.3	<10	52.9

Table 4. Maximum growth rate (μ_{max}) of each *Coolia* spp. isolated in this study, the level of irradiance (µmol photons · m^{-2} · s^{-1}) at which maximum growth occurred (I μ_{max}), the optimum iradiance range for growth (I_{opt}) and the minimum and maximum irradiance levels where growth ceased (I_{min} and I_{max}) calculated from the irradiance reaction norm function.

Species	**μ_{max} (d^{-1})**	**I μ_{max} (**µmol photons · m^{-2} · s^{-1}**)**	**I_{opt} (**µmol photons · m^{-2} · s^{-1})	**I_{min} (**µmol photons · m^{-2} · s^{-1})	**I_{max} (**µmol photons · m^{-2} · s^{-1})
***C. tropicalis* (UTSHI1D5)**	0.17	137.1	64.8- >200	0	>200
***C. tropicalis* (UTSHI2D4)**	0.14	175.2	82.3- >200	0	>200
***C. tropicalis* (UTSHI2D1)**	0.17	130.6	63.5- >200	0	>200
***C. palmyrensis* (UTSHI3C5)**	0.25	>200	104.2- >200	0	>200
***C. malayensis* (UTSMER17A5)**	0.16	>200	111.05- >200	0	>200
***C. malayensis* (UTSMER17A6)**	0.17	138.0	66.0- >200	0	>200

Figures

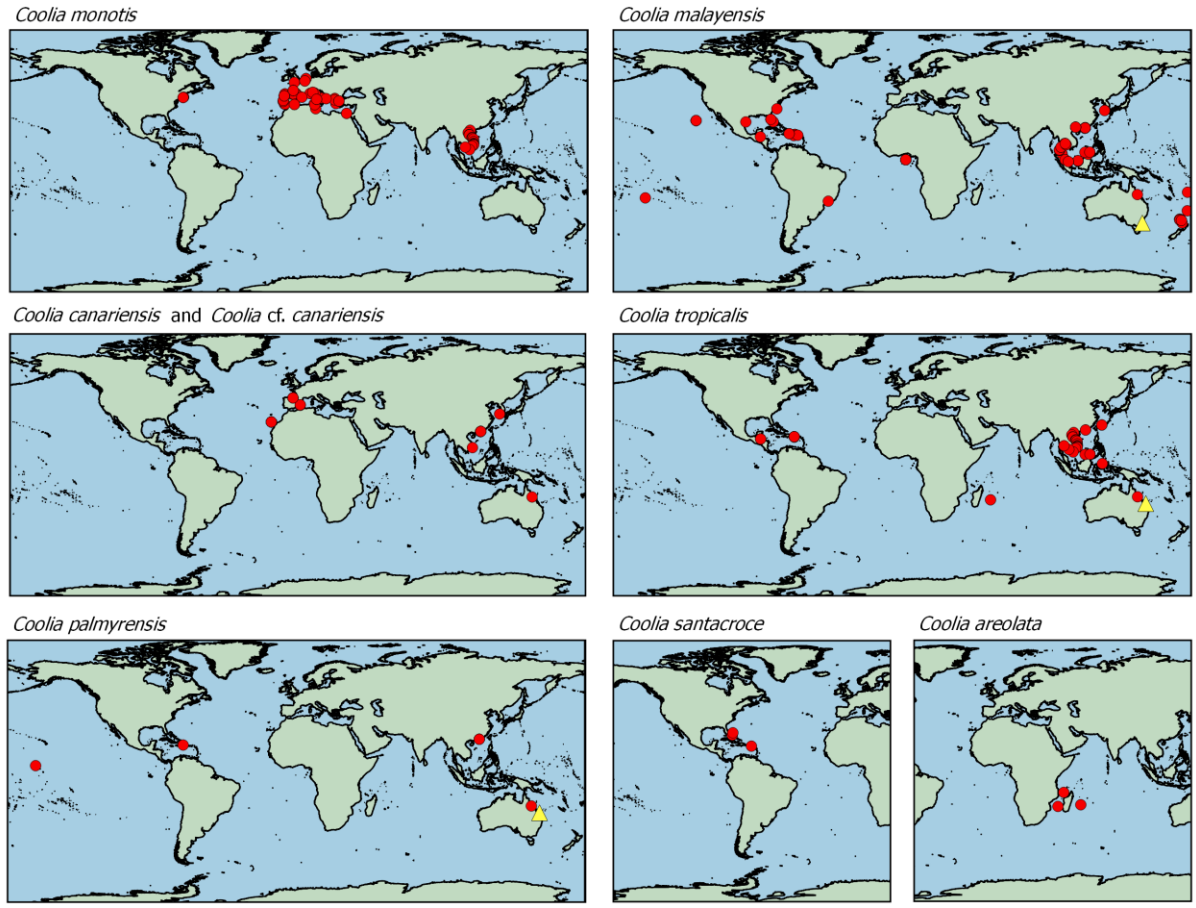


Figure 1. Global distribution of *Coolia* species shown with red circles and contributions from this study with yellow triangles, summarised from the following literature sources: Faust 1995, Rhodes et al., 2000, Ten-Hage et al., 2000, Simoni et al., 2004, Penna et al., 2005, Aligizaki and Nikolaidis 2006, Dolapsakis et al., 2006, Fraga et al., 2008, Armi et al., 2010, Leaw et al., 2010, Laza-Martinez et al., 2011, Cohu and Lemée 2012, Jeong et al., 2012, Pagliara and Caroppo 2012, Mohammad-Noor et al., 2013, Momigliano et al., 2013, David et al., 2014, Feki-Sahnoun et al., 2014, Nguyen 2014, Rhodes et al., 2014, Karafas et al., 2015, Karafas and Tomas 2015, Wakeman et al., 2015, 2016, Cruz and Okolodkov 2016, Gómez et al., 2016, Leaw et al., 2016, David et al., 2017, Leung et al., 2017.

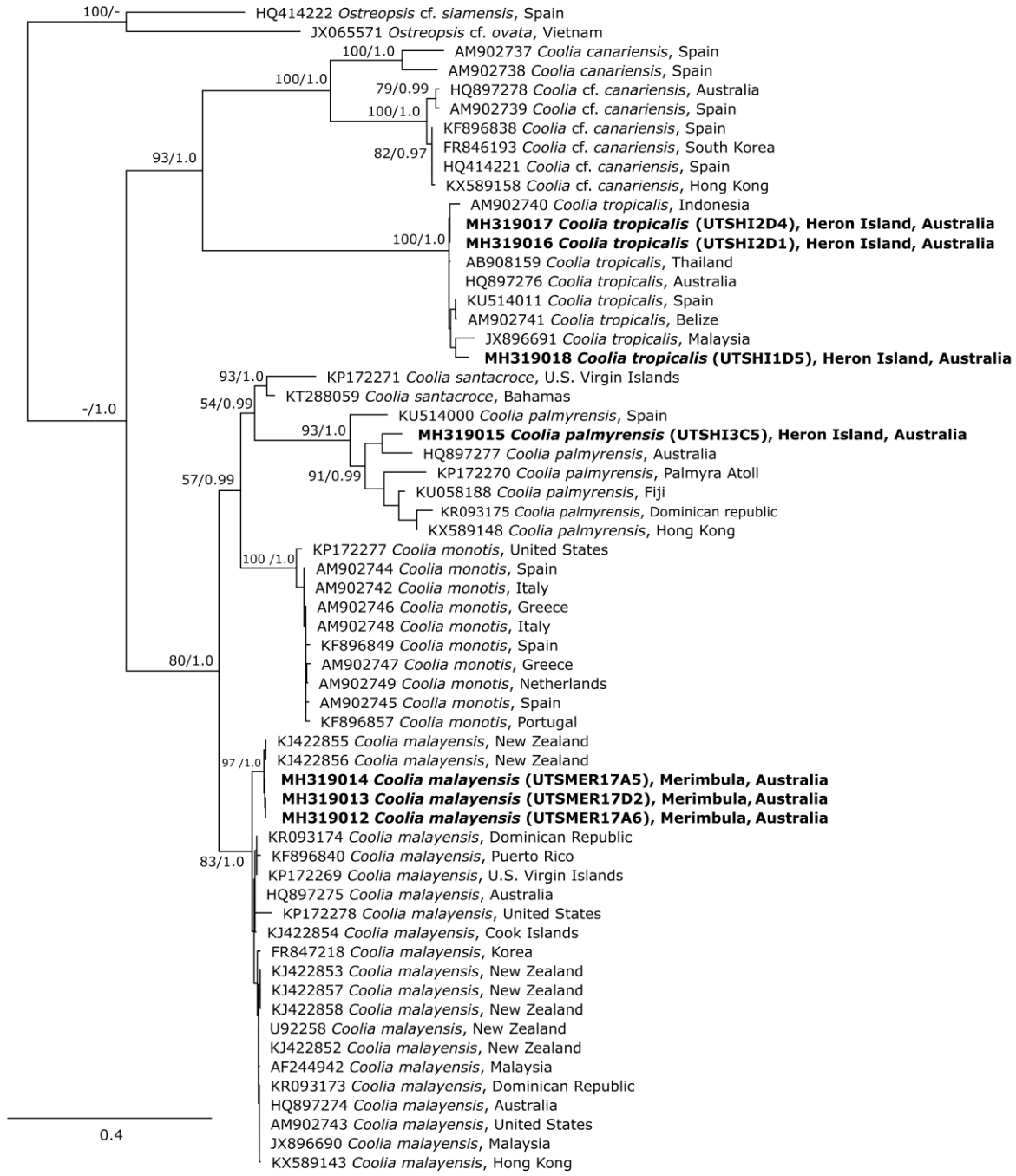


Figure 2. *Coolia* phylogenetic analysis based on the LSU rDNA (D1-D3 region). Maximum Liklihood tree of *Coolia* isolates globally, with strains from this study shown in bold. Values at nodes represent Maximum Likelihood bootstrap and Bayesian posterior probability support. Scale bar represents substitutions per site.

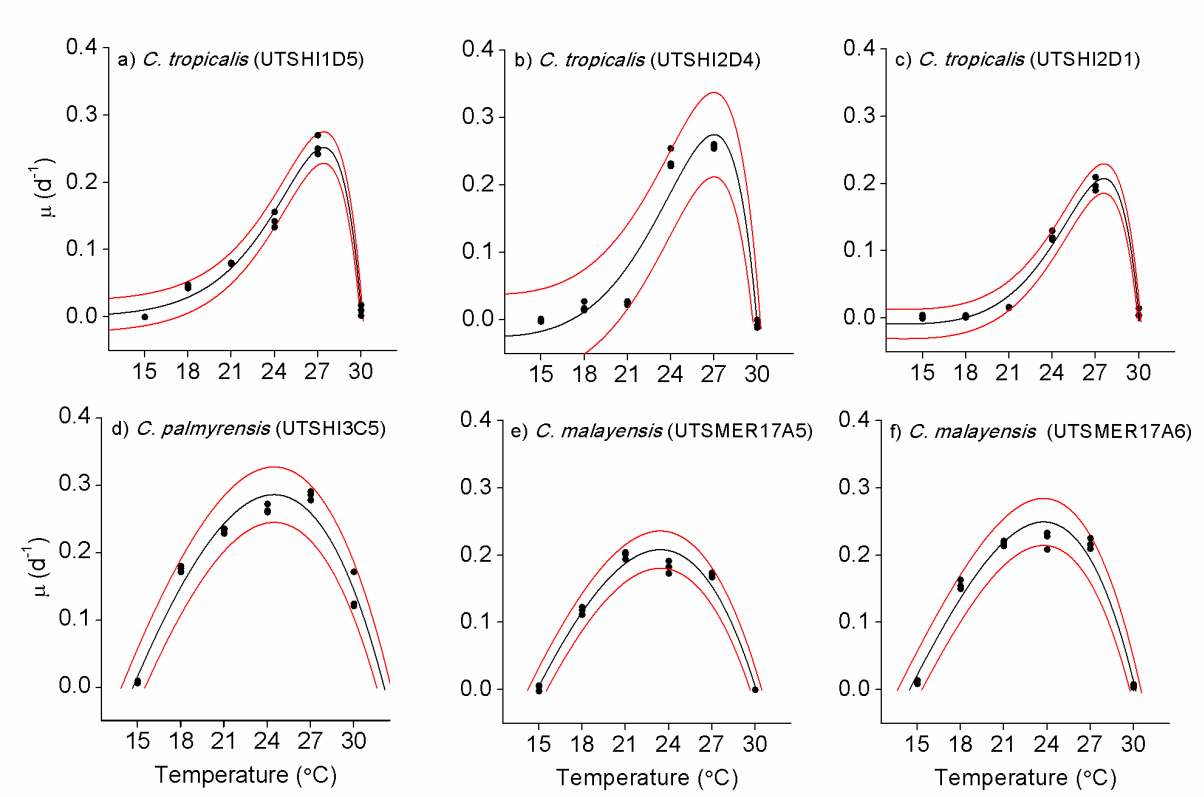


Figure 3. Temperature reaction norms for strains of *Coolia* spp. isolated in this study. Black line indicates line of best fit, red lines indicate 95% confidence limits.

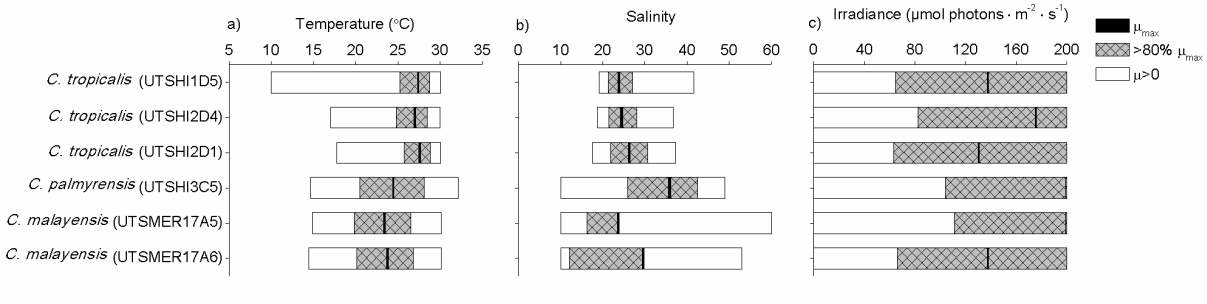


Figure 4. Comparison of the temperature, salinity and irradiance niche of *Coolia* spp. strains established in this study.

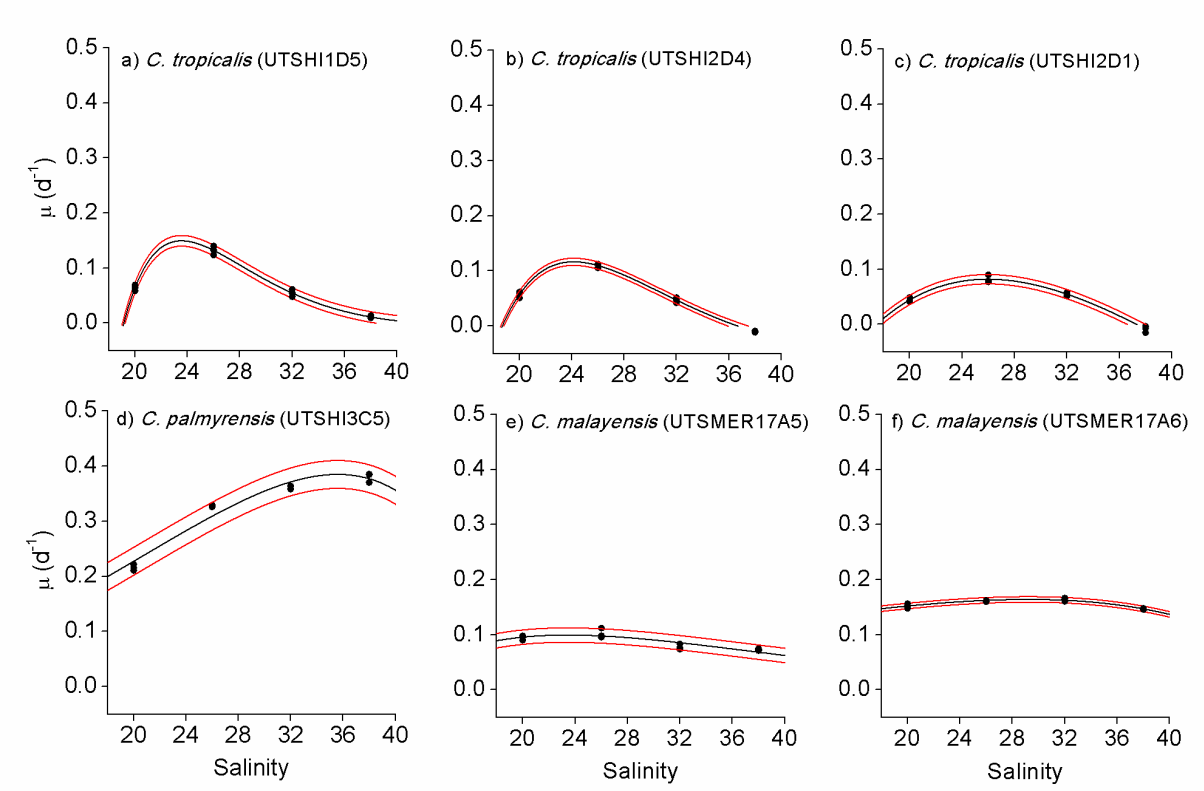


Figure 5. Salinity reaction norms for strains of *Coolia* spp. isolated in this study. Black line indicates line of best fit, red lines indicate 95% confidence limits.

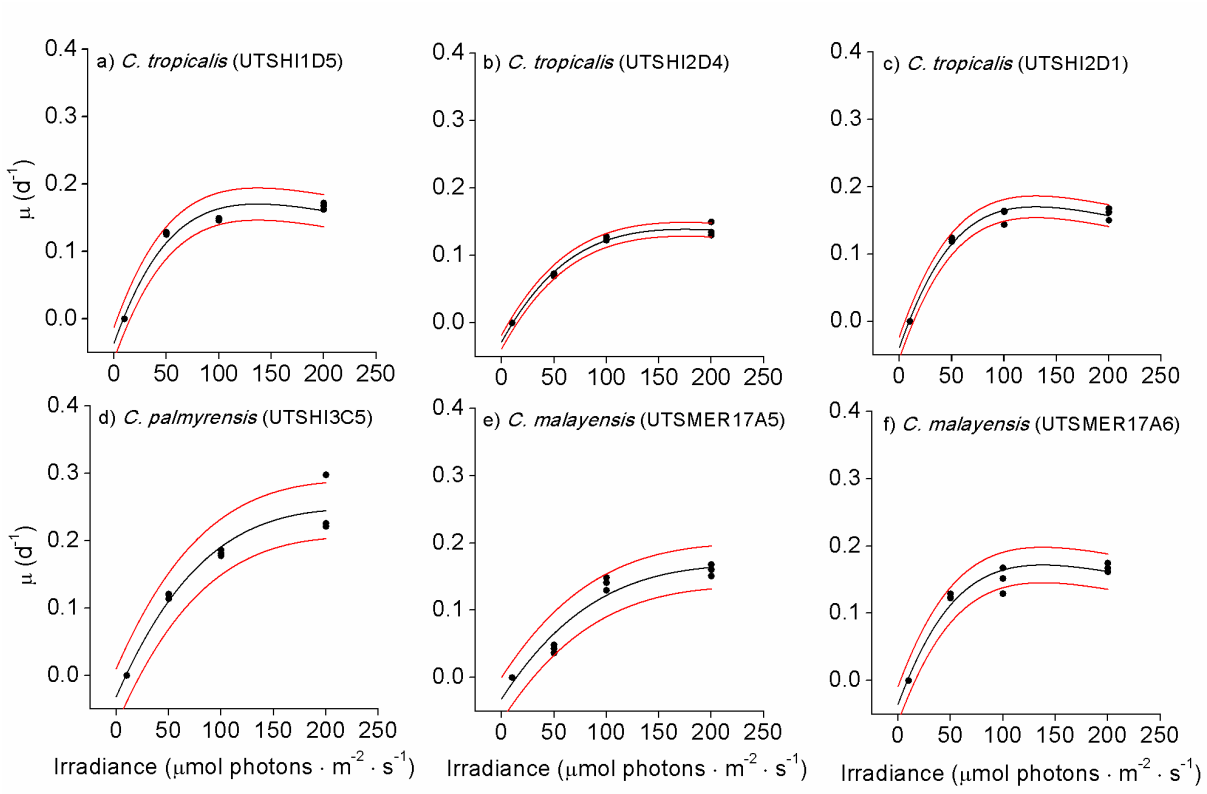
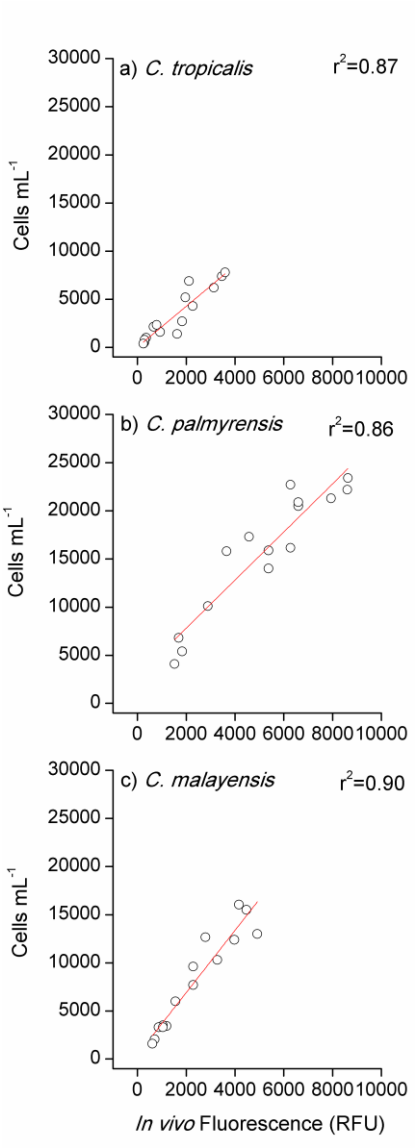


Figure 6. Irradiance reaction norms for strains of *Coolia* spp. isolated in this study. Black line indicates line of best fit, red lines indicate 95% confidence limits.

Supplementary Figure Captions



Supplementary Fig. 1. Relationship between *in vivo* chlorophyll a fluorescence (relative fluorescence units) and cell concentration (mL^{-1}) for *C. tropicalis* (a), *C. palmyrensis* (b), and *C. malayensis* (c), indicating that fluorescence is a suitable measure for estimating exponential growth.