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Extraction and quantification of microphytobenthic Chl *a* within calcareous reef sands

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Abstract

Calcareous reef sands are characterized by high concentrations of photosynthetic pigments that extend well below the sediment surface, as well as by high variability in concentrations between study sites. An important contributor to the observed variability may be differences in extraction protocols, further complicated by variability in calcareous sand characteristics. We tested the effects of freeze-drying, grinding, sonication, extraction temperature, and extraction time on quantification of Chl *a* content within calcareous sands. The resulting optimized extraction protocol consists of freeze-drying, grinding with a mortar and pestle for 30 s, and extracting with 100% acetone at –20°C for at least 20 h, yielding a 39% increase in Chl *a* content over frozen, unground samples. Using this protocol, we measured and compared ten sedimentary Chl *a* profiles taken in close proximity to test for relationships between surface and sub-surface concentrations. Sedimentary Chl *a* content at a back-reef location on the south shore of O'ahu varied between 4.33-14.25 μg g^{-1} dw, with distinctly higher values occurring in a relatively enriched surface layer (0-1 cm). Surface Chl *a* concentrations varied between 86-307 mg m^{-2}, depending on the depth of integration (0.5-2 cm), with 73% of the full-core (0-8 cm) Chl *a* concentration occurring below 2 cm. The concentrations of surface and subsurface layers were significantly correlated between cores, allowing for the use of plug sampling when profile generation is not feasible or necessary to determine the magnitude of the subsurface microphytobenthic biomass and its variability over scales of meters.

Unconsolidated, highly permeable sediments are ubiquitous in the euphotic shallow ocean (Emery 1968) and are characterized by high exchange rates and high metabolic activity (Huettel and Webster 2001). Whereas attention has mostly focused on heterotrophic processes in sands (e.g., Rusch et al. 2006), evidence is accumulating for the important role of microphytobenthos (MPB) in the functioning of permeable sediments. MPB in sands may (a) provide food for benthic and benthopelagic fauna, (b) reduce sediment-to-water nutrient fluxes by assimilating nutrients and immobilizing them in the sediment, (c) act as an indicator of nutrient pollution, (d) ameliorate hypoxia through oxygen production, and (e) maintain water clarity by stabilizing the sediment (see reviews in Roelfsema et al. 2002; Heil et al. 2004).

Highly permeable sediments can be classified into two major categories: (a) terrigenous siliciclastic sediments originating mainly from the erosion of continental rocks; and (b) biogenic calcareous sands produced by the breakdown of the mineral tissue of coral, macroalgae, and other organisms (Wild et al. 2005). Per unit of seafloor surface area, other communities associated with these sediments overshadow the contribution of MPB to seafloor primary production. For example, MPB gross primary production (GPP) rates in siliceous permeable sediments (e.g., 400 mg C m^{-2} d^{-1}, Jahnke et al. 2000), are relatively low compared with the GPP of seagrass or macroalgal meadows (e.g., 2850 mg C m^{-2} d^{-1}, Gattuso et al. 1998). Similarly, coral GPP driven by endosymbiotic zooxanthellae has been estimated at 7200 mg C m^{-2} d^{-1} (Kinsey 1985), whereas the most recent calculations indicate that the GPP of the MPB in calcareous reef sands ranges between 1600-3000 mg C m^{-2} d^{-1} (Werner et al. 2008). However, an assessment of the relative contributions of different communities, such as sand patches, macroalgal forests, and corals, to the productivity of coral reef systems must not rely solely on GPP per unit area of seafloor, but also on the areal extent of each community. Calcareous reef sands can occupy up to twice the seafloor area of coral and

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macroalgae in a typical reef setting (Roelfsema et al. 2002), and consequently MPB in sands could account for the same amount of productivity as the latter do over the scale of the whole coral reef ecosystem (Clavier and Garrigue 1999; Werner et al. 2008). The potential magnitude of MPB contribution to ecosystem-wide benthic primary productivity, especially in view of the anticipated decline in dissolved O_2 due to climate change (Keeling et al. 2010), necessitates further investigation into their role as important components of calcareous reef sand communities and habitats.

Pigment distribution in sediments

A key parameter in any study of benthic primary productivity is MPB biomass. However, the enumeration and identification of MPB is a time-consuming process, and consequently, photosynthetic pigments, and especially Chl *a*, are commonly used as proxies of MPB biomass (MacIntyre et al. 1996). Typically, a sedimentary sample of known dry weight is collected using a core of known cross-sectional area, is analyzed, and the amount of recovered pigment is normalized to (a) the dry weight of sediment to calculate the content, *M*, defined as μg of pigment per g of dry sediment, or μg g^{-1} dw, and (b) the cross-sectional area of the core to calculate the concentration, *C*, which is defined as mg of pigment per unit area of seafloor, or mg m^{-2} (*see* "Materials and procedures" for calculation details). Infrequently, *M* is converted to *C* using sedimentary weight-to-volume conversion factors, even though such conversions must be avoided when based on assumptions and not measurements of these factors (e.g., Tolhurst et al. 2005). Two major sampling approaches for microphytobenthic pigment determination are commonly reported in the literature: profiling and plug sampling.

Profiling consists of sediment coring, sectioning of the core (typically every 0.5-1 cm), and analysis of each section for *M*, followed by integration over a layer ranging from the sediment-water interface (SWI) down to a chosen depth (hereby defined as the depth of integration), and then dividing by the core cross-section area to obtain a *C* value (e.g., Werner et al. 2008). Selection of the depth of integration is a crucial step. Integrating over a depth of several mm to 1 cm below the SWI is commonly considered reasonable (Table 1), since availability of scalar irradiance (both visible and infrared, 400-880 nm) that can be used for photosynthesis in sands is restricted to the top few millimeters of the sediment column (Lassen et al. 1992; Kühl et al. 1994), and most of the actively photosynthesizing cells are expected to aggregate there. Whereas depth-relevant information provided by profiles can be useful, the number of samples needed, especially after factoring in the need for replicates, can make this a time-consuming and costly approach.

Plug sampling may greatly minimize the number of samples needed to effectively determine *C* at a given location. It consists of obtaining a single section that extends from the SWI down to a predetermined depth, essentially replacing the depth of integration with a sampling depth (e.g., Roelfsema et al. 2002; Grinham et al. 2007). Grinham et al. (2007) demonstrated that 8 plug samples down to 2 cm are sufficient to generate a reliable measure of *C* on a scale of meters, but also pointed out that *M* can be underestimated if high-value surface layers (e.g., 0-0.5 cm) are diluted by deeper poorer layers (e.g., 1.5-2 cm). Moreover, *C* can be underestimated if the sampling depth excludes deeper layers where pigments are present.

Plug sampling may be best suited for cases where pigments are detectable only a few mm to 1 cm below the SWI, as is commonly the case in quiescent locations such as estuaries and in finer-grained sediments (e.g., Cartaxana et al. 2006). In high-energy settings that are characterized by the presence of permeable, coarse-sand sediments, viable pigment-containing MPB can be distributed for centimeters to decimeters below the SWI (MacIntyre et al. 1996; Roelfsema et al. 2002; Werner et al. 2008). Accordingly, available profiles of pigment content in calcareous reef sediments feature significant amounts of pigment (e.g., >3 μg g^{-1} dw, Fig. 1) down to 10 cm below the SWI, well below scalar irradiance penetration depths. Another common feature of the limited available data are high variability (×2-5) both between replicate samples at the same depth as well as with depth in the same profile, with com-

Table 1. Concentration of sedimentary Chl *a* for different sediment types. The depth from the sediment-water interface (SWI) indicates the thickness of the sedimentary layer from the sediment-water interface over which *C* is calculated, and corresponds to the depth of integration and sampling depth for profiling and plug sampling, respectively.

Location (sediment type)	Chl *a* concentration (mg m^{-2})	Depth from SWI (cm)	Reference
Southwest lagoon, New Caledonia (calcareous sand)	59 ± 4–62 ± 5	1	Clavier and Garrigue (1999)
Heron Reef (calcareous sand)	36–1153	1.5–2	Roelfsema et al. (2002)
Heron Reef (calcareous sand)	92–995	1	Heil et al. (2004)
Heron Reef (calcareous sand)	31 ± 3–84 ± 10	1	Werner et al. (2008)
Cooloolo Passage (sand)	47 ± 2	2	Grinham et al. (2007)
Oxley Creek (silt)	33 ± 3	2	Grinham et al. (2007)
Georgia shelf (siliceous sand)	6–37.7	0.5	Nelson et al. (1999)
Florida shelf (siliceous sand)	10.6–40.9	0.5	Nelson et al. (1999)
Tagus estuary (siliceous sand)	59 ± 18	0.2	Cartaxana et al. (2006)
Tagus estuary (mud)	61 ± 20	0.2	Cartaxana et al. (2006)

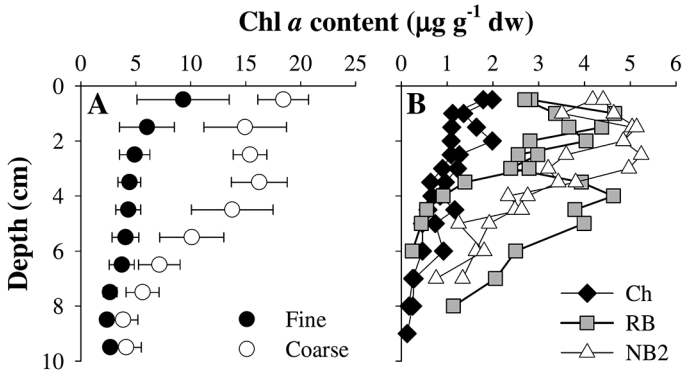

Fig. 1. Chlorophyll *a* (Chl *a*) content (dw = dry weight) profiles in calcareous reef sediments of Heron Reef, Great Barrier Reef, Australia (data replotted from the literature): (A) means and standard deviations of triplicates at each one of two stations: coarse sand on the reef flat (mean grain size, d_{mean} = 897 μm), and fine sand on the reef edge (d_{mean} = 590 μm) (Rao et al. 2012); (B) duplicate profiles from three stations: NB2 on the reef flat (d_{mean} = 591 μm), RB on the reef edge (d_{mean} = 426 μm), and Ch in the channel (d_{mean} = 227 μm) beyond the reef edge (Werner et al. 2008).

monly occurring sub-surface maxima (Fig. 1). These patterns in calcareous reef sands may reflect dynamic processes shaping pigment distributions in the vertical dimension, e.g., sediment mixing, cell filtration, and cell motility (MacIntyre et al. 1996; Rao et al. 2012). When coupled with high irradiance and low turbidity, which commonly characterize reef habitats (Clavier and Garrigue 1999), these processes result in high content variability and deep penetration below the SWI.

Because subsurface MPB (2-6 cm below SWI) has been shown to be viable (i.e., if given light, it has the potential to photosynthesize; Werner et al. 2008), sampling only surface sediment or integrating only over surficial depths to estimate *C* may exclude the majority of viable MPB biomass from the calculation. Consequently, given the concentration of subsurface pigments in calcareous reef sediments, subsurface sediment sampling and analysis is necessary to obtain a more comprehensive understanding of MPB-driven processes such as benthic primary productivity and photosynthesis/respiration coupling. Also, given the variability between replicate profiles (Fig. 1), the number of replicates necessary to characterize a station must be chosen carefully, and the logistical burden of generating more than 2-3 profiles per station must be considered. Moreover, the existence of a relationship between surface and sub-surface content has not been explicitly assessed and is far from certain, particularly given the aforementioned variability and the dynamic nature of calcareous reef sediment settings. These considerations dictate a need to compare profiling with plug sampling, while at the same time counter-balancing statistical rigor with logistical constraints.

Sample processing and analytical considerations

One potential reason for the observed variability between studies of pigments in calcareous reef sediments may be the extraction efficiency achieved with different analytical protocols (Heil et al. 2004). Whereas methods of pigment quantification in the water column are well-established (e.g., Jeffrey et al. 1997; Roy et al. 2011), similar methods for sediments are under debate (Reuss and Conley 2005; Hagerthey et al. 2006). Moreover, the nature of permeable calcareous reef sediments complicates the issue. Compared with siliciclastic grains, biogenic calcareous grains are irregularly shaped and marked by various microtopographic features such as pits, crevices, and crevasses, resulting in poorer sorting, a higher grain surface-area-to-volume ratio, and consequently greater microbial abundances (Wild et al. 2005; Schöttner et al. 2011). The more complex nature of the substrate, therefore, results in the necessity for multiple methodological steps to detect more accurately various microbial characteristics, such as cell numbers (Wild et al. 2006; Rusch et al. 2009).

The extraction efficiency of pigments from complex substrates, such as sediment, is commonly enhanced by freeze-drying, grinding, and sonication, used in isolation or sequentially (Pinckney et al. 2011). Freeze-drying a sample before extraction significantly improves the extraction efficiency of pigments compared with "wet-frozen" extraction (Buffan-Dubau and Carman 2000), and also improves peak resolution during HPLC, irrespective of the solvent used for the extraction (Hagerthey et al. 2006; van Leeuwe et al. 2006). Moreover, water contained in sandy sediment samples can induce dilution effects that differ from sample to sample, due to high but variable permeability and poor sorting (Buffan-Dubau and Carman 2000). Changes in the solvent solution concentration due to water in the sample may also significantly shift the wavelength at which pigment absorption maxima occur (Porra 2011). Therefore, removal of water by freeze-drying may improve analytical performance and precision.

Mechanical disruption via sonication is considered indispensable for calcareous reef sediments because the complex grain surface allows for the development of complex biofilms and the persistent attachment of cells and detrital materials (Wild et al. 2006; Rusch et al. 2009). Sonication has been shown to improve extraction irrespective of the solvent used, and allows shorter extraction times (Cartaxana and Brotas 2003). Sonication duration may vary from 8-10 short bursts of 3-6 s (Hagerthey et al. 2006) to several (7-15) minutes of continuous treatment (Grinham et al. 2007; Werner et al. 2008). In the case of long sonication times, the concomitant temperature increase may cause pigment degradation (Metaxatos and Ignatiades 2002), and consequently, it has been recommended to sonicate in an ice bath using cold solvents and a succession of short bursts (Reuss and Conley 2005; Hagerthey et al. 2006; van Leeuwe et al. 2006).

Grinding is typically put forth as an alternative to freeze-drying and sonication. Whereas no effects have been observed if samples are previously freeze-dried (Hagerthey et al. 2006), grinding can significantly improve extraction efficiency in the case of frozen samples (Heil et al. 2004). Calcareous reef sedi-

ment grain surfaces are characterized by rough microtopography that may protect pigment-containing cells from lysis during extraction. Thus, abrasion during grinding may release the cell-rich layer of the grain surfaces and dramatically increase exposure to the solvent.

In addition to procedures involving freeze-drying, sonication, and grinding, various extraction durations and temperatures have been reported. Long extraction times of 24 h or more are not uncommon (Clavier and Garrigue 1999; van Leeuwe et al. 2006; Werner et al. 2008), however they are thought to result in pigment degradation, isomerization, and allomer formation (Cartaxana and Brotas 2003). On the other hand, very short extraction times of less than an hour may not be sufficient. For example, cells with shells, such as testate diatoms, may take longer to digest than cyanobacteria (Jeffrey et al. 1997). For siliceous sandy sediments, studies have indicated that extraction times greater than 3 h do not result in significantly improved efficiencies (Buffan-Dubau and Carman 2000; Cartaxana and Brotas 2003), especially when the samples have been freeze-dried and mechanically disrupted (usually by sonication). The optimal duration of extraction also depends on the extraction temperature, which must be low enough to slow down pigment degradation but high enough to induce fast and efficient dissolution (van Leeuwe et al. 2006). Sedimentary and/or calcareous matrix samples have either been extracted at 2-4°C (Heil et al. 2004; Hagerthey et al. 2006; Chevalier et al. 2010) or at –20°C (Grinham et al. 2007; Werner et al. 2008), and a study on microalgal cultures demonstrated no observed difference between the two temperatures (van Leeuwe et al. 2006).

Objectives of this study

The preceding overview leads to two outstanding issues in the study of MPB in calcareous reef sediments: the isolated and synergistic effects of various methodological steps on pigment extraction efficiency, and the quantification of subsurface *C* and its relation to surface-layer *C*. In this study, we developed an optimized pigment extraction protocol for calcareous reef sands by investigating the effects of freeze-drying, sonication, grinding, and extraction duration and temperature on the final *M* measured. Using the optimized protocol, we analyzed and compared multiple sedimentary pigment profiles taken in close proximity to test for relationships between surface and sub-surface *C* values when investigating distributions on the lateral scale of meters. Based on our results, we propose a sampling strategy that captures the full-core pigment inventory (in mg m^{-2}) and its lateral variability without the time-consuming and resource-intensive requirements of fine-scale sediment profiling, where the latter is not needed.

Materials and procedures

Sediment sampling and sub-sampling

We obtained samples for this study from the shallow (0.5 m depth) near-shore back-reef of Waialae Beach Park (21° 16.1′ N, 157° 46.5′ W) on the south shore of Oahu, Hawaii. Surface sediment (down to 1 cm below the SWI) for the optimization of the extraction protocol was collected using several 50-mL Blue Max modified-polystyrene conical ("Falcon") tubes (BD). Cores for profiling were taken using 60-mL cut-off syringes (BD) with an inner diameter of 2.6 cm. Overlying water samples were collected in amber 250-mL Nalgene HDPE bottles (Thermo Fisher Scientific). All samples were placed on ice and kept in the dark (Buffan-Dubau and Carman 2000; Grinham et al. 2007) until arrival at the laboratory, which typically occurred within 30 min of sampling.

Upon arrival to the laboratory, the syringe cores were immediately stored upright in an ultra-cold (–80°C) freezer (Cartaxana and Brotas 2003; Reuss and Conley 2005), and the surface sediment from all Falcon tubes was combined and gently homogenized with a spatula in an ice-cooled beaker. Subsamples (1-2 g, 0.5-1 mL) of the pooled, homogenized sediment were transferred to pre-weighed 13 × 100 mm borosilicate tubes (VWR), which were covered with Parafilm (Pechiney, Plastic Packaging), and stored in an ultra-cold (–80°C) freezer until analysis.

The syringe cores were sectioned as soon as they were frozen (typically within 12-24 h after storage) at 0.5-cm depth intervals using a clean metal blade, and the sections were stored at –80°C in pre-weighed borosilicate tubes covered with Parafilm. Water samples were filtered onto 25-mm Whatman GF/F filters (GE Healthcare Life Sciences), which were folded in aluminum foil and stored at –80°C until extraction. The period between sampling and analysis did not exceed 2 weeks, thus minimizing storage effects. Sample processing and subsequent analysis were conducted under dim light (Jeffrey et al. 1997).

Sedimentary characteristics

Sedimentary characteristics at the study site (Table 2) were determined on sediment collected down to 10 cm below the SWI using a 5-cm diameter core. Grain-size distribution was determined by wet-sieving, and mean grain size, sorting, and skewness were calculated using the equations in McManus (1988). Porosity was determined gravimetrically (Breitzke 2000), whereas permeability was determined by the constant-head method (Klute and Dirksen 1986).

Table 2. Characteristics of the back-reef sediment (0-10 cm below the sediment-water interface) of South Oahu, Hawaii, used in this study (n=3). Results indicate the average and one standard deviation. The dimensionless measures for sorting (σ_1) and skewness (SK_1) are determined using the phi system (McManus 1988).

Mean grain size (phi, μm)	0.98 ± 0.11, 509 ± 39
Median grain size (phi, μm)	1.08 ± 0.27, 480 ± 90
Sorting, σ_1	1.78 ± 0.02
Classification (Wentworth scale)	Poorly sorted
Skewness, SK_1	–0.23 ± 0.12
Classification (McManus)	Negatively skewed
Porosity	0.45 ± 0.02
Permeability ($\times 10^{-11}$ m^2)	1.86 ± 0.7

Freeze-drying

All samples, except those used as frozen controls, were freeze-dried in the dark using a Virtis Benchtop K freeze drier (SP Scientific) at –80°C and pressure < 3 Pa (Hagerthey et al. 2006; van Leeuwe et al. 2006). Sediment samples weighing 1-2 g were typically dry within 5-10 h. Freeze-dried samples were immediately processed to avoid pigment degradation during storage (Reuss and Conley 2005).

Grinding

Samples were ground with a mortar and pestle for 30 s (Heil et al. 2004). The mortar and pestle were cleaned between samples using a brush and laboratory tissue paper, rinsed with 100% acetone, and allowed to dry before reuse.

Extraction solvent

We extracted pigments using 100% acetone, as it has been shown to recover all pigments (especially pheopigments) from sedimentary matrices more efficiently than other solvents, and results in better-resolved peaks during HPLC (Buffan-Dubau and Carman 2000; Werner et al. 2008). A known volume (3 mL) of cold (2-4°C) 100% reagent grade acetone (Thermo Fisher Scientific) was added to the 1.5-2 g freeze-dried, ground sediment subsamples, to yield a sediment-to-solvent volume ratio between 1:2 and 1:3 (Grinham et al. 2007). Such a ratio has been determined as optimal because it maximizes the amount of extracted pigment without diluting the signal significantly.

Sonication

After solvent addition, the samples were briefly mixed with a vortexer, and then sonicated in a bath-style Branson 200 Ultrasonic cleaner (Branson Ultrasonics) filled with chipped ice (Hagerthey et al. 2006) for a 30-s period (van Leeuwe et al. 2006; Chevalier et al. 2010). When testing required longer sonication times, these were accomplished by sonicating for 30-s periods interspersed with 15-s pauses.

Extraction duration and temperature

Following sonication, the samples were placed in the dark in a refrigerator at 2-4°C (Heil et al. 2004; Hagerthey et al. 2006; Chevalier et al. 2010) or in a freezer at –20°C (Grinham et al. 2007; Werner et al. 2008; Ruivo et al. 2011) for periods of time between 2 and 60 h, depending on the treatment. During extraction periods longer than 12 h, the samples were mixed by vortexing at least once every 12 h (e.g., Arar and Collins 1997; van Leeuwe et al. 2006).

Chlorophyll *a* analysis

Upon conclusion of the extraction period, the samples were vortexed, followed by centrifugation at 1750×*g* for 5 min (Grinham et al. 2007). A measured volume of the supernatant was removed, diluted if necessary, and Chl *a* and pheopigment content determined by fluorometry before and after acidification (Yentsch and Menzel 1963; Holm-Hansen et al. 1965) using a Turner TD700 fluorometer (Turner Scientific). Fluorometry was selected for its high analytical sensitivity and speed, as well as low cost, and is considered sufficient in detecting relative differences in Chl *a* (Pinckney et al. 1994).

Selected samples were also analyzed by HPLC to determine the relationship between the two methods for our samples. Mixtures of 1-mL extract (spiked with canthaxanthin as an internal standard) plus 0.3-mL HPLC grade water were prepared in opaque autosampler vials, and 200 µL were injected into a Varian 9012 HPLC system (Varian/Agilent) equipped with a Varian 9300 autosampler (Varian/Agilent), a Timberline column heater (26°C; Timberline Instruments), and a Waters Spherisorb® 5-µm ODS-2 analytical (4.6 × 250 mm) column and guard cartridge (7.5 × 4.6 mm). Pigments were detected with a ThermoSeparation Products UV2000 detector (λ_1 = 436, λ_2 = 450; Thermo Fisher Scientific). A ternary solvent system was used for pigment analysis: Eluent A (methanol:0.5 M ammonium acetate, 80:20, v/v), Eluent B (acetonitrile:water, 87.5:12.5, v/v), and Eluent C (100% ethyl acetate). Solvents A and B contained an additional 0.01% 2,6-di-ter-butyl-*p*-cresol (0.01% BHT, w/v; Sigma-Aldrich) to prevent the conversion of Chl *a* into Chl *a* allomers. The linear gradient used for pigment separation was a modified version of the Wright et al. (1991) method: 0.0 min (90% A, 10% B), 1.00 min (100% B), 11.00 min (78% B, 22% C), 27.50 min (10% B, 90% C), 29.00 min (100% B), 30.00 min (100% B), 31.00 min (95% A, 5% B), 37.00 min (95% A, 5% B), and 38.00 min (90% A, 10% B) (Bidigare et al. 2005). Eluent flow rate was held constant at 1.0 mL min^{-1}. Pigment peaks were identified by comparison of retention times with those of pure standards and extracts prepared from algal cultures of known pigment composition. A dichromatic equation was used to resolve mixtures of monovinyl and divinyl Chl *a* spectrally (Bidigare and Trees 2000).

After removing the supernatant, the remaining sediment samples were dried in a drying oven at 60°C for 72 h and weighed, to calculate sedimentary Chl *a* content M_{Chla} (µg g^{-1} dw):

$$M_{Chla} = \frac{C_{extr} \times V_{extr} \times DF}{W_{drysed}} \quad (1)$$

where C_{extr} is Chl *a* concentration in the extract (µg L^{-1}), V_{extr} is the volume of extract (L), *DF* is the dilution factor, and W_{drysed} is the dry mass of extracted sediment (g). Similarly, concentration, $C_{z1\text{-}z2}$ (mg m^{-2}), for each section (between depths *z1* and *z2*) was calculated by dividing by the cross-sectional area of the collected core, *A* (5.3 × 10^{-4} m^2), instead of W_{drysed}:

$$C_{z1\text{-}z2} = \frac{C_{extr} \times V_{extr} \times DF}{A} \quad (2)$$

Each section of our cores was typically 0.5 cm thick (i.e., *z2* – *z1* = 0.5). To obtain *C* for broader depth layers, we added the corresponding *C* values for the inclusive sections.

Data analysis

Comparisons of means of the various data sets (e.g., treatments, depth intervals) and other statistical analyses were conducted with SPSS (IBM) using the methods in Sokal and Rohlf (1994), whereas data were graphed using the rules outlined in Cumming et al. (2007). When separate treatments

consisted of four or fewer replicates, data were plotted as distinct data points and compared by a Mann-Whitney U-test in the case of only two treatments, and by a Kruskal-Wallis test in the case of more than two treatments. Where five or more replicates per treatment were available, data were plotted using box plots, and two treatments were compared by a Student *t* test, while more than two treatments were compared by analysis of variance (ANOVA) and post-hoc analysis by a Student-Newman Keuls (SNK) test. Potential linear relationships were examined by least-squares linear regression to determine the Pearson correlation coefficient, *r*, and an equation for the relationship between the two parameters. Unless otherwise noted, all of the above tests were evaluated at a confidence interval of 95% ($\alpha = 0.05$).

The cumulative variation about a single M_{Chla} measurement, including natural variation between sub-samples, as well as analytical errors associated with weighing, dilution, and analysis, was estimated by analyzing and comparing replicate sub-samples of the same sediment sample. The cumulative variation was calculated by correcting the coefficient of variation, CV, which is defined as:

$$\mathrm{CV} = \frac{\text{standard deviation}}{\text{mean}} \times 100 \quad (3)$$

The correction of the CV as defined above is necessary because it is biased by the number of replicates (*n*) used in calculating the mean and standard deviation (Sokal and Rohlf 1994). To correct for this bias and to calculate the variation about one single measurement (n=1), as is frequently the case in single-profile analyses, the corrected CV, CV*, was calculated as:

$$\mathrm{CV}^* = \mathrm{CV} \times \left(1 + \frac{1}{4}\right) \quad (4)$$

Assessment

Extraction efficiency optimization

Degree of sonication

Freeze-dried, unground samples in triplicate were sonicated for 30-s intervals (with 15-s pauses in-between) for total sonication times of 0 s, 30 s, 1 min, 3 min, 5 min, and 7 min, and then extracted for 2.25 h at –20°C. The results (Fig. 2A) show that a short sonication period (30 s) significantly improves Chl *a* extraction efficiency in calcareous reef sediments (H = 13.55, df = 5, P = 0.019). Longer sonication periods (1-7 min) caused a slight but statistically significant decline in M_{Chla} (r = –0.83, t = –4.75, P = 0.001), most likely due to Chl *a* degradation (Metaxatos and Ignatiades 2002).

Temperature and duration of extraction

We determined the optimum extraction time and temperature by analyzing triplicates extracted at either 2-4°C or at –20°C for extraction times of 0.2 h, 2.35 h, 11.0 h, 19.25 h, 33.9 h, 44 h, and 57.8 h (Fig. 2B). At both temperatures of extraction, maximum M_{Chla} values were achieved after 20-34 h of extraction, significantly longer than reported for siliceous sandy sediments (Buffan-Dubau and Carman 2000; Cartaxana and Brotas 2003). Higher M_{Chla} were recorded when extracting at –20°C, and remained relatively stable for at least another 24 h, compared with a detectable decline when extracting at 2-4°C over the same time interval. The difference in M_{Chla}

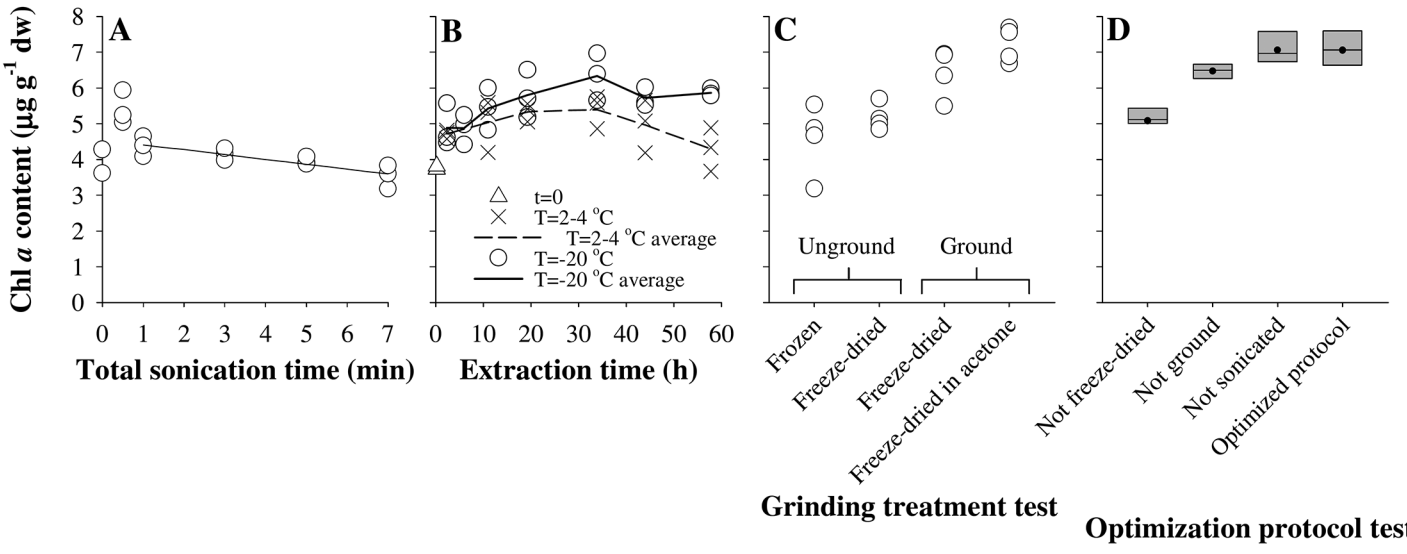

Fig. 2. (A) Effect of total sonication time (min) in 30-s intervals interspersed with 15-s pauses on sedimentary Chl *a* content (M_{Chla}). The solid line indicates a linear regression fit of the data for total sonication times of 1-7 min (r = –0.83); (B) Relationship between extraction time (h) and M_{Chla} for two extraction temperatures: 2-4°C and –20°C; (C) Effect of sediment treatment, grinding, and grinding conditions on M_{Chla}; (D) Comparison of optimized protocol with treatments omitting freeze-drying, grinding, and sonication (n = 7 per treatment). Boxes indicate the 5 to 95 percentile about the median (horizontal line), and dots indicate the mean.

between the two extraction temperatures at 20-34 h was marginally significant (t = 2.29, df = 10, P = 0.045), and this result, along with the stability of M_{Chla} values after 20 h of extraction, makes –20°C the preferred extraction temperature.

Grinding

Samples were ground with a mortar and pestle for 30 s (Heil et al. 2004) after freeze-drying in the presence or absence of 1.5 mL of 100% cold acetone (Buffan-Dubau and Carman 2000). The two ground treatments were compared with two unground treatments: frozen and freeze-dried sediment. Samples (4 replicates per treatment) were sonicated for 30 s and extracted for 24 h at –20°C. The results (Fig. 2C) indicate that grinding of calcareous reef sands yields significantly higher M_{Chla} ($F_{1,14}$ = 28.45, P < 0.001). Grinding in the presence of acetone does not yield statistically significantly higher M_{Chla} than does grinding in the absence of acetone (U_s = 1.155, df = 8, P = 0.343), despite the means being different (6.4 and 7.2 μg g^{-1} dw, respectively). Loss of acetone by evaporation during grinding with a mortar and pestle may concentrate the extract. Therefore, other methods of grinding, e.g., using drill-driven polyethylene rods (Grinham et al. 2007) and Teflon/glass homogenizers (Hagerthey et al. 2006), may lower this potential source of variation, especially if grinding can safely take place in the extraction vessel itself (i.e., without a transfer step).

To further investigate the effect of grinding, a frozen surface sediment (0-1 cm) sample was split and freeze-dried. One half was ground, while the other half was left unground. Both subsamples were sieved and weighed to obtain grain-size distributions, and the different size-fractions were analyzed for Chl *a*. A comparison of the grain size distribution of the two subsamples reveals subtle but noteworthy effects (Fig. 3). Specifically, grinding decreases the relative weight of particles with diameters of > 1 mm and 0.125-0.25 mm by 2.7% and 1.1%, respectively, whereas it increases the relative weight of particles with diameters of 0.5-1 mm and < 0.125 mm by 1.3% and 2.6%, respectively. The shift in contribution to total Chl *a* from larger (>1 mm) to smaller (<0.125 mm) particle size fractions by 8.9%, without a concomitant change in the weight of the larger size-fractions, is consistent with the pulverizing of the surface layers of grains where most of the extractable Chl *a* is likely to be found.

Simultaneous comparison

After the conclusion of the assessment tests described above, we divided 28 replicate samples into the following four treatments to simultaneously test for the combined effects of freeze-drying, sonication, and grinding: (1) Frozen samples were ground and sonicated for 30 s ("not freeze-dried"); (2) Freeze-dried samples were left unground and sonicated for 30 s ("not ground"); (3) Freeze-dried samples were ground ("not sonicated"); (4) Freeze-dried samples were ground and sonicated for 30 s ("optimized protocol").

All samples were extracted for 24 h at –20°C before analysis. The results (Fig. 2D) indicate that whereas freeze-drying and grinding after freeze-drying improve M_{Chla} by 27% (t = –7.56, df = 12, P < 0.001) and 39% (t = 7.51, df = 12, P < 0.001), respectively, over grinding a frozen sample, sonication does not have a significant effect (t = 0.030, df = 12, P = 0.977).

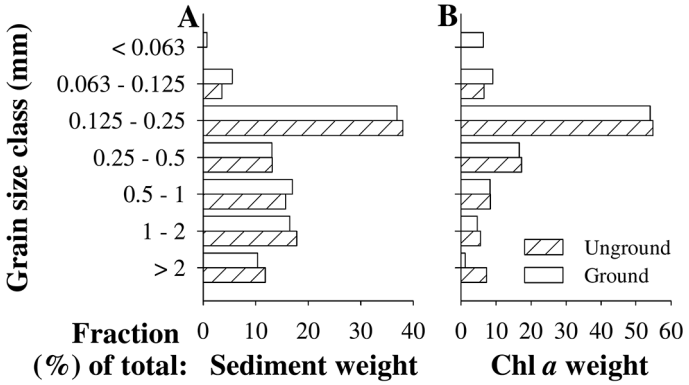

Fig. 3. Comparison of ground (n = 1) and unground (n = 1) freeze-dried sediment: (A) Grain size distribution, (B) Relative contribution of the different grain size fractions to the total Chl *a* pool.

A subsequent analysis of 17 replicate sub-samples with the optimized protocol (treatment d above) yielded a Chl *a* concentration mean and standard deviation of 8.04 ± 0.63 μg g^{-1} dw. Consequently, the cumulative variation (CV) of the method (including natural variation between sub-samples as well as minor variability associated with weighing, storage, and analysis) was 7.8% (n = 17) or CV* = 9.8% for n = 1 at this study site.

Values of M_{Chla} obtained by both fluorometry and HPLC were characterized by a very strong linear correlation ($M_{Chla\text{-}Flu}$ = 1.25 × $M_{Chla\text{-}HPLC}$ - 0.71, r = 0.987, t = 10.64, df = 3, P = 0.002) and a 25% overestimation of M_{Chla} by fluorometry relative to HPLC. This linear relationship is in accordance with previous studies (Redden et al. 1993; Pinckney et al. 1994; Nelson et al. 1999) and confirms that fluorometric measurements are adequate for the purposes of this study.

Optimized protocol

Based on our findings, Chl *a* extraction from calcareous reef sands in 100% acetone is maximized with the following sample processing sequence: (1) freezing at –80°C, followed by freeze-drying at –80°C in < 3 Pa; (2) grinding with a mortar and pestle for 30 s, or, if grinding is not possible, sonication for a brief period (≤30 s) in an ice bath; and (3) extraction with 100% acetone for 20-30 h at –20°C;

Even though we didn't test for the impact of vortexing during this extraction period, we recommend doing so once (at 12 h) as this is relatively common practice (e.g., Arar and Collins 1997; van Leeuwe et al. 2006).

Sampling strategies

To test the extent and variability of Chl *a* content with depth in calcareous reef sands, we collected 10 syringe cores within an area of 20 × 15 cm (300 cm^2), resulting in a distance of 3-5 cm between adjacent cores. All 10 syringes were first inserted into the sediment before retrieval began to ensure that retrieval of one syringe barrel did not disturb sediment

collected by a nearby barrel. The cores were processed as previously described (*see* "Methods"), yielding a total of 177 discrete sediment samples.

Profiling

The resulting M_{Chla} varied between 4.33 and 14.25 μg g^{-1} dw, compared with overlying water concentrations of 0.17-0.18 mg m^{-3} that are typical of surface waters in the area (e.g., Vedernikov et al. 2007). The ten profiles (Fig. 4A) were examined to determine the depths at which maximum and minimum M_{Chla} were recorded (Table 3). As anticipated, seven of the ten profiles exhibited maxima in the top-most centimeter, a common depth of integration (Table 1). The presence of a distinct

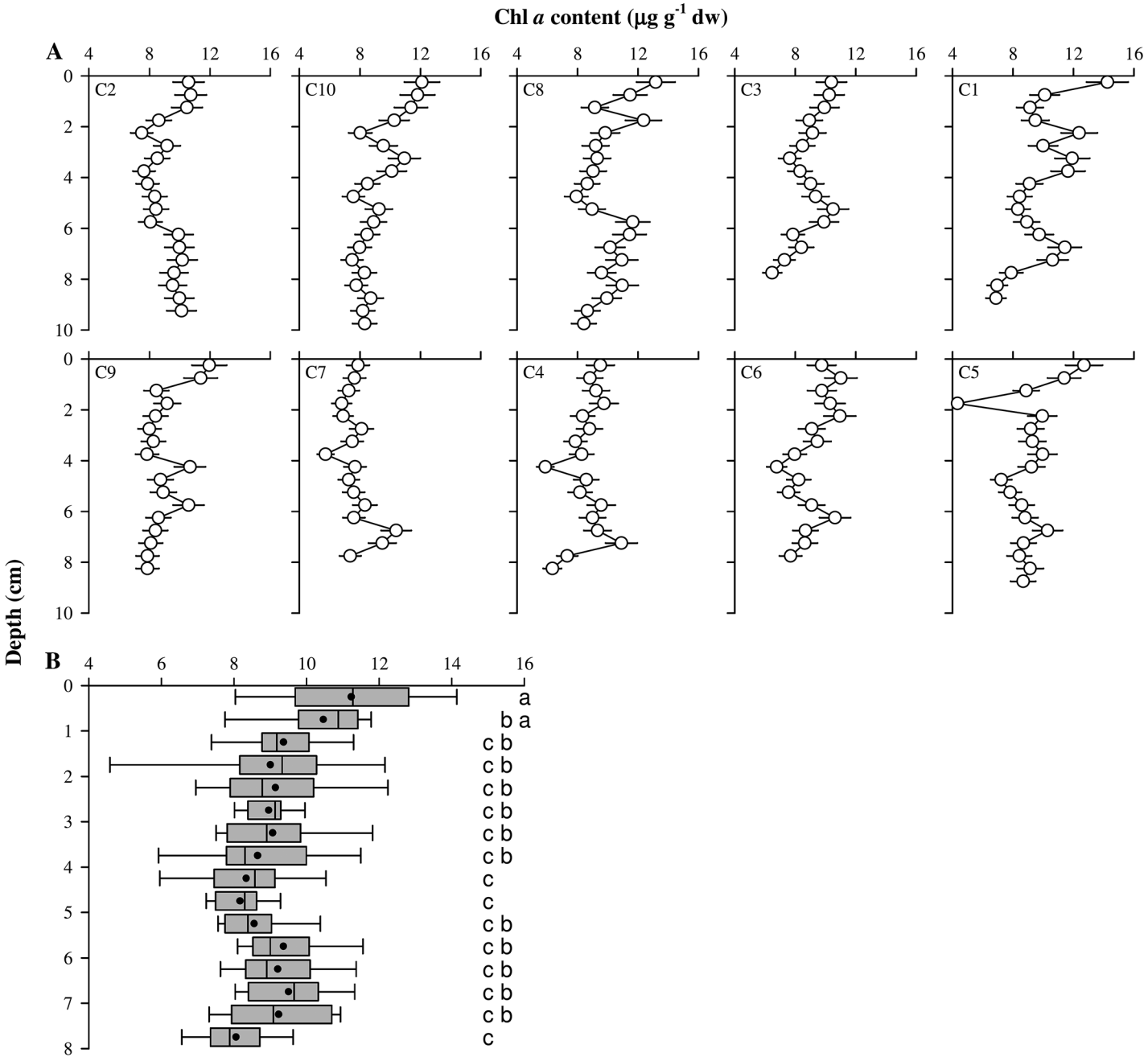

Fig. 4. (A) Ten Chl *a* content (M_{Chla}) profiles (C1-C10) taken in close proximity within an area of 300 cm^2 in the near-shore back reef. The symbol diameter is representative of the thickness of the analyzed sediment layer (0.5 cm). The horizontal bars indicate the corrected cumulative variation of the method for a single sample (CV* = 9.8%); (B) M_{Chla} distributions for 0.5 cm-deep layers (n = 10 for each layer). Boxes indicate the 25 to 75 percentile about the median (vertical line), whiskers indicate the 5 to 95 percentile, and dots indicate the mean. The symbols "a," "b," and "c" group together depths at which M_{Chla} values are not significantly different ($P > 0.05$).

Table 3. Characteristics of Chl *a* content (M_{Chla}) with depth in ten profiles taken in close proximity within an area of 300 cm^2 in the near-shore back reef (Fig. 4A).

Core number	Depth of maximum M_{Chla} (cm)	Maximum M_{Chla} (μg g^{-1} dw)	Depth of minimum M_{Chla} (cm)	Minimum M_{Chla} (μg g^{-1} dw)	Deepest M_{Chla} as a fraction of the top-most M_{Chla} (%)
C2	0.5–1	10.73	2–2.5	7.49	95.6
C10	0–0.5	12.10	7–7.5	7.48	68.7
C8	0–0.5	13.17	4.5–5	7.92	63.9
C3	5.25	10.50	7.5–8	6.47	62.3
C1	0–0.5	14.24	8.5–9	6.87	48.3
C9	0–0.5	11.95	3.5–4	7.84	65.8
C7	6.5–7	10.40	3.5–4	5.73	93.5
C4	7–7.5	10.90	4–4.5	5.85	66.6
C6	0.5–1	11.01	4–4.5	6.77	78.9
C5	0–0.5	12.69	1.5–2	4.33	68.3
Average ± std. dev.		11.8 ± 1.2	—	6.7 ± 1.1	71.2 ± 13.9

Table 4. Chl *a* concentrations, $C_{z1\text{-}z2}$ (mg m^{-2}) for various depth ranges between depths *z1* and *z2*. The last row lists the average fraction of *C* for each depth range as a percentage of the whole-core *C*, $C_{0\text{-}8}$.

	Surface *C* (mg m^{-2})				Sub-surface *C* (mg m^{-2})						
Core number	$C_{0\text{-}0.5}$	$C_{0\text{-}1}$	$C_{0\text{-}1.5}$	$C_{0\text{-}2}$	$C_{0.5\text{-}8}$	$C_{1\text{-}8}$	$C_{1.5\text{-}8}$	$C_{2\text{-}8}$	$C_{2\text{-}5}$	$C_{5\text{-}8}$	Whole-core $C_{0\text{-}8}$ (mg m^{-2})
2	81	163	244	310	1036	954	873	807	376	431	1117
10	93	184	271	350	1063	972	885	806	419	387	1156
8	101	189	259	354	1148	1060	990	895	414	481	1249
3	80	158	234	303	1008	930	854	785	398	387	1088
1	109	187	257	329	1144	1066	996	924	487	437	1253
9	92	179	244	314	1023	936	871	801	398	402	1115
7	60	119	175	227	887	828	772	720	331	389	947
4	73	141	212	287	996	928	857	782	366	416	1069
6	75	159	234	313	1042	958	883	803	402	401	1117
5	97	185	253	286	1013	925	857	824	420	403	1110
Average	86	166	238	307	1036	956	884	815	401	414	1122
St. dev.	15	23	28	36	75	69	66	57	41	30	88
Fraction of whole-core concentration, $C_{0\text{-}8}$ (%)											
	8 ± 1	15 ± 1	21 ± 1	27 ± 2	92 ± 1	85 ± 1	79 ± 1	73 ± 2	36 ± 2	37 ± 2	—

surface layer (0-1 cm) was verified by the results of ANOVA ($F_{15,144} = 3.552$, $P < 0.001$) followed by a post-hoc SNK test (symbols "a" and "c" in Fig. 4B). In addition, a sub-surface maximum between 5-7.5 cm below the SWI was also detected (symbol "b" in Fig. 4B), and contributes in part to high variability in the depth of minimum M_{Chla} between cores (Table 3). In each profile, the deepest values are 48% to 96% of the top-most values (Table 3), which is significantly higher than the 7-40% differences documented previously over similar depths in other calcareous reef sediments (Fig. 1). Whereas the reasons for this difference are not the focus of this study, our results share the findings of previous investigations which demonstrated that the majority of MPB pigments in calcareous reef sediments are found well below the top few millimeters of the euphotic surficial sediment layer (Werner et al. 2008; Rao et al. 2012).

Surface versus subsurface concentrations

To test whether *C* assessed at the surface is representative of sub-surface *C*, *C* values were calculated for selected depth layers (Table 4). The surface layers between 0-0.5 cm ($C_{0\text{-}0.5}$), 0-1 cm ($C_{0\text{-}1}$), 0-1.5 cm ($C_{0\text{-}1.5}$), and 0-2 cm ($C_{0\text{-}2}$) were selected because they correspond to commonly used depths of integration for *C* calculations (Table 1) or depth ranges for plug sampling (Grinham et al. 2007), and matching subsurface layers were chosen for comparison down to 8 cm depth, i.e., 0.5-8 cm ($C_{0.5\text{-}8}$), 1-8 cm ($C_{1\text{-}8}$), 1.5-8 cm ($C_{1.5\text{-}8}$), and 2-8 cm ($C_{2\text{-}8}$). Finally, values for the layers between 2-5 cm ($C_{2\text{-}5}$) and 5-8 cm

Table 5. Results of Pearson correlation analysis between surface and sub-surface Chl *a* concentrations $C_{z1\text{-}z2}$ (mg m^{-2}) for various depth ranges between depths *z1* and *z2* in Table 4: *r* = Pearson coefficient; *t* = t-statistic; *P* = significance (df = 8 in all cases). Asterisks indicate cases where $0.01 < P < 0.05$ (*), $0.001 < P < 0.01$ (**), and $P < 0.001$ (***). All cases involving $C_{5\text{-}8}$ were non-significant (n.s.) with *P* ranging between 0.135–0.342.

	$C_{0.5\text{-}8}$	$C_{1\text{-}8}$	$C_{1.5\text{-}8}$	$C_{2\text{-}8}$	$C_{2\text{-}5}$	$C_{5\text{-}8}$
$C_{0\text{-}0.5}$	$r = 0.85$;	$r = 0.83$;	$r = 0.83$; *	$r = 0.90$;	$r = 0.90$;	n.s.
	$t = 4.61$;	$t = 4.16$;	$t = 4.22$;	$t = 5.74$;	$t = 5.72$;	
	**$P = 0.002$	**$P = 0.003$	*$P = 0.003$	***$P < 0.001$	***$P < 0.001$	
$C_{0\text{-}1}$	—	$r = 0.78$;	$r = 0.76$;	$r = 0.81$;	$r = 0.83$;	n.s.
		$t = 3.52$;	$t = 3.31$;	$t = 3.86$;	$t = 4.14$;	
		**$P = 0.008$	*$P = 0.011$	**$P = 0.005$	**$P = 0.003$	
$C_{0\text{-}1.5}$	—	—	$r = 0.71$;	$r = 0.73$;	$r = 0.78$;	n.s.
			$t = 2.89$;	$t = 3.01$;	$t = 3.56$;	
			*$P = 0.020$	*$P = 0.017$	**$P = 0.007$	
$C_{0\text{-}2}$	—	—	—	$r = 0.73$;	$r = 0.68$;	n.s.
				$t = 3.03$;	$t = 2.64$;	
				*$P = 0.016$	*$P = 0.030$	

($C_{5\text{-}8}$) were calculated to test whether the subsurface maximum between 5-7.5 cm (Fig. 4B) affects the tested relationship.

Subsurface *C* accounts for a significant proportion of whole-core *C*, ranging from 73-92% depending on the depth of integration (Table 4). Linear regression indicates statistically significant relationships ($P < 0.05$) between all tested *C* (Table 5), with the exception of the sub-surface maximum layer (5-8 cm). All correlations between surface and sub-surface *C* values down to 8 cm were statistically significant. The results suggest that surface M_{Chla}, as well as surface-layer *C* of calcareous reef sands, are proportional to sub-surface values in calcareous reef sands, even though they are a small fraction (8-27%) of the Chl *a* present in the top 8-10 cm of the sediment column. Thus, in situations when profiles cannot be generated due to logistical limitations, our results support the approach of only sub-sampling a core in 1-2 subsurface sections, e.g., 2-8 cm, or 2-5 and 5-8 cm (instead of a finer-resolution profile), in addition to a surface section (e.g., 0-2 cm). These slices can then be analyzed to provide a quantitative measure of the subsurface *C* without increasing the number of samples 10-fold or more, as would be in the case of profiling.

Discussion

Permeable sediments remain relatively undersampled despite constituting a significant proportion of the seafloor area of the global coastal ocean. Whereas organic matter decomposition and nutrient regeneration rates are being progressively better constrained (Rocha 2008), MPB biomass and activity in these sediments are still poorly characterized, especially in the case of calcareous reef sands. The relatively few studies conducted thus far indicate that MPB biomass extends many centimeters into the sediment column and is highly heterogeneous in space, raising methodological questions in relation to sampling strategies. Moreover, high cell abundances and irregular grain-surface microtopography suggest that multiple sample processing steps may be required to effectively extract the pigments present in calcareous sands. Our study thus aimed to optimize the Chl *a* extraction protocol for calcareous sediments, and to propose a suitable sampling approach in view of high subsurface pigment inventories.

Extraction efficiency

We have demonstrated that the extraction efficiency of Chl *a* in calcareous sediments can be dramatically improved (by 39%) if frozen samples are freeze-dried and ground before extraction. Freeze-drying alone increases the M_{Chla} of frozen samples by 27%, whereas grinding the freeze-dried samples increases M_{Chla} by a further 12%. Freeze-drying and grinding may have the combined effect of better exposing pigments or pigment-containing cells to the extraction solvent, perhaps by removing water-rich mucous matrices surrounding the cells as well as increasing the surface area over which extraction takes place. The highest M_{Chla} and *C* values in carbonate reef sands previously reported (Table 1) were obtained on non-dried samples that were either previously frozen (Heil et al. 2004) or analyzed fresh (Roelfsema et al. 2002; Rao et al. 2012). Our results suggest that these values could be increased by 27% had these samples been freeze-dried. Moreover, the M_{Chla} values reported by Rao et al. (2012) were obtained on unground sediment, thus suggesting that a further 12% increase would be possible. Moreover, it is likely that the proposed protocol may enhance the extraction of other photosynthetic pigments, as documented by other similar extraction efficiency studies (e.g., Buffan-Dubau and Carman 2000; Hagerthey et al. 2006). These proposed corrections amplify the already high documented abundance of MPB in reef settings (Table 1) and suggest that a renewed investigation into their role in reef ecosystem function is due.

Sampling strategies

Fine-scale profiles of Chl *a* in calcareous reef sands (Fig. 1) highlight variations that may reflect physical and ecological

processes driving MPB distributions in the sediment column, e.g., sediment mixing, cell filtration, cell motility, etc. (MacIntyre et al. 1996; Rao et al. 2012). On the other hand, where the objective is the quantification of lateral distributions on the scale of m, plug sampling may be adequate (Grinham et al. 2007). Our study confirmed the previously documented (Fig. 1) persistence of substantial Chl *a* several centimeters below the SWI (Fig. 4), which, in our case, accounted for a substantial proportion of the whole core *C*, between 73-92% depending on the depth of integration. Therefore, we propose that, if mm-to-cm scale information is not necessary, fine-scale profiling may be replaced by one surface (0-2 cm) and 1-2 subsurface samples (down to 8-10 cm below SWI) for several cores collected within a few m^2 (e.g., 8 cores, as suggested by Grinham et al. 2007). The lower limit of 10 cm below SWI is proposed simply due to technical considerations, as it is evident from previous studies as well as ours that significant Chl *a* can still be detected at such a depth. It is probable that measureable Chl *a* content extends well below 10 cm. While the study we report here doesn't investigate the reasons behind the deep presence of MPB within the sediment column, it highlights the fact that investigations of MPB in calcareous reef sediments must be extended to sub-surface sediments to obtain a broader understanding of the role of sediments in coral reef ecosystem function.

Comments and recommendations

In summation, we propose that microphytobenthic Chl *a* within calcareous sand samples should be extracted by freeze-drying at –80°C in < 3 Pa, grinding for 30 s with a mortar and pestle (or, if grinding is not possible, sonicating for ≤30 s in an ice bath), and extracting with 100% acetone for 20-30 h at –20°C. Vortexing once at 10-15 h during extraction is recommended, even though we did not test its impact on extraction efficiency during our study.

When estimates of *C* on the scale of meters are sought, fine-scale (mm-cm) profiling may be unnecessary. Instead, plug surface samples (e.g., 0-2 cm) and one or two sub-surface layers (down to 8-10 cm below SWI) should be used to determine the magnitude and variability of the subsurface MPB biomass.

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