Vol. 12: 165–176, 2011
doi: 10.3354/ab00334

AQUATIC BIOLOGY
Aquat Biol

Published online May 5

Chlorophyll *a* and intertidal epilithic biofilms analysed *in situ* using a reflectance probe

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ABSTRACT: Spectrometry of biofilms in microhabitats (e.g. rock crevices) is difficult using natural light. An alternative approach was evaluated using a reflectance probe with an artificial light source to measure the abundance and pigment composition of intertidal biofilms *in situ*. Measurements made by the probe were compared with those made under sunlight. To determine whether the probe could be used to measure amounts of chlorophyll *a* (chl *a*, as an index of biomass), reflectance spectra (350–1050 nm) were acquired from microalgae grown on sandstone discs. Spectra were acquired under natural light and using the probe (artificial light) with or without an intervening layer of plastic. Two chlorophyll indices (a ratio of reflectances at 750 and 672 nm and the Phytobenthos Index) were used to estimate amounts of chl *a* from the spectra. Strong linear relationships were found between laboratory measures of chl *a* and both chlorophyll indices (R^2 ranging from 0.85 to 0.94). Spectra of natural intertidal biofilms were used to determine whether there were any differences between spectra measured under natural and artificial light in terms of the shapes of their curves and the wavelengths and heights of absorption peaks caused by photosynthetically active pigments. No differences in shapes of curves were found between spectra measured under natural and artificial light. The wavelength positions of absorption peaks were similar between spectra measured under sunlight and artificial light but there were differences in their height. Taken together, results show that the reflectance probe presented a reliable and rapid method for providing quantitative and qualitative information about intertidal biofilms *in situ*.

KEY WORDS: Microalgae · Reflectance · Intertidal · Chlorophyll · Pigments · Biofilm · Spectra

INTRODUCTION

Field spectrometry has enabled the abundance and composition of intertidal microalgae on rocky (Murphy et al. 2005a) and soft sedimentary (Paterson et al. 1998, Meleder et al. 2003, Murphy et al. 2005b) substrata to be determined *in situ*, allowing specific hypotheses to be tested about the roles of microalgae in ecological processes (as exemplified in detail by Murphy et al. 2008, Jackson et al. 2009, Murphy & Tolhurst 2009, Chapman et al. 2010, Iveša et al. 2010). The advantages of field spectrometry over other methods are that it is not destructive or intrusive, less laborious and less expensive. Ambient sunlight is usually the source of light for field spectrometry. The amount of light reflected from a surface is then measured relative to a reflectance standard. Most often, measurements are first made from the calibration panel, immediately before the target (biofilm) surface. This approach requires stable environmental conditions so that there is no change in incident illumination (irradiance) between measurements of the calibration panel and the substratum. Large changes in the shape and intensity of the spectral curve can result from even minor changes in atmospheric conditions (Salisbury 1998, Milton et al. 2009). Variations in sun–sensor–target viewing geometry between measurements may also affect reflectance and the chlorophyll indices derived from them (Castro-Esau et al. 2006). In experimental ecology, measurements often need to be made at a specific time and

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place. Methods therefore need to be tested that allow measurements to be made independently of prevailing environmental conditions. To maximise comparability, spectra should be acquired under a standard viewing geometry and illumination.

In the present study, we evaluated the use of a reflectance probe, using an integrated, artificial light source, to obtain information about intertidal biofilms *in situ*. The use of artificial illumination for spectroscopy is not new; it has been used in the laboratory and in the field to obtain information from geological and biological materials (Clark et al. 1993, Merry & Pontual 1999, Kühl & Polerecky 2008, de Backer et al. 2009, Serôdio et al. 2010). Artificial illumination opens up the possibility of acquiring measurements in places where topographic variation makes the use of natural light difficult, e.g. in crevices, around boulders, on seawalls or on the sides of rockpools. The stable light source allows several measurements to be made in a very short interval of time. Despite these advantages, artificial light sources, with a constant viewing geometry, have not been evaluated for studying intertidal benthic microalgae *in situ*. Artificial light may introduce additional artefacts into the data and has a different spectral distribution from that of natural light, with greater noise in some parts of the spectrum. The probe must be in contact with the surface in order to obtain reproducible measurements. Measurements on damp substrata or on soft sediments might contaminate the reflectance probe. The manufacturers of the probe (Analytical Spectral Devices) suggested that, to protect the probe from contamination, measurements could be made through a layer of transparent plastic film. If, despite such a protective layer, reliable data can still be acquired, this would solve the problem of contaminated probes. We therefore compared measurements made using the reflectance probe with or without an intervening plastic film. Measurements made with the probe (artificial light) were then compared with those made with the bare spectrometer fibre-optic under solar illumination (natural light).

The shape of a reflectance curve contains information about the amount and identity of photosynthetic pigments in biofilms (Bidigare et al. 1989), providing information about the composition of the assemblage (Millie et al. 1993, Jeffrey & Vesk 1997). The shape of a reflectance curve also affects ratio-based indices (Murphy et al. 2005b). Reflectance is the basic measurement used in spectrometric studies, and it is important to understand the differences in spectra acquired under artificial versus natural light. Our first objective was to quantify reflectances of different biofilms measured under natural and artificial light to determine whether any differences affected indices of chlorophyll. Ratio-based indices are commonly used to quantify amounts of chl *a* in biofilms measured with natural light (Murphy et al. 2005a,b,c). Our second objective was therefore to determine whether such a relationship was true for the indices generated from spectra acquired using artificial versus natural light. Two indices were investigated: a simple ratio of reflectance at near infrared (750 nm) and red (672 nm) wavelengths (Murphy et al. 2005a,b) and the phytobenthos index (PI; Meleder et al. 2003).

Many photosynthetic pigments have weak or overlapping absorptions, and these are superimposed on a background or continuum which can be of varying brightness. Variations in brightness of the spectrum are caused by broad non-specific absorption and/or scattering of light by the substratum, causing decreasing and increasing brightness, respectively (Clark & Roush 1984). Spectra may also be vertically offset (i.e. show a uniform increase or decrease in reflectance across wavelengths) as a consequence of errors in calibration. Derivative analysis is therefore used to enhance subtle, weak or overlapping absorptions against a sloping spectral background (Bidigare et al. 1989, Demetriades-Shah et al. 1990) and expressing them as separate peaks above a zero baseline (Butler & Hopkins 1970). The identity of pigments can be inferred from the wavelength positions of peaks, thus providing information on the composition of the biofilm. The derivative reflectance at the centre of a peak is indicative of the amount of pigment which absorbs at that wavelength (Bidigare et al. 1989, Murphy et al. 2005a). The method used to measure reflectance may affect the positions or heights of peaks of wavelengths. Our third objective was therefore to determine whether the positions and height of peaks of wavelengths in derivative spectra were different for spectra acquired with artificial or natural light.

Comparison of an 'unknown' spectrum with 'known' library spectra is often done using derivative spectra (e.g. Kirkpatrick 2000, Jackson et al. 2010). Our fourth objective was therefore to determine whether there were any differences in the shape of derived curves between spectra acquired under artificial and natural light. If the shape of the derivative curve was consistent between methods, spectra of different biofilms should have similar distributions in multivariate space.

We used 5 naturally-occurring intertidal biofilms (3 micro- and 2 macroalgal biofilms). Macroalgal biofilms presented either as very thin (<1 mm), uniform, encrusting layers as in the case of *Hildenbrandia rubra* (Rhodophyta) or as a very thin and uniform covering of *Ulva* sp. (Chlorophyta). We included the macroalgal biofilms because they contain a range of different pigments and exhibited quite different spectral characteristics, allowing comparison of several different features of absorption.

MATERIALS AND METHODS

Growth of microalgae on sandstone discs. Microalgae were grown on sandstone discs (2.5 cm diameter) in a recirculating seawater aquarium under artificial light (36 W; Sylvania Grolux) for 15 h d^{-1} at 17 to 19°C. Natural seawater, obtained from a clean area of the New South Wales (NSW), Australia, coastline was used in the aquarium to provide the algal spores. Four replicate sandstone discs were placed into the aquarium at each of 9 intervals of 4 d to ensure a range of amounts of algae. The last discs were added at the end of the experiment to provide a reference without microalgae.

Reflectance measurements. ***Methods:*** Reflectance is the wavelength-specific amount of light reflected from an object as a function of the amount of incoming light; it is therefore a dimensionless unit ranging between 0 and 1. Spectra in reflectance units are henceforth described as 'reflectance spectra'. A reflectance probe (Analytical Spectral Devices), measured reflectance over a small (2 cm diameter) area using an internal quartz-halogen light source. To acquire comparable measurements, reflectance was measured at the same distance from the target and calibration panel (2.5 cm) by placing the probe into direct contact with the substratum or calibration panel. Reflectance was measured using 3 different methods (henceforth termed 'treatments'):

(1) bare spectrometer fibre-optic using direct solar illumination: 'natural light',

(2) reflectance probe in direct contact with the substratum: 'artificial light (– plastic)',

(3) reflectance probe with a thin (25 µm thick) PVC film placed between the probe and the calibration panel or substratum: 'artificial light (+ plastic)'.

A spectrum from a calibration standard (~99% Spectralon) was taken prior to each spectrum of biofilm.

The photosynthetically active irradiance emitted by the probe between 400 and 700 nm at the sample surface was calculated from 5 replicate radiance measurements made from the calibration standard. The photosynthetically active irradiance (mean ±SE) emitted by the probe was 373.80 (2.61) µmol m^{-2} s^{-1}.

Natural biofilm: Measurements were made on the rocky shores at the Cape Banks Scientific Marine Research Area (Australia). Horizontal sandstone surfaces were chosen at midtidal level. Five different areas (Areas 1–5) (30 × 30 cm) were selected corresponding to 5 biofilms differing from each other in colour and thickness. Three biofilms were dominated by microalgae (grains of sandstone could be seen with a magnifying glass) and 2 by macroalgae (substratum completely covered by algae; Table 1). For each biofilm, 9 replicate measurements were made for each of the 3 different treatments (natural light, artificial light +/– plastic).

Sandstone discs: Spectra were acquired (see 'Methods' above) from all sandstone discs for the natural light and artificial light (– plastic) measurements (n = 4 disks for each interval of time). For the artificial light (+ plastic) treatment, half of the discs were randomly selected for spectral analysis (n = 2 disks for each interval of time).

Laboratory estimates of chl *a*. Laboratory estimates of chl *a* were made from the sandstone discs using spectrophotometry, in accordance with Thompson et al. (1999).

Spectral analysis. ***Differences in reflectance between treatments:*** To quantify any differences in reflectance between the natural and artificial light (+/– plastic) treatments, the average spectrum for each natural biofilm was calculated from the n = 9 spectra, for for each treatment. This therefore resulted in 3 average spectra for each natural biofilm (i.e. 1 average spectrum for each treatment). Differences in reflectance at each wavelength measured under artificial light relative to that measured in natural sunlight were calculated as:

$$\ln[\text{reflectance (+ or – plastic)} / \text{reflectance natural light}] \quad (1)$$

Zero indicates no difference in reflectance relative to natural light; an increase or decrease in reflectance measured by artificial light would be a positive or negative value, respectively.

Generation and analyses of chlorophyll indices: The simple ratio of reflectance was constructed by dividing the reflectance (R) at 750 nm by the reflectance

Table 1. Intertidal substrata identified for spectral analysis

Area	Substratum	Algae type	Description
1	Tile (sandstone)	Microalgae	Red. Attached to rock platform
2	Tile (sandstone)	Microalgae	Brown. Attached to rock platform
3	Rock platform (sandstone)	Microalgae	Red-brown
4	Rock platform (sandstone)	Encrusting macroalgae *Hildenbrandia rubra*	Pink-red. Thin layer tightly bound to the substratum
5	Rock platform (sandstone)	Thin covering of macroalgae, probably *Ulva* sp.	Green. Thin but dense film, completely covering the substratum

at 672 nm (R_{750}/R_{672}; Murphy et al. 2005a,b). The PI (Meleder et al. 2003) was calculated as:

$$PI = \frac{R_{750} - R_{636}}{R_{750} + R_{636}} \quad (2)$$

This was done for spectra acquired from the discs and from the natural biofilms. The former were used to explore the relationships between each index and laboratory estimates of chl *a*, using linear regression. The latter were used to test the hypothesis that there was no significant difference in index values between treatments.

Analyses of spectral derivatives: Spectra were converted to units of pseudo-absorbance (log[1/reflectance]), and fourth-order derivatives were calculated using a 26 nm smoothing window (Savitzky & Golay 1964). Absorption maxima of pigments in these derivative spectra are represented as peaks above the zero baseline.

If spectra measured with artificial light had peaks at different wavelengths from those in the natural light treatment, this would make it more difficult to identify unambiguously any pigments using this criterion. Differences between treatments in the heights of peaks could cause amounts of pigments to be under- or over-estimated. To address our third objective, i.e. to determine whether there were any differences in the positions and heights of the peaks of wavelengths in spectra acquired using artificial and natural light, these variables were extracted for 3 peaks in the spectra from each biofilm. The largest peaks were selected because they represented absorptions by the major pigments in the algal assemblages.

Our fourth objective was to determine whether there were any differences in the shape of curves between spectra measured with natural and artificial light. If the shapes of the spectra of biofilms did not differ among the 3 treatments, they would have similar distributions in multivariate space. Non-metric multidimensional scaling (nMDS) ordination and analysis of similarity (ANOSIM), based on Euclidean distances between spectra, were used to visualise the distribution of multivariate spectra in 2D plots and to detect potential differences between treatments (Clarke & Warwick 2001). Wavelengths outside of the range 500 to 750 nm were not used in this analysis because of noise (see below). nMSD ordination was first done using all data (i.e. micro- and macroalgal spectra). A second ordination was done using only the microalgal spectra.

Quantification of noise in spectra: The amount of noise in spectra of each biofilm was quantified as follows: (1) average reflectance spectra were calculated separately for each treatment (3 treatments, n = 9) for each biofilm; (2) from these, standard deviations were calculated for the reflectance at each wavelength and its adjacent wavelengths; (3) the standard deviations from spectra measured using artificial light (+/– plastic) were divided by those from spectra measured using natural light to remove effects of background slope caused by pigment absorptions. Thus, for each biofilm, the amount of noise was estimated for spectra acquired in artificial light (+ or – plastic), relative to that in spectra measured with natural light.

RESULTS

Differences in reflectance spectra of natural biofilms

For all biofilms, spectra acquired using artificial light (+ plastic) showed the greatest differences from spectra measured using natural light (Fig. 1). For wavelengths greater than 460 nm (i.e. the spectral region not affected by noise), the greatest differences from natural light were found in the encrusting macroalgal biofilms. For microalgal biofilms, the spectra measured under artificial light (+ or – plastic) had more reflectance at wavelengths shorter than about 470 to 530 nm than did spectra measured under natural light (Fig. 1). The opposite was found at wavelengths longer than about 470 to 530 nm.

Differences in chlorophyll indices from spectra of natural biofilms

For microalgae, there were no significant differences in the R_{750}/R_{672} ratio generated from spectra in each treatment (Fig. 2). For the macroalgae, the R_{750}/R_{672} ratio using artificial light (+ plastic) was significantly smaller than in the other treatments (SNK tests, $p < 0.01$, n = 9). There were no significant differences in PI among treatments (Fig. 2).

Relationships between chlorophyll indices and laboratory estimates of chlorophyll

Each chlorophyll index showed a strong linear relationship with laboratory estimates of chl *a* (Fig. 3). For all treatments, relationships were marginally stronger for the PI than for R_{750}/R_{672}. The largest difference in correlation was found in the artificial light (– plastic) treatment, where PI was associated with 4% more variance than was R_{750}/R_{672}. The weakest relationships between chlorophyll indices and laboratory estimates of chl *a* were found in the artificial light (– plastic) treatment, but results were significant and were associated with 85% and 89% of the variance in the data for R_{750}/R_{672} and PI, respectively.

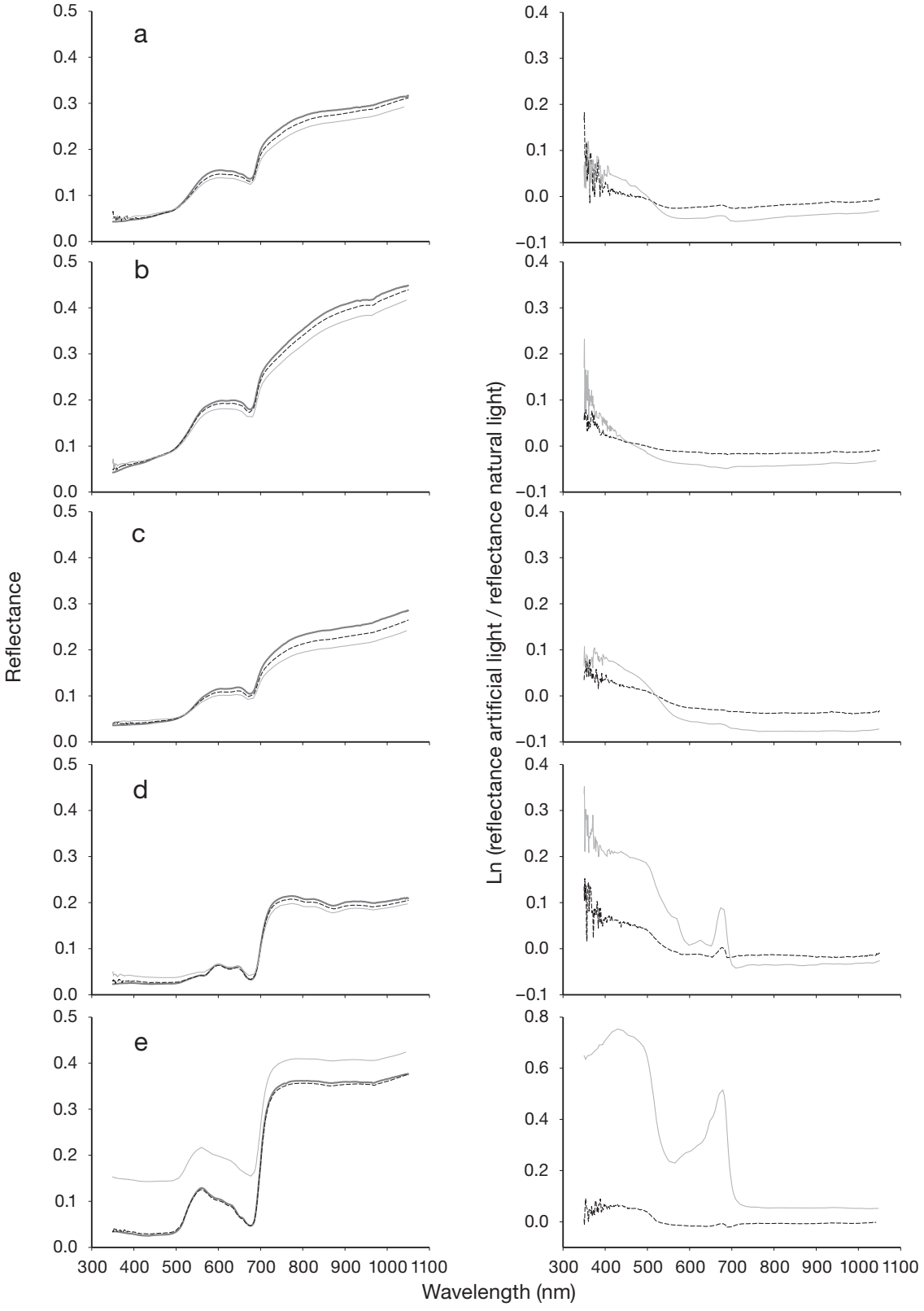

Fig. 1. Reflectance spectra (left panels) of intertidal biofilms measured using natural light (dark grey thick line), artificial light (– plastic) (black dashed line) or artificial light (+ plastic) (light grey line). Change in reflectance of spectra (right panels) measured using artificial relative to natural light: artificial light (– plastic) (dashed line) and artificial light (+ plastic) (thin grey line). (a) Red-coloured microalgae; (b) brown-coloured microalgae; (c) red-brown-coloured microalgae; (d) *Hildenbrandia rubra* and (e) *Ulva* sp.

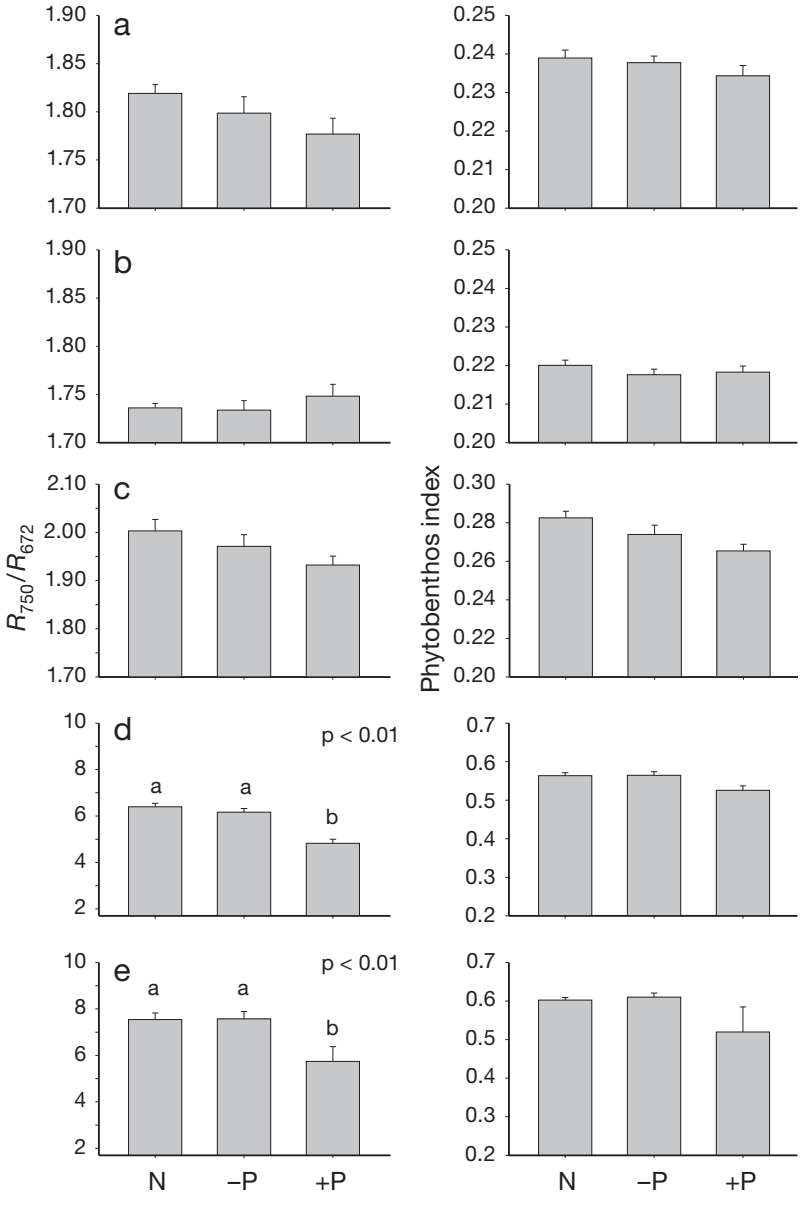

Fig. 2. R_{750}/R_{672} ratio of spectra of intertidal biofilms (mean ± SE; n = 9) shown in Fig. 1. Natural light (N); artificial light (– plastic, –P); artificial light (+ plastic, +P). (a) Red microalgae; (b) brown microalgae; (c) red-brown microalgae; (d) *Hildenbrandia rubra* and (e) *Ulva* sp. Significant differences among treatments (SNK tests) are indicated by letters above the bars

Positions of wavelengths and heights of derivative peaks

The modal wavelengths of the largest peaks in the replicate spectra acquired from the biofilms were calculated (n = 9; Table 2). The pigments causing these peaks were determined by matching the locations of their wavelengths with the known locations of pigment absorptions *in vivo* from the literature (e.g. see survey in Murphy et al. 2005a). Maximal shifts in wavelength of each of the peaks tested were determined amongst replicate spectra for each biofilm. Amongst treatments, the modal wavelengths of peaks varied by only 1 nm. Spectra acquired using artificial light (– plastic) and artificial light (+ plastic) had, respectively, the smallest and largest ranges in wavelength position of the features.

Significant differences were found in the heights of each peak in derivative spectra among treatments (Table 3). In macroalgal spectra, the heights of peaks were smallest in spectra acquired under artificial light (+ plastic). In the majority (5 of 6) of cases, there were no significant differences in the heights of peaks in spectra acquired under natural versus artificial light (– plastic). For microalgae (Areas 1–3; Table 3), the heights of peaks did not vary in a consistent way among treatments. For example, the heights of some peaks were smaller when measured under artificial than under natural light (e.g. pigment peak 3 in spectra from Area 1). The heights of other pigment peaks, however, were greater when measured under artificial than under natural light (pigment peak 1 in spectra from Area 3).

Differences in derivative spectra measured by the three methods

Derivatives of spectra acquired with artificial light were compared with those measured with natural light in terms of: (1) the wavelength position and height of the different peaks in the spectrum which provide information on pigment type and abundance and (2) the shape of the derivative spectra (500 to 750 nm) and their distributions in multivariate space.

Shape of the derivative curve and distributions in multivariate space

The shapes of average 4th-derivative spectra were consistent among the different treatments (Fig. 4). Peaks representing the centres of absorption of different pigments were located at similar wavelengths and had similar amplitudes. On average, the largest absolute differences from spectra measured in natural light were observed in spectra measured with artificial light (+ plastic).

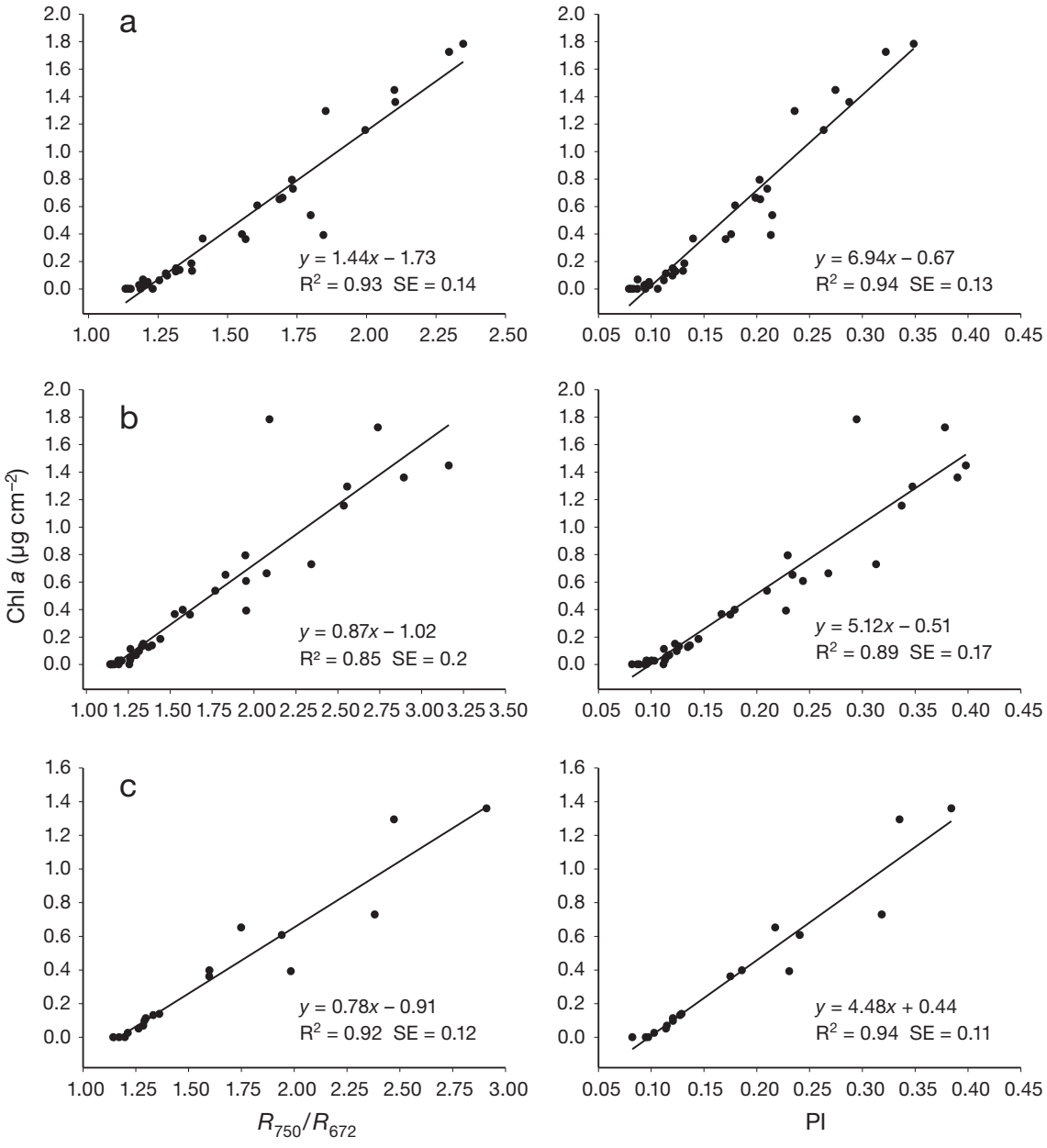

Fig. 3. Regression of chl *a* on R_{750}/R_{672} (left column) and chl *a* on phytobenthos index (PI) (right column): (a) natural light; (b) artificial light (– plastic) or (c) artificial light (+ plastic). Summary of regression analyses is shown in the bottom right of each graph; $p < 0.0001$ for all graphs

nMDS ordination showed that spectra were tightly distributed into 3 clusters, representing (1) *Hildenbrandia rubra*, (2) *Ulva* sp. and (3) the different microalgal biofilms (Fig. 5; left panel). The distribution of the biofilms was similar for all treatments. Dissimilarities among the spectra of the microalgal biofilms were not observed in these plots because the microalgal biofilms were much more similar to each other than they were to either of the macroalgal spectra (Table 4). To allow clearer representation of the microalgal biofilms, they were examined separately from the macroalgal samples. This nMDS ordination (Fig. 5, right panel) showed that spectra of microalgal biofilms formed into groups, each of which occupied different regions of the nMDS plot. The patterns of distribution, however, were similar for each of the 3 treatments.

ANOSIM confirmed the interpretations of the nMDS plots. Spectral data of microalgae, *Hildenbrandia rubra* and *Ulva* sp. were significantly different (Table 5). The 3 microalgal assemblages were also

Table 2. Wavelengths and maximal difference (shift in wavelength) of the 3 largest peaks in 4th-derivative spectra acquired under natural light (N), artificial light (– plastic, –P) or artificial light (+ plastic, +P), n = 9 spectra. Chl: chlorophyll, PEC: phyco-erythrocyanin, PE: phyco-erythrin

Area	Pigment peak	Modal wavelength (nm)			Maximal difference (nm)		
		N	–P	+P	N	–P	+P
1	1 Chl *b*	650	649	650	2	1	1
	2 Chl *a*	668	667	668	1	1	1
	3 Chl *a*	683	683	684	1	0	0
2	1 Chl *b*	649	649	649	1	0	1
	2 Chl *a*	668	668	668	1	1	0
	3 Chl *a*	683	683	683	1	1	1
3	1 PEC	575	574	575	4	1	2
	2 Chl *a*	668	668	669	3	1	1
	3 Chl *a*	684	684	684	0	1	2
4	1 PE	570	571	570	2	0	5
	2 Chl *a*	665	666	666	1	2	2
	3 Chl *a*	684	685	685	1	1	1
5	1 Chl *b*	649	649	649	0	0	5
	2 Chl *a*	667	667	667	0	0	8
	3 Chl *a*	684	685	685	0	1	10

Table 3. Results (SNK tests) from a 1-factor analysis of variance of heights of peaks in spectra (n = 9) in the different treatments: natural light (N); artificial light (– plastic; –P); artificial light (+ plastic; +P). Chl: chlorophyll, PEC: phyco-erythrocyanin, PE: phyco-erythrin. $^{\star}p < 0.05$; $^{\star\star}p < 0.01$; =: not significantly different, ns: $p > 0.05$

Area	Pigment peak	Significance
1	1 Chl *b*	ns
	2 Chl *a*	ns
	3 Chl *a*	+P = –P < N*
2	1 Chl *b*	ns
	2 Chl *a*	N < –P < +P**
	3 Chl *a*	ns
3	1 PEC	N = –P < +P*
	2 Chl *a*	ns
	3 Chl *a*	+P = –P < N**
4	1 PE	+P < –P = N**
	2 Chl *a*	+P < N = –P**
	3 Chl *a*	+P < –P < N**
5	1 Chl *b*	+P < –P = N**
	2 Chl *a*	+P < N = –P*
	3 Chl *a*	+P < –P = N**

significantly different from each other. Pairwise ANOSIM tests showed that there were no differences among treatments: natural versus artificial light (+ plastic; R = 0.10, p = 0.001); natural versus artificial light (– plastic; R = 0.10, p = 0.001); artificial light (+ plastic) versus artificial light (– plastic; R = 0.05, p =

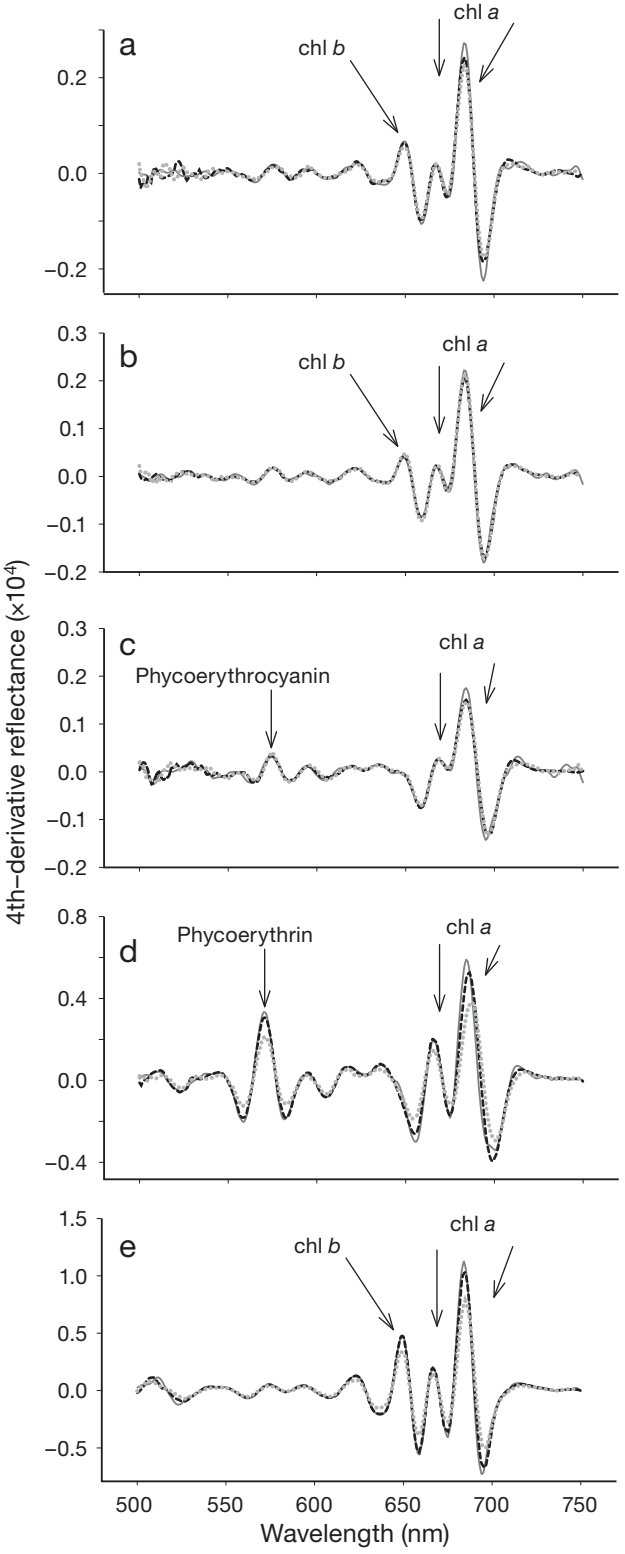

Fig. 4. Derivative spectra of natural biofilms measured using natural light (dark grey line), artificial light (– plastic) (black dashed line) or artificial light (+ plastic) (light grey dotted line). (a) Red-coloured microalgae; (b) brown-coloured microalgae; (c) red-brown-coloured microalgae; (d) *Hildenbrandia rubra* and (e) *Ulva* sp.

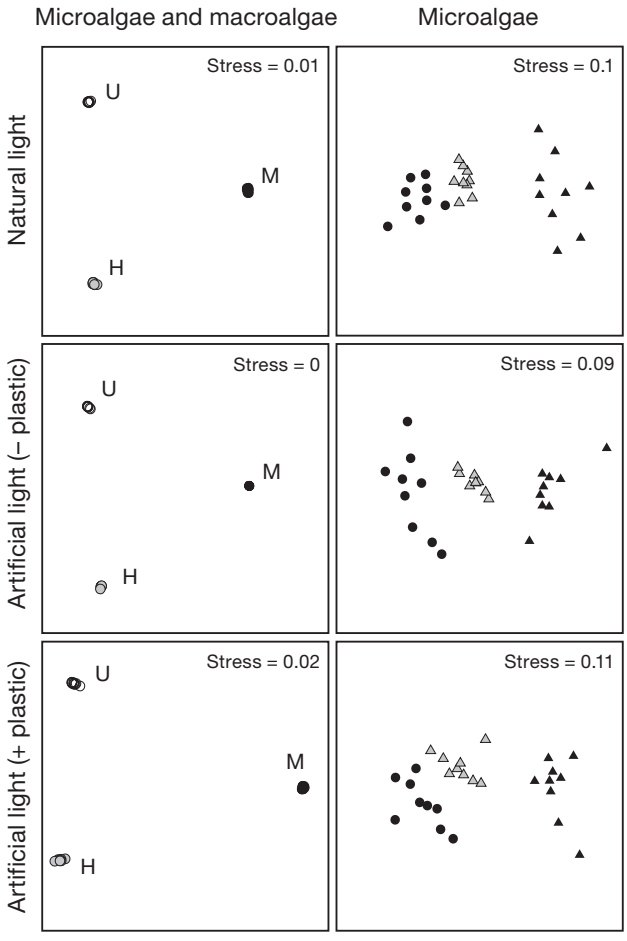

Fig. 5. Non-metric multidimensional scaling plots generated from the derivative spectra (500–750 nm) of natural biofilms acquired under natural or artificial light, indicating similar differences between the biofilms among the different treatments. Left panel: micro- (M) and macroalgal (H = *Hildenbrandia rubra*; U = *Ulva* sp.) biofilms. Right panel: microalgal biofilms: (black triangles, area 1; grey triangles, area 2; black circles, area 3). See also Tables 4 & 5

Table 4. Mean values of dissimilarities (Euclidean distances) within and between the groups of biofilms calculated from the derivative spectra (500–750 nm). 1–3: Microalgae; 4: *Hildenbrandia rubra*; 5: *Ulva* sp. (see non-metric multidimensional scaling plot in Fig 5). The top diagonal row shows within-group dissimilarities

	1	2	3	4	5
1	8.47				
2	8.89	5.77			
3	10.61	8.67	7.97		
4	27.30	27.16	25.79	12.53	
5	28.58	28.9	30.30	30.19	8.28

noise increased. This was similar for the artificial light (+ plastic) and artificial light (– plastic) treatments. Noise also increased at wavelengths longer than 760 nm, particularly in the artificial light (+ plastic) treatment. Amounts of noise in the spectra corresponded to parts of the spectrum where there are smaller amounts of incident light as measured by the probe or using natural light (the former are measured relative to the latter).

DISCUSSION

Increasing use of field spectrometry in ecological experiments requires that measurements are made using standard protocols and under standard conditions of observation. Because measurements are made relative to the amount of incoming light, the reflectance of a particular surface should be consistent, independent of the source of illumination. Factors that affect reflectance include the geometry of measurement (i.e. the relative positions of the source of illumination, the target and the sensor) and the wavelength-intensity distribution of the source of illumination. In

0.008). Results from nMDS and ANOSIM therefore indicate that the 3 methods of measuring reflectance provided similar information and can be used to distinguish between the different intertidal biofilms.

Spectral noise

The amount of noise in spectra measured by the reflectance probe showed consistent patterns for all biofilms (as shown for microalgae in Fig. 6). At wavelengths less than 480 to 500 nm,

Table 5. Analyses of similarity (ANOSIM) of the derivative spectra (500–750 nm) of biofilms from the different areas (see also Fig 5). Each line shows the R statistic (R) and the significance level from the pairwise tests of the different areas acquired under natural or artificial light (+/– plastic)

Area	Natural		Artificial light (+ plastic)		Artificial light (– plastic)	
	R	p	R	p	R	p
1–2	0.55	0.001	0.91	0.001	0.82	0.0001
1–3	0.80	0.001	0.85	0.001	0.95	0.001
2–3	0.74	0.001	0.82	0.001	0.9	0.001
All others	1	0.001	1	0.001	1	0.001
Global R	0.84	0.001	0.86	0.001	0.87	0.001

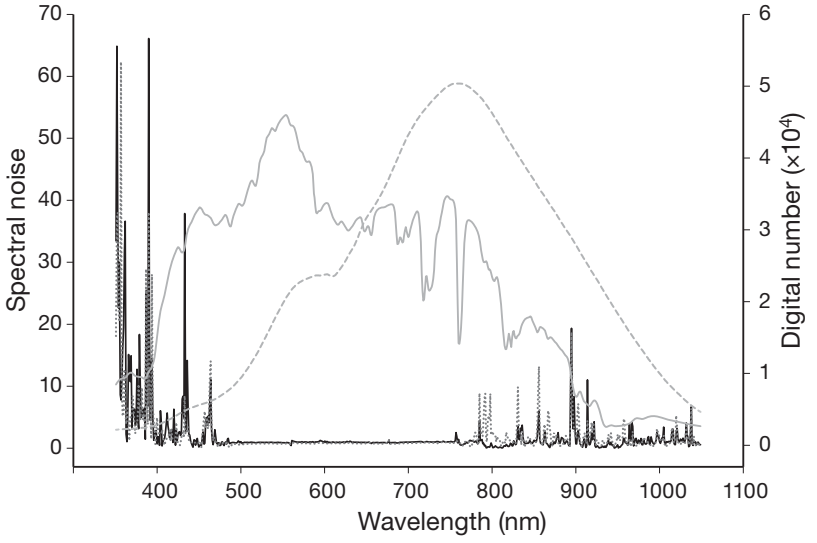

Fig. 6. Local amounts of variation (noise) in spectra measured under artificial relative to natural light. Artificial light (– plastic) (solid line); artificial light (+ plastic) (dotted line). The distribution of incident illumination at different intensities and wavelengths is shown for comparison. Natural light (grey solid line); artificial light (grey dashed line)

intertidal areas, specular reflectance (sun glint) from the substratum can affect reflectance to different degrees, depending on the viewing geometry (Coelho et al. 2009). Our reflectance probe acquired spectra with a standard geometry and with a stable light source which illuminated the target from all directions, minimising specular effects.

The R_{750}/R_{672} and PI indices of chlorophyll derived from spectra measured by the probe were strongly and linearly related to laboratory measures of chl *a*. The relationship between chl *a* and indices generated from spectra in the artificial light (– plastic) treatment were slightly weaker than was the case for natural light. The reasons for this are unclear. A similarly strong relationship was found between chl *a* and R_{750}/R_{672} from spectra acquired with artificial light (+ plastic), indicating that measurements can be made through a plastic film so that the probe is not contaminated by material from the substratum (as in the case of soft sediments).

Although the relationships between chl *a* and the R_{750}/R_{672} and PI indices are strong, there were differences among treatments in the slopes of the regressions. Therefore, to convert chlorophyll indices to amounts of chl *a*, a different regression equation must be used, specific to the method that was used to measure reflectance. Amounts of chl *a* on rocky substrata are generally much less than in soft sediments, with values from several studies generally not exceeding 12 µg cm^{-2} (Underwood 1984a,b, Dye & White 1991, Lasiak & White 1993, Boaventura et al. 2003, Skov et al. 2010). Murphy et al. (2006) found that amounts of chl *a* in microflora in Sydney, Australia, did not exceed 2 µg cm^{-2}. The use of chlorophyll indices at red wavelengths can underestimate amounts of chl *a* at concentrations greater than ~19.2 µg cm^{-2}, because absorption by chl *a* is offset by increasing fluorescence, at least in the case of sediments (Serôdio et al. 2009). The range of concentrations found here was well below such critical amounts.

Variability in the wavelength positions of absorption peaks was greatest amongst spectra acquired under artificial light (+ plastic). The range of variability (≤10 nm) is of small consequence for identifying pigments from derivative spectra. The reason for the greater variability in the wavelength position in the artificial light (+ plastic) treatment is unclear, but may be an artefact of measurement caused by small droplets of condensation forming on the underside of the plastic film. This was not seen during measurement of the microalgal biofilms with plastic because the film was flush with the substratum. Condensation may also be the cause of the significant differences found for R_{750}/R_{672} in the artificial light (+ plastic) treatment for macro- but not microalgal biofilms.

For macroalgal biofilms, the height of peaks in derivative spectra were consistently smaller for spectra acquired with artificial light (+ plastic) than in the other treatments. Differences in heights of peaks in the microalgal biofilms were not significantly different among treatments in the majority of cases. If, however, the heights of peaks are to be used as indices for abundances of pigments, it is recommended that a single method should be used for all spectral measurements within an experiment, e.g. using the reflectance probe without plastic or with plastic, but not a mixture of both.

Amounts of noise in the reflectance spectra below 480 nm and above 760 nm make these parts of the spectrum unsuitable for analysis using higher-order derivatives. Because absorptions by most photosynthetically-active pigments in algae occur between 500 and 700 nm, this does not pose a problem. Taken together, our findings show that a reflectance probe with an integrated, artificial light source can provide quantitative information on amounts of chlorophyll *a* and on relative abundances of other photosynthetically-active pigments in intertidal biofilms *in situ*. Measurements can be made at a small fraction of the cost of conventional sampling. Furthermore, the method is extremely rapid (~5 s per measurement), compared with natural sunlight or conventional sampling methods.

Using sunlight for illumination has limited the application of field spectrometry in experimental intertidal ecology. The present study shows how measurements can be made under varying environmental conditions and in microhabitats where complex topography precludes the use of natural sunlight.

Acknowledgements. This research was supported by the Australian Research Council through its Special Research Centre Scheme and the Australian Centre for Field Robotics. J.C.K. was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, KL-2264/1-1). This work conforms to the policies on ethics in research of the University of Sydney, the Australian Centre for Field Robotics and the Centre for Research on Ecological Impacts of Coastal Cities. We are grateful to 3 anonymous reviewers for their comments which improved this paper.

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Editorial responsibility: Hans Heinrich Janssen, Oldendorf/Luhe, Germany

Submitted: October 18, 2010; Accepted: February 28, 2011
Proofs received from author(s): April 19, 2011