# LINKING BENTHIC ALGAL BIOMASS TO STREAM SUBSTRATUM TOPOGRAPHY[1]

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The physical properties of substrata significantly influence benthic algal development. We explored the relationships among substratum surface texture and orientation with epilithic microphytobenthic biomass accumulation at the whole-substratum and micrometer scales. Unglazed clay tiles set at three orientations (horizontal, vertical, and 45°), and six substrata of varying surface roughness were deployed in a prairie stream for 3 weeks. Substrata were analyzed for loosely attached, adnate, and total benthic algal biomass as chl *a*, and confocal laser scanning microscopy was used to measure substrata microtopography (i.e., roughness, microscale slope angles, and three-dimensional surface area). At the whole-substratum level, vertical substrata collected significantly ($P < 0.05$) less algal biomass, averaging 34% and 36% less than horizontal and 45° substrata, respectively. Benthic algal biomass was also significantly less on smoother surfaces; glass averaged 29% less biomass than stream rocks. At the microscale level, benthic algal biomass was the greatest at intermediate values, peaking at a mean roughness of approximately 17 μm, a mean microscale slope of 50°, and a projected/areal surface area ratio of 2:1. The proportion of adnate algae increased with surface roughness (26% and 67% for glass and brick, respectively), suggesting that substratum type changes the efficiency of algal removal by brushing. Individual substrata and microsubstrata characteristics can have a strong effect on benthic algae development and potentially affect reach scale algal variability as mediated by geomorphology.

*Key index words:* confocal microscopy; microphytobenthos; orientation; periphyton; roughness; surface area

*Abbreviations:* CLSM, confocal laser scanning microscopy; CV, coefficient of variation; D, fractal dimension

Stream bottoms are often thought of and studied as two-dimensional (2-D) planes. A 2-D approach is evident by many measurements quantified and reported in areal units (e.g., mg chl $a \cdot m^{-2}$, Dodds et al. 2002). When examined at a smaller scale, such as that experienced by microorganisms (i.e., benthic algae), a stream bottom becomes a complex three-dimensional (3-D) terrain (Bott et al. 1997), and to algae, edges and pits on a substratum may represent ''mountains and valleys'' during assemblage development. As a result of the small scale in which microorganisms grow, relatively minor changes in the physical environment affect colonization processes and assemblage structure (Burkholder 1996, Bergey 2005). Determining the relationships between benthic algal accumulation and substrata physical properties at the pit/crevice (microscale) level, in addition to the whole-substratum (macroscale) level, could further elucidate the heterogeneous nature of benthic communities (Robson and Barmuta 1998) and improve sampling efficiency and standardization. The most prominent physical differences among substrata are surface texture and orientation, and these properties potentially have a large effect on benthic algal development.

Considerable research has been conducted on the effects of the overlying abiotic environment on benthic algae (Stevenson et al. 1996, Wetzel 2001, Murdock et al. 2004); yet, the physical attributes of the underlying substratum have been studied much less frequently. Substratum research has focused mainly on qualitative measurements (Dudley and D'Antonio 1991, Clifford et al. 1992, Johnson 1994, Cattaneo et al. 1997) because of technical limitations of microscale measurement (but see Sanson et al. 1995, Bergey and Weaver 2004, Bergey 2006). Although confocal laser scanning microscopy (CLSM) is used to assess aquatic biofilms (Lawrence et al. 2002, 2004, Larson and Passy 2005, Leis et al. 2005), to our knowledge there have been few attempts to use it to quantify the characteristics of the substrata on which benthic algae grow.

Whole-substratum surface properties affect benthic algal development. Algal accumulation is consistently greater on horizontal surfaces than on vertical surfaces (Vandermeulen and DeWreede 1982, Baynes 1999, Kralj et al. 2006) and on rougher, textured surfaces (Clifford et al. 1992, Sanson et al. 1995). Reasons cited for the greater biomass with texture include increased sedimentation efficiency (Johnson 1994) and cell adhesion (Sekar et al. 2004, Scardino et al. 2006), protection from disturbances, such as scour and grazing (Dudley and D'Antonio 1991, Bergey and Weaver 2004), and alteration of flow around a substratum (DeNicola and McIntire 1990, Jørgenson 2001). As a result of

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the fractal nature of surface topography, it is likely that the surface characteristics evident at the whole-substratum level, such as texture, are influenced by the microscale surface properties of that substratum.

Micrometer-scale surface irregularities such as pits, crevices, and protrusions can also reduce algal susceptibility to grazing and scouring (Lubchenco 1983, Bergey 1999, Bergey and Weaver 2004) and alter diffusion boundary layer thickness (Vogel 1994, Dade et al. 2001, Dodds and Biggs 2002). Additionally, as texture changes the physical dimensions of substratum surfaces, it should also change the availability of resources regulated by these dimensions, such as colonization area and light. Ultimately, though, the assemblage itself will change surface topography during development, providing substrata for further colonization and reducing light and nutrient availability near the substratum surface (Dodds 1991). Thus, surface influence should change during the successional sequence.

The physical properties of texture at the micrometer (i.e., individual algal) scale can create heterogeneous microhabitats across a single substratum, which may lead adjacent algal assemblages to become dominated by species with different adaptive traits. For example, small adnate species can obtain refuge from disturbances in pits (Bergey 2005), but this environment may decrease nutrient and light availability. Larger, loosely attached species can acquire more light and nutrients through vertical growth, but this strategy makes them more susceptible to loss from grazing or scouring (Biggs and Thomsen 1995, Biggs et al. 1998). As a result, physically diverse benthic algal assemblages may develop on different areas of the same or adjacent substrata, markedly increasing local assemblage heterogeneity. Amplified heterogeneity in substrata physical properties due to texture should therefore increase benthic algae microscale, and macroscale, heterogeneity (Downes et al. 1998).

Understanding the basic ecology of colonization and accumulation of benthic algae in streams is vital because of the nonequilibrium nature of most lotic habitats. Scouring floods and inundation/drying oscillations regularly reduce algal biomass and reset successional processes. We hypothesize that changes in micrometer-scale physical properties of a substratum's surface will affect the physical properties (i.e., growth forms) of the accumulating benthic algal assemblage. Our objective was to assess the relationships among surface characteristics and epipelic algal biomass based on coarse, physical groupings of loose and adnate forms. Confocal laser scanning microscopy was used to measure 3-D substratum microtopography, and algal/substratum relationships were evaluated at both micrometer and whole-substratum levels. Microscale quantification also allowed us to address two possible physically based factors that may regulate accumulation (i.e.,

increased surface area due to texture, and reduced light availability due to 3-D sloping and shading). We also briefly assessed the percentage of algal biomass left behind after brushing in relation to its roughness and discuss implications for collection.

## MATERIALS AND METHODS

*Study site.* Experiments were conducted in lower Kings Creek on the Konza Prairie Biological Station, located in the Flint Hills region of the Great Plains, approximately 10 km south of Manhattan, Kansas, USA (39°6.34′ N, 96°36.31′ W). Kings Creek is a headwater stream with an upper watershed of pristine tallgrass prairie and a lower watershed containing a mixture of prairie and agricultural land with oak woodland as the riparian vegetation (Gray et al. 1998, Gray and Dodds 1998). Three successive runs (<30 m between runs) were used to achieve similar current velocities (20–30 cm · s$^{-1}$), depth (15–30 cm), and light regimes (~60% shade duration) among all substrata used in the experiments. The runs were physically similar, with a mean depth of 0.3 m, mean width of 2.5 m, and mean length of 7 m. Stream substratum in the study reach was primarily limestone cobble (5–10 cm).

*Substratum deployment and algal collection.* Benthic algal chl *a* levels were monitored in the study reach after a severe flood (165 m$^3$ · s$^{-1}$) 3 months prior to the experiment to assess the accumulation rate of loosely attached algae and determine the duration of the substrata colonization experiment. The flood scoured all visible algae from the reach. Beginning 5 d after the flood, five rocks were randomly collected every 2 d for the first 2 weeks and then every 4 d for the next 3 weeks. Algae were brushed into a bottle and processed as described below for the natural rock substrata. Algal biomass accumulation began to level off at ~30 d (Fig. 1); therefore, a 3-week colonization period was chosen to achieve a benthic algal mat with both a developed understory and overstory while minimizing sloughing losses.

Thirty 6.5 cm × 20.5 cm unglazed clay tiles (133.3 cm$^2$) were deployed in the middle run from 18 October to 10 November 2004. A stream reach was used that positioned the tiles in an east–west orientation to ensure a similar daily light regime for all tiles [~11:13 light:dark (L:D)]. During the study period, the sun was in the southern half of the sky, with an average declination of 25.4° and maximum declination of 40.4°. Tiles were placed in one of three orientations: horizontal (0°),
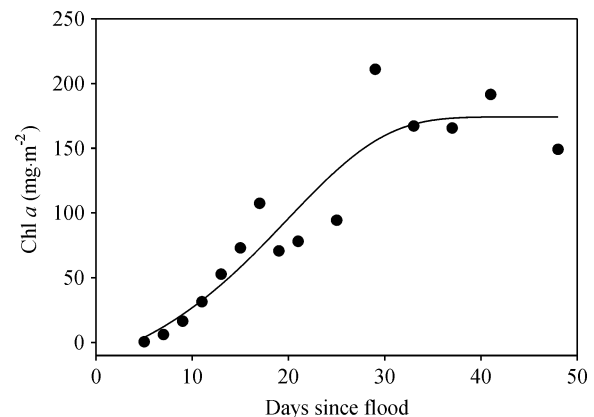


FIG. 1. Benthic algal accumulation in the study reaches following a scouring flood. A second-order sigmoidal curve fit ($r^2 = 0.87$, $P = 0.001$), versus a linear fit ($r^2 = 0.78$, $P = <0.001$), suggests algal biomass began to level off at ~4 weeks after the flood.

vertical (90° from horizontal, facing north, because of heavy sunlight interception shading from south bank vegetation), or 45° from horizontal (facing north) with a total of 10 replicates per orientation. A block design was used, placing five 5.1 cm diameter poly-vinyl chloride (PVC) pipe frames into rows, with each row containing six systematically oriented tiles with two angle replicates per row. The frames were positioned length-wise across the stream, approximately 50 cm apart, and placed in similar current velocities, depth, and light conditions to reduce environmental variability.

Five additional substrata types of varying roughness (glass slides, two glazed tile types, bricks, and sterilized rocks taken from the site) were tested, for a total of six roughness values with the horizontal unglazed tile. Ten replicate PVC sheets, each containing one of each substrata type were deployed in the adjacent runs. Substrata were randomly positioned on each sheet, and all substrata in this experiment were positioned horizontally.

All substrata were removed after 23 d, and individual tile contents were divided into loosely attached and adnate benthic algae. Loose algae are defined as those relatively easily removed by brushing, and adnate as those tightly attached to the substrata (most likely some adnate algae were removed by brushing). Algae were brushed from the top surface of each tile with a stiff nylon brush and rinsed into a bottle (loose algae). Brushing consisted of short strokes (approximately 4 cm per stroke) with moderate pressure over the entire top surface, with each particular area receiving approximately 10 strokes. During substrata brushing, the loosened content was rinsed into the bottle after each third of the surface was brushed. The ''loose algae'' bottle was placed on ice in the field. The brushed substrata, containing the remaining ''adnate algae,'' were placed in individual plastic bags and put on ice.

Samples were analyzed for chl $a$ in the laboratory within 4 h of collection. Each bottle of loosely attached algae was homogenized for 1 min with a hand blender, and a subsample filtered onto a GFF filter (Whatman, Middlesex, UK). Filters and brushed tiles were frozen until chl analysis could be performed. For chl $a$ extraction, filters and tiles were immersed in 95% ethanol:water and placed into a hot water bath at 78°C for 5 min (Sartory and Grobbelaar 1984). Samples were then placed in the dark at 4°C for 12 h. Extracts were analyzed for chl $a$ with a Turner Model 112 fluorometer (Turner Designs Inc., Sunnyvale, CA, USA) using an optical configuration optimized for the analysis of chl $a$ without phaeophyton interference (Welschmeyer 1995). Benthic algae growing on the sides of the substrata were removed as much as possible with brushing, wiping, and rinsing before whole-substratum extraction.

*Substratum microscale measurements.* Substratum surfaces were analyzed using CLSM (Zeiss Axioplan 2 LSM 500 CLSM; Carl Zeiss, Jena, Germany). Images were collected with a 10× plan-neofluar objective (×100, 0.85 mm² per image), with a 488 nm laser, recording all wavelengths of the reflected light. Measurements were taken from five areas on each substrata type of the more homogeneous manufactured substrata, and three areas on five rocks. Each measurement consisted of a series of images of incrementing depth (Z-stack) with vertical resolution ranging from 0.9 μm for glass to 10 μm for brick. Slice thickness was set to optimize vertical resolution while still maintaining a practical scanning time. For example, scanning time for glass with a peak-to-pit distance of 4 μm with an incrementing thickness of 0.9 μm would be similar to scanning a brick with a peak-to-pit distance of 400 μm at a thickness of 10 μm (~10 min).

Z-stacks were analyzed with Image J digital image analysis software (Abramoff et al. 2004; U. S. National Institute of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/) and

an associated plugin, SurfCharJ (Chinga et al. 2003), for mean surface roughness (the average distance from the bottom of all pits, and the top of all peaks to a middle plane that is equidistant from the deepest pit and highest peak), mean microscale orientation (the angle of pit walls and sides of peaks), and increased surface area resulting from increased texture. Fractal dimensions (D) of each surface type were calculated from the CLSM images with ImageJ and the MapFractalCount plugin (http://rsb.info.nih.gov/ij/plugins/index.html), which calculates fractals for 3-D topography, to assess scale-independent surface characteristics among substrata.

In addition to CLSM surface area measurement, surface area was measured using the soapy water method (Harrod and Hall 1962) to compare it with a currently used and more readily conducted technique. Ten replicates of each substratum were weighed, the top surface dipped in soapy water for 20 s, allowed to drip for 20 s, and then reweighed. The weight of the water was then correlated to the 3-D surface area. Projected (2-D) surface areas for each substratum were determined by measuring the length and width of the top surface of the substratum. Areal rock surface area was measured by scanning the outline of a rock into a computer to produce a digital image and then calculating the planar surface area using SigmaScan 5.0 (Systat Software Inc., Richmond, CA, USA). The glass surface was used as the reference value (i.e., 1 mm² of 2-D glass equals 1 mm² of 3-D area). All other surfaces were set to a proportional increase in mass of soapy water retained relative to glass.

*Minimum microscale determination.* Confocal microscopy allows microtopography measurements at a submicron scale; thus, it is possible to focus on a scale smaller than is relevant to benthic algae. For instance, the slope of a 3.2 μm segment on a rock (two pixel widths in our images) would most likely be irrelevant to a 50 μm alga. Finding and applying the most relevant lower scale for image analysis is necessary because changes in this scale can alter microscale measurements. Different minimum scales were evaluated to determine their impact on the resulting microtopography measurements by using a fast Fourier transform (FFT) band-pass filter on the image prior to analysis. This filter enables the user to set a lower and upper limit of a given linear segment used for measurement. We used a minimum segment length of 15 μm and no maximum limit on segment length to analyze substrata images.

We obtained the relevant linear segment length by estimating the median algal size accumulating on the experimental substrata. The smoothest (glass), roughest (brick), and a midroughness (red tile) substrata were redeployed in the same reach, for the same colonization time, during the same month in the following year. Algal cell length was measured from cells growing directly on each substratum with CLSM. Images of algal fluorescence (excitation 488 nm and emission > 650 nm) were collected from three areas on each substratum (20× plan-neofluar objective, 0.21 mm² per area), and 600–900 cells/colonies/filaments were measured per substratum type. The minimum cell length measurable with this method was ~2 μm.

*Data analysis.* At the whole-substratum scale, two-way analysis of variance (ANOVA) was used to compare benthic algae biomass among surface orientations and row placement, and among surface texture and run placement using SPSS 11 (SPSS Inc., Chicago, IL, USA). Multiple comparisons were performed using Tukey's HSD post hoc procedures when significant differences were determined among algal biomass and substrata type or orientation. Linear and nonlinear regression analyses were used to correlate the microscale characteristics of surface roughness, microscale slope, and 3-D surface area to accumulated algal chl using SigmaPlot8 (Systat Software Inc.). Note that the same chl measurements (i.e., loosely attached,

adnate, and total chl from each substratum type) are used for comparison at both whole-substratum and microscopic scales.

Chlorophyll values were adjusted to the 3-D area available for colonization for each substratum type to assess the effects of increased surface area on benthic algae accrual. For example, if a tile had an areal surface area of 100 cm², but its roughness increased the 3-D area to 150 cm², the chl content was calculated as mg chl per 150 cm² and then adjusted to m⁻². Changes in the intensity of direct light hitting a given point due to texture (i.e., changing angles of incidence on substrata) were calculated using Lambert's Law, $E = \cos(\Phi)$, where ($E$) is the proportion of illumination hitting a surface, which varies as the cosine of the angle of incidence ($\Phi$, in radians).

## RESULTS

*Minimum scale determination.* Confocal microscopy revealed substantial physical differences among substrata at an algal-relevant micrometer scale. Figure 2 illustrates these differences, showing 3-D surface plots and a random profile of each surface. Values for surface roughness, microslope orientation, surface area, and D of each substratum increased with surface texture and are listed in Table 1. Detailed trends for each physical component will be discussed below. Microscale measurement differed depending on the minimum segment length used to base the image analysis. As a finer scale is used, mean roughness decreases, mean microslope orientation gets closer to 90°, and surface area greatly increases (Table 2).

Algal cell/filament/colony lengths were similar among glass, red tile, and brick substrata. Across substrata, algal cells had a median length of 13 µm, a mean of 21 µm, a standard deviation of 34, and a range of 546 µm. As CLSM analysis identifies algal chloroplasts, some cell lengths may have been underestimated—especially pennate diatoms, which dominated assemblages during both deployments. Therefore, we adjusted for the underestimation of the dominant cell type and used a minimum substrata segment length of 15 µm for image analysis. The diatom genera *Achnanthes* and *Cocconeis* dominated adnate forms, while loosely attached forms were dominated by stalked diatoms from the genera *Gomphonema* and *Cymbella*, the filamentous green alga *Cladophora*, and filamentous cyanobacterium *Oscillatoria*.

*Orientation.* Benthic algal chl *a* concentrations were significantly different among whole-substratum orientations ($\alpha = 0.05$, $P = 0.001$ for loose, adnate, and total algae), but not different between rows ($P = 0.465$, $P = 0.879$, and $P = 0.490$ for loose, adnate, and total algae, respectively). No significant orientation by row interaction effects was observed for any algal form ($P > 0.05$ for all forms). The 0° and 45° tiles accumulated significantly greater loose and adnate algae than the 90° tiles but were not significantly different from each other (Fig. 3a). Total chl variability was also greater within the 0° and 45° tile replicates than in the 90° replicates (Levene test

of constant variance, $P = 0.011$). Although differences were observed in biomass and variability, the proportion of loose versus adnate forms was not significantly different among orientations ($P = 0.363$). Loosely attached algae averaged 53%, 57%, and 54% of the total algae for 0°, 45°, and 90° tiles, respectively.

Microslope orientation varied from a 1° mean slope for glass, to 63° for brick (Table 1). Adnate, loose, and total benthic algae biomass increased in a Gaussian pattern with increasing microscale slope (adnate: $r^2 = 0.84$, $P < 0.0001$; loose: $r^2 = 0.42$, $P < 0.0001$; total: $r^2 = 0.77$, $P < 0.0001$; Fig. 4), and peak biomass occurred at a slope of ~50°. Above 50°, adnate chl did not change (ANOVA, Tukey's post hoc comparisons, $P = 0.42$), but loose and total chl decreased (Tukey's post hoc, $P = 0.002$ and $P = 0.001$, respectively). The greatest change in chl with microslope angle occurred at an intermediate orientation (16°–50° mean slope). In this portion of the curve, adnate chl increased linearly at roughly twice the rate (1.3 mg chl $a \cdot$ m⁻² $\cdot$ degree⁻¹) as loose chl (0.55 mg chl $a \cdot$ m⁻² $\cdot$ degree⁻¹), and these trends were stronger for adnate ($r^2 = 0.72$, $P < 0.001$) than loosely attached forms ($r^2 = 0.24$, $P < 0.001$). The mean proportion of loose to adnate algae decreased linearly ($r^2 = 0.96$, $P < 0.0001$) with increased microscale slope at a rate of 0.7% per degree, and assemblages switched from a dominance of loose forms to adnate forms at a mean microslope angle of ~50°.

*Surface roughness.* At a qualitative whole-substratum level, texture significantly influenced the accumulation of both growth forms ($P < 0.001$ for adnate, loose, and total algae; Fig. 3b). Loosely attached algae differed between runs ($P = 0.001$), but adnate and total algae did not ($P = 0.379$ and $P = 0.056$, respectively). There were no substrata-by-run interaction effects ($P > 0.05$ for all types). Smoother substrata (i.e., glass and glazed tiles) collected less adnate and total algae than the rougher unglazed tile, natural rock, and brick. Maximum loosely attached chl was observed on an intermediate roughness, but no strong pattern was exhibited. The proportion of loose to adnate algae decreased on increasingly rougher surfaces, with loose forms comprising 78%, 71%, 65%, 53%, 45%, and 33% on glass, white tile, red tile, unglazed tile, rock, and brick, respectively.

Microscale surface roughness differed by two orders of magnitude between the smoothest surface, glass (0.87 µm), and the roughest surface, brick (53.8 µm; Table 1). Benthic algal biomass exhibited a right-skewed peak (best fit with a Weibull distribution, SigmaPlot 8, Systat Software Inc.) over the range of roughness used (adnate: $r^2 = 0.85$, $P < 0.0001$; loose: $r^2 = 0.42$, $P < 0.0001$; total: $r^2 = 0.74$, $P < 0.0001$; Fig. 5). Algal biomass increased linearly with increasing surface roughness,
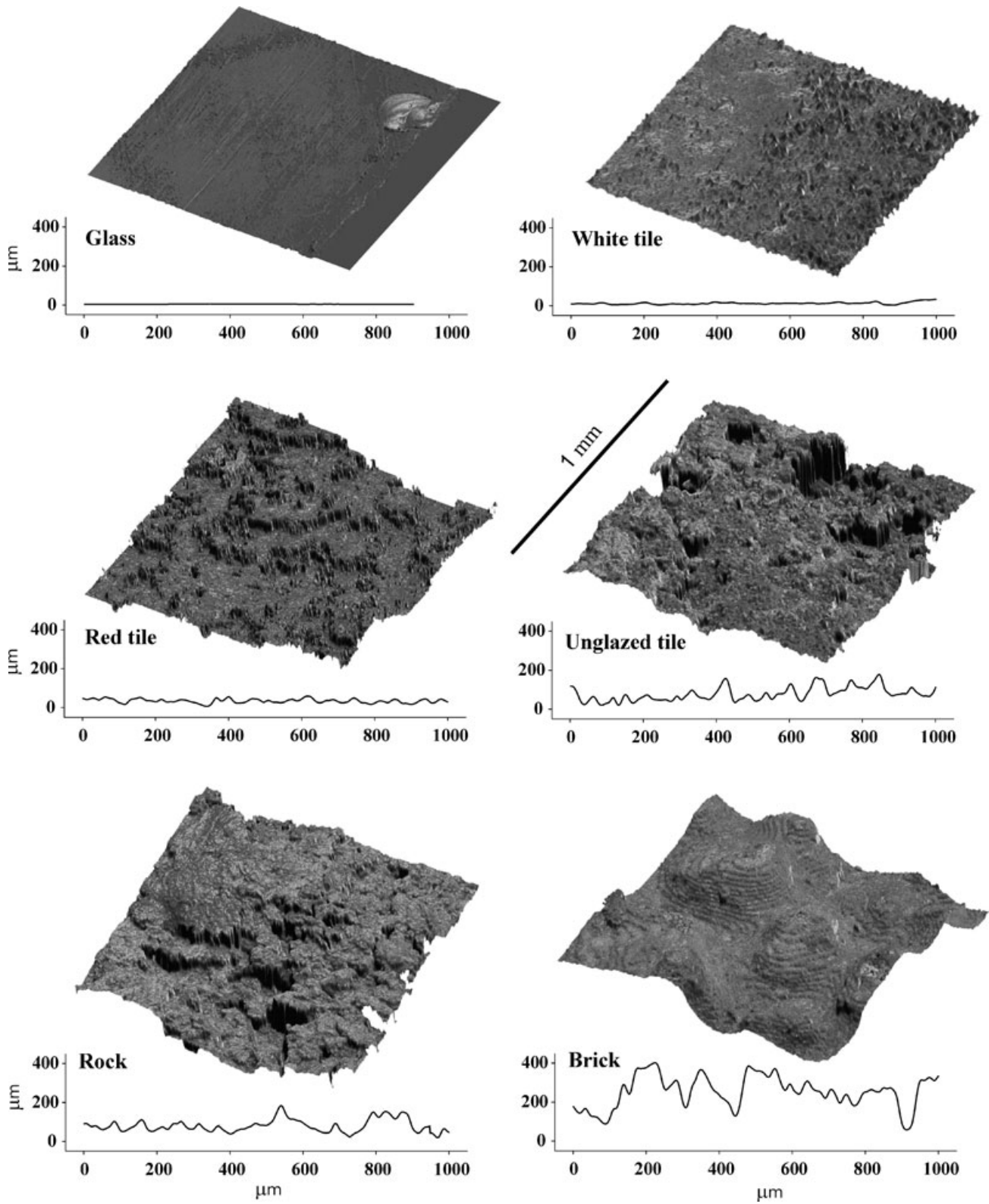
FIG. 2. Representative 3-D surface plots and 2-D profiles of experimental substrata used. Images collected from confocal laser scanning microscopy.

TABLE 1. Substratum surface characteristics for the six substrata used in the experiment.

| Substratum | Mean roughness (μm) | Surface area increase | | Mean microslope orientation (degrees) | % of microslopes <45° | Fractal dimension (D) |
|---|---|---|---|---|---|---|
| | | Soapy water | Confocal | | | |
| Glass | 0.87 (0.05) | 1.00 | 1.00 | 1.05 (0.12) | 99.8 (0.48) | 1.983 (0.035) |
| White tile | 3.19 (0.45) | 2.80 | 1.07 | 16.4 (2.28) | 97.8 (1.10) | 2.478 (0.085) |
| Red tile | 5.99 (1.36) | 1.41 | 1.25 | 31.5 (2.85) | 80.6 (6.77) | 2.250 (0.068) |
| Unglazed tile | 15.2 (9.53) | 4.14 | 1.88 | 43.6 (16.0) | 51.6 (33.1) | 2.419 (0.168) |
| Rock | 17.1 (9.62) | 6.52 | 2.00 | 49.7 (10.0) | 37.4 (21.2) | 2.047 (0.163) |
| Brick | 53.8 (25.0) | 5.93 | 3.53 | 63.1 (6.94) | 15.6 (8.71) | 2.445 (0.167) |

Numbers in parentheses indicate one standard deviation. Surface area increase is the proportion of 3-D projected surface area on a substratum compared with the 2-D area using the soapy water and confocal laser scanning microscopy methods. Microslope is the angle of the pits walls.

TABLE 2. Changes in microscale measurements with increasing lower-limit resolutions on a single surface image (brick).

| Smallest segment length measured (μm) | Mean roughness (μm) | Mean microslope orientation (degrees) | Surface area (mm$^2$) |
|---|---|---|---|
| 1.8 | 61.8 | 84.1 | 15.2 |
| 5 | 40.2 | 75.9 | 5.4 |
| 10 | 36.5 | 63.8 | 2.7 |
| 15 | 34.7 | 54.6 | 1.9 |
| 20 | 33.9 | 48.3 | 1.6 |
| 50 | 28.5 | 29.5 | 1.1 |
| 100 | 23.2 | 18.6 | 0.9 |

As more surface detail is accounted for, the measured values of each category increase and change disproportionately to one another. (1.8 μm is the digital image pixel size.)

up to a mean roughness of ~17 μm. In the linear portion of the curve, adnate algae increased at twice the rate as loose algae, increasing at 3.9 and 1.8 mg chl $a \cdot m^{-2} \cdot μm^{-1}$ roughness, respectively. This trend was stronger for adnate forms ($r^2 = 0.84$, $P = 0.001$) but was still significant for loose algae ($r^2 = 0.31$, $P = 0.001$). The proportion of loosely attached forms decreased linearly at a rate of 0.8% ($r^2 = 0.81$, $P = 0.001$) with each μm increase in mean roughness. Loosely attached dominance gave way to adnate dominance at ~17 μm.

Substrata with greater heterogeneity in surface roughness (coefficient of variation, CV) collected more total chl (linear regression, $r^2 = 0.91$, $P = 0.03$; Fig. 6). However, chl heterogeneity (CV)
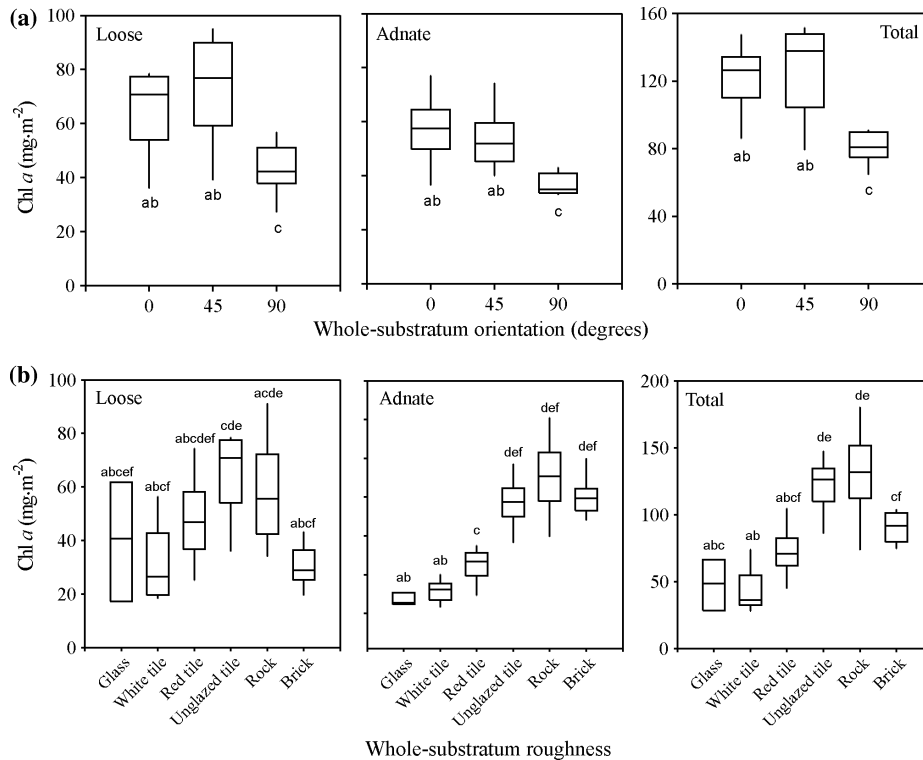


FIG. 3. (a) Distribution of benthic algal chl $a$ on tiles set out at 0°, 45°, and 90° relative to stream bottom and divided into loosely attached, adnate, and total chl. (b) Benthic algal chl $a$ values for substrata of varying surface roughness. Boxes represent the median, and 25th and 75th quartiles. Whiskers show values within 1.5 times the interquartile range. Boxes with the same letter indicate no significant difference, $P < 0.05$.
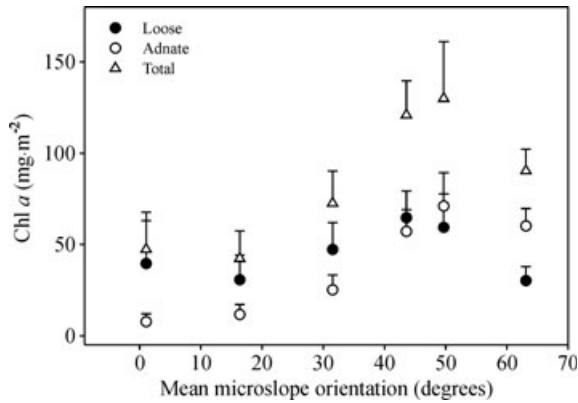
FIG. 4. Chlorophyll *a* concentration versus mean microslope angle (i.e., angle of pit walls) for loose, adnate, and total benthic algae. Biomass increases with increased pit wall angle up to approximately 50° and then decreases. Bars are 95% confidence intervals, and only the top interval is shown.
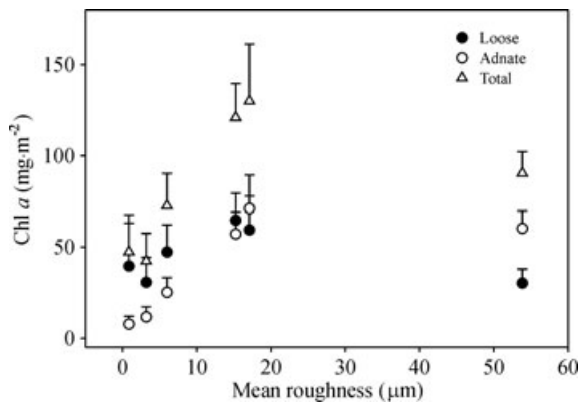


FIG. 5. Chlorophyll *a* concentration versus mean roughness for loose, adnate, and total benthic algae. Biomass increases linearly with roughness until approximately 17 μm and then begins to decrease. A shift in dominance from loosely attached to adnate forms occurs around a roughness of 15–17 μm. Bars are 95% confidence intervals.
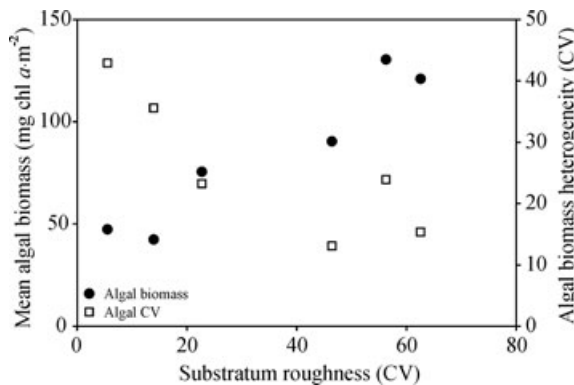


FIG. 6. Comparison of the variation of roughness on a substratum (coefficient of variation, CV) with the mean algal biomass and biomass variability (CV) on each substratum type. Algal biomass increased with roughness heterogeneity across a surface, but algal biomass variability decreased with roughness heterogeneity.

on a particular substratum type decreased linearly ($r^2 = 0.71$, $P = 0.04$) with increasing heterogeneity in surface roughness. Substrata D generally increased with increasing roughness (Table 1), but no significant trends were found with D and benthic algal chl.

*Surface area and light intensity.* Both the CLSM method and the soapy water method showed an increase in the 3-D surface area with increasing roughness; however, the soapy water measurements were consistently greater than the CLSM results (Table 1). Regression analysis of surface roughness versus CLSM measured area increase showed a linear gain in surface area of approximately 0.047 mm$^2$ · μm$^{-1}$ average roughness ($r^2 = 0.89$, $P < 0.001$). The soapy water method showed a linear gain in surface area of 0.098 mm$^2$ · μm$^{-1}$ increase in average roughness ($r^2 = 0.69$, $P = 0.05$). Confocal microscopy results were used for further comparative analysis for consistency in the method used to collect substrata measurements.

Surface-area-adjusted chl differed significantly across substratum types for adnate and total algae ($P < 0.001$ and $P < 0.001$, respectively; Fig. 7), showing a similar trend to the nonadjusted values, and peaked at a 3-D to 2-D area ratio of 2:1. Loosely attached chl, though, was only significantly different on the roughest surface.

Increasing surface area enlarged the angle and length of pit walls. Table 3 shows the calculated availability of direct light on the smoothest, an intermediate, and the roughest substratum used in this experiment with varying substratum and microscale slopes, and incoming light directions. Direct light intensity on a given area of substrata was reduced 45% from glass to brick.
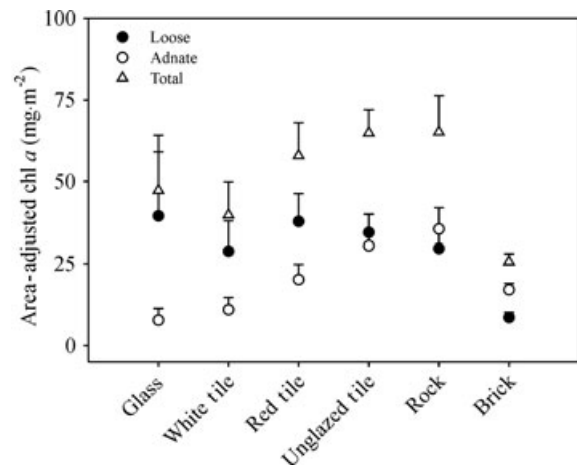


FIG. 7. Chlorophyll *a* values for loose, adnate, and total benthic algae adjusted for increased surface area due to increased roughness. This equalizes the surface area and chl relationships among all substrata. Loose forms only differed significantly on the roughest surface, while adnate and total forms still showed a pattern similar to before adjustment. Bars are 95% confidence intervals.

TABLE 3. Example of how direct light intensity on a given point on a surface changes with whole-substratum orientation and microslope angle.

| Substratum (mean microslope angle) | Whole substratum orientation | Light hitting surface (% of full intensity) | | |
|---|---|---|---|---|
| | | Incident light directly above (all surfaces) | Incident light at 45° angle | |
| | | | Surfaces facing toward | Surfaces facing away |
| Glass (1.1°) | Horizontal | 99.9 | 72.0 | 69.4 |
| | Vertical | 0.0 | 69.4 | 0.0 |
| Unglazed tile (44°) | Horizontal | 72.4 | 99.9 | 2.4 |
| | Vertical | 0.0 | 2.4 | 0.0 |
| Brick (63°) | Horizontal | 45.2 | 95.1 | 0.0 |
| | Vertical | 0.0 | 0.0 | 0.0 |

Calculated values (from Lambert's cosine law) are given for a substratum horizontal to the stream bottom and one rotated 90° (vertical), for situations where the light is coming from directly overhead, and from a 45° angle in the same direction as the 90° tilt.

## DISCUSSION

*Algal biomass.* Algal biomass was significantly affected by substratum texture. At the whole-substratum scale, our results generally support the previous findings that rougher (Clifford et al. 1992, Johnson 1994, Sanson et al. 1995) and more horizontal surfaces (Knott et al. 2004, Kralj et al. 2006) collect more algae. Yet our results differed in that biomass peaked at an intermediate roughness, suggesting that the stimulatory effects of increased texture on algal biomass accrual decrease as roughness increases past a certain point. Confocal microscopy measurements identified this peak at about 17 μm, which was similar to the roughness of the natural rocks found in this stream. It is tempting to speculate that more algal species were adapted to the surface roughness of the rocks, and that is why the peak biomass accrual occurred at a roughness approximately equal to natural rock roughness. The true biomass peak though, may lie in the roughness range between unglazed tiles and bricks, as there was a large gap between these roughness values. Algal biomass has been linked to other physical substratum characteristics, such as size (Watermann et al. 1999) and stability (Cattaneo et al. 1997), and it is likely that roughness is equally important in regulating a substratum's physical effect in streams.

Surface roughness is fractal in nature, and microscale roughness affects whole-substratum roughness. Thus, similar mechanisms may be regulating algal growth at both macro- and microscales. For example, edges of substrata tend to accumulate more algae than the interior surface (Comte et al. 2005), and rougher textured substrata (i.e., those with more microscale edges) tend to collect more algae (Clifford et al. 1992, Sanson et al. 1995). Additionally, algal cell attachment efficiency has been observed to increase with roughness at scales ranging from 1 to 14 μm (Scardino et al. 2006), from 0.1 to 1.2 mm (Johnson 1994), and from 2.0 to 4.0 mm (Sanson et al. 1995). Therefore, our focus on roughness was not necessarily a comparison between scales, but an illumination and quantification of roughness that can be applied to both scales. Our data support the theory that in addition to macroscale texture (i.e., stream bed roughness, Quinn et al. 1996), microscale roughness has significant effects on algal accumulation. Additionally, we conclude that qualitatively assigning texture as smooth and rough decreases the accuracy of cross-site comparisons of substrata effects as these classifications are subjective and scale dependent.

More algal accrual on horizontal than vertical surfaces in our study was consistent with findings in other systems and on varying substrata, such as glass slides in a Croatian lake (Kralj et al. 2006); asbestos plates in a British Columbian bay (Vandermeulen and DeWreede 1982); and pontoons, concrete breakwalls, and rocky reefs in Sydney bays (Glasby and Connell 2001, Knott et al. 2004). Studies looking at surface angles between 0 and 90° are rare; however, the accumulation of the green macroalga *Cladophora* in Tosa Bay, Japan, on 0, 45°, and 90° acrylic tiles (Somsueb et al. 2001) followed very similar accumulation patterns to loosely attached algal growth on the angled tiles in Kings Creek.

Surface orientation is distinct at both the substratum and microscale levels and can be quantified independently at both scales. Unlike chl and orientation at the substratum scale, which was only significantly different at 90°, chl at the microscale orientation peaked at an intermediate level. However, comparison with whole-substratum orientation is limited because none of the substrata had a mean microscale slope close to 90°; but it is unlikely that many natural substrata have a mean microscale slope close to 90°. A larger gradient of orientations at the substratum scale may have made microscale differences more apparent. Nevertheless, significant differences between 0° and 45° at the microscale level suggest that effects of orientation may change with scale. Determining whether surface angles have more influence at the scale of an individual alga, or if substratum orientation overrides the effect of microscale angles, will require further study.

Consistent patterns at the scale of individual algal cells showed that microtopography differences can

structure assemblages during development at the coarse separation of loose and adnate forms, and this may be partially a result of differential grazer removal abilities with texture (Dudley and D'Antonio 1991, Bergey and Weaver 2004, Hutchinson et al. 2006). Rougher surfaces appear to benefit both forms but favor the accumulation of tightly attached forms. There also appeared to be an optimal roughness and orientation range for both forms, with a wider optimal range for adnate forms, as shown by their smaller decline from the unglazed tile to the brick. All trends were consistently the strongest for adnate benthic algae, which is logical because this group is more closely associated with the surface.

The mechanisms behind changes in algal biomass and form with varying texture are not well understood. Microtopography measurements allow us to further examine three aspects of surface texture that may regulate resource availability and therefore influence algal accumulation: (i) overall surface heterogeneity, (ii) surface area available for colonization, and (iii) light availability on the surface.

*Microscale heterogeneity.* Habitat heterogeneity is a major driver of community diversity in lotic ecosystems and has been observed at the scale of watersheds (Vannote et al. 1980, Griffith et al. 2002), pools and riffles (Stevenson 1997), and substrata (Taniguchi and Tokeshi 2004). As long as the environment varies on a scale relevant to the organism in question, we should expect a biological response to changing physical conditions, even at a microscopic scale. The positive correlation of algal biomass with substratum roughness (CV) conforms to this presumption. Decreased variability in algal biomass with increasing variability in substratum roughness was unexpected and deserves additional study. The decrease in variability in algal biomass may be the result of grazer/cell immigration/roughness interactions. At the riffle scale, Poff and Nelson-Baker (1997) observed no effect of substratum surface heterogeneity on algal biomass (CV) in an ungrazed system, but different (albeit increased) biomass variability in snail-grazed systems. Biofilm abundance patchiness also increased with individual substratum roughness in marine rocky intertidal biofilms because of grazing and algal recruitment efficiency (Hutchinson et al. 2006).

Rougher surfaces have deeper and steeper sloped pits, resulting in portions of the "extra" area becoming partially or completely shaded. This shading creates a mosaic of light intensities across a small area, possibly allowing species with different light requirements to coexist and increase overall assemblage variability and diversity (Steinman 1992). Also, light heterogeneity increases as the sun moves across the sky and regions that were shaded become lit and vice versa. Deeper pits also increase the distance of the diffusion boundary layer between a resident algal cell and the overlying water (Jørgenson 2001). As a result, living in a pit may

reduce nutrient availability and slow waste removal (Hart and Finelli 1999). Still, deep pits are not completely uninhabitable by benthic algae and may provide benefits. Motile algae such as pennate diatoms and flagellates can move vertically, maximizing resource availability and protection from the substrata (Consalvey et al. 2004, Underwood et al. 2005). Fish grazers, such as *Campostoma anomalum,* which were common during the study, have mouthparts that scrape the substratum's surface but may not be able to get into the smaller crevices (Matthews et al. 1986, Bergey and Weaver 2004). Increased roughness traps more detritus (Bergey 1999, Taniguchi and Tokeshi 2004), which could serve as a nutrient source for colonizing algal cells when mineralized by heterotrophic microorganisms. Finally, greater algal cell deposition on a rougher substratum may provide a larger initial base of cells to start reproducing, and increased sediments may provide additional nutrients to feed benthic algae growth.

*Surface area.* As surface roughness increased, surface area increased. If the increase in algal biomass was solely because of the increased area, then the adjusted chl values would be the same for each substratum type. This was generally true for loosely attached algae. Adjusted values were only different for the roughest surface, suggesting surface area availability is important for loose forms. Adnate forms (as well as total algae), though, were not strongly regulated by surface area. For tightly attached forms, it appears that after a certain 3-D increase (2:1 in this study) other factors appear to negate the positive effects of more surface area. A similar trend of decreasing algal biomass per area with increasing surface complexity at a centimeter scale was observed by Robson and Barmuta (1998). This trend was attributed to the added area creating lower quality attachment sites through less nutrient availability and more shading.

*Light.* Light often limits benthic algae growth (Hill et al. 1995, Roberts et al. 2004), and substrata texture changes light availability. Microscale texture increases surface area but decreases the intensity of light across that surface area (Table 3) because the same amount of light energy is distributed across a larger area (Lambert's Law). Additionally, direct light intensity changes with whole-substratum orientation and light direction because of microscale slope angle. For example, when the substratum (mean microscale slope of 63°) is horizontal and the light is directly overhead, all surfaces receive approximately the same amount of light, which is 45% of the incoming intensity. If that surface is rotated 90° and the light source changes to a 45° incident angle, surfaces facing the light source receive almost 100% of the incoming intensity, while surfaces facing away from the light receive no direct light. The influence of whole-substratum tilting is greater on rougher surfaces, as increasing

roughness increases the mean microscale slope of a surface. Our model is simplistic and does not take into consideration other factors that affect light availability to benthic phototrophs, such as light that is scattered, refracted, or absorbed by organism pigments (Kuhl et al. 1996), or differential properties related to variation in wavelength (DeNicola et al. 1992, Kelly et al. 2003). The model is presented to highlight the potential heterogeneity in light imparted by surface roughness characteristics.

We assume that chl content is equally related to algal biomass for both loose and adnate forms. When light becomes limiting, benthic algae can increase chl content (Thomas et al. 2006), and adnate biomass may be overestimated. In the future, CLSM methods could be used to measure algal biovolume relationships with surface texture, which would address this limitation. The above influence of texture might also be limited to adnate forms and/or early to midsuccessional assemblages. Once benthic algal mats become thicker and grow further from the substratum's surface, surface influence should diminish, with other factors dominating growth and loss dynamics.

*Implications for collection.* Artificial substrata are frequently used to assess stream benthic algae assemblages (Aloi 1990, Cattaneo and Amireault 1992) and have advantages over sampling natural substrata, such as reduction in algal variability and known time of colonization (Meier et al. 1983), and some (e.g., glass slides) can allow direct examination of benthic algal structure. Our study divided algae into loosely attached (mostly green filaments) and adnate (mostly diatoms) categories, and our data are concordant with the previous findings that artificial substrata can produce different green algae, cyanobacteria (Cattaneo and Amireault 1992), and diatom abundances (Barbiero 2000). We show that gross growth forms can also be significantly affected by substrata, and a component of this difference may be the result of microscale texture.

The adnate portion of benthic algae in this study is analogous to that left behind after algae are removed during collection. A common collection technique is to brush or scrape rocks rather than extracting the entire rock for chl. On all surfaces, adnate forms were a large proportion of total biomass and exhibited more variability among substrata types than loose forms. Several studies have looked at the efficiency of brushing or scraping rock (Jones 1974, Cattaneo and Roberge 1991) and have also observed that significant amounts of algae can be left after scrubbing. This collection bias can potentially underestimate biomass as well as alter the proportion of adnate algae during identification.

To get more accurate estimates of benthic algae biomass, we recommend using substrata that are similar in texture and orientation to that of the nat-ural stream and to extract the entire substratum for chl measurements, when possible. Ideally, it would be best to identify algal species on the surfaces they grow, and this has been suggested for some time (Jones 1974). However, currently this is neither easily done nor economically feasible for most researchers. Scraping and brushing substrata can leave behind significant amounts of adnate algae, which may differ with substrata type and brushing effort. Benthic algal data should therefore be collected, analyzed, and interpreted with knowledge of this limitation.

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