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The effect of bacteria on the sensitivity of microalgae to copper in laboratory bioassays

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Abstract

Although single-species laboratory toxicity tests with microalgae are sensitive and highly reproducible, they lack environmental realism. Interactions between algae and their associated bacteria, either in the plankton or in biofilms, may alter algal sensitivity to contaminants, which are not mimicked in laboratory toxicity tests. This study investigated the effects of simple algal-bacterial relationships on the sensitivity of laboratory-cultured algae to copper using 72-h algal growth-rate inhibition bioassays. Four species of microalgae were used, two isolates of each; a strain of algae with no microscopically visible and no culturable bacteria present (operationally defined as axenic) and a non-axenic strain. The four algae used were the marine diatom Nitzschia closterium, the freshwater green alga Pseudokirchneriella subcapitata and two tropical Chlorella spp. Under control conditions (no copper), N. closterium and P. subcapitata grew better in the presence of the bacterial community. Sensitivity to copper (assessed as the concentration to inhibit the growth rate by 50% after 72-h (IC50)) was not significantly different for the axenic and non-axenic strains of N. closterium, P. subcapitata or for Chlorella sp. (PNG isolate). At pH 5.7, the axenic Chlorella sp. (NT isolate) had a 72-h IC50 of 46 μg Cu L−1, while in the presence of bacteria the IC50 increased (i.e., sensitivity decreased) to 208 μg Cu L−1. However, when the bacterial status of both the operationally defined axenic and non-axenic cultures of N. closterium and Chlorella sp. (NT isolate) was investigated using polymerase chain reaction (PCR) amplification of 16S rRNA followed by DNA fingerprinting using denaturing gradient gel electrophoresis (DGGE), it was found that bacteria were actually present in all the algal cultures, i.e. the axenic cultures were not truly bacteria-free. Based on sequence information, the bacteria present were nearly all identified as alphaproteobacteria, and a number of isolates had high similarity to bacteria previously identified as symbionts or species endophytically associated with marine organisms. The “axenic” cultures contained less bacterial phylotypes than the non-axenic cultures, and based on band-intensity, also contained less bacterial DNA. This supported the findings of few differences in copper sensitivity between strains, and suggests that standard microalgal toxicity tests probably inadvertently use non-axenic cultures in metal assessment.

Keywords

effect, bacteria, sensitivity, microalgae, copper, laboratory, bioassays

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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The effect of bacteria on the sensitivity of microalgae to copper in laboratory bioassays

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Abstract

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than the non-axenic cultures, and based on band-intensity, also contained less bacterial DNA. This supported the findings of few differences in copper sensitivity between strains, and suggests that standard microalgal toxicity tests probably inadvertently use non-axenic cultures in metal assessment.

Keywords: bacteria, phytoplankton, microalgae, 16S rRNA, Cu, axenic

1. Introduction

Algae are useful organisms to assess metal contamination and bioavailability in aquatic systems, as they are sensitive to metal contaminants at environmentally relevant concentrations (Stauber and Davies, 2000). Algae are primary producers and affect the cycling of nutrients through marine, freshwater and aquaculture ecosystems (Sakata and Sakata, 1996; Azam and Malfatti, 2007). As such, algae are regarded as ecologically significant organisms and ideal candidates for ecotoxicological studies. Usually toxicity tests use single species (monoculture) testing under controlled laboratory conditions to reduce variability and to simplify the interpretation of results. However, these tests lack environmental realism because algae rarely occur in isolation, but rather as part of complex planktonic or biofilm communities. One of the drivers for this research was the concern that laboratory-cultured algae may have sensitivities to metal contaminants that differ from that of their field counterparts because of the presence of natural bacteria and other biofilm components. This could mean that guideline trigger values based on laboratory toxicity testing could be overly conservative if the field species are less sensitive, or vice versa if the sensitivities were higher.

The environmental relevance of laboratory-based toxicity tests could be improved by incorporating multi-species or multi-taxa tests. Several studies have used pollution-induced community tolerance (PICT) responses of phytoplankton, periphyton, benthic algae or biofilms to toxicants (Blanck and Dahl, 1996; Admiraal et al., 1999; Knauer et al, 1999; Schmitt-Jansen and Altenburger, 2005), focusing on changes in community structure in response to pollutants. Alteration of community structure can influence overall function (e.g. respiration, photosynthesis) and the sensitivity of the community to toxicants. Recent

research has attempted to develop multi-species algal tests in toxicity-based metals assessment (Franklin et al., 2004, Yu et al., 2007). These studies have explored the toxicological response of individual algal species when exposed in combination with one or two other algal species. Such studies have previously been hampered by practical difficulties associated with counting individual species and assessing their responses in the presence/absence of toxicants. Furthermore, little attention has been given to how algal interactions with non-algal species influences metal uptake and toxicity.

Bacteria may have either a positive or negative effect on algae in polluted environments. For example, the tolerance of the green macroalga *Enteromorpha compressa* to copper in a polluted coastal environment in Chile may have been attributable to a community of epiphytic bacteria colonising its surface (Riquelme et al., 1997). Bacterial biofilms can mediate toxicity of metals to the host organism, e.g. through diffusion limitation of toxicants, protective effects of high concentrations of extracellular polymeric substances, protective effects of trapped nutrient stores, and effects due to a greater surface area (less toxicant per cell). While the effects of metals on biofilms are quite widely reported (Morel and Palenik, 1989; Barranguet et al., 2000, 2002, 2003, Massieux et al., 2004, García-Meza et al., 2005), few studies on the effects of biofilms on metal toxicity to algae have been reported.

In natural habitats, bacteria are always associated with algae and can have both beneficial or deleterious effects on algal growth. Interactions between algae and bacteria are complex and include competition for resources (Grossart, 1999), production of anti-microbial agents (Fukami et al., 1997; Steinberg et al., 1997; Gross 2003), stress protection via production of extracellular polymeric substances, and metal binding or transformation through exudate

production (Koukal et al., 2007). Algal cells may associate with a range of bacterial communities (Shafer et al., 2002; Grossart et al., 2005). This association varies from general habitat sharing, to direct colonisation of bacteria on the algal surface (epiphytic biofilm) and endophytic association of bacteria within algae cells.

Despite the importance of bacteria in nature and their relationship to algae, toxicity testing protocols usually use axenic cultures, i.e., unialgal cultures free from bacteria. The aim of this paper was to examine the influence of bacteria on the growth of a number of algal species under controlled laboratory conditions, and on the toxicity of copper to these algae.

2. Materials and Methods

2.1 General

All general glassware and plasticware was cleaned in a laboratory dishwasher (GW 3050, Gallay, Auburn, NSW, Australia) with a phosphate-free detergent (Clean A Powder Detergent, Gallay), then acid-washed in HNO_3 (30% v/v; Merck, Kilsyth, VIC, Australia) and rinsed three times with MilliQ® water (>18 MΩ cm^{-1}, Millipore, North Ryde, NSW Australia). All glassware used in bioassays was pre-soaked in 10% HNO_3 overnight and then washed thoroughly five times with demineralised water and five times with MilliQ® water. All chemicals were AR grade or better, and solutions were prepared with high purity MilliQ® water. For DNA analysis, all plasticware was sterile, DNA- and RNA-free (CellStar polypropylene centrifuge tubes, Greiner Bio-One, Frickenhausen, Germany or other DNA-grade plastic-ware, LabServ, BioLab Australia, Clayton, VIC, Australia).

2.2 Algal stock cultures

A culture of the marine diatom, *Nitzschia closterium* (Ehrenberg) W. Smith (Bacillariophyceae), originally isolated from the Port Hacking River in NSW, Australia, was obtained from the CSIRO Collection of Living Microalgae (Strain CS-5). It was maintained in a modified f medium, with reduced iron and trace element concentrations (Guillard and Ryther, 1962). Three freshwater species were also selected. *Pseudokirchneriella subcapitata* (Korshikov) Hindak (formerly *Selenastrum capricornutum* Printz) (Chlorophyceae) was obtained from the American Type Culture Collection (ATCC 22662) and is a temperate isolate from the Nitelv River, Askershus, Norway used widely in toxicity testing (USEPA, 2002). It was maintained in USEPA media (USEPA, 2002). *Chlorella* sp. isolate 6 (1b) (Chlorophyceae) is a tropical species isolated from Lake Aesake, Papua New Guinea. It was maintained in half strength MBL media (Stein, 1973) and is denoted as *Chlorella* sp. (PNG isolate) throughout this paper. A second *Chlorella* sp. was obtained from the Environmental Research Institute of the Supervising Scientist (ERISS, NT, Australia). It was originally isolated in Kakadu National Park, Northern Territory, Australia, in very soft, acidic water (2-4 mg $CaCO_3$ L^{-1} hardness, pH 6) and was maintained in MBL medium (Franklin et al., 2000). It is denoted as *Chlorella* sp. (NT isolate) throughout this paper. The two tropical *Chlorella* species were kept at 27(±2)°C on a 12 h light:12 h dark cycle, at a light intensity of 70 µmol photons m^{-2} s^{-1} (Philips TL 40-W cool-white fluorescent lighting). *N. closterium* was maintained at 21°C on a 12 h light:12 h dark cycle, at a similar light intensity as the tropical species. The temperate *P. subcapitata* was kept at 21(±2)°C on a continuous light cycle at a light intensity of 70 µmol photons m^{-2} s^{-1}, as per the standard protocol (USEPA, 2002). A continuous light cycle is required for this test species to ensure exponential cell division (minimum one division per day) throughout the test period.

To establish (operationally defined) axenic strains of the four algae, cultures were streaked onto agar plates containing normal medium plus 2% bacto-agar (Oxoid, Bacto Laboratories, Liverpool, NSW, Australia). After 7-10 days incubation (conditions described above) single colonies were isolated onto a new medium agar plate. This process was repeated until no bacterial colonies were visible on the plates (i.e. only algal colonies visible). A single algal colony was then transferred into fresh liquid algal medium and checked for bacterial colonies. After culturing for at least 14 days, this new liquid culture was spread onto either fresh or marine-water Peptone Yeast Extract Agar (PYEA: 2% agar (Oxoid), 0.1% peptone (Oxoid), 0.1% yeast extract (Oxoid), in either MilliQ® or filtered natural seawater). Cultures were operationally-defined as axenic if no bacteria were observed in the liquid cultures under a phase-contrast microscope.

2.3 Growth-rate inhibition bioassays.

The chronic toxicity of copper to four species of microalgae was tested using 72-h growth-rate inhibition bioassays (details below). A batch method was used for the bioassays using 250-mL borosilicate glass Erlenmeyer flasks, treated with Coatasil silanising solution (APS Ajax Finechem, Seven Hills, NSW, Australia) to prevent adsorption of copper to the glass.

Copper stock solutions (5 and 100 mg L^{-1}) were prepared from copper sulfate ($CuSO_4 \cdot 5H2O$, AR grade, APS Ajax Finechem) using Milli Q water and acidified with HCl (0.2% v/v, pH < 2, Tracepur, Merck). The medium used to prepare test treatments for the marine test species (*N. closterium*) was filtered seawater (0.45 μm pore size, cartridge filter, Millipore). The media used to prepare test treatments for the freshwater species are listed in Table 1. *P. subcapitata* test-treatments were prepared in USEPA media without EDTA as per the

standard protocol (USEPA, 2002) (pH 7.4 - 7.5). *Chlorella* sp. (PNG isolate) test-treatments were prepared in softwater (80-90 $CaCO_3$ mg L^{-1} hardness, pH adjusted to pH 7.4 - 7.5) (Table 1). Bioassays using *Chlorella* sp. (NT isolate) were conducted in a very softwater (2-4 mg $CaCO_3$ L^{-1} hardness) at low pH (pH 5.7 and pH 6.5) to match the water quality conditions of the region from which this alga was isolated (Table 1) (Franklin et al., 2000). For tests with *Chlorella* sp. (NT isolate), the pH of the treatment solutions was initially adjusted to either 5.7 or 6.5 by drop-wise addition of 0.1 M HCl or NaOH, prior to dispensing the solution in flasks. The tests with *Chlorella* sp. (NT isolate) were done at two pH values because previous research had shown that small changes in pH had a large effect on copper toxicity to this species (Franklin et al., 2000). Manual pH adjustment rather than pH buffers were used, as some buffers have previously been shown to affect control growth rates in some algae and/or to complex copper, reducing its bioavailability (Wilde et al., 2006). At least five different copper treatments and a control (copper-free filtered test media) were prepared from the stock solutions in triplicate (or quadruplicate for some controls) and 50 mL dispensed into 250-mL Erlenmeyer flasks. Nominal copper exposure concentrations varied in each test, and were 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 40 and 45 µg Cu/L in *N. closterium* bioassays; 0.5, 1, 2, 2.5, 4, 5, 6, 7.5, 10, 25 and 50 µg Cu/L in *P. subcapitata* bioassays; 2, 2.5, 3, 3.5, 4, 6, 12 and 24 µg Cu/L in *Chlorella* sp. (PNG isolate) bioassays; 1.5, 6, 24, 60, 100, 400 and 600 µg Cu/L in *Chlorella* sp. (NT isolate) pH 5.7 bioassays, and; 0.5, 1, 2, 4, 5, 6, 8, 10, 20 and 30 µg Cu/L in *Chlorella* sp. (NT isolate) pH 6.5 bioassays. Each flask was supplemented with 15 mg NO_3^- L^{-1} (*Chlorella* spp. and *N. closterium* tests) ($NaNO_3$, AR grade, APS Ajax Finechem) and 0.15 mg PO_4^{3-} L^{-1} (*Chlorella* spp. tests) or 1.5 mg PO_4^{3-} L^{-1} (*N. closterium* tests) (KH_2PO_4, AR grade, APS Ajax Finechem). No further nutrient additions to the USEPA medium were required for *P. subcapitata*.

Cells in exponential growth phase (5-6 d old; or for *Chlorella* sp. (NT isolate) 4-5 d old), were used to inoculate the test treatments after centrifugation (1200 *g*, 7 min, Spintron GT-175BR, Spintron, Melbourne, VIC, Australia) and three subsequent washes in test medium (seawater, softwater or USEPA medium (-EDTA)) to remove residual culture medium. The test flasks were then inoculated with 2-4 × 10^3 cells mL^{-1}. A 5 mL sub-sample was immediately filtered through an acid-washed 0.45 µm membrane filter (MiniSart, Sartorius, Oakleigh, VIC, Australia), acidified with 0.2% HNO_3 (TracePur, Merck), and dissolved copper was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Spectro Flame-EOP, Spectro Analytical Instruments, Kleve, Germany). The flasks were incubated for 72 h in 12:12 h light/dark conditions at 140 µmol photons m^{-2} s^{-1} at 27°C (for tropical species) or 21°C (temperate species) (except *P. subcapitata* which was kept under constant illumination for the duration of tests as per the standard protocol). Test flasks were rotated within the light cabinet and shaken twice daily by hand to ensure sufficient gas exchange. The pH was recorded initially and after 72 h. For *Chlorella* sp. (NT isolate), the pH was monitored and adjusted daily to pH 5.70 ± 0.05 or pH 6.50 ± 0.05 using drop-wise additions of 0.005 or 0.01 M HCl (TracePur, Merck) and NaOH (APS Ajax FineChem).

The cell density in each treatment was measured daily using flow cytometry (BD-FACSCalibur, Becton Dickinson BioSciences, San Jose, CA, USA). Cells were excited with blue light (488 nm) and chlorophyll *a* autofluorescence was measured as light emission > 600 nm (long pass filter). TruCount fluorescent beads (BD Trucount™ Tubes, BD Biosciences) were added to each sample as an internal counting standard. Further details on the flow cytometric method are detailed in Franklin et al. (2004). The growth rate (cell division; µ), was calculated as the slope of the regression line from a plot of $\log_{10}$ (cell density) versus time

(h). Growth rates for treatment flasks ($24 \times \mu \times 3.32$, doublings day^{-1}) were expressed as a percentage of the control growth rates.

2.4 Copper analyses

The concentration of dissolved copper in samples was measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). Copper concentrations were calculated from a matrix-matched calibration curve (Milli Q water or filtered seawater, acidified with 0.2% HNO_3) using serial dilution of a mixed metal standard (QCD Analysts, Eaglewood, FL, USA) and a drift standard incorporated into the analysis procedure. The detection limit for copper was typically 0.5 to 1 $\mu g\ L^{-1}$ for individual analytical runs. Measured copper concentrations were used in all calculations of toxicity endpoints.

2.5 Statistics

The inhibitory concentration to reduce the growth rate by 50% (72-h IC50) was calculated using linear interpolation (ToxCalc, Ver 5.0.23C, Tidepool Software, San Francisco, CA, USA). The data were tested for normality and homogenous variance, and Dunnett's multiple comparison test was used to determine which treatments differed significantly from controls (1 tailed, $p \leq 0.05$) and to estimate the no-observable effect concentration (NOEC) and the lowest-observable-effect concentration (LOEC). Where data were pooled to gain a single IC50 value based on multiple tests, the Bonferroni t test or the Wilcoxon Rank Sum Test were used to determine which treatments differed significantly from controls (due to greater replicates of control versus treatment samples).

Control growth rates for single species of algae grown either axenically or with bacteria present were compared using *t*-tests. Differences were significant if $p \leq 0.05$. The sensitivity of individual algal species grown either axenically or with bacteria present were compared using the method of Sprague and Fogels (1976). A parameter $f_{1,2}$ was calculated where $f_{1,2} = \sqrt{\{[\log (\text{upper } 95\% \text{ CL}_1/\text{IC50}_1)]^2 + [\log (\text{upper } 95\% \text{ CL}_2/\text{IC50}_2)]^2\}}$ (where CL = confidence limit) and compared to the ratio of the largest IC50/smallest IC50. If the ratio was larger than $f_{1,2}$, the IC50s were deemed to be significantly different.

2.6 Characterisation of bacteria associated with N. closterium *and* Chlorella *sp. (NT isolate) cultures*

Samples of *N. closterium* and *Chlorella* sp. (NT isolate) cultures were transferred to 50 mL sterile, RNA- and DNA-free polypropylene centrifuge tubes (BD Falcon, Bacto Laboratories, Liverpool, NSW, Australia) under sterile conditions and frozen at -80°C until analysis.

For DNA extraction, the sample tubes were thawed and cellular material collected by centrifugation (12000 *g*, 20 min, 25°C). The pellet of biological material was collected using a flame-sterilised stainless steel laboratory spoon and DNA extracted using the MoBio PowerSoil™ DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA). Extraction of nucleic acids from the cells was aided by the use of mechanical disruption via bead beating (FP120; Q-biogene Inc, USA). DNA was extracted into 50 µL of Tris EDTA buffer and stored at -20°C.

Polymerase chain reaction- denaturing gradient gel electrophoresis (PCR-DGGE) was used to establish the presence of bacterial DNA in the axenic and non-axenic *N. closterium* and

Chlorella sp. algal cultures and to estimate the potential number of species present. PCR is used to amplify a specific type of nucleic acid present in a sample, in this case bacterial 16S rRNA, after which further separation (e.g. DGGE) can be used for DNA fingerprinting of samples or identification of species present. Bacterial specific PCR targeted the 16S rRNA gene with primers 27F (Lane, 1991) and 534R (Muyzer et al., 1993) – primer 27F was modified with the addition of a 42 base pair GC-rich 'clamp' (Muyzer et al., 1993). PCR followed the method described in Wakelin et al. (2008) and was based on Qiagen™ Hot Start PCR reagents. PCR products (2 µL) were electrophoretically separated in 1.5% agarose gels, stained with ethidium bromide (0.5 µg mL^{-1}), and visualised under UV light to check for single-banding, or success of the PCR process. The remaining PCR products were used for DGGE analysis.

DGGE separation of 16S rRNA PCR products was performed in the Ingeny PhorU system using a 30-60% urea-formamide denaturing gradient range in a 7% acrylamide:bis-acrylamide gel. Electrophoresis was conducted at 60°C and 110 V for 14 h. Gels were stained with SYBR gold (Molecular Probes, Invitrogen, Mount Waverley, VIC, Australia), visualised on a dark-reader (Clare Chemical Research, Dolores, CO, USA) and photographed with an Olympus SLR digital camera. Each individual band on gels observed following DGGE was indicative of a bacterial phylotype. More intense DGGE bands generally indicate a greater bacterial DNA density and thus a greater number of bacteria.

DNA sequencing was used to phylogenetically characterise bacterial species present in the algal cultures. Using the extracted DNA, bacterial 16S rRNA genes were specifically amplified as before, but using primers F968 and R1401 (Duineveld et al., 1998). PCR

chemistry and conditions were as described in Wakelin et al. (2007), and agarose-gel electrophoresis as described before. PCR products were overnight ligated at 4°C into the pGEMT vector and heat-shock transformed into *E. coli* JM109 competent cells (Promega). Following blue-white screening on X-gal/IPTG plates containing ampicillin, bacterial colonies were picked onto a library plate and sent to the Australian Genome Research Facility (Adelaide) for capillary sequencing from the M13 region. Eight random colonies from each of the four samples were sequenced. Following removal of flanking vector regions, the 16S rRNA sequences were compared against those in the GenBank database using the Blastn search tool. Phylogenetic affiliations were based on the consensus of data within the distance tree view of the Blastn search results. Where possible, sequence information present from characterised type-strains of bacteria was used to support the phylogenetic affiliations.

3. Results

3.1 Growth-rate inhibition bioassays

Initial pH for *N. closterium* bioassays was between 8.10 and 8.30 (± 0.03 pH units) for individual tests, with increases of up to 0.5 pH unit (in controls where growth rates were higher). The pH in *P. subcapitata* bioassays was initially 7.4 ± 0.1, with pH of individual treatments increasing by ≤ 0.2 units over the course of bioassays. Initial pH of *Chlorella* sp. (PNG isolate) bioassays was 8.05 ± 0.10, with increases of ≤ 0.2 units over the course of 72 h. The pH for the *Chlorella* sp. (NT isolate tests) was adjusted by hand to within 0.05 of pH 5.7 or pH 6.5 daily, but often adjustment was not required where growth was minimal. However, pH values in controls were up to 0.2 units higher at the end of the test due to growth in the final few hours of the test.

Control growth rates for all species were within typical ranges: 1.2 to 1.9 doublings day^{-1} for the three freshwater algae, and 2.1 to 2.5 doublings day^{-1} for the marine alga *N. closterium*. Variability in the control growth rates was ≤ 7% for all species except *P. subcapitata*, where variability in control growth rates was < 20%, but still within test acceptability limits. Control growth rates in individual toxicity tests were significantly higher in non-axenic cultures of *N. closterium* and *P. subcapitata* compared to the axenic cultures (*t*-tests, $p < 0.05$). Control growth rates in the axenic *Chlorella* sp. (NT isolate) bioassays were not significantly different from the non-axenic bioassays (Table 2). The control growth rate of the non-axenic *Chlorella* sp. (PNG isolate) was significantly higher than the axenic isolate in one individual test, but lower in the second test.

As the concentration of copper in solution increased, the growth rate (as a % of controls) decreased for all species (Figure 1). The no-observable effect concentration (NOEC), the lowest-observable-effect concentration (LOEC) and 72-h IC50 values for each algal species and for each of the two culture types, axenic and non-axenic, are given in Table 2. These results were calculated based on the pooled data from two definitive toxicity bioassays (*N. closterium*, *Chlorella* sp. (PNG isolate) and *Chlorella* sp. (NT isolate) at pH 5.7) or three definitive toxicity bioassays (*P. subcapitata*, *Chlorella* sp. (NT isolate) at pH 6.5).

For *N. closterium*, *Chlorella* sp. (PNG isolate) and *P. subcapitata*, there was no significant difference in the sensitivity of the alga in the axenic culture when compared to the non-axenic culture in individual tests, or when data from multiple toxicity tests for an individual species was pooled. The 72-h IC50 values were 7 and 8 µg Cu L^{-1} for the axenic and non-axenic *N.*

closterium cultures, respectively, 3 μg Cu L^{-1} for *Chlorella* sp. (PNG) isolate (both cultures) and 0.8 μg Cu L^{-1} for *P. subcapitata* (both cultures) (Table 2).

When the NT isolate of *Chlorella* sp. was exposed to copper at pH 5.7, differences in IC50 values were significant, with the axenic culture being more sensitive to copper than the non-axenic culture (72-h IC50 values of 46 and 208 μg Cu L^{-1}, respectively) (Table 2). Differences were significant in both the individual toxicity tests and when the data from the tests were pooled to obtain a single IC50 value. Concentrations of copper as low as 5 μg L^{-1} caused significant reduction in growth rate of algae in the axenic culture, whereas in the non-axenic culture the LOEC was higher (55 μg Cu L^{-1}), i.e., a decrease in the sensitivity of the alga was found when bacteria were present. The NT *Chlorella* sp. isolate was more sensitive to copper at pH 6.5 than at pH 5.7, however, the bacteria had no protective effect at pH 6.5, with no significant difference in 72-h IC50 values of 28 and 19 μg Cu L^{-1} for the axenic and non-axenic cultures, respectively. Calculated LOEC values were also similar for both axenic and non-axenic cultures at pH 6.5.

3.2 Bacterial analysis

The similar sensitivity of algae to copper in the presence/absence of bacteria was unexpected. As such, the association of bacteria with two algal species - *N. closterium* and *Chlorella* sp. (NT isolate) - was investigated further.

16S rRNA genes were present in both the axenic and non-axenic cultures of *N. closterium* and *Chlorella* sp. (NT isolate). DGGE separation of the PCR products revealed a number of ribosomal genotypes present in each sample (Figure 2). The axenic algal cultures were

dominated by a single bacterial ribotype each (DGGE band; Figure 2), however the non-axenic cultures had 2 major ribotypes present in each sample. A similar bacterial ribotype was present in both the axenic and non-axenic *Chlorella* sp. (NT isolate) cultures (Fig 2).

16S rRNA sequence information was used to explore the phylogenetic association of bacteria within the algal cultures. Selected sequences representative of bacteria found in the algal cultures were loaded onto GenBank under accession numbers EU650654 to EU650661 (Table 3). From the axenic *N. closterium* culture, all of the clones sequenced were highly similar to *Mesorhizobium* spp., a genera of alphaproteobacteria (Table 3). Sequences from the non-axenic culture of *N. closterium* were more varied, but again were all from the alphaproteobacteria phylum. The majority of the sequences (half) were from a poorly described alphaproteobacterial lineage (sequence EU650658; Table 3), however alphaproteobacterial sequences in the Rhodobacteriaceae and Sneathiellaceae families were also present. Similarly, the axenic *Chlorella* sp. (NT isolate) culture had sequences associated with Rhodobacteriaceae (Table 3), however most sequences were from origins which indicated contamination of the DNA (e.g. skin-associated bacteria). In the non-axenic *Chlorella* sp. (NT isolate) culture, sequences from the Methylobacteriaceae and Aurantimonadaceae were present (Table 3).

4. Discussion

The presence of bacteria, or rather, the presence of greater numbers and diversity of bacteria associated with the algal cultures (as more intense DGGE bands indicate a greater bacterial DNA density and a greater number of bacteria), was found to enhance the growth of two out of four of the algal species tested in controls (no copper). This increase in growth rate

suggests that the relationship between algae and bacteria in these cultures is beneficial to the algal species. Grossart et al. (2006) also found that the cell density of *Skeletonema costatum* in exponential growth phase was significantly higher in the presence of bacteria. The ability of bacteria to augment algal growth has been shown to vary with the growth phase of the algae (Grossart et al, 2006). Grossart et al. (2006) found that cell densities of *Thalassiosira rotula* remained high if exposed to bacteria in the exponential phase of growth, but if exposed in stationary phase, algal cell densities decreased rapidly. Algal growth rates in this study were measured only in the exponential phase, in keeping with standard toxicity testing protocols which use exponentially dividing cells. The relationship between bacteria and algae in laboratory bioassays has also been shown to vary with phosphate concentrations and light intensity, with algae generally out-competing bacteria in high phosphate and high light conditions (Gurung, 1999), as in our bioassays. The response of the alga will also depend on the species of bacteria present, and the media in which the alga are grown (e.g. nutrients and vitamins) (Grossart and Simon, 2007). Bacteria specifically isolated from the surface of marine diatoms were found to have a greater positive effect on algal growth than when marine bacteria isolated from ocean water were added to algal cultures (Grossart, 1999), suggesting that the spatial relationship between bacteria and algae may be important. Rier and Stevenson (2002) suggested that bacteria tended to be more effective competitors for resources due to (i) faster growth rates, (ii) a greater surface area to volume ratio and (iii) faster uptake rates of phosphorus. Competition effects to the detriment of the algal species were not observed in the current study, with algal species generally either gaining no benefit from the bacteria, or positive effects in the form of increased growth rates in both controls and copper-treatments, possibly due to nutrient remobilisation of essential elements for algal growth through bacteria-mediated degradation processes (Grossart, 1999).

The impact of algal growth, alone or in combination with copper, on the bacterial population was not assessed in this study, as the primary aim was to determine the differences in sensitivity of algae to copper in the presence/absence of natural bacteria. However, bacteria may also benefit from association with algae. In oligotrophic open ocean conditions the algal-bacterial relationship is strengthened because non-algal derived dissolved organic matter is very low in concentration and bacteria largely rely on algal-derived carbon as an energy source (Morán et al., 2002, Gurung et al., 1999). Dissolved organic matter in laboratory algal bioassays has been found to be lower when bacteria are present, indicating rapid bacterial solubilisation and decomposition of algal-born organic matter (Grossart et al., 2006). Increased extracellular polymeric substance (EPS) production, either as a natural defence against colonisation (Steinberg et al., 1997) or as a result of nutrient or trace metal stress, can increase the sedimentation or sinking rate of algae through greater aggregation of cells, which is of primary importance in driving carbon circulation in oceanic systems (Azam and Malfatti, 2007). EPS is also important for the cycling of trace metals in aquatic systems, as metals bound to bacterial and algal agglomerates, and to colloidal material/EPS, will be removed from surface waters as the large particles sink (Morel and Price, 2003; Koukal et al., 2007). Bacterial colonisation has been shown to be higher on stressed algal cells than healthy algal cells (Grossart, 1999), which could be related to the release of organic material from the cell upon cell lysis as part of the natural senescence process or under conditions of induced stress, such as exposure to metal contaminants.

The sensitivity of the axenic algal species to copper ranged over three orders of magnitude from 0.8 µg Cu L^{-1} for *P. subcapitata* to 46 µg Cu L^{-1} for *Chlorella* sp. (NT isolate) at pH 5.7. The *Chlorella* sp. (NT isolate) was less sensitive to copper at pH 6.5 (28 µg Cu L^{-1}) than reported previously (1.5 µg Cu L^{-1}, Franklin et al., 2000), but similarly sensitive at pH 5.7

(46 µg Cu L^{-1} this test; 35 µg Cu L^{-1} Franklin et al., 2000). In contrast, previous tests with *P. subcapitata* reported 72-h IC50 values of 6.6 to 17 µg Cu L^{-1} at cell densities of 10^2–10^5 cells mL^{-1} (Franklin et al., 2000, 2002), compared to this study in which it was much more sensitive to copper (72-h IC50 of 0.8 µg Cu L^{-1} at an initial cell density of 10^3 cells mL^{-1}).

The presence of bacteria (or the greater concentration of certain bacteria in culture) was found to decrease the sensitivity of only one algal species to copper, the NT *Chlorella* sp. isolate. However, this protective effect was only observed at pH 5.7. The presence of additional bacteria did not have any protective effect against copper for *N. closterium*, the PNG *Chlorella* sp. isolate or for *P. subcapitata*. This was unexpected, as it was hypothesised that the presence of bacteria would increase the surface area, thereby providing a greater number of binding sites for copper and subsequently a decrease in copper uptake and toxicity. It is likely that the two latter axenic strains, not characterised by DGGE, also contained bacteria. This potentially explains why the sensitivities of the axenic and non-axenic algae to copper were similar.

DNA of bacterial origin was detected in both the axenic and non-axenic *N. closterium* and *Chlorella* sp. (NT isolate) cultures. Such DNA can, however, be present regardless of whether the viable or active bacteria exist in culture. The autoclaving process used to maintain algal cultures from week to week, while destroying live bacteria, liberates DNA into the culture media. Furthermore, bacterial DNA present in batch seawater or MilliQ® water used as the culture medium base can also be potentially detected by DNA-based measures. For both algae, however, the axenic *vs.* non-axenic cultures (having been made from the same culture medium), differed in bacterial community structure as shown by both DGGE fingerprinting and sequence information. The phylogenetic identity of dominant bacterial

species present in the axenic cultures, *Mesorhizobium* sp. for *N. closterium* and a *Ruegeria* sp. for the *Chlorella* sp. (NT isolate), is also significant. In particular, the *Ruegeria* sequence closely matched others which originated from studies assessing bacterial diversity associated with laboratory cultures of marine algae (e.g. GenBank accession DQ486504). Bacterial sequences from the non-axenic *Chlorella* sp. (NT isolate) culture also showed high levels of similarity to submissions of bacteria intimately (symbiotically and even endophytically) associated with marine organisms. In particular, this was true for sequence EU650658 which could not be reliably assigned to a known family of alphaproteobacteria. Sequences with high similarity to EU650658 originated from tropical sponge tissue (EF092174), coral (DQ416480), and oligochaetes (EU287331). Similar sequences have been found within bulk marine sediment (EU287331, EU287307, EU491873) and may have been present in symbiosis with higher organisms in this habitat. Finally, the *Chlorella* sp. (NT isolate)-associated *Aurantimonas* sequence was most similar to a 16S rRNA sequence originating from the phycosphere of a *Chlorella vulgaris* isolate (AM286549). Together, these results provide overwhelming evidence for the presence of specific algal- bacterial communities to be present in both the axenic and non-axenic cultures.

The inability to visibly detect or culture bacteria from the axenic cultures may be due to either the very close phycosphere association of the bacterium with the algal cell wall, or the bacteria existing endophytically within the algal cell. Where bacteria are closely associated with the algal cell, it may be impossible to remove the bacteria from the alga using physical techniques. Moreover, the algal species may benefit from the presence of bacteria, as shown by the increased growth rate in the current tests when more bacteria were present. Other techniques previously used to render algal cultures "bacteria free" have included the use of antibiotics. Antibiotics were not used here, as previous work has found that antibiotics were

toxic to algae, leading to changes in cell morphology and reduced growth (J. Stauber, unpublished results). Recently, the toxicity of a suite of antibacterial agents to *P. kirchneriella* was tested, individually and in mixtures, and toxicity of these antibiotics to the alga was found (Yang et al., 2008) at μg L^{-1} concentrations.

The interactions in mixed species toxicity testing are complex and, as in any particular community, some species may exhibit greater, decreased or no change in sensitivity to metals (Franklin et al., 2004). In multispecies toxicity tests with freshwater algae *Microcystis aeruginosa*, *P. subcapitata* and *Trachelomonas* sp., the toxicity of copper to *Trachelomonas* sp. was greater when other species were present, with the 72-h IC50 decreasing from 9.8 μg Cu L^{-1} in individual assays to 2.8 μg Cu L^{-1} in multispecies assays (Franklin et al., 2004). The toxicity to *M. aeruginosa* and *P. subcapitata* was not affected. In contrast, in a multispecies test with the marine algae *Micromonas pusilla*, *Phaeodactylum tricornutum* and *Heterocapsa niei*, toxicity to *P. tricornutum* was reduced while that for *H. niei* was not (Franklin et al., 2004). As cells were added in quantities to give equivalent surfaces areas, these differences could not be simply related to a decrease in copper binding to cells (Franklin et al., 2004). Similarly, in tests combining *M. aeruginosa* and either *Chlorella pyrenoidosa* or *Scenedesmus obliquus*, the addition of other algae in surface-area equivalent and cell number equivalent tests provided a protective effect against short-term (24-h) toxicity of copper to *M. aeruginosa* (based on esterase activity) (Yu et al., 2007). Intracellular concentrations of reactive oxygen species were also higher for *M. aeruginosa* in single species assays compared to multispecies assays. This suggests significant algal-algal interactions, beyond surface area effects (Yu et al., 2007).

Previous work on *P. subcapitata* has shown that the exudates it produces (7-d old culture) decreased the toxicity of cadmium, copper, lead and zinc to the alga based on the inhibition of photosynthesis after 1 h of exposure to high concentrations of metals. The protective effect of the exudates was metal- and concentration-specific: at very high metal concentrations the exudates no longer ameliorated toxicity due to saturation of exudate binding sites (Koukal et al., 2007). Exudate production of mixed bacterial/algal biofilm communities has been shown to increase upon metal exposure (García-Meza et al., 2005). These exudates may be of bacterial or algal origin, but they can be a survival mechanism for these organisms under stress. Boivin et al. (2007) did a comprehensive study that investigated the genetic and physiological structure of algal and bacterial communities across a natural contaminant gradient in flood-plain sediments. Although they found that the structure and physiology of the bacterial communities correlated with the algal community structure, there was no pattern to changes in the bacterial and algal communities based upon metal contamination. Due to the complexity of mixed species toxicity testing, the majority of research has focussed on PICT responses of phytoplankton, bacteria or biofilm communities (Blanck and Dahl, 1996; Boivin et al., 2006; Admiraal et al., 1999; Massieux et al., 2004); or functional changes brought about by metals for entire communities (Lehmann et al., 1999; Boivin et al., 2005) and not on the specific individual responses of algae or bacteria within a community to a metal.

As the presence of bacteria had little effect on copper sensitivity of these algae, this work shows that algal cultures do not necessarily need to be axenic to be useful in toxicity testing. This research suggests that algal toxicity tests that use non-axenic algal cultures are acceptable for metals assessment, where contamination is low, as it is likely to be difficult to obtain a culture that is truly bacteria-free. The findings indicate that for the algal species

examined, laboratory tests are an acceptable mimic for the effects that might be observed in the field.

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Table 1. Final concentration of reagents (mg L^{-1}) used in the media for freshwater toxicity tests[a]

Chemical Reagent Added	Test Media		
	Chlorella sp. (NT isolate) Magela Creek Softwater (2-4 mg $CaCO_3$ L^{-1} Hardness)	*Chlorella* sp. (PNG isolate) Softwater (80-90 mg $CaCO_3$ L^{-1} Hardness)	*Pseudokirchneriella subcapitata* USEPA Media (No EDTA)
$NaNO_3$	15	15	25.5
K_2HPO_4	0.15	0.15	1.04
$MgSO_4.7H_2O$	6.13	123	14.7
$MgCl_2.6H_2O$			12.2
$CaSO_4.2H_2O$		60	
$CaCl_2.2H_2O$	1.65		4.41
$NaHCO_3$	3.62	96	15.0
KCl	0.71	8	
$FeCl_3.6H_2O$	0.44		0.160
$Al_2(SO_4)_3.18H_2O$	0.87		
$MnSO_4.H_2O$	0.0297		
$MnCl_2.4H_2O$			0.416
$CuSO_4.5H_2O$	0.00275		
$CuCl_2.2H_2O$			0.000012
$ZnSO_4.7H_2O$	0.00308		
$ZnCl_2$			0.00328
$UO_2SO_4.3H_2O$	0.00017		
H_3BO_3			0.185
$CoCl_2.6H_2O$			0.00144
$Na_2MoO_4.2H_2O$			0.00728

[a] Filtered seawater (0.45 µm) collected from Oak Park, Cronulla (NSW, Australia) was used for the test medium for the marine alga *Nitzschia closterium*. For all bioassays using either filtered seawater, Magela Creek softwater or the 80-90 hardness softwater, 15 mg NO_3^- L^{-1} and 0.15 mg PO_4^{3-} L^{-1} were added to each test flask just prior to inoculation, while for the USEPA media for testing *P. subcapitata,* no further nutrient additions were required.

Table 2. Comparison of 72-h IC50 values (µg Cu L^{-1}) for four algal species for both axenic and non-axenic cultures.

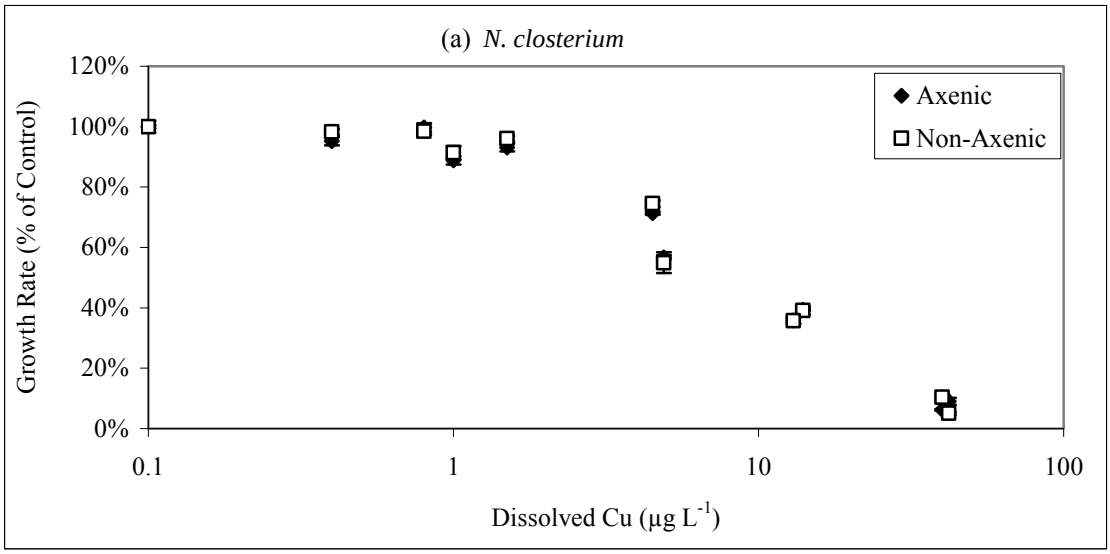
Alga	Culture type	Control Growth Rate (doublings day^{-1}) [c]	NOEC	LOEC	72-h IC50
			µg Cu L^{-1}		
Nitzschia closterium [a]	Axenic	2.12 ± 0.05	0.8	1	7 (6-8)
	Non-axenic	2.37 ± 0.05	1	1.5	8 (4-10)
Chlorella sp. (PNG isolate) [a]	Axenic	1.52 ± 0.09	2.3	2.8	3.0 (2.9-3.0)
	Non-axenic	1.38 ± 0.05	2.3	3	3.1 (3.0-3.2)
Pseudokirchneriella subcapitata [b]	Axenic	1.28 ± 0.04	0.3	0.6	0.8 (0.8-0.9)
	Non-axenic	1.59 ± 0.14	[d]	[d]	0.8 (0.5-1.1)
Chlorella sp. (NT isolate) (pH 5.7) [a]	Axenic	1.75 ± 0.07	4	5	46 (36-57)
	Non-axenic	1.68 ± 0.10	26	55	208 (114-289) *[e]
Chlorella sp. (NT isolate) (pH 6.5) [b]	Axenic	1.62 ± 0.04	< 1	1	28 (27-28)
	Non-axenic	1.65 ± 0.08	< 1	1	19 (0-43)

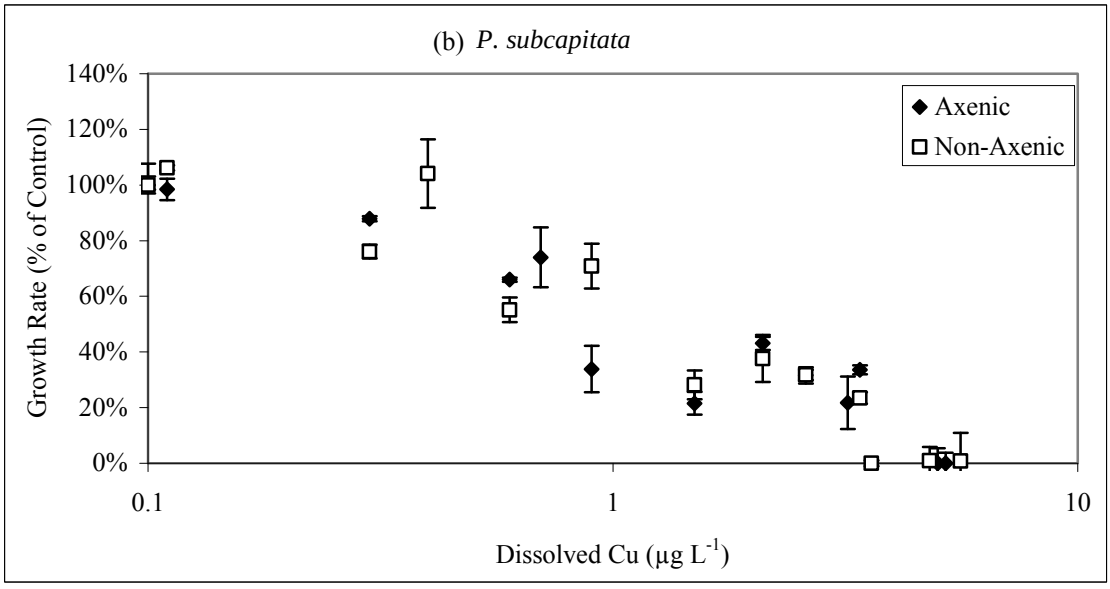
[a] Results obtained by pooling the data from two definitive toxicity tests.
[b] Results obtained by pooling the data from three definitive toxicity tests.
[c] Control growth rate is the mean (± standard error) growth rate calculated from all control replicates from 2 or 3 toxicity tests. Tests for significant differences in control growth rates were only done on individual results for any particular test week, and not on the pooled data presented here.
[d] Calculated NOEC and LOEC values higher than the IC50, therefore not reported.
[e] Asterisk indicates significant difference in IC50 values for the axenic and non-axenic cultures.

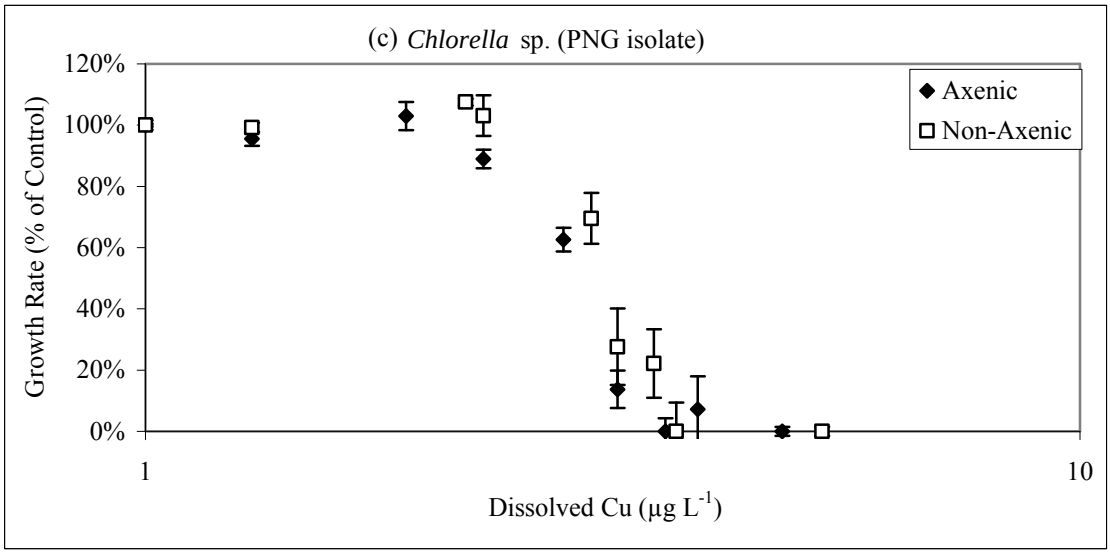
Table 3. Origin and putative phylogenetic association of algae-associated bacterial 16S rRNA sequences submitted to GenBank

Accession number	Origin	Phylogenetic association [a]
EU650654	*Nitzschia closterium* axenic culture	Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae; *Mesorhizobium*
EU650655	*Nitzschia closterium* non-axenic culture	Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; *Ruegeria*
EU650656	*Nitzschia closterium* non-axenic culture	Alphaproteobacteria; Sneathiellales; Sneathiellaceae; *Sneathiella*
EU650657	*Nitzschia closterium* non-axenic culture	Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae; *Thalassospira*
EU650658	*Nitzschia closterium* non-axenic culture	Alphaproteobacteria
EU650659	*Chlorella* sp. (N.T. isolate) axenic culture	Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; *Ruegeria*
EU650660	*Chlorella* sp. (N.T. isolate) non-axenic culture	Alphaproteobacteria; Rhizobiales; Methylobacteriaceae; *Methylobacterium*
EU650661	*Chlorella* sp. (N.T. isolate) non-axenic culture	Alphaproteobacteria; Rhizobiales; Aurantimonadaceae; *Aurantimonas*

[a] Based on the consensus of phylogenetic affiliation generated using the "distance tree view" of the Blastn search results.


(a) N. closterium
120%
100%
80%
60%
40%
20%
0%
Growth Rate (% of Control)
Axenic
Non-Axenic
0.1
1
10
100
Dissolved Cu (µg L-1)


(b) P. subcapitata
140%
120%
100%
80%
60%
40%
20%
0%
Growth Rate (% of Control)
Axenic
Non-Axenic
0.1
1
10
Dissolved Cu (µg L-1)


(c) Chlorella sp. (PNG isolate)
120%
100%
80%
60%
40%
20%
0%
Growth Rate (% of Control)
Axenic
Non-Axenic
1
10
Dissolved Cu (µg L-1)

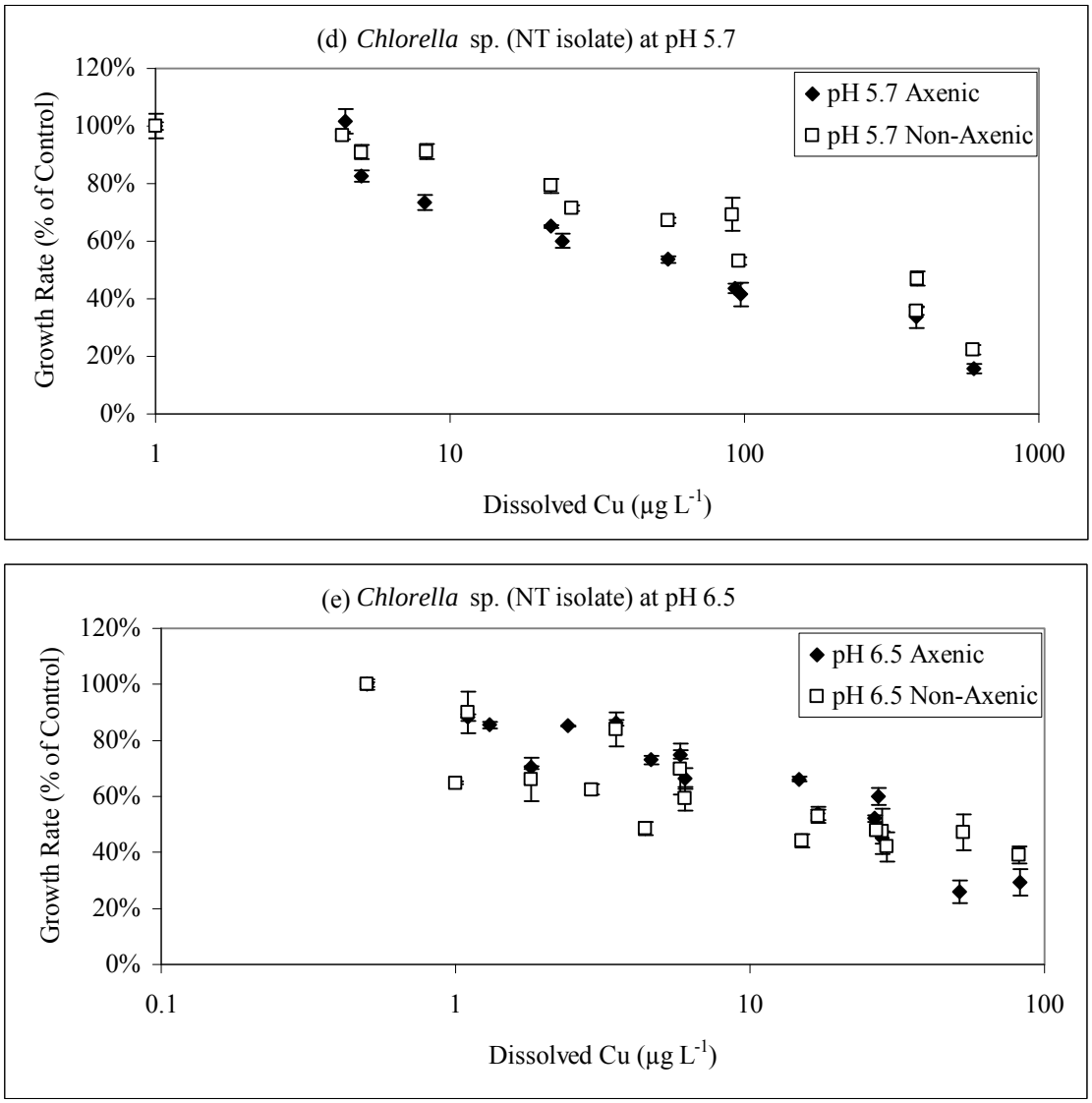


Figure 1. Concentration-response curves for four algal species, (a) *N. closterium*, (b) *P. subcapitata*, (c) *Chlorella* sp. (PNG isolate) and (d, e) *Chlorella* sp. (NT isolate), exposed to copper for 72-h (◆ axenic culture; □ non-axenic culture). Data for *Chlorella* sp. (NT isolate) are presented at two pH values, pH 5.7 (d) and pH 6.5 (e). Results for each alga have been pooled from ≥ two bioassays. Each data point is the mean of ≥ three replicates with the bars representing the standard error. Initial measured copper concentrations were used. Note that the x axis is on a log scale, the scale of which varies for each concentration-response curve.

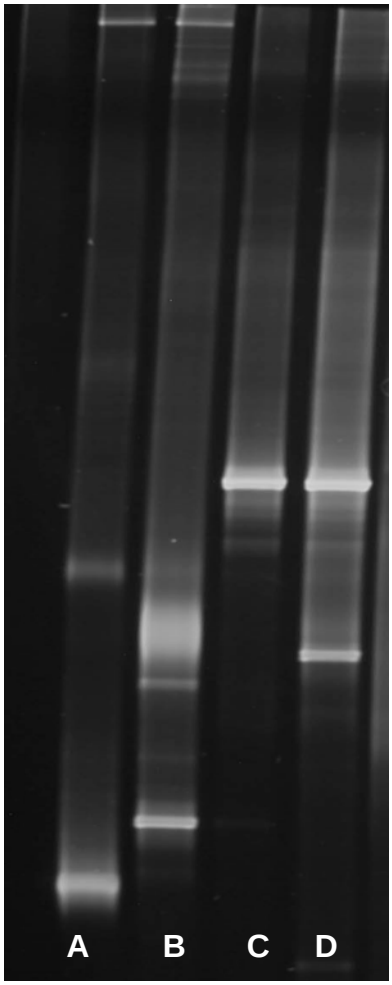


Figure 2. Bacteria in algal cultures using denaturing gradient gel electrophoresis (DGGE). Cultures were operationally defined as axenic or non-axenic (See Methods). Lane A. Axenic *Nitzschia closterium*. Lane B. Non-axenic *Nitzschia closterium*. Lane C. Axenic *Chlorella* sp. (NT isolate). Lane D. Non-axenic *Chlorella* sp. (NT isolate). Each band represents an operationally distinct taxonomic unit, i.e., species of bacteria. Note that band intensity was quantified using image analysis and that a higher band intensity can indicate greater bacterial DNA density and thus the presence of more bacteria.