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Accurately measuring the abundance of benthic microalgae in spatially variable habitats

Alistair R. Grinham,[1] Tim J. B. Carruthers,[2] Paul L. Fisher,[3] James W. Udy,[1] and William C. Dennison[2]
[1]School of Engineering, The University of Queensland, Brisbane, Australia
[2]University of Maryland Center for Environmental Science, Cambridge MD, USA
[3]Centre for Marine Studies, The University of Queensland, Brisbane, Australia

Abstract

Although many studies measure the abundance of benthic microalgae (BMA), at the meters squared scale, comparing these studies is difficult due to the variety of sampling, extraction, and analysis techniques. This difficulty is exacerbated by the fact that BMA abundance has high spatial and temporal variability, at all spatial scales. A suitable standard sampling regimen would reduce variation in estimates due to different sample collection and processing greatly facilitating comparisons between studies. This study examined the effect of varying the volume of extraction solvent, sampling core diameter, and sample replication on BMA biomass estimates. Key findings, applicable to all spatial scales, to accurately determine biomass were the use of a minimum sediment to extraction solvent ratio of 1:2 and use of a sampling core diameter of 19 mm. Across a wide range of sediment types, at the meters squared scale and using spectrophotometric techniques, a minimum replication number of 8 was found to be appropriate. We report the significant effect coring depth and units of expression have on BMA biomass estimates across a range of sediment types, highlighting the potential pitfalls when comparing studies.

Introduction

Benthic microalgae are a major component of shallow estuarine/marine ecosystems (MacIntyre et al. 1996; Cahoon 1999; Underwood and Kromkamp 1999). Studies of these systems often require measurement of benthic microalgal biomass, commonly estimated as chlorophyll *a* content per unit of sediment. Because of high spatial and temporal variability in BMA abundance, determining reliable, repeatable, and comparable estimates can often be problematic (Light and Beardall 1998; Sandulli and Pinckney 1999; Underwood and Kromkamp 1999; Kelly et al. 2001). Traditionally, 3 methods are used to quantify chlorophyll *a* concentration of sediment extracts: spectrophotometric or fluorometric for routine analysis and high-performance liquid chromatography (HPLC) for greater precision and when quantification of other pigments is required (Pinckney et al. 1994). A number of studies focusing on the comparative merits or deficiencies of these techniques have been published (Brown et al. 1981; Sartory 1985; Daemen 1986; Pinckney et al. 1994; Jeffrey et al. 1997; Reuss and Conley 2005). Generally a constant and linear relationship exists between each, and this holds true over a wide range of pigment concentrations and sediment types (Pinckney et al. 1994; Jeffrey et al. 1997).

Samples should be stored at a minimum of –20°C as soon as possible after sampling, with additional treatment (e.g., freeze drying) performed just before analysis (Reuss and Conley 2005). Extraction solvents differ in both type and concentration, but the most commonly reported are acetone, methanol, and dimethyl-formamide, all found to be suitable for chlorophyll *a* (Wellburn 1994; Jeffrey et al. 1997), although acetone should be used if no freeze drying treatment of samples occurs before extraction (Buffan-Dubau and Carmen 2000). Reliable recovery of chlorophyll *a* requires extraction under dark conditions at –20°C over 24 h; extraction time may be reduced by physical disruption of sediment (e.g., sonication or grinding) (Jeffrey et al. 1997; Buffan-Dubau and Carmen 2000).

Very few studies have examined the effect of differing sediment collection methods on chlorophyll *a* concentration, which has the potential to be an important oversight. Core

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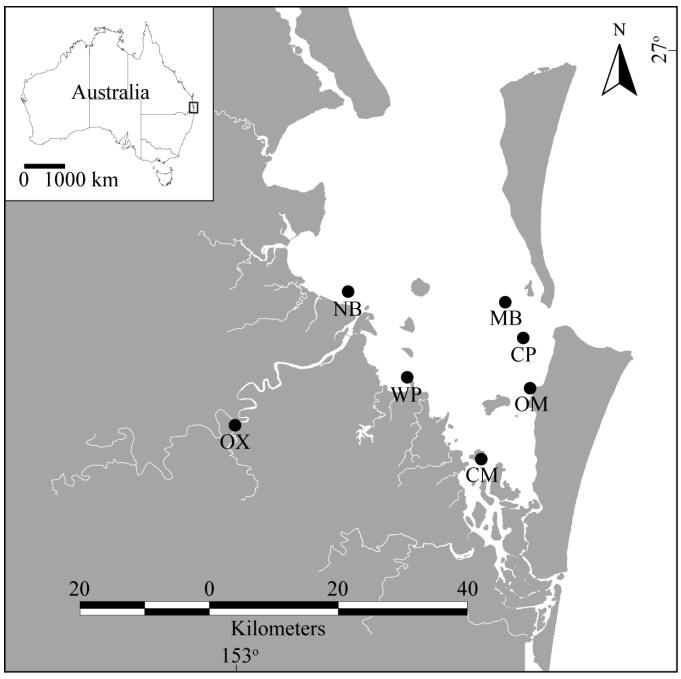

Fig. 1. Study sites in Moreton Bay: NB, Nudgee Beach; MB, Moreton Banks; CP, Cooloolo Passage; OX, Oxley Creek; WP, Wellington Point; OM,One Mile Flat; and CM, Coochiemudlo Island.

diameter can range from at least 46 mm (Mitbavkar and Anil 2002) to 10 mm (Welker et al. 2002), core depth from 50 mm (Wardle et al. 2001) to 2 mm (Underwood et al. 1998), and replication number from 20 (de Jonge and Colijn 1994) to 3 (Hansen et al. 1992). Units to express biomass are usually concentration of chlorophyll *a* per unit area (mg m^{-2}) or chlorophyll *a* content per weight of sediment (µg g^{-1} dry or wet sediment) (Tolhurst et al. 2005). The potential pitfalls associated with expressing sediment chlorophyll *a* as concentration or content have been demonstrated when comparing estimates between different sediment types (Tolhurst et al. 2005) and core depth with differing water content (Perkins et al. 2003). The combined effect of sediment type and core depth has not been examined.

The sources of variation in biomass are many, ranging from natural patchiness of the community on spatial and temporal scales to methodologies using differing replicate numbers, core diameter and sampling depth, sample storage, extraction methods and solvents, instruments to estimate chlorophyll *a* concentrations, and units to express the biomass. It was not the purpose of this study to address the broader issue of scales of natural variation in BMA communities, but rather to clarify some methodological issues and therefore help reduce sampling error, so that these important broader questions can be successfully addressed. Specifically, we asked the following questions:

- What is a suitable sediment to extraction solvent ratio?
- What is a suitable core diameter?
- How many replicates are required to accurately determine biomass at the meters squared scale?

Table 1. Summary of different sediment core diameters and volumes (assuming sample depth 20 mm).

Syringe (mL)	Internal diameter (mm)	Sampling area (cm^2)	Volume (mL)	Core SA:vol ratio
5	13	1.33	2.65	4.1
10	16	2.01	4.02	3.5
20	19	2.84	5.56	3.2
60	29	6.60	13.2	2.4

- How well does spectrophotometry compare with HPLC?
- Do sediment type and sample core depth influence estimates of BMA content and concentration?

Materials and procedures

Sediment sampling—This study was conducted at 7 sites within Moreton Bay, Queensland, Australia (Fig. 1). Samples for establishing a suitable core diameter and number of replicates were taken from fine- to medium-grained sand at One Mile Flat (OM, Fig. 1) and Moreton Banks (MB, Fig. 1) from a 6- by 3-m quadrat. Samples were taken randomly, moving across the quadrat, to ensure that no area was resampled. Sediment cores were sampled using 5-, 10-, 20-, and 60-mL syringes (13, 16, 19, and 29 mm internal diameter) with the tip end removed (Table 1). Sediment cores were all taken to 20 mm depth, capped with rubber stoppers, and stored on ice in the dark until frozen at –20°C.

A subsequent sampling of 5 sites throughout Moreton Bay provided the opportunity to assess this sampling regimen over a wide range of sediment types (Table 2). A 20-mL cutoff syringe was used for sediment collection, and 8 replicate samples were taken at each of 3 depths (2, 5, and 20 mm). Cores were immediately transferred to cryotubes and placed on liquid nitrogen in the field. These were transported back to the lab and stored at –70°C. All samples were analyzed for chlorophyll *a* within 30 days of collection (Jeffrey et al. 1997; Reuss and Conley 2005). A further 9 cores (60-mL syringe to 20 mm depth) were taken at each of the 5 sites for sediment grain size analysis.

Chlorophyll analysis—Frozen sediment samples were thawed in the dark before analysis, and all subsequent work was carried out under dim light (4 µmol m^{-2} s^{-1}) (as per Jeffrey et al. 1997). Samples from the initial Moreton Banks and One Mile Bank samplings were transferred to 60-mL polyethylene tubes and ground in 2 mL cold 90% acetone using a sharpened polyethylene rod attached to an air-drill for 60 s. Further 90% cold acetone was added to attain the required extraction volume, and samples were extracted at –20°C for 12 h. Samples were shaken and centrifuged (1620*g* for 5 min), and a Pharmacia LKB Ultraspec III was used to measure absorbance at 665 and 750 nm. Samples were acidified with 2 drops of 0.1 N HCl, mixed, and left for 60 s; absorbance was read at 665 and 750 nm. Concentrations of corrected chlorophyll *a* and phaeopigments were calculated using the spectrophotometric equations of

Table 2. Physical parameters of study sites from baywide study.

Site	Median ϕ	Median grain size diameter (µm)	Classification (Wentworth scale)	Moisture content (%)	Silt content (%)
Coolooloo Passage	2.17 ± 0.04	223 ± 6.7	Fine Sand	19.6 ± 1.46	0.1 ± 0.03
Nudgee Beach	2.44 ± 0.01	184 ± 1.4	Fine sand	22.7 ± 0.62	2.6 ± 0.42
Coochiemudlo Island	3.19 ± 0.12	112 ± 10.5	Muddy sand	27.6 ± 6.27	13.9 ± 0.83
Wellington Point	3.27 ± 0.04	103 ± 2.9	Muddy sand	37.2 ± 4.38	19.8 ± 2.0
Oxley Creek	4.48 ± 0.02	45 ± 0.7	Silt	92.7 ± 0.4	69.1 ± 1.19

Data are mean ± 1 SE; $n = 9$ per site.

Lorenzen (1967), which calculate the concentration of pigment in the extract (C_E). An additional calculation is required to calculate abundance of chlorophyll *a* concentration (mg m^{-2}) and content (mg g^{-1}) of sediment as follows:

$$\text{BMA biomass (mg m}^{-2}\text{ or g}^{-1}) = [C_E * (A/B)]/C$$

where C_E = chlorophyll *a* concentration (mg L^{-1}) calculated from Lorenzen (1967), A = total extract volume (mL), B = 1000 (conversion from mL to L), and C = required units in this case area (m^2) or weight of sediment sampled (g).

Samples collected during the baywide study were transferred to 60-, 15-, or 5-mL polyethylene tubes depending on core depth (20, 5, and 2 mm, respectively), and the wet weight was recorded. Different volumes of cold 90% acetone were added depending on core depth but equivalent to a sediment:acetone ratio of 2:5. Samples were vortexed and sonicated for 15 min in ice and placed at –20°C for 12 h, vortexed, and centrifuged (1620*g* for 5 min) again. The supernatant was decanted and filtered through a 0.2-µm pore syringe filter (Millex-GV Filter Unit, Millipore). The sediment pellet was dried at 60°C for at least 48 h, and the weight was recorded. Chlorophyll *a* concentration of these extracts was determined using reverse-phase HPLC. Pigment separation was achieved following the method of Zapata et al. (2000). Extract (100 µL) was mixed with 300 µL 100% methanol and diluted with 80 µL water just before injection to avoid peak distortion (Zapata et al. 2000). Samples were placed in a refrigerated autosampler (SIL-10AD) of a Shimadzu VP Series HPLC system, injection volume 250 µL, Waters Symmetry C8 3.5 µm column. Chlorophyll *a* peaks were detected by a diode-array detector (SPD-M10A) at 430 nm and quantified against DHI standard chlorophyll *a* (PPS-CHLA). Chlorophyll *a* was then expressed as concentration or content (dry and wet) and calculated as follows:

$$\text{BMA biomass (mg m}^{-2}\text{ or g}^{-1}) = [(C_E * A) * B)/C]/D$$

where C_E = Chl *a* content of injection volume (ng), A = convert Chl *a* content to injection extract volume (480 µL/250 µL = 1.92), B = convert Chl *a* content to total extract volume (extract volume µL/100 µL), C = required units in this case area (m^2) or weight of sediment sampled (g), and D = factor to convert Chl *a* content (ng) to appropriate units (for mg, D = 1 000 000)

In addition, absorbance of the supernatant was determined as above using a Pharmacia LKB Ultraspec III, and BMA biomass was determined using the spectrophotometric method described above. To test the resolving power of each method, supernatant from each sample was run 3 times through both methods.

Sediment grain analysis—Grain size analysis followed methods described in Folk (1974), using a combination of wet and dry sieving. For each sample, the silt fraction and moisture content were measured and sediment type was classified according to the Wentworth scale using the median ϕ value (Table 2).

Statistical analysis—Data were analyzed using the software program Statistica v. 7.1 (StatSoft, Inc.). Categorical predictors were sediment:acetone ratio, core diameter, and core depth, and the dependent variable was BMA biomass. Biomass values were log or double log transformed where necessary to ensure normality of distribution and homogeneity of variance (Cochran test) (Zar 1984). Post-hoc tests were performed using Fisher LSD test (Zar 1984).

Assessment

Extraction ratio—To determine the optimal sediment:extraction solvent volume, replicate cores of different diameters were collected randomly from the same 18-m^2 region of an intertidal sand flat. The extraction volume of acetone was between 4 and 5 mL. All cores were taken to a depth of 20 mm, and thus the volume of sediment sampled within the different core diameters varied. This resulted in the largest cores (29 mm diameter; a total of 13.2 mL sediment) having proportionately less extraction acetone than the smallest cores (13 mm diameter; 2.65 mL sediment) (Table 1). Chlorophyll *a* estimates for the largest core size (29 mm; Table 1) were approximately 25 mg m^{-2}, which was around one-third the value in the smaller core sizes (60 to 80 mg m^{-2}) ($F_{(3,36)}$ = 112.51; $P < 0.001$) (Fig. 2). As all samples were taken randomly, it was hypothesized that these large differences may have been due to incomplete extraction of chlorophyll in the larger cores, perhaps resulting from difference in the ratio of sediment to extraction solvent volumes.

The next set of analyses was to determine the minimum sediment:extraction solvent (acetone) volume ratio to obtain a

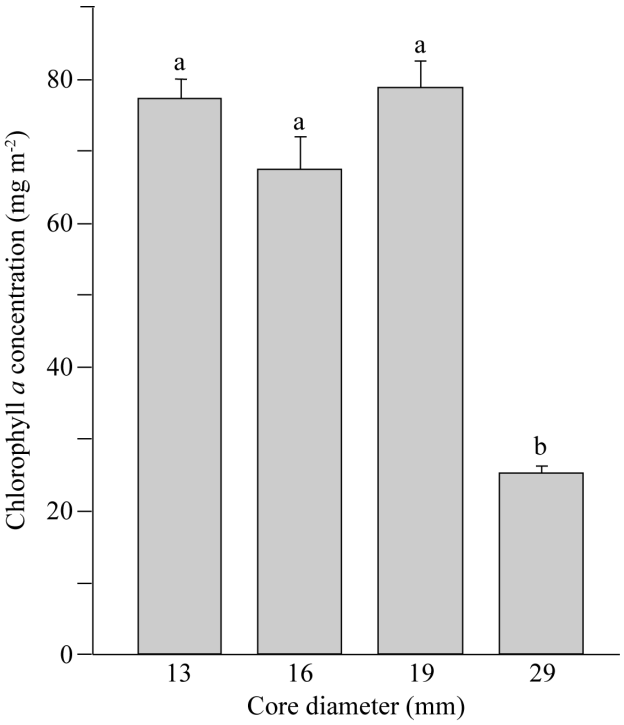

Fig. 2. Estimates of BMA biomass obtained using corers of different diameter, sampled to 20 mm depth (see Table 1 for sediment volume) and extracted with constant volumes of 90% acetone (approximately 5 mL) (mean ± 1 SE). Samples from One Mile Harbour (n = 12). Treatments with different letters are significantly different (P < 0.05).

consistent estimate of chlorophyll *a* abundance. This was carried out for 19-mm-diameter cores by analyzing replicate cores under a range of extraction solvent volumes, 2:1 up to 1:4 (sediment volume:extraction solvent volume) (Fig. 3). At low extraction solvent volumes, an increase in solvent resulted in an increase in measured chlorophyll *a*; however, at ratios greater than approximately 1:1.5, a consistent chlorophyll *a* concentration was measured (Fig. 3). Many studies do not report the volume of acetone used for extractions, but many different core diameters are used for sampling BMA. The current results suggest that for any given sediment sample volume, twice that volume of extraction solvent (e.g., acetone) will ensure that the maximum amount of chlorophyll *a* is extracted.

Core diameter—Once the issue of appropriate volume of extraction acetone had been addressed, a direct comparison was made between 4 different core diameters to assess any other potential sampling variation due to core size; 13, 16, 19, and 29 mm diameter (equivalent to 5-, 10-, 20-, and 60-mL syringes; Table 1). All cores were sampled to 20 mm depth and extracted with a sediment:acetone ratio of 1:2. Cores with a larger core diameter (19 and 29 mm) had significantly higher concentrations of chlorophyll *a* ($F_{(3,115)}$ = 16.24; P < 0.01) and significantly lower concentrations of phaeopigments ($F_{(3,115)}$ = 4.35; P < 0.01) than cores with a smaller diameter (13 and 16 mm) (Fig. 4). When these pigments were combined, the total pigment (chlorophyll *a* plus phaeopigments) was not significantly different between different core diameters ($F_{(3,115)}$ = 2.00; NS) (Fig. 4). Chlorophyll *a* may readily degrade to phaeopigment if exposed to light or heat (Jeffrey et al. 1997;

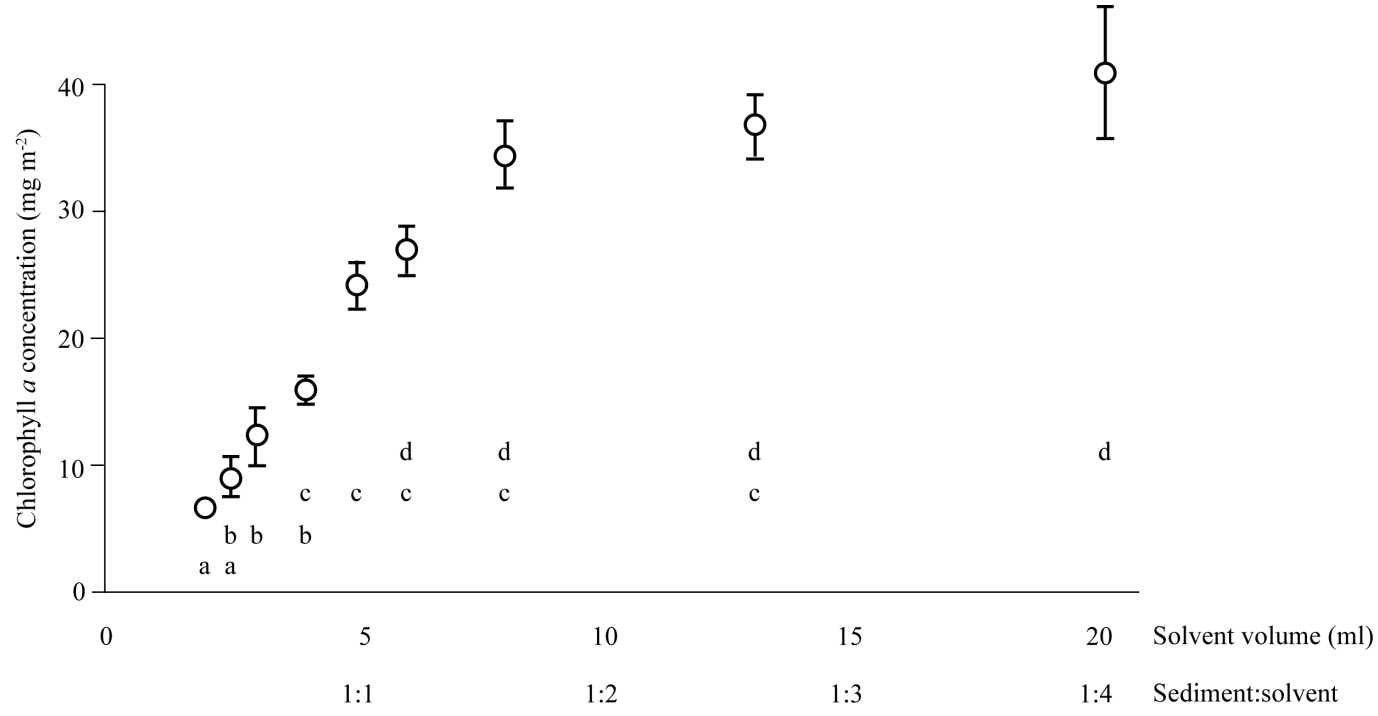

Fig. 3. Series of BMA biomass estimates from 19-mm-diameter cores sampled to a depth of 20 mm and extracted using different sediment:solvent (90% acetone) volumes (mean ± 1 SE). Samples taken from One Mile Harbour. Treatments with different letters are significantly different (P < 0.05).

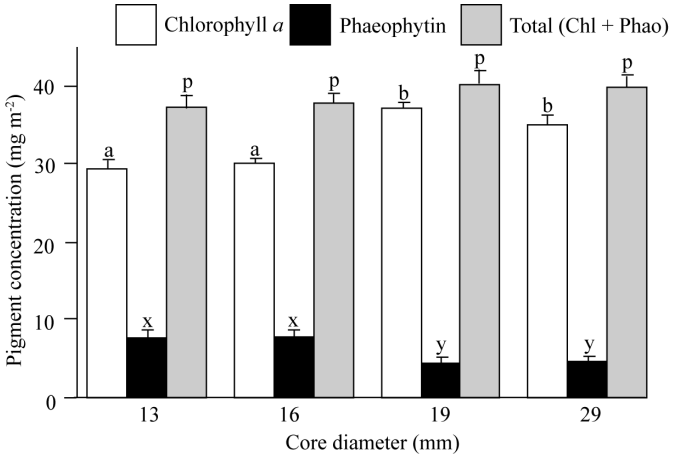

Fig. 4. Influence of core diameter on BMA biomass estimates using a constant sediment:solvent (90% acetone) extraction ratio of 1:2 (n = 30; mean ± 1 SE). Samples taken from Moreton Banks. Letters indicate significant differences (P < 0.05), with each parameter analyzed using a separate 1-way ANOVA.

Reuss and Conley 2005), and these results suggest that in the smaller cores the chlorophyll *a* was degrading more rapidly, even with the same sampling and storage treatment as the larger cores. The surface area:volume ratio of the smaller cores was 4.1 and 3.5 as opposed to 3.2 and 2.4 in the larger cores (Table 1), suggesting that this greater ratio exposes more of the BMA to physical disturbance during coring (BMA cells physically scraping along the side of the core), as well as greater light and temperature exposure, resulting in a higher proportion of chlorophyll *a* breakdown. In conclusion, when sampling BMA cores to 20 mm depth, a core diameter of at least 19 mm is recommended to maintain chlorophyll *a* integrity.

Required replication at meters squared scale—For the subsequent analyses, a 19-mm-diameter core sampled to 20 mm depth was used to ensure maximum integrity of chlorophyll *a* and extracted in 12 mL of 90% acetone (sediment:extraction solvent ratio 1:2) to ensure maximal extraction of chlorophyll *a*. Thirty cores were analyzed for chlorophyll *a* concentration and assessed for sample size using the Monte-Carlo sampling technique of Day and Quinn (1989). Ten replicate sets of each sample size were randomly selected from the 30 samples and the standard error calculated for each set (10 SEs per sample size). The mean, maximum, and minimum SEs per sample size are presented in Figure 5. As sample size increases, the maximum and minimum approach the mean standard error and become increasingly balanced around the mean value, indicating that a minimum of 8 (certainly >6) replicate samples are required (Fig. 5).

Spectrophotometer vs. HPLC and sediment type—For comparison of measurement methods, samples from different sediment types throughout Moreton Bay were compared. Extracts were run on both the HPLC and the spectrophotometer and graphed against each other (Fig. 6). Overall, a strong linear relationship was found between the spectrophotometer and HPLC methods

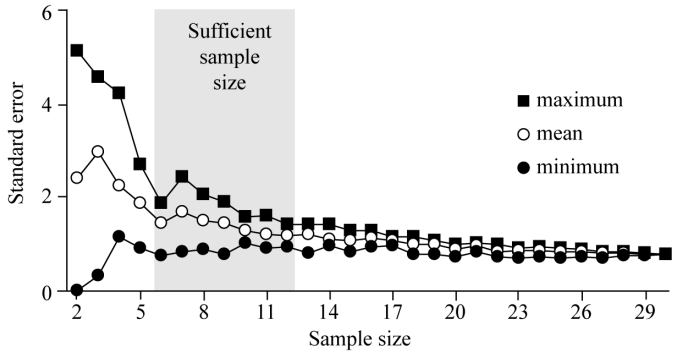

Fig. 5. Assessment of required sample number for determining benthic microalgal abundance at the meters squared scale (Day and Quinn 1998). Samples from Moreton Banks.

(r^2 = 0.96; P < 0.001, F test), and this was consistent over all sediment types (Fig. 6). Comparisons of relative values using either method were found to be equally valid, and the sediment type did not affect the accuracy of measurement, or comparison between methods, of chlorophyll *a* concentration. In this study, however, estimates from the spectrophotometer were consistently higher than those from the HPLC, which agrees with the findings of Daemen (1986) and Pinckney et al. (1994) and suggests that careful standardization and calibration is required to determine absolute BMA abundances. The two methods also differed in their resolving power, estimates using HPLC showed less variation with a lower mean standard error for replicate samples irrespective of sediment types. When expressed as a proportion of the mean chlorophyll *a* concentration extracts, HPLC showed a mean standard error of 1.69% compared with 4.09% for the spectrophotometer.

Core depth and units of expression—Having established a suitable sampling regimen, the effects of coring depth and units of expression were investigated. Biomass units are usually

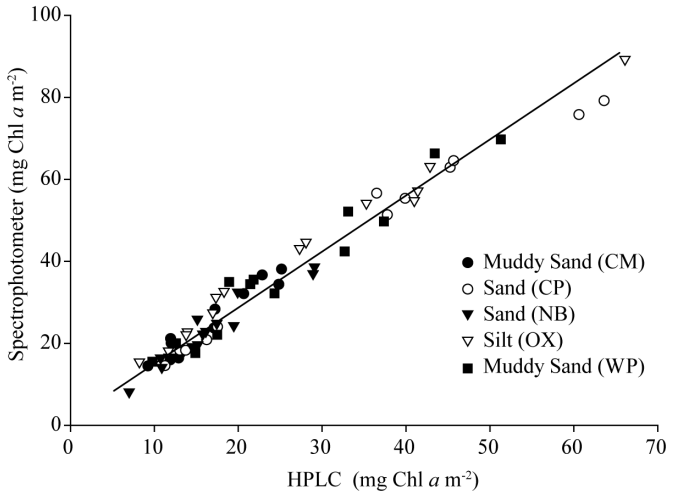

Fig. 6. Simple linear regression of BMA biomass of differing sediment types using spectrophotometry and HPLC (n = 66). Line indicates regression slope (spectrophotometer = 2.877 + 1.329 * HPLC, r^2 = 0.96).

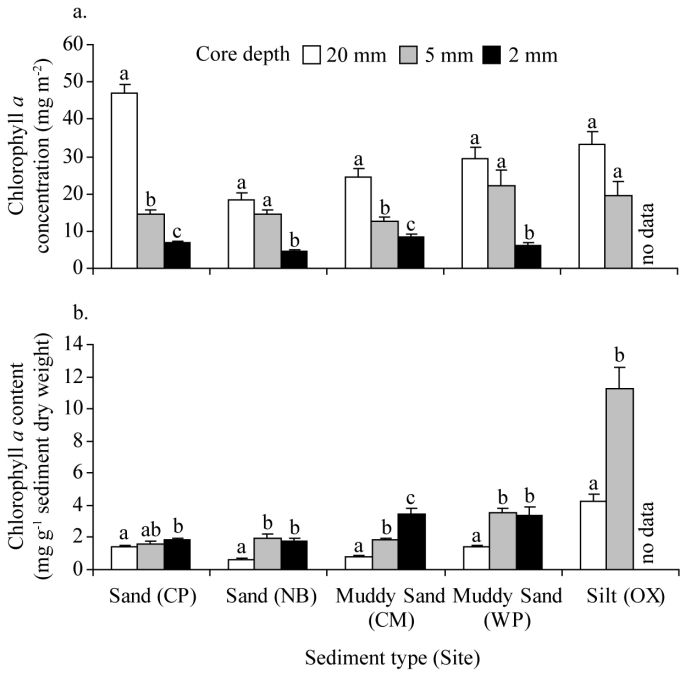

Fig. 7. Comparison of core depths from different sediment types (mean ± 1 SE). Units of biomass expressed as concentration (mg m^2) (a) or content (µg g^{-1} sediment dry weight) (b). Letters indicate significant differences ($P < 0.05$), with each site analyzed using a separate 1-way ANOVA.

expressed either as concentration (mg m^{-2}) or content (mg g^{-1}) sediment, and conversion of one to the other can be problematic. Shallow cores will tend to have lower biomass when expressed in spatial terms, as "buried" chlorophyll will not be sampled. In terms of chlorophyll *a* per unit sediment, shallow cores will generally have far higher biomass estimates, as there will be no dilution effect from the lower sediment layers, which usually have lower chlorophyll content.

When expressed as concentration, cores taken to a 2 mm depth had a significantly lower biomass than those to 5 or 20 mm depth (Fig. 7a). The highest average biomass of Chl *a* was 47 mg m^{-2} from 20 mm depth at the sandy site of Cooloolo Passage (Table 2). This trend was consistent across all sediment types. When biomass was expressed as content, the trend between depths was reversed (Fig. 7b). Cores taken from a 20 mm depth had a significantly lower biomass than cores from a 5 or 2 mm depth. With respect to sediment type, the silty site (OX) had a higher biomass of Chl *a* (11.29 µg g^{-1} sediment dry weight from 5 mm depth). This may have been due to a higher silt fraction reducing light penetration into the sediment, concentrating the BMA community in the upper layers, and the unconsolidated nature of the sediment resulting in a higher moisture content, which has the effect of reducing the sediments mass per unit volume (Table 2). The unconsolidated nature of the sediment and the high water content found at site OX made cores shallower than 5 mm extremely difficult to collect, and the 2 mm depth was not possible (Fig. 7). For fine-scale studies in these silty environments, alternative sample collection methods such as the "Cryolander" (Wiltshire et al. 1997) or contact coring (Anderson and Black 1980) should be used.

Discussion

The current state of knowledge on benthic microalgae, knowledge gaps, and key areas for future research have been clearly identified in 3 comprehensive reviews (MacIntyre et al. 1996; Cahoon 1999; Underwood and Kromkamp 1999). All 3 note the diversity of methods in biomass estimation and emphasize the need for accurate biomass determination. The current study aimed to clarify issues of estimation of benthic microalgal biomass in 2 ways. First, to provide those designing a study, using chlorophyll *a* concentration for biomass estimation, with the minimum effective sampling regimen, and second, to highlight 3 sources of potential variation when comparing studies: sediment type, depth of core, and units of biomass expression. We believe this is especially important when comparing studies of widespread total sediment chlorophyll estimates to those focused on estimates of photosynthetically active chlorophyll in the upper sediment layers. The current study was focused on measurements at the scale of meters; extra caution should be exercised when comparing between spatial scales (mm, m, km).

To design an effective sampling regimen, one should attempt to extract the maximum amount of chlorophyll with minimum degradation from each sample and ensure that the number of samples collected captures the natural variation within the community, at the scale being sampled. To ensure maximum chlorophyll *a* extraction, the ratio of sediment to extraction solvent volume should be at least 1:2. When sampled to a depth of 20 mm, a core diameter of 19 mm was found to be optimal, as the chlorophyll *a* does not suffer from degradation to the same extent as smaller cores and requires smaller solvent volume for extraction than larger diameter cores while still giving accurate total pigment estimates. This was supported by the findings of Snow et al. (2000), who also determined 20 mm wide cores as optimal. Finally, detecting changes in benthic chlorophyll *a* between locations or in time, at the scale of meters, requires at least 8 replicates, in agreement with the study of Kendrick et al. (1996), who found that 8 replicates would have had a power of 80% to detect 50% change.

Therefore, regardless of the spatial scale of the study, samples should be collected using a core of at least 19 mm diameter, and chlorophyll extraction should be performed with a solvent to sediment ratio no less than 2:1. For studies at the meters squared scale, 8 replicates was sufficient over a range of sediment conditions, which is greater than the 3 to 5 replicates used in many previous studies. Results from this study suggest either HPLC or spectrophotometry may be used to provide a reliable estimate of benthic chlorophyll *a*; however, HPLC has a greater resolving power than spectrophotometry. Finally, great care must be taken when comparisons are made between studies using different sediment core depths and/or sediment types.

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