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A comparison of two methods of obtaining densities of zooxanthellae in *Acropora millepora*

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Abstract Quantification of zooxanthellae densities in tissues of reef-building corals aids in the assessment of the extent and severity of coral bleaching. Various methods are available to quantify zooxanthellae densities; however, a direct comparison of these techniques has yet to been done. Here, we compare estimates of zooxanthellae densities obtained using conventional airbrushing coupled with post-tissue-blasting surface area determination, versus a technique whereby zooxanthellae densities are quantified from a known area (0.25 cm^2) of tissue after corals have been fixed and decalcified. Estimates of zooxanthellae densities obtained were correlated across replicate colonies ($R^2 = 0.40$, $n = 81$), and both techniques revealed similar patterns of variation among locations. The main benefit of the decalcification technique was reduced processing time, because the technique eliminates the time-consuming process of tissue blasting and retrospective estimates of surface area. We estimate that decalcification halves the processing time per sample, and produces a more accurate estimate of zooxanthellae density.

Keywords Bleaching, Coral reefs, Decalcification technique, Zooxanthellae densities

Introduction

The occurrence and severity of bleaching among natural coral populations is often quantified using indirect proxies for zooxanthellae densities, such as conspicuous paling of coral tissues (Marshall and Baird 2000). Non-intrusive techniques are useful to quantify major changes in coral health and condition, and facilitate rapid sampling across a significant number and high diversity of corals (Fitt et al. 2001). However, there is also a critical need to validate indirect proxies of zooxanthellae loss (Siebeck et al. 2006). For example, paling or whitening of coral tissues provides limited resolution to assess changes in zooxanthellae density, which might be necessary to predict and forewarn the occurrence of bleaching-related mortality (Jones 2008, but see Baird and Marshall 2002). Direct quantification of zooxanthellae densities within known samples of coral tissue provides the most unambiguous and definitive measure of changes in zooxanthellae densities, thereby providing high resolution for measuring the extent of bleaching (Fitt et al. 2001).

The purpose of this study was to compare two methods for directly measuring zooxanthellae densities in host coral tissues: one the more commonly used method of

airbrushing coupled with post-tissue-blasting surface area determination (e.g. Johannes and Wiebe 1970) vs a second method based on fixing and decalcifying host coral tissues. This second method has been used previously by Drew (1972), Stimson (1997) and Stimson et al. (2002). The greatest benefit that the decalcification technique is that it eliminates the time-consuming step of blasting tissue from the intact coral skeleton. Moreover, it eliminates the need to retrospectively measure the surface area of coral samples from which tissues were removed. Stimson (1997) used the decalcification technique to measure the natural variation of zooxanthellae densities within *Pocillopora damicornis* and the results (annual range of 0.8–1.6 $\times 10^6$ cells/cm^2) were within the range of estimates obtained using airbrushing (D'Croz and Mate 2004; Schloder and D'Croz 2004) and waterpiking (Li et al. 2008). In this study, we directly compared estimates of zooxanthellae densities obtained for paired coral samples using both the decalcification technique and airbrushing. The two techniques are compared in terms of the relative measure of zooxanthellae densities, as well as the overall time required to process coral samples.

Methods

In order to compare the two methods of estimating zooxanthellae densities, i) airbrushing tissues from intact coral skeletons and ii) fixing and decalcifying coral samples, two replicate branches were collected from each of 81 tagged colonies of the stony coral *Acropora millepora* from between 1–3 m depth in July 2007. Colonies were sampled from three sites; two from Orpheus Island (Pioneer Bay and Cattle Bay), and one at the southwest corner of Pelorus Island, all part of the Palm Islands Group, Great Barrier Reef, Australia (18°35′S, 146°29′E). All coral branches were snap frozen in liquid nitrogen, and maintained at −30℃ until further laboratory analysis.

For air-brushed samples, tissues were removed from frozen coral branches using a modified airgun connected to a dive cylinder containing compressed air. Coral tissues were airbrushed into a plastic bag filled with 15mL of 0.5 μm filtered seawater until all tissue was removed (the time for this varied dependent on the size of the coral branch; from five to ten minutes). The resultant slurry was then homogenized at 11 rotations/minute for thirty seconds. Nine mL of the suspension was immediately fixed in 1 mL of formaldehyde. Each of the 8 replicate subsamples were processed in the following manner: the vial was shaken vigorously; then, using a clean pipette, the sample was placed onto a Neubauer Improved Tiefe Depth Profoundeur (0.100 mm) haemocytometer, and viewed under 40x magnification with an Olympus CX31 light microscope. To mitigate 'edge effects' (i.e. counting cells lying on quadrat margins more than once) only the cells which touched the top and left-hand side of each square were counted. There were eight replicate counts from each branch.

Zooxanthellae densities (number per cm^2) were determined by multiplying the number of zooxanthellae counted in each sample (N) by 10^4 (to account for 0.0001 ml sampled in haemocytometer chamber) and 16.67 (to account for dilution with 15 ml of water used when airbrushing), and then divided by the estimated surface area (cm^2) of the branch from which tissue was removed. The surface areas of respective branches were determined using the aluminum foil method (Marsh 1970); whereby branches were carefully wrapped with a uniform single layer of aluminum foil, which was then weighed to establish the surface area of the foil. A calibration curve of the surface area to mass ratio was constructed based on pieces of aluminum foil with known area ($y = 0.3427x$, $r^2 = 0.9996$, $n = 15$), which was then used to back- calculate the surface area of aluminum pieces wrapped around each coral sample.

For decalcified samples, branches were removed from the freezer and fixed in 10% buffered formalin for 4 days. Each sample was then placed in an individual container with 5% HCL solution to gently decalcify the sample over a period of 5 days. The HCL within each container was refreshed on days 3 and 4. Once the skeleton was dissolved, the remaining tissue samples were triple rinsed and stored in 70% ethanol. Two replicate 5 $\times$ 5 mm sections were cut from the surface of each coral sample. Sections were taken 1–2 cm from the apical tip, thereby avoiding areas of tissue that may be devoid of zooxanthellae (Gladfelter et al. 1989; Li et al. 2008). These sections were then placed in individual vials with 1 mL of

70% ethanol. The sample was then mixed with an Ultra Turrax T25 Basic homogenizer (Crown Scientific) for two minutes. 0.0025 ml aliquots of this homogenate were immediately placed on to Neubauer Improved Tiefe Depth Profoundeur (0.100 mm) haemocytometer to quantify zooxanthellae densities as described above.

A paired t-test was used to test for differences in estimates of zooxanthellae densities obtained using the two techniques directly comparing branches from each of 81 colonies. The relationship between the two techniques was also tested using correlation analysis. Finally, resolution of the two methods was compared based on the detection of significant differences in zooxanthellae densities among coral populations from distinct locations. One way ANOVA was used to test for differences in the mean zooxanthellae densities in corals from each location (Cattle Bay, Pioneer Bay and Southwest Pelorus). A separate ANOVA was conducted for each technique.

Results and discussion

This study revealed highly significant differences in zooxanthellae estimates obtained using standard airbrushing of coral samples collected from replicate colonies of *Acropora millepora*, versus estimates obtained following decalcification of coral samples. Decalcification provided significantly higher estimates of mean zooxanthellae densities, compared to airbrushing (Paired t-test, $t=11.92$, df$=80$, $p<0.01$). These differences are most likely caused by differences in the extent of tissue sampling using each technique. Following decalcification, a small (0.25 cm^2) section of coral tissue was taken from well below the apical tip, whereas during airbrushing, tissue was removed from the entire length of coral branches (including the tip). This can cause discrepancy, because the zooxanthellae densities in *Acropora* are generally much lower towards the tip (Gladfelter et al. 1989; Li et al. 2008), leading to lower estimates of zooxanthellae densities when averaging over the entire branch length. Further, differences may arise because water-blasting and airbrushing do not remove tissues that perforate throughout the coral skeleton of *Acropora* corals (and other corals with perforate skeletons).

Estimates of zooxanthellae densities obtained from decalcified coral samples versus those samples from the same colonies that were airbrushed were correlated ($R=0.40$, Fig. 1). However, the estimated zooxanthellae densities were much higher for decalcified coral samples, and this discrepancy increased with increasing densities of zooxanthellae (Fig. 1). Consequently, the two techniques are not directly comparable, but either technique could be used independently to test for changes in zooxanthellae densities within and among coral populations. The maximum density of zooxanthellae (3.85×10^6 versus 2.77×10^6 zooxanthellae per cm^2), as well as the range in estimates of zooxanthellae densities (3.06×10^6 versus 2.37×10^6), were much higher for the decalcification technique, which may increase resolution for detecting significant differences in zooxanthellae densities. For this study, both techniques revealed significant variation in zooxanthellae densities among corals at each location (ANOVA, df$=1/78$, $p<0.01$), whereby the average zooxanthellae density for corals from southwest Pelorus, was significantly higher than Cattle Bay or Pioneer Bay (Fig. 2).

The primary benefit of using decalcification instead of the more commonly used tissue blasting technique (e.g. D'Croz and Mate 2004; Li et al. 2008), is the time it takes to process samples. Decalcifying *Acropora* samples in mild hydrochloric acid takes up to 5 days, but there is very limited handling time during this process. Following decalcification, the time taken to prepare one branch by sectioning tissues, preparing a homogeneous solution, and counting the zooxanthellae in four replicate aliquots was less than 10 minutes. Importantly, this process removes the time-consuming step of blasting tissues from intact coral skeletons, which takes 5–10 minutes per sample. Moreover, it negates the need to retrospectively measure the surface area of the intact coral sample, which is also time-consuming. There are numerous methods available to measure the surface area of coral samples, which vary in their accuracy (Jones et al. 2008; Naumann et al. 2009), but all are time-consuming. This study used the foil wrapping technique (Marsh 1970), which aside from developing the required calibration curve, took up to 8 minutes to wrap and cut, and then weigh the foil for each coral branch. Consequently, the average time for processing samples using the decalcification technique is

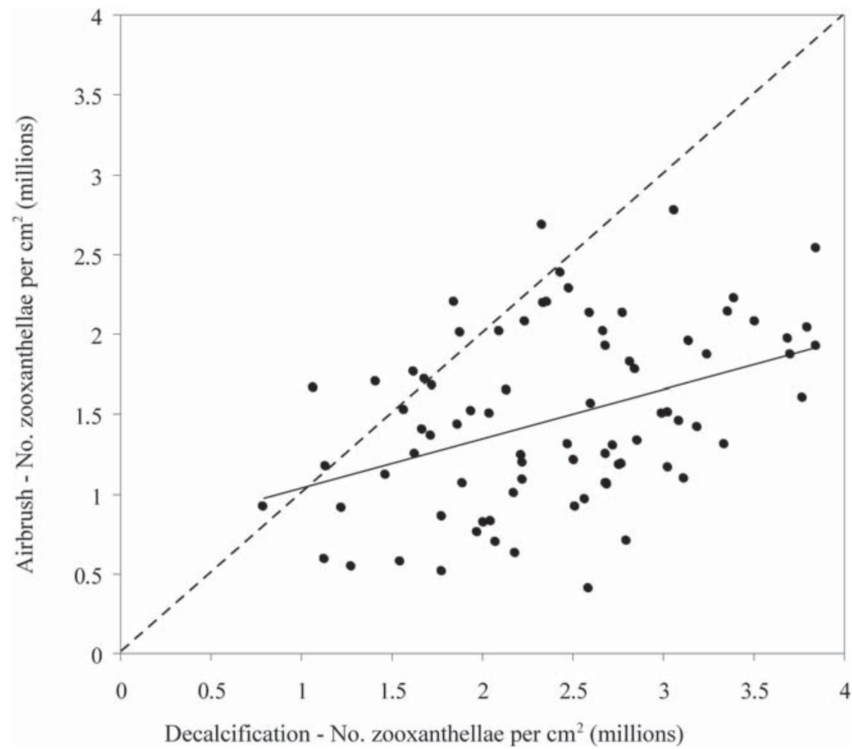

Fig. 1 Comparative estimates of zooxanthellae densities obtained using standard airbrushing of coral samples collected from replicate colonies of *A. millepora*, versus estimates obtained following decalcification of coral samples. While there was a significant correlation in the two estimates (R=0.403), the line of best diverges greatly from a 1:1 relationship (as indicated by the dashed line)

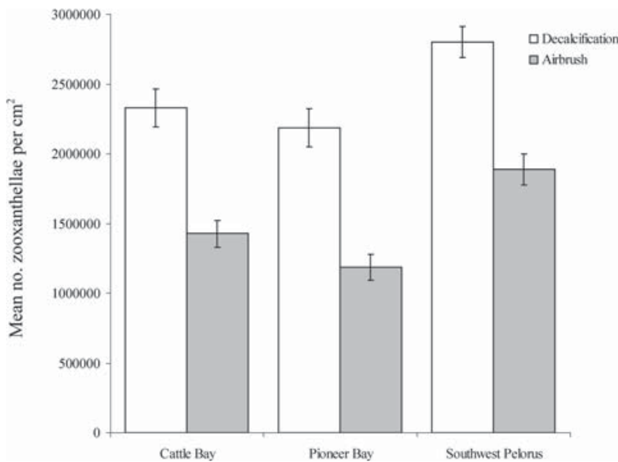

Fig. 2 Mean (±SE) zooxanthellae densities for replicate colonies of *A. millepora* from three different locations in the Palm Islands, central Great Barrier Reef. Paired samples were collected from each colony (N=81 colonies) and standard airbrushing versus a decalcification technique

approximately half that for the airbrushing technique.

Irrespective of the increased efficiency in processing samples, the fewer steps involved in the decalcification technique may reduce inaccuracies in measuring zooxanthellae densities in coral tissues. The primary concern identified in the decalcification process is the accuracy with which small sections can be cut from the decalcified tissues, because of their elasticity, flexibility, and variability among locations and species. Increasing the size of the coral sample (up to 1 cm^2) will eliminate some of the error due to extrapolation when scaling up to determine the number of zooxanthellae per cm^2, but further improvements could also be made by embedding tissue sections in paraffin wax, prior to cutting precise sections. In comparison, there are a number of potential inaccuracies associated with standard tissue blasting methods, including loss of zooxanthellae due to spillage, and incomplete tissue removal during water-picking and airbrushing (Johannes and Wiebe 1970). Methodologies

used to retrospectively measure the surface area of intact coral samples will also introduce a further source of error. In foil wrapping, the surface area of irregular coral samples is likely to overestimate tissue area due to difficulties in getting smooth, non-overlapping coverage of the entire sample (Hoegh-Guldberg 1988), which would further reduce the resulting estimate of zooxanthellae densities.

Accurate quantification of zooxanthellae densities in tissue samples from corals (and other zooxanthellate organisms) is critical for establishing the extent and severity of bleaching, which is increasingly becoming a major threat to coral reefs, globally (Hughes et al. 2003). This study presents an effective method for measuring zooxanthellae densities based on decalcification of coral samples, which requires less handling-time, and is more accurate, than techniques based on blasting tissues from intact coral samples. Moreover, tissue samples can be immediately fixed in 10% buffered formalin (rather than freezing) prior to processing, and much less tissue is required for analyses, which is important if repeatedly sampling corals through time. Further refinements of this technique may be required to obtain accurate estimates of zooxanthellae densities that are comparable within and among corals, especially for non-*Acropora* corals. However, this study has shown that it is both possible and much more efficient to estimate zooxanthellae densities in coral tissues that have been decalcified, rather than physically removed from intact coral skeletons.

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