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Quantitative imaging to measure photosynthetic biomass on an intertidal rock-platform

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ABSTRACT: Conventional methods for measuring amounts of epilithic photosynthetic biomass in intertidal habitats by estimating amounts of chlorophyll in rock samples are imprecise, laborious and destructive. An alternative remote sensing method for quantifying chlorophyll amounts at small spatial scales using field-based digital colour-infrared (CIR) imagery is presented. CIR images were obtained from 4 areas of the emersed rock-platform. Experimental plots were initially scraped to remove any macro- or micro-algae. After different periods of recolonization of micro-algae, images of the plots were acquired under full sunlight or under artificial shade, before and after the rocks were uniformly wetted. Samples of rock were taken for laboratory determination of amount of chlorophyll. Relative absorption by chlorophyll was estimated from CIR data using a ratio of near-infrared (NIR) and red bands. The image ratios were validated by comparing them to data from a field spectrometer. Measurements of the amount of chlorophyll extracted from rock samples were linearly related to estimates from the NIR:red ratio. Ratios derived from images acquired under full sunlight, before and after wetting the surface, had the strongest relationship with chlorophyll (r^2 = 0.84 and 0.83, respectively). Artificial shading of the rock reduced the strength of the relationship between chlorophyll and the NIR:red ratio before and after wetting (r^2 = 0.79 and 0.63, respectively). Micro-algae in scraped areas and micro- and macro-algal mixtures could be estimated using the same equation. The technique enables rapid contiguous *in situ* measurements of chlorophyll to be made without the microflora being destroyed and will facilitate more comprehensive studies of competitive interactions among intertidal grazing animals.

KEY WORDS: Epilithic · Micro-algae · Intertidal · Chlorophyll · Remote sensing

INTRODUCTION

Autotrophic micro-algal biofilms are essential to the functional ecology of rocky intertidal habitats (Underwood 1979, Hawkins & Hartnoll 1983, Thompson et al. 2004). They provide attachment for germinal stages of algae, and influence the settlement of invertebrate larvae (Morse et al. 1984, Wahl 1989, Rodriguez et al. 1993). Of primary importance is their role as the major food of herbivorous grazing animals, through which they contribute to higher tropic levels in the food-web (Castenholz 1961, Underwood 1976, Creese & Underwood 1982, Underwood 1984a). Micro-algal biofilms are comprised of variable assemblages of diatoms, cyanobacteria and the spores and germinal stages of macro-algae. Conventionally, their abundance is estimated either directly by counting individual algal cells using light, scanning electron or confocal laser scanning microscopy (e.g. Nicotri 1977, Underwood 1984b, Hill & Hawkins 1990, 1991, Nagarkar & Williams 1997, Norton et al. 1998, Neu et al. 2004), or indirectly by measuring the amount of chlorophyll in samples (see review by Thompson et al. 1999). These techniques are laborious and involve the removal of algae from the rock surface or destructively sampling the surface itself. Random-point sampling strategies cannot pro-

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vide contiguous measurements at small spatial scales. Therefore, new techniques that can rapidly make contiguous quantitative measurements of chlorophyll *in situ* without destructive sampling of the microflora are needed. Saravia et al. (1999) used digitized colour photographs to estimate chlorophyll in periphyton grown in the laboratory on artificial surfaces (polystyrene tiles). This technique would be ineffective on natural substrata because of the dominance of colour variations in the rock surface which are unrelated to the amount of chlorophyll present.

Remote sensing using colour-infrared (CIR) photography has been shown to be effective for the estimation of chlorophyll in soft sediments (Murphy et al. 2004), but this has not been demonstrated for chlorophyll in epilithic algae. Quantifying chlorophyll in epilithic algae is potentially more difficult because it is often present in smaller quantities than that found in soft sediments, where values are known to exceed 50 µg cm^{-2} (see review by MacIntyre et al. 1996). Rock surfaces are also generally brighter than mud and this can mask small changes in reflectance due to chlorophyll.

This paper describes a novel approach to quantifying amounts of chlorophyll at very small (<1 mm) spatial scales over large (654 by 488 mm) areas of rocky intertidal habitat using digital CIR imagery. Chlorophyll is estimated from a ratio of reflectance at near-infrared (NIR) and red bands (Jordan 1969). The NIR:red ratio works by detecting absorption of chlorophyll using the reflectance at NIR wavelengths (where chlorophyll does not absorb) as a reference. Reflectance at red wavelengths (where chlorophyll is maximally absorptive) is used as the denominator. A ratio, rather than individual camera bands, was used because it removes variations in brightness due to the inherent spectral qualities of the rock in addition to differences in illumination caused by small scale variations in surface topography. This ratio is strongly related to amount of chlorophyll on rock surfaces (Murphy et al. 2005).

Intertidal ecologists often measure epilithic chlorophyll to estimate the amount of micro-algal food available to grazing animals. Chlorophyll is, however, also present in mature encrusting macro-algae which do not constitute a primary food source of many grazing animals. Most studies which measure the total amount of chlorophyll from samples make tacit assumptions that the chlorophyll in samples originates from micro-algae and is therefore readily available to microphagous grazers (e.g. Dye & White 1991, Lasiak & White 1993, Boaventura et al. 2002, 2003). In areas where there is great variability in amounts of mature encrusting macro-algae, it is first necessary to scrape the rock surface free of macro-algae and to then allow micro-algae to recolonize the surface before samples are taken for chlorophyll analysis (e.g. Underwood 1984a,b). Alternatively, in situations where encrusting algae can be visually distinguished on the rock surface, sampling could be stratified to avoid the algal crusts, provided that such stratification is consistent with the hypotheses being tested.

The study sites used for this experiment are typical for southeastern Australia in that there is some cover of encrusting macro-algae present on all intertidal rock surfaces. To measure all the chlorophyll present, it is not necessary to scrape the rock surface for the presented techniques to work. To determine amounts of chlorophyll in the micro-algae themselves, it was necessary to have previously removed all encrusting macro-algae from the surface of the rock.

Remote sensing of chlorophyll in rocky intertidal areas is complicated by variable amounts of surface moisture. Surface moisture can affect the reflectance at each pixel in the image in 2 ways. First, wetting a surface can reduce reflectance. Light reflected from the surface of the rock is 'internally reflected' from the water-air interface back towards the rock surface, thus increasing the chance that light will be further absorbed (Angstrom 1925). Second, damp surfaces increase specular reflectance (sun-glint), where the direct solar beam is reflected back from the upper surface of the water on the rock to the observer. This reduces the amount of useable information in the image because specular reflectance contains no information about the substratum. Variations in surface moisture can be minimised by uniformly wetting the surface using a fine spray. Specular effects can be minimised by artificially shading the rock surface from direct sunlight or by acquiring images under overcast skies. Collecting CIR images under these conditions would also reduce the variability in illumination in the image caused by small shadows cast by small scale variations in topography of the rock surface. Wetting and artificially shading the rock surface before imaging therefore has the potential to improve chlorophyll estimates by reducing the variability in reflectance within the image due to factors other than chlorophyll. These methods are tested in this study.

Absorption by small amounts of chlorophyll is subtle, resulting in very small changes in the NIR:red ratio. The digital CIR camera used in this study was initially developed to detect hydrogen fires and is now commonly used for agricultural applications and for food inspection. Before this study the ability of CIR imagery to detect small changes in amount of chlorophyll on the rock surface was not known. The CIR camera quantizes data at 8 bits, therefore pixels in each band can have a range of integer values between 0 and 255. To determine whether the CIR camera has sufficient radiometric sensitivity and that data quantised at 8 bits are able to detect small changes in chlorophyll, the

NIR:red ratios generated from the CIR data were compared with the same ratio generated from data obtained from a field spectrometer. Because the field spectrometer used in this study has a much greater radiometric sensitivity than the CIR camera and quantizes data at 16 bits, it can detect subtle changes in reflectance due to absorption by small amounts of chlorophyll.

MATERIALS AND METHODS

Study area and experimental setup. The study area is located in a small bay in the Cape Banks Scientific Marine Research Area, Botany Bay. Relatively few data exist about the nature of the micro-algal communities at Cape Banks but they are thought to be dominated by cyanobacteria (P. Range unpubl. data). MacLulich (1987) found that the micro-algal assemblages on a rock-platform north of the study area consisted of (1) a dominant cover of cyanobacteria (*Anacystis* sp.), which were present in greater densities in low-shore areas during winter, (2) diatoms, that increased during late spring and late autumn, particularly on the lower shore, and (3) spores and sporelings of macro-algae. Very few diatoms have been observed in samples taken from the Cape Banks study area (Underwood 1984b). Algal assemblages on the rock surfaces used in this study are therefore considered to be composed of mixtures of encrusting macro-algae (mainly *Hildenbrandia rubra* and *Ralfsia verucosa*), macro-algal sporelings, and micro-algae (mainly cyanobacteria).

The rock platform was composed of medium-grained sandstone which ranged in colour from cream to orange-red. The colour of the sandstone varied significantly over small (cm) spatial scales, due mainly to variable amounts of iron minerals in the rock. Calibrated CIR data showed that the shape of the spectral curve between green and NIR wavelengths was consistent with the spectral observations of sandstone made by Hunt & Salisbury (1976).

The experiment was initiated in December 2004 and data were collected in February 2005 (Austral summer time). Four sites (~0.7 × 0.4 m) that were completely emersed at low tide and did not have standing pools of water were randomly chosen on the rock-platform at the mid-shore height. The surface at each site was characterised by small pits and hollows and had a topographical relief (i.e. the maximal difference in height of the surface) of about 3 to 5 cm. Thin layers of encrusting macro-algae were distributed in variable amounts on the rock surface at each site. Ten replicate plots, each measuring approximately 8 × 10 cm, were randomly located within each site. Macro-algae were removed by scraping away the surface layers (~2 to 4 mm) of the substratum until the natural yellow-brown colour of the rock was observed. To allow different amounts of micro-algae to repopulate the surface, 2 replicate plots were scraped on each of 4 dates. Two plots were left unscraped. When sampled, plots had accumulated micro-algae for periods of 0 (unscraped plots), 3, 16, 30 or 45 d. These time intervals were chosen because the density of micro-algal cells had been shown to increase rapidly in the first 28 d after scraping, after which density remains fairly constant (MacLulich 1986a). At the time of sampling, between 1 and 3 gastropods were present in ~30% of the scraped areas.

CIR image acquisition and field spectrometry. Digital CIR images of the rock-platform were acquired using a MS3100 digital CIR camera (Redlake MASD). This camera records images at green, red and infrared wavelengths using 3 separate detector arrays (Table 1). To ensure that images were obtained at the same height and camera angle, the camera was mounted on a black metal stand, 1.5 m in height, and was oriented normal to the surface of the rock. The camera was fitted with a Sigma 14 mm f/2.8 ES/HSM super wide angle lens. The height of the camera above the rock-platform and the size of the camera lens determined the size of the area of ground imaged by the camera, which was 654 × 488 mm. The resulting images were 1392 × 1039 pixels in size and the ground spatial resolution of each square pixel was 0.46 mm. For calibration, a reflectance standard (~30% reflective spectralon) was placed in the lower corner of the area being imaged. The camera integration (i.e. exposure time) used to acquire each image was optimised for ambient light conditions so that no object in the image saturated the detector arrays. Camera data were recorded at 8-bit resolution onto a laptop PC using a PCI frame-grabber interface (PCI 1428, National Instruments).

To determine if wetting and/or shading had an effect on the relationship between image and laboratory estimates of chlorophyll, 4 CIR images (treatments) were acquired from each site of: (1) the 'dry' (i.e. not artificially wetted) rock surface under full sunlight; (2) the 'dry' rock surface artificially shaded from direct sun-

Table 1. Wavelength ranges and centre wavelength for each band of the colour-infrared (CIR) digital camera. NIR: near-infrared

CIR camera band	Wavelength range (nm)	Centre wavelength (nm)
Green	525 – 575	550
Red	645 – 689	667
NIR	758 – 833	795

light; (3) the rock surface which had been uniformly wetted under full sunlight; and (4) the rock surface which had been uniformly wetted and artificially shaded from direct sunlight. These image treatments are subsequently termed dry-full, dry-shade, wet-full and wet-shade, respectively. When the images for the dry-full and dry-shade treatments were acquired, the rock surface had variable amounts of surface moisture, from completely dry to damp. Shading of the rock surface was done using a wooden placard. This was held some distance away from the area being imaged so that the rock surface would be adequately illuminated by indirect sunlight. A final CIR image was taken after samples of rock had been removed (see below) in order to register the exact locations in the image from where the samples had been taken.

A reflectance spectrum was recorded from the centre of each plot using a FieldSpec Pro field spectrometer (Analytical Spectral Devices). The spectrometer measures radiance (μW cm^{-2} sr^{-1} nm^{-1} [sr: steridan]) between 350 and 1050 nm at a sampling interval of 1.4 nm, giving a full width half maximum spectral resolution of about 3 nm. The fibre optic cable to the spectrometer was fitted with an 8° foreoptic lens. Spectra were recorded from a height of 29 cm above the surface, thus measuring an area of rock 4 cm in diameter. Prior to the acquisition of each rock spectrum a reference spectrum was recorded from a ~99% reflective spectralon standard.

To reduce the amount of noise in the spectra, 20 consecutive spectra were recorded and averaged into a single spectral measurement. All spectra were converted to absolute reflectance (ρ) using:

$$\rho = (\text{target spectrum/reference spectrum}) \times \text{panel calibration factor} \quad (1)$$

Spectra were recorded from the rock surface using the same experimental treatments as for the CIR data (i.e. dry-full, dry-shade, wet-ful, wet-shade). The time interval between acquisition of the CIR image and the corresponding treatment for the spectrometer data was about 1 min.

Laboratory analysis of rock samples. To calibrate/validate estimates of chlorophyll derived from the CIR data, samples of rock were taken for laboratory determination of amount of chlorophyll. Depending on the friability of the rock surface, between 3 and 5 rock chips were collected from each plot using a hammer and chisel. After the sample was prised free of the surface the resulting hole was filled to its exact dimensions with coloured modelling clay and labelled. After all samples had been taken, a further CIR image was acquired to record their locations.

Chlorophyll was extracted from the individual rock chips using the cold methanol method (Thompson et al. 1999). The efficacy with which chlorophyll is extracted from rock chips depends upon the state of hydration of the rock chip and its biofilm. To minimise variability due to this effect, the rock chips were soaked in filtered seawater for 1 h prior to extraction. The rock chips were placed into individual jars with tight fitting lids. The jar, lid and chip were weighed. Depending upon the size of each chip, between 2 and 3 ml of methanol was then added, the jars were then placed in the dark for 15 h. The jar was then reweighed and the volume of remaining methanol was calculated by subtraction. The absorbances at 665 and 750 nm were measured using a spectrophotometer and the amount of chlorophyll was calculated using the equation presented in Thompson et al. (1999).

Image analysis. Image analysis consisted of 4 stages: (1) calibration of CIR data to reflectance, (2) derivation of the NIR:red ratio, (3) collocation and extraction of NIR:red ratio data (pixel values) that represent areas in the image from where rock samples were taken, and (4) statistical analyses of matched chlorophyll and NIR:red ratio data.

In order to be able to compare NIR:red ratios derived from different CIR images, they must be derived from CIR images that have been standardised to relative reflectance. In uncalibrated CIR data, the pixel values (or brightness) in each band are largely a product of the intensity of sunlight incident upon the target and the integration (exposure time) that is used to acquire the image. To reduce the amount of noise in the data and to allow the maximal possible range of pixel values, the CIR data collected for this experiment were optimised to ambient light conditions by changing the integration time. Reflectance calibration was therefore essential and this was done using a simple flat-field calibration method (Roberts et al. 1986). This procedure, originally devised to calibrate data obtained from aircraft and satellites, and modified for use here, standardises the pixel values in each band to the reflectance factor of the calibration panel in that band. For each CIR band, the image pixel values (Digital Number; *DN*) over the ~30% reflective calibration panel were averaged and the image calibrated to relative reflectance using:

$$\rho(\text{image}) = \frac{DN(\text{image})\ \rho(\text{panel})}{DN(\text{panel})} \quad (2)$$

where ρ (image) is the relative reflectance at each image pixel, ρ (panel) is the reflectance of the panel, *DN* (image) the *DN* at each image pixel, and *DN* (panel) the average *DN* of the pixels over the panel. NIR:red ratios were created from the reflectance-calibrated data by dividing the NIR band by the red band. A ratio image was created for each of the 4 images (treatments) acquired at each site (16 images in total).

For each plot in each site, direct measures of chlorophyll were obtained for 3 to 5 chips of rock. These were averaged, to give the mean and SE for each plot (i.e. 10 values per site). The exact positions of the chips in each plot were marked, and thus the means (and SEs) were calculated for the NIR:red ratio values of pixels in the area of each chip. This produced 10 paired values per site, representing the average amount of chlorophyll and the average NIR:red ratio of the samples acquired within each plot. This was done separately for each of the 4 treatments at each site.

Data from 3 of the 4 sites were randomly selected to establish a relationship between chlorophyll and the NIR:red ratio. These data are termed the 'predictor data'. Data from the remaining site (termed the 'test data') was used to provide an independent test of the ability of this relationship to estimate amount of chlorophyll. Plots of NIR:red ratio versus chlorophyll were constructed individually for sites in the predictor data. Two outlying data points were evident in these plots; these are considered further in the Results section, but they were excluded from the following analyses. Linear regression analysis of amount of chlorophyll on the NIR:red ratio was done using all of the predictor data. To determine if the relationship between chlorophyll and the NIR:red ratio was being influenced significantly by the unscraped plots (i.e. the plots which contained encrusting macro-algae) analysis of covariance was done on data which included and excluded data from the unscraped plots. Independent, paired chlorophyll and NIR:red ratio data were randomly assigned into 1 of 2 groups of equal size, 1 of which contained only data from the scraped plots.

To test the hypotheses that shading and wetting of the rock surface would influence the relationship between chlorophyll and the NIR:red ratio, the slopes and intercepts of the regression equations of the following pairs of treatments were compared: (1) dry-full and dry-shade, (2) wet-full and wet-shade, (3) dry-full and wet-full, and (4) dry-shade and wet-shade. All data (i.e. scraped and unscraped plots) were used in this analysis. Data for the analysis of covariance were obtained by randomly selecting half of the data from the first image in each of the above image pairs and half from the second.

To test the ability of the relationship between chlorophyll and the NIR:red ratio from the predictor data to estimate the amount of chlorophyll from the NIR:red ratio, chlorophyll was calculated from the test data using the regression coefficients from the predictor data for each of the 4 image treatments. The accuracy of the resulting estimates of chlorophyll was determined by calculating the mean difference from measured amounts of chlorophyll.

Comparison of ratios from spectrometer and CIR data. Pixel values representing the central area in each plot measured by the spectrometer were extracted from the NIR:red ratio image along with the statistics (mean, SE) generated from them (n = 6512). This was done for each of the 4 image treatments.

In order to directly compare the NIR:red ratios from the spectrometer with those from the CIR data it was first necessary to convert the reflectance detected by the numerous narrow bands of the spectrometer to the reflectance as it would be detected by the 3 broader bands of the CIR camera. A simple average of the spectrometer bands over the wavelength range detected by the camera would not provide an accurate estimate of reflectance detected by the camera because the sensitivity of each camera band is not uniform across these wavelengths. Reflectance spectra acquired by the field spectrometer were therefore convolved to the broader bands of the CIR data using the within-band sensitivity functions of each camera band provided by the manufacturer. NIR:red ratios were created from these convolved data. NIR:red ratios from the CIR data acquired at each site and for each treatment were compared with the NIR:red ratios from the convolved spectrometer data collected under the same treatment. This was done by correlation and by calculating the absolute mean difference between these data.

RESULTS

Example images

The image of NIR reflectance is strongly influenced by variations in brightness of the rock surface (Fig. 1a). Red reflectance is smaller with increasing amounts of algae due to absorption by chlorophyll (Fig. 1b). Neither of these images show any clear patterns associated with variations in amount of chlorophyll because differences in rock brightness are generally greater than variations in reflectance due to absorption and scattering by algal cells. The NIR:red ratio effectively removes variations in rock brightness and is indicative of the amount of chlorophyll at the surface. Variations in the amount of chlorophyll can be seen in the chlorophyll image derived from the NIR:red ratio (Fig. 1c). Replicate plots in the image which have been allowed to accumulate micro-algae for the longest period of time (45 d, 'D' in Fig. 1c) show the greatest amount of chlorophyll (i.e. they have brighter pixels in the image). Plots that have been allowed to accumulate algae for the shortest period of time (3 d, 'A' in Fig. 1c) have the smallest amounts of chlorophyll and appear black. Plots left to accumulate for intermediate periods of time (30 and 16 d, 'B' and 'C', respectively, in Fig. 1c)

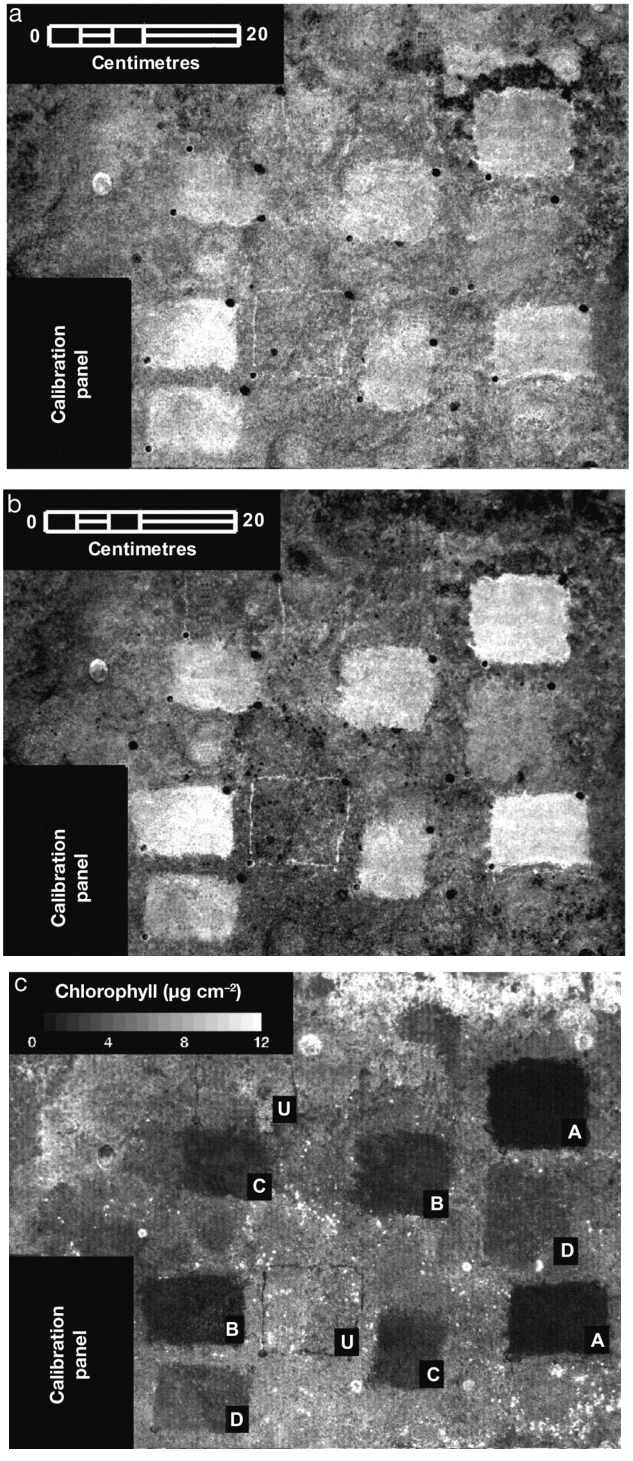

Fig. 1. Example images. (a) Near-infrared (NIR) reflectance. (b) Red reflectance. (c) Image of chlorophyll derived from ratio of NIR:red bands using coefficients from regression of amount of chlorophyll on NIR:red ratio from full-wet treatment. Replicate scraped plots are clearly visible as darker areas in the image because they have smaller amount of chlorophyll than unscraped areas. Amount of time that areas were left to acquire micro-algae after scraping is indicated in lower right hand corner of each plot: (A) 3 d, (B) 16 d, (C) 30 d, (D) 45 d, and (U) unscraped areas

show gradations in amount of chlorophyll between these extremes. Unscraped plots ('U' in Fig. 1c) have the largest amounts of chlorophyll in the image because they contain the encrusting macro-alga *Hildenbrandia rubra* as well as micro-algae.

Comparison of ratios from spectrometer and CIR data

The strength of relationship between the NIR:red ratio derived from the CIR data and the NIR:red ratio derived from the convolved spectrometer data was different for each of the 4 treatments (Table 2). The strongest relationships between CIR and spectrometer ratios were observed where data was collected under full sunlight, and the weakest where data was collected in the shade. Wetting the surface reduced the strength of the relationship for data collected under full sunlight or under shade. The relationship between the NIR:red ratio from CIR and spectrometer data was linear for all treatments (see, for example, the dry-full treatment in

Table 2. Correlation (r), absolute mean difference between NIR:red ratios from CIR and convolved field spectrometer data, and mean difference as a percentage of measured range of spectrometer data

Treatment	r	Mean	Range %
Dry-full	0.99	0.05	5.1
Dry-shade	0.93	0.12	12.1
Wet-full	0.98	0.06	4.4
Wet-shade	0.89	0.14	11.6

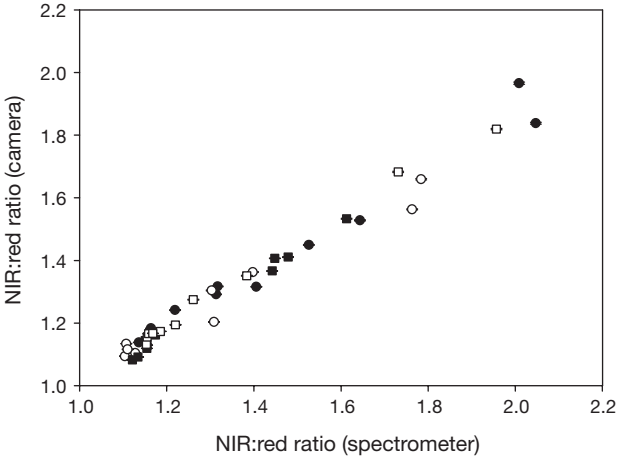

Fig. 2. Comparison of the NIR:red ratio from colour-infrared (CIR) data and from convolved spectrometer data. Data are from the dry-full treatment (r = 0.99; n = 40). (●) Site 1, (○) Site 2, (■) Site 3, (□) Site 4. Horizontal bars: SEs of means for NIR:red ratios created from camera data

Fig. 2). On average, the ratio was smaller in the CIR data than in the spectrometer data, but the absolute difference was small (0.05 or 5.06% of the measured range of spectrometer values). The linear increase and small absolute difference between the 2 methods indicates that the calibration of CIR data to reflectance was effective. Without effective calibration, the CIR ratios would have shown a large site-dependent offset on the vertical axis, because the data were collected under different conditions of solar illumination and periods of camera integration. The strong linear relationship between NIR:red ratios derived from CIR and spectrometer data indicate that the CIR camera and spectrometer can detect similar amounts of change in this ratio.

Relationships between chlorophyll and NIR:red ratio from CIR data

At each site, in the predictor data, the NIR:red ratio increased linearly with increasing amounts of chlorophyll for all treatments. Individual sites in the predictor data for the dry-full treatment are shown in Fig. 3, as an example. At each site, the amount of chlorophyll shows a larger SE than does the NIR:red ratio. Outlying data points are evident in Fig. 3a,b. The outlying point in Fig. 3a shows a much smaller ratio value than is expected for the amount of chlorophyll measured from the rock samples. This particular point represents a plot that was scraped and left to accumulate algae for only 3 d prior to the acquisition of the CIR data. The amounts of measured chlorophyll for this plot were greater than for all other scraped plots in the site, including those which had been left to accumulate micro-algae for 47 d. The replicate of this plot had the smallest amount of chlorophyll (0.09 µg cm^{-2}) for that site. The second outlying point (Fig. 3b) had an SE for amount of chlorophyll more than twice as large as any other point. It also had a larger amount of measured chlorophyll than sites which were unscraped. It is therefore likely that these 2 points are true outliers, thus they were not included in further analyses.

Linear regression analysis of chlorophyll on the NIR:red ratio showed that there were differences in the strength of this relationship between the 4 image treatments (Fig. 4; Eqs. 3 to 6). The dry-full treatment had the largest correlation between chlorophyll and the NIR:red ratio. There was no significant difference in the strength of this relationship between the dry-full and wet-full treatments. The relationship between chlorophyll and the NIR:red ratio was weaker for dry-shade and wet-shade treatments:

Dry-full

$$\text{Chlorophyll (µg cm}^{-2}) = 3.46 \times (\text{NIR:red}) - 3.56 \quad (3)$$

Dry-shade

$$\text{Chlorophyll (µg cm}^{-2}) = 3.50 \times (\text{NIR:red}) - 3.37 \quad (4)$$

Wet-full

$$\text{Chlorophyll (µg cm}^{-2}) = 3.13 \times (\text{NIR:red}) - 3.25 \quad (5)$$

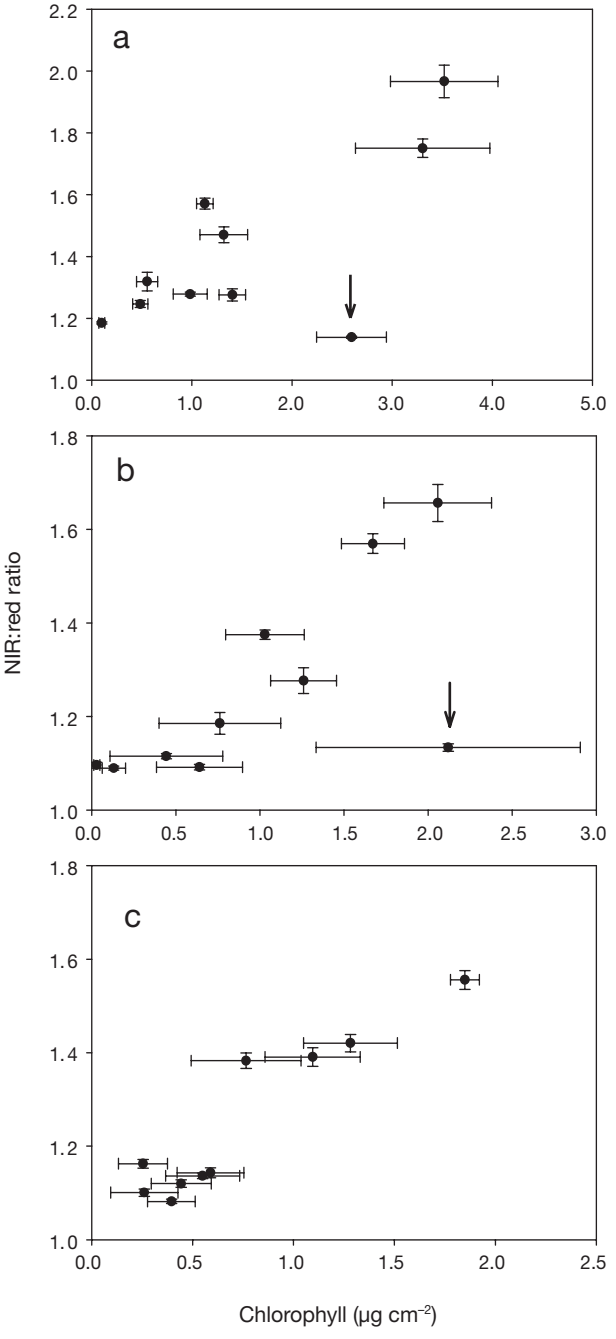

Fig. 3. Relationship between chlorophyll and the NIR:red ratio derived from CIR data. (a) Site 1 (r^2 = 0.48), (b) Site 2 (r^2 = 0.46), (c) Site 3 (r^2 = 0.87). SEs of the means are shown for each point. Arrows: outlying data points (see text, this page). Scales are set to maximize separation of data points

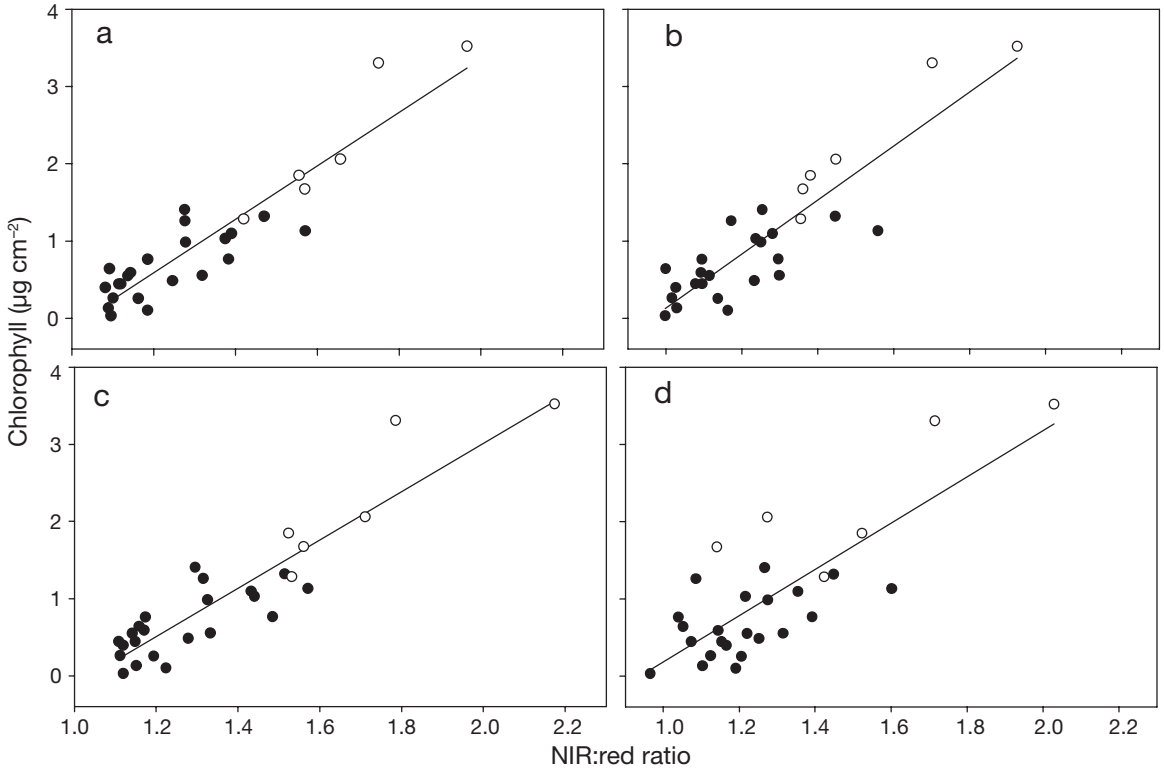

Fig. 4. Linear regression of chlorophyll on NIR:red ratio generated from predictor data (n = 28; outliers removed; see text in 3rd section of 'Results'). (a) Dry-full ($r^2 = 0.84$; $p < 0.001$; SE = 0.34), (b) dry-shade ($r^2 = 0.79$; $p < 0.001$; SE = 0.39), (c) wet-full ($r^2 = 0.83$; $p < 0.001$; SE = 0.35), (d) wet-shade ($r^2 = 0.63$; $p < 0.001$; SE = 0.53). (●) Scraped plots, (○) unscraped plots

Wet-shade

$$\text{Chlorophyll (µg cm}^{-2}) = 3.00 \times (\text{NIR:red}) - 2.82 \quad (6)$$

Across all treatments, there was no significant difference in the slopes and intercepts of data which included or excluded the unscraped plots. Chlorophyll in micro- and encrusting macro-algae within the range of values measured (0.02 to 3.51 µg cm^{-2}) can therefore be estimated using a single equation.

Although shading reduced the correlation between chlorophyll and the NIR:red ratio, it did not significantly influence the slope and intercept of the regression equation compared with the corresponding treatments where data were acquired under full sunlight. There were also no significant differences in the slopes and intercepts between the dry and the wet treatments.

Test data

Amounts of chlorophyll predicted from the NIR:red ratio in the test data for each treatment were strongly correlated with measured amounts of chlorophyll (Table 3). The smallest difference between the measured and predicted values were observed in the dry-full and wet-shade treatments (–1.53 and –0.41%, respectively). The difference between measured and predicted values for the dry-shade and wet-full treatments was 4.28 and –5.18%, respectively. On average, the predicted values of chlorophyll for dry-full, wet-full and wet-shade treatments were greater than the measured values.

DISCUSSION

Estimation of micro-algal standing crop in rocky intertidal habitats is usually significantly more difficult and costly than for soft sediments. It is most commonly

Table 3. Statistics comparing measured and predicted (test data) amounts of chlorophyll (n = 10)

Treatment	r	Mean	SE	Range %
Dry-full	0.79	–0.05	0.20	–1.53
Dry-shade	0.81	0.15	0.20	4.28
Wet-full	0.82	–0.18	0.19	–5.18
Wet-shade	0.82	–0.01	0.20	–0.41

done indirectly, by measuring the amount of chlorophyll in samples of rock which have been removed from the rock surface. Several stages in collection and processing of samples can introduce errors and variability into the estimates of chlorophyll. An alternative method, presented here, has several advantages over conventional sampling methods: (1) only chlorophyll at the rock surface is measured. From an ecological perspective, chlorophyll at the rock surface is of most relevance because it is readily accessible to assemblages of grazers (Nicotri 1977, Underwood 1984a). Other methods, which require removal of micro-algae (and the rock surface), often sample variable amounts of endolithic chlorophyll; (2) chlorophyll is measured without destroying the micro-algae and without physically interacting with the rock surface, so the same area of rock surface could be measured on subsequent occasions, or during experiments about grazing. Independent measurements of chlorophyll can be made from experimental areas by spatially sub-sampling the image data; (3) collection of data is remarkably rapid, each image takes only a few minutes to acquire; (4) the area being measured and the area of ground represented by each pixel in the image can be easily varied by changing the height at which the camera is mounted above the rock surface; (5) the data can be sampled at a hierarchy of spatial scales in the laboratory, making it possible to measure variability in amounts of chlorophyll over different spatial scales; (6) images of chlorophyll can be easily integrated with other measurements such as fluorescence; and (7) species of gastropods can be readily identified within the CIR images, thus opening up the possibilities of studying the spatial association of different gastropods to food supply.

The acquisition and analysis of the data were kept deliberately simple. Calibration of the images was done using a 'flat-field' procedure using a reflectance standard. This method was selected because it did not require data from other sources and is computationally simple. Chlorophyll was estimated from CIR data using a ratio of NIR and red bands. It was known that the spectrometer would be able to detect small differences in amount of chlorophyll because of its greater radiometric sensitivity and higher 16-bit resolution. The CIR camera allows amounts of chlorophyll to be estimated at very small spatial scales and preserves the spatial position of amounts in each pixel. The results demonstrate that the radiometric sensitivity and the 8-bit resolution of the CIR camera were sufficient to distinguish the same small changes in the NIR:red ratio that were detected by the spectrometer.

Of the 4 treatments, data collected from 'dry' rock surfaces under full sunlight (i.e. the dry-full treatment) had the strongest relationship with chlorophyll. The surface moisture plots sampled for the dry-full treatment was variable and ranged from dry to damp. Uniformly wetting the rock surface did not improve the relationship between the NIR:red ratio and chlorophyll. There was no significant difference in the regression slopes and intercepts between the dry-shade and wet-shade treatments, but the difference between dry-full and wet-full treatments might have been significant with larger samples ($p = 0.06$ for the slope and intercept). Although variable amounts of surface moisture were observed at the time that the images for the full-dry treatment were acquired, the maximal amount of surface moisture was always much smaller than was the case for the full-wet treatment (i.e. after the same areas were wetted). Extreme variation in amount of surface moisture can therefore change the relationship between the NIR:red ratio and chlorophyll. In areas with large variations in surface moisture, wetting of the rock surface to uniform amounts may be appropriate.

Shading the rock surfaces from direct sunlight was done in an attempt to reduce variability in the image due to specular reflectance and shading due to microtopography. Artificial shading of the rock surface more than doubled the mean absolute difference between NIR:red ratios derived from spectrometer and from the CIR data, and both the dry-shade and wet-shade treatments had a weaker relationship with chlorophyll than the unshaded treatments. This suggests that artificially shading the rock surface introduces variability into the data which is unrelated to the spectral properties of the substratum. The correlation between chlorophyll and the spectral estimates in the dry-shade treatment was still relatively large ($r^2 = 0.79$). Artificial shading could therefore be used where it was necessary to collect data under full sunlight in areas with a lot of microtopographical variation or specular reflectance. Results also demonstrate that data can be collected under overcast skies, where the diffuse light field would largely remove these effects.

Validation or calibration of remotely sensed data can only be done by reference to direct measures of the amount of chlorophyll. Direct measurements of chlorophyll are, however, more variable than the NIR:red ratios in the same sample area (compare the SEs in Fig. 3). A large amount of variability in estimates of chlorophyll extracted from replicate rock chips has been observed in other studies (e.g. MacLulich 1986b, Thompson et al. 1999). Several factors contribute to this variability. During sampling, small flakes of biofilm can be removed and lost from the surface of the rock sample by percussion during chiseling. Rock samples not only contain chlorophyll at the surface, but also endolithic chlorophyll from deeper layers in the sample. The amount of endolithic

algae and the efficacy with which it is extracted from the rock chips depends mainly on the type of rock and its cohesion. The physical characteristics of the rock sample (e.g. its surface area to volume ratio, its rugosity and porosity) all influence the efficiency of the extraction of chlorophyll and are important sources of variability (Thompson et al. 1999). Variability in direct estimates of chlorophyll makes it difficult to make a true estimate of the accuracy of the NIR:red ratio in predicting the amount of chlorophyll. Given this problem, test data indicate that the average difference between measured and predicted amounts of chlorophyll was smallest for the dry-full and wet-shade treatments. Logically, the most useful method would be to collect CIR data as in the wet-shade treatment, because the conditions under which the measurements were acquired could then be standardised. However, data for the dry-full treatment were collected with variable amounts of moisture on the rock surface, but the NIR:red ratio from these data, nevertheless, had the strongest relationship with chlorophyll and a small average difference between measured and predicted values.

Many ecological studies do not require absolute measurements of chlorophyll. A relative measure of amount of chlorophyll would suffice in order to test hypotheses about differences in biomass over time or space. In these cases, a NIR:red ratio (providing it was generated from reflectance-calibrated data and was within the range of values presented here) would provide a linear, relative measure of amount of chlorophyll.

Intertidal rock-platforms often have variable amounts of pooled water on their surface. Water is an effective absorber of light, particularly at longer NIR wavelengths (Kirk 1996). With increasing depth there is a progressive increase in the absorption of light. Standing water on the rock surface, even if only a few millimeters in depth, can significantly reduce NIR reflectance relative to red reflectance, causing a decrease in the NIR:red ratio which is unrelated to the amount of chlorophyll on the rock surface. Rock surfaces used in this study ranged from completely dry to wet but did not have standing water on the surface. To correct for the absorption of water it would be necessary to know its depth and its inherent optical properties. From this information, it would be possible to predict the amount of reflectance at the surface by modelling the underwater light field. This is beyond the scope of this paper, but is currently being investigated and will be reported elsewhere in order to improve the usefulness of these methods.

Calibrated CIR reflectance measurements showed that the bare rock surface had large variations in colour due to variable amounts of iron. Despite this, the NIR:red ratio showed a strong correlation with amount of chlorophyll on the rock surface, indicting that the method was robust across a broad range of rock colours. It must be noted, however, that these observations were made from a single rock type (sandstone). Where comparisons are to be made across a variety of different rock types that exhibit very large variations in brightness (for example bright limestone and dark shale) it would be advisable to establish a separate calibration between chlorophyll and the NIR:red ratio, for each rock type.

The method described here quantifies the total amount of chlorophyll at the rock surface – i.e. chlorophyll in micro- and encrusting macro-algae. In areas where there are large variations in amount of encrusting macro-algae it would be necessary to remove encrusting macro-algae and then to allow colonization by micro-algae before taking samples. The technique presented here works for situations where there are only micro-algae or mixtures with underlying macro-algae. Red reflectance decreases with increasing amounts of chlorophyll; however, where large amounts of chlorophyll are present, red reflectance remains constant. This reflectance saturation effect occurs where chlorophyll is present at amounts greater than ~10 µg cm^{-2} (Buschmann & Nagel 1993, Meleder et al. 2003). The amount of epilithic chlorophyll in intertidal habitats is generally much smaller than in sediments or in terrestrial vegetation. A survey of published papers indicates that the amount of epilithic chlorophyll measured in intertidal habitats ranged between 0 and 12 µg cm^{-2} (Underwood 1984a,b, MacLulich 1986a, Dye & White 1991, Lasiak & White 1993, Boaventura et al. 2002, 2003). Red reflectance (and hence the NIR:red ratio) would still be sensitive to changes in chlorophyll within these ranges.

The difficulties in sampling chlorophyll on emersed rocky intertidal habitats have limited the scope of ecological research into the spatio-temporal distribution of epilithic algal biomass and, in particular, grazing and competitive interactions between grazers. To the best of our knowledge, this is the first time that epilithic chlorophyll in an intertidal habitat has been quantitatively imaged at sub-millimetre scales. The ability to make *in situ*, spatially contiguous measurements of chlorophyll opens up new possibilities for research into competition between grazing animals and their spatial relationships with food supply in rocky intertidal environments.

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LITERATURE CITED

Angstrom A (1925) The albedo of various surfaces of ground. Geogr Ann 7:323

Boaventura D, Cancela L, da Fonseca LC, Hawkins SJ (2002) Analysis of competitive interactions between the limpets *Patella depressa* Pennants and *Patella vulgata L.* on the northern coast of Portugal. J Exp Mar Biol Ecol 271: 171–188

Boaventura D, da Fonseca LC, Hawkins SJ (2003) Size matters: competition within populations of the limpet *Patella depressa*. J Anim Ecol 72:435–446

Buschmann C, Nagel E (1993) *In vivo* spectroscopy and internal optics of leaves as basis for remote sensing of vegetation. Int J Remote Sens 14:711–722

Castenholz RW (1961) The effect of grazing on marine littoral diatom populations. Ecology 42:783–794

Creese RG, Underwood AJ (1982) Analysis on inter- and intra-specific competition amongst intertidal limpets with different methods of feeding. Oecologia 53:337–346

Dye AH, White DRA (1991) Intertidal microalgal production and molluscan herbivory in relation to season and elevation on two rocky shores on the east coast of southern Africa. S Afr J Anim Sci 11:483–489

Hawkins SJ, Hartnoll RG (1983) Grazing of intertidal algae by marine invertebrates. Oceanogr Mar Biol Annu Rev 21: 195–282

Hill AS, Hawkins SJ (1990) An investigation of methods for sampling microbial films on rocky shores. J Mar Biol Assoc UK 70:77–88

Hill AS, Hawkins SJ (1991) Seasonal and spatial variation of epilithic microalgal distribution and abundance and its ingestion by Patella vulgata on a moderately exposed rocky shore. J Mar Biol Assoc UK 71:403–423

Hunt GR, Salisbury JW (1976) Visible and near infrared spectra of minerals and rocks. XI. Sedimentary rocks. Mod Geol 5:211–217

Jordan CF (1969) Derivation of leaf area index from quality of light on the forest floor. Ecology 50:663–666

Kirk JTO (1996) Light and photosynthesis in aquatic ecosystems. Cambridge University Press, Cambridge

Lasiak TA, White DRA (1993) Micro-algal food resources and competitive interactions among the intertidal limpets *Cellana capensis* (Gmelin, 1791) and *Siponaria concinna* Sowerby, 1824. S Afr J Mar Sci 13:127–134

MacIntyre HL, Geider RJ, Miller DC (1996) Microphytobenthos: the ecological role of the 'secret garden' of unvegetated, shallow-water marine habitats. 1. Distribution, abundance and primary production. Estuaries 19: 186–201

MacLulich JH (1986a) Colonization of bare rock surfaces by microflora in a rocky intertidal habitat. Mar Ecol Prog Ser 22:91–96

MacLulich JH (1986b) Experimental evaluation of methods for sampling and assaying intertidal epilithic microalgae. Mar Ecol Prog Ser 34:275–280

MacLulich JH (1987) Variations in the density and variety of intertidal epilithic microflora. Mar Ecol Prog Ser 40: 285–293

Meleder V, Barille L, Launeau P, Carrere V, Rince Y (2003) Spectrometric constraint in analysis of benthic diatom biomass using monospecific cultures. Remote Sens Environ 88:386–400

Morse ANC, Froyd CA, Morse DE (1984) Molecules from cyanobacteria and red algae that induce larval settlement and metamorphosis in the mollusc *Haliotis rufescens*. Mar Biol 81:293–298

Murphy RJ, Tolhurst TJ, Chapman MG, Underwood AJ (2004) Estimation of surface chlorophyll on an exposed mudflat using digital colour infrared (CIR) photography. Estuar Coast Shelf Sci 59:625–638

Murphy RJ, Underwood AJ, Pinkerton MH, Range P (2005) Field spectrometry: new methods to investigate epilithic micro-algae on rocky shores. J Exp Mar Biol Ecol 325:111–124

Nagarkar S, Williams GA (1997) Comparative techniques to quantify cyanobacteria dominated epilithic biofilms on tropical rocky shores. Mar Ecol Prog Ser 154:281–291

Neu TR, Woelfl S, Lawrence JR (2004) Three-dimensional differentiation of photo-autotrophic biofilm constituents by multi-channel laser scanning microscopy (single-photon and two-photon excitation). J Microbiol Methods 56: 161–172

Nicotri ME (1977) Grazing effects on four marine intertidal herbivores on the microflora. Ecology 58:1020–1032

Norton TA, Thompson RC, Pope J, Veltkamp CJ, Banks B, Howard CV, Hawkins SJ (1998) Using confocal laser scanning microscopy, scanning electron microscopy and phase contrast light microscopy to examine marine biofilms. Aquat Microb Ecol 16:199–204

Roberts DA, Yamaguchi Y, Lyon RJP (1986) Comparison of various techniques for calibration of AIS data. In: Vane G, Goetz AFH (eds) Second Airborne Imaging Spectrometer Data Analysis Workshop. NASA Jet Propulsion Laboratory, Pasadena, CA, p 21–30

Rodriguez SR, Ojeda FP, Inestrosa NC (1993) Settlement of benthic marine invertebrates. Mar Ecol Prog Ser 97:193–207

Saravia LA, Giorgi A, Momo FR (1999) A photographic method for estimating chlorophyll in periphyton on artificial substrata. Aquat Ecol 33:325–330

Thompson RC, Tobin ML, Hawkins SJ, Norton TA (1999) Problems in extraction and spectrophotometric determination of chlorophyll from epilithic microbial biofilms: towards a standard method. J Mar Biol Assoc UK 79:551–558

Thompson RC, Norton TA, Hawkins SJ (2004) Physical stress and biological control regulate the producer-consumer balance in intertidal biofilms. Ecology 85:1372–1382

Underwood AJ (1976) Food competition between age-classes in the intertidal neritacean *Nerita atramentosa* Reeve (Gastropoda: Prosobranchia) in S.E. Australia. J Exp Mar Biol Ecol 23:145–154

Underwood AJ (1979) The ecology of intertidal gastropods. Adv Mar Biol 16:111–210

Underwood AJ (1984a) Microalgal food and the growth of the intertidal gastropods *Nerita Atramentosa* Reeve and *Benbicium nanum* (Lamarck) at four heights on a shore. J Exp Mar Biol Ecol 79:277–291

Underwood AJ (1984b) The vertical distribution and seasonal abundance of intertidal microalgae on a rocky shore in New South Wales. J Exp Mar Biol Ecol 78:199–220

Wahl M (1989) Marine epibiosis. I. Fouling and antifouling: some basic aspects. Mar Ecol Prog Ser 58:175–189

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