16S rRNA assessment of the influence of shading on early-successional biofilms in experimental streams

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Abstract

Elevated nutrient levels can lead to excessive biofilm growth, but reducing nutrient pollution is often challenging. There is therefore interest in developing control measures for biofilm growth in nutrient-rich rivers that could act as complement to direct reductions in nutrient load. Shading of rivers is one option that can mitigate blooms, but few studies have experimentally examined the differences in biofilm communities grown under shaded and unshaded conditions. We investigated the assembly and diversity of biofilm communities using *in situ* mesocosms within the River Thames (UK). Biofilm composition was surveyed by 454 sequencing of 16S amplicons (~400 bp length covering regions V6/V7). The results confirm the importance of sunlight for biofilm community assembly; a resource that was utilized by a relatively small number of dominant taxa, leading to significantly less diversity than in shaded communities. These differences between unshaded and shaded were either because of differences in resource utilization or loss of diatom-structures as habitats for bacteria. We observed more co-occurrence patterns and network interactions in the shaded communities. This lends further support to the proposal that increased river shading can help mitigate the effects from macronutrient pollution in rivers.

Introduction

Seasonal algal and cyanobacterial blooms have become regular occurrences in many watersheds (Dodds, et al. 2002, Paerl, et al. 2011), and are predicted to increase in frequency as a result of human population growth and climate change (Paerl, et al. 2011, Johnson *et al.*, 2009). The increasing frequency of algal blooms in rivers worldwide could have substantial economic and ecological consequences, there is hence much interest in mitigating their impacts. The general consensus is that the primary driver of algal blooms is concentration of macronutrients, which is increasing due to growing human populations, agricultural intensification and increased collection and release of urban wastewater (Bowes, et al. 2012, Mainstone and Parr 2002).

Options to reduce harmful blooms have focused on reducing effluent fluxes (Kelly and Wilson 2004) (Neal, et al. 2010). The addition of tertiary treatment to

many sewage treatment works has led to substantial reductions in river macronutrient contamination. However, reductions in macronutrient concentration are typically costly, and do not always result in reductions in riverine biofilm (or periphyton) growth or in improvements to other proxies of overall river health. What is more, the relationship between harmful algal blooms and macronutrient enrichment is not always a linear one. As Smayda (2008) notes, it is often difficult to trace harmful algal blooms back to nutrient enrichment. In addition, some harmful blooms are caused by nitrogen-fixers that thrive under nutrient-limited conditions (Paerl, et al. 2011). In rivers, catchment area, residence time and temperature are also important factors (Desortova and Puncochar 2011, Bowes, et al. 2012). Notable examples where nutrient management alone appears to be insufficient to control blooms are the Rivers Thames in the UK and Berounka in the Czech Republic. In both rivers, annual means of soluble reactive phosphorus concentrations have declined over the last decades (from ca 1000 $\mu g\ l^{-1}$ to ca 200 $\mu g\ l^{-1}$ and ca 430 $\mu g\ l^{-1}$ to ca 160 $\mu g\ l^{-1}$ respectively). These concentrations are still high enough for the algal biomass (measured as chlorophyll-*a*) to remain similar to that observed before phosphorus mitigation measures were introduced (Bowes, et al. 2012, Desortova and Puncochar 2011). There is therefore a need for complementary controls that enhance the impact of nutrient reductions.

One possibility is to use artificial or natural shading to reduce algae growth rates in catchments with elevated nutrient concentrations. The rationale behind that is that light can be as important in limiting growth of primary producers as macronutrients (Rosemond 1993). Light-limited conditions can also prevent dominance by a few fast growing species, particularly constrain the growth of low-diversity communities consisting of filamentous species that can rapidly take advantage of the high-nutrient environments and create a thick biomass mat that in itself limits the growth of a range of organisms that grow in deeper biofilm layers (Steinman 1992). In support of this, several experiments have shown that light limitation mitigates the impact of eutrophication, even in nutrient rich environments (Burrell, et al. 2013, Colijn and Cadée 2003, Hill and Knight 1988, Hill, et al. 1995, Hutchins, et al. 2010, Mosisch, et al. 2001, Sanches, et al. 2011, Triska, et al. 1983, Winterbourn 1990). Numerous studies have proposed a range of growth limiting factors for controlling eutrophication. Most often cited is phosphorus, however, just

as important is flow velocity, grazing pressure, nitrogen pollution and light (McCall, et al. 2014). "Natural" experiments have been particularly helpful in elucidating the role of sunlight, whereby increased and decreased periods of natural sunlight on rivers has directly translated into a corresponding increases and decreases in the intensity of the resulting algal bloom (Read et al. 2014).

While there have been many studies on the effects of shading on overall measures of biofilm growth, there has been relatively little research on how algal and bacterial biofilm composition is affected by shading. The bacterial biofilm component is less directly affected by light availability, but light levels could affect heterotrophs through changes in UV radiation (Kahn & Wetzel 1999, Yoshikuni 2005, Thomas*, et al.*, 2009), or through indirect effects caused by changes to the autotrophic component (Rier & Stevenson, 2002). This study uses an experimental approach to compare riverine biofilm communities grown under shaded and unshaded conditions in the River Thames. The Thames catchment is already heavily impacted by anthropogenic activities. High and rising population density in the catchment are projected to put additional pressure on water quality in the Thames, which might be exacerbated by declining river flows and higher water temperatures brought about by climate change (EA 2009, Evans, et al. 2003, Johnson, et al. 2009, Neal and Jarvie 2005). Algal and bacterial biofilm communities were characterized using a molecular approach. Having observed in previous experiments that increased algal growth prompted by excess nutrients lead to biofilms which were dominated by few organisms and were less diverse than those grown under nutrient-limiting conditions, we hypothesized that a similar effect could be observed when light, another strongly limiting factor, was restricted - as high light levels would allow a few fast-growing species from rapidly dominating the communities. We tested two linked hypotheses:

1) biofilm grown in nutrient rich, shaded conditions assemble significantly different biofilm communities than unshaded communities. Assuming that many members of a biofilm community interact with each other in competitive, predatory or symbiotic ways, any shift in one component of the population would lead to a shift in the others. In that context, we assumed that 2) the biofilm communities that assemble in the shade are more diverse than those that assemble in unshaded conditions, similar to effects observed in nutrient experiments. The reason for the greater diversity might be

due to a reduced abundance of algal keystone species, which lead to a more complex habitat (Bruno et al., 2003).

Methods

Study site

The study was conducted in experimental flumes placed within Seacourt Stream, a side-branch of the Thames at Wytham in Oxfordshire, southern England (Supplementary Figure 1; 51.786413, -1.317073 Lat/Long, Decimal Geographic Coordinates). Seacourt Stream is a disused millstream directly fed by the Thames (100m upstream). The site was selected due to its lack of natural shading. Macronutrient composition at the start of the experiment was similar to that in the main Thames branch, and showed little change during the study period (7th to 17th September 2010; see Results).

Experiment methodology

We installed 12 experimental flow-through flumes at the study site (Supplementary Figure 2). The mesocosms are described in Bowes *et al*. (2010: 384-9). Briefly, they are constructed as blocks of 3 flumes that float directly in the river, allowing river water to flow through freely. Each flume has a gate to standardise flow rates at the upstream end and a sump to collect river debris ¾ of the length from the inlet (Bowes, et al. 2010). The flumes are made of polyvinyl chloride sheeting set in an aluminium frame, with each flume measuring 5 m x 0.3 m. For this experiment, 4 sets of flumes (i.e. 12 flumes in total) were tethered to the riverbank and positioned 0.5 m above the streambed, held by scaffolding poles. Floats ensured a constant water depth of ~6 cm inside the flumes. The gap between the riverbed and mesocosms limits invertebrate colonizers from entering the flumes.

Before the start of the experiment, we measured midday light intensity with a SunScan SS1 light probe in both direct sunlight and full tree shade. In full tree shade, the intensity of direct sunlight was reduced by 71%. We used layers of greenhouse shading mesh, positioned directly above the flumes, to create light intensities equivalent to those measured in full tree shade over parts of the flumes. Dividing each channel in half, we grew shaded and unshaded biofilm next to each. A similar

151 approach has been used in other studies investigating the effect of light on biofilm
152 (Hill, et al. 2009, Hill, et al. 2011, Rier, et al. 2006). We positioned temperature
153 loggers (iButton, Maxim, San Jose, CA, USA) next to each set of tiles to determine
154 whether the shading also reduced water temperature. We set the flow rates in each
155 channel to 0.1 m s^{-1} (measured using a Valeport 801 flow meter) at the start of the
156 experiment.
157
158 **Sample collection and DNA sequencing**
159 We grew biofilm on 2 x 2 cm limestone tiles anchored to the bottom of the channels
160 for 9 days. This might have lead to a community that was not fully mature, but when
161 left longer, biofilms in previous experiment invariable sloughed off the tiles and
162 floated downstream. In the upstream half of each flume, there were 3 shaded tiles and
163 3 unshaded tiles. On day 9, we harvested the biofilms in all flumes and extracted the
164 DNA, pooling the 3 tiles within each treatment/flume. Briefly, we added 300 µl of
165 lysis buffer (100 mmM NaCl, 500 mM Tris (pH 8), 10% (w/v) sodium dodecyl
166 sulfate, 2 mg ml^{-1} proteinase K, 2 mg ml^{-1} lysing enzyme mix (both Sigma-Genosys,
167 Gillingham, UK)) and 300 µl of NaH_2PO_4 (pH 8.0) to the pooled sample, incubated
168 the DNA in a 55°C water bath for 30 min and mixed every 10 min., added 80 µl of
169 prewarmed 10% CTAB solution (65°C), incubated in 65°C for 10 minutes, added
170 680µl chloroform:isoamyl alcohol (24:1 vol/vol). The tubes were centrifuged for 5
171 minutes at 14000 rpm. The aqueous top layer was aspirated into a new tube and the
172 DNA precipitated by adding 300% (w/v) TE Buffer, pH 8.0 (10 mM TRIS-HCl, 1
173 mM EDTA, pH 8.0) and 200% (w/v) PEG/$MgCl_2$ mix (30% (w/v) PEG 8000, 30 mM
174 $MgCl_2$), leaving the samples overnight at 5°C (Paithankar and Prasad 1991). We then
175 centrifuged the replicates (12 per treatment) for 10 min at 14000 rpm, discarded the
176 supernatant and washed the DNA pellets by adding 300 µl 70% chilled ethanol. We
177 centrifuged the tubes again, discarded the ethanol and left the tubes to dry in a laminar
178 flow cabinet until the ethanol had evaporated. We added 50 µl ultrapure water and left
179 the DNA to resuspend for 1 h on the bench. We used the 454 GS-FLX TITANIUM
180 platform (Roche 454 Life Sciences, Branford, CT, USA) to produce tag-encoded 16S
181 amplicons of ~400 bp length. We targeted a fragment of the 16S ribosomal RNA
182 (rRNA), comprising the V6 and V7 regions. Primers used for the PCR were 967F, 5'-
183 CNACGCGAAGAACCTTANC-3', and 1391R, 5'- GACGGGCGGTGTGTRCA-3'

184 (Huber, et al. 2009: 1292-302, Huse, et al. 2008). These universal primers are
185 designed to amplify a large variety of 16S sequences, but, as with all universal
186 primers, it cannot be excluded that some OTUs (both chloroplast and bacterial 16S
187 rRNA) did not get captured. The sequencing libraries were generated through a one-
188 step PCR with a total of 30 cycles, a mixture of Hot Start and Hot Start high fidelity
189 taq polymerases and amplicons extending from the forward primers. DNA
190 amplification and pyrosequencing were carried out at Research and Testing
191 Laboratory (Lubbock, TX, USA).

192 **Bioinformatics**

193 We used CloVR 1.0 RC4 (Angiuoli, et al. 2011) on the Data Intensive Academic Grid
194 (DIAG, University of Maryland, USA) to run the QIIME workflow
195 'pick_otus_through_otu_tables.py' (Caporaso, et al. 2010). Within the QIIME
196 workflow: (1) we set the minimum and maximum sequence length to 100 bp and
197 2000 bp respectively, the maximum homopolymer length to 8 bp and maximum
198 number of ambiguous calls to zero; (2) clustering was performed using UCLUST with
199 a nucleotide sequence identity threshold within each cluster at 97% and alignment
200 against the Greengenes 16S database with PyNAST; (3) taxonomy assignment of each
201 OTU-representing sequence through the RDP classifier with a confidence threshold of
202 0.8. After quality control, the data set consisted of 101,617 combined reads for all 12
203 flume channels of the experiment. Clustering and chimera removal left 97,065
204 combined reads. Following from earlier studies (Lindemann, et al. 2013, Pillet, et al.
205 2011) we used the chloroplast 16S rRNA to focus on the algal communities.
206 Therefore, of those OTU's that were identified to Genus level, we divided the
207 community into algal-derived chloroplast reads and bacterial (including
208 cyanobacterial) reads. We equilibrated the number of sequences per sample by
209 randomly sampling without replacement (Hamady and Knight 2009, Koren, et al.
210 2013), resulting in 290 algal sequences per sample, and 732 bacterial sequences per
211 sample. OTUs that are discussed on the species level were blasted individually against
212 the RDP database (Cole, et al. 2009). Only fragments that could be matched at 97% or
213 above were classified to species level. The rarefied OTU tables were imported into
214 Primer (PRIMER-E Ltd, Ivybridge, UK), MEGAN (Huson, et al. 2007) and R for
215 further analysis.
216

Statistics

We were interested in how shading altered biofilm community composition and diversity. We calculated diversity, tested that the diversity data was normally distributed, then compared diversity across the treatments using analysis of variance (ANOVA). We included both treatment and channel as factors. We added the location of the flumes within the river channel as an additional factor/error, because not all flumes could be placed next to each in the river channel and location effects could not be excluded. We compared dissimilarity in community composition by calculating Bray-Curtis dissimilarities between pairs of communities (Bray and Curtis 1957). We tested for differences between treatments using PERMANOVA, a multivariate permutation test analogous to ANOVA (Anderson 2005). The PERMANOVA design was two-factorial, including treatment and channel. We used non-metric multidimensional scaling (NMDS) (Kruskal 1964) to visualize differences between the communities. We then used similarity percentages (SIMPER) to explore the contribution of each species (Clarke 1993). SIMPER assesses the contribution of each species to the observed dissimilarity between communities. PCR-based data cannot be used to accurately assess abundances in the original samples, but given that all samples were amplified in the same way, we assume that abundances can be compared between our samples (but not with samples from other datasets). Lastly, we used network analysis of co-occurrence patterns (Barberan, et al. 2012) to explore possible connections between biofilm components. We used network analysis implemented in the MEGAN software package (Huson, et al. 2007) to visualise co-occurrence patterns in our data. The visualisation connects OTUs (here at the taxonomic level of Class) that exceed a prescribed probability of co-occurrence. We set the following threshold values: the threshold required for a taxon to be considered present in a sample was 0.5%; the minimum and maximum percentage of samples in which a taxon could occur was set to 15% (2 samples) and 100% (12 samples); the minimum probability that a co-occurrence between two taxa had been observed was set to 95%. All of the statistical results and figures were produced using CloVR (Angiuoli, et al. 2011), MEGAN or the R environment (www.r-project.org). Significance thresholds of $p < 0.05$ were used throughout to validate the results.

Results

250 The taxonomic distribution of the data shows that a large proportion of taxa were of
251 algal origin (Fig. 3). 19% of total number of sequences could not be identified to
252 Genus level, but only 1% of the 44% that were identified as algae were not
253 identifiable to Genus level. The Shannon diversity (Fig. 4) of both the algal and
254 bacterial communities was significantly higher in the shaded communities ($F_{1,1}$ = 36.4
255 (Algae) and 7.1 (Bacteria), p = 1.26e-04 (Algae) and 0.02 (Bacteria)). In the algal
256 component of the biofilm, the community was dominated by *Amphora sp. C10*,
257 *Melosira varians* and *Amphiprora paludosa str. CCMP 125 C52*, which accounted for
258 62% of overall relative abundance (Fig. 5). In contrast, in the shaded community
259 these 3 species were still dominant but accounted for only 44% of the overall relative
260 abundance (Fig. 5). For the bacterial component of the unshaded communities, the
261 most abundant species were *Curvibacter sp. str. HMD2015* (2.4%) and
262 *Steroidobacter sp. str. ZUMI 37* (2.4%). These two species were also the most
263 abundant in the shaded community (3.4% and 1.7%), but in both cases they accounted
264 for only approximately 5% of the total community (Fig. 5). Applying Pielou's
265 evenness measure to the samples confirms that the bacterial components of the
266 biofilm were significantly more even in composition than the algal ones, in both the
267 unshaded and shaded replicates (0.95 for the bacterial components, 0.7 for the algal
268 component, F=460, p< 2e-16).
269 Ordination of the communities using NMDS (Fig. 6) indicates distinct clusters of
270 unshaded and shaded communities for both the eukaryotes (PERMANOVA:
271 eukaryotes, Pseudo F = 7.60, p = 0.002 and bacteria, Pseudo F = 2.52, p = 0.006).
272 The unshaded communities are less variable than the shaded ones.
273 We used SIMPER analysis (Clarke 1993) to investigate which OTUs
274 contributed most to the observed dissimilarity between the shaded and unshaded
275 assemblages. In the algal component, the three most abundant diatoms also
276 contributed most to the observed dissimilarity: *Amphora sp. C10* contributed 8.6%,
277 *Melosira varians* 8.4% and *Amphiprora paludosa str. CCMP 125 C52* 6.5%. All three
278 were more abundant in the unshaded treatment and together accounted for 23% of the
279 overall observed difference. The next six important algal contributors, however, were
280 more abundant in the shaded treatment than in the unshaded treatment, and accounted
281 for 14% of the overall observed difference. Due to the greater evenness of the
282 bacterial biofilm component, the 10 major contributors in the bacterial replicates only

283 accounted for 6% of the overall dissimilarity, with the two most abundant species
284 (*Steroidobacter sp. str. ZUMI 37* and *Curvibacter sp. str. HMD2015*) contributing just
285 2% of dissimilarity.
286
287 We used co-occurrence network analysis to explore the relationship between bacterial
288 and algal taxa (Fig. 7). The figure shows one main network in the unshaded samples,
289 consisting of common bacteria and diatoms found in all twelve replicates (Fig. 7:
290 *Bacillariophyceae, Gemmatimonadetes, Rhodobacter, Bacteroidetes,*
291 *Prosthecobacter, Acidobacteria, Anaerolineae*). This major network was also
292 detectable in the shaded replicates, consisting of four of the nodes that were present in
293 the unshaded samples (Fig. 7: *Bacillariophyceae, Bacteroidetes, Acidobacteria,*
294 *Anaerolineae*). These were joined by four additional nodes, differing from the ones in
295 the unshaded samples (Fig 7: *Methylobacter, Variovorax, Polaromonas,*
296 *Planctomycetes*). That means this network included one additional member in all
297 twelve replicates of the shaded treatment.
298 In eleven of the unshaded replicates there was also a small network consisting
299 of just two nodes, *Frateuria* and *Planctomycetes*. This 2-node-network containing
300 *Frateuria* was also detectable in ten of the shaded samples, but the *Planctomycetes*
301 node was replaced by *Verrucomicrobia*.
302 In eleven of the shaded samples, there were also two small networks that were
303 absent in the unshaded samples (*Actinobacteria* and *Nitrospira, Rhodobacter* and
304 *Rhodoferax*), and in two of the replicates there was a network consisting of
305 *Acidovorax* and *Shewanella*.
306 Throughout the experiment, Seacourt Stream had nutrient concentrations of
307 around 234 μg l^{-1} soluble reactive phosphorus (SRP), 5.18 mg l^{-1} nitrate (N), and the
308 dissolved reactive silicon 3.05 mg l^{-1}, which is considered high for SRP, low for N,
309 and below average for silicon (EA 2012, Neal, et al. 2005). Silicon is typically
310 depleted at periods when diatoms are 'in bloom', which is consistent with this period
311 of study. The water temperature of the shaded areas was at all times identical to that
312 of the unshaded areas and in all channels (averaging at 15.5°C) throughout the
313 experiment.
314
315 **Discussion**

316 This study joins a number of other studies in observing significant changes to algal
317 assemblages under reduced light conditions (Bowes, et al. 2012, Guariento, et al.
318 2011, O'Driscoll, et al. 2006). Uniquely, however, our results show that reduced light
319 conditions affect both algal and bacterial components of biofilm.

320 A marked result of shading is a change of dominance in the most prevalent
321 organisms. Whilst PCR can skew abundance-patterns found in the original sample,
322 the decrease in dominance of the three most dominant diatoms, *Amphora sp. C10,*
323 *Melosira varians,* and *Amphiprora paludosa str. CCMP 125 C52,* under shaded
324 conditions is consistent with findings by Hill, et al. (2011), who note in addition, that
325 light is a more limiting factor for autotrophs than nutrient availability. In another
326 study, Sanches, et al. (2011) confirm and expand on these findings by showing that
327 low light availability does not only limit biofilm growth, but also nutrient propagation
328 within the biofilm, thereby affecting the autotrophic to total biofilm biomass ratio. In
329 the Sanches *et al.* experiment, autotrophic biomass was highest under high light
330 conditions and N enrichment, whereas heterotrophic biomass increased under
331 enrichment for both P and N (Sanches, et al. 2011). To relate that back to the Seacourt
332 Stream experiment: the macronutrient content in Seacourt Stream measured at the
333 beginning of the experiment was sufficiently enriched enough to allow for an increase
334 in abundance of autotrophs. We have shown this increase in a parallel experiment,
335 which measured biomass and chlorophyll a content conducted in the downstream area
336 of the same flumes (published as Bowes *et al.*, 2012b). The parallel experiment
337 certainly showed higher algal biomass accrual in the unshaded treatment, and that is
338 probably reflected in the observed increase of the dominant diatom. Bowes *et al.*
339 (2012) further support the findings of this study in as much as the biomass tiles of the
340 shaded treatment showed greater algal diversity than the unshaded tiles.

341 The changes in the bacterial community components were less marked than
342 for the algae, but again the most visible pattern was a reduction in the relative
343 abundance of the most prominent dominant organisms in the shaded samples. One
344 potential explanation is that this is driven partly by the correlated changes in the algal
345 community. A possible reason for this is that specific bacteria utilize organic
346 compounds excreted by algal species (Ylla, *et al.* 2009). A greater diversity of algae
347 might provide a higher diversity of exudates, which would in turn support a higher
348 diversity of heterotrophs. Kritzberg, et al. (2006) have stated that 30-65% of bacterial

349 production in lakes is supported by algae-derived autochthonous carbon. A reduction
350 in algae due to less light is also likely to have lead to a reduction in autochthonous
351 carbon availability on the tiles, to which the bacterial community responded with a
352 shift in structure. Chang (2010) has hypothesized that such structural changes could
353 be due to a shift from heterotrophic consumers, to consumers that are more likely
354 adapted to allochthonous carbon sources. Finally, many bacteria colonize the
355 exoskeletons of diatoms and their diffusive boundary layer, a thin layer of fluid
356 directly surrounding the diatom known as the 'phycosphere' (Bell and Mitchell 1972,
357 Rier and Stevenson 2002, Znachor, et al. 2012). This layer contains extracellular
358 products produced by the diatom, and bacteria living in the vicinity might provide the
359 diatom with products it cannot produce itself (Amin, et al. 2012). A reduction of
360 Baccilariophyceae numbers would also reduce the number of such colonizers. The co-
361 occurrence network analysis suggests, that there were indeed a number of bacterial
362 classes in our samples with occurrence patterns that matched those of the
363 Baccilariophyceae in the experiment.

364 It is not possible to infer much about the nature of taxon interactions from our
365 data, but the result of the co-occurrence analysis could suggest that the influence of
366 light is so great, that interactions between taxa are less important when enough light is
367 available. To expand on that, under shaded conditions, a reduced amount of freely
368 available DOM and other metabolites might make it more important for biofilm
369 organisms to interact with co-habitants of the biofilm, as possibly indicated by the
370 increase of co-occurrence networks in the shaded replicates. At the same time, light
371 limitation might forge relationships that differ from those in light non-limited
372 conditions, as seen in the changing *Frateuria*-networks. A possible cause for the
373 network formations observed between *Frateuria* and other organisms could be that
374 *Frateuria* is unable to synthesize some of the compounds required for its growth
375 (Hashidoko 2005). In the unshaded samples, *Frateuria* appears to form a network
376 with *Planctomycetes*, but in the shaded samples, *Planctomycetes* are part of the main,
377 bigger network, whereas *Frateuria* forms a network with *Verrucomicrobia*.

378 The *Verrucomicrobia* are found in a greater number of the shaded samples than the
379 unshaded samples, but it is probably more relevant that in the shaded samples, the
380 *Planctomycetes* are part of the main network. One possible cause for this is that the
381 shaded and unshaded replicates harbor different species of *Planctomycetes* and

382 *Verrucomicrobia*. Another cause could, however, be that in the shaded samples, the
383 *Planctomycetes* rely on a close relationship with other organisms to obtain products,
384 which under non-limited light conditions, are easily available, leaving *Frateuria* to
385 seek out another organism to form a relationship with. Any statement on the nature of
386 the exchanges is purely speculative, but it may be interesting to note that *Frateuria*
387 have been linked to methanogens in the past (Romanovskaya and Titov 1992), and
388 that both *Verrucomibrobia* and *Planctomycetes* include methanogens amongst their
389 groups of species (Chistoserdova, et al. 2004, Dunfield, et al. 2007). A possible
390 outcome of such a change in interactions could be that there are functional differences
391 between shaded and unshaded communities.

392 All of the dominant diatom species in this study can cause blooms (Hillebrand
393 and Sommer 1997, Ohtsuka 2005, Khare and Chaurasia 2009, Vanelslander, et al.
394 2009, Dorigo, et al. 2010, Paerl, et al. 2011). Even though we observed that a shaded
395 environment led to less dominance of diatom taxa, it cannot be excluded that these
396 diatom taxa could adjust to shady conditions by reaching saturation levels at lower
397 light intensities, as previously described (Rier, et al. 2006). Notably, there were two
398 possible blooming species, *Haslea nipkowii* and *Synedra hyperborea str. CCMP* that
399 were more abundant under shaded than under unshaded conditions. Likewise, 15 of
400 the 29 observed cyanobacteria species were more abundant under shaded conditions,
401 too. This means that riparian shading can only be one tool in managing algal and
402 cyanobacterial blooms.

403 Lastly, it is important to consider what effect riparian shade has on other riverine
404 organisms. Invertebrates, for example, have been shown to decline when shading is
405 increased by more than 60% (Quinn, et al. 1997). Hence, mitigating measures for
406 'algal' blooms, such as shading, could have wider ecosystem implications.

407 There was a surprisingly low abundance of algal genera other than diatoms. It
408 is possible that the experiment was stopped before a significant number of
409 filamentous algae could establish themselves. Inferences drawn in this study might
410 therefore only be applicable to early succession biofilms. Alternatively, the low
411 number of filamentous species could have been the result of the exclusion of snails
412 from the flumes, which selected for diatoms (Rosemond 1993). It is also unclear
413 whether the universal primers that were used to amplify our 16S sequences might
414 have been more suitable for diatoms than for filamentous algae (Chung and Staub

2003). The shaded replicates had two outliers (S1, S2), which probably received more sun during the experiment due to the sun's angle at particular times of the day, however, these did not affect the statistical significance of the overall results.

Conclusions

Shading has a marked effect on the structure and diversity of both algal and bacterial assemblages in biofilm. It significantly reduces the prevalence of diatoms that are known to cause nuisance blooms under nutrient-enriched conditions, and creates communities that are more even and diverse. Our algal results support findings e.g. by Hill, et al. (2009), Ghermandi, et al. (2009) and Bowes, et al. (2012) that suggest riparian shading is an effective tool in controlling biofilm growth rates and managing the effects of eutrophication. Whilst it may not seem practicable to have extensive riparian planting schemes, the need to mitigate climate change might make such schemes more palatable. Recent management practice in the UK had begun to advertise how to create riparian shade where it is absent (Lenane, 2012). The obvious advantages that shading has in reducing algal blooms and keeping water temperatures low (Warner and Hendrix, 1984, Lenane, 2012) seem to make the planting of shading desirable even if it is a longer term project (Lenane, 2012).

Even if, however, riparian shading presents itself as a useful tool to manage eutrophic streams that experience blooms, more research needs to be conducted to assess if shading becomes ineffective as diatoms adjust to lower light levels (Rier, et al. 2006) or are replaced by species better suited to shade. It is also necessary to investigate if shading has a negative effect on the function of biofilm and on invertebrate grazers.

To understand the observed differences between the communities, it would be useful to investigate any changes in function. This could be an examination to determine if bacterial assemblages in shaded rivers are less equipped to process glycosate, which is produced by periphytic algae, or simply transcriptomic analysis of the whole communities, as transcriptomics would identify if the communities are functionally different. It would also be interesting to test whether the diverse and even communities created by riparian shading prove to be more resilient to stress and resistant to pollution events. In that context, it should be tested what effect different community assemblages have on nutrient cycling and biofilm function. Another question is to what degree changes to biofilm nutrient stoichiometry (Cross, et al.

2005) cause changes to higher trophic levels. Whilst shading might shift biofilm community structure in such a way that harmful blooms are reduced, it might produce unexpected effects on higher trophic levels in the river.

Acknowledgements

Many thanks to Alan Grafen for his advice on the statistical methods. This work was funded by the NERC Centre for Ecology & Hydrology National Capability funding through the CEH Thames Initiative and the NERC–Knowledge Transfer (PREPARE) Initiative contract NE/F009216/1.

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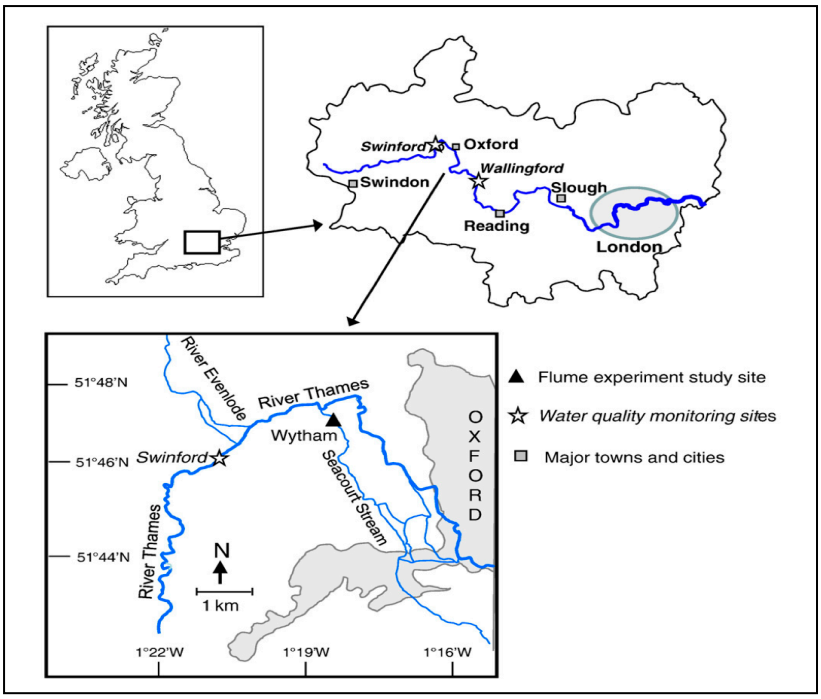

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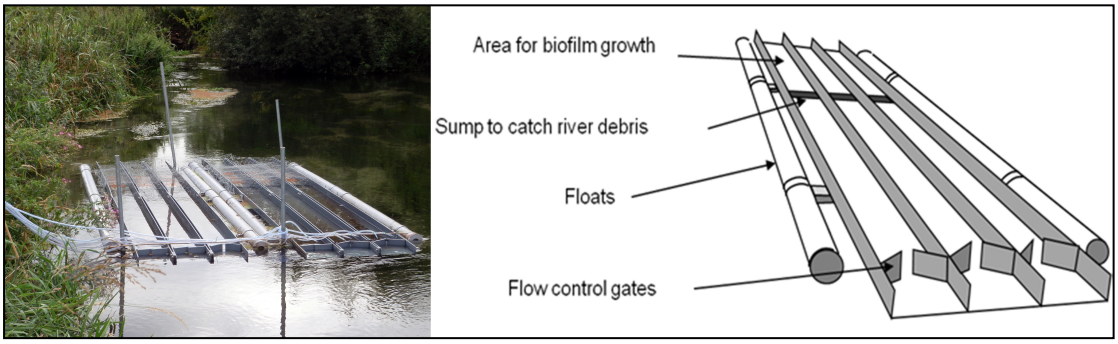
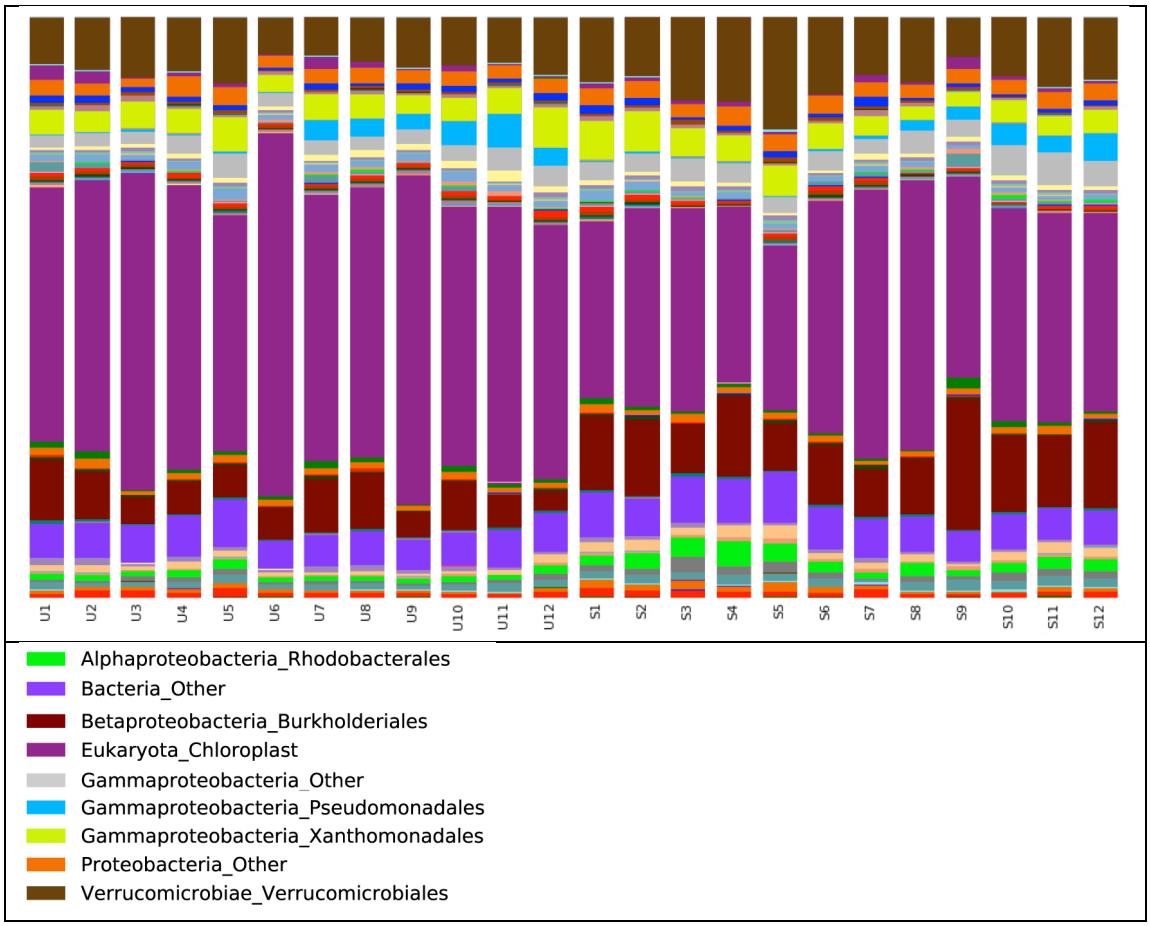


Fig. 2: design and photo of 6 flumes (facing downstream).



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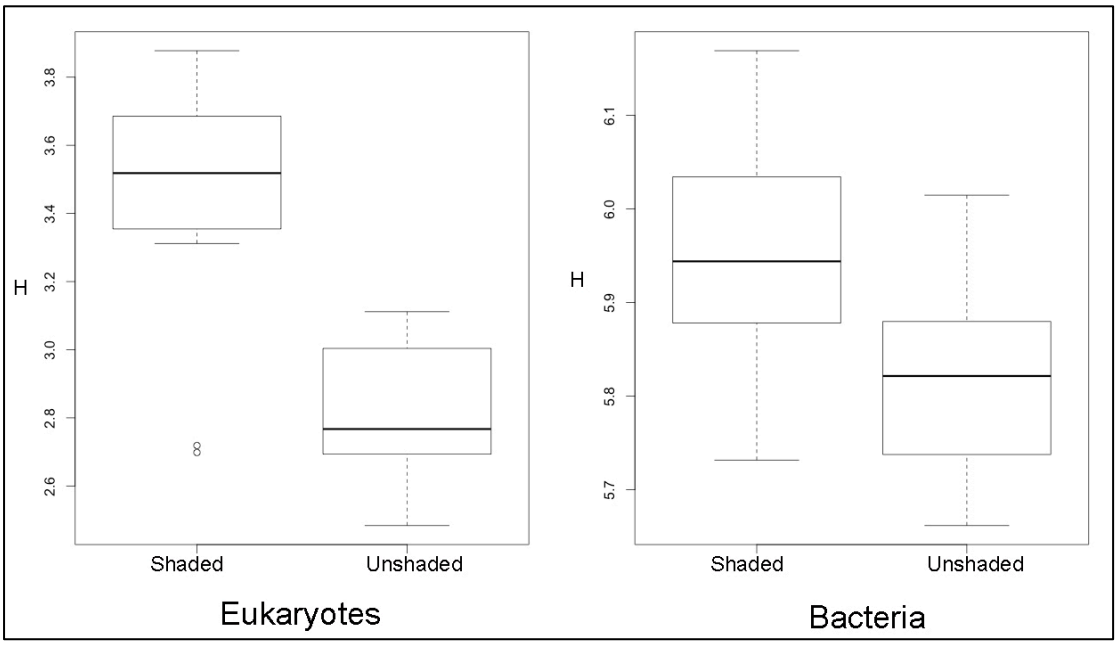

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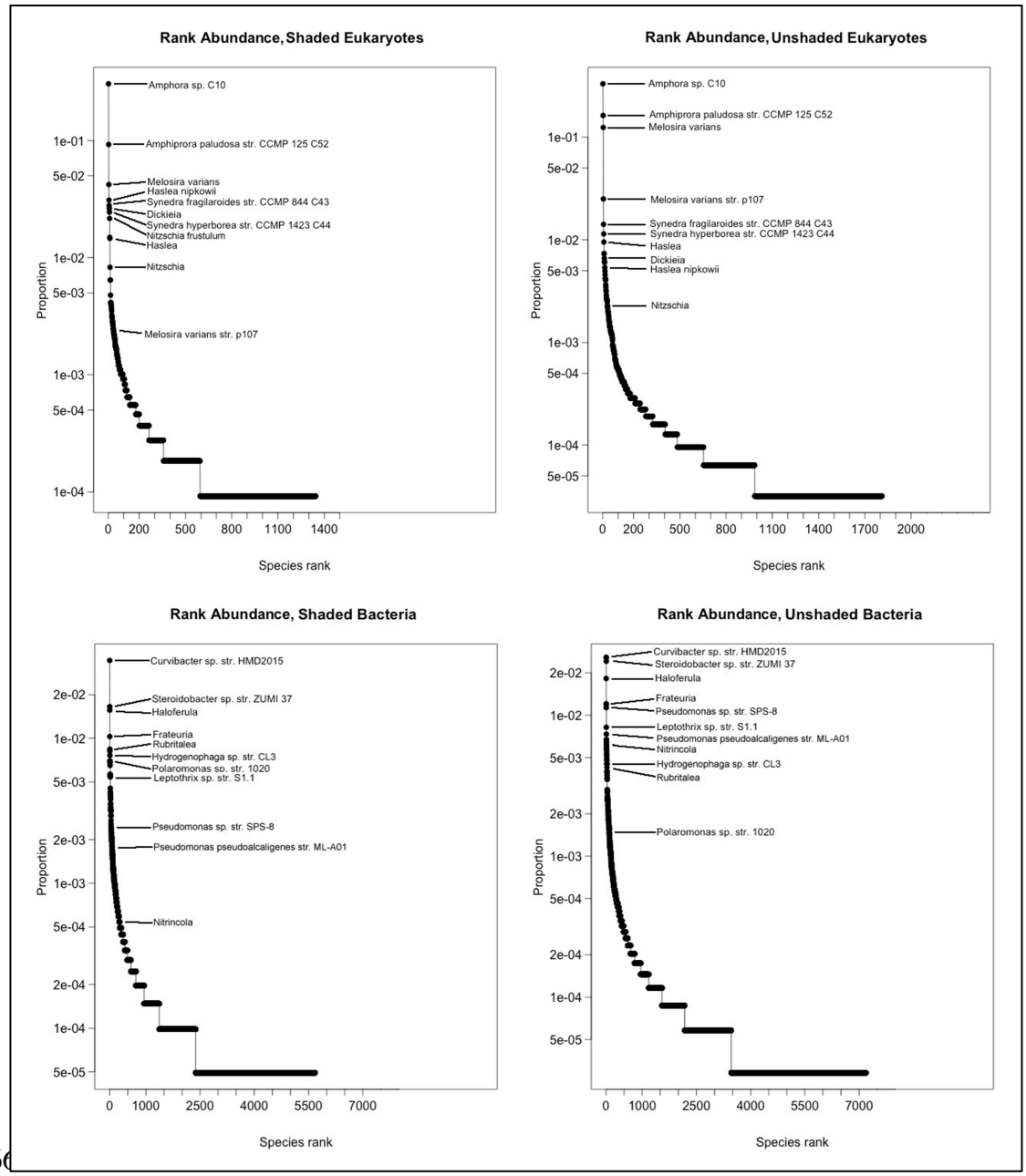

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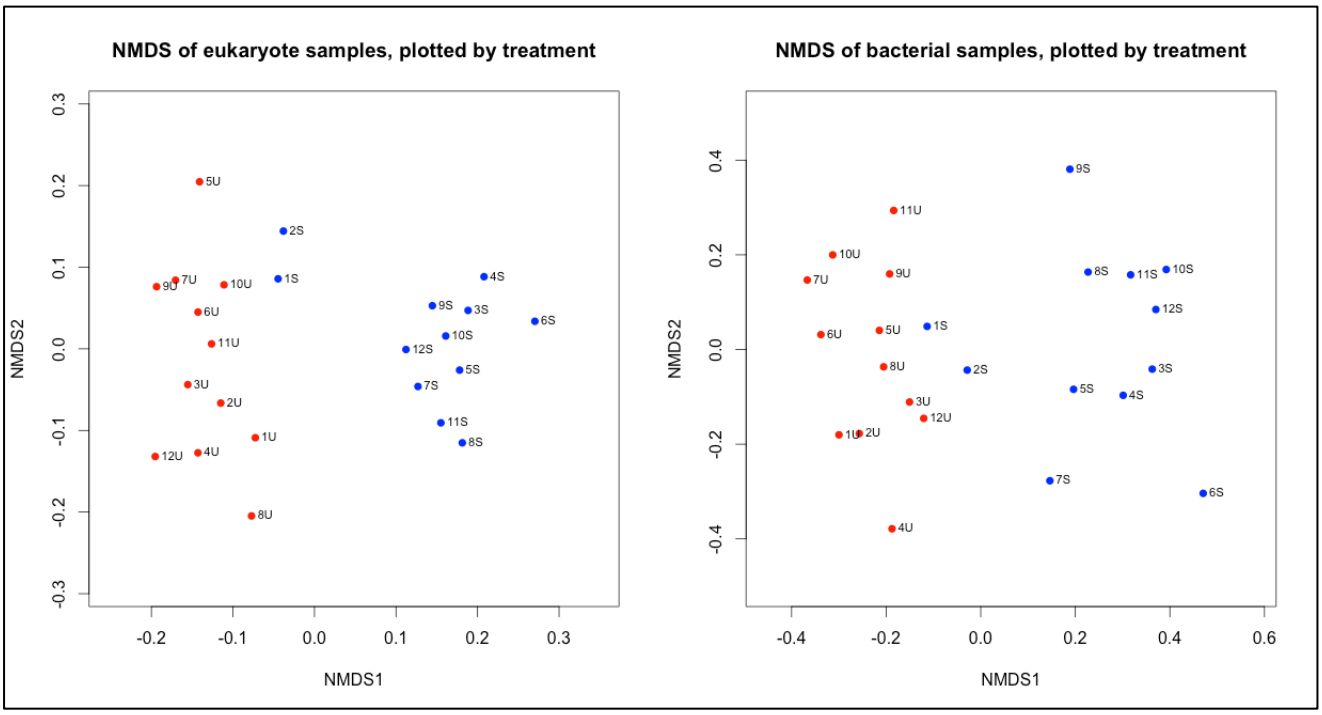

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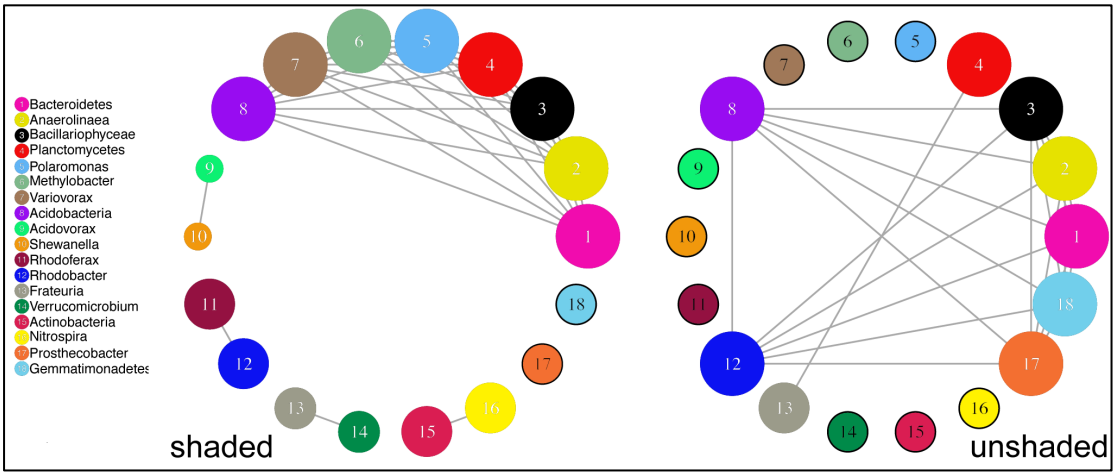

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