1-1-2011

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Abstract

Standard algal growth rate inhibition bioassays can lack environmental realism and may over- or underestimate 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 72h 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 72h IC_{50} values; the copper concentration to inhibit population growth by 50%) in the presence of a blended biofilm inoculum varied 2-fold and was independent of the amount of biofilm added. However, increases in IC_{10} and IC_{20} 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.

Keywords

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

Disciplines

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

Publication Details

Levy, J. L., Stauber, J. L., Wakelin, S. A. & Jolley, D. F. (2011). The effect of field-collected biofilms on the toxicity of copper to a marine microalga (Tetraselmis sp.) in laboratory bioassays. Marine and Freshwater Research, 62 (12), 1362-1372.

The effect of field-collected biofilms on the toxicity of copper to a marine microalga (*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 *Tetraselmi*s 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 plastic-ware, 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 m^2 harvested; surface area based on number of slides and a surface area of 0.004 m^2 per slide). In spring, material from 60 slides was harvested (0.241 m^2). 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.

175

176 Particle and fluorescent cell counts

177 Flow cytometry (4-colour BD-FACSCalibur™, Becton Dickinson Biosciences, San Jose,
178 CA, USA) was used to determine the number of fluorescent cells and total particles
179 (bacterial and algal cells) in the biofilms. General instrument details are outlined in Levy *et*
180 *al.* (2007). The method was modified by setting the counting threshold to > 35 arbitrary
181 units of side scatter of light (SSC) so that bacterial cells were included in the counts.
182 Discrimination of *Tetraselmis* sp. from biofilm material was best obtained using a plot of
183 side scatter (SSC) against chlorophyll *a* autofluorescence (FL3) (Figure 1). The cell counts
184 for fluorescent biofilm material were obtained by selecting any cells with an FL3 signal >
185 2 arbitrary fluorescence units (i.e. an operationally defined parameter). Total biofilm cell
186 counts were obtained by selecting all particles (i.e., including particles with FL3 < 2)
187 (Figure 1) and subtracting background particle counts for seawater (averaging 60×10^4 cell
188 mL^{-1}).

189

190 Chlorophyll *a* determination

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

198

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

201 DNA was extracted using a PowerSoil™ DNA extraction kit (MoBio Laboratories) as
202 described previously (Levy *et al.* 2009) using a glass spreader to scrape the biofilm from
203 the slide, following ultrasonication of defrosted samples. Polymerase Chain Reaction-
204 Denaturing Gradient Gel Electrophoresis (PCR-DGGE) (Muyzer *et al.* 1993) was used to
205 visualise changes in the bacterial community structure as per Wakelin *et al.* (2008). PCR
206 products (2 µL) were electrophoretically separated in 1.5% agarose gels, stained with
207 ethidium bromide (0.5 µg mL^{-1}), and visualised under UV light to check for single-banding
208 or success of the PCR process. The remaining PCR products were used for DGGE

209 analysis. Each band on the gel represents a distinct operational taxonomic unit (i.e. a
210 phylotype of an individual bacterial species). The relative intensity of the band is used to
211 assess abundance. DGGE-band intensity data was down-weighted using a square-root
212 transformation, then the similarity of winter and spring communities was compared using
213 analysis of Bray-Curtis similarities (ANOSIM) (Clarke 1993). Margalef's species diversity
214 index was also calculated. These calculations were all conducted in the Primer6 software
215 package (PrimerE Ltd., U.K.).
216
217 DNA sequence libraries were created for each season's samples. Given the relatively low
218 number of bacterial DGGE bands, indicating low diversity, only small libraries were
219 constructed; 23 sequences from the spring samples and 18 from the winter samples.
220 Sequencing was conducted on the same 16S rRNA gene region as for DGGE
221 fingerprinting; however, primers were used without the GC-clamp. PCR products were
222 clones into the pGEM-T vector (Promega) and capillary sequencing conducted through the
223 Australian Genome Research Facility (Brisbane). The two sequence libraries were
224 compared using the Ribosomal Database Project (RDP release 10; Michigan State
225 University) library compare tool. Taxonomy was assigned to sequences and changes in
226 bacterial taxa across the two sampling times were determined.
227
228 *Algal culture*
229 The marine microalga *Tetraselmis* sp. was cultured as previously reported (Levy *et al.*
230 2008). Preliminary experiments showed that this alga could easily be distinguished from
231 marine biofilm material using flow cytometry due to its higher chlorophyll *a* fluorescence
232 (FL3 parameter) and larger forward scatter (FSC) compared to the fluorescent bacterial and
233 algal species present in biofilms collected from Bass Point (Figure 1).
234
235 *Growth rate inhibition bioassays*
236 Growth rate inhibition bioassays were used to assess the chronic toxicity of copper to
237 *Tetraselmis* sp. and were prepared as described previously (Levy *et al.* 2008). Each
238 bioassay consisted of a copper-free treatment (control) and a minimum of five different
239 copper concentrations, with three replicates per treatment. Flasks were inoculated with
240 *Tetraselmis* sp. cells to give initial cell densities of 1.5, 3 or 4.5×10^4 cells mL^{-1}. The effect
241 that the addition of biofilm had on the toxicity of copper to *Tetraselmis* sp. was approached
242 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 15×10^4 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 ≤ 2 µg 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 (± standard error) initial cell density was 3.9 (± 0.3) × 10^9 fluorescent cells m^{-2} and 140 (± 10) × 10^9 total cells m^{-2}. In spring, cell numbers were significantly higher (*t* tests, $P < 0.001$) with values of 36 (± 1) × 10^9 fluorescent cells m^{-2} and 1440 (± 90) × 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 ± 13 and 1.8 ± 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 ± 3 and 4.8 ± 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°C, dark; Figure 2c).

311 However, the volume of inoculum required to give initial biofilm fluorescent cell densities
312 of 1.5×10^4 fluorescent cells mL^{-1} did increase slightly over this time. This suggests that
313 the algal component of the biofilm was changing over time, so subsequent tests with the
314 spring biofilm were performed within 10 d of collection. Ideally, toxicity tests with
315 biofilms should be completed as soon as possible after collection.
316
317 *Comparison of winter and spring biofilms: Community structure*
318 Results of both PCR-DGGE fingerprinting and 16S rRNA gene shotgun sequencing
319 revealed significant differences in bacterial community composition between the spring
320 and winter samples (Figure 3). The winter biofilm community had fewer species
321 (Margalef's index d = 0.3) than the spring samples (d = 0.94). Twenty-three different
322 DGGE bands were detected overall, with a number of phylotypes present in both winter
323 and spring. The number of phylotypes per sample ranged from 2 to 8 (average 4) in winter,
324 and 9 to 14 (average 12) in spring.
325
326 Analysis of the 16S rRNA sequence libraries provided insight into the taxonomy of the
327 biofilm communities. In winter, the community was completely dominated by
328 gammaproteobacteria (Figure 3b). Most of these sequences were classified as *Francisella*
329 spp. (Thiotrichales: Francisellaceae), which are often endosymbionts or parasites of other
330 organisms. However, the most highly related sequences were most homologous to those
331 found during a survey of marine bacteria in the Salton Sea, a saline lake in California (e.g.
332 accession EU592368.1) (Dillon *et al.* 2009). In spring, the biofilm community was more
333 diverse (Figure 3c). Although gammaproteobacteria were present, they were classified in
334 the family Vibrionaceae. The majority of isolates were of Verrucomicrobia origin; all were
335 assigned to the Verrucomicrobiales order, but further classification was hampered by both
336 the partial 16S rRNA gene fragments and the relative lack of information on
337 Verrucomicrobia sequence taxonomy (it is a recently described phylum).
338
339 *Growth-rate inhibition tests with biofilm as a homogenate*
340 For *Tetraselmis* sp. alone (i.e. no added biofilm) at initial cell densities of 1.5×10^4 cells
341 mL^{-1}, the control growth rates ranged from 1.10 ± 0.08 to 1.42 ± 0.06 doublings day^{-1}.
342 Control growth rates over 72 h were similar at all initial cell densities from 1.5 to 4.5×10^4
343 cells mL^{-1} (Table 1, rows 4-6). Addition of biofilm material either improved or had no
344 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 1.5×10^4 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 3×10^4 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 4.5×10^4 *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 (1.5×10^4 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 (15×10^4 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 15×10^4 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 µmol 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 µmol 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 µg 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 61×10^4 cells mL^{-1} by Day 3) than when *Tetraselmis* was present (average of 13×10^4 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 ~2×10^3 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

447 and minimal nutrient test media) has been shown to ameliorate the toxicity of cadmium,
448 zinc, copper and lead to the green alga *Pseudokirchneriella subcapitata* (Koukal *et al.*
449 2007). This was likely due to decreases in the free ionic metal in solution due to binding to
450 exudates or due to specific interactions between the exudates and algal cells that altered
451 metal uptake (Koukal *et al.* 2007). Barranguet *et al.* (2000) found that field-isolated species
452 grown as mono-specific biofilms (diatoms, cyanobacteria) were less sensitive to toxicants
453 than their planktonic forms, and that natural biofilms were more tolerant than the
454 laboratory-produced mono-specific biofilms (derived from those natural biofilms). The
455 decrease in toxicity of metals to algae in biofilms may partially be due to the higher pH in
456 biofilms that can decrease the bioavailability of metals through formation of insoluble
457 species (Morel and Palenik 1989; Barranguet *et al.* 2000).
458
459 It was hypothesised that the small protective effects that the biofilms exerted on copper-
460 toxicity to *Tetraselmis* sp. were due to the increased binding of copper to cell surfaces and
461 cell exudates when biofilms were present (i.e. a surface area effect). The large increase in
462 cell density and in cell exudates for biofilms when compared to plankton is possibly the
463 most important explanation for the potential protective effect of biofilms against metal
464 toxicity (Morel and Palenik 1989). Previous research has shown that increasing the initial
465 algal cell density (10^2-10^5 cells mL^{-1}) in growth rate inhibition bioassays with
466 phytoplankton significantly decreased the toxicity of metals, including copper, to algae
467 (Franklin *et al.* 2002). In our research, a three-fold increase in the initial cell density of
468 *Tetraselmis* sp. significantly decreased the toxicity of copper (Table 1). Attempts to
469 determine if inactivated biofilms could also ameliorate toxicity were unsuccessful as the
470 heat-treatment and freeze-thaw treatment did not totally inactivate the biofilms, with
471 growth of cells observed after 72 h. Some organisms are resistant to rupture using this
472 approach, especially when accumulated intracellular solutes prevent freezing (Harris and
473 Angal 1989). Chemical treatment of the biofilm was not attempted as the addition of
474 glutaraldehyde or formalin to kill the biofilm cells would have resulted in carry-over of the
475 fixative to the test species, *Tetraselmis* sp. Subsequently, it could not be determined if the
476 small protective effect exerted by the biofilm in some tests was due to abiotic effects (i.e. a
477 surface area effect where a greater number of binding sites for copper gives less toxicant
478 per cell and therefore a decrease in sensitivity) or due to biotic effects (i.e. specific cell-cell
479 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.

Acknowledgments

We thank Monique Binet, Merrin Adams, Jenny Boge and Anthony Platts-Baggs for technical assistance in maintaining the algal cultures used in this project. Thanks also to George Woolmer, Christine Levy, John Levy, Anthony Chariton, David Strom and Brad Angel for their assistance in the field, Adrienne Gregg for the bacterial community

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

518

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520 **References**

521 Adams, M. S., and Stauber, J. L. (2004). Development of a whole-sediment toxicity test
522 using a benthic marine microalga. *Environmental Toxicology and Chemistry* **23**, 1957-
523 1968.
524
525 Adler, N. E., Schmitt-Jansen, M., and Altenburger, R. (2007). Flow cytometry as a tool to
526 study phytotoxic modes of action. *Environmental Toxicology and Chemistry* **26**, 297-306.
527
528 Admiraal, W., Blanck, H., Buckert-De Jong, M., Guasch, H., Ivorra, N.*, et al.* (1999).
529 Short-term toxicity of zinc to microbenthic algae and bacteria in a metal polluted stream.
530 *Water Research* **33**, 1989-1996.
531
532 APHA/AWWA/WEF (2006). 'Standard Methods for the Examination of Water and
533 Wastewater.' (American Public Health Association, American Water Works Association,
534 Water Environment Federation: Washington D.C.)
535
536 Barranguet, C., Charantoni, E., Plans, M., and Admiraal, W. (2000). Short-term response
537 of monospecific and natural algal biofilms to copper exposure. *European Journal of*
538 *Phycology* **35**, 397-406.
539
540 Barranguet, C., Plans, M., van der Grinten, E., Sinke, J. J., and Admiraal, W. (2002).
541 Development of photosynthetic biofilms affected by dissolved and sorbed copper in a
542 eutrophic river. *Environmental Toxicology and Chemistry* **21**, 1955-1965.
543
544 Biggs, B. J. F., and Kilroy, C. (2000). 'Stream Periphyton Monitoring Manual.' (National
545 Institute of Water and Atmospheric Research: Christchurch)
546
547 Boivin, M.-E. Y., Massieux, B., Breure, A. M., van den Ende, F. P., Greve, G. D.*, et al.*
548 (2005). Effects of copper and temperature on aquatic bacterial communities. *Aquatic*
549 *Toxicology* **71**, 345-356.
550
551 Bradac, P., Wagner, B., Kistler, D., Traber, J., Behra, R.*, et al.* (2010). Cadmium
552 speciation and accumulation in periphyton in a small stream with dynamic concentration
553 variations. *Environmental Pollution* **158**, 641-648.
554
555 Burmølle, M., Webb, J. S., Rao, D., Hansen, L. H., Sørensen, S. J.*, et al.* (2006). Enhanced
556 biofilm formation and increased resistance to antimicrobial agents and bacterial invasion
557 are caused by synergistic interactions in multispecies biofilms. *Applied and Environmental*
558 *Microbiology* **72**, 3916-3923.
559
560 Burns, A., and Ryder, D. S. (2001). Potential for biofilms as biological indicators in
561 Australian riverine systems. *Ecological Management and Restoration* **2**, 53-64.

562
563 Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community
564 structure. *Australian Journal of Ecology* **18**, 117-143.
565
566 Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., and Lappinscott, H. M.
567 (1995). Microbial biofilms. *Annual Review of Microbiology* **49**, 711-745.
568
569 Dillon, J. G., McMath, L. M., and Trout, A. L. (2009). Seasonal changes in bacterial
570 diversity in the Salton Sea. *Hydrobiologia* **632**, 49-64.
571
572 Franklin, N. M., Adams, M. S., Stauber, J. L., and Lim, R. P. (2001). Development of an
573 improved rapid enzyme inhibition bioassay with marine and freshwater microalgae using
574 flow cytometry. *Archives of Environmental Contamination and Toxicology* **40**, 469-480.
575
576 Franklin, N. M., Stauber, J. L., Apte, S. C., and Lim, R. P. (2002). Effect of initial cell
577 density on the bioavailability and toxicity of copper in microalgal bioassays.
578 *Environmental Toxicology and Chemistry* **21**, 742-751.
579
580 Franklin, N. M., Stauber, J. L., and Lim, R. P. (2004). Development of multispecies algal
581 bioassays using flow cytometry. *Environmental Toxicology and Chemistry* **23**, 1452-1462.
582
583 Fuchs, S., Haritopoulou, T., Schafer, M., and Wilhelmi, M. (1997). Heavy metals in
584 freshwater ecosystems introduced by urban rainwater runoff - Monitoring of suspended
585 solids, river sediments and biofilms. *Water Science and Technology* **36**, 277-282.
586
587 García-Meza, J. V., Barranguet, C., and Admiraal, W. (2005). Biofilm formation by algae
588 as a mechanism for surviving on mine tailings. *Environmental Toxicology and Chemistry*
589 **24**, 573-581.
590
591 Gold, C., Feurtet-Mazel, A., Coste, M., and Boudou, A. (2003). Impacts of Cd and Zn on
592 the development of periphytic diatom communities in artificial streams located along a
593 river pollution gradient. *Archives of Environmental Contamination and Toxicology* **44**,
594 189-197.
595
596 Grossart, H. P. (1999). Interactions between marine bacteria and axenic diatoms
597 (*Cylindrotheca fusiformis, Nitzschia laevis, and Thalassiosira weissflogii*) incubated under
598 various conditions in the lab. *Aquatic Microbial Ecology* **19**, 1-11.
599
600 Harris, E., and Angal, S. (Eds) (1989) 'Protein Purification Methods - A Practical
601 Approach.' (Oxford University Press: Oxford)
602
603 Holding, K. L., Gill, R. A., and Carter, J. (2003). The relationship between epilithic
604 periphyton (biofilm) bound metals and metals bound to sediments in freshwater systems.
605 *Environmental Geochemistry and Health* **25**, 87-93.
606
607 Hu, Z. Q., Jin, J., Abruna, H. D., Houston, P. L., Hay, A. G., *et al.* (2007). Spatial
608 distributions of copper in microbial biofilms by scanning electrochemical microscopy.
609 *Environmental Science & Technology* **41**, 936-941.
610

611 Ivorra, N., Barranguet, C., Jonker, M., Kraak, M. H. S., and Admiraal, W. (2002). Metal-
612 induced tolerance in the freshwater microbenthic diatom *Gomphonema parvulum*.
613 *Environmental Pollution* **116**, 147-157.
614
615 Koukal, B., Rosse, P., Reinhardt, A., Ferrari, B., Wilkinson, K. J.*, et al.* (2007). Effect of
616 *Pseudokirchneriella subcapitata* (Chlorophyceae) exudates on metal toxicity and colloid
617 aggregation. *Water Research* **41**, 63-70.
618
619 Levy, J. L., Stauber, J. L., and Jolley, D. F. (2007). Sensitivity of marine microalgae to
620 copper: The effect of biotic factors on copper adsorption and toxicity. *Science of the Total*
621 *Environment* **387**, 141-154.
622
623 Levy, J. L., Angel, B. M., Stauber, J. L., Poon, W. L., Simpson, S. L.*, et al.* (2008). Uptake
624 and internalisation of copper by three marine microalgae: Comparison of copper-sensitive
625 and copper-tolerant species. *Aquatic Toxicology* **89**, 82-93.
626
627 Levy, J. L., Stauber, J. L., Wakelin, S. A., and Jolley, D. F. (2009). The effect of bacteria
628 on the sensitivity of microalgae to copper in laboratory bioassays. *Chemosphere* **74**, 1266-
629 1274.
630
631 Mages, M., Ovari, M., von Tumpling, W., and Kropfl, K. (2004). Biofilms as bio-indicator
632 for polluted waters? Total reflection X-ray fluorescence analysis of biofilms of the Tisza
633 river (Hungary). *Analytical and Bioanalytical Chemistry* **378**, 1095-1101.
634
635 Massieux, B., Boivin, M. E. Y., van den Ende, F. P., Langenskiold, J., Marvan, P.*, et al.*
636 (2004). Analysis of structural and physiological profiles to assess the effects of Cu on
637 biofilm microbial communities. *Applied and Environmental Microbiology* **70**, 4512-4521.
638
639 Meylan, S., Behra, R., and Sigg, L. (2003). Accumulation of copper and zinc in periphyton
640 in response to dynamic variations of metal speciation in freshwater. *Environmental Science*
641 *& Technology* **37**, 5204-5212.
642
643 Morel, F. M. M., and Palenik, B. (1989). The aquatic chemistry of trace metals in biofilms.
644 In 'Structure and Function of Biofilms'. (Eds W. G. Characklis and P. A. Wilderer) pp.
645 289-300. (John Wiley and Sons: Chichester)
646
647 Muyzer, G., Dewaal, E. C., and Uitterlinden, A. G. (1993). Profiling of complex microbial
648 populations by denaturing gradient gel electrophoresis analysis of polymerase chain
649 reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*
650 **59**, 695-700.
651
652 Palmer, R. J., and White, D. C. (1997). Developmental biology of biofilms: implications
653 for treatment and control. *Trends in Microbiology* **5**, 435-440.
654
655 Rodgers, J. H., Dickson, K. L., and Cairns, J. (1979). A review and analysis of some
656 methods used to measure functional aspects of periphyton. In 'Methods and Measurements
657 of Periphyton Communities: A Review, ASTM STP 690'. (Ed. R. L. Weitzel) pp. 142-167.
658 (American Society for Testing and Materials: Philadelphia)
659

660 Serra, A., Guasch, H., Admiraal, W., Van der Geest, H. G., and Van Beusekom, S. A. M.
661 (2010). Influence of phosphorus on copper sensitivity of fluvial periphyton: the role of
662 chemical, physiological and community-related factors. *Ecotoxicology* **19**, 770-780.
663
664 Soldo, D., and Behra, R. (2000). Long-term effects of copper on the structure of freshwater
665 periphyton communities and their tolerance to copper, zinc, nickel and silver. *Aquatic*
666 *Toxicology* **47**, 181-189.
667
668 Sprague, J. B., and Fogels, A. (1976). Watch the Y in bioassay. In 'Proceedings of the 3rd
669 Aquatic Toxicity Workshop'. Halifax, Nova Scotia, Canada, 2 - 3 November, 1976. (Eds
670 W. R. Parker, E. Pessah, P. G. Wells and G. F. Westlake) pp. 107-118. (Environmental
671 Protection Service, Environment Canada: Ottawa)
672
673 Stal, L. J., and Défarge, C. (2005). Structure and dynamics of exopolymers in an intertidal
674 diatom biofilm. *Geomicrobiology Journal* **22**, 341-352.
675
676 Stauber, J. L., and Davies, C. M. (2000). Use and limitations of microbial bioassays for
677 assessing copper bioavailability in the aquatic environment. *Environmental Reviews* **8**,
678 255-301.
679
680 Sutherland, I. W. (2001). The biofilm matrix - an immobilized but dynamic microbial
681 environment. *Trends in Microbiology* **9**, 222-227.
682
683 Wakelin, S. A., Macdonald, L. M., Rogers, S. L., Gregg, A. L., Bolger, T. P.*, et al.* (2008).
684 Habitat selective factors influencing the structural composition and functional capacity of
685 microbial communities in agricultural soils. *Soil Biology & Biochemistry* **40**, 803-813.
686
687 Wilkinson, K., and Buffle, J. (2004). Critical evaluation of physicochemical parameters
688 and processes for modelling the biological uptake of trace metals in environmental
689 (aquatic) systems. In 'Physicochemical Kinetics and Transport at Biointerfaces'. (Eds H. P.
690 van Leeuwen and W. Köster) pp. 445-533. (John Wiley and Sons: Chichester)
691
692 Yu, Y., Kong, F. X., Wang, M. L., Qian, L. L., and Shi, X. L. (2007). Determination of
693 short-term copper toxicity in a multispecies microalgal population using flow cytometry.
694 *Ecotoxicology and Environmental Safety* **66**, 49-56.
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696
697

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

Season	Date	Initial cell density [a]		*Tetraselmis* sp.	72-h effect concentrations [c]		
		Tetraselmis sp. ($\times 10^4$ cells mL^{-1})	Biofilm cells ($\times 10^4$ cells mL^{-1})	control growth rate [b] (doublings day^{-1} ± 1SD)	IC10 (µg Cu L^{-1})	IC20 (µg Cu L^{-1})	IC50 (µg Cu L^{-1})
Winter	July 28	1.5	-	1.21 ± 0.06	7.9 (6.1 - 10) ^{A}	16 (12 - 20) ^{A}	67 (56 - 77) ^{A}
		1.5	1.5	1.29 ± 0.05	23 (18 - 29) ^{B}	30 (25 - 36) ^{B}	107 (95 - 120) ^{B}
	August 4	1.5	-	1.10 ± 0.08	11 (1.8 - 17) ^{A}	16 (10 - 22) ^{A}	66 (40 - 86) ^{AB}
		3.0	-	1.01 ± 0.10	24 (2.0 - 42) ^{BCD}	32 (10 - 56) ^{BC}	97 (67 - 135) ^{B}
		4.5	-	0.97 ± 0.08	18 (7.2 - 44) ^{ABCD}	35 (14 - 81) ^{ABC}	123 (88 - 176) ^{C}
		1.5	1.5	1.30 ± 0.11 *	23 (14 - 27) ^{C}	29 (22 - 34) ^{B}	59 (42 - 72) ^{A}
		1.5	3	1.49 ± 0.05 *	15 (12 - 18) ^{AB}	29 (24 - 36) ^{B}	69 (60 - 72) ^{A}
		1.5	6	1.43 ± 0.02 *	40 (33 - 48) ^{D}	52 (46 - 59) ^{C}	97 (86 - 107) ^{BC}
	August 19	1.5	-	1.30 ± 0.02	25 (0.0 - 37) ^{A}	49 (41 - 58) ^{A}	136 (110 - 162) ^{AB}
		1.5	1.5	1.58 ± 0.12 *	49 (7.8 - 68) ^{B}	67 (48 - 84) ^{B}	124 (108 - 136) ^{A}
		1.5	6	1.50 ± 0.12 *	85 (59 - 109) ^{C}	110 (91 - 129) ^{C}	175 (154 - 214) ^{B}
Spring	October 9	1.5	-	1.42 ± 0.06	49 (0.0 - 60) ^{AB}	62 (51 - 71) ^{A}	133 (112 - 152) ^{B}
		1.5	1.5	1.56 ± 0.01 *	40 (3.6 - 57) ^{A}	56 (46 - 62) ^{A}	97 (76 - 114) ^{A}
		1.5	15	1.58 ± 0.01 *	63 (52 - 73) ^{B}	105 (86 - 173) ^{B}	175 (160 - 184) ^{C}
		1.5	15 (heat treated)	1.53 ± 0.02 *	73 (59 - 99) ^{B}	101 (72 - 120) ^{B}	167 (162 - 173) ^{C}
	October 16	1.5	-	1.25 ± 0.07	20 (2.30 - 46) ^{A}	39 (11 - 68) ^{A}	98 (71 - 125) ^{A}
		1.5	15	1.51 ± 0.07 *	76 (59 - 107) ^{B}	102 (82 - 120) ^{C}	158 (146 - 168) ^{B}
		1.5	15 (heat treated)	1.51 ± 0.03 *	62 (58 - 67) ^{B}	82 (73 - 91) ^{B}	149 (142 - 157) ^{B}
		1.5	15 (freeze-thaw treated)	1.52 ± 0.02 *	79 (61 - 103) ^{B}	106 (90 - 140) ^{BC}	160 (150 - 169) ^{B}

[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 ($\alpha < 0.05$).

[c] Data in each toxicity test were found to be normally distributed (Shapiro-Wilks test), and to have equal variance (Bartlett 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.

706 **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
707 has been added attached to a slide.

	Tetraselmis sp. mean (±1SD) growth rate (doublings day^{-1})	(% of control)	ANOVA Group	Biofilm sp. (centric diatom) mean (±1SD) growth rate (doublings day^{-1})	(% of control)	ANOVA Group
Slide test 1 (August 9)						
Control (Tet, no slide)	1.42 ± 0.04	100 ± 3	a			
Control (Tet + clean slide)	1.46 ± 0.02	102 ± 1	a			
Control (+ biofilm slide)				2.37 ± 0.13	100 ± 6	a
Control (Tet + biofilm slide)	1.34 ± 0.02	95 ± 1	a,b	0.80 ± 0.38	34 ± 16	b
100 µg L^{-1} Cu (Tet, no slide)	0.70 ± 0.03	49 ± 2	c			
100 µg L^{-1} Cu (Tet + clean slide)	0.67 ± 0.02	47 ± 2	c			
100 µg L^{-1} Cu (+ biofilm slide)				0.79 ± 0.64	33 ± 27	b
100 µg L^{-1} Cu (Tet + biofilm slide)	1.26 ± 0.01	89 ± 8	b	0.75 ± 0.46	32 ± 19	b
Slide test 2 (August 16)						
Control (Tet, no slide)	1.23 ± 0.13	100 ± 10	d			
Control (Tet + clean slide)	1.24 ± 0.04	101 ± 3	d			
Control (+ biofilm slide)				2.41 ± 0.05	100 ± 2	a
Control (Tet + biofilm slide)	1.56 ± 0.03	127 ± 3	e	1.69 ± 0.14	70 ± 6	a
100 µg L^{-1} Cu (no slide)	0.40 ± 0.07	32 ± 6	f			
100 µg L^{-1} Cu (+ clean slide)	0.36 ± 0.01	29 ± 1	f			
100 µg L^{-1} Cu (+ biofilm slide)				0.69 ± 0.39	29 ± 16	b
100 µg L^{-1} Cu (Tet + biofilm slide)	0.99 ± 0.05	80 ± 4	g	0.53 ± 0.43	22 ± 18	b

708 Note: Based on cell counts of the homogenate of 2.7×10^7 fluorescent cells mL^{-1} and 100×10^7 total cells mL^{-1} (see Table 1), number of cells per slide were 1.6×10^7
709 fluorescent cells per slide and 59×10^7 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
710 $\times 10^5$ fluorescent cells mL^{-1} (similar to inoculum used in some homogenate tests) and 59×10^5 total cells mL^{-1}.
711

712 **Figure 1**. Flow cytometry plots defining biofilm material (R2) from *Tetraselmis* sp. (R1).
713 R2 indicates (a) the total cell count for biofilm cells or (b) the fluorescent biofilm cell
714 count with FL3 > 2. Note that *Tetraselmis* sp. cells are easily defined from the biofilm cells
715 and that there is a region of growth of biofilm cells at FL3 10-100 and SSC 10-100 that is
716 likely to be a centric diatom based on microscopic observations.
717
718 **Figure 2**. Winter and spring biofilm characterisation. Mean (± standard error) (a)
719 fluorescent cell count, (b) total cell count and (c) concentration of chlorophyll *a* and
720 pheophytin pigments. Winter samples are closed data points, spring samples are open data
721 points.
722
723 **Figure 3.** Bacterial community composition for marine biofilm samples. (a) DGGE profile
724 showing change in community composition (banding patterns) between winter (July 2007)
725 and spring (October 2007). (b) and (c) Comparison of 16S rRNA sequence analysis at the
726 two sampling times. In winter, the community was dominated by Gammaproteobacteria.
727 In spring, the community was more complex and dominated by Verrucomicrobia.
728
729 **Figure 4.** Concentration-response curves for the growth of *Tetraselmis* sp., at an initial
730 cell density of 1.5×10^4 cells mL^{-1}, in the presence of (a) winter biofilm material (0, 1.5, 3
731 and 6×10^4 fluorescent cells mL^{-1}) or (b) spring biofilm material (0, 1.5 and 15×10^4
732 fluorescent cells mL^{-1}). Winter tests were run in parallel (Aug 4-7 2007; 8 days post-
733 collection). Spring tests were run in parallel (Oct 9-12 2007; 1 day post-collection). Points
734 with error bars are average ± 1 standard deviation (n=3). Initial copper concentrations have
735 been used. Tet only = *Tetraselmis* sp. (initial cell density of 1.5×10^4 cells mL^{-1}), no
736 biofilm. Tet + BF(n)= *Tetraselmis* sp. (initial cell density of 1.5×10^4 cells mL^{-1}) plus a
737 biofilm addition with an initial cell density of $n \times 10^4$ fluorescent cells mL^{-1}. Non-linear
738 regression was used to fit a 4-parameter sigmoidal curve to each data set, with R^2 giving
739 the goodness of fit.

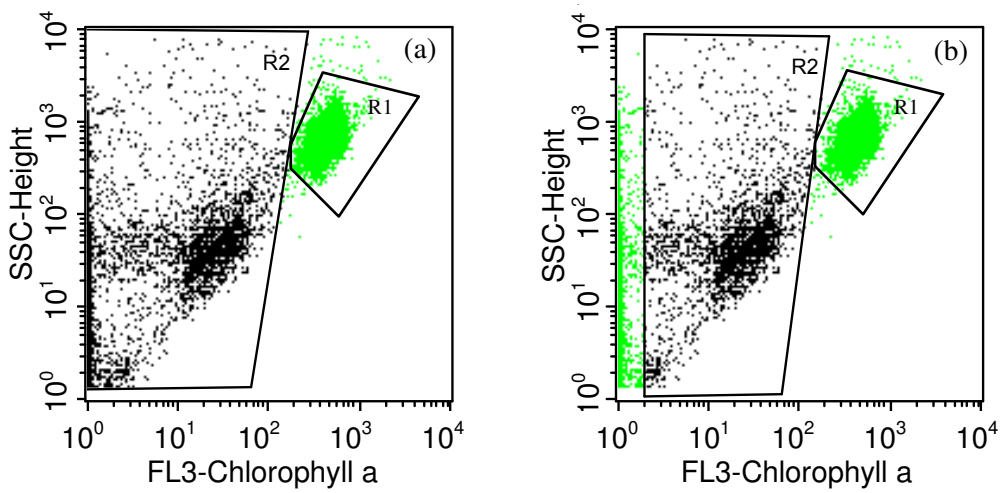
(a)
R2
R1
SSC-Height
FL3-Chlorophyll a
(b)
R2
R1
SSC-Height
FL3-Chlorophyll a

Figure 1

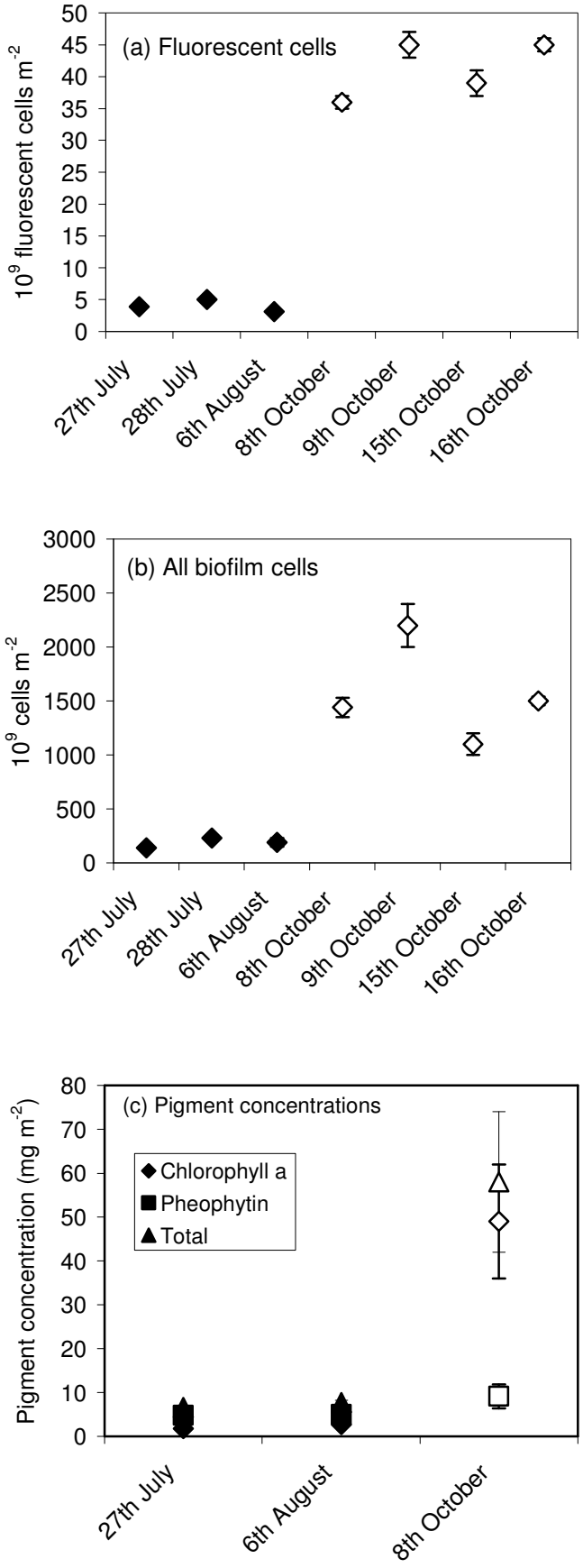
(a) Fluorescent cells
10^9 fluorescent cells m^{-2}
0
5
10
15
20
25
30
35
40
45
50
27th July
28th July
6th August
8th October
9th October
15th October
16th October
(b) All biofilm cells
10^9 cells m^{-2}
0
500
1000
1500
2000
2500
3000
27th July
28th July
6th August
8th October
9th October
15th October
16th October
(c) Pigment concentrations
Pigment concentration (mg m^{-2})
Chlorophyll a
Pheophytin
Total
0
10
20
30
40
50
60
70
80
27th July
6th August
8th October

Figure 2.

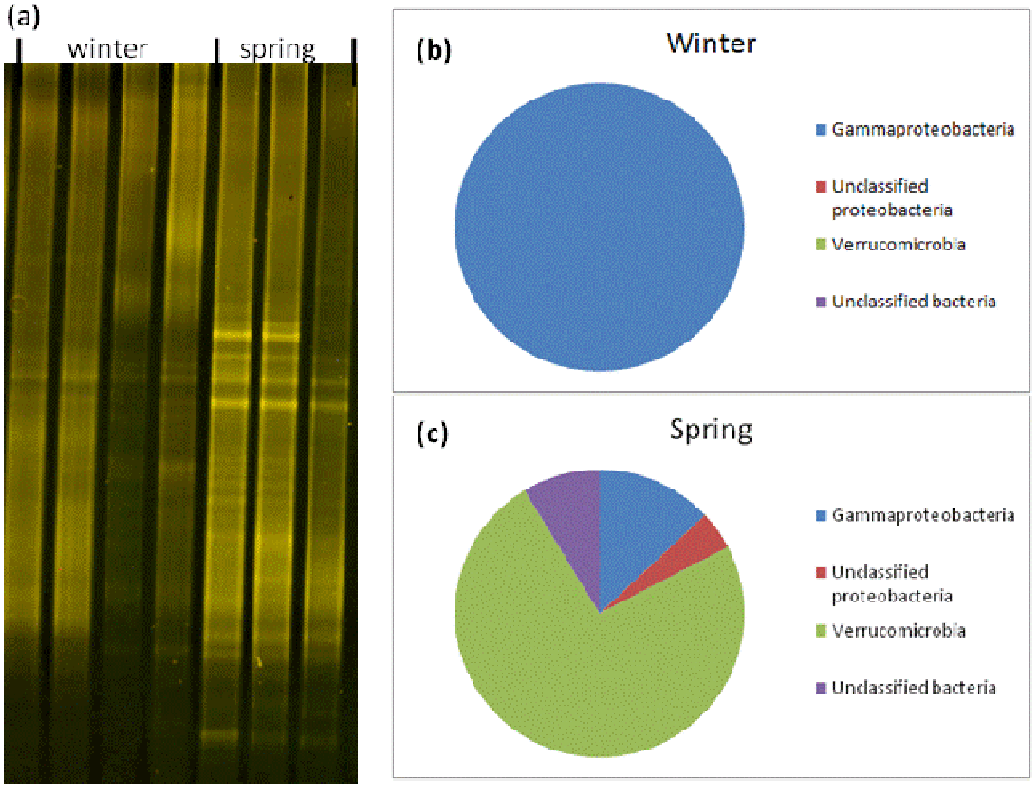
(a)
winter
spring
(b)
Winter
Gammaproteobacteria
Unclassified proteobacteria
Verrucomicrobia
Unclassified bacteria
(c)
Spring
Gammaproteobacteria
Unclassified proteobacteria
Verrucomicrobia
Unclassified bacteria

Figure 3

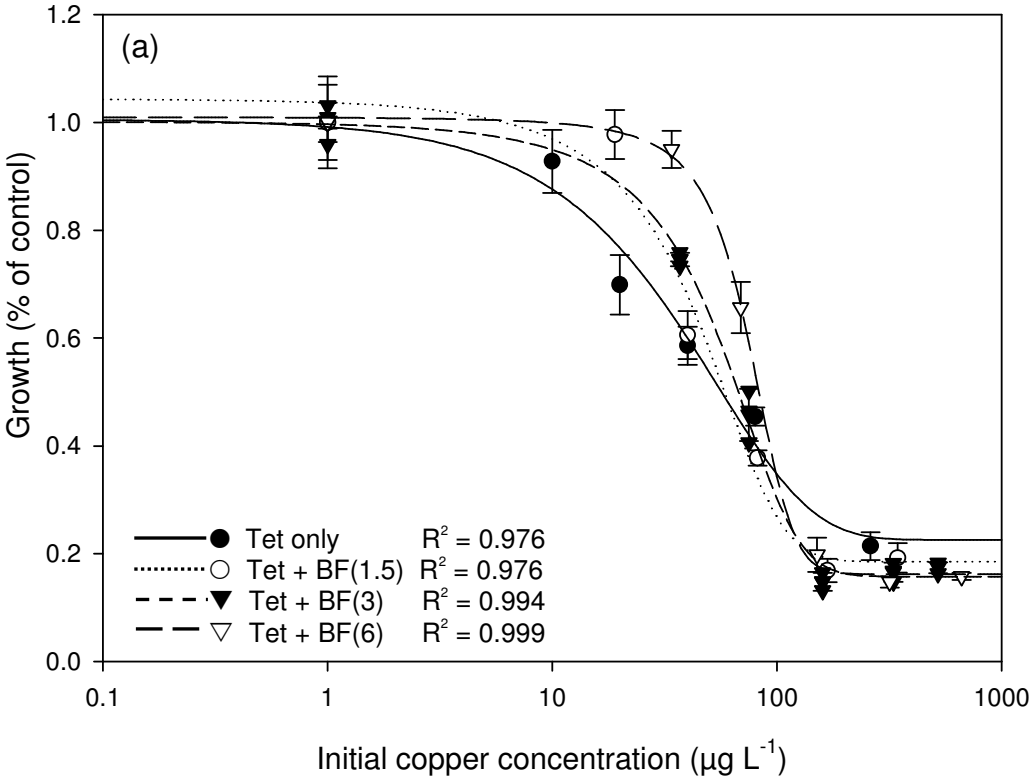
(a)
Growth (% of control)
Initial copper concentration (µg L-1)
Tet only R2 = 0.976
Tet + BF(1.5) R2 = 0.976
Tet + BF(3) R2 = 0.994
Tet + BF(6) R2 = 0.999
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.1
1
10
100
1000

Figure 4a

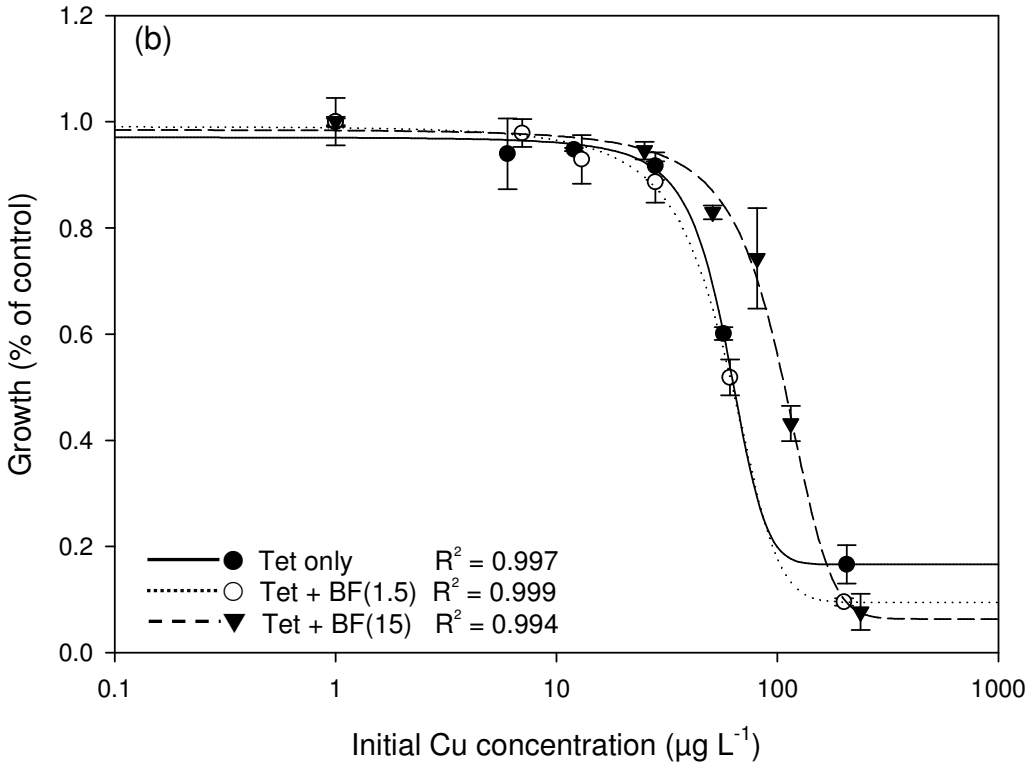
(b)
Growth (% of control)
Initial Cu concentration (µg L-1)
Tet only R2 = 0.997
Tet + BF(1.5) R2 = 0.999
Tet + BF(15) R2 = 0.994
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.1
1
10
100
1000

Figure 4b