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Keywords
effect, field, collected, biofilms, toxicity, copper, marine, microalga, Tetraselmis, laboratory, bioassays

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(Tetraselmis sp.) in laboratory bioassays

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Key words:
biofilm, marine, phytoplankton, bacteria, PCR-DGGE, Cu, toxicity
Abstract

Standard algal growth rate inhibition bioassays can lack environmental realism and may over- or under-estimate metal bioavailability in natural systems. In aquatic environments, algal species interact with other biota, including other algae, bacteria and biofilms. In this work, the feasibility of incorporating marine biofilms into 72-h algal growth inhibition toxicity tests was explored. The effects of copper on *Tetraselmis* sp. were tested in the absence and presence of characterised field-collected biofilms. We hypothesised that the addition of biofilm would prevent copper toxicity to the alga primarily through interactions of the metal with other cells and biofilm exudates. The sensitivity of *Tetraselmis* sp. to copper (based on 72-h IC50 values; the copper concentration to inhibit population growth by 50%) in the presence of a blended biofilm inoculum varied two-fold and was independent of the amount of biofilm added. However, increases in IC10 and IC20 values indicated some amelioration of copper toxicity. When intact biofilms were added to the bioassays, amelioration of toxicity was more consistent, probably due to increased binding of copper to cell surfaces or exudates. Difficulties in characterising biofilms and distinguishing that material from the test alga need to be overcome before biofilms can be routinely incorporated into laboratory bioassays.
Introduction

Biofilms (or periphyton in freshwater systems) are an integral part of aquatic systems. In combination with planktonic algae, they form the basis of the food chain and are of particular importance for grazers (Barranguet et al. 2003, 2005; Zippel and Neu 2005). A biofilm is a community of microorganisms, and their associated extracellular products, growing on a living or non-living substrate. The microorganisms may be heterotrophic or autotrophic and can include bacteria, algae, fungi or protozoa (Palmer and White 1997). Biofilms and periphyton represent useful biomonitors of pollution because they are sessile, have short generation times, are species-rich and may accumulate contaminants over time (Rodgers et al. 1979; Biggs and Kilroy 2000; Burns and Ryder 2001). They are often the first community to respond to and recover from stress (Fuchs et al. 1997; Burns and Ryder 2001) and may reflect both sediment and water quality (Holding et al. 2003; Meylan et al. 2003; Stal and Défarge 2005). Metals can accumulate in biofilms, adsorbing or binding to cells and the extracellular polymeric substances (EPS) in the biofilm matrix, i.e. they may be a potential source of nutrition or toxicity to higher-order organisms (Mages et al. 2004; García-Meza et al. 2005).

The growth of cells in a biofilm matrix can be a mechanism of survival for individual species in contaminated aquatic environments (García-Meza et al. 2005) or a defence against naturally occurring microbial agents (Burmølle et al. 2006). Factors that may provide protection for biofilms against metals include:

(i) higher cell densities in biofilms, compared to planktonic populations, could protect individual cells from high toxicant exposure (Morel and Palenik 1989; Wilkinson and Buffle 2004);
(ii) the community matrix can be a store for nutrients which can protect against metal toxicity (Serra et al. 2010);
(iii) toxicants may be diffusion-limited due to the physical structure of the biofilm, protecting cells deeper in the biofilm structure (Hu et al. 2007); and,
(iv) metals may adsorb to inorganic material within the biofilm or bind to sites associated with the extracellular polymeric substances (EPS; i.e. exudates), decreasing metal availability to cells (Morel and Palenik 1989; Wilkinson and Buffle 2004).

Most work on the interaction of metals and aquatic biofilms has focussed on the impact of metals on the whole biofilm (e.g. in terms of biomass and chlorophyll a concentrations
Barranguet et al. 2002; Gold et al. 2003). These types of studies may incorporate changes in community function (e.g., bacterial and/or algal respiration, photosynthesis, and ability to use carbon substrates), changes in structure (taxonomic shifts), or combinations of the two. They may include pollution-induced community tolerance (PICT), in which shifts in community structure towards more tolerant species often account for increased community tolerance (e.g., Admiraal et al. 1999; Soldo and Behra 2000; Massieux et al. 2004).

However, research on the toxicity of metals to individual species, either in a biofilm matrix, in the presence of biofilm exudates or in whole biofilms, is limited. One key finding has shown that production of exudates may help to ameliorate metal toxicity because metals will bind to the exudates in solution, decreasing the amount of metal binding to, and being taken up by, cells (Koukal et al. 2007). Other research has shown that natural biofilms with a mixture of algal species are more tolerant to copper when compared to planktonic algae or biofilms made up of only one algal species (Barranguet et al. 2000; Ivorra et al. 2002). Another direction for the study of metal-biofilm interactions has focused on the role of metal speciation in the water column on the accumulation of metal in biofilms/periphyton (Meylan et al. 2003; Bradac et al. 2010).

While it is known that in natural environments cell-cell and cell-exudate interactions may help alleviate metal stress to algal cells, in general the effect of metals on algal growth is determined using laboratory toxicity tests with single species of planktonic algae (Stauber and Davies 2000). Benthic algae (Adams and Stauber 2004), mixed planktonic algal species (Franklin et al. 2004; Yu et al. 2007) and mono-specific algal and cyanobacterial biofilms (Barranguet et al. 2000; Ivorra et al. 2002) have also been used. However, the logistics of studying the impact of a toxicant on a single species within a natural mixed population, particularly within, or in the presence of, a natural biofilm, is very difficult. Collectively, this research has shown that current toxicity test protocols are likely to overestimate toxicity and that the role of biofilms needs further study.

We investigated the impact of copper on a laboratory alga, *Tetraselmis* sp., in the absence and presence of field-collected marine biofilm using 72-h growth inhibition bioassays. *Tetraselmis* sp. was selected due to its moderate sensitivity to copper (Levy et al. 2008) and because, using flow cytometry, *Tetraselmis* sp. cells could be easily distinguished from biofilm cells over the 72-h exposure period (± copper). Protection against toxicity is
hypothesised to occur due to copper binding to the additional algal and bacterial cells provided by the biofilm and the EPS provided by the biofilm. Copper binding to these additional sites instead of the surface of *Tetraselmis* sp. cells will reduce the total dissolved copper and the amount of copper uptake into *Tetraselmis* sp., both of which will decrease the likelihood of toxic effects.

**Methods**

**General**

All glassware and plasticware were cleaned in a laboratory dishwasher (GW 3050, Gallay Medical and Scientific, Auburn, NSW, Australia) with a phosphate-free detergent (Clean A Powder Detergent, Gallay Medical and Scientific), then acid-washed in HNO$_3$ (30% v/v; Merck, Kilsyth, VIC, Australia) and rinsed three times with Milli-Q water (> 18 MΩ cm$^{-1}$, Millipore, North Ryde, NSW Australia). All glassware used in bioassays had been pre-treated with silanising solution to help prevent metal adsorption to the glassware. Prior to use, bioassay glassware was pre-soaked in 10% HNO$_3$ overnight and then washed thoroughly five times with demineralised water and five times with Milli-Q 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 plasticware, LabServ, BioLab Australia, Clayton, Australia). Care was taken at all stages of biofilm collection, harvesting and DNA analysis to use sterile procedures (autoclaved glassware, 70% ethanol sterilised equipment, a UV-laminar flow cabinet). All chemicals were Analytical Reagent grade or better, and solutions were prepared with high purity Milli-Q water.

**Sampling site**

Biofilms were collected at Beaky Bay, Bass Point, New South Wales, Australia (34°35.6 S, 150°53.9 E). The site is on the north side of the Bass Point headland, adjacent to a marine protection area known as Bass Point Coastal Marine Reserve (Bushrangers Bay). The estimated depth of the bay at the point of collection was 14 m.

**Biofilm collection**

Field sampling dates for biofilm material used in the toxicity tests were July (winter) and October (spring) 2007. Customised devices known as “periphytometers” (supplied by Curtin University, Australia) were used to collect the biofilms. They consisted of Perspex
chambers with grooves to fit ten glass microscope slides (76.2 × 25.4 × 1.0 mm plain unfrosted pathology grade slides), secured using fishing line. Multiple periphytometers were attached to a polypropylene rack and the rack suspended from a buoy, 50 m from shore, at a depth of 2 m. After 12 days, the periphytometers were collected, placed in a clean container filled with site seawater and transported back to the laboratory on ice.

Two 2-L Nalgene containers were filled with seawater and returned to the laboratory on ice. The seawater was immediately filter-sterilised (0.2 µm). This water was used to prepare the biofilm homogenate and for blanks in subsequent analyses.

Harvesting the biofilm

Biofilm material was harvested into a homogenate on the day of collection. The term homogenate is used, but in reality the biofilm is a heterogeneous entity and processing it in this way is unlikely to create a truly homogenous inoculum. The material on the slides was scraped into a sterile container using a Teflon-coated stainless steel blade. One mL of filter-sterilised seawater was used to rinse the slides. In winter, material from 59 slides was combined (0.237 m² harvested; surface area based on number of slides and a surface area of 0.004 m² per slide). In spring, material from 60 slides was harvested (0.241 m²). In addition, several slides were retained intact and placed in sterile centrifuge tubes containing filter-sterilised seawater. Some slides were stored overnight (4°C, dark) for use in whole-slide toxicity tests. Other slides were sent on ice to CSIRO Land and Water in Urrbrae where they were frozen in the dark at -80°C for later DNA analysis.

The pooled biofilm material was blended using a laboratory blender (19000 rpm, 500 W multi-speed X10/25 fitted with a 6-mm microshaft, Ystral, Ballrechten-Dottingen, Germany) then sonicated (3 × 30 s) in an ultrasonic bath (UniSonics, Manly Vale, NSW, Australia). Sub-samples of homogenate for initial cell counts were analysed immediately. Triplicate subsamples for chlorophyll a analyses were stored overnight (4°C, dark) and analysed the following day.

Characterisation of the biofilm

Characterisation of biofilm material from the winter and spring collections included particle and fluorescent cell counts, chlorophyll a content and analysis of bacterial DNA including denaturing gel gradient electrophoresis (DGGE) and community fingerprinting.
Particle and fluorescent cell counts

Flow cytometry (4-colour BD-FACS Calibur™, Becton Dickinson Biosciences, San Jose, CA, USA) was used to determine the number of fluorescent cells and total particles (bacterial and algal cells) in the biofilms. General instrument details are outlined in Levy et al. (2007). The method was modified by setting the counting threshold to > 35 arbitrary units of side scatter of light (SSC) so that bacterial cells were included in the counts. Discrimination of *Tetraselmis* sp. from biofilm material was best obtained using a plot of side scatter (SSC) against chlorophyll *a* autofluorescence (FL3) (Figure 1). The cell counts for fluorescent biofilm material were obtained by selecting any cells with an FL3 signal > 2 arbitrary fluorescence units (i.e. an operationally defined parameter). Total biofilm cell counts were obtained by selecting all particles (i.e., including particles with FL3 < 2) (Figure 1) and subtracting background particle counts for seawater (averaging 60 × 10⁴ cell mL⁻¹).

Chlorophyll *a* determination

Chlorophyll *a* concentrations in biofilms were determined spectrophotometrically after extraction in 90% acetone as outlined for phytoplankton in APHA/AWWA/WEF (2006), and adapted for biofilms (Biggs and Kilroy 2000). The absorbance at 750 nm was subtracted from that at 665 nm to correct for turbidity. Absorbance was measured a second time, following an HCl acidification step, to allow calculation of pheophytin, a major degradation product of chlorophyll *a*. The concentrations of chlorophyll *a* (mg m⁻²) and pheophytin (mg m⁻²) were calculated as per Biggs and Kilroy (2000).

Bacterial DNA extraction, PCR amplification of 16S-rRNA gene fragments and community analyses

DNA was extracted using a PowerSoil™ DNA extraction kit (MoBio Laboratories) as described previously (Levy et al. 2009) using a glass spreader to scrape the biofilm from the slide, following ultrasonication of defrosted samples. Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) (Muyzer et al. 1993) was used to visualise changes in the bacterial community structure as per Wakelin et al. (2008). 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. Each band on the gel represents a distinct operational taxonomic unit (i.e. a
phylogtype of an individual bacterial species). The relative intensity of the band is used to
assess abundance. DGGE-band intensity data was down-weighted using a square-root
transformation, then the similarity of winter and spring communities was compared using
analysis of Bray-Curtis similarities (ANOSIM) (Clarke 1993). Margalef’s species diversity
index was also calculated. These calculations were all conducted in the Primer6 software
package (PrimerE Ltd., U.K.).

DNA sequence libraries were created for each season’s samples. Given the relatively low
number of bacterial DGGE bands, indicating low diversity, only small libraries were
constructed; 23 sequences from the spring samples and 18 from the winter samples.
Sequencing was conducted on the same 16S rRNA gene region as for DGGE
fingerprinting; however, primers were used without the GC-clamp. PCR products were
clones into the pGEM-T vector (Promega) and capillary sequencing conducted through the
Australian Genome Research Facility (Brisbane). The two sequence libraries were
compared using the Ribosomal Database Project (RDP release 10; Michigan State
University) library compare tool. Taxonomy was assigned to sequences and changes in
bacterial taxa across the two sampling times were determined.

**Algal culture**

The marine microalga *Tetraselmis* sp. was cultured as previously reported (Levy et al.
2008). Preliminary experiments showed that this alga could easily be distinguished from
marine biofilm material using flow cytometry due to its higher chlorophyll *a* fluorescence
(FL3 parameter) and larger forward scatter (FSC) compared to the fluorescent bacterial and
algal species present in biofilms collected from Bass Point (Figure 1).

**Growth rate inhibition bioassays**

Growth rate inhibition bioassays were used to assess the chronic toxicity of copper to
*Tetraselmis* sp. and were prepared as described previously (Levy et al. 2008). Each
bioassay consisted of a copper-free treatment (control) and a minimum of five different
copper concentrations, with three replicates per treatment. Flasks were inoculated with
*Tetraselmis* sp. cells to give initial cell densities of 1.5, 3 or 4.5 × 10^4 cells mL^-1. The effect
that the addition of biofilm had on the toxicity of copper to *Tetraselmis* sp. was approached
using: (1) addition of prepared homogenate (preparation described above); and (2) addition
of whole biofilm-colonised slides. In each test, the concentrations of copper to inhibit
growth rate by 10, 20 and 50% (IC10, IC20 and IC50) were used to compare the toxic
response of *Tetraselmis* sp., with and without biofilm.

Three separate tests with homogenate were conducted with the winter biofilm (start dates
of Jul 28, Aug 04 and Aug 19 in Table 1). Two separate tests with homogenate were
conducted with the spring biofilm (start dates of Oct 8 and Oct 16 in Table 1). Flasks were
inoculated with initial densities of 0, 1.5, 3.0, 6.0 or $15 \times 10^4$ fluorescent biofilm cells mL$^{-1}$. In a number of tests, biofilms were treated to try and inactivate cells prior to addition to
the flasks. Heat-treated biofilm cells were prepared by placing a tube of biofilm inoculum
in boiling water for 30 min. Alternatively, biofilms were frozen then thawed ten times
(Harris and Angal 1989).

In two tests, field-collected biofilms were added directly to flasks while still attached to the
glass slides that had been colonised in the field. The slides were cut in half using a
diamond cutter in a laminar-flow cabinet before addition to the flasks. Clean half-slides
were also added to control (no copper) flasks and to copper-spiked treatment flasks as an
additional control (no biofilm).

After inoculation, the standard assay protocol was followed (Levy *et al.* 2008), with sub-
samples for dissolved copper taken initially, and daily thereafter. The test flasks were
incubated for 72 h in 12:12 h light/dark conditions at 140 µmol photons m$^{-2}$ s$^{-1}$ at 21°C. For
biofilm control samples, a lower light intensity of 70 µmol photons m$^{-2}$ s$^{-1}$ was also used to
assess the effect of light intensity on the response of the biofilms in the test media. Cell
densities for *Tetraselmis* sp., total biofilm and fluorescent biofilm were measured daily
using flow cytometry. Three seawater blanks were also incubated for the 72-h test period
and counted daily to permit adjustment for background particle counts. The growth rate
and the 72-h IC10, IC20 and IC50 concentrations were calculated as previously described
(Levy *et al.* 2008).

*Copper analyses*

Copper was analysed by inductively coupled plasma-atomic emission spectroscopy (ICP-
AES). Copper concentrations were calculated from a matrix-matched calibration curve
(clean 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 $\leq 2 \, \mu g \, L^{-1}$. Initial copper concentrations were used in all toxicity calculations.

**Statistical analyses**

Comparisons of biofilm cell counts and pigment content were done using Student’s $t$-tests or one-way analyses of variance (ANOVA), respectively ($\alpha < 0.05$), in the statistical package SPSS (SPSS, Version 14.0 for Windows), following testing for equal variance using Levene’s test. As previously described, the PCR-DGGE data was analysed using Primer 6. The Shapiro-Wilks test for normality and the Bartlett test for equal variance were used to initially assess the toxicity data (ToxCalc, Version 5.0.23 C). Linear interpolation was then used to calculate IC10, IC20 and IC50 values, and their 95% confidence intervals. IC50 values were compared using the method of Sprague and Fogels (1976) to test for differences in toxic effects. Non-linear regression was used to fit a 4-parameter sigmoidal curve to each data set using SigmaPlot 8.0, with $R^2$ values giving the goodness of fit.

**Results**

**Comparison of winter and spring biofilms: Cell density and chlorophyll a**

Spring biofilms colonising slides were observably thicker and had higher cell counts and chlorophyll $a$ concentrations than biofilms collected in winter (Figure 2). In winter, the average ($\pm$ standard error) initial cell density was $3.9 \times 10^9$ fluorescent cells $m^{-2}$ and $140 \times 10^9$ total cells $m^{-2}$. In spring, cell numbers were significantly higher ($t$ tests, $P <0.001$) with values of $36 \times 10^9$ fluorescent cells $m^{-2}$ and $1440 \times 10^9$ total biofilm cells $m^{-2}$, approximately 10-fold higher than the winter biofilm. Chlorophyll $a$ concentrations were higher in spring compared to winter ($49 \pm 13$ and $1.8 \pm 0.2$ mg $m^{-2}$ chlorophyll $a$, respectively; one way ANOVA $F_{2,6} = 37.4$, $P <0.001$). There was also a slightly higher pheophytin content in spring biofilms ($9 \pm 3$ and $4.8 \pm 0.4$ mg $m^{-2}$ pheophytin in spring and winter, respectively; $F_{2,6} = 6.5$, $P = 0.031$) (Figure 2c).

Preliminary work, including analysis of carbohydrate, protein and chlorophyll $a$ content and total counts, had shown that there was little change in biofilms over a storage period of one month. In the winter test, the concentrations of chlorophyll $a$ and pheophytin in the homogenate were not significantly different after 10 d storage ($4^\circ C$, dark; Figure 2c).
However, the volume of inoculum required to give initial biofilm fluorescent cell densities of $1.5 \times 10^4$ fluorescent cells mL$^{-1}$ did increase slightly over this time. This suggests that the algal component of the biofilm was changing over time, so subsequent tests with the spring biofilm were performed within 10 d of collection. Ideally, toxicity tests with biofilms should be completed as soon as possible after collection.

*Comparison of winter and spring biofilms: Community structure*

Results of both PCR-DGGE fingerprinting and 16S rRNA gene shotgun sequencing revealed significant differences in bacterial community composition between the spring and winter samples (Figure 3). The winter biofilm community had fewer species (Margalef’s index $d = 0.3$) than the spring samples ($d = 0.94$). Twenty-three different DGGE bands were detected overall, with a number of phylotypes present in both winter and spring. The number of phylotypes per sample ranged from 2 to 8 (average 4) in winter, and 9 to 14 (average 12) in spring.

Analysis of the 16S rRNA sequence libraries provided insight into the taxonomy of the biofilm communities. In winter, the community was completely dominated by gammaproteobacteria (Figure 3b). Most of these sequences were classified as *Francisella* spp. (Thiotrichales: Francisellaceae), which are often endosymbionts or parasites of other organisms. However, the most highly related sequences were most homologous to those found during a survey of marine bacteria in the Salton Sea, a saline lake in California (e.g. accession EU592368.1) (Dillon et al. 2009). In spring, the biofilm community was more diverse (Figure 3c). Although gammaproteobacteria were present, they were classified in the family Vibrionaceae. The majority of isolates were of Verrucomicrobia origin; all were assigned to the Verrucomicrobiales order, but further classification was hampered by both the partial 16S rRNA gene fragments and the relative lack of information on Verrucomicrobia sequence taxonomy (it is a recently described phylum).

*Growth-rate inhibition tests with biofilm as a homogenate*

For *Tetraselmis* sp. alone (i.e. no added biofilm) at initial cell densities of $1.5 \times 10^4$ cells mL$^{-1}$, the control growth rates ranged from $1.10 \pm 0.08$ to $1.42 \pm 0.06$ doublings day$^{-1}$. Control growth rates over 72 h were similar at all initial cell densities from $1.5$ to $4.5 \times 10^4$ cells mL$^{-1}$ (Table 1, rows 4-6). Addition of biofilm material either improved or had no effect on the growth rate of *Tetraselmis* sp. under control conditions (no Cu; Table 1,
column 5). For *Tetraselmis* sp., at initial cell densities of $1.5 \times 10^4$ cells mL$^{-1}$ in the absence of biofilms, the 72-h IC50 values varied by a factor of 2, from 66 to 136 µg Cu L$^{-1}$ (Table 1, Column 8). The IC10 values (Table 1, column 6) ranged from 7.9 to 49 µg Cu L$^{-1}$, while the IC20 values ranged from 16 to 62 µg Cu L$^{-1}$ (Table 1, column 7).

An increase in the initial cell density of *Tetraselmis* sp. from 1.5 to $3 \times 10^4$ cells mL$^{-1}$ had no significant effect on its sensitivity to copper, with 72-h IC50 values (with 95% confidence limits) of 66 (40-86) and 97 (67-135), respectively. A further increase in cell density to $4.5 \times 10^4$ *Tetraselmis* sp. cells mL$^{-1}$ significantly decreased copper toxicity, with a 72-h IC50 of 123 (88-176) µg Cu L$^{-1}$ (Table 1). However, a change in sensitivity based on the threshold effects parameters (IC10 and IC20) was not evident, with large, overlapping 95% confidence intervals.

Addition of small amounts of biofilm homogenate ($1.5 \times 10^4$ fluorescent biofilm cells mL$^{-1}$) made little difference to the sensitivity of *Tetraselmis* sp. to copper, as only one of four definitive bioassays had significantly lower copper toxicity than *Tetraselmis* sp. alone (based on statistically significant increases in 72-h IC50 values) (Table 1; Figure 4). Under our test conditions, the 72-h IC50 values for the toxicity of copper to *Tetraselmis* sp. varied from 59 (42-72) µg Cu L$^{-1}$ to 124 (108-136) µg Cu L$^{-1}$. Where the addition of winter biofilm did result in a decrease in the sensitivity of *Tetraselmis* sp., the IC50 increased from 67 (56-77) µg Cu L$^{-1}$ (in the absence of biofilm) to 107 (95-120) µg Cu L$^{-1}$ (in the presence of biofilm).

Larger additions of biofilm ($15 \times 10^4$ fluorescent biofilm cells mL$^{-1}$) did help ameliorate copper toxicity to *Tetraselmis* sp., but only in two of four tests (based on 72-h IC50 values). In the October 9th bioassays, the IC50 increased from 133 (112-152) to 175 (160-184) µg Cu L$^{-1}$, while in the October 16th bioassays the IC50 increased from 98 (71-125) to 158 (146-168) µg Cu L$^{-1}$. IC10 and IC20 values increased with the addition of biofilm in all tests when biofilm was added at a concentration $\geq 6 \times 10^4$ cells mL$^{-1}$ (Table 1).

To determine if inactivated biofilms could also ameliorate toxicity, heat-treated and frozen/thawed biofilms were added to toxicity tests. The treatments did not totally inactivate the biofilms, with growth of cells observed after 72 h. The 72-h IC50 values for the “inactivated” and non-treated biofilm tests were not significantly different (Table 1).
While the aim of this study was not to investigate the effect of copper on the biofilm itself, biofilms did not grow under laboratory bioassay test conditions unless an initial biofilm inoculum of $15 \times 10^4$ fluorescent cells mL$^{-1}$ was used. Where growth of biofilm material did occur, it appeared to be due to one algal species. This species was smaller and contained less chlorophyll $a$ than *Tetraselmis* sp., based on FL3 and SSC flow cytometry measurements. Microscopic observation confirmed that this species was a small centric diatom. It was hypothesised that the higher light conditions used in the toxicity tests (140 $\mu$mol photons m$^{-2}$s) compared to the light intensity at the depth of biofilm colonisation (2 m) may have been phytotoxic. However, use of low light conditions (70 $\mu$mol photons m$^{-2}$s) did not improve the growth of biofilm cells (data not shown).

**Growth-rate inhibition tests with biofilms attached to slides**

The growth of *Tetraselmis* sp. was measured over 72 h in two bioassays where whole attached biofilms were added to test solutions (Table 2). These bioassays used slides from the winter colonisation period. Addition of a washed slide, free of biofilm, as a control, did not affect the growth of *Tetraselmis* sp., either in the presence or absence of 100 $\mu$g Cu L$^{-1}$. Addition of biofilm slides to copper-free solutions increased the *Tetraselmis* sp. control growth rate from 1.34 ± 0.02 to 1.46 ± 0.02 doublings day$^{-1}$ in the first test and from 1.24 ± 0.04 to 1.56 ± 0.03 doublings day$^{-1}$ in the second test.

In the first test, the growth rate of *Tetraselmis* sp. cells exposed to copper (percentage of control growth rates) increased from 47 ± 2% in the absence of biofilms to 89 ± 8% in the presence of biofilms (Table 2). In the second test, the values increased from 29 ± 1% (biofilm-absent) to 80 ± 4% (biofilm-present), i.e. partial amelioration of toxicity was occurring (Table 2). In these tests, some cells detached from the biofilm and began to grow in the test solution. As discussed for the homogenate tests, these were small centric diatom species, although other species were also present in smaller numbers. When *Tetraselmis* sp. was absent, the growth rate and yield of this biofilm alga in solution was much higher (up to $61 \times 10^4$ cells mL$^{-1}$ by Day 3) than when *Tetraselmis* was present (average of $13 \times 10^4$ cells mL$^{-1}$ by Day 3). This suggests competition for nutrients between the species. The addition of copper inhibited the growth rate of the other algal species.

**Discussion**
Seasonal differences in the biofilm

The substantial increase in biofilm cell density, pigment concentrations, bacterial community diversity and bacterial abundance (over a 10-d colonisation period) was expected in the spring samples when the increased light incidence, potential nutrient upwelling and higher water temperatures promote a peak in primary production. Temperature has been shown to influence biofilm productivity, with higher growth rates for bacteria and algae at higher temperatures (Rodgers et al. 1979; Palmer and White 1997). Temperature also influences community structure and the sensitivity of communities to toxicants (Boivin et al. 2005). Despite the differences in the biofilms, the biofilm composition appeared to make little difference to the toxicity tests with Tetraselmis sp. and copper. While there is a potential for different cells to produce different exudates and thus modify toxicity, the primary reason for a change in toxicity is likely to be simply a surface area/adsorption effect.

Toxicity of copper to Tetraselmis sp. in the presence of biofilm

The addition of biofilm to bioassays often increased the control growth rate of Tetraselmis sp. (no Cu, Table 1, column 5). The biofilms may provide nutrients (e.g. iron) to Tetraselmis sp. that are not otherwise present in the minimal nutrient test medium (seawater with nitrate and phosphate). Biofilms can act as a sink for nutrients, trapping them within the EPS matrix, while the close interactions of species within the dense biofilm can encourage the breakdown of algal products by bacteria, remobilising nutrients for growth (Costerton et al. 1995; Grossart 1999; Sutherland 2001). Similarly, Levy et al. (2009) showed that increases in bacteria associated with algae in culture increased control growth rates for some algae, but not others.

Previous assessments of the toxicity of copper to Tetraselmis sp. under similar conditions had given 72-h IC50 values of 47 µg L\(^{-1}\) (Levy et al. 2008) and 146 µg L\(^{-1}\) (Franklin et al. 2001), with initial cell densities of \(\sim 2 \times 10^3\) and \(10^4\) cells mL\(^{-1}\), respectively. The IC50 values for Tetraselmis sp. in this study ranged from 66 to 136 µg Cu L\(^{-1}\) (i.e. in the range of previous studies).

No previous reports in the literature have assessed the toxicity of copper to algae in laboratory tests in the presence of natural biofilms. However, the addition of exudates or EPS derived from algae under environmentally relevant conditions (i.e. low cell densities...
and minimal nutrient test media) has been shown to ameliorate the toxicity of cadmium, zinc, copper and lead to the green alga *Pseudokirchneriella subcapitata* (Koukal *et al.* 2007). This was likely due to decreases in the free ionic metal in solution due to binding to exudates or due to specific interactions between the exudates and algal cells that altered metal uptake (Koukal *et al.* 2007). Barranguet *et al.* (2000) found that field-isolated species grown as mono-specific biofilms (diatoms, cyanobacteria) were less sensitive to toxicants than their planktonic forms, and that natural biofilms were more tolerant than the laboratory-produced mono-specific biofilms (derived from those natural biofilms). The decrease in toxicity of metals to algae in biofilms may partially be due to the higher pH in biofilms that can decrease the bioavailability of metals through formation of insoluble species (Morel and Palenik 1989; Barranguet *et al.* 2000).

It was hypothesised that the small protective effects that the biofilms exerted on copper-toxicity to *Tetraselmis* sp. were due to the increased binding of copper to cell surfaces and cell exudates when biofilms were present (i.e. a surface area effect). The large increase in cell density and in cell exudates for biofilms when compared to plankton is possibly the most important explanation for the potential protective effect of biofilms against metal toxicity (Morel and Palenik 1989). Previous research has shown that increasing the initial algal cell density (10²-10⁵ cells mL⁻¹) in growth rate inhibition bioassays with phytoplankton significantly decreased the toxicity of metals, including copper, to algae (Franklin *et al.* 2002). In our research, a three-fold increase in the initial cell density of *Tetraselmis* sp. significantly decreased the toxicity of copper (Table 1). Attempts to determine if inactivated biofilms could also ameliorate toxicity were unsuccessful as the heat-treatment and freeze-thaw treatment did not totally inactivate the biofilms, with growth of cells observed after 72 h. Some organisms are resistant to rupture using this approach, especially when accumulated intracellular solutes prevent freezing (Harris and Angal 1989). Chemical treatment of the biofilm was not attempted as the addition of glutaraldehyde or formalin to kill the biofilm cells would have resulted in carry-over of the fixative to the test species, *Tetraselmis* sp. Subsequently, it could not be determined if the small protective effect exerted by the biofilm in some tests was due to abiotic effects (i.e. a surface area effect where a greater number of binding sites for copper gives less toxicant per cell and therefore a decrease in sensitivity) or due to biotic effects (i.e. specific cell-cell or cell-exudate interactions). In addition, it was not possible to assess the concentration of
metal in and on *Tetraselmis* sp. cells in the presence of biofilms, because it was impossible to separate the test species from the biofilm, once added.

While the current testing protocol was suitable for following the growth of *Tetraselmis* sp. in the presence of biofilm (± copper), the impact of copper on the biofilm itself could not be assessed. Upon addition of *Tetraselmis* sp., it was difficult to measure the true biofilm cell density, because as *Tetraselmis* sp. became unhealthy due to copper toxicity, the chlorophyll *a* fluorescence signal of the cells decreased (i.e. the autofluorescence signal shifted to the left on plots like Figure 1, < FL3), placing the unhealthy *Tetraselmis* sp. cells in the region used to define the biofilm cells. Further research is required to enable the assessment of toxicity to biofilms or any mixed community using flow cytometric methods, such as the use of fluorescent dyes to monitor crucial metabolic pathways (Adler *et al*. 2007) or the development of non-toxic dyes that can tag specific species and follow their growth over time (Franklin *et al*. 2004).

**Conclusion**

The addition of biofilms to growth rate inhibition bioassays provided some amelioration of copper toxicity to the laboratory-cultured species (*Tetraselmis* sp.), effectively increasing both IC10 and IC20 values. However, increases in the 72-h IC50 values were not always significant, suggesting that at higher copper concentrations the protective effects of the biofilm were overcome, possibly due to saturation of binding sites within the biofilm. When whole biofilms attached to slides rather than as a homogenate were added to tests, there was significant amelioration of toxicity at copper exposures of 100 µg Cu L⁻¹. Further work is required to determine if the amelioration of toxicity by the addition of biofilm is due to abiotic factors influencing copper speciation, e.g. an increase in sorption to inactive binding sites due to a greater surface area, or due to specific biotic factors. Importantly, the results indicate that laboratory-based algal bioassays have the potential to over-estimate copper toxicity when compared to field conditions where biofilms may be present.

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profiling, Mira Durr for help in interpreting DGGE profiles, and Graeme Batley and Stuart Simpson for edits of earlier versions of this manuscript. The helpful comments of the reviewers and the editor are also greatly appreciated. JLL was funded by an Australian Postgraduate Award and a CSIRO postgraduate scholarship.

References


Table 1. The effect of copper on *Tetraselmis* sp. in the absence and presence of field-collected biofilms.

<table>
<thead>
<tr>
<th>Season</th>
<th>Date</th>
<th>Initial cell density a</th>
<th><em>Tetraselmis</em> sp. control growth rate b (doublings day -1 ± 1SD)</th>
<th>72-h effect concentrations c (µg Cu L -1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Tetraselmis</em> sp. Biofilm cells control growth rate</td>
<td>IC10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(× 10⁴ cells mL -1)</td>
<td>(× 10⁴ cells mL -1) (µg Cu L -1) (µg Cu L -1) (µg Cu L -1)</td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>July 28</td>
<td>1.5</td>
<td>-</td>
<td>1.21 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>-</td>
<td>1.29 ± 0.05</td>
</tr>
<tr>
<td>August</td>
<td>4</td>
<td>1.5</td>
<td>-</td>
<td>1.10 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>-</td>
<td>1.01 ± 0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.5</td>
<td>-</td>
<td>0.97 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>1.30 ± 0.11 *</td>
<td>23 (14 - 27) C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>3</td>
<td>1.49 ± 0.05 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>6</td>
<td>1.43 ± 0.02 *</td>
</tr>
<tr>
<td>August</td>
<td>19</td>
<td>1.5</td>
<td>-</td>
<td>1.30 ± 0.02 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>1.58 ± 0.12 *</td>
<td>49 (7.8 - 68) B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>6</td>
<td>1.50 ± 0.12 *</td>
</tr>
<tr>
<td>Spring</td>
<td>October 9</td>
<td>1.5</td>
<td>-</td>
<td>1.42 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>1.56 ± 0.01 *</td>
<td>40 (3.6 - 57) A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>15</td>
<td>1.58 ± 0.01 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>15 (heat treated)</td>
<td>1.53 ± 0.02 *</td>
</tr>
<tr>
<td>October</td>
<td>16</td>
<td>1.5</td>
<td>-</td>
<td>1.25 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>15</td>
<td>1.51 ± 0.07 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>15 (heat treated)</td>
<td>1.51 ± 0.03 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>15 (freeze-thaw treated)</td>
<td>1.52 ± 0.02 *</td>
</tr>
</tbody>
</table>

a A dash indicates the absence of biofilm.

b An asterisk (*) indicates a significantly higher control growth rate for *Tetraselmis* sp., compared with the no biofilm test from the same date (α < 0.05).

c Data in each toxicity test were found to be normally distributed (Shapiro-Wilk's test), and to have equal variance (Bartlett's test) using ToxCalc software, with the exception of the Aug 19, T + BF(6) test where equal variance could not be verified (one treatment had only 1 replicate). 72-h IC10, IC20 and IC50 are the concentrations of copper to inhibit the growth rate of *Tetraselmis* sp. by 10, 20 or 50%, respectively with 95% confidence limits given in brackets. These were calculated using linear interpolation in ToxCalc. Superscript capital letters (A-D) indicate significant difference in 72-h IC50 values (for an individual test) using pair-wise Sprague and Fogel's (1976) tests.
Table 2. Growth of *Tetraselmis* sp. and a centric diatom (released from the biofilm) in control and 100 µg Cu L$^{-1}$ treatments, where the biofilm has been added attached to a slide.

<table>
<thead>
<tr>
<th>Slide test 1 (August 9)</th>
<th>Tetraselmis sp. mean (±1SD) growth rate (doublings day$^{-1}$)</th>
<th>ANOVA Group</th>
<th>Biofilm sp. (centric diatom) mean (±1SD) growth rate (doublings day$^{-1}$)</th>
<th>ANOVA Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Tet, no slide)</td>
<td>1.42 ± 0.04</td>
<td>100 ± 3</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Control (Tet + clean slide)</td>
<td>1.46 ± 0.02</td>
<td>102 ± 1</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Control (+ biofilm slide)</td>
<td>1.34 ± 0.02</td>
<td>95 ± 1</td>
<td>a,b</td>
<td>2.37 ± 0.13</td>
</tr>
<tr>
<td>100 µg L$^{-1}$ Cu (Tet, no slide)</td>
<td>0.70 ± 0.03</td>
<td>49 ± 2</td>
<td>c</td>
<td>0.80 ± 0.38</td>
</tr>
<tr>
<td>100 µg L$^{-1}$ Cu (Tet + clean slide)</td>
<td>0.67 ± 0.02</td>
<td>47 ± 2</td>
<td>c</td>
<td>0.79 ± 0.64</td>
</tr>
<tr>
<td>100 µg L$^{-1}$ Cu (+ biofilm slide)</td>
<td>1.26 ± 0.01</td>
<td>89 ± 8</td>
<td>b</td>
<td>0.75 ± 0.46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Slide test 2 (August 16)</th>
<th>Tetraselmis sp. mean (±1SD) growth rate (doublings day$^{-1}$)</th>
<th>ANOVA Group</th>
<th>Biofilm sp. (centric diatom) mean (±1SD) growth rate (doublings day$^{-1}$)</th>
<th>ANOVA Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Tet, no slide)</td>
<td>1.23 ± 0.13</td>
<td>100 ± 10</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Control (Tet + clean slide)</td>
<td>1.24 ± 0.04</td>
<td>101 ± 3</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Control (+ biofilm slide)</td>
<td>1.56 ± 0.03</td>
<td>127 ± 3</td>
<td>e</td>
<td>2.41 ± 0.05</td>
</tr>
<tr>
<td>100 µg L$^{-1}$ Cu (no slide)</td>
<td>0.40 ± 0.07</td>
<td>32 ± 6</td>
<td>f</td>
<td>1.69 ± 0.14</td>
</tr>
<tr>
<td>100 µg L$^{-1}$ Cu (+ clean slide)</td>
<td>0.36 ± 0.01</td>
<td>29 ± 1</td>
<td>f</td>
<td>0.69 ± 0.39</td>
</tr>
<tr>
<td>100 µg L$^{-1}$ Cu (+ biofilm slide)</td>
<td>0.99 ± 0.05</td>
<td>80 ± 4</td>
<td>g</td>
<td>0.53 ± 0.43</td>
</tr>
</tbody>
</table>

Note: Based on cell counts of the homogenate of $2.7 \times 10^7$ fluorescent cells mL$^{-1}$ and $100 \times 10^7$ total cells mL$^{-1}$ (see Table 1), number of cells per slide were $1.6 \times 10^7$ fluorescent cells per slide and $59 \times 10^7$ total cells per slide (59 slides in 35 mL seawater). i.e., in these tests the initial inoculum was ½ a slide in 50mL of test solution, so 1.6 $\times 10^7$ fluorescent cells mL$^{-1}$ (similar to inoculum used in some homogenate tests) and $59 \times 10^7$ total cells mL$^{-1}$. 

708 709 710 711
Figure 1. Flow cytometry plots defining biofilm material (R2) from *Tetraselmis* sp. (R1). R2 indicates (a) the total cell count for biofilm cells or (b) the fluorescent biofilm cell count with FL3 > 2. Note that *Tetraselmis* sp. cells are easily defined from the biofilm cells and that there is a region of growth of biofilm cells at FL3 10-100 and SSC 10-100 that is likely to be a centric diatom based on microscopic observations.

Figure 2. Winter and spring biofilm characterisation. Mean (± standard error) (a) fluorescent cell count, (b) total cell count and (c) concentration of chlorophyll *a* and pheophytin pigments. Winter samples are closed data points, spring samples are open data points.

Figure 3. Bacterial community composition for marine biofilm samples. (a) DGGE profile showing change in community composition (banding patterns) between winter (July 2007) and spring (October 2007). (b) and (c) Comparison of 16S rRNA sequence analysis at the two sampling times. In winter, the community was dominated by Gammaproteobacteria. In spring, the community was more complex and dominated by Verrucomicrobia.

Figure 4. Concentration-response curves for the growth of *Tetraselmis* sp., at an initial cell density of $1.5 \times 10^4$ cells mL$^{-1}$, in the presence of (a) winter biofilm material (0, 1.5, 3 and $6 \times 10^4$ fluorescent cells mL$^{-1}$) or (b) spring biofilm material (0, 1.5 and $15 \times 10^4$ fluorescent cells mL$^{-1}$). Winter tests were run in parallel (Aug 4-7 2007; 8 days post-collection). Spring tests were run in parallel (Oct 9-12 2007; 1 day post-collection). Points with error bars are average ± 1 standard deviation (n=3). Initial copper concentrations have been used. Tet only = *Tetraselmis* sp. (initial cell density of $1.5 \times 10^4$ cells mL$^{-1}$), no biofilm. Tet + BF(n)= *Tetraselmis* sp. (initial cell density of $1.5 \times 10^4$ cells mL$^{-1}$) plus a biofilm addition with an initial cell density of $n \times 10^4$ fluorescent cells mL$^{-1}$. Non-linear regression was used to fit a 4-parameter sigmoidal curve to each data set, with $R^2$ giving the goodness of fit.
Figure 1
Figure 2.
Figure 3
Figure 4a

**Figure 4a**

**Initial copper concentration (µg L$^{-1}$)**

**Growth (% of control)**

- Tet only $R^2 = 0.976$
- Tet + BF(1.5) $R^2 = 0.976$
- Tet + BF(3) $R^2 = 0.994$
- Tet + BF(6) $R^2 = 0.999$
Figure 4b