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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 IC₅₀ 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 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.

Keywords

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

Disciplines

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1 **The effect of field-collected biofilms on the toxicity of copper to a marine microalga**
2 **(*Tetraselmis* sp.) in laboratory bioassays**

3

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16

17 **Key words:**

18 biofilm, marine, phytoplankton, bacteria, PCR-DGGE, Cu, toxicity

19

20

21 **Abstract**

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

38

39 **Introduction**

40 Biofilms (or periphyton in freshwater systems) are an integral part of aquatic systems. In
41 combination with planktonic algae, they form the basis of the food chain and are of
42 particular importance for grazers (Barranguet *et al.* 2003, 2005; Zippel and Neu 2005). A
43 biofilm is a community of microorganisms, and their associated extracellular products,
44 growing on a living or non-living substrate. The microorganisms may be heterotrophic or
45 autotrophic and can include bacteria, algae, fungi or protozoa (Palmer and White 1997).
46 Biofilms and periphyton represent useful biomonitors of pollution because they are sessile,
47 have short generation times, are species-rich and may accumulate contaminants over time
48 (Rodgers *et al.* 1979; Biggs and Kilroy 2000; Burns and Ryder 2001). They are often the
49 first community to respond to and recover from stress (Fuchs *et al.* 1997; Burns and Ryder
50 2001) and may reflect both sediment and water quality (Holding *et al.* 2003; Meylan *et al.*
51 2003; Stal and Défarge 2005). Metals can accumulate in biofilms, adsorbing or binding to
52 cells and the extracellular polymeric substances (EPS) in the biofilm matrix, i.e. they may
53 be a potential source of nutrition or toxicity to higher-order organisms (Mages *et al.* 2004;
54 García-Meza *et al.* 2005).

55

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

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

70

71 Most work on the interaction of metals and aquatic biofilms has focussed on the impact of
72 metals on the whole biofilm (e.g. in terms of biomass and chlorophyll *a* concentrations

73 Barranguet *et al.* 2002; Gold *et al.* 2003). These types of studies may incorporate changes
74 in community function (e.g. bacterial and/or algal respiration, photosynthesis, and ability
75 to use carbon substrates), changes in structure (taxonomic shifts), or combinations of the
76 two. They may include pollution-induced community tolerance (PICT), in which shifts in
77 community structure towards more tolerant species often account for increased community
78 tolerance (e.g., Admiraal *et al.* 1999; Soldo and Behra 2000; Massieux *et al.* 2004).

79

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

90

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

101

102 We investigated the impact of copper on a laboratory alga, *Tetraselmis* sp., in the absence
103 and presence of field-collected marine biofilm using 72-h growth inhibition bioassays.
104 *Tetraselmis* sp. was selected due to its moderate sensitivity to copper (Levy *et al.* 2008)
105 and because, using flow cytometry, *Tetraselmis* sp. cells could be easily distinguished from
106 biofilm cells over the 72-h exposure period (\pm copper). Protection against toxicity is

107 hypothesised to occur due to copper binding to the additional algal and bacterial cells
108 provided by the biofilm and the EPS provided by the biofilm. Copper binding to these
109 additional sites instead of the surface of *Tetraselmis* sp. cells will reduce the total dissolved
110 copper and the amount of copper uptake into *Tetraselmis* sp., both of which will decrease
111 the likelihood of toxic effects.

112

113 **Methods**

114 *General*

115 All glassware and plasticware were cleaned in a laboratory dishwasher (GW 3050, Gally
116 Medical and Scientific, Auburn, NSW, Australia) with a phosphate-free detergent (Clean A
117 Powder Detergent, Gally Medical and Scientific), then acid-washed in HNO₃ (30% v/v;
118 Merck, Kilsyth, VIC, Australia) and rinsed three times with Milli-Q water (> 18 MΩ cm⁻¹,
119 Millipore, North Ryde, NSW Australia). All glassware used in bioassays had been pre-
120 treated with silanising solution to help prevent metal adsorption to the glassware. Prior to
121 use, bioassay glassware was pre-soaked in 10% HNO₃ overnight and then washed
122 thoroughly five times with demineralised water and five times with Milli-Q water. For
123 DNA analysis, all plasticware was sterile, DNA- and RNA-free (CellStar polypropylene
124 centrifuge tubes, Greiner Bio-One, Frickenhausen, Germany or other DNA-grade plastic-
125 ware, LabServ, BioLab Australia, Clayton, Australia). Care was taken at all stages of
126 biofilm collection, harvesting and DNA analysis to use sterile procedures (autoclaved
127 glassware, 70% ethanol sterilised equipment, a UV-laminar flow cabinet). All chemicals
128 were Analytical Reagent grade or better, and solutions were prepared with high purity
129 Milli-Q water.

130

131 *Sampling site*

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

136

137 *Biofilm collection*

138 Field sampling dates for biofilm material used in the toxicity tests were July (winter) and
139 October (spring) 2007. Customised devices known as “periphytometers” (supplied by
140 Curtin University, Australia) were used to collect the biofilms. They consisted of Perspex

141 chambers with grooves to fit ten glass microscope slides ($76.2 \times 25.4 \times 1.0$ mm plain
142 unfrosted pathology grade slides), secured using fishing line. Multiple periphytometers
143 were attached to a polypropylene rack and the rack suspended from a buoy, 50 m from
144 shore, at a depth of 2 m. After 12 days, the periphytometers were collected, placed in a
145 clean container filled with site seawater and transported back to the laboratory on ice.

146

147 Two 2-L Nalgene containers were filled with seawater and returned to the laboratory on
148 ice. The seawater was immediately filter-sterilised ($0.2 \mu\text{m}$). This water was used to
149 prepare the biofilm homogenate and for blanks in subsequent analyses.

150

151 *Harvesting the biofilm*

152 Biofilm material was harvested into a homogenate on the day of collection. The term
153 homogenate is used, but in reality the biofilm is a heterogeneous entity and processing it in
154 this way is unlikely to create a truly homogenous inoculum. The material on the slides was
155 scraped into a sterile container using a Teflon-coated stainless steel blade. One mL of
156 filter-sterilised seawater was used to rinse the slides. In winter, material from 59 slides was
157 combined (0.237 m^2 harvested; surface area based on number of slides and a surface area
158 of 0.004 m^2 per slide). In spring, material from 60 slides was harvested (0.241 m^2). In
159 addition, several slides were retained intact and placed in sterile centrifuge tubes
160 containing filter-sterilised seawater. Some slides were stored overnight (4°C , dark) for use
161 in whole-slide toxicity tests. Other slides were sent on ice to CSIRO Land and Water in
162 Urrbrae where they were frozen in the dark at -80°C for later DNA analysis.

163

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

170

171 *Characterisation of the biofilm*

172 Characterisation of biofilm material from the winter and spring collections included
173 particle and fluorescent cell counts, chlorophyll *a* content and analysis of bacterial DNA
174 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 $\mu\text{g mL}^{-1}$), 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

243 of whole biofilm-colonised slides. In each test, the concentrations of copper to inhibit
244 growth rate by 10, 20 and 50% (IC10, IC20 and IC50) were used to compare the toxic
245 response of *Tetraselmis* sp., with and without biofilm.

246

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

255

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

261

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

272

273 *Copper analyses*

274 Copper was analysed by inductively coupled plasma-atomic emission spectroscopy (ICP-
275 AES). Copper concentrations were calculated from a matrix-matched calibration curve
276 (clean seawater acidified with 0.2% HNO₃) using serial dilution of a mixed metal standard

277 (QCD Analysts, Eaglewood, FL, USA) and a drift standard incorporated into the analysis
278 procedure. The detection limit for copper was typically $\leq 2 \mu\text{g L}^{-1}$. Initial copper
279 concentrations were used in all toxicity calculations.

280

281 *Statistical analyses*

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

293

294 **Results**

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

296 Spring biofilms colonising slides were observably thicker and had higher cell counts and
297 chlorophyll *a* concentrations than biofilms collected in winter (Figure 2). In winter, the
298 average (\pm standard error) initial cell density was $3.9 (\pm 0.3) \times 10^9$ fluorescent cells m^{-2} and
299 $140 (\pm 10) \times 10^9$ total cells m^{-2} . In spring, cell numbers were significantly higher (*t* tests, P
300 < 0.001) with values of $36 (\pm 1) \times 10^9$ fluorescent cells m^{-2} and $1440 (\pm 90) \times 10^9$ total
301 biofilm cells m^{-2} , approximately 10-fold higher than the winter biofilm. Chlorophyll *a*
302 concentrations were higher in spring compared to winter (49 ± 13 and $1.8 \pm 0.2 \text{ mg m}^{-2}$
303 chlorophyll *a*, respectively; one way ANOVA $F_{2,6} = 37.4$, $P < 0.001$). There was also a
304 slightly higher pheophytin content in spring biofilms (9 ± 3 and $4.8 \pm 0.4 \text{ mg m}^{-2}$
305 pheophytin in spring and winter, respectively; $F_{2,6} = 6.5$, $P = 0.031$) (Figure 2c).

306

307 Preliminary work, including analysis of carbohydrate, protein and chlorophyll *a* content
308 and total counts, had shown that there was little change in biofilms over a storage period of
309 one month. In the winter test, the concentrations of chlorophyll *a* and pheophytin in the
310 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,

345 column 5). For *Tetraselmis* sp., at initial cell densities of 1.5×10^4 cells mL⁻¹ in the
346 absence of biofilms, the 72-h IC50 values varied by a factor of 2, from 66 to 136 $\mu\text{g Cu L}^{-1}$
347 (Table 1, Column 8). The IC10 values (Table 1, column 6) ranged from 7.9 to 49 $\mu\text{g Cu L}^{-1}$,
348 while the IC20 values ranged from 16 to 62 $\mu\text{g Cu L}^{-1}$ (Table 1, column 7).

349

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

357

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

367

368 Larger additions of biofilm (15×10^4 fluorescent biofilm cells mL⁻¹) did help ameliorate
369 copper toxicity to *Tetraselmis* sp., but only in two of four tests (based on 72-h IC50
370 values). In the October 9th bioassays, the IC50 increased from 133 (112-152) to 175 (160-
371 184) $\mu\text{g Cu L}^{-1}$, while in the October 16th bioassays the IC50 increased from 98 (71-125) to
372 158 (146-168) $\mu\text{g Cu L}^{-1}$. IC10 and IC20 values increased with the addition of biofilm in
373 all tests when biofilm was added at a concentration $\geq 6 \times 10^4$ cells mL⁻¹ (Table 1).

374

375 To determine if inactivated biofilms could also ameliorate toxicity, heat-treated and
376 frozen/thawed biofilms were added to toxicity tests. The treatments did not totally
377 inactivate the biofilms, with growth of cells observed after 72 h. The 72-h IC50 values for
378 the “inactivated” and non-treated biofilm tests were not significantly different (Table 1).

379

380 While the aim of this study was not to investigate the effect of copper on the biofilm itself,
381 biofilms did not grow under laboratory bioassay test conditions unless an initial biofilm
382 inoculum of 15×10^4 fluorescent cells mL^{-1} was used. Where growth of biofilm material
383 did occur, it appeared to be due to one algal species. This species was smaller and
384 contained less chlorophyll *a* than *Tetraselmis* sp., based on FL3 and SSC flow cytometry
385 measurements. Microscopic observation confirmed that this species was a small centric
386 diatom. It was hypothesised that the higher light conditions used in the toxicity tests (140
387 $\mu\text{mol photons m}^{-2}\cdot\text{s}$) compared to the light intensity at the depth of biofilm colonisation
388 (2 m) may have been phytotoxic. However, use of low light conditions ($70 \mu\text{mol photons}$
389 $\text{m}^{-2}\cdot\text{s}$) did not improve the growth of biofilm cells (data not shown).

390

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

392 The growth of *Tetraselmis* sp. was measured over 72 h in two bioassays where whole
393 attached biofilms were added to test solutions (Table 2). These bioassays used slides from
394 the winter colonisation period. Addition of a washed slide, free of biofilm, as a control, did
395 not affect the growth of *Tetraselmis* sp., either in the presence or absence of $100 \mu\text{g Cu L}^{-1}$.
396 Addition of biofilm slides to copper-free solutions increased the *Tetraselmis* sp. control
397 growth rate from 1.34 ± 0.02 to 1.46 ± 0.02 doublings day^{-1} in the first test and from $1.24 \pm$
398 0.04 to 1.56 ± 0.03 doublings day^{-1} in the second test.

399

400 In the first test, the growth rate of *Tetraselmis* sp. cells exposed to copper (percentage of
401 control growth rates) increased from $47 \pm 2\%$ in the absence of biofilms to $89 \pm 8\%$ in the
402 presence of biofilms (Table 2). In the second test, the values increased from $29 \pm 1\%$
403 (biofilm-absent) to $80 \pm 4\%$ (biofilm-present), i.e. partial amelioration of toxicity was
404 occurring (Table 2). In these tests, some cells detached from the biofilm and began to grow
405 in the test solution. As discussed for the homogenate tests, these were small centric diatom
406 species, although other species were also present in smaller numbers. When *Tetraselmis*
407 sp. was absent, the growth rate and yield of this biofilm alga in solution was much higher
408 (up to 61×10^4 cells mL^{-1} by Day 3) than when *Tetraselmis* was present (average of $13 \times$
409 10^4 cells mL^{-1} by Day 3). This suggests competition for nutrients between the species. The
410 addition of copper inhibited the growth rate of the other algal species.

411

412 **Discussion**

413 *Seasonal differences in the biofilm*

414 The substantial increase in biofilm cell density, pigment concentrations, bacterial
415 community diversity and bacterial abundance (over a 10-d colonisation period) was
416 expected in the spring samples when the increased light incidence, potential nutrient
417 upwelling and higher water temperatures promote a peak in primary production.
418 Temperature has been shown to influence biofilm productivity, with higher growth rates
419 for bacteria and algae at higher temperatures (Rodgers *et al.* 1979; Palmer and White
420 1997). Temperature also influences community structure and the sensitivity of
421 communities to toxicants (Boivin *et al.* 2005). Despite the differences in the biofilms, the
422 biofilm composition appeared to make little difference to the toxicity tests with *Tetraselmis*
423 sp. and copper. While there is a potential for different cells to produce different exudates
424 and thus modify toxicity, the primary reason for a change in toxicity is likely to be simply
425 a surface area/adsorption effect.

426

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

428 The addition of biofilm to bioassays often increased the control growth rate of *Tetraselmis*
429 sp. (no Cu, Table 1, column 5). The biofilms may provide nutrients (e.g. iron) to
430 *Tetraselmis* sp. that are not otherwise present in the minimal nutrient test medium
431 (seawater with nitrate and phosphate). Biofilms can act as a sink for nutrients, trapping
432 them within the EPS matrix, while the close interactions of species within the dense
433 biofilm can encourage the breakdown of algal products by bacteria, remobilising nutrients
434 for growth (Costerton *et al.* 1995; Grossart 1999; Sutherland 2001). Similarly, Levy *et al.*
435 (2009) showed that increases in bacteria associated with algae in culture increased control
436 growth rates for some algae, but not others.

437

438 Previous assessments of the toxicity of copper to *Tetraselmis* sp. under similar conditions
439 had given 72-h IC₅₀ values of 47 µg L⁻¹ (Levy *et al.* 2008) and 146 µg L⁻¹ (Franklin *et al.*
440 2001), with initial cell densities of ~2 × 10³ and 10⁴ cells mL⁻¹, respectively. The IC₅₀
441 values for *Tetraselmis* sp. in this study ranged from 66 to 136 µg Cu L⁻¹ (i.e. in the range
442 of previous studies).

443

444 No previous reports in the literature have assessed the toxicity of copper to algae in
445 laboratory tests in the presence of natural biofilms. However, the addition of exudates or
446 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

480 metal in and on *Tetraselmis* sp. cells in the presence of biofilms, because it was impossible
481 to separate the test species from the biofilm, once added.

482

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

494

495 **Conclusion**

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

508

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518

519

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695
696
697

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

Season	Date	Initial cell density ^a		<i>Tetraselmis</i> sp. control growth rate ^b (doublings day ⁻¹ ± 1SD)	72-h effect concentrations ^c		
		<i>Tetraselmis</i> sp. (× 10 ⁴ cells mL ⁻¹)	Biofilm cells (× 10 ⁴ cells mL ⁻¹)		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

699 ^a A dash indicates the absence of biofilm.

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

701 ^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
702 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
703 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
704 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
705 (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.

	<i>Tetraselmis</i> sp.		ANOVA Group	Biofilm sp. (centric diatom)		ANOVA Group
	mean (±1SD) growth rate (doublings day ⁻¹)	(% of control)		mean (±1SD) growth rate (doublings day ⁻¹)	(% of control)	
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 (\pm 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 \pm 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 R² giving
739 the goodness of fit.

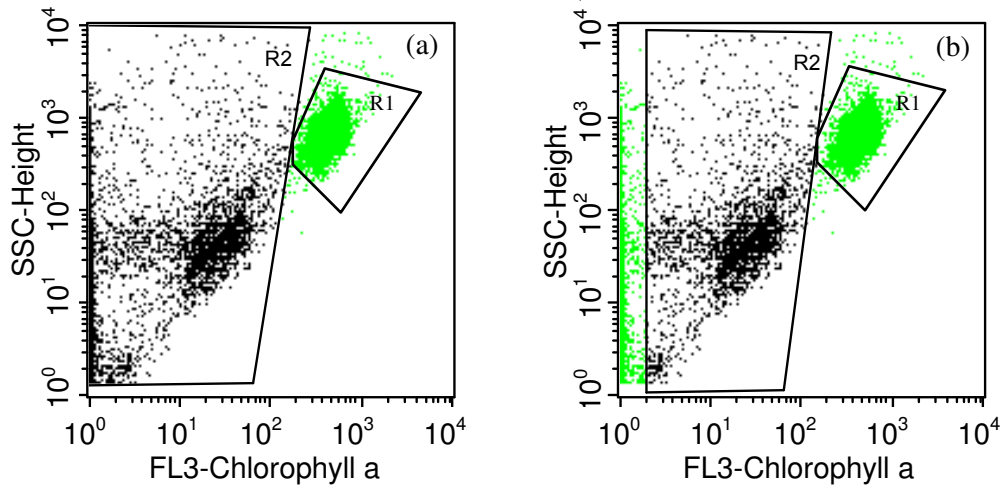


Figure 1

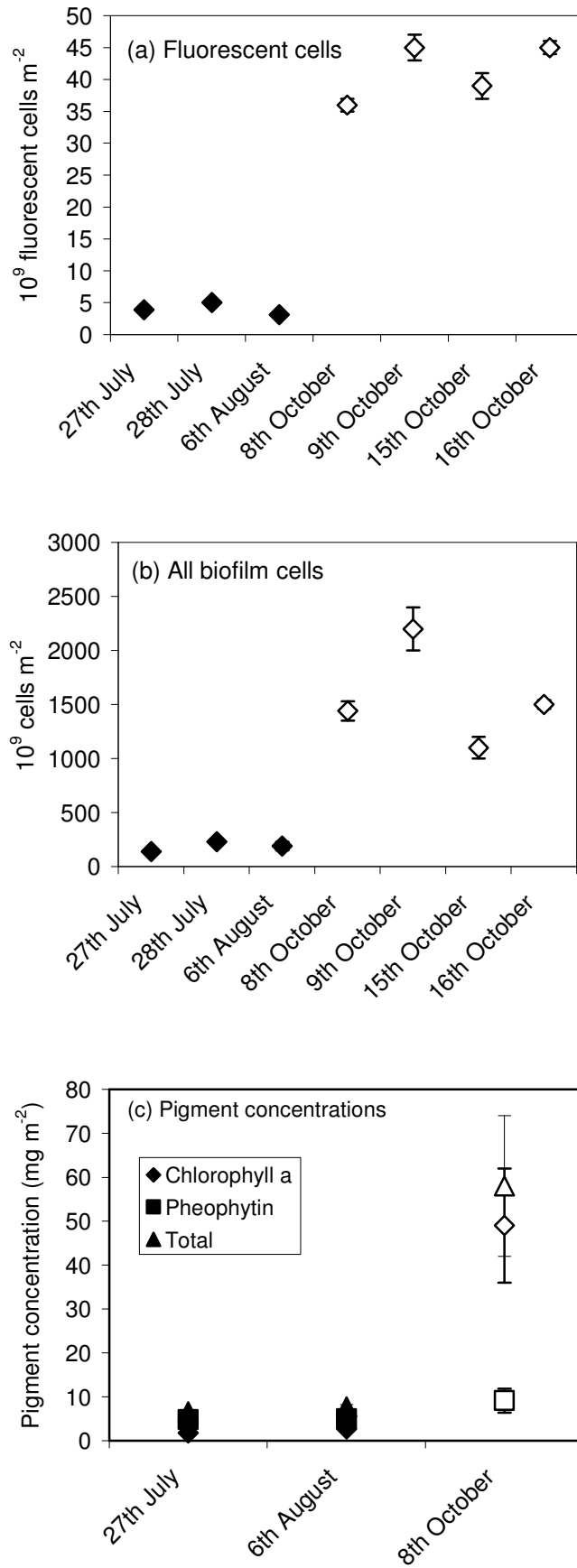


Figure 2.

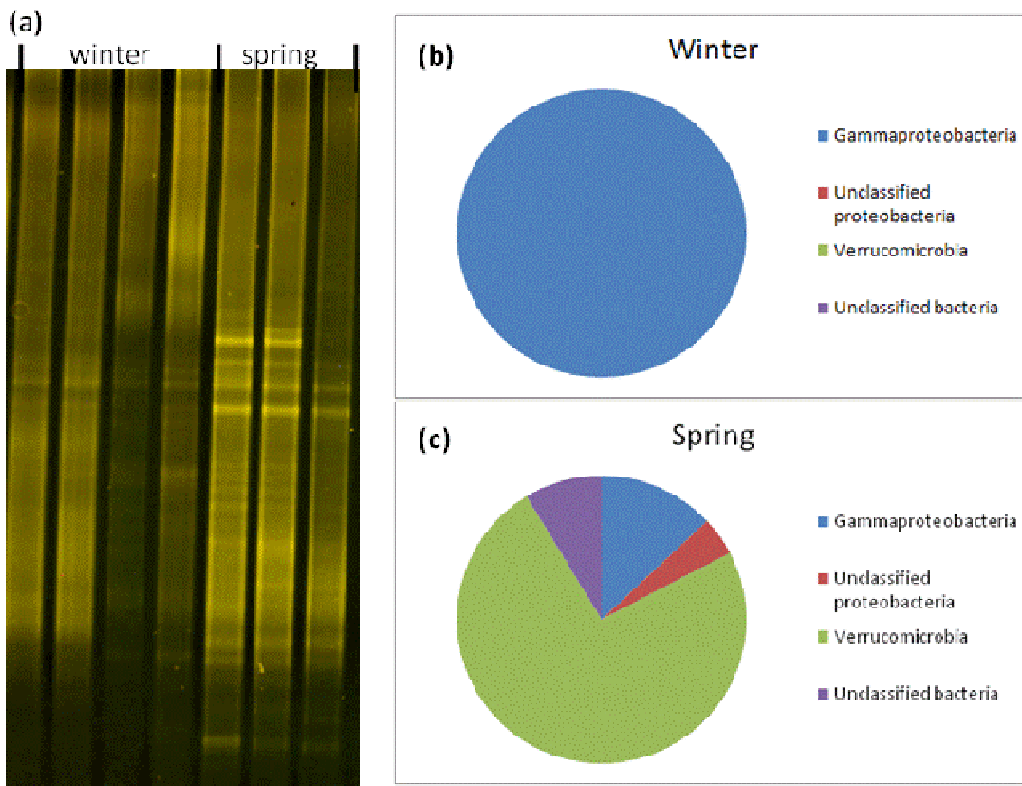


Figure 3

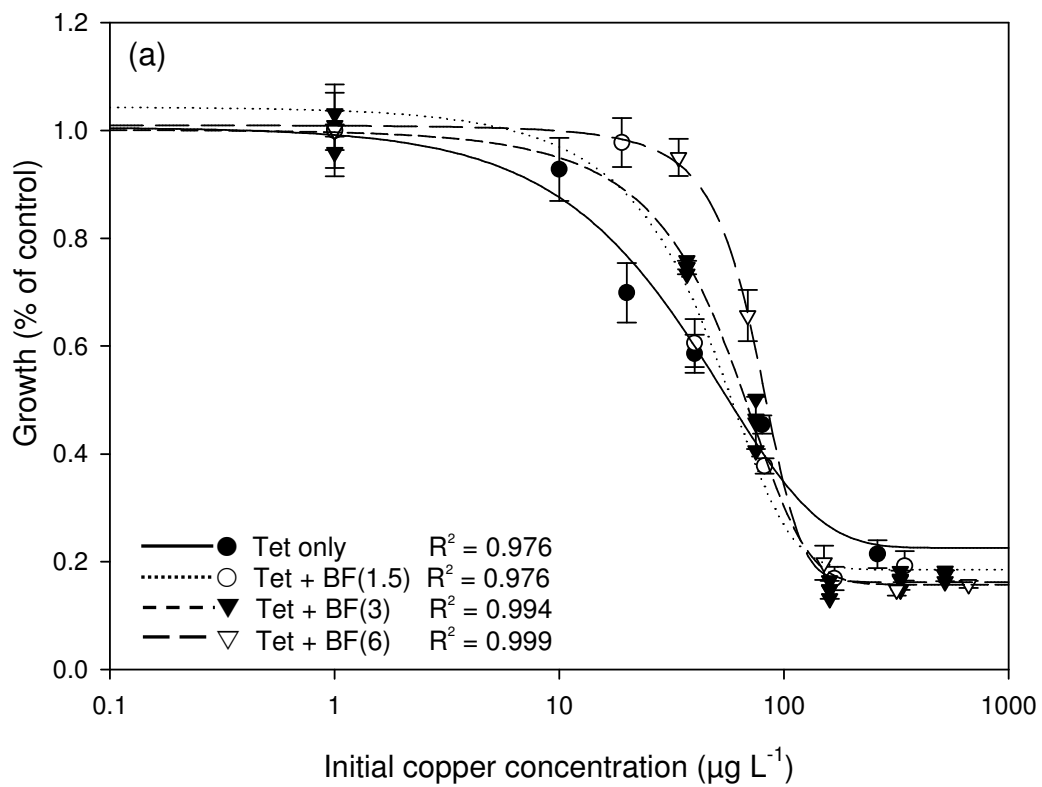


Figure 4a

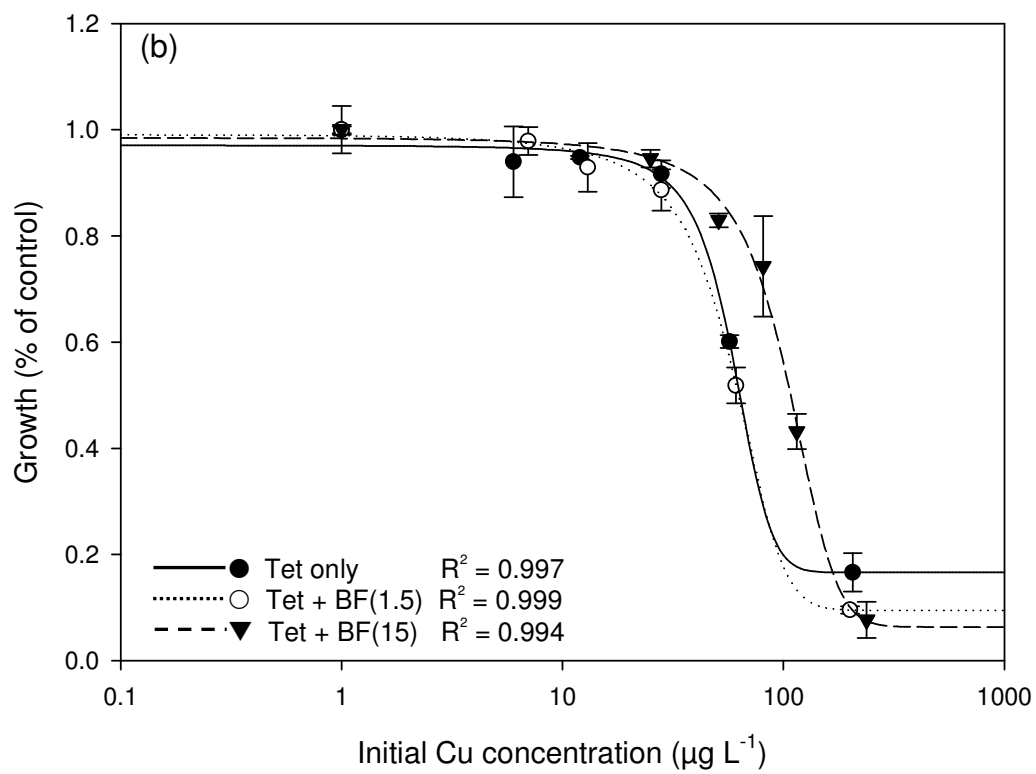


Figure 4b