

RESEARCH ARTICLE

Understanding decay in marine calcifiers: Micro-CT analysis of skeletal structures provides insight into the impacts of a changing climate in marine ecosystems

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Abstract

1. Calcifying organisms and their exoskeletons support some of the most diverse and economically important ecosystems in our oceans. Under a changing climate, we are beginning to see alterations to the structure and properties of these exoskeletons due to ocean acidification, warming and accelerated rates of bioerosion. Our understanding has grown as a result of using micro-computed tomography (μ CT) but its applications in marine biology have not taken full advantage of the technological development in this methodology. We present a significant advancement in the use of this method to studying decalcification in a marine calcifier.
2. We present a detailed workflow on best practice for μ CT image processing and analysis of marine calcifiers, designed using coral skeletons subjected to acute, short-term microbial bioerosion. This includes estimating subresolution microporosity and describing pore space morphological characteristics of macroporosity, in perforate and imperforate exoskeletons. These metrics are compared between control and bioeroded samples, and are correlated with skeletal hardness as measured by nanoindentation.
3. Our results suggest that using subresolution microporosity analysis improves the spatiotemporal resolution of μ CT data and can detect changes not seen in macroporosity, in both perforate and imperforate skeletons. In imperforate samples, the mean size and relative number of pores in the macroporous portion of the images changed significantly where total macroporosity did not. The increased number of pores and higher microporosity are both directly related to a physical weakening of the calcareous exoskeletons of imperforate corals only. In perforate corals, increased macroporosity was accompanied by an overall widening of pore spaces though this did not correlate with sample hardness.
4. These novel techniques complement traditional approaches and in combination demonstrate the potential for using μ CT scanning to sensitively track the process

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of decalcification from a structural and morphological perspective. Importantly, these approaches do not necessarily rely on ultra-high resolution (i.e. single micron) scans and so maintain the accessibility of this technology. The continued optimization of these tools for a variety of marine calcifiers will advance our understanding of the effect of climate change on marine biogenic calcified structures.

KEYWORDS

bioerosion, calcification, exoskeleton, hardness, micro-computed tomography, morphology, ocean acidification, porosity

1 | INTRODUCTION

Biogenic calcification is a critically important global process (Frankignoulle, Pichon, & Gattuso, 1995). For all benthic calcifiers, the exoskeleton is a support structure, protecting them from predation and providing the basis for a variety of ecosystem goods and services. For example, one of the major calcifiers in tropical ecosystems are hard corals; the structural complexity of the reefs they build also supports fish biomass, reduces coastal erosion and promotes recovery from environmental disturbance (Coker, Graham, & Pratchett, 2012; Graham, Jennings, MacNeil, Mouillot, & Wilson, 2015; Graham & Nash, 2013; Vergés, Vanderklift, Doropoulos, & Hyndes, 2011). Similarly, oyster reefs stabilize shorelines and improve estuarine water quality (Grabowski et al., 2012), while ubiquitous coccolithophores underpin global oceanic food webs (Legendre & Le Fevre, 1995). Calcifying organisms are also some of the most economically important marine organisms; scleractinian corals are the primary reef builders of an ecosystem valued globally at 10 trillion USD (Costanza et al., 2014). However, these organisms are under threat from ocean warming and acidification that accelerate bioerosion (Reyes-Nivia, Díaz-Pulido, Kline, Guldborg, & Dove, 2013; Silbiger, Guadayol, Thomas, & Donahue, 2014; Tribollet, Godinot, Atkinson, & Langdon, 2009), disrupting calcification in the marine environment and degrading existing carbonate structures, with far-reaching economic and ecological impacts (Costanza et al., 2014; Davidson, Altieri, Ruiz, & Torchin, 2018; Orr et al., 2005).

A decrease in exoskeletal durability and strength has broad consequences for ecosystem structure and function. For pelagic and benthic calcifiers, alterations to their shell structure will affect their ability to capture light, and protect themselves from predation and UV damage (Enríquez, Méndez Eugenio, Hoegh-Guldborg, & Iglesias-Prieto, 2017; Meng et al., 2018; Monteiro et al., 2016; Orr et al., 2005). Reef-building organisms will suffer a loss of structural stability, compromising key ecosystem services such as commercial fishing and coastal protection (Fantazzini et al., 2015; Graham & Nash, 2013; Meng et al., 2018). Impairment to these critical functions has the potential to affect primary productivity over large areas (e.g. the Caribbean sea; Allgeier, Valdivia, Cox, & Layman, 2016). Therefore, the nature and progression of climate change impacts in

these marine systems are directly related to changes in the physical and morphological properties of biogenic CaCO_3 structures. Despite this recognized link, we still lack a comprehensive knowledge base to predict changing structural properties of calcifiers in the Anthropocene. This is, in part, due to limitations in the tools we use to study decalcification (i.e. the degradation or dissolution of CaCO_3 structures; see Table 1).

Micro-computed tomography (μCT) is a non-destructive 3D imaging technique that uses the same principles as 'macro'-CT scanning but at a considerably higher resolution (i.e. resolution is measured in micrometres; Gutiérrez, Ott, Töpperwien, Salditt, & Scherber, 2018). Computed tomography, and especially μCT , has provided insights into inter-annual growth patterns (Barkley et al., 2018; Oakes & Sessa, 2019; Ross, Falter, Schoepf, & McCulloch, 2015), historical environmental stress events (Barkley & Cohen, 2016; DeCarlo et al., 2017), environmental drivers of bioerosion (Enochs, Manzello, Tribollet, et al., 2016; Li et al., 2014; Silbiger, Guadayol, Thomas, & Donahue, 2016) and skeletal micromorphology (Johnson, Harianto, Thomson, & Byrne, 2020; Roche, Abel, Johnson, & Perry, 2010). However, the application of this method in marine biology stands to benefit from improvements in μCT technology in other disciplines. Recently, we applied advanced μCT methodology to estimate the subresolution porosity (termed microporosity) of coral skeletons subjected to accelerated microbial bioerosion by resident endoliths during a marine heatwave (Leggat et al., 2019). Endolithic microbes are near ubiquitous in biogenic carbonates (Tribollet, 2008; Tribollet & Golubic, 2011) and are highly effective bioeroders (Carreiro-Silva, McClanahan, & Kiene, 2005; Chazottes, Campion-Alsumard, & Peyrot-Clausade, 1995; Perry et al., 2014). Short-term microbial bioerosion is an ideal process off which we can design sensitive methods for measuring decalcification because the small-scale changes are hard to detect and because reef-wide microbial bioerosion is expected to significantly increase as a result of climate change (Leggat et al., 2019; Reyes-Nivia et al., 2013). Here we present a major advance in the application of tomographic (μCT) analyses, adapting three geological techniques that can be applied to investigate decay and dissolution in calcified structures. We also provide a case study of quantifying imperforate and perforate pore space morphology in two species of scleractinian coral at a high spatiotemporal resolution. Included is a step-by-step protocol (Figure 1) that provides a guide for tomographic image processing and analyses.

TABLE 1 Common methods used to study the structural properties of marine calcifiers

Method	Description	Advantages	Limitations	Source(s)
By Archimedean principles	Uses the difference in weight of an object when dry versus immersed in seawater to estimate the microdensity of a sample. Can be combined with geometry or paraffin wax dipping to estimate bulk density and porosity	Easy to implement with variations for improved high accuracy (e.g. vacuum infiltration) or in situ measurements. Bulk density data are useful for long-term studies and readily comparable to past literature. Non-destructive. Cost-effective for high biological replication	Can overestimate bulk density depending on the presence and prevalence of occluded pores in a sample. Low spatiotemporal resolution. Cannot identify where change is happening (i.e. internal or external). Measure net growth or degradation (i.e. cannot separate processes of calcification and dissolution)	Bucher, Harriott, and Roberts (1998), Herler and Dirnwöber (2011) and Fantazzini et al. (2015)
Visual assessment or imaging	Range of methods from measuring linear extension or ecological volume, to high-precision 3D photogrammetry. Can be in situ measurements; more commonly done using a camera	Easy to implement and cost-effective. Can quantify and describe external morphology	Cannot measure internal porosity or morphological features, including structural complexity. Cannot determine variation in skeletal density	Cruz, Villanueva, and Baria (2014), Ferrari et al. (2017) and Kim, Lecours, and Frederick (2018)
Scanning electron microscopy	Thin sections are prepared by cutting with a diamond-blade saw, mounting the sample and coating in gold or platinum. An electron beam is fired at the sample and the electromagnetic signals produced as a result are measured to determine surface composition and topography	High-resolution images of corallite microstructure. High spatiotemporal resolution. Can calculate the rate of CaCO ₃ removal from a 2D image	Cannot produce 3D images. Measurement is highly dependent upon where the 2D image is taken. Destructive	Tribollet, Decherf, Hutchings, and Peyrot-Clausade (2002), Milner, Langer, Grelaud, and Ziveri (2016), Bylenga, Cummings, and Ryan (2017), Meng et al. (2018) and Leggat et al. (2019)
Nuclear magnetic resonance imaging	Samples are saturated with water and held inside a constant magnetic field. A second magnetic 'pulse' is applied to excite hydrogen protons in the water. Their subsequent relaxation energy is measured as the time taken for energy to dissipate transversely. This relaxation time is greater in larger pores because of the greater dissipation distance before fluid-solid boundaries	Non-destructive. Pore size distribution is inferred from relaxation curve in time-domain NMR. Can additionally provide information about sample permeability	Cannot distinguish between pore and pore throats (i.e. connection points between pores) as it relies upon transverse energy dissipation. Does not directly measure pore size. Isolated pores inside the sample cannot be measured	Fantazzini et al. (2013, 2015) and Anovitz and Cole (2015)
X-ray radiography	A 2D X-ray radiograph is taken of a cut surface or slice of the sample. Growth bands can be used to measure calcification and in sclerochronology, erosion scars can be used to measure dissolution/degradation	Can be used to measure inter-annual growth patterns and identify historical stress events. Cost-effective for high biological replication	Destructive of whole sample. Measurement is dependent upon where the 2D image is taken. Images artefacts can arise from 3D variation in density	Barnes, Lough, and Tobin (1989), Le Tissier, Clayton, Brown, and Davis (1994) and Duprey, Boucher, and Jiménez (2012)
Computed tomography (macro and micro)	See Section 2	Non-destructive 3D imaging technique. Depending upon the sampling design, it can be used to distinguish between calcification/accretion and decalcification/bioerosion. Can identify structural changes at several scales. Can describe the growth of individual polyps	See Section 4.3	Ross et al. (2015), DeCarlo and Cohen (2016), Iwasaki et al. (2016), Silbiger et al. (2016), Gooday et al. (2018) and Meng et al. (2018)

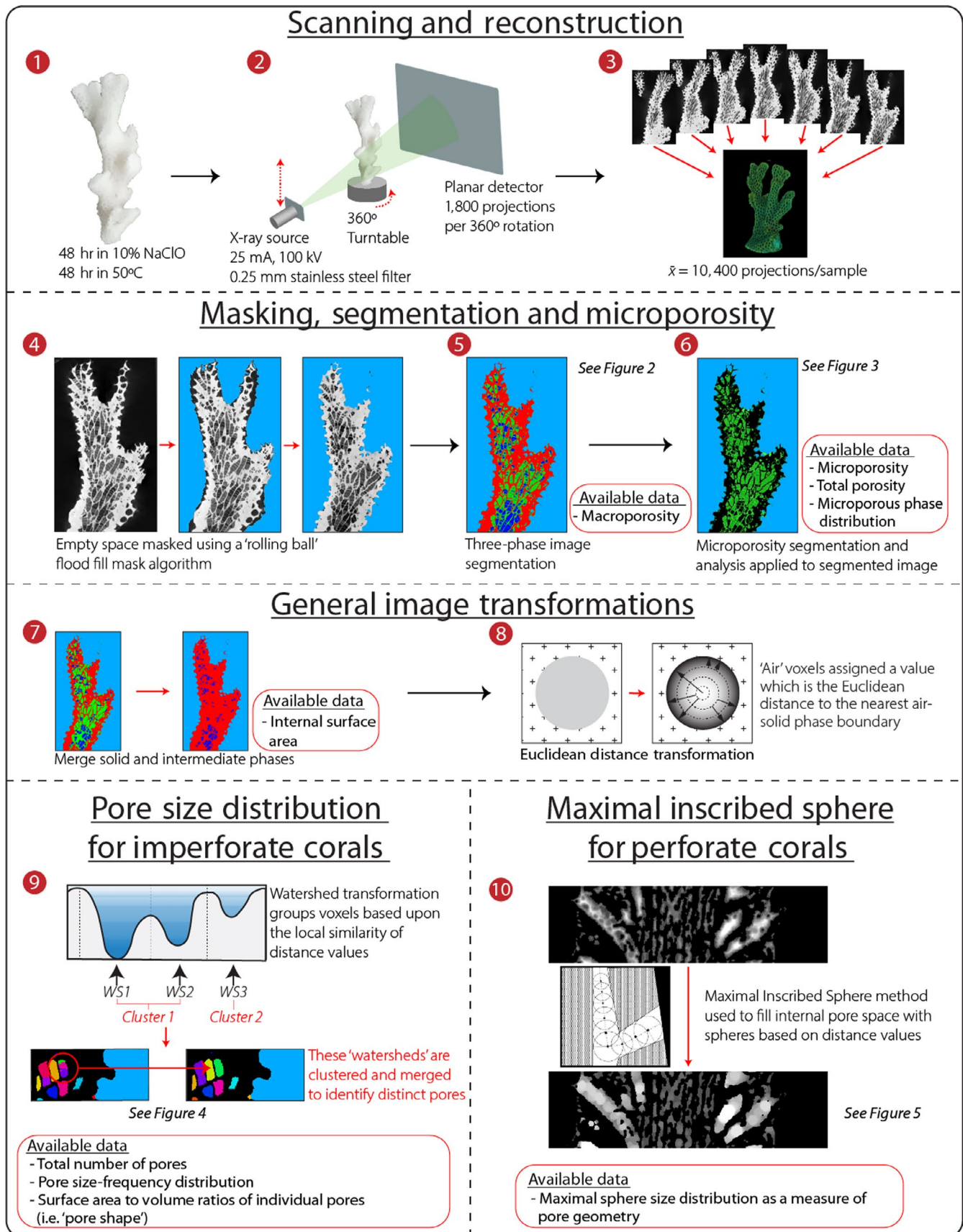


FIGURE 1 Schematic illustrating the analytical pipelines used in this study

2 | MATERIALS AND METHODS

2.1 | Protocol

2.1.1 | Sample preparation (Step 1, Figure 1)

To illustrate these techniques, we present a case study of their application to the skeletons of two scleractinian corals: (a) *Pocillopora damicornis* ($n = 18$ nubbins from 18 parent colonies; 6 at time point 0 before heat stress, 12 at time point 1 following heat stress), an imperforate coral and (b) *Acropora aspera* ($n = 18$ from 6 parent colonies; same regime as *P. damicornis*), a perforate coral. For detailed sample handling, collection and experimental procedures see Leggat et al. (2019) from which we use an expanded dataset. Whole coral colonies were collected from the Heron Island reef flat (23.4385°S, 151.9084°E) and acclimatized to tank conditions for 1 week. Skeletal samples were taken prior to applying simulated heat stress (time point 0, TP₀). The heating trajectory, including daily variation, was designed based on in situ and satellite temperature measurements at Lizard Island during the 2016 mass bleaching event. Corals were heated at a rate of 1°C per day, to a daily maximum of 34°C. After 6 weeks of heating, during which coral bleaching, mortality and skeletal degradation occurred, a second set of skeletal samples were taken (time point 1, TP₁). Skeletons were prepared following the methods of Fantazzini et al. (2015): (a) submerged in 10% sodium hypochlorite for 48 hr to remove organic matter; (b) washed with deionized water; (c) and dried at a maximum temperature of 50°C for 48 hr to prevent crystalline phase shifts (Caroselli et al., 2011). Skeletons were packed in 50 ml centrifuge tubes with cotton wool to prevent damage during storage and transport. For scanning, two samples were mounted on top of each other inside a 50 ml falcon tube, separated by a disk of clear plastic.

2.1.2 | Scanning (Step 2, Figure 1)

Samples were scanned using a HeliScan MicroCT system with an optimized space-filling trajectory (Kingston et al., 2018) to yield sharp images (Latham, Varslot, & Sheppard, 2008; Myers, Kingston, Varslot, & Sheppard, 2011) with an isotropic voxel size of 26.2 µm (Table 2). In samples with variable thickness and density, beam hardening can lead to the misrepresentation of the true structure and properties of the object (DeCarlo, 2017; Roche et al., 2010) as well as poor image quality (Barrett & Keat, 2004). To correct for this, the polychromatic X-ray source was passed through a 0.25-mm stainless steel filter. This removes this artefact without sacrificing the improved resolution associated with polychromatic versus monochromatic X-ray sources (Barrett & Keat, 2004).

2.1.3 | Reconstruction (Step 3, Figure 1)

Image reconstruction refers to the process of recombining two-dimensional information contained in sinograms into a three-dimensional

tomogram (Table 2). The optimization of reconstruction algorithms requires analytical expertise to ensure a theoretically exact reconstruction; in this case, a Katsevich 1PI inversion formula was used (Kingston, Sakellariou, Varslot, Myers, & Sheppard, 2011; Varslot, Kingston, Latham, et al., 2011; Varslot, Kingston, Myers, & Sheppard, 2011). This was performed in Mango image analysis software.

2.1.4 | Image masking (Step 4, Figure 1)

All tomographic image analyses were performed using WebMango, an online implementation of Mango image analysis software; however, ImageJ could also be used. The software NCViewer was used to examine 2D slices from tomograms in netcdf format; any netcdf viewer can be used in its place.

One challenge in quantifying skeletal morphology in tomograms of irregular objects is defining the region of interest by excluding external space outside of the object (Roche et al., 2010). To achieve this, we used a three-step process. First, we manually 'shrank' the external space to be subsequently masked algorithmically (first panel, Step 4, Figure 1). In practice, this involves choosing a 2D slice of the tomogram on the z-axis and defining the smallest possible circle about the sample that excludes the maximum empty space without cropping the sample. We then cycle through the rest of the z-slices, growing the circle as necessary to ensure none of the tomogram is cropped. We then applied a flood fill algorithm (FFM) in order to mask the empty external space within our theoretical cylinder (second panel, Step 4, Figure 1). The algorithm grows from user-defined voxels with a particular intensity value (Table 2), replacing these seeded voxels with a pre-defined replacement intensity. Here, we replaced zero-intensity external voxels with the max value 65,335, the maximum value on the 16-bit scale (Table 2), as no part of our sample had this intensity, and we wanted to partition internal air space (i.e. porosity) from external air space. The process is the same as using the 'fill' function in digital image analysis/design programs to replace one colour with another across a whole image. The FFM grows along connections between neighbouring voxels with the same intensity; therefore, how you define a connection between two voxels affects the outcome. There are two variations based on whether we consider voxels touching at the corners as connected: eight-way (faces and corners) and four-way (faces only). Here we used eight-way connections and constrained the 'invasion radius' of the FFM to six voxels (157.2 µm) wide. The FFM tests if it can grow at least six voxels in each direction before making any replacements; this prevented it from incorrectly masking pore space that was connected externally. Finally, we shrank the resulting mask by two voxels (third panel, Step 4, Figure 1); this was a trade-off between excluding external space and removing some small features of the skeleton.

2.1.5 | Image segmentation (Step 5, Figures 1 and 2)

Image segmentation, also known as thresholding (Gooday, Sykes, Góral, Zubkov, & Glover, 2018; Roche et al., 2010), is arguably the

Term	Definition	Source (s)
Sobel gradient	The gradient of a slope representing an increase or decrease in greyscale intensity as you move from one voxel to its neighbours. The bigger the difference between neighbours, the greater the gradient	Sobel and Feldman (1968)
Isotropic voxel size	The length of a cubic 3D pixel. Akin to the image resolution of our tomogram (though resolution and voxel size can be different; see source)	du Plessis, Broeckhoven, Guelpa, and le Roux (2017)
Greyscale intensity	The intensity of X-ray attenuation by a voxel or group of voxels. Related to sample density but using a variable scale from 8-bit (0 to 255) to 16-bit as presented here (0 to 65,335)	Ketcham and Carlson (2001)
Macroporosity	The porosity defined as air that is 'visible' at the chosen scan resolution (i.e. resolvable porosity)	Leggat et al. (2019)
Microporosity	Subresolution porosity due to pore spaces that are smaller than the isotropic voxel size but affect X-ray attenuation	Leggat et al. (2019)
Pore size distribution (PSD)	Size–frequency distribution of pores inside the sample. See Section 2	Lawrence and Jiang (2017) and Sheppard, Sok, et al. (2006)
Maximal inscribed sphere (MIS)	A method for describing pore space geometry using maximally inscribed, overlapping theoretical spheres. See Section 2	Sheppard, Sok, et al. (2006) and Fanwua et al. (2011)
Seeded voxel	A voxel or group of voxels designated as a particular phase by the operator. The starting point for adaptive algorithms to grow from	n/a
Sinogram	2D array of data containing information about X-ray attenuation along that plane	Varslot, Kingston, Myers, et al. (2011), Varslot, Kingston, Latham, et al. (2011)
Tomogram	The result of reconstructing sinograms into a 3D image	du Plessis et al. (2017)
Euclidean distance	The straight-line distance between two points in Euclidean space	Schlüter et al. (2014)
Watershed transformation	A clustering algorithm based conceptually on the idea of geological watersheds. Here implemented on the distance-transformed tomogram. Clusters voxels based upon the similarity of their distance values	Schlüter et al. (2014)

TABLE 2 Terms and definitions used in this study

most important step in image pre-processing as it defines the physical composition of the sample based on greyscale intensity (i.e. density). Historically, μ CT imaging of marine calcifiers has employed binary segmentation, in which a single greyscale intensity threshold is used to distinguish between solid CaCO_3 and air (DeCarlo, 2017; Gooday et al., 2018; Meng et al., 2018; Roche et al., 2010; Silbiger et al., 2016). This threshold can be manipulated to produce the most realistic operator-perceived segmentation (Veal, Holmes, Nunez, Hoegh-Guldberg, & Osborn, 2010) or may be calibrated based on objective measures such as density measured by Archimedean principles (Silbiger et al., 2016) or using Otsu's thresholding (Otsu, 1979) which can automatically segment images based on maximizing variance between the classes of voxels (e.g. pore and skeleton). Binary

objective segmentation is valuable because it overcomes a phenomenon known as 'partial voxel filling', in which voxels in a tomogram are neither wholly solid nor air (Barrett & Keat, 2004; DeCarlo, 2017; Silbiger et al., 2016). This occurs at the boundaries between the two phases or due to subresolution porosity, where pore spaces are smaller than one voxel. It also allows us to convert greyscale values on, for example, a 16-bit scale to Hounsfield Units (DeCarlo, 2017), which can help us measure and compare densities within or between samples (i.e. densitometry; Enochs, Manzello, Kolodziej, et al., 2016). However, depending upon the method used to calibrate the 16-bit scale it can introduce uncertainties. For example, using Archimedean principles can lead to small but consistent underestimations of bulk density due to the presence of occluded pores in the skeleton

(Table 1; Fantazzini et al., 2015). Otsu's thresholding is a straightforward and objective method but its performance depends upon how well separated the peaks in the intensity histogram are and how deep the 'valley' is between them (Kittler & Illingworth, 1985). Binary thresholding addresses the problem of partial voxel filling at phase boundaries; but partial voxel filling does not always represent an artefact of the method but might also represent variation in sub-resolution porosity (Figure 2b–d). Therefore, the accuracy of objective thresholding may be context-dependent and it limits inferences about sub-resolution characteristics of the sample.

A challenge with biological samples is pore size variability relative to voxel size, creating a need to correct for boundary effects while retaining information about sub-resolution porosity. Our proposed solution was to apply three-phase segmentation, a technique designed for the geosciences that can be used to simultaneously define and 'grow' phases from multiple materials with variable density in polymineralic samples (Prodanović, Mehmani, & Sheppard, 2015; Sheppard, Arns, et al., 2006; Sheppard, Sok, & Averdunk, 2004). We defined three phases in our monomineralic sample: air, solid and an air–solid mixed

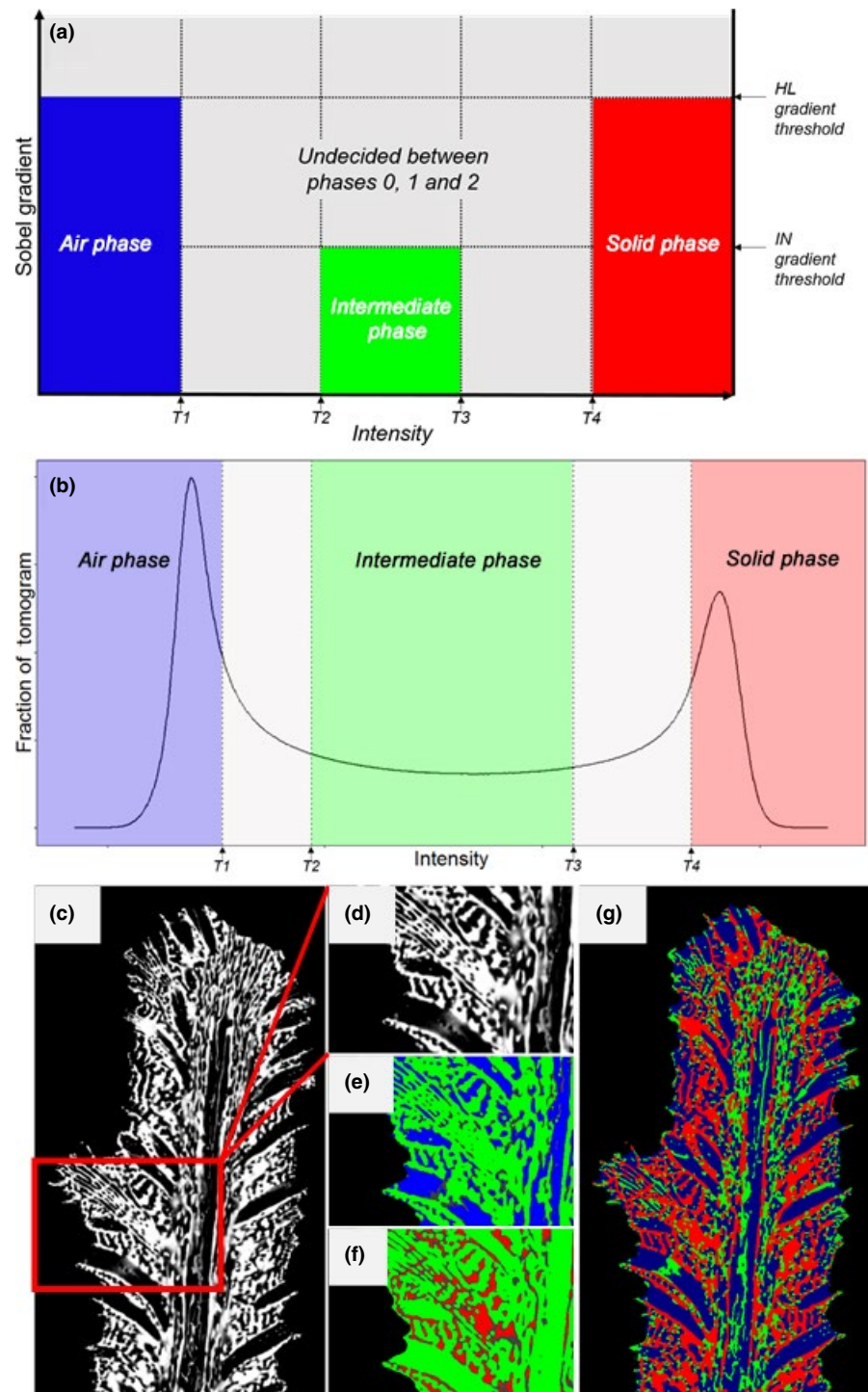


FIGURE 2 Three-phase segmentation of a single *Acropora aspera* skeleton. (a) Voxels in an image are seeded or undecided based on their greyscale intensity and sobel gradient (Table 2). (b) The application of this framework to a greyscale intensity histogram that corresponds to images. The three phases are simultaneously grown algorithmically. (c) An unsegmented 2D y-slice. (d) Zoomed in subsection. Note the range of greyscale intensities representing microporosity. (e) Having set thresholds T_1 and T_2 ; blue is air below T_1 and green is intermediate and solid above T_2 . Grey is undecided. (f) Subsection having set thresholds T_3 and T_4 ; red is solid above T_4 and green is combined intermediate and air below T_3 . Grey is undecided. (g) Fully segmented slice; blue is air, red is solid calcium carbonate and green is intermediate

intermediate phase comprised of partially filled voxels. We call this intermediate phase microporosity (see Section 2.1.6 below). This phase is akin to that used to estimate microbial bioerosion over long time frames (>1 year) by Enochs, Manzello, Kolodziej, et al. (2016) and Silbiger et al. (2016), which was referred to as variable skeletal density. Three-phase segmentation uses a combination of greyscale intensity and sobel gradients (Figure 2a; Table 2). Using sobel gradients includes additional information about the average change in density in all directions of 3D space, which indicates a possible phase boundary (i.e. high gradient), to distinguish between true microporosity and partially filled voxels at the boundary between air and solid. First, we set the seeded voxels and the 'undecided' voxels (Figure 2a,b). The seeded voxels were then used to grow each of the three phases into the 'undecided' fractions, using a converging active contours (CAC) algorithm (Sheppard et al., 2004). The speed at which this algorithm grows each phase is defined by the sobel gradient value. Near a true phase boundary, the algorithm will slow down due to a higher intensity difference between adjacent voxels. This generates a sharp, well-defined image.

Segmentation steps:

1. The operator defined two intensity thresholds, T_1 and T_4 . All voxels below T_1 are assigned as air; all voxels above T_4 are assigned as solid (Figure 2a,b). In Figure 2e, air voxels are represented as blue; in Figure 2f, solid voxels are represented as red.
2. The operator then needs to define the lower threshold for the intermediate phase, T_2 (Figure 2a,b). As per Figure 2e, we set T_1 to highlight pore space (blue) and set T_2 to highlight combined intermediate and solid (green). In Figure 2e, there are uncoloured voxels which represent the areas of uncertainty which will be assigned algorithmically (see below; Figure 2a,b).
3. The inverse of this process was then used to define the upper threshold for the intermediate phase, T_3 (Figure 2a,b). As per Figure 2f, T_4 is set and solid voxels highlighted in red; T_3 is set to highlight combined intermediate and air (green). Once again, there are areas of uncertainty (Figure 2f).
4. Next, we set the gradient thresholds. The gradient threshold for pore and solid CaCO_3 voxels (HL threshold; Figure 2a) was set at the maximum possible intensity value. The gradient threshold for the intermediate phase (IN threshold; Figure 2a) is the gradient value above which microporosity voxels are re-assigned to the 'undecided' portion. It is particularly important, because it prevents partially filled voxels at boundary layers being incorrectly assigned as microporosity; a sobel gradient above the IN threshold indicates a rapid rate of change in density and a probable phase boundary. Using the IN threshold avoids 'halos', where the air and solid phases are consistently separated by a layer of intermediate phase; setting a HL threshold maintains true air-solid phase boundaries.
5. Once these thresholds are set, then the CAC algorithm grows all three phases simultaneously into the undecided fractions of the tomograms (Figure 2a,b).

The operator needs to examine the segmented image and compare it in detail to the original greyscale tomogram. In order to optimize the segmentation process, we needed to go through multiple iterations of steps 1–5 during which thresholds are adjusted one at a time. As a result, three-phase segmentation is a time-consuming process. It is essential that the same bias (under- or overestimation) be applied to all the samples. It is useful for final segmentations to be checked against the original tomogram by a third party, with a focus on phase boundaries, in order to identify potentially consistent operator bias. At the end of this process, we could quantify the macroporosity (i.e. resolvable porosity) of our samples as the volume of voxels in the air phase (Figure 1). While this method of segmentation uses thresholds that are initially defined by the user which can introduce subjectivity, it also allows us to make inferences about subresolution characteristics of the sample.

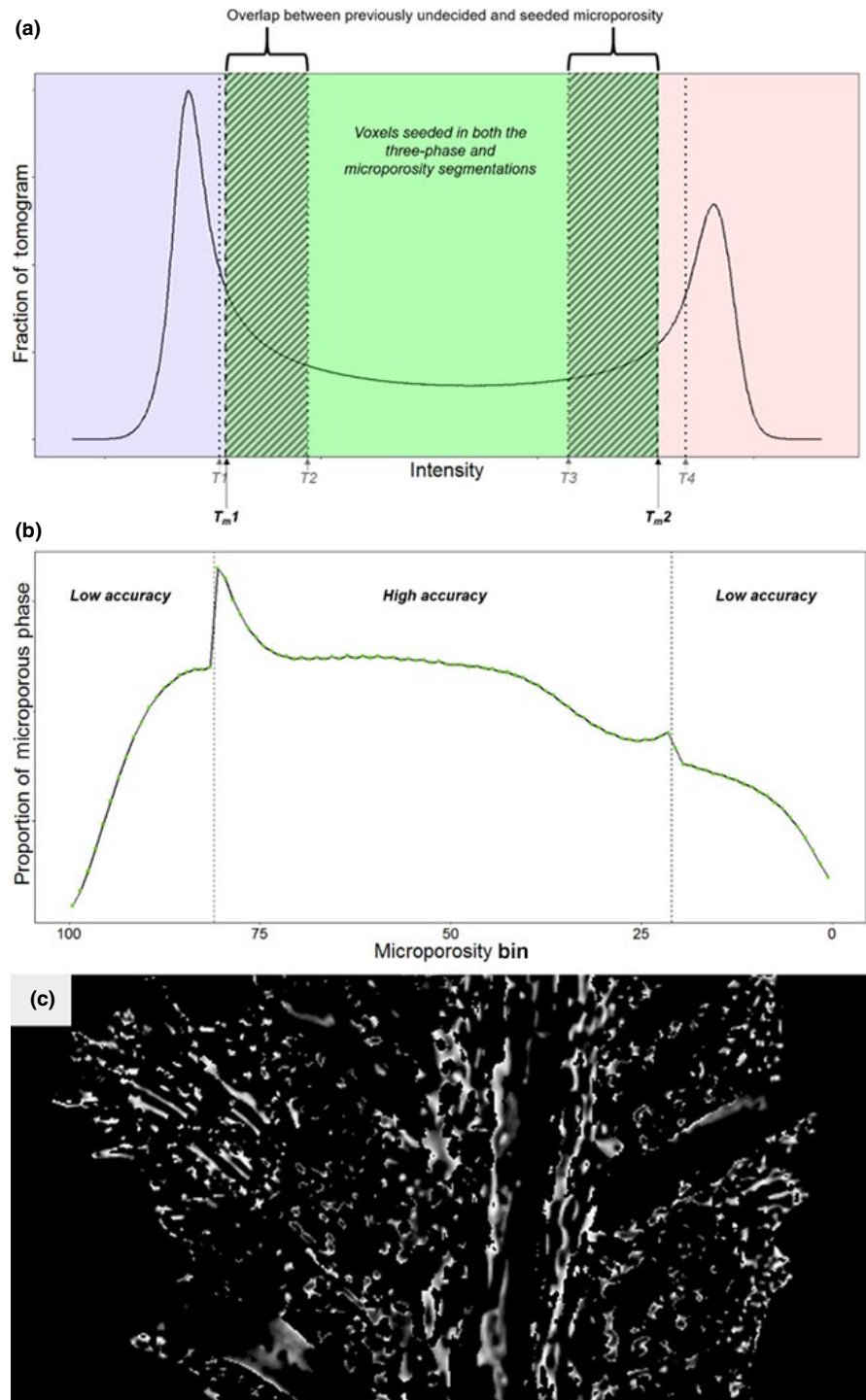
2.1.6 | Microporosity analysis (Step 6, Figures 1 and 3)

We used microporosity analysis to estimate the total volume of air contained within all of subresolution pore spaces in the intermediate phase (Sheppard, Arns, et al., 2006; Varslot, Kingston, Myers, et al., 2011). This can then be summed with macroporosity to obtain a more precise value for skeletal porosity. Microporosity analysis uses the greyscale range of the intermediate phase from the three-phase segmentation to inform how to apply another segmentation to the original greyscale tomogram. It relies on the assumption that, within the intermediate phase, there is a linear relationship between the greyscale intensity of an intermediate voxel and the volume of air within it (Sheppard, Arns, et al., 2006). Here that is a reasonable assumption because our samples are, for practical purposes, monomineralic. It is well known that calcite is present in small amounts in coral skeletons but its absolute density (2.71 g/cm^3) is close to the dominant CaCO_3 isomorph, aragonite (2.93 g/cm^3), and not distinguishable through μCT scanning at this resolution.

Microporosity steps:

1. The operator defined two thresholds, T_{m1} and T_{m2} , for microporosity based upon the thresholds used in, and results from, the preceding three-phase segmentation ($T_1 < T_{m1} < T_2$ and $T_3 < T_{m2} < T_4$; Figure 3a).
2. To visualize the range of greyscale intensities captured by T_{m1} and T_{m2} , we set the voxels below T_{m1} and above T_{m2} to a neutral colour such as black (Figure 3b) and compared this to the original and segmented tomograms. We identified originally 'uncertain' areas, noted how the CAC algorithm assigned these voxels and compared whether these agreed with our microporosity thresholds.
3. We then categorized the greyscale values captured by the microporosity thresholds into 100 bins. Based upon the linear relationship between greyscale intensity and microporosity, these

FIGURE 3 Microporosity analysis of a single *Acropora aspera* skeleton. (a) The green section of the intensity histogram shows the defined microporous phase based on the results of the three-phase segmentation and the original tomogram. (b) Voxels in the microporous phase are binned according to an assumed linear relationship between greyscale intensity and percent microporosity. Low accuracy areas represent areas that were segmented by the CAC algorithm and so were originally defined as uncertain by the user. (c) A 2D y-slice with the solid/air phases set to black to highlight to range of greyscale intensities within the microporous phase



correspond to 0.5% microporous at the boundary with the solid phase (T_{m1}) to 99.5% microporous at the boundary with the air phase (T_{m2}).

4. Given that we know the volume of a single voxel ($26.2 \mu\text{m}^3$), we can then quantify the total volume of air contained within the microporous phase by multiplying the number of voxels in each bin (i) by their volume and microporosity value:

$$\sum_{i=0.005}^{0.995} i(26.2 \times \text{number of voxels in bin } i).$$

We also examined the distribution of binned microporous voxels in order to identify possible shifts in distribution towards either threshold, which might indicate either a widening or narrowing of microporous spaces. While modern μCT scanners can achieve single-micron resolutions, this requires a significant reduction in the physical size of your sample which would reduce the generalizability of results. It would also require longer scanning times and cost, likely at the expense of biological replication. Therefore, we consider microporosity analysis a cost-effective trade-off between sample size, cost and resolution.

2.1.7 | Image transformations (Steps 7 and 8, Figure 1)

The analyses in steps 9 and 10 are approaches to describing the morphology of the resolvable pore space. Prior to these, we necessarily merged the solid and intermediate phases to produce a two-phase tomogram (Figures 4b and 5c). At this stage you can also calculate the internal surface area of the resolved void space using the methods described by Ohser and Mücklich (2000). Following this, we applied a Euclidean distance transformation (Step 8, Figure 1) to voxels in the air phase. Each voxel is revalued based upon the straight-line distance to the nearest phase boundary in

3D space which represents its position within the pore space. The resultant 'distance maps' appear as in Figures 4c and 5d.

2.1.8 | Particle size distribution analysis for imperforate corals (Step 9, Figures 1 and 4)

TD-NMR has been previously used to describe the size–frequency distribution curves of pores within coral skeletons in three dimensions (Table 1; Fantazzini et al., 2013, 2015). But it only describes the shape of, and shifts, in distribution (Lawrence & Jiang, 2017), as opposed to

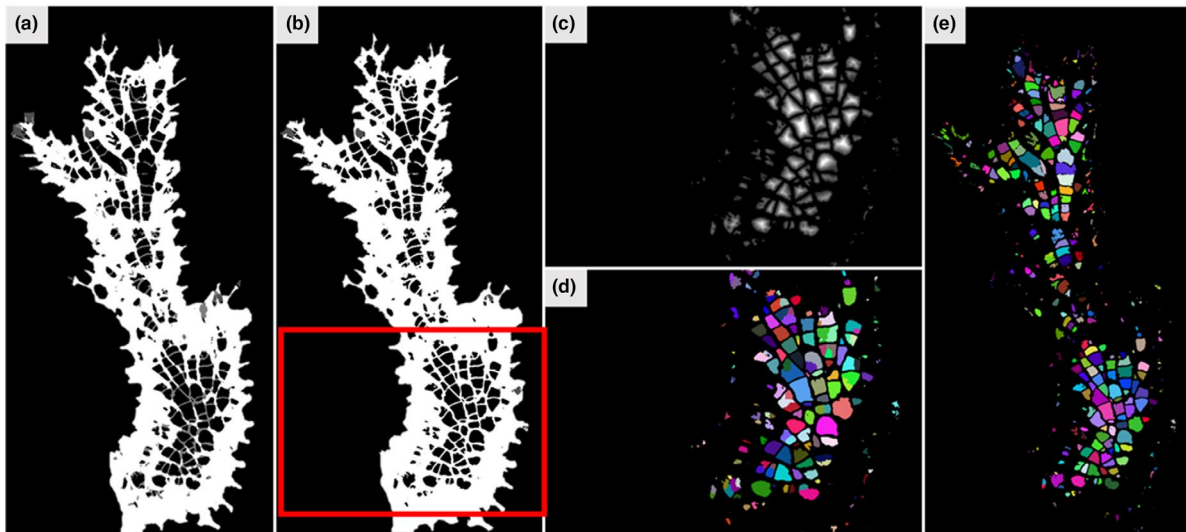


FIGURE 4 Particle size distribution method. (a) A 2D y-slice of *Pocillopora damicornis* showing evidence of solid, air and intermediate phases. (b) After having merged the solid and intermediate phases. (c) A Euclidean distance transformation is applied to the tomogram; each voxel is revalued according to the minimum distance to the nearest phase boundary. (d) A watershed transformation clusters voxels based on the similarity of their distance values and spatial gradients. (e) Secondary clustering, based on the strength of separation in watershed clusters, produces the final transformed tomogram

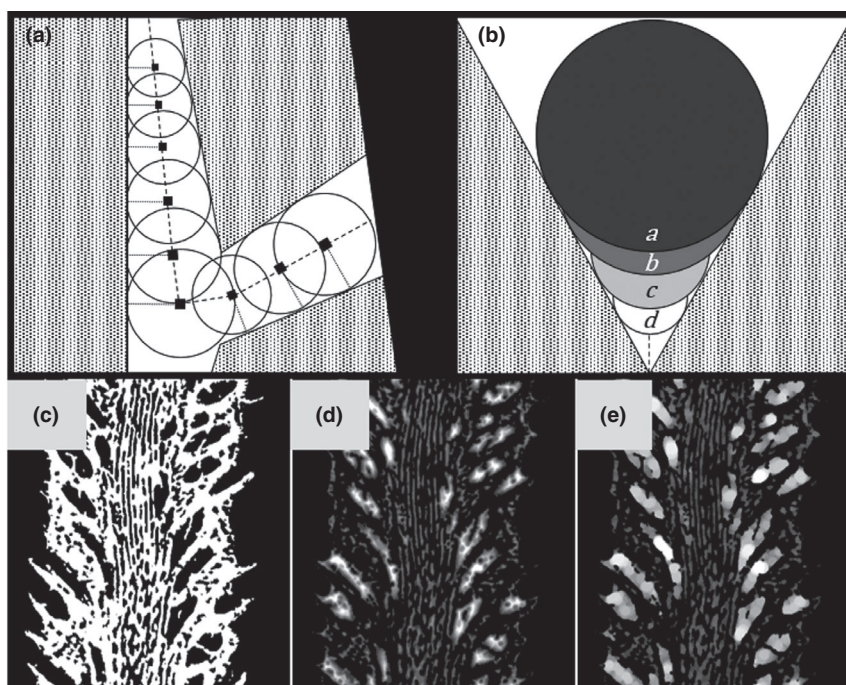


FIGURE 5 Maximal Inscribed Sphere method. (a) Conceptual diagram illustrating the revaluing of voxels based on the radius of the maximum inscribed sphere size that can be theoretically constructed around them. (b) A size-dependent hierarchy is used to resolve conflict where voxels can take multiple values. (c) A 2D y-slice of *Acropora aspera* showing merged solid and intermediate phases. (d) After applying a Euclidean distance transformation. (e) The final output, showing transformation according to the maximum inscribed sphere (MIS) method

quantifying the volumetric size of individual pores. Here we applied a common technique from the geosciences, particle size distribution (PSD) analysis (Lawrence & Jiang, 2017). Even in the geosciences, μ CT is traditionally not the most common technology used in PSD (du Plessis, Sperling, Beerlink, du Preez, & le Roux, 2018; Lawrence & Jiang, 2017), but recent improvements in this technology have allowed researchers to generate and measure true 3D images (du Plessis et al., 2018), overcoming the biases and limitations inherent to inferred 3D or 2D data (Van Dalen & Koster, 2012).

Working upon the transformed tomogram, our steps for applying PSD to biogenic carbonates were as follows:

1. Applied a watershed transformation (Schlüter, Sheppard, Brown, & Wildenschild, 2014; Sheppard, Sok, Averdunk, Robins, & Ghous, 2006; Figure 1, Step 9; Figure 4d). This clusters voxels based on the similarity of their Euclidean distance values and the strength of local gradients between distance values of neighbouring voxels, *sensu* sobel gradients.
2. These watershed clusters were then merged based on the strength of their separation to prevent over-partitioning (Sheppard, Sok, et al., 2006; Figure 4e). This is based on two parameters: the constriction and length-width ratios of the separator. Setting these ratios is best achieved through multiple iterations with a focus on how it affects the presence/absence of small intrusions into the void space. For example, a thin intrusion might lead to two separate clusters after the watershed transformation when in fact, they are part of the sample pore.
3. Once completed, data are generated on the number of pores and their size–frequency distribution. Using the methods of Ohser and Mücklich (2000), we also calculated pore-wise surface area to volume ratio as a proxy for pore shape. Finally, by normalizing the number of pores to the total volume of the sample, we calculated pore density (i.e. no. of pores/mm³).

2.1.9 | Maximal inscribed sphere analysis (Step 10, Figures 1 and 5)

We described the pore geometry of complex perforate corals through the maximum inscribed sphere technique (Fanwua, Chunguang, Haiming, & Juan, 2011; Sheppard, Arns, et al., 2006; Sheppard, Sok, et al., 2006). This approach assigns a value to each pore voxel based on the radius of the largest possible theoretical sphere that can be formed around it while still fully contained within the pore space (Figure 5a). In isolation, the greatest sphere size for each voxel has a radius equal to its Euclidean distance value (Figure 5a). Each voxel within this ‘inscribed’ sphere is labelled with this value. When applied to all the voxels in our sample simultaneously, this produces a series of overlapping spheres. The problem then is that voxels can take multiple values; either that of the sphere formed about them, or of the spheres formed by neighbouring voxels that encompass them. This is resolved by a size-dependent hierarchy, wherein voxels that exist in an overlap take the value of the larger sphere (Figure 5b), hence the term ‘maximum inscribed sphere’ (MIS). It allows us to describe both maximum and minimum geometric features of pore space. The output is in the form of a relative proportion of all voxels that are in each sphere size class, expressed as binned radii measured in voxels (Figure 8).

2.2 | Statistical analyses

All statistical analyses were conducted using R 3.6.0 (R Core Team, 2019). The statistical tests and packages used were based upon the formats and distributions of the data output by the above analyses (Table 3; Table S1). Almost certainly the data generated will vary by taxa, species, resolution and possibly by the

TABLE 3 Data types and descriptions, as well as the statistical model used to analyse them in this study

Response variable	Species	Data type	Description	Statistical model
Macroporosity	<i>Pocillopora damicornis</i> and <i>Acropora aspera</i>	Percentage/proportion	% of whole tomogram that is in the ‘air’ phase	Beta regression
Whole-sample S.A.: volume ratio	<i>P. damicornis</i> and <i>A. aspera</i>	Numeric	Internal surface area of the air–solid phase boundaries	Ordinary least squares regression
Total microporosity	<i>P. damicornis</i> and <i>A. aspera</i>	Percentage/proportion	Percentage of sample that is air within the microporosity phase	Beta regression
Microporous distribution	<i>P. damicornis</i> and <i>A. aspera</i>	Percentage/Proportion	Distribution of voxels in microporosity phase across 100 bins (0.5, 1.5, 2.5, ... 99.5)	Beta regression
Pore size distribution	<i>P. damicornis</i>	Numeric	Pore size–frequency data. Heavily right skewed non-negative with narrow peak and large right tail	Mixed effects gamma regression
Pore S.A.: volume ratio	<i>P. damicornis</i>	Numeric	Surface area to volume ratio for individual pores. Heavily right skewed, non-negative data with a narrow peak and large right tail	Mixed effects gamma regression
Pore density	<i>P. damicornis</i>	Numeric	The number of pores per sample divided by total sample volume	Ordinary least squares regression
Maximal inscribed sphere	<i>A. aspera</i>	Percentage/proportion	Size–frequency distribution of imaginary spheres used to fill the ‘air’ phase in the tomogram	Beta regression

number of biological replicates. The statistics applied here are not definitive approaches for analysing μ CT data but can help guide users.

2.2.1 | Beta regression

This type of regression is designed to examine data in the form of relative proportions (or percentages), bounded between 0 and 1 (0% and 100%; Table S5; Cribari-Neto & Zeileis, 2009; Ferrari & Cribari-Neto, 2004). It was the most common type of regression used here (Table 3). To perform this, we used the *betareg* package (Cribari-Neto & Zeileis, 2009). This model cannot take zero values which were present in low numbers in our microporosity phase and MIS data. Therefore we applied the following transformation recommended by Cribari-Neto and Zeileis (2009), where n is the number of samples in your analysis:

$$y = \frac{(n - 1) + 0.5}{n}.$$

2.2.2 | Regression with gamma distribution

The PSD and pore 'shape' data were non-negative and extremely skewed, suggesting that a gamma distribution would be the optimum choice for modelling these data (Table 3; Table S5; Figures S1 and S2). We used the *fitdistrplus* (Delignette-Muller & Dutang, 2015) and *DescTools* (Signorell, 2016) to compare the goodness-of-fit for gamma, Weibull and log-normal models (Figure S2). In particular, the Weibull distribution was a good candidate given its historical application to PSD data (Vesilind, 1980). However, here we judged Gamma regression to be a similar, if not superior, fit to the data and easier to interpret. We use the shape (α) and rate (β) parameters to describe distributions. α relates to the skewness of the data (skewness = $2/\sqrt{\alpha}$) while β describes the 'rate of decay', essentially the gradient of the slope between the peak and the tail. Low values of α correspond to high skewness; high values of β indicate a narrow peak. A total of 174,679 pores were analysed for size and shape. To avoid pseudoreplication and account for the unequal sample-wise contribution to model variance, we included sample ID as a random effect.

2.2.3 | Correlating with hardness

To elucidate the relationship between these metrics and mechanical properties, we used the data on sample hardness ($n = 5$ per species per treatment) from TP₁, as measured by nanoindentation in Leggat et al. (2019). Total sample hardness is represented on a scale between 0 and 100 and is an average of three locations in the skeleton: outer surface, inner skeleton and internal pore surface. We used multiple imputation by chained equations (Buuren

& Groothuis-Oudshoorn, 2010) to account for the unequal sample sizes, generating 20 imputed datasets for each component of hardness. We then ran beta regression models on each dataset and pooled results. For *P. damicornis*, we included macroporosity, microporosity, median pore size and pore density as covariates. Pore shape was excluded due to its high variance inflation factor (>10) and strong correlation with median pore size ($r^2 = 0.92$). For *A. aspera*, we included the sphere size class mode and median as well as macroporosity and microporosity.

3 | RESULTS

Here we provide the results of our case study, in which we applied this μ CT workflow to two scleractinian corals, *P. damicornis* and *A. aspera*. We compare the analysis results of heat-stress impacted, eroded corals to healthy, control corals. We used imperforate and perforate coral growth forms to demonstrate the variations in applying and interpreting μ CT analysis depending on exoskeletal structure and also exposure to microbial bioeroders. Outliers within each analysis were identified by their Cook's distance relative to a cut-off of $4/n$ (0.222; Table S5; Cook, 1977) and the model outcomes with and without these outliers are presented.

3.1 | Imperforate coral, *P. damicornis*

3.1.1 | Microporous phase distribution

At time point 0 (TP₀), only the 83.5% microporous bin had significantly higher relative proportion for heat-exposed corals compared to controls (Figure 6a; $z = -2.055$, $p = 0.040$). In contrast, control and treatment groups had markedly different distributions at time point 1 (TP₁; Figure 6b). Voxels between 71.5% and 83.5% microporous in the treatment group were proportionally greater compared to the control ($p < 0.05$; Table S2). Concurrently, there was a significant ($p < 0.05$) decrease in the relative proportion of voxels in the range 32.5%–18.5% microporous for the heat-exposed corals compared to control corals.

3.1.2 | Total surface area to volume ratio

Graphically, it appeared that the ordinary least squares (OLS) model violated the assumption of homoscedasticity with respect to time point. This is likely due to different sample sizes for TP₀ and TP₁ ($n = 6$ vs. $n = 12$). We applied a Breusch–Pagan test (Breusch & Pagan, 1979) for heteroscedasticity which was non-significant ($\chi^2 = 1.183$; $p = 0.277$). At neither TP₀ nor TP₁ were the two groups significantly different (TP₀: $t = 2.097$, $p = 0.055$; TP₁: $t = -0.401$, $p = 0.695$). However excluding an outlier in the control group at TP₁ (Cook's distance = 0.375; surface area to

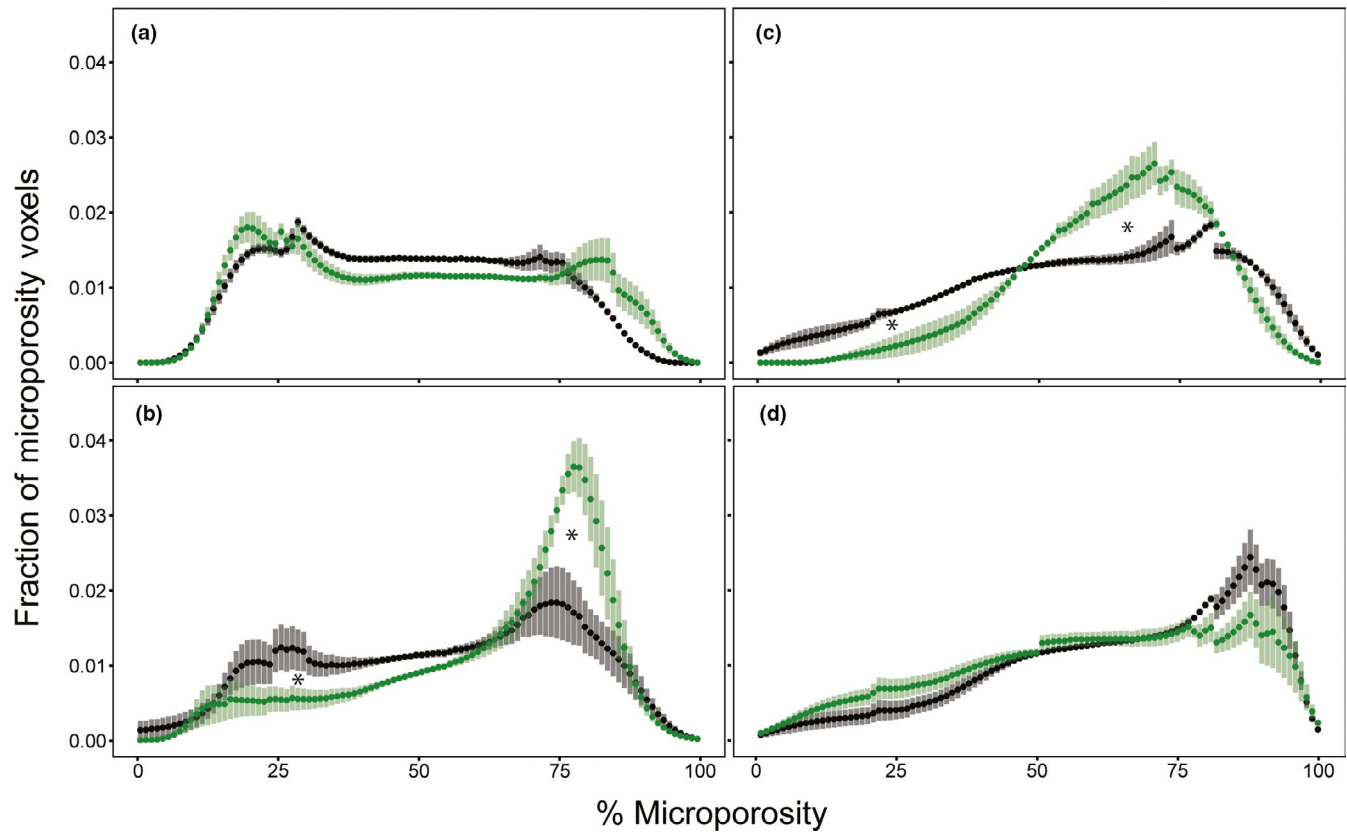


FIGURE 6 The microporosity phase distributions for *Pocillopora damicornis* (a and b) and *Acropora aspera* (c and d), at TP₀ (a and c) and TP₁ (b and d). Green curves represent treatment/eroded corals; black curves are controls. In c and b, *denotes a significant ($p < 0.05$) divergence in distribution. Bin-wise values and contrasts are in Tables S2 and S3

volume ratio = 3.252) affected these results: the model fit improved (Adjusted R^2 increased from 0.544 to 0.767) and control corals had a significantly higher ratio compared to heat-exposed corals at TP₀ (3.665 vs. 2.552; $t = 2.965$, $p < 0.05$) but not at TP₁ (1.302 vs. 1.777; $t = -1.708$, $p = 0.111$).

3.1.3 | Pore density

A potential deviation from normality arose due to two outliers, both from TP₁: one in the treatment group (Cook's distance = 0.303; density = 10.300 pores/mm³) and the other in the control group (Cook's distance = 0.285; density = 8.595 pores/mm³). When included in the OLS model, there were no significant differences between control and heat exposed at either time point (Figure 7a; TP₀: $t = 1.245$, $p = 0.234$; TP₁: $t = -1.099$, $p = 0.291$). When excluding the outliers, model fit improved (Adjusted R^2 increased from 0.237 to 0.807) and the results of post hoc analyses changed significantly (Figure 7a,b). The mean pore densities for both groups at TP₁ decreased from 3.322 to 2.267 pores/mm³ in the control group and 4.858 to 3.769 pores/mm³ in the treatment group. Post hoc contrasts highlighted significant differences at both time points (Figure 7b; TP₀: $t = 2.877$, $p < 0.05$; TP₁: $t = -2.267$, $p < 0.05$).

3.1.4 | Pore size–frequency distribution

The number of pores per sample ranged from 3,027 to 20,890. The size–frequency distribution curve of these was extremely right skewed. In all cases, the shape parameter α was < 0.3 (Figure 7c,d) which means the curve was effectively asymptotic to the y-axis. At TP₀, there was no significant difference ($z = -0.463$; $p = 0.644$) in mean pore size between control ($0.029 \pm 2.522e^{-6}$ mm³) and heat-exposed corals ($0.030 \pm 2.012e^{-6}$ mm³). They also had similar values for rate parameter β (C: 8.247 vs. H: 7.980). In contrast, the mean pore size for the heat impacted group at TP₁ ($0.026 \pm 1.211e^{-6}$ mm³) was significantly smaller ($z = 2.075$, $p = 0.038$) compared to control corals ($0.036 \pm 1.448e^{-6}$ mm³; Figure 7c,d). Additionally, β was about 50% greater in the treatment group (C: 6.438 vs. H: 9.748).

3.1.5 | Pore-wise surface area to volume ratio

Graphically, it appeared that high volume pores in TP₁ treatment corals had a lower ratio compared to controls and better approximated the ratio of a sphere (Figure 7e,f). However, the mixed effects gamma regression revealed this apparent change to be non-significant.

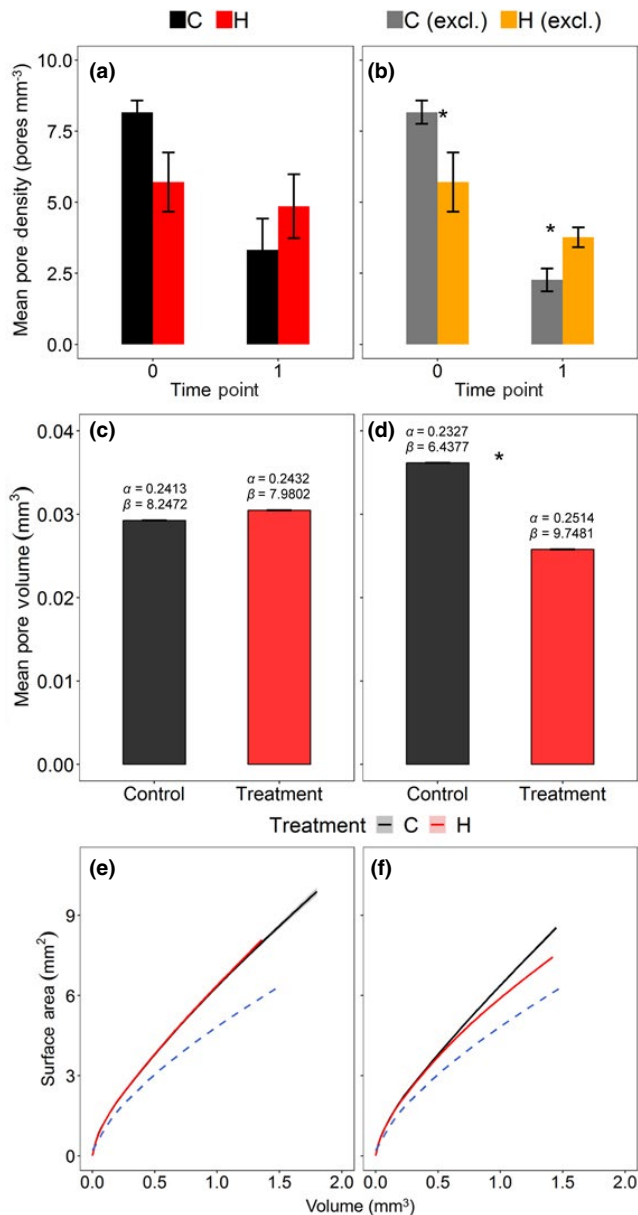


FIGURE 7 Results from the particle size distribution analysis (black represents the control group in all cases; red/orange represents the treatment group). In all cases, *denotes a significant difference ($p < 0.05$) between treatment groups. (a and b) Changes in mean pore density (pores/mm³), including (a) or excluding (b) two outlier (one in each treatment group). (c and d) Mean volumes of individual pores in control and treatment groups at TP₀ (c) and TP₁ (d). α and β refer to the shape and rate parameters, respectively, of the gamma distributions for each group. Low values of α correspond to highly skewed data; high values of β correspond to a steep slope between the distribution peak and tail. (e and f) Pore-wise surface area to volume relationships for control and treatment groups at time points zero (e) and one (f). The blue dotted line is the surface area to volume ratio of a sphere

3.1.6 | Hardness versus porosity

Pore density was significantly negatively related to inner skeletal hardness in all of our samples ($z = -3.048$, $p < 0.05$). Outer skeletal

hardness was not significantly related to any of the porosity metrics used here. Finally, the hardness of the inner surface of pores was negatively correlated with sample microporosity ($z = -3.516$, $p < 0.05$) with an estimated 3.8% decrease in hardness per % increase in microporosity.

3.2 | *Acropora aspera*

3.2.1 | Microporous phase distribution

At TP₀, there were considerable differences between the microporosity phase distributions of control and heat-exposed corals (Figure 6c). Forty-four of the 100 microporosity bins were significantly different between the two groups (Table S3). Voxels that were 93.5%–89.5% or 37.5%–21.5% were proportionally more common in control compared to heat-exposed corals ($p < 0.05$). At TP₁, these differences were no longer evident (Figure 6d). Voxels between 94.5% and 79.5% were of greater relative proportion in control compared to heat-exposed corals ($p < 0.05$).

3.2.2 | Total surface area to volume ratio

The assumptions of the OLS model were confirmed graphically and results indicated that there was no significant difference in surface area to volume ratio at TP₀ ($t = 0.312$, $p = 0.7595$). But at TP₁, the surface area to volume ratio in treatment corals was almost double than that in control corals (12.8 vs. 6.62; $t = -2.284$, $p = 0.039$).

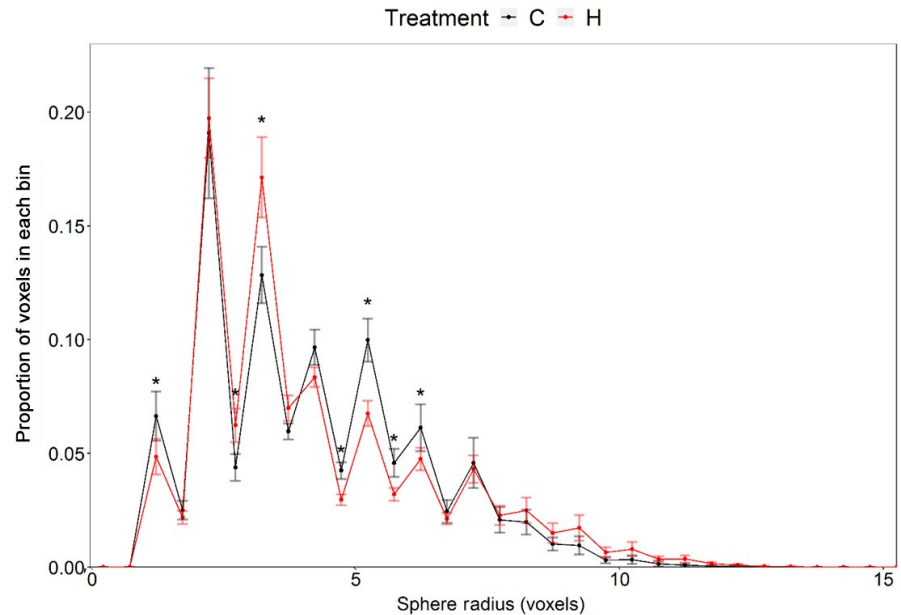
3.2.3 | MIS distribution

The distribution of sphere radii was, as with PSD, right skewed though to a lesser extent (Figure 8). At TP₀, there were no significant differences in the relative proportions of each bin between heat exposed and control (Table S4; $p > 0.05$). In contrast, seven of the bins were significantly different at TP₁, following microbial bioerosion ($p < 0.05$; Leggat et al., 2019). The relative proportions of spheres with radii 1.25 and 4.75–6.25 voxels decreased in treatment groups while the relative proportions in bins 2.75 and 3.25 increased. This latter bin in particular accounted for 17.13% of all air voxels compared to 12.84% to control corals (Table S4).

3.2.4 | Hardness versus porosity

We found no significant correlations between measures of skeletal hardness, porosity and pore space morphology, likely reflecting the lack of significant changes in hardness for this species (Leggat et al., 2019).

FIGURE 8 Results from maximum inscribed sphere (MIS) analysis. Shown for TP₁ only as there were no significant differences at TP₀. It is the distribution of voxels across different sphere radii, normalised to a relative proportion for each size class. *Denotes a statistically significant ($p < 0.05$) difference between groups in a particular size class



4 | DISCUSSION

4.1 | Interpretation, *P. damicornis*

Using a single density threshold to distinguish between air and solid overcomes artefacts such as partial voxel filling but invariably ignores information contained in the tomogram by partitioning and assigning voxels with variable density to one phase or the other (Roche et al., 2010; Scherf & Tilgner, 2009). By using three-phase segmentation, we make an attempt to quantify this information by considering this range of densities as a distinct phase we call microporosity. In this case study, this is particularly useful as we are studying biological behaviour occurring below scan resolution. The results of *P. damicornis* highlight this. The macroporosity of heat-treated corals showed no significant increase in an 8-week period while the microporosity of our samples doubled. Interestingly, this overall increase was accompanied by a shift in the relative distribution of voxels towards a higher voxel-wise percentage of microporosity (Figure 6). We find evidence of this crossover when we use PSD analysis. The decrease in mean pore size (Figure 7c,d) in eroded samples is counterintuitive to that we might expect but our working hypothesis is that it can be explained by an increased number of small pores that were previously too small to appear at our resolution.

There are no significant changes in pore shape despite apparent shift towards a relationship characteristic of a sphere. As this change seemed most evident in larger pores, we re-ran the analysis on pores $\geq 0.5 \text{ mm}^3$ but this did not affect the results. It is possible that pores undergo alternating roughening and smoothing in relation to our scan resolution. Subtle features first eroded, leading to an apparent smoothing of pore surfaces; this may also explain the non-significant difference in whole sample surface area to volume ratio at TP₁ compared to TP₀, in that model which excluded outliers. The 'smoother' surface might then be subsequently roughened;

this is supported by the increase in surface area to volume ratio in *A. aspera* whose macroporosity increased more than that of *P. damicornis*. Different measures of pore shape should be explored in the future as this is a known determinant of the mechanical properties, including hardness, of manufactured materials (Speirs, Humbeeck, Schrooten, Luyten, & Kruth, 2013; Torres-Sanchez, McLaughlin, & Bonallo, 2018).

We found that the hardness of internal pore surfaces was significantly correlated with microporosity but not macroporosity, highlighting the potential for this measure to predict changes in exoskeletal mechanical properties where macroporosity cannot. This is consistent with the findings of both geological (Torres-Sanchez et al., 2018) and medical (Wu, Adeeb, & Doschak, 2015; Zhang, Fan, Dunne, & Li, 2018) studies. In nanoporous materials, Esqué-de Los Ojos, Pellicer, and Sort (2016) show that for a fixed degree of porosity, material deformation by nanoindentation was greater when the material was composed of many small pores than those with fewer big pores. The inner pore surface is effectively the reaction substrate for CaCO_3 dissolution given that microbial bioerosion is generally accepted to occur due to decreases in pore water pH resulting from microbial respiration (Garcia-Pichel, 2006; Tribollet, 2008). Further we show that increased pore density, concurrent with decreased mean pore size, correlated with decreased hardness inside the coral exoskeletons. These results suggest that pore density and microporosity could be useful avenues for better predicting a loss in skeletal hardness compared to macroporosity.

4.2 | Interpretation, *A. aspera*

In addition to the increase in macroporosity reported by Leggat et al. (2019), we found a significant change in sample microporosity in our extended analysis. Unexpectedly, bioeroded corals had lower

microporosity. One possible explanation is that the increase in microporosity, which presumably precedes increased macroporosity in *P. damicornis*, happened earlier in the experimental period in *A. aspera*. We are possibly seeing a later stage of the same process, hence the flattening of the distribution for *A. aspera* at TP₁ (Figure 6d). This has interesting implications for combining micro- and macroporosity to measure fine-scale rates of carbonate dissolution in different species/organisms.

The overall decrease in microporosity also indicates that voxels in this phase were not 'replaced' by the erosion of solid carbonates (i.e. the emergence of a new peak at the solid-intermediate threshold). We suggest this is due to a nonlinear relationship between the rates of microbioerosion of solid and microporous carbonates. Microbioerosion is a chemical process driven by the acidification of the microbes' external environment (Tribollet & Golubic, 2011). Comparing solid and microporous carbonates, the latter has a greater surface area for carbonate dissolution, suggesting that the rate of dissolution increases exponentially with microporosity. But this is potentially limited to very small pore spaces due to higher water volumes in larger pores, given that we did not see an increase in macroporosity in *P. damicornis* despite higher microporosity.

4.3 | Limitations

A potential confounding factor is the presence of a scaffolding proteinaceous organic matrix (OM) in calcified exoskeletons (Weiner, Traub, & Lowenstam, 1983). Soaking in bleach is intended to remove OM from the skeleton, but the success of this depends on how well the bleach saturates the skeleton. For the same reason that buoyant weight and TD-NMR underestimate porosity (Table 3), it is likely that some intra-crystalline OM remains within the skeleton. This will attenuate low energy X-rays and so may appear as very low-density areas of skeleton (i.e. high microporosity). Fortunately, OM tends to constitute <1% of the total skeletal weight (Caroselli et al., 2011). Nonetheless, it remains a possible source of variation between organisms and/or species.

We find that the microporous phase distribution does not precisely match the shape of the original intensity histogram for the same sample (Figure 3). Specifically, in the original intensity histogram we see the curve rising towards the air/solid peaks while the microporosity distribution falls to zero. This is due to the 'uncertain' areas assigned by the CAC algorithm. Microporosity analysis ignores uncertain voxels that were assigned as solid or air by the CAC algorithm and only displays those assigned as 'intermediate'. As the greyscale intensity approaches either peak, the algorithm is more likely to assign an uncertain voxel as air or solid. Thus the curve approaches zero at the tail ends of the microporosity distribution. In some cases, the location of the T2 and T3 thresholds from the segmentation is visible in the microporosity distribution as sudden jumps in the curve (Figure 3b); however, in Figure 6 they are less clear due to averaging across samples. Given that these areas were

previously uncertain, we consider the bin-wise distribution here to retain some of that uncertainty.

4.4 | Further applications and future directions

The biomechanical strength of carbonate structures built by marine calcifiers underpins the stability of the physical habitats these organisms build (Byrne & Fitzer, 2019), and directly affects population scale dynamics such as survivorship and productivity (Davidson et al., 2018; Orr et al., 2005). It is therefore vital that we are able to precisely quantify how environmental change in the Anthropocene is affecting the strength and decay rate of calcified structures. μ CT scanning is a powerful tool that has so far been used to advance our understanding of the micromorphology of calcified exoskeletons, but its full potential in ecological research remains untapped (Gutiérrez et al., 2018). It has been employed for very high-resolution (i.e. single microns) studies of a range of calcifying taxa including pteropods, gastropods, rhodophytes, bivalves and annelids, quantifying density, shell thickness and volume in the context of biomechanical change (Chatzinikolaou, Grigoriou, Keklikoglou, Faulwetter, & Papageorgiou, 2016; Li et al., 2014; Meng et al., 2018; Oakes & Sessa, 2019). But a lack of advanced analytical techniques to complement this means that certain features of carbonate structures, such as pore space morphology, are still predominantly measured using 2D microscopy such as SEM (Byrne & Fitzer, 2019; Li et al., 2014; Meng et al., 2018).

The results of published studies which have used μ CT scanning strongly suggest that the techniques presented in this study are generally applicable to studying the internal structure of other marine calcifiers. For example, a 2D cross-section of the short-spined sea urchin *Heliocidaris erythrogramma* from a 3D tomogram displays a highly connected and complex pore space (Johnson et al., 2020), similar to that of *Porites cylindrica* (Video S1), which would make it amenable to MIS analysis. The SEM images of a Portuguese oyster shell *Magallana angulata* presented by Meng et al. (2018) indicate that the combination of pores and fissures in this case would require jointly applying PSD and MIS analysis on different regions of interest. But in both instances, SEM was used to describe the widening of more spaces. Examining the results of Li et al., 2014 who employed μ CT and SEM to study the effect of ocean acidification on the mechanical and micromorphological change in calcareous tubes of serpulid worms, we also see an opportunity to use microporosity analysis to indirectly examine skeletal ultrastructure. Their micrographs highlight an increase in the number and size of submicron pores as a result of short-term exposure to CO₂-enriched water. There was a concomitant decrease in the hardness of the affected layers as measured by nanoindentation, similar to the weakening of the internal pore surface in our study. This suggests that microporosity analysis might be a viable alternative to ultra-high resolution SEM, at least in identifying losses in biomechanical strength. Further developing these methods signifies that μ CT could replace SEM as the foremost method for studying biomechanical change, which would reduce the cost

of analysis compared to using both methods to study 2D and 3D changes respectively.

The methods described here could also be used to study the nature of biotic interactions between ubiquitous endolithic microbes and their calcified hosts. These microbes play an important role in primary production and biogeochemical cycling (Mason et al., 2009; Tribollet, Langdon, Golubic, & Atkinson, 2006), including the exchange of nitrogen and carbon with their host (Barille et al., 2017; Pernice et al., 2019). Different skeletomorphs will impose restrictions on the biomass and composition of this community, through limitations in physical space and/or hydrodynamic diffusion of pore water; this variation can be modelled using these μ CT analyses. For example, the permeability of abiogenic carbonates has been successfully quantified in carbon sequestration research and involves the application of microporosity and pore size analysis (Shah, Yang, Crawshaw, Gharbi, & Boek, 2013; Sheppard, Arns, et al., 2006). Understanding the relationship between skeletal structure and the endolithic microbiome could improve our knowledge of the ecological consequences of evolutionary divergence in calcification patterns.

5 | CONCLUSIONS

Our ever-improving ability to effectively measure change in marine ecosystems is an important driver of our growing understanding of the natural world. μ CT is a tool that is growing in its power and popularity in the medical and geological sciences and has the potential to provide new insights through its application to new areas of research and new ecosystems. Here we have described in detail three analytical techniques that are common to the geosciences and adapted them for the first time to studying the impact of climate change upon marine calcifiers. The successful transfer of these methods across disciplines suggests that these tools, as well as others, can be applied to the study of marine calcifiers in general due to commonalities in the features of all carbonate structures (Prodanović et al., 2015; Shah et al., 2013). By relating these measures to the mechanical properties of our samples, we highlight their potential for predicting change in the functional ecology of marine calcifiers.

These tools can advance our understanding of how the physical structures built by marine calcifiers are changing in the Anthropocene as oceans become warmer and more acidic. Combined with further investigations that link the morphological and mechanical properties of these structures, we can begin to model shifts in their ecological function and how this will affect the ecosystems they reside. Interdisciplinary approaches such as these allow ecological researchers to benefit from the technological advancement in a seemingly unrelated field.

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AUTHORS' CONTRIBUTIONS

A.J.F., T.D.A. and W.L. conceived the study; L.B. and M.T. optimized and performed the μ CT scanning; A.J.F. and L.K. designed and implemented the image analysis workflow; A.J.F. performed the statistical analyses; A.J.F., L.K., T.D.A. and W.L. wrote and finalized the manuscript.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/2041-210X.13439>.

DATA AVAILABILITY STATEMENT

Data and associated R code (Fordyce, 2020) are available on Github, and can be accessed through Zenodo <https://zenodo.org/record/3899501#.XvFD6WgzBdC>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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