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Assessing pteropod shell dissolution to advance ocean monitoring techniques: a methods comparison of SEM, CT, and light microscopy

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Pteropods are marine planktonic snails that are used as bioindicators of ocean acidification due to their thin, aragonitic shells, and ubiquity throughout the world's oceans; their responses include decreased size, reduced shell thickness, and increased shell dissolution. Shell dissolution has been measured with a variety of metrics involving light microscopy, scanning electron microscopy (SEM), and computed tomography (CT). While CT and SEM metrics offer high resolution imaging, these analyses are cost- and time-intensive relative to light microscopy analysis. This research compares light microscopy, CT, and SEM shell dissolution metrics across three pteropod species: *Limacina helicina*, *Limacina retroversa*, and *Heliconoides inflatus*. Sourced from multiple localities, these specimens lived in tropical to subpolar environments and were exposed to varying aragonite saturations states due to oceanographic differences in these environments. Specimens were evaluated with light microscopy for the *Limacina* Dissolution Index (LDX), with SEM for percent of pristine shell coverage and maximum dissolution type, and with CT for whole-shell thickness. LDX and the percentage of pristine shell determined via SEM were highly correlated in all three species' datasets. For *L. retroversa*, LDX was also significantly correlated to SEM maximum dissolution type. Although the genera *Heliconoides* and *Limacina* have different shell microstructures, the relationship between LDX and SEM dissolution did not vary by species. The CT metric for shell thickness was not significantly correlated to any other dissolution metrics for any species. However, severely dissolved areas apparent in SEM were visually discernible in CT thickness heatmaps. While CT may not detect minor shell dissolution, previous studies have used CT to detect reduced calcification in response to ocean acidification. SEM is ideal for detecting the onset of dissolution, but SEMing large numbers of specimens may not be practical due to monetary and time constraints. LDX, on the other hand, is a fast and cost-effective metric that is strongly correlated with

SEM metrics, regardless of the oceanographic conditions that those species experienced. These results suggest that an efficient ocean acidification monitoring strategy is to evaluate all pteropod specimens via LDX and to then SEM a subset of those specimens.

KEYWORDS

pteropod, limacina, ocean acidification, shell dissolution, climate change, scanning electron microscopy, light microscopy, micro-computed tomography

1 Introduction

Human activities including fossil fuel burning, deforestation, and cement production have emitted 560 billion tons of CO₂ to the atmosphere since the onset of the Industrial Revolution (e.g., Doney et al., 2009a; Le Quéré et al., 2009). The world's oceans are responsible for absorbing one 20–40% of all anthropogenic CO₂ emissions (Ciais et al., 2013; Gruber et al., 2019; Sabine et al., 2004), making it the largest sink of anthropogenic carbon. This uptake is causing ocean acidification: a decrease in pH which causes a decline in calcium carbonate saturation (Ω ; Whitfield, 1975; Guinotte and Fabry, 2008; Doney et al., 2009b; Orr, 2011; Zeebe, 2012). Particularly at risk are calcifying organisms, as it will be increasingly difficult and energy-demanding to precipitate and maintain calcium carbonate shells and skeletons as saturation state decreases (Doney et al., 2009a; Feely et al., 2004), though protection against dissolution has been documented from the body parts of living organisms, such as the periostracum of mollusks (Peck et al., 2016a; Miller et al., 2023). Even when waters are saturated or supersaturated, many (but not all) calcifying taxa are observed to calcify less as saturation state decreases (e.g., Feely et al., 2004; Ries et al., 2009; Mekkes et al., 2021b; Bednaršek et al., 2012b). Both polymorphs of calcium carbonate, calcite and aragonite, will be subject to changing saturation state in future oceans, but aragonite is 50% more soluble than calcite (Mucci, 1983; Orr, 2011), making organisms with aragonitic shells more vulnerable to the effects of acidification (Fabry, 2008; Orr et al., 2005).

'Bioindicators' are species that are used to assess local environmental conditions and to monitor ocean acidification and its impacts on marine organisms (e.g., Howes et al., 2017; Bednaršek et al., 2017b; Marshall et al., 2019; Wall-Palmer et al., 2021; Gaylord et al., 2018; Shinn, 2008; Widdicombe et al., 2023; Maas et al., 2023). Pteropods, especially those within the superfamily Limacinoidea (Figure 1), are used as bioindicators for ocean acidification (e.g., Bednaršek et al., 2014; 2017b; Mekkes et al., 2021a; Oakes and Sessa, 2020; Orr et al., 2005) because they secrete thin (10–15 μ m) aragonitic shells, are ubiquitous throughout the world's oceans, and have high abundance in polar regions where acidification is predicted to have the most severe impacts. These marine pelagic gastropods contribute ~500 Tg to the carbon cycle annually and

occupy a key node in marine food webs, consuming phytoplankton and small zooplankton while being consumed by krill, zooplankton, and economically important fishes (Armstrong et al., 2005; Bednaršek et al., 2012b; Gilmer and Harbison, 1986; Hunt et al., 2008). As diurnal migrators, pteropods are exposed to a range of water chemistry conditions at various ocean depths (Fabry and Deuser, 1992; Juranek et al., 2003; Keul et al., 2017; Oakes et al., 2021).

Pteropods and their responses to acidification have been extensively studied over the past two decades. These responses include changes in shell size (Lischka et al., 2011), shell surface dissolution (Lischka and Riebesell, 2012; Busch et al., 2014; Niemi et al., 2021), shell thickness (Mekkes et al., 2021a; Roger et al., 2012; Howes et al., 2017), metabolic rates (Lischka and Riebesell, 2017; Maas et al., 2018; Moya et al., 2016), respiration (Maas et al., 2018), and gene expression patterns (Moya et al., 2016). Shell responses are the most common metrics for acidification impacts because they can be assessed after organismal death and without preservation of genetic material. These responses are measured in a variety of ways. The most common method of evaluating dissolution in pteropods is a semi-quantitative SEM-based method that grades shell surface dissolution on a scale of 0 (pristine) to 3 (dissolution of the external shell layer and inner layers) and assigns a percent coverage of shell surface to each dissolution "type" (Bednaršek et al., 2012a). This method has been previously used to quantify dissolution at various stages of life (Niemi et al., 2021; Mitchell, 2019) and to characterize pteropod shell microstructure for future application in biomaterials research (Ramos-Silva et al., 2021). The *Limacina* Dissolution Index (LDX) is a light microscopy method that grades shell dissolution on a semi-quantitative scale from pristine (0) to highly dissolved (5) based on shell luster and transparency (Gerhardt et al., 2000; Gerhardt and Henrich, 2001). As described in these publications, at LDX 0, shells are transparent and lustrous. At LDX 1, shells are lustrous but cloudy. Luster is maintained at LDX 2, but shells are completely white and opaque. At LDX 3, shells begin to lose their luster and become totally lustreless by LDX 4 due to the complete loss of the upper shell layer, LDX 5 is characterized by opaque, white, totally lustreless shells, as in LDX 4, but also includes additional shell damage such as perforations. LDX was developed as a dissolution proxy to track aragonite preservation in sediment cores via fossil specimens (Gerhardt et al., 2000). It has also been

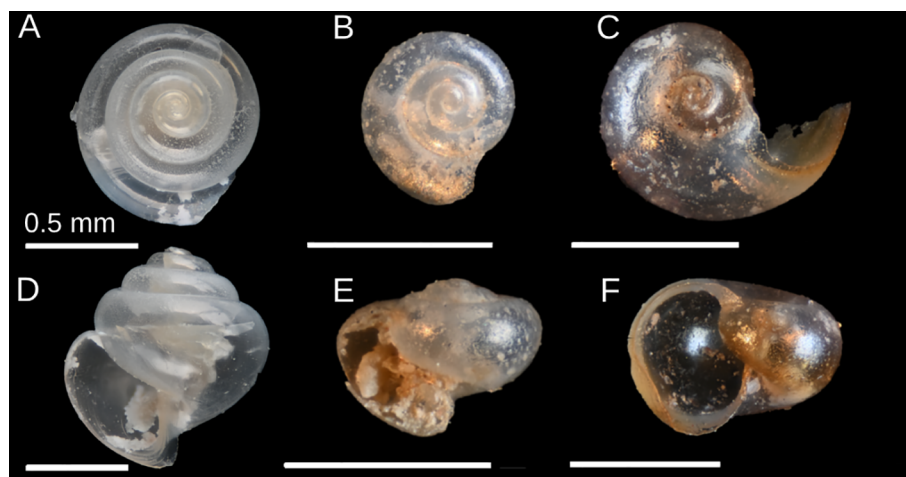


FIGURE 1

Photographs of species used in the study: *Limacina retroversa* (A, D), *Limacina helicina pacifica* (B, E), and *Heliconoides inflatus* (C, F). Shells are oriented apically (A–C) and aperturally (D–F). Scale bars represent 0.5 mm.

used in the fossil record to document past variability in ocean carbonate concentration and supersaturated environments (Wall-Palmer et al., 2013; Hallenberger et al., 2022). Additionally, studies assessing dissolution on modern pteropods have utilized LDX and similar semi-quantitative scales (Lischka et al., 2011; Lischka and Riebesell, 2012; Manno et al., 2012; Maas et al., 2023). Alternatively, micro-computed tomography (micro-CT) imaging produces shell thickness and volume data and is one of the few ways to obtain fully quantitative data on the impact of acidification on pteropod shell thickness. Micro-CT has been used to generate time series of how shell thickness has decreased over time in response to increasingly acidic conditions (e.g., Howes et al., 2017; Mekkes et al., 2021a).

Significant progress has been made in understanding and measuring the diversity of pteropod responses to acidification. However, establishing best practices in utilizing these responses for ocean acidification monitoring is an ongoing process. For example, a systematic comparison of light microscopy, CT, and SEM methods has not been completed. Previous research that has employed more than one of these metrics often compared only two methods (ex., Lischka et al., 2011 compared dissolution in light microscopy and SEM), or used one method only for visualization. Additionally, light microscopy methods like LDX have been largely underutilized in previous research. This lack of comparative information can make deciding which method(s) to implement in a monitoring program challenging. Compared to LDX, SEM and micro-CT methods produce higher resolution data, but they are time-intensive, less accessible with respect to equipment availability, and potentially cost-prohibitive. To access an SEM, researchers must undergo training and either pay \$50–\$110 per hour at an imaging lab or invest in their own SEM, which can cost hundreds of thousands of dollars (Mitchell, 2019). CT work involves either similar fees or limited availability at institutions offering free CT services (see discussion in Oakes et al., 2020). In contrast, LDX

assessment only requires a binocular microscope, a common component of biological and earth science labs that is familiar to researchers of all levels. If LDX can effectively evaluate dissolution, it would be an ideal addition or substitute in ocean acidification monitoring programs; it is fast, essentially free, and immediately implementable within most research labs.

This paper compares light microscopy, CT, and SEM methods for assessing dissolution across the same specimens of three pteropod species that are commonly used in ocean acidification studies. In particular, this research seeks to elucidate the relationships among the different dissolution assessment methods and to determine the comparable effectiveness of light microscopy relative to more time- and cost-intensive techniques. This work will inform acidification monitoring efforts in two primary ways: by comparing the various dissolution methods and by investigating how these methods may be applied across multiple species.

2 Methods

2.1 Species studied

Higher-level pteropod taxonomy is complicated due to the lack of distinguishing shell morphological characteristics across taxa and rate heterogeneity, leading to similar yet conflicting classifications (Klussmann-Kolb and Dinapoli, 2006; Corse et al., 2013). For example, the gastropod classification of Bouchet et al. (2017) lists the order Pteropoda as consisting of three suborders: Euthecosomata (shelled), Gymnosomata (non-shelled), and Psuedothecosomata (variable), while acknowledging that Burrige et al. (2017), using molecular markers, found that Thecosomata (mucus-web feeders; Gilmer, 1974; Lalli and Gilmer, 1989; Conley et al., 2018) and Gymnosomata (active predators; Lalli and Gilmer, 1989; Dadon and

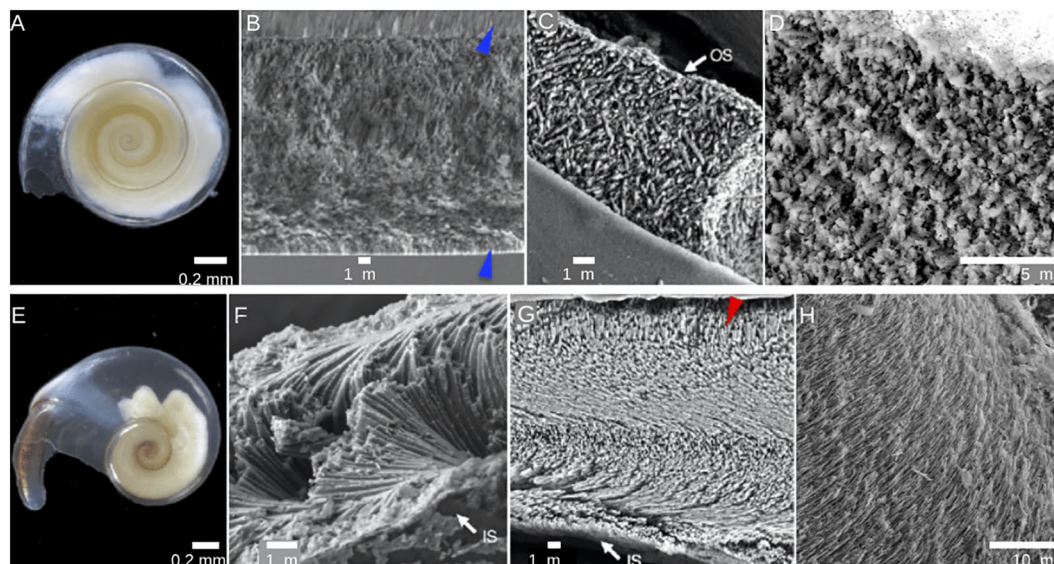


FIGURE 2

Light microscopy and SEM images of *Limacina helicina* (A–D) and *Heliconoides inflatus* (E–H). SEM images (B–D, F–H) demonstrate the differing shell structures between the two genera. Blue arrows indicate the prismatic layer. Images A–C, E, F–G are from Ramos-Silva et al., 2021, and have been adapted into this figure in compliance with its license (<https://creativecommons.org/licenses/by/4.0/>). (D,H) are original to this paper.

Chauvin, 1998) were orders, with the former containing the suborders Euthecosomata and Psuedothecosomata. Most recently, a comprehensive phylogenomic study that included fossil data found support for the traditional taxonomic classification of the order Pteropoda consisting of the suborders Thecosomata and Gymnosomata, in which the former contains the infraorders Euthecosomata and Psuedothecosomata (Peijnenburg et al., 2020). Within the Euthecosomata, the superfamily Limacinoidea was recovered as a monophyletic group containing the genera *Limacina* and *Heliconoides* (Peijnenburg et al., 2020), the genera studied here.

This project focuses on the *Limacina helicina* species complex Phipps, 1774, *Limacina retroversa* J. Fleming, 1823, and *Heliconoides inflatus* d'Orbigny, 1835. The *L. helicina* species complex is composed of two subspecies: *L. helicina* Phipps, 1774 and *L. helicina pacifica* Dall, 1871 (Hunt et al., 2010; Janssen et al., 2019). While many studies do not identify pteropods down to the subspecies level, the *L. helicina* specimens used in this study were taxonomically evaluated in 2019 by a pteropod specialist who identified all specimens as *L. helicina pacifica* (Janssen et al., 2019).

All three species included in this study are well researched with respect to ocean acidification. The *L. helicina* (Figures 1B, E) species complex is one of the most common taxa used in ocean acidification studies; documented negative responses to acidified conditions include reduced shell thickness and increased shell dissolution and degradation for both juvenile and adult stages (Bednaršek et al., 2017a; Lischka et al., 2011; Busch et al., 2014; Mekkes et al., 2021a). *L. retroversa* (Figures 1A, D) responses to ocean acidification include changes in metabolism, calcification, early development survivorship, and the expression of genes related to

biomineralization, metabolism, and neural function (Thabet et al., 2015; Maas et al., 2018; Mekkes et al., 2021b). This species also displays compounding impacts from ocean acidification and nanoplastics in laboratory conditions (Manno et al., 2022). *H. inflatus* (Figures 1C, F) responses to acidification include downregulated genes associated with metabolism and upregulated genes associated with calcification (Moya et al., 2016).

2.2 Shell structure

Like other shelled mollusks, pteropods produce shells using a biomineralization process controlled by the mantle (e.g., Marin et al., 2012). This process produces a series of two to five superposed calcified shell layers (Marin et al., 2012) and an outermost organic layer known as the periostracum, which can provide protection against dissolution (Peck et al., 2016a; Miller et al., 2023). Each calcified shell layer is an organo-mineral biocomposite comprised of aragonite crystals arranged in varying microstructures and a 0.1–5% organic fraction (e.g., Checa, 2018). For *L. helicina* and *L. retroversa*, the crystal structure of the adult shell consists of an outer and inner prismatic layer and an internal crossed lamellar layer (Figures 2A–D; Ramos-Silva et al., 2021). A helicoidal crystal structure may also occur beneath the prismatic layer in the protoconch (the embryonic shell) of *Limacina* species (Glacon et al., 1994). For *H. inflatus*, a simple helicoidal internal layer (Figures 2E–H; Ramos-Silva et al., 2021) is bounded by an inner prismatic layer (Glacon et al., 1994) and an external granular prismatic layer. Externally located crossed lamellar structures may also be present (Ramos-Silva et al., 2021),

but were not observed in this study. Differences in shell structures among the species in this study impact their relative vulnerability to ocean acidification. Cavolinid pteropods and *H. inflatus* have a helical shell microstructure that has been found to be more susceptible to dissolution than the cross-lamellar microstructure of other pteropods, such as *Limacina* (Bé and Gilmer, 1977; Wall-Palmer et al., 2013).

2.3 Specimen selection and handling

This study used 89 specimens from previous research and 56 newly studied specimens. Specimens missing more than approximately $\frac{1}{4}$ whorl were not used. The amount of missing whorl was determined by signs of breakage and remnant shell material where the outer whorl was fused to the inner whorl. Juvenile specimens were also excluded. Only specimens that were stored dry long term were used in this study to avoid post-mortem dissolution caused by storage in ethanol and other preservatives (following recommendations of Oakes et al., 2019a). Specimens were handled and manipulated using fine detail paintbrushes, modeling clay, and SEM tape, and never with tweezers or any metal instruments, to avoid damaging the shell. During shipments, specimens were packaged individually in microscope slide wells with pieces of nitrile gloves surrounding the specimens to reduce collisions with the glass slide cover and well walls.

Thirty *L. helicina pacifica* (hereafter *L. helicina*) specimens and 14 *H. inflatus* specimens were obtained on loan from the Natural History Museum of Los Angeles County (LACM) Malacology Collection. These specimens were collected at multiple localities from 1931–1981 within the California Current; detailed collection data are provided in the [Supplementary Materials](#) ([Supplementary Table S1](#)). Seasonal upwelling of cold subsurface water causes the California Current to be naturally prone to ocean acidification. In combination with biological processes like plankton blooms and sinking of organic matter, this produces trends in pH and aragonite saturation state that vary with depth, distance to shore, and time; pH can range from ~8.1 to ~7.7 due to these factors (Hauri et al., 2013 and references therein; Bednaršek et al., 2014 and references therein; Takeshita et al., 2021). pH was not measured during the cruises that collected the California Current specimens analyzed here. This poses no problem to the current study, since it compares how three dissolution metrics are interrelated, not how pteropods respond to pH or calcium carbonate saturation state.

Twenty-two *H. inflatus* specimens from Oakes and Sessa (2020) study of the Cariaco Basin were also included. The pH conditions of the Cariaco Basin vary from 7.57 to 8.04, depending on depth and latitude (Scranton et al., 2001) and waters remain saturated with respect to aragonite through the year (Oakes and Sessa, 2020). Due to the aragonite supersaturation, nutrient availability during periods of upwelling is the determining factor for shell strength and growth (Oakes and Sessa, 2020). These specimens were collected in 2013

and 2014 via a sediment trap deployed at a depth of 150 m. This trap collected specimens and debris falling in open water into one of 13 cups filled with borate-buffered formalin solution (described in Thunell et al., 2000). Each cup captured a 2-week interval of falling material, and the trap was collected and redeployed every six months as part of the CARIACO (Carbon Retention In A Colored Ocean) time series project (Thunell et al., 2000). The trap contents were washed, split, picked and stored as described in Thunell et al. (2000) and Tedesco and Thunell (2003). Pteropod specimens were picked from the washed and dried faunal samples in 2019 and were subjected to light microscopy and CT scanning as described in Oakes and Sessa (2020) and are reposit in the Academy of Natural Sciences of Philadelphia (ANSP) Malacology Collection ([Supplementary Table S2](#)).

Eight *H. inflatus* and two *L. retroversa* specimens from the Atlantis II cruise and two *L. retroversa* specimens from the H.M.S. Challenger expedition were borrowed from the Foraminifera collection of the Paleobiology Department of the Smithsonian National Museum of Natural History (NMNH). H.M.S. Challenger and Atlantis II specimens were collected using a plankton tow (Fox et al., 2020; Cifelli and McCloy, 1983). Due to the age and unspecified collection location of these specimens, pH conditions and aragonite saturation could not be specified.

Finally, sixty-seven *L. retroversa* specimens from St. Magnus Bay, Shetland Islands, UK were also assessed for this study. This region is rarely undersaturated, with an aragonite saturation ranging from 1.0 - 3.1 (Hartman et al., 2019). It has a surface pH that varies seasonally from 8.09 to 8.19 (Hartman et al., 2019), with the lower pH values generally seen during spring and summer (Bresnan et al., 2016; Findlay et al., 2022). The specimens were collected at 5 of 17 sampling stations on a transect running from the Olne Firth inlet to the open shelf in May through June of 2018 (see detailed description of sites in Dees, 2021). Specimens were collected via vertical plankton tows with a 125 μ m-mesh bongo net (C. Angus, personal communication, May 16, 2018) deployed to maximum depths ranging from 107 to 145 meters (P. Dees, personal communication, June 4, 2020). The specimens were temporarily kept in 70% buffered ethanol during the cruise and shipping phases and were then picked and dried within 6 months of collection for long term storage to reduce any dissolution from the ethanol (i.e., Oakes et al., 2019a). All are reposit in the Academy of Natural Sciences of Philadelphia (ANSP) Invertebrate Paleontology Collection ([Supplementary Table S2](#)).

2.4 Light microscopy dissolution assessment

The LDX method utilizes the six preservation stages from Gerhardt et al. (2000), in which a score of 0 indicates a pristine shell and a score of 5 indicates severe corrosion ([Figure 3](#)). When examining pteropod specimens either through photographs or



FIGURE 3

The *Limacina* Dissolution Index (LDX) from 0 (no dissolution) to 4 (highest dissolution of specimens observed in this study). Specimens on the left are *Heliconoides inflatus*, specimens in the middle column are *Limacina retroversa*, and specimens on the right are *Limacina helicina*. From LDX 0 to 2, specimen shells progress from transparent at LDX 0, to milky, and then to fully opaque and white at LDX 2. From LDX 2 to 4, shells progress from lustrous to no luster and no outer shell layer. Shells are not to scale.

under a microscope, the following characteristics are taken into consideration for scoring: transparency, luster, and, in severely dissolved specimens, the removal of the entire surface layer and deeper layers (Gerhardt et al., 2000). As the specimens move up the LDX scale, transparency and luster decreases while (in more severe cases) exposure of inner shell layers increases.

Light microscopy was employed to photograph specimens using a standard photography rig containing a Nikon Z50 with two PK-13 auto extension tubes attached to a Bausch & Lomb monocular microscope, which produced higher resolution images relative to the Leica built-in camera on the monocular scope. This setup was then connected to a monitor that is operating Nikon Camera Control Pro (version 2.34.1, Nikon Inc., Tokyo, JPN; Camera Control Pro, 2021). Polarizers on the light source were used to minimize overexposure in photos due to the shiny nature of pteropod shells. Individual image slices were photographed manually using the microscope's stage wheel and stacked in Helicon Focus 8 (version 8.2.2, Helicon Soft, Kharkiv, UA; Helicon Focus 8, 2022). LDX was then assessed based off these specimen images (Supplementary Plate 1). To determine whether LDX score is affected by using photographs versus when assessing via light microscope, Koester scored 105 shells both ways.

Taphonomic grading systems like the LDX are widely used across both paleontological and neontological disciplines, and studies

emphasize the need for the training of new operators and of assessing inter-operator error (Kidwell, 2001; Rothfus, 2004). Several studies have found that greater inter-operator error is associated with less experienced operators (Rothfus, 2004; Soficaru et al., 2014; Wilczak et al., 2017; Ziegler and van Huet, 2021), and as such training new operators is essential to ensure reproducible results. Two of the co-authors, Koester and Mercado, had not previously worked with the LDX method. They were trained by first studying the LDX scale and images in Gerhardt and Henrich (2001) and other studies that employed the LDX method (Oakes et al., 2019a; Oakes and Sessa, 2020). They then practiced assigning scores and reviewed their scores with Oakes and Sessa, both of whom are experienced in assigning LDX scores. To assess whether there are differences in scores from different researchers, 142 shells were assessed by 2–4 authors (Supplementary Table S3).

2.5 Micro-computed tomography dissolution assessment

For most California Current specimens, CT scanning was conducted by lab technicians at the CT facilities at the University of Texas (UTCT) using a Zeiss Versa 620 scanner. Scans were run with a voltage of 80kV, a current of 10W, and an acquisition time ranging from 0.5–1.0 seconds. The resulting scan resolution from these parameters ranged from 0.7- to 2.2-micron voxels. The computer software programs Dragonfly (version 2022.1, ORS, Montreal, CAN; Dragonfly, 2022) and VG Studio Max (version 2023.2, Volume Graphics, Heidelberg, GER; VG Studio Max, 2023) were used to digitally remove non-shell materials, such as sediment or other organisms within or adhering to the shell and remnants of the pteropod body, from the resulting scan data and to measure shell width and height and calculate shell thickness. In Dragonfly, the Otsu method was utilized for density thresholding to separate the denser shell material from less-dense organic material and background air so that the shell could be isolated as a Region of Interest (ROI; Otsu, 1979). Some specimen scans required manual adjustments to the Otsu threshold to more accurately differentiate between shell and non-shell material. For the specimens processed with VG Studio Max, sediment and organic material were made into a ROI using the 'Draw' tool and then subsequently removed from the area of analysis (e.g., the shell) using the ROI 'Rendering' tool. With the non-shell ROI removed, a 'surface determination' was performed to select only the shell material for thickness analysis.

All Cariaco and Shetland specimens were CT scanned using the methods described in Oakes et al. (2020) and Oakes and Sessa (2020), at the American Museum of Natural History in New York, New York, or at General Electric Inspection Technologies in Lewistown, PA, with voxel resolution ranging from 1–2 microns. The Cariaco specimen CT data had been previously processed by Oakes and Sessa (2020) using VG Studio MAX. The Shetland CT data were processed using the same procedure described above for the California Current specimens.

For all specimens, shell thickness was determined in VG Studio Max, using the Sphere Method under Wall Thickness Analysis and then exporting the data from the Histogram tab. Bin sizes for histograms were set according to the specific specimen's voxel size. Shell ROIs were converted into a masked version of the original scan and saved as a DICOM file for future reference and analysis. All scans are available for download on Morphosource under the project IDs: 000623860 (California Current Pteropod Scans Project), 00000C908 (Oakes and Sessa Pteropod Variability Project), and 000640972 (Academy of Natural Sciences Invertebrate Paleontology Collection Project).

2.6 Scanning electron microscopy dissolution assessment

SEM imaging was conducted by Koester at the Singh Laboratory at the University of Pennsylvania using a Quanta 600 FEG ESEM (Supplementary Plate 2). Specimens were kept in an unaltered state without sputter coating. Following the commonly employed pteropod dissolution method of Bednaršek et al. (2012a), Koester ranked dissolution from pristine (Type 0) to highly dissolved (Type 3) based on the amount of dissolution of the surface prismatic layer and the underlying shell layer (Figure 4). Dissolution is either reported as the maximum dissolution type observed throughout the entire shell (Bednaršek et al., 2014; Mitchell, 2019) or as a percentage of the surface area of each dissolution type (Bednaršek et al., 2012a; Mekkes et al., 2021a). Both evaluations were included in this study and were assessed when the specimen was oriented apically, as in Figure 3, and percentage of surface area was determined visually, in a manner similar to estimating mineral composition of a petrographic thin section. Chemical baths and plasma etching are still common practice for preparing specimens for SEM analysis. Following the recommendations of Peck et al. (2016a; 2016b), these methods were not employed here because they could remove the shells' inter- and intra-crystalline organic matrix, thereby increasing the appearance of dissolution. Initial SEM scans indicated that the shell surface and any dissolution could be sufficiently observed without subjecting specimens to these methods.

2.7 Specimen measurements

All specimens were measured to provide information on size. Height and width were taken from CT scans using the 'Ruler Tool' in Dragonfly. Height was measured along the shell columella, the axis around which the shell coils as it grows (Supplementary Figure S1). Width was measured perpendicular to the height measurement at the widest point of the specimen on the apertural plane, a plane aligned with the aperture of the shell that bisects the shell and columella (Supplementary Figure S1).

Whorl count is used as a proxy for gastropod specimen age (Kuznik-Kowalska, 2006; Schöne et al., 2007) and thus pteropod

age. Under ambient oceanic conditions, pteropods will increase their entire shell size over time (Mekkes et al., 2021b), subsequently increasing their whorl count. Following the methods of Janssen (2007), whorls are counted when viewing the shell in an apical orientation. The first whorl begins at a visualized straight line that divides the semicircular nucleus of the protoconch (protoconch-1) from subsequent shell growth. Each full whorl is completed when a 360° rotation is made from the visualized line; total whorl count is estimated to the nearest 1/8 whorl when the aperture is reached (Supplementary Figure S1).

2.8 Statistical analysis

Because Oakes and Sessa (2020) found that larger shells of *H. inflatus* specimens from the Cariaco Basin were thicker, they used a simple linear regression model of shell thickness and width (referred to as diameter in their study) to remove the influence of size on thickness (Supplementary Text 1). These normalized thickness values were referred to as 'residual thickness'. Here, Spearman correlation tests between shell thickness and width are used to determine if size influences thickness in the *L. helicina*, *L. retroversa*, and expanded *H. inflatus* datasets (Supplementary Table S4). For any species where a significant correlation was found, the 'residual thickness' of Oakes and Sessa (2020) is used instead of modal thickness.

The Spearman correlation test accounts for potential non-linear relationships between variables and therefore was used to test for significant relationships among all dissolution metrics (Supplementary Text 1). Eighteen correlation tests were conducted. A Bonferroni correction was applied to the resulting *p*-values (Bonferroni, 1936), adjusting the threshold of significance from 0.05 to 0.0028 to account for the increased risk of a Type I error, i.e., a spurious significant correlation due to the large number of correlations performed (Abdi, 2007). Any correlation that is highly significant ($p < 0.001$) before Bonferroni correction ($p < 0.0028$) will therefore remain significant after.

Using beta regression, locality and species were examined as potential influencing variables on the most common significant relationship from the Spearman correlation tests (Supplementary Text 2). Models were fit two ways, by maximum likelihood using the R-package 'betareg' (Cribari-Neto and Zeileis, 2010) and Bayesian simulation (Markov Chain Monte Carlo) using 'brms' (Bürkner, 2017). Fits from both methods were assessed using appropriate methods, inspecting plots of residuals for the maximum likelihood analysis and posterior predictive checks for the Bayesian analysis, respectively. For Bayesian fits, the R-package 'loo' was used, which performs a "leave one out" simulation of fits and predictions to estimate an expected log pointwise probability density (ELPD; Vehtari et al., 2024). For the maximum likelihood fits, the AIC method of model ranking was utilized. Raw AIC values are random and subject to sampling error.

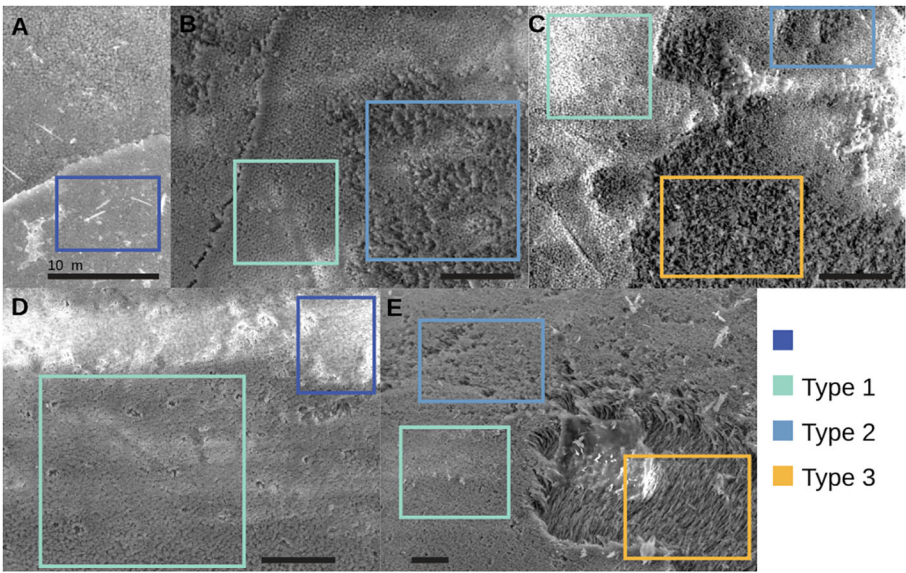


FIGURE 4
SEM images of Type 0 to 3 dissolution examples on *Limacina helicina* (A–C) and *Heliconoides inflatus* (D, E) specimens. All scale bars are 10 microns. Colored boxes highlight examples of the different dissolution types.

3 Results

3.1 Data overview

Some shells were lost or destroyed during various stages of analysis and so were not analyzed using all three dissolution methods – 145 specimens were analyzed via light microscopy; 141 were CT scanned; and 134 were imaged with SEM. All dissolution assessments are listed by specimen in [Supplementary Materials](#) ([Supplementary Table S2](#)).

During SEM imaging, abnormal structures were observed on the surface of all studied specimens from the Atlantis II and Challenger expeditions (4 *L. retroversa* and 8 *H. inflatus* specimens). These structures, which ranged from superficial nodules to altered crystal structures of the shell layers, covered substantial portions of each specimen. These features do not fall within the SEM dissolution method and could alter LDX values and were therefore removed and not analyzed further. Additionally, one *L. helicina* specimen lot (LACM-1951-65.2) is from ~800 m, while

all other specimens are from <200 m, creating a significantly different taphonomic history for this deep lot relative to the rest of the dataset. These deep lot specimens were very likely collected dead after falling through the water column for hundreds of meters. This would provide ample opportunity for the organic body’s decay to cause internal dissolution (as described in [Oakes et al., 2019b](#)), which would increase LDX values but not the dissolution measured by SEM on the external shell. This lot strongly diverged from negative relationship between LDX and SEM Type 0 displayed by all other specimens across the three species ([Supplementary Figure S2](#)), highlighting the importance of standardized collection and preservation techniques, and was therefore removed from analyses (see [Table 1](#) for finalized specimen counts).

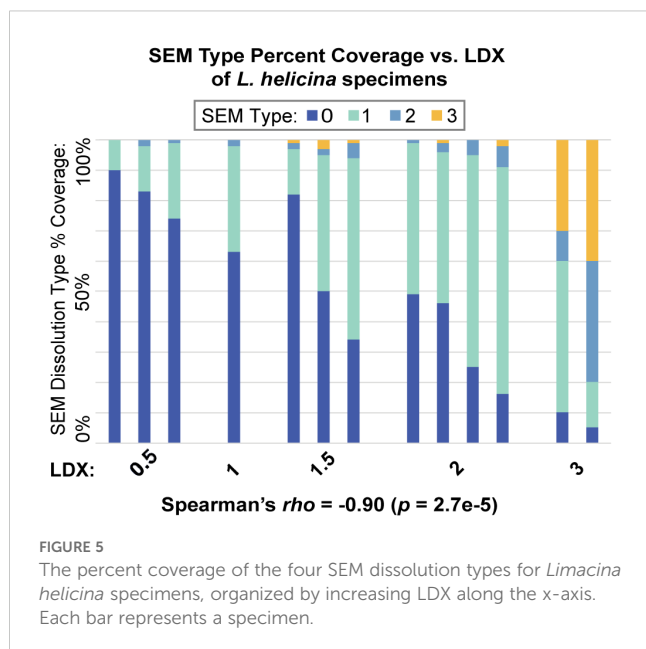
TABLE 2 Number of specimens in each LDX category by species.

LDX	<i>L. helicina</i>	<i>L. retroversa</i>	<i>H. inflatus</i>
0 LDX	0	15	3
0.5 LDX	3	17	5
1 LDX	1	13	6
1.5 LDX	3	6	3
2 LDX	4	2	10
2.5 LDX	0	4	1
3 LDX	2	8	7
3.5 LDX	0	1	1
4 LDX	0	1	0

TABLE 1 Number of specimens in each analysis by species.

Species	Light Microscopy	CT	SEM
<i>L. helicina</i>	13	10	13
<i>L. retroversa</i>	67	67	65
<i>H. inflatus</i>	36	36	34
Total	116	113	112

Specimens that broke after only completing one of the three analyses and those that had unique taphonomic histories relative to the rest of the dataset have been removed.

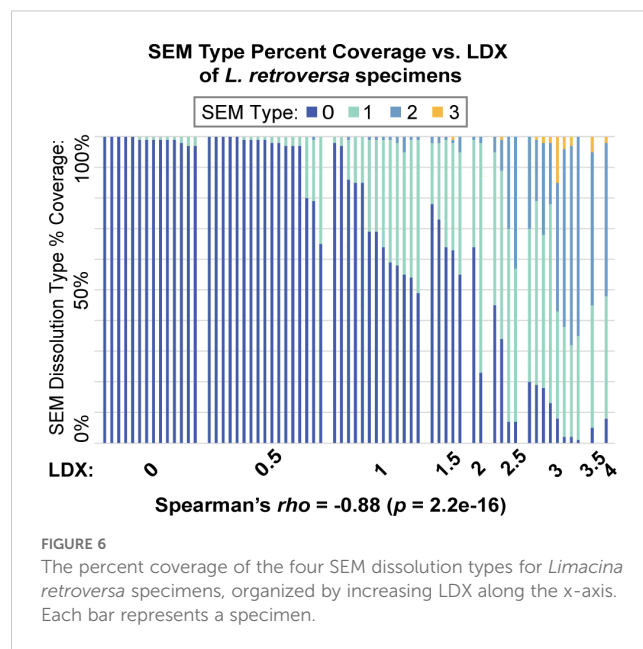


LDX values in *L. helicina* specimens ranged from 0.5 - 3; *H. inflatus* values ranged from 0 - 3.5 (Table 2). *L. retroversa* had the largest range, from LDX 0 - 4. Modal shell thickness ranged from 5 - 10 μm , 3 - 13 μm , and 5 - 16 μm , for *L. helicina*, *L. retroversa*, and *H. inflatus* respectively. For SEM maximum type dissolution, *L. helicina* ranged from 0 - 3, *L. retroversa* ranged from 0 - 3, and *H. inflatus* ranged from 2 - 3. *H. inflatus* specimens had the largest amounts of Type 3 dissolution overall, while *L. retroversa* had an average Type 3 percent coverage of 0.6%.

Four researchers evaluated and assigned LDX values to specimens. These values were compared to evaluate how this metric varies by researcher, and the average absolute difference among LDX assignments was 0.27 units (Supplementary Table S3). The average difference between LDX assignments made using a light microscope versus viewing a specimen photograph was also small (0.35 LDX units; Supplementary Table S3). Luster and transparency did not change in lockstep for every specimen. Thus, specimens with the same score can have slightly different appearances regarding these characteristics, which could contribute to variability in LDX assignments.

3.2 *Limacina helicina*

When comparing the SEM type percent composition to the LDX of each specimen, there is a trend of decreasing pristine shell (Type 0) and increasing proportions of Type 1, 2, and 3 dissolution as LDX dissolution increases (Figure 5). A Spearman analysis shows that LDX is significantly correlated to SEM Type 0 ($\rho = -0.90$, $p < 0.001$) both before and after Bonferroni correction ($p < 0.05$ to $p < 0.0028$; Supplementary Figure S3). SEM maximum dissolution was correlated to SEM Type 0 ($\rho = -0.59$, $p < 0.05$) and LDX ($\rho = 0.59$, $p < 0.05$), but only before the Bonferroni correction. Modal shell thickness was not correlated with shell width ($\rho = 0.52$, $p =$



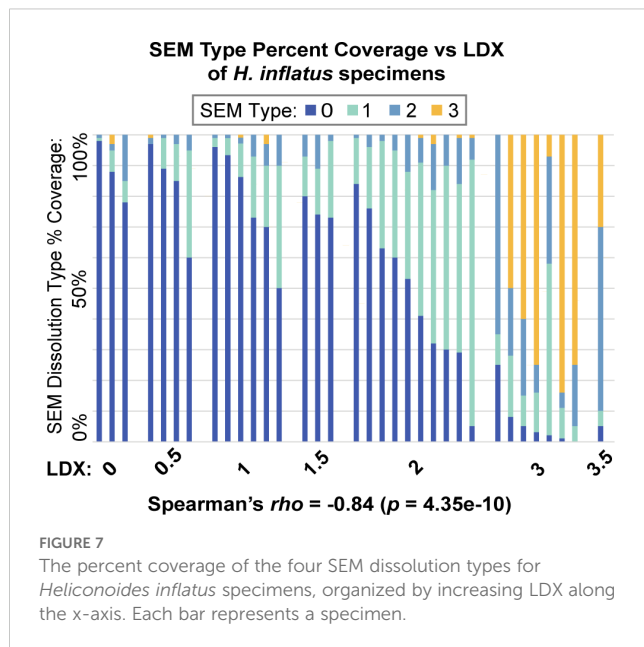
0.13), and therefore residual thickness was not used to replace the raw thickness values (Supplementary Table S4). Raw thickness was not significantly correlated to any other dissolution metric in the Spearman ρ analysis.

3.3 *Limacina retroversa*

When comparing the SEM type percent composition of each specimen to LDX (Figure 6), a general trend of increasing proportions of Type 1–3 dissolution and decreasing proportions of pristine shell (Type 0) as LDX increases was again observed. The Spearman correlations among LDX and SEM metrics quantitatively support this finding (Supplementary Figure S4). Both before and after Bonferroni correction, LDX and percent shell coverage of SEM Type 0 were negatively correlated ($\rho = -0.88$, $p < 0.001$) and LDX and SEM maximum dissolution type were positively correlated ($\rho = 0.85$, $p < 0.001$). Percent shell coverage of SEM Type 0 and SEM maximum dissolution type were also negatively correlated after Bonferroni correction ($\rho = -0.92$, $p < 0.001$). Overall, these results indicate that increased dissolution as measured by LDX equates to increased dissolution as measured by the SEM type metrics. Within the *L. retroversa* dataset, CT modal shell thickness was not correlated to width ($\rho = -0.10$, $p = 0.41$; Supplementary Table S4), and therefore residual thickness did not replace raw thickness in the Spearman ρ analysis.

3.4 *Heliconoides inflatus*

When comparing the SEM type percent composition to the LDX of each *H. inflatus* specimen (Figure 7), proportions of Type 1–3 dissolution generally increase as LDX dissolution increases. Modal thickness was significantly correlated to shell diameter ($\rho =$



0.38, $p < 0.05$) but not whorl count ($\rho = 0.09$, $p = 0.61$; [Supplementary Table S4](#)). Due to this correlation, residual thickness replaced raw thickness in this dataset. Like *L. retroversa*, *H. inflatus* had a strong, significant, and negative correlation between LDX and percent coverage of pristine shell (SEM Type 0; $\rho = -0.84$, $p < 0.001$; [Supplementary Figure S5](#)). LDX was also positively correlated to SEM maximum dissolution type ($\rho = 0.41$, $p < 0.05$), but this correlation was not maintained after Bonferroni correction. Percent coverage of SEM Type 0 and SEM maximum dissolution type were also negatively correlated before and after Bonferroni correction ($\rho = -0.50$, $p < 0.01$).

3.5 Comparison of SEM. dissolution variability vs. LDX across species and localities

A negative correlation between LDX and SEM Type 0 (pristine) percent shell coverage was the most common significant correlation among the three methods. To investigate the influence of species and locality on this relationship, a beta regression with a logit link function was used. Using both maximum likelihood estimation and Bayesian simulation, four models were considered: 1) a model with

LDX and no other interactions, 2) a model with all three species separated, 3) a model with *L. helicina* and *L. retroversa* combined and *H. inflatus* separate (to assess influence of generic-level differences and their associated shell structure variations), and 4) a model with all three species separated and *H. inflatus* further divided by locality. Both maximum likelihood and Bayesian approaches produced the same ranking of the four models by accuracy of fit ([Table 3](#)). The maximum likelihood results indicate model 4 ([Figure 8](#)), where all species are distinct and *H. inflatus* is further divided by locality, is most supported, while the Bayesian results show models 4, 1, and 2 as equally plausible, since their ELPD difference is less than 4 ([Sivula et al., 2020](#)). Results for this analysis with lot LACM-1951-65.2 included can be found in the [Supplementary Materials](#) ([Supplementary Table S5](#), [Supplementary Figure S6](#)). Other potential influences on the data, such as shell size and thickness, were assessed for influence in each species dataset, but did not appear to influence the relationship between LDX and SEM Type 0.

3.6 Visual comparison of metrics

Light microscopy photos, SEM images, and CT heatmaps were compared side by side for a qualitative visual analysis ([Figure 9](#); [Supplementary Figure S7](#)). SEM dissolution Type 3 was often visible in CT shell thickness heatmaps, but smaller patches of both Type 3 and 2 dissolution were often lost because of the lower resolution of the shape meshes that are used to produce the heatmaps ([Figure 9](#)). Patches of Type 3 dissolution were also often visible in light microscopy as gouges in the shell surface ([Figures 9A](#); [Supplementary Figure S8B](#)). Additionally, differentiating between types of dissolution severity observed in SEM was often possible with light microscopy, when the patches of dissolution were large enough to be visible under the magnification of the light microscope ([Supplementary Figure S8A](#)).

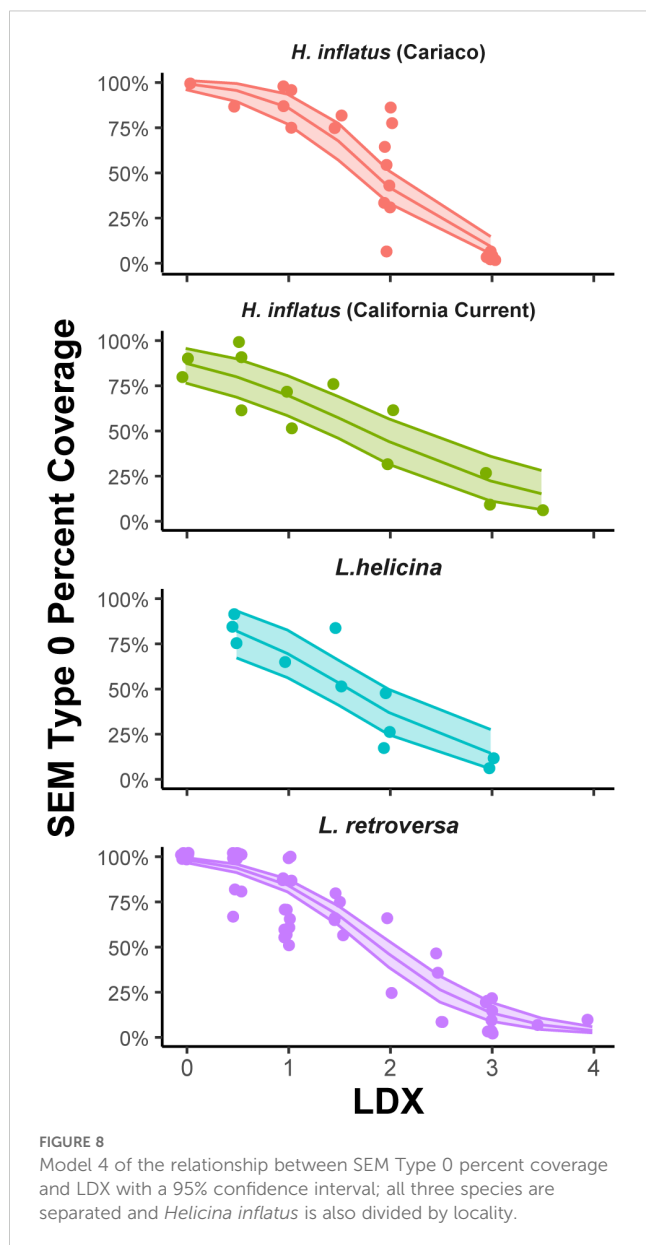
4 Discussion

4.1 Light microscopy LDX as a substitute for SEM metrics

Implementing LDX in ocean acidification studies requires minimal training and results in consistent scoring amongst

TABLE 3 Maximum likelihood and Bayesian simulation beta regression results comparing various models. A $\Delta AIC > 2$ or a $\Delta ELPD > 4$ between models is taken to be significant.

Analysis:	Max. Likelihood		Bayesian Simulation	
Model	AIC	dAIC	ELPD_diff	se_diff
4: SEM Type 0% ~ Species + LDX : Species + Locality	-281.868	0	0	0
1: SEM Type 0% ~ LDX	-276.683	5.185	-2.926	3.409
2: SEM Type 0% ~ Species + LDX : Species	-275.657	6.211	-3.334	2.799
3: SEM Type 0% ~ Species + LDX : Species (<i>Limacina</i> species combined)	-274.608	7.26	-4.053	3.203



researchers (Supplementary Table S3). Researchers new to this method will be able to become proficient within a day of reading and practice, provided that they can train with a researcher experienced in assigning LDX, since there are multiple criteria for dissolution. Additionally, LDX is significantly faster and cheaper than SEM based on the equipment time and costs for this study (Table 4).

Among the five dissolution metrics, a negative relationship between LDX and SEM Type 0 percent coverage was the most common significant correlation, occurring in all three species. This relationship persists regardless of shell microstructure type (i.e., crossed lamellar vs. helical; see Section 1.3). Furthermore, these taxa were collected in locations with different oceanographic regimes, ranging from a relatively shallow subpolar bay (*L. retroversa*) to the tropical, aragonite-supersaturated Cariaco Basin (*H. inflatus*; Astor et al., 2013; Oakes et al., 2020) and the subtropical/temperate, aragonite-undersaturated California Current (*H. inflatus* and *L.*

helicina; Feely et al., 2016, 2008). Despite this wide variety of oceanographic conditions, the correlation between LDX and SEM Type 0 percent coverage was retained. This correlation is also maintained across a variety of collection and preservation methods. Even if this study removed collection and preservation as potentially confounding variables by only analyzing *L. retroversa*, a significant negative correlation between LDX and SEM Type 0 percent coverage is still demonstrated. This resilience of this result supports the hypothesis that the faster and less expensive LDX metric can perform as a substitute for SEM-based dissolution assessment. The authors suggest using a combination of LDX and SEM for analyzing shell dissolution—where LDX is used to assess the entirety of specimens and SEM is used on a minority of specimens determined by the time and resources available.

Further research is still merited to understand why some pteropod specimens do not show a relationship between LDX and SEM dissolution; perhaps the opacity method (Bergan et al., 2017), a light microscopy dissolution metric that quantitatively assesses changes in shell transparency, could provide additional insights.

4.2 Influence of location and species on LDX and SEM Type 0 relationship

Both types of beta regression showed that separating the dataset by species and locality produced the best model fits for the relationship between LDX and SEM Type 0. While these results are statistically significant, they may not be scientifically important, due to the inherent variability in both LDX and SEM type dissolution as semi-quantitative methods (i.e., Supplementary Figure S9), the differences in imaging resolution among the techniques, and the small number of specimens in many LDX categories. In particular, *L. helicina* and *H. inflatus* have small sample sizes when split by locality. Additionally, the *L. helicina* LDX dataset ranges from 0.5 to 3, with 14 of the 30 specimens scoring an LDX of 2, whereas the other species encompass a larger and more distributed range of LDX values (Table 2). Furthermore, the confidence regions of the different species in model 4 largely overlap with one another (Figure 8). While the California Current and the Cariaco Basin specimens of *H. inflatus* experienced different conditions, it is unclear how this could have led to the locations having different relationships between LDX and SEM Type 0. Therefore, we consider model 1, that of no species or locality, which is ranked as the second most plausible, to most accurately represent the relationship between LDX and SEM Type 0 based on the data currently available. Future studies should increase the sample size for all species, and particularly for *L. helicina*, across the LDX and SEM Type scales.

4.3 SEM maximum type dissolution as a potential substitute for SEM percent coverage metrics

SEM maximum dissolution may serve as a substitute for SEM Type 0 percent coverage, saving SEM lab time and usage fees.

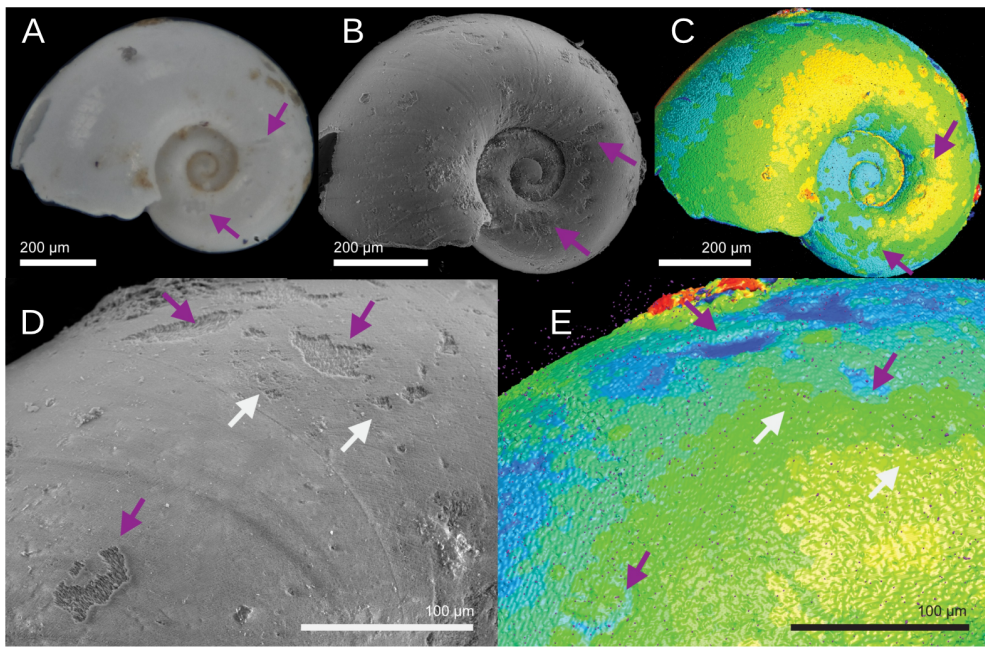


FIGURE 9
Comparison of a *Heliconoides inflatus* specimen (ANSP_477914_09) across light microscopy (A), SEM (B, D), and CT (C, E). In the CT thickness heatmaps, warmer colors equate to the thickest portions of the shell and cooler colors represent thinner areas. In the SEM images, purple arrows indicate examples of SEM type dissolution visible with other imaging methods and white arrows indicate patches of SEM type dissolution not visible with other imaging methods.

However, the significance of this correlation was only maintained after Bonferroni correction in *L. retroversa*, so this relationship needs additional data (in the form of increasing specimen numbers) and testing. In this study, a thorough evaluation of all SEM dissolution Type percent coverages, including that of SEM Type 0, took 60–80 minutes per specimen, while a max dissolution assessment would have likely taken ~5–30 minutes per specimen, since Type 2 and Type 3 are relatively easy and quick to identify (Table 4). Previous research (Mitchell, 2019) reported that SEM type surface area assessment only took 20 minutes per specimen, but Koester found that more time was required, both as a new SEM user and to detect early stages of Type 1 dissolution. Regardless, substituting SEM maximum type dissolution for Type 0 would save research time and costs if a strong relationship between the two SEM metrics is established.

TABLE 4 Time and cost per specimen for each tool of dissolution analysis, based on this study’s processing rates and equipment costs at UTCT and Singh Center for Nanotechnology.

Tool	Time per specimen	Cost per specimen
Light microscopy	1-5 mins	free
SEM (max. vs. % coverage)	5-30 mins/60 - 80 mins	~\$12/~\$47
CT	35 mins scanning & 50 mins processing	\$75

SEM is divided into assessing only maximum dissolution type versus assessing percent coverage of each SEM dissolution type.

4.4 CT is not sensitive to minor dissolution

Across all species, modal shell thickness was not correlated to any dissolution metric after Bonferroni’s correction. Modal shell thickness can be influenced by shell size and it was correlated to diameter in one of the three species (*H. inflatus*). Residual thickness, which accounts for the impact of shell size on shell thickness, did not produce any correlations with other dissolution metrics in this species. Either a different method of normalizing shell thickness was required, or whole-shell thickness was not significantly correlated to dissolution within the generally lower range of dissolution severity displayed by these specimens. It is likely that the surface dissolution caused by lived ocean conditions does not thin the shell enough to cause a significant change in modal thickness.

Unlike the shell-averaged thickness results, CT thickness heatmaps display patches of severe surface dissolution. At the scan resolution range achieved in this study (generally 1.2 microns), CT shell thickness heatmaps detected SEM Type 3 dissolution but lacked sensitivity to detect Type 1 SEM dissolution and smaller patches of Type 2 dissolution (Figure 9). Although heatmaps captured surface dissolution better than modal thickness, CT remains less sensitive than SEM to surface dissolution.

While CT may not be effective at capturing shell thinning caused by *dissolution*, it can detect shell thinning caused by *reduced calcification* across temporal and geographic acidification gradients. One study in the Mediterranean found that the shell thickness of *Styliola subula* significantly decreased from 1921 to 2012 as pH decreased ~0.1 units (Howes et al., 2017). Another study from the California Current showed that shell thickness declined as

aragonite saturation decreased along an acidification gradient caused by regional upwelling (Mekkes et al., 2021a). This aligns with the use of CT in detecting reduced calcification in other mollusks (Wall-Palmer et al., 2021; Chatzinikolaou et al., 2017; Meng et al., 2018; Barclay et al., 2020), in foraminifera (Kinoshita et al., 2022), and hard corals (Fantazzini et al., 2015; Fordyce et al., 2020). Additionally, CT has been used to detect areas of repair in pteropod shells in response to various types of damage (Peck et al., 2018). Furthermore, CT analyses have been useful in modeling saturation states and dissolution rates to model benthic calcium carbonate preservation (Sulpis et al., 2022). Due to these findings, CT is recommended for assessing the loss of calcification in aragonite-oversaturated regions, where the primary response is likely to be a loss of calcification, as opposed to shell dissolution, which is more likely to occur in areas experiencing saturation and undersaturation.

5 Conclusion

As the first study to quantitatively compare LDX and SEM dissolution methods on modern pteropods, this study provides a promising indication that light microscopy methods can act as a viable substitute for the more cost- and time-intensive SEM-based techniques. Further work incorporating larger sample sizes than what was possible here may illuminate whether there are different responses by species and by locality. Results show that implementing LDX requires minimal training and results in consistent scoring amongst researchers. Furthermore, this study demonstrates that the relationship between LDX and SEM metrics persists across multiple species and oceanographic conditions, which strengthens its use on a global scale. CT, SEM, and light microscopy all have strengths and drawbacks; this study will hopefully help ocean acidification monitoring efforts that use shell dissolution as a bioindicator save time and money by understanding and strategically utilizing available resources. By determining how these metrics are related, detection of ocean acidification impacts via pteropod dissolution can become more efficient while still producing informative data. The more accessible monitoring practices are, the more we will be able to understand the impacts of ocean acidification at both the local and global scales. In turn, the more accessible the data from this research becomes, the easier it will be to formulate effective, inclusive policy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author/s.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

BK: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. JH: Formal analysis, Methodology, Writing – review & editing, Writing – original draft. MM: Writing – original draft, Writing – review & editing, Formal analysis, Visualization. OG: Writing – review & editing, Formal analysis. RO: Data curation, Methodology, Resources, Writing – review & editing, Supervision. JS: Conceptualization, Methodology, Resources, Software, Supervision, Writing – review & editing, Project administration, Funding acquisition, Data curation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2025.1473333/full#supplementary-material>

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