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ORIGINAL ARTICLE

Using Hydrofluoric Acid for Morphological Investigations of Zoanthids (Cnidaria: Anthozoa): A Critical Assessment of Methodology and Necessity

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Abstract Zoanthids comprise an order of benthic, generally colonial cnidarians, which can usually be distinguished from other hexacorallians by embedded sand and detritus in their mesoglea to help strengthen their structure. These animals are becoming increasingly important research subjects in biochemistry and other research fields. Their inclusion of both calcium and silica results in the need for both decalcification and desilification for internal morphological examinations. Since the methodology of hydrofluoric acid (HF) desilification has rarely been documented in zoanthids, histological surveys for zoanthid taxonomy have often been abandoned and their taxonomy is often problematic. Recent investigations utilizing molecular methods have brought a clearer understanding of zoanthid diversity, but standardization of HF treatments are still needed to provide a link between molecular and more traditional techniques, and to properly examine specimens for which molecular methods may not be an option (e.g., formalin-preserved specimens, etc.). Here, we use both "straight" HF and, for the first time with zoanthids, buffered HF (BHF) treatments at different treatment lengths (1–48 h) on polyps from three different species of zoanthids for histological examination. Section conditions were judged based on the presence/absence of embedded detritus, drag marks, and tissue condition. Results show that the BHF treatment resulted in slightly better tissue conditions for all specimens, and suggest that desilification works well regardless of treatment time for species with smaller (polyp diameter <0.5 cm), less heavily encrusted polyps. Desilification of heavily encrusted *Palythoa mutuki* polyps were still problematic, with at least 24 h treatment needed. To aid future research, we provide guidelines for HF treatments of zoanthid specimens.

Keywords Diagnostic characters · Morphology · Encrustation · Zoantharia · Histological section · Sand

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Introduction

Zoanthids comprise a hexacorallian order (Zoantharia or Zoanthidea) of generally colonial cnidarians found worldwide in various marine environments from shallow waters

to the deep sea. Most zoanthid taxa encrust sand and other hard materials obtained from the water column into their ectoderm and mesoglea to help strengthen their structure (e.g., Haywick and Mueller 1997). The amount of encrustation may vary among different zoanthid species and genera, with some taxa (e.g., the genus *Palythoa*) known to contain almost 45% encrustation by weight (Haywick and Mueller 1997). It has also been shown that although apparently zoanthids incorporate materials of a selected size range, they apparently do not select what kind of material they incorporate into their mesoglea, incorporating all available minerals (Haywick and Mueller 1997).

Zoanthids are an important research subject for a wide variety of research, particularly in biochemistry-related investigations. The toxic compound palytoxin was originally isolated from *Palythoa* (Moore and Scheuer 1971), and norzoanthamine, which has shown promise in treating osteoporosis, was originally isolated from a *Zoanthus* sp. (Fukuzawa et al. 1995). As zoanthids are still relatively under-researched, they are likely to harbor other undiscovered bioresources, and are becoming an increasingly important research subject (Behenna et al. 2008). Despite increasing interest in zoanthids as bioresources, most studies of this kind either do not include a species identification for the zoanthid utilized (e.g., Hoffman et al. 2008) or include a dubious identification as zoanthid taxonomy is still chaotic and in need of clarification. This problem can be attributed to several different causes.

Zoanthids have relatively simple morphologies, with few diagnostic morphological characters. Although external appearance and/or micro-habitat/substrate of zoanthids can often allow easy identification of some species in the field (e.g., Burnett et al. 1997; Reimer 2007; Fig. 1), for proper species descriptions and in the case of species that look very similar, examination of internal morphological characters is necessary. The status (complete or incomplete) of the fifth mesentery from the dorsal directive (Fig. 2) is used to divide zoanthids into the suborders Brachycnemina (incomplete; the families Sphenopidae and Zoanthidae) and Macrocnemina (complete; the families Epizoanthidae and Parazoanthidae). Other diagnostic internal morphological characters include the presence or absence of lacunae in the mesoglea and their frequency and size, whether the sphincter muscle is endodermal or mesodermal, as well as the number of mesenteries. Sexual reproductive characteristics of species (i.e., gonochoric, asexual, or hermaphroditic polyps) are also determinable from internal morphological examinations, and can also allow for an ecological component of zoanthid species description.

However, histological examination of zoanthid internal morphology is very difficult as most specimens contain hard materials, e.g., sand encrustation. Without treatment,

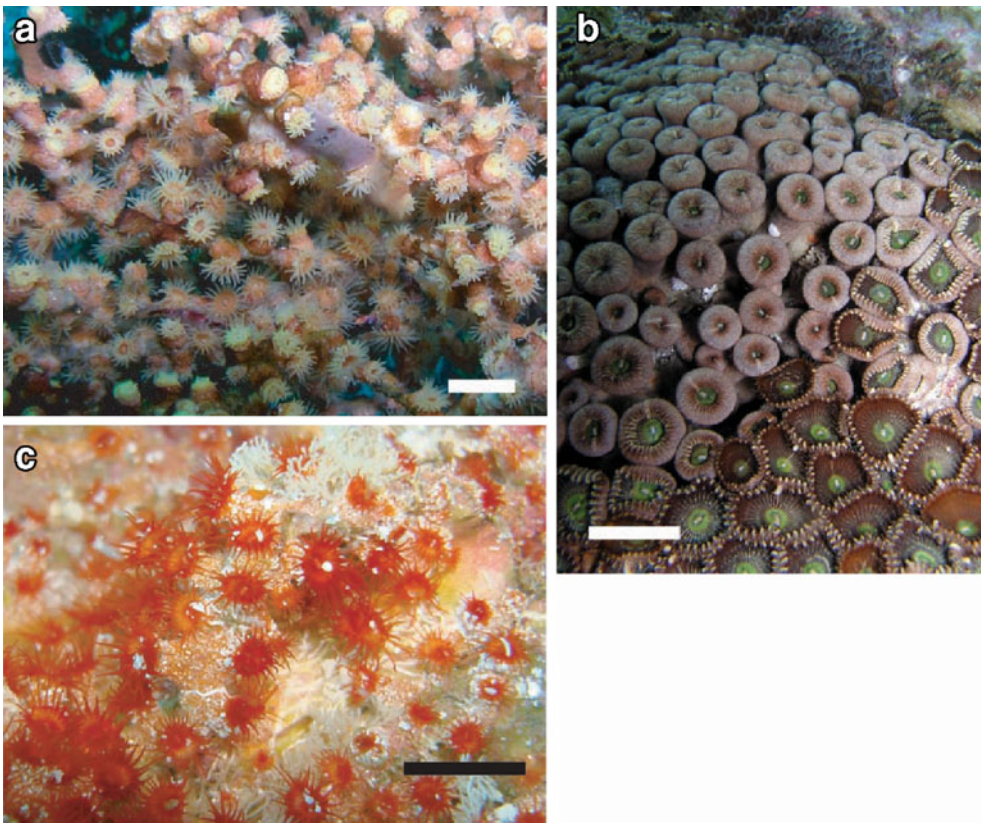

Fig. 1 **a** *Parazoanthus* sp. G1 in situ at Santiago Island, Galapagos, Ecuador; **b** *Parazoanthus* sp. G3 in situ at Darwin Island, Galapagos, Ecuador; and **c** *Palythoa mutuki* at Nishidomari South, Otsuki, Kochi, Japan. All images taken by JDR. All scales = 2 cm

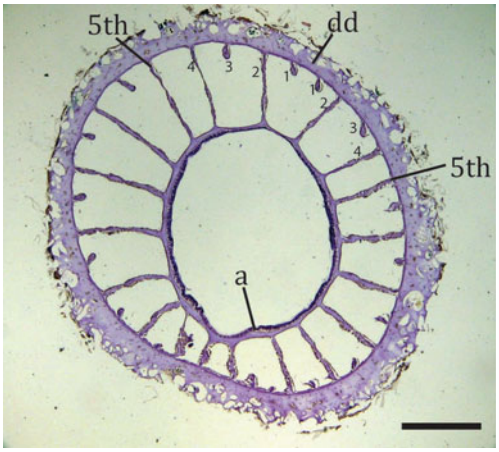

Fig. 2 An example of a cross-section of a zoanthid polyp (*Parazoanthus* sp. G3) at the actinopharynx region demonstrating some major features of zoanthids. *Abbreviations dd* dorsal directives, *numbers* mesentery numbers from the dorsal directive, *5th* fifth mesentery, complete in this case, therefore specimen is in the suborder Macrocnemina, *a* actinopharynx. *Scale* = 500 μm

these hard particles damage surrounding soft tissue during sectioning, making detailed observations of such internal characters as mesentery structure and patterns difficult if not impossible. Additionally, often paraffin or resin does not permeate hard materials. Thus, morphological examinations of zoanthids face a serious problem; if the specimen is encrusted it is a zoanthid, but to properly describe specimens' morphology in detail the encrustations must be somehow removed or otherwise taken into account.

The most common method of treating encrusted zoanthid specimens has been desilification of specimens with hydrofluoric acid (HF; e.g., Ryland and Babcock 1991; Ryland and Westphalen 2004; Sinniger et al. 2005; Swain 2009). Despite being a weak acid, hydrofluoric acid is extremely dangerous if mishandled (Peters and Miethchen 1996). Additionally, results using HF to desilicate encrustations in zoanthids are often less than desirable (F. Sinniger, personal communication), with much variation in the quality of results, and no set methodology of HF treatment in place. Furthermore, the fluoride ion in HF strongly binds with free calcium ions, creating insoluble CaF_2, and thus zoanthid specimens from coral reefs and other environments, which may have much calcium embedded in their mesoglea, need an additional step to decalcify specimens (e.g., Walsh and Bowers 1971) before HF treatment. The lack of set protocol combined with increasing concerns and regulations regarding the use of HF has led to the abandonment, for the large part, of the examination of zoanthid internal morphology. These issues combined with high levels of intraspecific morphological variation in many zoanthid species (Reimer et al. 2004) has resulted in the current chaotic situation of zoanthid taxonomy. To solve these taxonomic problems, molecular techniques have been proven to be useful (e.g., Burnett et al. 1997; Reimer et al. 2004; Sinniger et al. 2005), and a large-scale reassessment of zoanthid diversity is now underway (e.g., Reimer et al. 2006, 2008a; Sinniger and Haussermann 2009).

Thus, in order to link traditional internal morphological examination with modern DNA identification techniques and provide easier species identification for other studies using zoanthids as subject materials (e.g., biochemistry-related work, etc.), standardized guidelines and protocol for dealing with zoanthid encrustation should be established. In this study, we subjected polyps from three different-sized species of zoanthids under two different HF treatment protocols (HF alone, buffered HF/NH_4F) exposed for varying lengths of time to address the following questions:

1. Buffered HF treatments (BHF) are often used in industry due to a better controlled rate of desilicification and constant pH (Cui 2006), but this has yet to be applied to zoanthid histology. Do buffered HF/NH_4F treatments give better results than simple HF treatments?
2. Does the amount of time polyps are subjected to treatment influence the cross-section results?
3. Does the size of the polyp and its encrustations influence which treatment is better and/or the optimal amount of desilification time?

Based on the outcome of the answers to these questions it is hoped some guidelines for HF treatment of zoanthid specimens can be established.

Materials and Methods

Specimens

Three species of zoanthid were utilized in this experiment (Fig. 1, Table 1). Specimens of species *Parazoanthus* sp. G1 and *Parazoanthus* sp. G3 were collected as described in Reimer et al. (2008b). Additionally, the much larger polyps of *Palythoa mutuki* were also examined; these specimens were collected from Tatsukushi, Kochi, Japan, on September 26, 2006. Subject species were chosen to reflect different sizes and amounts of encrustation seen in zoanthids; small polyps with fine sediment encrustation (*Parazoanthus* sp. G3), slightly larger and longer polyps with fine sediment encrustation (*Parazoanthus* sp. G1), and large polyps with heavy large sediment encrustation (*P. mutuki*).

Decalcification and Hydrofluoric Acid Desilification Protocol

The two protocols used in this experiment were decided after examination of previous results and consultation with other zoanthid researchers (Table 2). While some research

Table 1 Summary of zoanthid specimens used in hydrofluoric acid desilification experiments in this study

Species	Sample number/ name	Sampling date	Sampling location	Collected by	Encrustation? (characters[a])	Avg. polyp size (oral disk diameter; height; mm)	Number of tentacles in species	Specimen colony weight (g)	Reference
Parazoanthus sp. G1	04-343	Dec. 3, 2004	Caleta Iguana, Isabela I., Galapagos	G. Edgar	Yes, fine sediment	6–12; 6–15[b]	40[b]	4.69	Reimer et al. 2008b
	04-345	Dec. 3, 2004	Caleta Iguana, Isabela I., Galapagos	C. Hickman	Yes, fine sediment	6–12; 6–15[b]		2.03	Reimer et al. 2008b
Parazoanthus sp. G3	03-539	Aug. 25, 2003	Cousins Rock, Galapagos	C. Hickman	Yes, fine sediment	4–12; <20[b]	32–40[b]	1.74	Reimer et al. 2008b
Palythoa mutuki	PmTa1	Sept. 26, 2006	Tatsukushi, Kochi, Japan	J.D. Reimer	Yes, large sediment	Up to 30; up to 40[c]	50–80[d]	3.92	Reimer 2007

[a] Fine=<50 μm; large=up to 150 μm

[b] From Reimer et al. 2008b

[c] From Reimer 2007

[d] From Reimer, unpublished data

has used 20% HF (Ryland and Babcock 1991; Ryland and Westphalen 2004; Swain 2009), we chose 15% HF (and 12.5% BHF, which is approximately the same strength) for three reasons: (1) higher percentages of HF can cause more damage to tissue, and thus it is desirable to use lower HF percentages when possible, (2) previous research has used a large variety of HF percentages (Table 2), so we wished to use a relatively "medium" value, and (3) HF becomes increasingly dangerous (or even fatal) to use at higher percentages. Thus, when possible, lower percentages of HF are much more desirable. Initial colony fixation and decalcification was performed at the Biological Institute on Kuroshio (BIK), Otsuki, Kochi, Japan from January 7 to 9, 2007, and HF desilification was conducted at the Stella Sanpo Factory in Sakai, Osaka, Japan from January 10 to 13, 2007.

Initially, four colonies each of the three species (*Parazoanthus* sp. G3 colonies 04-343 and 04-345; *Parazoanthus* sp. G1 colony 03-539; *P. mutuki* colony PmTa1; see Table 1) were fixed in 75% ethanol, and then subsamples of each colony were transferred to 10% formalin–seawater for 1 day.

Next, as all samples were collected from coral reef environments, polyps were decalcified by chelation with a 1:1 mixture of 20% citric acid and 50% formic acid subsequently diluted 50% with distilled water (DW). Thirty-two polyps of *Parazoanthus* sp. G3, and ten polyps each of *Parazoanthus* sp. G1 and *P. mutuki* were decalcified; polyps were decalcified in groups of four (*Parazoanthus* sp. G3) or two (*Parazoanthus* sp. G1, *P. mutuki*). Polyps were decalcified for 2 h to 3 days depending on their size with the decalcification mix changed twice daily; decalcification was stopped when bubbles no longer emitted from the polyp(s) (Ravindran et al. 2001). Colonies were then rinsed overnight in DW, with water changed multiple times.

Polyps were then divided into two treatments; one buffered HF treatment (hereafter BHF treatment) of 12.5% NH_4F and 10% HF (n=16 polyps of *Parazoanthus* sp. G3; n=4 of *Parazoanthus* sp. G1 and *P. mutuki*) with a pH of 1~2, and one treatment of 15% HF (n=16 polyps of *Parazoanthus* sp. G3; n=4 of *Parazoanthus* sp. G1 and *P. mutuki*; hereafter HF treatment) with a pH of <1. BHF treatments have the advantage of maintaining a constant and more basic pH and a more controlled "etch" or desilicification rate (Kikuyama et al. 1991; Cui 2006).

Polyps in both treatments were subjected to 1, 6, 24, or 48 h desilification (n=2 polyps/treatment/time for *Parazoanthus* sp. G3, n=1 polyp/treatment/time for *Parazoanthus* sp. G1 and *P. mutuki*; Table 3) following all safety protocol (triple gloves, safety goggles, conducted in a fume hood, etc.). Desilification times were decided based on previous research, consultation (Table 2), and estimation based on both the dissolution rates of HF and BHF treatments (estimated at 120~130 nm/min for HF treatment; 230~250 nm/min for BHF treatment) and particle sizes in zoanthid specimens (see Discussion for more details on particle size). After desilification, polyps were rinsed overnight with multiple changes of DW until pH was approximately 7.0 (checked with Advantec Test (Tokyo) pH test paper—Universal Grade {pH 1–11}), and then stored in 70% ethanol until sectioning.

Histology

The specimens were dehydrated through an ethanol–xylene series. Some specimens in 100% ethanol were placed in

Table 2 A comparison of hydrofluoric acid treatments of zoanthid specimens in previous studies with the current study

Reference	Swain 2009; Swain personal communication	Sinniger et al. 2005; Sinniger personal communication	Hickman pers. comm. (based on Ryland)	Ryland and Westphalen 2004	Ryland and Babcock 1991	This study
Species used	*Isozoanthus antumbrosus*	*Parazoanthus axinellae*	Various including *Parazoanthus*, *Zoanthus*	*Parazoanthus parasiticus*	*Protopalythoa* (*Palythoa*) sp.	*Parazoanthus* sp. G1; *Parazoanthus* sp. G3; *Palythoa mutuki*
Polyp oral disk diameter; length (mm)	Up to 4.8; up to 8.9	Not given	Various	Not given	10; 10	See Table 1
Initial sample fixation	4% zinc formalin; 70% ethanol	4% formalin–seawater	75% ethanol	Bouin's fluid; 70% ethanol	10% formalin–seawater	75% ethanol; 10% formalin–seawater
Rinse	Not given	Overnight in DW	None	Not given	Not given	Overnight in DW
Decalcification agent; time	Formical-4^{tm}; 4 h (repeated twice)	Chelating solution; few hours	None	Not performed	Performed after desilification; 1:1 formalin and saturated formic acid diluted 1:9 with DW; time not given	1:1 20% citric acid and 50% formic acid diluted 1:1 with DW; 2 h to 3 days
Rinse	Not given	Overnight in DW	None	Not performed	Not given	Overnight in DW, DW changed once
Desilification procedure; time	20% HF; 24 h	5~15% HF; 30 min	15% HF; 48 h	20% HF; 24 h	20% HF; 24 h	15% HF; 1, 6, 24, 48 h 12.5% HF, 10% NH_4F; 1, 6, 24, 48 h
Rinse	Not given	Overnight in DW	20% sucrose in 10% formalin; 24 h	Not given	Not given	Overnight in DW, DW changed 2–3 times
Dehydration	Ethanol	Vacuum; 100 min; LMR solvent	Performed (method not given)	Performed (method not given)	Not given	70% ethanol; 90% ethanol, 3 h, twice; 100% ethanol, 1 h, twice
Clearing	Xylene	Not given	Not given	Histoclear	Not given	Ethanol–xylene, vacuum
Pre-embedding staining	None	None	HemoDe; 2 h	None	None	None
Embedding	Paraffin	Paraffin	Paraffin	Paraffin	Paraffin	Paraffin
Staining	Harris' hematoxylin, eosin Y	Masson's trichrome	Mallory's triple stain	Mallory's triple stain	Mallory's triple stain	Delafield's hematoxylin, eosin
Section thickness (μm)	13	8	10	8	8	5–10
Results	Generally good	Generally poor	Generally poor	Not given	Not given	See "Discussion"
Conclusions	Worked well	HF too short?	No decalcification?	Not given	Worked well? (based on figures)	See "Discussion"

Table 3 Summary of treatments and cross-section conditions of zoanthid specimens subjected to different hydrofluoric acid treatments and times

Treatment; species; specimen number[a]	Sample number[a]	Hydrofluoric acid or buffered hydrofluroic acid treatment time (h)	Overall tissue condition	Mesentery number[b]	Drag marks?[c]	Sand or debris?[c]	5th mesentery status	Other notes
BHF; *Parazoanthus* sp. G1; 04-343	1	1	Good	36	+	−	Brachycnemic	
	2	6	Good	37	+	−	Brachycnemic	
	3	24	Good	36	+	−	Brachycnemic	
	4	48	Good	36	+	−	Brachycnemic	
BHF; *Parazoanthus* sp. G3; 03-539	5	1	Good	28	−	−	Brachycnemic	
	6	6	Good	30	−	−	Brachycnemic	Gonads
	7	24	Good	30	−	−	Brachycnemic	
	8	48	Good	30	−	−	Brachycnemic	
BHF; *Parazoanthus* sp. G1; 04-345	9	1	Good	35	+	+	Brachycnemic	
	10	6	Good	36	−	+	Brachycnemic	
	11	24	Good	38	−	−	Brachycnemic	
	12	48	Good	36	−	−	Brachycnemic	
BHF; *Palythoa mutuki* PmTa1	13	1	Bad	NA	+++	+++	NA	
	14	6	Bad	74?	+++	+++	NA	
	15	24	Fair	59	+	+	NA	
	16	48	Fair	56	+	+	NA	Gonads
HF; *Parazoanthus* sp. G1; 04-343	17	1	Good	40	−	+	Brachycnemic	
	18	6	Good	37	+	+	Brachycnemic	
	19	24	Good	36	+	−	Brachycnemic	
	20	48	Good	36	+	−	Brachycnemic	
HF; *Parazoanthus* sp. G3; 03-539	21	1	Fair	27	−	−	Brachycnemic	
	22	6	Fair	19?	+	−	Brachycnemic	
	23	24	Good	30	−	−	Brachycnemic	
	24	48	Good	32	+	−	Brachycnemic	Gonads
HF; *Parazoanthus* sp. G1; 04-345	25	1	Good	36	+	+	Brachycnemic	
	26	6	Good	36	+	−	Brachycnemic	
	27	24	Fair	36	+	−	Brachycnemic	
	28	48	Good	39	−	−	Brachycnemic	
HF; *Palythoa mutuki* PmTa1	29	1	Bad	NA	+++	+++	NA	
	30	6	Bad	52?	++	++	NA	
	31	24	Bad	45?	++	+	NA	
	32	48	Bad	NA	++	+	NA	

NA not acquired, impossible to acquire, *BHF* buffered hydrofluoric acid treatment, *HF* hydrofluoric acid treatment, +++ present in very large amounts, significantly degrading condition of slide, ++ present in large amounts, somewhat degrading condition of slide, + present in small amounts, not significantly degrading condition of slide, − not present, no contribution to degradation of slide

[a] For treatments, specimen, and sample information refer to Table 1

[b] If mesentery number is countable, it is implied that the macrocnemic/brachycnemic status of cross-sections could also be inferred. Question marks indicate uncertain counts, and uncertain macrocnemic/brachycnemic status

[c] Expressed as relative amounts

vacuo for approximately 30 min to remove bubbles in the coelenteron. Then, they were embedded in paraffin. Serial sections of 5–10 μm thick were prepared with a rotary microtome and stained with Delafield's hematoxylin and eosin.

Obtained slides of HF-treated zoanthid specimens were examined with a light microscope (Nikon Express E50i). The following morphological characters and conditions were examined; mesentery condition, number, and form (in particular fifth mesentery from dorsal directive complete or incomplete); presence or absence of drag marks from debris; presence or absence of sand and debris in mesoglea; overall condition of tissue and

cells; and in particular ectoderm and endoderm; any other morphological characters of note (e.g., presence of gametes, etc.).

Results

Parazoanthus sp. G1

Slides resulting from the various treated polyps of *Parazoanthus* sp. G1 are shown in Fig. 3 (see also Table 3). In general, there appeared to be no large differences in the conditions of the cross-sections treated for different lengths of time; cross-sections for all treatments did not have any sand or debris remaining, with holes resembling lacunae remaining in the mesoglea (Fig. 3b). Mesentery form and shape were generally well preserved for both treatments and all treatment times. Specimens were all macrocnemic, with the fifth mesentery from the dorsal directive complete. The number of mesenteries ranged from 27 to 32, with a mean of 30 (Table 3). In some sections, oocytes were clearly visible (Fig. 3b). Although in some areas there was damage to the ectoderm (Fig. 3a), the endoderm was generally in fine condition (Fig. 3a, b).

Overall, the condition of the sections on the BHF sections appeared to be slightly better than that of sections in the HF treatment, with the condition of complete mesenteries appearing slightly less damaged in BHF sections. There were 13 damaged complete mesenteries in four sections from slides for BHF treated *Parazoanthus* sp. G1 sections, and over 30 damaged complete mesenteries in four sections from four slides for HF sections.

However, the largest problem encountered with *Parazoanthus* sp. G1 polyp sectioning was not related to demineralization. Polyps were very small and round, resulting in correct orientation during paraffin embedding being problematic. Thus, many sections range from slightly to very oblique in sectioning angle, often making mesentery observation difficult.

Parazoanthus sp. G3

Slides resulting from the variously treated polyps of *Parazoanthus* sp. G3 are shown in Fig. 4, with a summary also shown in Table 3. In general, there appeared to be no large differences in the conditions of the cross-sections treated for different lengths of time; cross-sections for all treatments did not have any sand or debris remaining, with holes resembling lacunae remaining in the mesoglea (Fig. 4a–c). Mesentery form and shape were well preserved for both treatments and all treatment times. Specimens were all macrocnemic, with the fifth mesentery from the dorsal directive complete. The number of mesenteries ranged from 35 to 40, with a mean of 36 (Table 3). In some areas damage to the ectoderm was clearly visible (Fig. 4a–c), but the endoderm was generally in fine condition (Fig. 4a, b). The sphincter muscle, a diagnostic character at the generic level in some zoanthids in longitudinal sections, was clearly visible and in good condition in sections cut near the oral end of polyps (Fig. 4d).

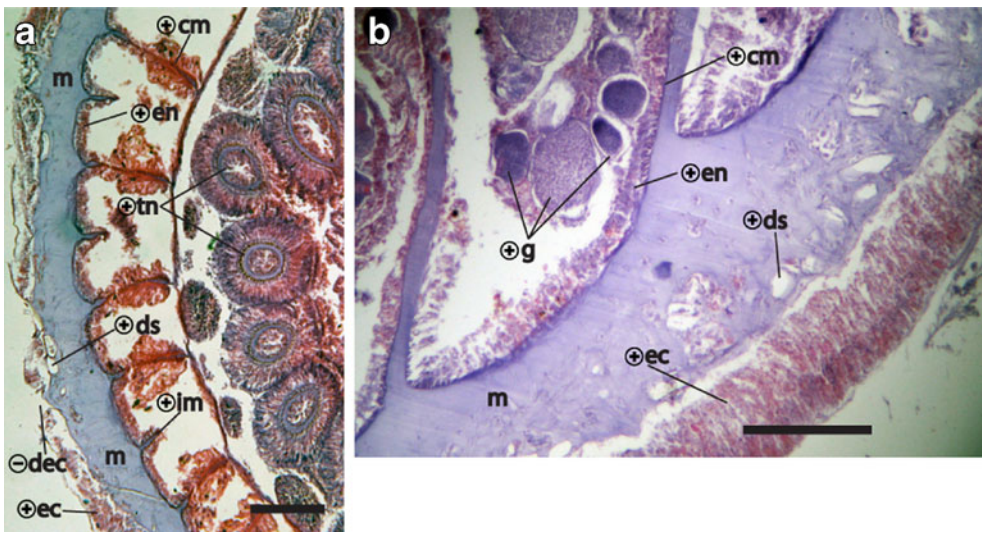

Fig. 3 **a**–**b** Cross-sections of *Parazoanthus* sp. G1 at the actinopharynx region showing both well-preserved histological features (*plus symbols*) and features with damage (*minus symbols*). *Abbreviations cm* complete mesentery, *dec* damaged/absent ectoderm, *ds* dissolved sand "holes", *ec* ectoderm, *en* endoderm, *g* gonads, *im* incomplete mesentery, *m* mesoglea, *tn* tentacles. **a** and **b** Sample 6 (see Table 3 for treatment details). **a** Image shows area from outer ectoderm (*left*) to near center of closed polyp with retracted tentacles on inner side of mesenteries (*right*); **b** shows close up of area from outer ectoderm (*lower right*) to mesenteries and gonads (*upper left*). *Scales* **a** 100 μm; **b** 50 μm

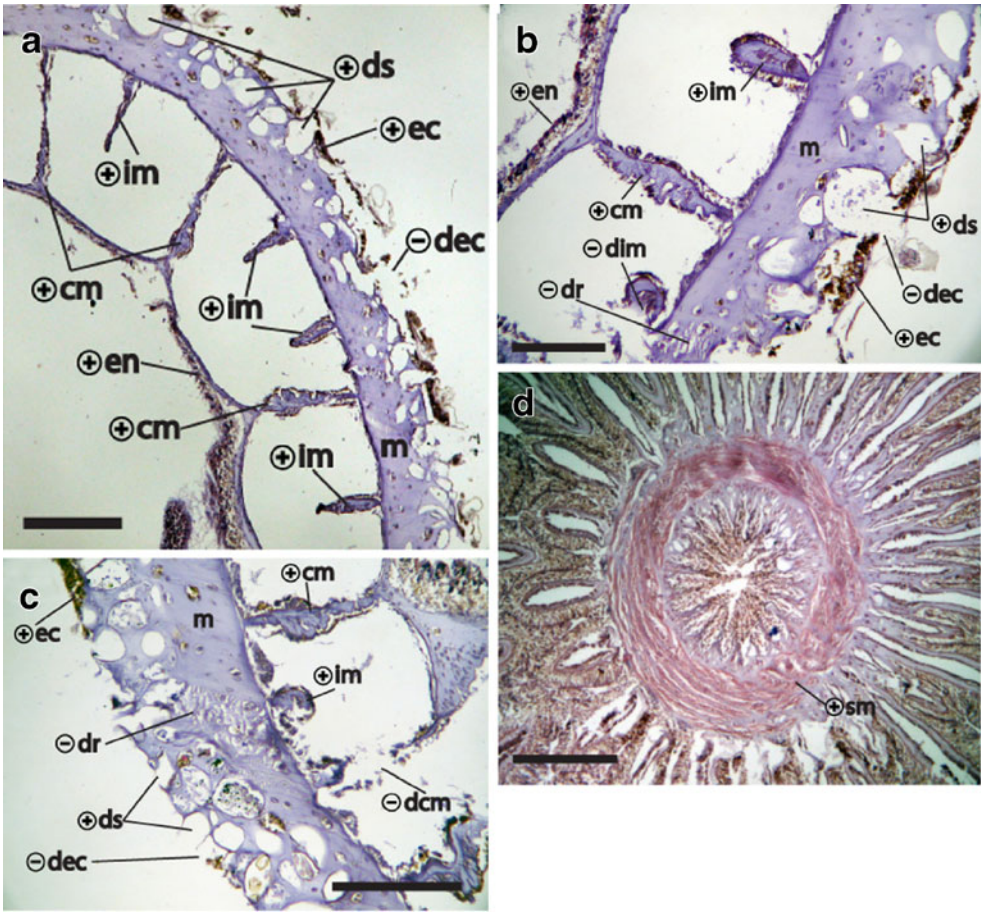

Fig. 4 **a**–**d** Various cross-sections of *Parazoanthus* sp. G3 at the actinopharynx region showing both well-preserved histological features (*plus symbols*) and features with damage (*minus symbols*). *Abbreviations* *cm* complete mesentery, *dcm* damaged complete mesentery, *dec* damaged/absent ectoderm, *dim* damaged incomplete mesentery, *dr* drag marks (in mesoglea), *ds* dissolved sand "holes", *ec* ectoderm, *en* endoderm, *im* incomplete mesentery, *m* mesoglea, *sm* sphincter muscle. **a**, **b**, **c** Sample 2 and **d** sample 11 (see Table 3 for treatment details). **d** Image shows sphincter muscle (*center*) at center of polyp surrounded by numerous mesenteries (status as complete or incomplete undeterminable). *Scales* **a** and **d** 100 μm, **b** and **c** 50 μm

Overall, the condition of the sections on the BHF sections appeared to be slightly better than that of sections in the HF treatment, with the condition of both mesenteries appearing slightly less damaged in BHF sections. There were five damaged complete mesenteries in eight sections from eight slides for BHF treated *Parazoanthus* sp. G3 sections, and 15 damaged complete mesenteries in eight sections from eight slides for HF sections.

No serious problems were encountered with oblique sections, unlike in *Parazoanthus* sp. G1, and thus HF/BHF treatment and resulting sectioning and examination proceeded most smoothly with polyps of this species.

Palythoa mutuki

Slides resulting from the various treated polyps of *P. mutuki* are shown in Fig. 5, with a summary also shown in Table 3. In general, polyps treated for short amounts of time (1, 6 h) had much debris remaining in their mesoglea (Fig. 5), and this debris damaged the surrounding soft tissue greatly during sectioning (Fig. 5b). On the other hand, cross-sections for longer treatments (24, 48 h) had no or comparatively little sand or debris remaining, with holes resembling lacunae remaining in the mesoglea (Fig. 5c). *P. mutuki* samples had holes much larger, more frequent, and more deeply embedded in the mesoglea than the other two zoanthid species examined. Additionally, some polyps treated for 48 h were observed to have "squished" oblique sections (Fig. 5c), and therefore polyps treated for 24 h in both HF and BHF treatments were most utilizable. Asides from a few sections (BHF at 24, 48 h; HF at 6, 24 h), it was impossible to observe mesentery form and count for most sections. It was virtually impossible to tell if specimens were macrocnemic or brachycnemic. The number of mesenteries ranged from 45 to 74 (Table 3), but these numbers are questionable for most sections, and only counts of 56 and 59 are considered reliable (Table 3). The ectoderm generally clearly had sustained heavy damage (Fig. 5c), with the endoderm in better shape. Mesogleal lacunae were visible in the few sections that were in overall good condition (Fig. 5c).

Overall, the condition of the sections on the BHF sections appeared to be slightly better than that of sections in the HF treatment, with mesenteries slightly more visible and in better condition, especially when observing BHF sections for 24 and 48 h. There were approximately ten damaged complete mesenteries in two sections from two slides (24, 48 h) for BHF-treated *P. mutuki* sections, and

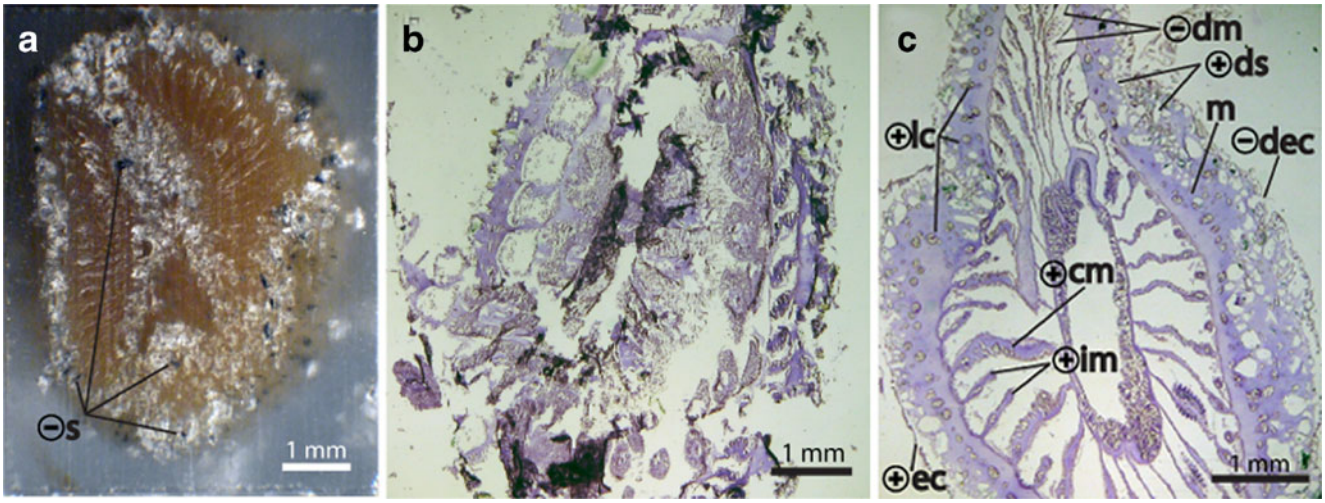

Fig. 5 **a**–**c** Cut surface on the paraffin block (**a**) and histological cross-sections (**b**, **c**) of *Palythoa mutuki* at the actinopharynx region showing both well-preserved histological features (*plus symbols*) and features with damage (*minus symbols*). *Abbreviations cm* complete mesentery, *dec* damaged/absent ectoderm, *dm* damaged mesentery (unknown if complete or incomplete), *ds* dissolved sand "holes", *ec* ectoderm, *im* incomplete mesentery, *lc* lacunae, *m* mesoglea, *s* sand particles. Note many sand grain(s) remaining in the paraffin-embedded specimen in **a**, and the overall poor condition of the section in **b** rendering the identification of most features impossible. **a** Sample 13, **b** sample 29, and **c** sample 15 (see Table 3 for treatment details). Scales = all 1 mm

over 25 damaged complete mesenteries in two sections from two slides for HF sections. Unlike with the other two species of zoanthids, the treatment and sectioning of *P. mutuki* did not produce many sections that were utilizable for internal morphological examination (Table 3).

Discussion

Necessity of Decalcification Treatment

HF binds strongly with free calcium ions to form insoluble CaF_2, and thus straight HF treatment of zoanthid polyps from environments with marine calcifers (e.g., scleractinian corals, shellfish, foraminiferans, etc.) without any decalcification step poses the risk of producing CaF_2 within the specimen(s). As calcifers are common not only in coral reef ecosystems but throughout most marine environments, and given that zoanthids apparently indiscriminantly take up debris into their mesoglea, the decalcification step performed both in previous studies (e.g., Sinniger et al. 2005; Swain 2009) and here is strongly recommended for all zoanthid specimens before any HF treatment. From our observations here, it appears that the decalcification step does not adversely affect the specimens.

Factors to Consider when Determining Optimum HF Treatment Times in Different Zoanthid Species

From the results of this study, it is clear that different species of zoanthids should be treated differently with HF. The two smaller species, *Parazoanthus* sp. G1 and G3, showed fair to excellent section results for all different treatment times from 1 to 48 h. These smaller species uptake sand and debris particles of a much smaller size than *Palythoa* as judged by the post-treatment holes remaining in mesogleal areas in cross-sections. Additionally, these two species did not become "soft" after prolonged (e.g., 48 h) treatment, perhaps due to the relatively small size of the polyps, or perhaps having less reliance on encrustations to make their structure. Thus, it appears that smaller, less encrusted (e.g., polyp diameter <0.5 cm) species of zoanthids are easier to treat with HF. Although some small amounts of detritus remained in 1 h treatments of these samples (Table 3), they had apparently little effect on the quality of the sections obtained. Based on the results of this study, if results from different treatment times are similar, a shorter treatment time may be more desirable to avoid a higher amount of potential tissue damage, although overnight desilification is possible if required by scheduling.

On the other hand, *P. mutuki* results clearly demonstrate the "classic" problems of zoanthid internal morphological examination. *Palythoa* spp. are notorious for their heavy uptake of debris (Haywick and Mueller 1997), and often produce very large (e.g., polyp diameter >1.0 cm) polyps. As these species are found primarily in coral reef environments, they contain not only silica and rock, but also calcareous sand grains. Results here show that desilification time that is too short (e.g., 1, 6 h) results in incomplete desilification of all particles, making sectioning difficult to impossible. Longer desilification times showed poor results, with sections treated for 48 h not useable, and 24-h results

resulting in less-than-ideal cross-sections, with still some amounts of debris remaining and occasional drag marks evident in sections, caused by either remaining detritus or formed CaF_2 (Table 3).

Evidently, *Palythoa* species gain much of their strength and rigidity from encrustation (Haywick and Mueller 1997). With their large polyps and heavy encrustation, as well as thick mesoglea (compared to Parazoanthidae species), HF treatment of *Palythoa* species remains a problematic issue. Many sand grains remained in the *Palythoa* polyps. This may be due to either or both of two possible reasons. Firstly, there may be impurities embedded in *Palythoa* mesoglea that are not digested by the HF and decalcification processes. Secondly, it may be that HF does not permeate into the *Palythoa* tissue (particularly the thick mesoglea) well, which could explain why longer treatments of 24 and 48 h seem to result in more desilification than 1 or 6 h. In the future, these problems may possibly be avoided by cutting the polyp (zooid) at the top or bottom to expose the coelenteron prior to desilification. This could possibly also reduce the presence of any remaining air bubbles in the coelenterons, although whether this process will solve the problems facing histological examination of *Palythoa* remain dubious as samples used in this experiment already had somewhat exposed coelenterons and yet bubbles remained despite desilification and in vacuo treatment. Cutting polyps at the top or bottom may also reduce effectiveness of histological examinations. Additionally, air bubbles alone are very likely not responsible for the drag marks and subsequent poor results seen in *Palythoa*. From results here and past investigations, it is clear histological examination of *Palythoa* polyps remains a difficult and inexact process.

When deciding on an appropriate time for HF desilification, mesogleal thickness, particle size, and HF acid strength all need to be taken into account. Obviously larger particles will take longer to digest than smaller particles, as will a thicker mesoglea with more particles more deeply embedded when compared to a specimen with a thinner mesoglea. Although likely these characters will vary group to group, a fair approximation of these two characters may be estimated solely by polyp size, although direct observation of embedded particles (e.g., Haywick and Mueller 1997; Reimer et al. 2008a) is the most certain method to confirm particle size.

Given known dissolution rates at different strengths and estimated particle size, a fair range of appropriate desilification times may be estimated. For example, the 10% HF treatment and the BHF treatment of 10% NH_4F and 12.5% HF have dissolution rates of 120–250 nm/min of silica (room temperature, 1 atm; S. Hashiguchi, personal communication). Therefore, if the largest particle sizes in a zoanthid's mesoglea are 300 μm, estimated desilification times are approximately 20~41.6 h.

Based on these dissolution rates and the results, we estimate that the two Parazoanthidae species in this study contained largest particles of no larger than approximately 50 μm in diameter (agrees with previous Parazoanthidae results—see Fig. 4 in Reimer et al. 2008a), while *P. mutuki* contained particles up to approximately 150 μm in diameter, which compares well with previous data acquired in Haywick and Mueller (1997; size, up to ca. 125 μm).

Buffered HF Treatment vs. HF Treatment

BHF treatments are often used instead of "straight" HF treatments in industry as they display a more controlled rate of desilicification and pH remains constant and more basic. BHF treatment may digest other, non-siliceous minerals such as iron more effectively than HF treatment due to a stable pH (see Cui 2006). From observing zoanthid cross-section slides from both BHF and HF treatments, it appears that the BHF treatment resulted in slightly more desirable results, with mesentery condition slightly better (see Results) than in the HF treatment. This may be partially due to the more basic nature of BHF, which could cause less tissue damage than HF (HF pH = <1 as opposed to BHF pH of 1~2). Therefore, we recommend future HF treatments of zoanthid specimens utilize BHF where possible, although the HF-alone treatment also produced utilizable cross-sections, particularly for the two Parazoanthidae species. Detailed morphological data obtained for the two undescribed Paraozoanthidae species from this study will be used in their formal descriptions, which will appear elsewhere.

Necessity of HF Treatment for Zoanthid Internal Morphological Examinations?

As discussed above, both HF treatments worked well on the smaller polyps of the two Parazoanthidae species from the Galapagos; *Parazoanthus* sp. G1 and *Parazoanthus* sp. G3. Both complete and incomplete mesenteries were clearly visible and countable in the majority of slides, despite problems with the orientation of the small rounded polyps of *Parazoanthus* sp. G1 during embedding in paraffin, which often resulted in slides being cut at oblique angles.

However, despite the success of the HF treatments and cross-sectioning, a critical discussion of these diagnostic characters is needed. Here, after HF treatments, the fifth mesentery from the dorsal directive was confirmed as complete in all slides from *Parazoanthus* sp. G1 and G3, as is expected for macrocnemic zoanthids including the family Parazoanthidae. It should be remembered, though, that macrocnemic species can be easily distinguished from

Table 4 Summary of advantages and disadvantages of different methods of internal morphological examination of zoanthids

Method	Advantages	Disadvantages	Example reference(s)
HF treatment, sectioning, optical microscope	Best way to see mesentery structure, staining possible	Invasive; inconsistent results, dangerous	This study; Ryland and Babcock 1991; Ryland and Westphalen 2004; Swain 2009
Scanning electron microscope	No need to account for encrustation, can see very fine details	Invasive; result interpretation often difficult; equipment expensive	Babcock and Ryland 1990; Reimer et al. 2008b
Dissecting microscope	Can manipulate in real time; no need to account for encrustation; staining possible	Invasive; low resolution	Shiroma and Reimer 2009
CT scanner	Non-invasive; no need to account for encrustation	Technology still developing, equipment expensive; result interpretation often difficult	Reimer et al. 2009

brachycnemic species by other, more easily distinguishable characteristics such as habitat, substrate, coloration, and (usually) the presence of zooxanthellae, and it is rare for experts to misidentify between these two suborders. As well, mesentery number generally fits within the range of tentacle numbers for two zoanthid species in this study (*Parazoanthus* sp. G3, *P. mutuki*), although differences do occur (*Parazoanthus* sp. G1). However, even in species such *Parazoanthus* sp. G1 where mesentery and tentacle number do not perfectly agree, the range of the variation is very similar to previously acquired tentacle number data (see Tables 1 and 3). We suggest that tentacle number data can potentially replace mesentery data except when ascertaining suborder assignment in zoanthids.

Given the inherent dangers of working with HF and for these reasons above, we do not strongly recommend treating zoanthids with any kind of HF desilification treatment, especially considering that other diagnostic data are usually more easily accessible and no less accurate in species identification. Additionally, with the advent of molecular identification work, which can provide an accurate placement of zoanthid specimens within the order Zoantharia, HF desilification treatment of polyps has become arguably less necessary than before in most cases.

There are, however, at least two cases in which we feel that HF desilification of species may be desirable. These situations are:

1. In original species descriptions, particularly when dealing with new or unique lineages of zoanthids for which suborder placement is questionable.
2. In examinations of the reproductive characteristics (gamete production) of specimens, particularly when dealing with specimens from difficult to access locations (deep sea, etc.) where access to multiple samples and/or numerous sampling opportunities or specimens do not exist.

For both cases listed above, internal morphological examination is necessary, and thus we can understand the need for HF desilification. There are other less dangerous techniques that could supplement or replace HF treatment in the future. These techniques include dissecting microscope examination (e.g., Shiroma and Reimer 2009), although fine details and resolution are lost; scanning electron microscope examination (e.g., Babcock and Ryland 1990; Reimer et al. 2008a), and high-resolution non-invasive CT scanning (Reimer et al. 2009). The various techniques currently available for the internal examination

Table 5 Recommended hydrofluoric acid protocol for internal morphological examination of zoanthid specimens

Order	Step	Notes
1	**Estimate size of largest mesogleal encrustations**	Can be performed with electron, optical, or dissecting microscope
2	**Calculate digestion time of largest encrustations**	Use chemistry/industry references
3	**Decalcification**	To remove potentially troublesome calcium before HF treatment. Use methods shown in Table 2
4	**Desilification using buffered HF solution**	Results in slightly better tissue condition than with non-buffered HF. Expose coelenterons if necessary to remove air bubbles
5	Sectioning and staining	As required based on experimental goals

New recommendations of methodology from this study in bold (step column)

Includes rinses in between steps as per Materials and Methods and Table 2.

of zoanthids along with their merits and drawbacks are summarized in Table 4. However, for now, in particular in the two situations described above, HF treatment remains the best option available for internal examination of zoanthid specimens.

Guidelines for Hydrofluoric Acid Desilification Treatment of Zoanthids

As no study has thus far examined and compared different HF methodologies in zoanthid histology, it is prudent to mention some simple guidelines (Table 5). First of all, estimation of the sizes of the largest encrustations can help establish optimum digestion time(s) of various zoanthid specimens, as over-digestion may lead to undesirable histological results such as extensive tissue damage. Calculation of digestion times should also take into account the strength of HF used and utilize industrial references on rates of digestion. After thorough decalcification and rinsing, desilification using BHF should be performed. Exposure of the inner coelenteron may be needed for heavily encrusted specimens, although this is unlikely needed for most zoanthids asides from Sphenopidae (*Palythoa*, etc.) species. After desilification and final rinsing, staining can be performed as needed based on experimental goals.

Conclusions

For all three species examined in this study, the more basic BHF treatment gave slightly better results regarding tissue condition in cross-section examination when compared to the HF treatment, and thus we recommend using buffered HF treatments where possible, combined with prior decalcification. The optimum time of BHF treatment should be roughly estimated before desilification by either examining encrusted maximum particle size or roughly estimating this from either polyp size or previous literature from similar or the same species. A summary of the recommended HF protocol is given in Table 5. Despite these recommendations, for some species or specimens (e.g., *Palythoa* spp.) BHF and HF treatment may not yield desirable results due to heavy encrustation. Various zoanthid taxa have very different mesogleal thicknesses, polyp sizes, and encrustation characters, and as shown here times needed for desilification may vary widely between species.

Additionally, while HF treatment of some kind remains a necessity for some internal morphological examinations of zoanthids, it is strongly recommended detailed in situ high-resolution images along with relevant habitat data (depth, environmental type, substrate) are collected as these data can greatly aid in species identification and description. As well, it is hoped that the continuing development of new and different techniques such as CT scanners and electron microscopy will allow for the necessity of dangerous HF treatment to be supplanted in the future. However, HF treatment is still necessary in some instances and thus this methodology should not be discarded. It is hoped that the research contained within this study will spur further interest in re-examining more traditional methodologies in order to strengthen links between past zoanthid examinations with current and future research. We believe that problematic taxa such as zoanthids are best properly identified and understood through combined morphological, ecological, and molecular approaches.

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