Diadema Response Network Tissue Sampling Instructions

Diadema Response Network. (Judith C. Lang, Ian Hewson, Yasunari Kiryu, Thierry M. Work, Ruth Francis-Floyd, Michelle M. Dennis, Esther C. Peters, Mya Breitbart, Isabel C Romero, S.M. Williams)

May 27, 2022

Citation. *Diadema* Response Network (JC Lang, I Hewson, Y Kiryu, TM Work, R Francis-Floyd, MM Dennis, EC Peters, M Breitbart, IC Romero, SM Williams). 2022. *Diadema* Response Network Tissue Sampling Instructions. 16 pp.

Contents

Background. The long-spined urchin, *Diadema antillarum*, is a key herbivore of benthic algae on Caribbean coral reefs. Its feeding activities clear space on solid substrata, allowing settlement of coral larvae and coral growth. *Diadema* populations began dying in early February 2022 in the U.S. Virgin Islands. By mid-March, mortality events had been independently observed in at least another five jurisdictions in the eastern Caribbean as well as Jamaica. The cause of these dispersed die-offs is currently unknown. However, the speed at which a majority of individually diseased *Diadema* are now dying on reefs that have been affected resembles the regional, mass mortality event of 1983-84.

Research into the cause of the 2022 die-off in *Diadema antillarum*, and possibly other sea urchins, is a goal of the *Diadema* Response Network. As described below, this effort includes collecting samples of *Diadema* tissues for histopathology, environmental contaminants and for microbial, molecular, and other genetic (hereafter "micro/molecular") analyses.

Statement of ethics for collected specimens and other data

Samples, and all information derived from their study, by the *Diadema* Response Network are the property of collectors and their providing organisations. Any remnant sample materials can be returned when no longer needed, if desired by the local research community or government. Collectors and organisations will be acknowledged on all resulting publications. Duplicate samples can be retained by the providers, for parallel analyses in home institutions or shared with other scientists in their networks.

Register. Thank you for having registered in the *Diadema* Response Network (DRN) Sampling program.

Permits. *Diadema* is not a CITES-listed species. However, some Caribbean jurisdictions require a permit before *Diadema* can be collected, or to sample within its protected areas. An export permit or other form of authorization may also be needed before any collected materials can be sent abroad for analyses. Local government authorities must be asked if any permits are required for non-commercial research into the cause of the current die-off. If any are necessary, immediately request an emergency permit (or permits), or at least have reliable assurances that such permission will be granted before starting to collect any *Diadema*. If needed, the *Diadema* Response Network can help initiate this contact. Have signed copies of any needed forms of permission for exporting preserved *Diadema* specimens before trying to send the collected materials to partner groups in other countries.

Customs. Basic lab supplies and fixatives to preserve tissues for transmission electron microscopy (TEM) and micro/molecular analyses, can be provided by the DRN. Please verify they can be imported through customs for non-commercial research purposes, and ensure that you can afford any import fees, prior to agreeing to receive a shipment. Fixatives for light microscopy (Z-Fix or formaldehyde, see p.) cannot be shipped by air. They must already be present and available for use.

Site selection. Choose sites with enough *Diadema* for sampling. If scarce on reefs and hardgrounds, larger populations may be found near ports and terminals, on seawalls, under wavecut notches along rocky shores, among mangroves or in seagrass meadows. If no vicinity near the site with the early-stage diseased urchins is suitable for a control, examine other reefs in the area using spot check dives or manta tows.

Control Site. All *Diadema* appear healthy (Fig. 1), and none show signs of disease or death. Need 6 urchins.

Diseased Site. *Diadema* either look healthy (Fig. 1) or are in the first two, early stages of the disease (Figs.2, 3). Avoid sites with many tests (skeletons) that are empty but intact. Need 3 healthy looking and 6 early-stage diseased urchins.

***Diadema* Disease signs.** It is very important to learn the signs of the disease because collections must be limited to *Diadema* that appear healthy or are in the early stages of the disease (Fig. 1). Sick urchins pass through several, externally visible stages of disease (Figs. 2-4), and they die within about 1-2 days of first looking ill (Fig. 5). Their internal tissues already are being decomposed in the later stages of disease, and no longer suitable for research on the causation of this disease (Figs. 4-5).

Healthy

Figure 1. *Diadema* lack visible lesions. they cling tightly to the substratum with their tube feet, regardless of its angle (a,b,c). Their spines are held erect, move quickly to and fro in response to gentle hand waving over the tips, or pull together in "a multi-spear-like" defense if touched lightly with a tool (*e.g.*, any kind of fork, skewer, forceps, hand rake). **Can collect.**

Diseased Stages

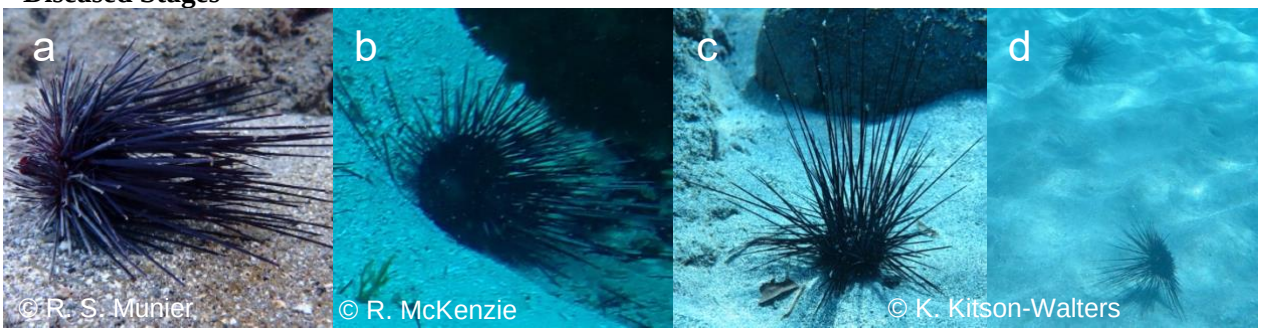

Figure 2. The tube feet lose their firm grip on the substratum and gradually stop moving. *Diadema* detach from vertical or inclined surfaces and fall into holes or open areas on a reef or hardground. They may even lie on their sides with all spines erect (a). Urchins are easily moved around by currents (b) or gentle prodding, and often drift into adjacent sandy areas (c, d). **Can collect.**

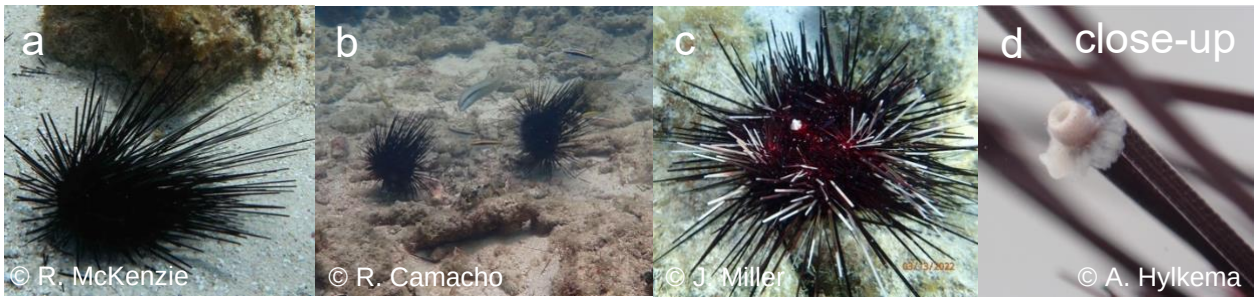

Figure 3. Soon the spines move slowly, even when repeatedly prodded; then barely respond to touch and droop (a). The urchins may drift (b) and land on their sides or up-side down (c). When the spine tips snap, the tissues around the broken ends of the tips come off as "mucous rings" (d). **Can collect.**

Figure 4. The spines completely stop moving, and, with the outer epidermis (skin) start to slough off, forming lesions (a, b, c) that expose the underlying white test (skeleton). Scavenging fishes (b, c) or invertebrates often appear, and the internal tissues have started to decompose. **Do not collect**.

Dead

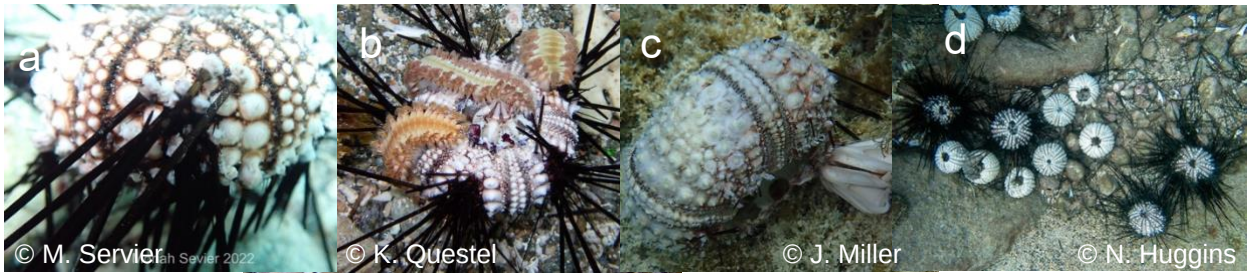

Figure 5. Spines detach from the tests as the urchins die (a). Fireworms (b) and other scavengers may be attracted to the decaying tissues. The tests start to break apart and the urchin's Aristotle's Lantern (c) may be exposed. Skeletal remains (d) are soon broken and dispersed. **Do not collect.**

Collection of Tissue Samples. If no *Diadema* have died in your area, limit collections to healthy urchins in Control Sites. If diseased *Diadema* already are present in the area, first collect at the Control Site and process these urchins before proceeding to the site with both healthy looking and early-stage diseased animals.
Whenever possible, plan to have a second team collecting the benthic environmental samples and recording habitat information while an urchin team is collecting the *Diadema.*

If the processing location is close to the control and diseased sites, it may be possible to finish the collection and processing of the control urchins in the morning, followed by the collection and processing of the urchins at the diseased site in the afternoon. Notice, however, that more urchin samples are needed at the diseased site (9 *versus* 6 controls). In general, teams may wish to spread collection and processing over two days.

Processing of Collected Samples. Keep all urchins in fresh changes of ambient seawater until ready for processing, which should be done *as soon as possible* after collection. Similarly process the urchins from the diseased site *as soon as possible* after their collection, starting with those that look healthy.

A table, countertop, or equivalent hard surface on a stable platform is needed when processing the bagged *Diadema* (no rocking boats–formalin is carcinogenic, as well as being an irritant!), along with a portable ice chest if a freezer is not immediately available. If collecting in a small boat, and more than about 2 hours at most away from wherever the samples will be stored, be prepared to process the urchins on shore somewhere close to the collection site and take a portable ice chest until you can locate a freezer!

Fixative Solutions for Light Microscopy. Avoid touching or inhaling the fixatives. Wear gloves when handling. If indoors, use a laboratory fume hood whenever possible, or turn on a fan or open windows. If outdoors, seek a shaded, breezy area.

For ~5% Z-fix solution, use a graduated cylinder to dilute 18.5% Z-Fix concentrate with seawater at a ratio of 1 part Z-Fix, and 4 parts clean seawater.
For 10% seawater formalin, use a graduated cylinder to dilute 37% formaldehyde with seawater at a ratio of 1 part 37% formaldehyde and 9 parts clean seawater.
Fixatives can be stored ready for use in a gallon jug at room temperature.

Healthy Control Site Collection.
Start here in a site without any signs of the *Diadema* disease to collect a total of 6 healthy urchins and some benthic samples. The control site should be sampled ***before the diseased site*** to reduce the chance of introducing the disease at a healthy site.

Materials

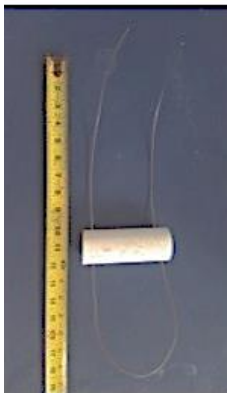
Fig. 6 © R. Francis-Floyd

- Snorkel and, if needed, scuba equipment
- UW camera
- Underwater slates and pencil (include spare pencils)
- Mesh collecting bag or equivalent for the collecting equipment
- Hand or cultivator rake, large fork, tongs or equivalent *(e.g.,* bended rebar wire or coat hanger with a PVC pipe handle, see Fig. 6) for moving urchins
- **1** milk crate or at least **1,** 2-gal. bucket for transporting the urchins underwater
- At least 6 large (2 gallon), numbered Ziploc bags (or equivalent) for urchins
- At least 6 small, numbered Ziploc bags (or equivalent) for benthic samples
- 2, 50 ml tubes or sandwich-sized Ziploc bags for sediments
- 2 Prenumbered Ziploc containers (or equivalent)
- At least **2** large, intact, marked, 5-gal. buckets or larger number of smaller buckets or 2 large coolers to hold urchins in seawater after collection
- 1 cooler with ice packs for benthic samples
- Sharpie or marker pen
- Roll of aluminum foil
- Squeeze bottles with freshwater (ideally deionized or distilled water)

***Diadema* Collection Method.** Six healthy urchins.

1. Locate 6 healthy *Diadema* that firmly grip the substrata and have "normally" mobile spines (Fig. 1).
2. Photograph one of the numbered collecting bags (this will serve as the urchin's unique identification number), the appearance of one of these urchins in its habitat and, if possible, make a close-up video showing the active movement of its spines.
3. Carefully detach this urchin from the substratum with the hand rake, fork or tongs and place it in the numbered plastic bag in the milk crate or in a bucket with holes. Write the bag no. on a slate or photo the urchin in the numbered bag.
4. Repeat steps 2 and 3 with another 2 urchins.
5. At the boat or shore and carefully transfer all 3 *Diadema* to fresh ambient seawater in at least 1 large bucket or a cooler. Keep in their individual plastic bags.
6. Collect 3 more healthy urchins and place them in a second large bucket or other container with fresh seawater (photography isn't necessary for these specimens).
7. Proceed to tissue preparation as soon as possible *(i.e.,* <2 hours) unless seawater is refreshed at frequent intervals in a large tank or live well in a boat to keep the *Diadema* from overheating during transit. If collecting in a small boat, and more than about 2 hours at most away from wherever the samples will be stored, be prepared to process the urchins on shore somewhere close to the collection site. Beach processing will probably be needed if the diseased site is some distance from the control.

Record the site (as control) and urchin condition (as healthy looking) for each numbered bag.

Benthic Samples Collection Method. *If possible, to be done by a second team.*

1. Collect and bag representative algal samples (~ 0.1kg for each taxon) from the same locations as urchins are collected. Include peyssonnelid algae if present (since sea urchins are the only animals known to feed on them).
2. Without touching the animal, carefully transfer any *Hermodice carunculata* (bearded fireworms) that are present in the nearby area to a prenumbered Ziploc plastic container (or equivalent) and close tightly.
3. Collect surface sediment samples by scooping into 1 or 2 50 ml conical tubes or 2 of the sandwich bags.
4. Collect any ambient *Diadema* fecal pellets as are present by scooping into a 50 ml conical tube or sandwich bag.
5. Return to the boat or shore and carefully rinse each algal taxon with fresh (preferably deionized) water, drain excess water, cover in aluminum foil, then put in a numbered plastic bag and place in the cooler with ice packs.
6. Collect a field blank by taking a piece of aluminum foil and rinsing with the same water as used for algae. Drain the excess water, fold, put in a numbered plastic bag and place in the cooler with ice packs.
7. Put the sediment and the *Diadema* fecal pellets and *Hermodice* (if any) in the cooler with ice packs.
8. Be sure to record the site corresponding to each numbered bag and record collection dates.

Habitat information. *If possible, to be done by a second team.* Before leaving be sure to note a few important characteristics of the habitat on your slate.

1. Estimate the density of urchins that are diseased, dying or dead as their approximate number/10m.^{2}
2. Note any sympatric organisms that also appear to be dying or dead– especially sponges, any other echinoderms (sea stars, other urchins, sea cucumbers).
3. Note water clarity – what is the approximate horizontal visibility underwater at the time of collection (in meters). Photo if unusual.
4. If a thermometer is available, note seawater temperature above the substratum.

Habitat Photos. *If possible, to be done by the second team.*

A good set of high-resolution, underwater photos of the general area is worth a thousand words. Highly recommended are some panoramic or wide-view photos of the general sampling area.

Processing Healthy Control Samples.

Materials

- Nitrile or other gloves not easily punctured by urchin spines
- Eye protection (glasses, goggles or dive mask)
- Fume hood or open windows or turn on a fan if indoors; seek breezy shade if outdoors
- Hand or cultivator rake, large fork, tongs or equivalent (see Fig.6 for a handmade tool)
- ENT shears (or heavy scissors),
- Sharp-ended scissors, *e.g.*, some nail scissors
- A way to clean and dry all shears and scissors used after each use (tissues or Kimwipes™)
- Labels
- Cutting board
- Digital camera and ruler (for scale bar)
- 3, 500 ml (or 16 oz.) wide-mouth jars or sealable food containers *(e.g.*, Ziploc)
- ~1.5 L of ~5% seawater buffered Z-Fix or 10% seawater buffered formalin
- 3, 25G needles, 3, new 1ml syringes, 3, 3ml cryovials pre-filled with 2 ml RNALater (in **Bag #1** if kit is provided)
- 3, 3ml cryovials pre-filled with 2 ml DNA/RNA Shield (or RNALater) (in **Bag #2** if kit is provided)
- 3, 0.5oz plastic cosmetic jars pre-filled with 3 ml of RNALater (in **Bag #3** if kit is provided)
- 6, 1.2ml cryovials pre-filled with 1.2 ml RNALater (in **Bag #4** if kit is provided)
- 9, 1.2ml cryovials pre-filled with 1 ml Trump's TEM fixative (in **Bag #5** if kit is provided)
- Pencil, or a chemically resistant, permanent marker pen (not a Sharpie®)
- Sharpies/marker pens (provided)
- Ziploc Bags

Processing the 3 Bagged *Diadema.* Start with one urchin. When finished, repeat in turn with the other two. Be careful that each animal retains the unique healthy number to which it was originally assigned in the field.

1. To avoid puncture wounds while handling, using the gloves and eye protection, take *Diadema* with tongs, large fork or small rake and put it on the cutting board.
2. (If provided, use the materials in **Bag # 1**.) Attach a new 25 G needle to a new 1ml syringe. One person should maintain the urchin upside down, with its ventral surface uppermost, on a small rake, large fork or equivalent tool, using one hand if needed to steady the urchin. While pointing away from the midline to avoid Aristotle's lantern and making sure to keep the penetration depth shallow to avoid puncturing the animal's digestive tract, a second person carefully inserts the needle through the test near the mouth at an angle of about 70^{o} to the urchin's vertical axis. Then the plunger is gently withdrawn to allow the collection of 0.5 ml of the coelomic fluid from its body cavity and the needle is removed. The fluid is transferred to a 3ml cryovial containing 2ml of RNALater, capped, and labeled with its unique urchin number. (See demonstration in Williams 2022).
3. (If provided, use the materials in **Bag # 2**.) While the Diadema is still lying upside down on the tool, pull upwards on one of its short, ventral spines to detach it along with some of the tissues around the base. Place in a 3ml cryovial prefilled with 3 ml of DNA/RNA Shield (or RNALater). Similarly remove another 2 spines with tissue bases, transfer them to the same cryovial, cap and label with its unique urchin number. (Use the pliers included in **Bag #2** if necessary to dislodge a spine that resists separation, or substitute with another spine that proves to be more easily removed.)
4. Very carefully trim the remaining spines with the ENT shears or heavy scissors, leaving only 2 -3 cm at their bases and avoiding personal injury from the spines.
5. Divide the *Diadema* vertically in two *(i.e.*, separating its left and right hemispheres) to reveal the internal organs (Fig. 7). Start by inserting the sharp-ended scissors into the peristomal membrane (the fleshy, soft ring around the lantern on the underside of the animal) at one side,

cutting toward the top of the animal and while avoiding the anus then down again to the peristomal membrane on the other side. Try to leave most of the Aristotle's lantern and peristomal membrane on whichever is the 'slightly larger' part.

6. Place the two sections on a cutting board with the test sides down (Fig. 8) and take a picture of their internal tissues (Fig. 8), along with one of its identification labels.

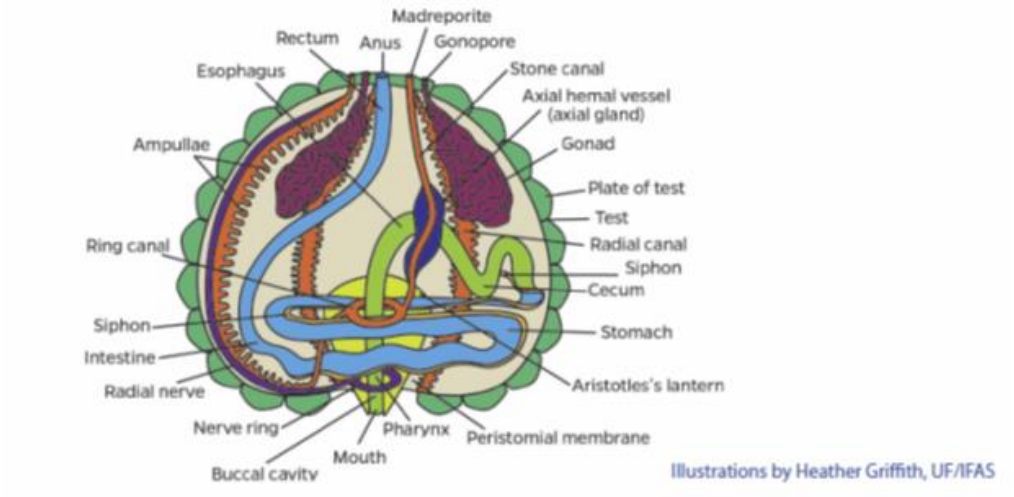

Fig. 7. Diagram of sea urchin anatomy. From Francis-Floyd et al. (2020).

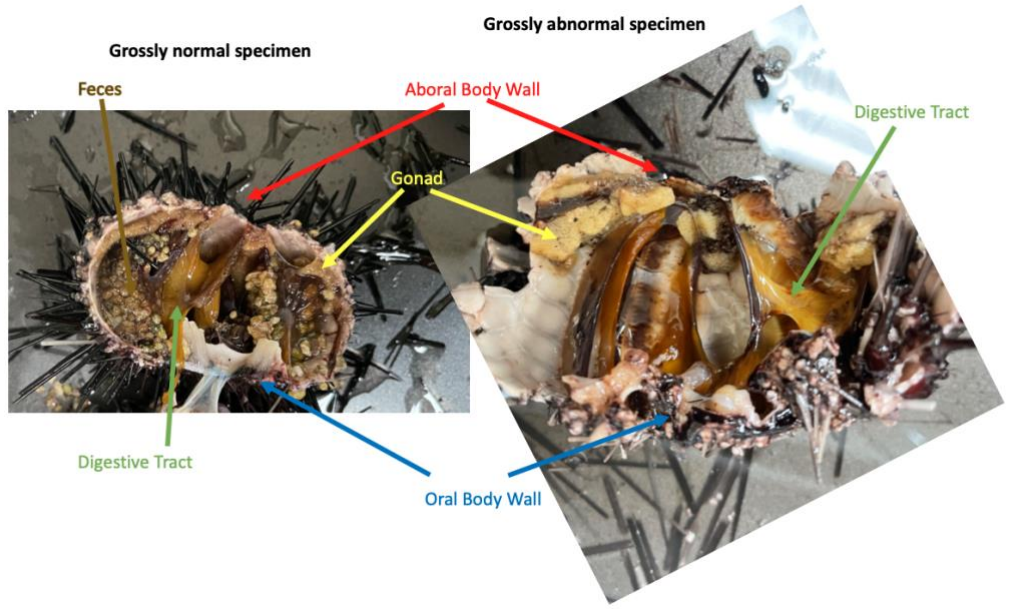

Fig. 8. Vertically sliced sections from a grossly normal *(i.e.*, healthy or healthy looking) *Diadema* (left) and a grossly abnormal *(i.e.*, early-stage diseased) *Diadema* (right). Photos © Ian Hewson.

Light Microscopy

1. Place the 'slightly larger' section in jar or a sealable container with its cut side up. Slowly add 5% Z-Fix or 10% seawater formalin to the jar or container to keep from disturbing the organs by gently filling at first from one side, then into the middle of the urchin, and finally by filling to the top. IMPORTANT: Make sure to use plenty of fixative (volume of tissue to fixative ratio should be **1:10**). Cap the jar lid tight or seal the container with its lid and use the **pencil or chemically resistant marker pen,** (but not a Sharpie) to add a label with its unique urchin

number and its condition (as healthy control), date collected, site name, and type of fixative used.
2. Store at **room temperature** for **at least 48 hours** before shipping. **Do not refrigerate or freeze.**

Microbial and Molecular. Target tissues to be dissected from the 'slightly smaller' section are the external epidermis (with test, ampullae, tube feet), plus the internal digestive tract (stomach and/or intestine), and gonads (see Figs. 7, 8).

1. (If provided, use the materials in **Bag # 3**.) For the epidermis, remove ~0.5 cm^2 pieces of the dorsal (aboral) and ventral (oral) surfaces of the specimen, using clean scissors or shears. Put these tissues in a labelled 0.5oz plastic cosmetic jar containing 0.3 ml of RNALater, cap and shears (=labelled cryovial). The volume of RNA Later should be at least 3X the volume of the sample.
2. (If provided, use the materials in **Bag # 4**.) Using the sharp-ended scissors, remove ~0.5cm^2 of the digestive tract and put in a labelled cryovial containing 2 ml of RNALater. Similarly remove ~0.5cm^2 of a gonad and put in a labelled cryovial with 2 ml of RNALater.
3. Put all samples in a marked Ziploc bag and **store in a freezer at -20 ºC until shipped.**

TEM. Target tissues to be dissected from the 'slightly smaller' section are the test with tube feet and digestive tract (see Figs. 7, 8).

1. (If provided, use the materials in **Bag # 5**.) Remove a small (~ 5 mm^2) portion of each of the following: any visible lesion on the test, the test with tube feet, and a section of the digestive tract. Place each in a cryovial containing 2 ml of Trump's fixative.
2. Put all samples in a marked Ziploc bag and **refrigerate**. **Do not freeze.**

Processing the Environmental Samples

1. Very carefully trim the spines from 1of the 3 unbagged *Diadema* as described above.
2. Cover the entire urchin with aluminum foil, place in a marked Ziploc bag and **store in a freezer at -20 ºC until shipped.**
3. Repeat with each of the other 2 unbagged *Diadema*.
4. Put all algal samples, the field blank, sediment and any other benthic samples in marked Ziploc bags and **store in a freezer at -20 ºC until shipped.**

Disinfection. After processing, disinfect all field gear and materials with a 10-minute, 5% bleach soak before any reuse

Metadata. Be sure to keep careful records for all collected samples. Create a backup and store in a secure location.

Shipping instructions will be provided at a later date.

Diseased Site Collection.

After processing the urchins from the control site (pp. 8-10), continue to the diseased site by collecting a total of 3 healthy looking urchins, 6 urchins in early stages of the disease, and some benthic samples at the diseased site.

Materials

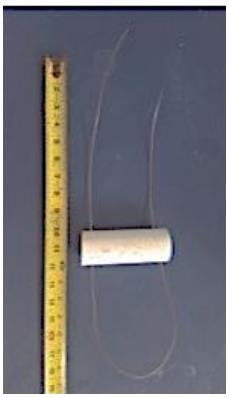

Fig. 6 © R. Francis-Floyd

- Snorkel and, if needed, scuba equipment
- UW camera
- Underwater slates and pencil (include spare pencils)
- Mesh collecting bag or equivalent for the collecting equipment
- Hand or cultivator rake, large fork, tongs or equivalent *(e.g.,* bended rebar wire or coat hanger with a PVC pipe handle, see Fig. 6) for moving urchins
- **2** milk crates or at least **2,** 2-gal. buckets for transporting the urchins underwater
- At least 9 large (2 gallon), numbered Ziploc bags (or equivalent) for urchins, with "healthy" addeded to nos. 1-3 and "diseased" added to nos. 4-9.
- At least 6 small, numbered Ziploc bags (or equivalent) for benthic samples
- 2, 50 ml tubes or sandwich-sized Ziploc bags for sediments
- 2 Prenumbered Ziploc containers (or equivalent)
- At least **3** large, intact, marked, 5-gal. buckets or a larger number of smaller buckets or 2 large coolers to hold urchins in seawater after collection
- 1 cooler with ice packs for benthic samples
- Sharpie or marker pen
- Roll of aluminum foil
- Squeeze bottles with freshwater (ideally deionized or distilled water)

Healthy Looking *Diadema* Collection Method. Three urchins. Do first.

1. Locate 3 healthy looking *Diadema* that firmly grip the substrata and have "normally" mobile spines (Fig. 1).
2. Photograph one of the numbered healthy collecting bags (this will serve as the urchin's unique identification number), the appearance of one of these urchins in its habitat and, if possible, make a close-up video showing the active movement of its spines.
3. Carefully detach this urchin from the substratum with the hand rake or tongs and place it in a numbered plastic bag in the milk crate or in a bucket with holes. Write the bag number on a slate or photo the urchin in the numbered bag.
4. Repeat steps 2 and 3 with another 2 urchins.
5. At the boat or shore and carefully transfer all 3 of these bagged *Diadema* to fresh ambient seawater in at least 1 large, marked bucket or a cooler.
6. Proceed to tissue preparation as soon as possible *(i.e.,* <2 hours) after collecting the early-stage diseased urchins.

Early-stage Diseased *Diadema* Collection Method. Six urchins. Do second.

1. Locate and select 6 diseased urchins that still show some movement in their spines when gently prodded, but which are no longer clinging to the substratum (Figs. 2, 3). Do not choose any individuals with drooping or detached spines or lesions (Figs. 4, 5).
2. Photograph one of the numbered diseased collecting bags, the appearance of one of these urchins in its habitat and, if possible, make a close-up video showing the active movement of its spines.
3. Carefully detach this urchin from the substratum with the hand rake or tongs and place it in the plastic bag in the milk crate or in a bucket with holes. Write the bag number on a slate or photo the urchin in the numbered bag.
4. Repeat steps 2 and 3 with another 2 urchins.
5. At the boat or shore, carefully transfer all 3 of these bagged *Diadema* to fresh ambient seawater in at least 1 other large, marked bucket or a cooler.

6. Collect 3 more early-stage diseased urchins and place them in a second large bucket or other container with fresh seawater (photography isn't necessary for these specimens).
7. Proceed to tissue preparation as soon as possible *(i.e.,* <2 hours) unless seawater is refreshed at frequent intervals in a large tank or live well in a boat to keep the *Diadema* from overheating during transit. If collecting in a small boat, and more than about 2 hours at most away from wherever the samples will be stored, be prepared to process the urchins on shore somewhere close to the collection site. Beach processing will probably be needed if the diseased site is some distance from the control.

Record the site (as diseased) and the condition (as healthy looking or diseased) for each numbered bag.

Benthic Samples Collection Method. *If possible, to be done by a second team.*

1. Collect and bag representative algal samples (~ 0.1kg for each taxon) from the same locations as the urchins are collected. Include peyssonnelid algae if present (since sea urchins are the only animals known to feed on them).
2. Without touching the animal, carefully transfer any *Hermodice carunculata* (bearded fireworms) that are present in the nearby area to a prenumbered Ziploc plastic container (or equivalent) and close tightly.
3. Collect surface sediment samples by scooping into 1 or 2 50 ml conical tubes or 2 of the sandwich bags.
4. Collect any ambient *Diadema* fecal pellets as are present by scooping into 1 50 ml conical tube or sandwich bag.
5. Return to the boat or shore and carefully rinse each algal taxon with fresh (preferably deionized) water, drain excess water, cover in aluminum foil, then put in a numbered plastic bag and place in the cooler for environmental samples.
6. Collect a field blank by taking a piece of aluminum foil and rinsing with the same water as used for algae. Drain the excess water, fold, put in a numbered plastic bag and place in the cooler for environmental samples.
7. Put the sediment and the *Diadema* fecal pellets and *Hermodice* (if any) in the cooler.
8. Be sure to record the site corresponding to each numbered bag and label date collected.

Habitat information. *If possible, to be done by a second team.*

Before leaving be sure to note a few important characteristics of the habitat on your slate.

1. Estimate the density of urchins that are diseased, dying or dead as their approximate number/10m.^{2}
2. Note any sympatric organisms that also appear to be dying or dead– especially sponges, any other echinoderms (sea stars, other urchins, sea cucumbers).
3. Note water clarity – what is the approximate horizontal visibility underwater at the time of collection (in meters). Photo if unusual.
4. If a thermometer is available, note seawater temperature above the substratum.

Habitat Photos. *If possible, to be done by the second team.*

A good set of high-resolution, underwater photos of the general area is worth a thousand words. Highly recommended are some panoramic or wide-view photos of the general sampling area.

Processing Diseased Site Samples

Materials

- Nitrile or other gloves not easily punctured by urchin spines
- Eye protection (glasses, goggles or dive mask)
- Fume hood, open windows or turn on a fan if indoors; seek breezy shade if outdoors
- Hand rake or tongs (see Fig.6 for a handmade tool)
- ENT shears (or heavy scissors),
- Sharp-ended scissors, *e.g.*, nail scissors
- A way to clean and dry all shears and scissors used after each use (tissues or Kimwipes™)
- Labels
- Cutting board
- Digital camera and ruler (for scale bar)
- 6, 500 ml (or 16 oz.) wide-mouth jars or sealable food containers *(e.g.,* Ziploc)
- ~1.5 L of ~5% seawater buffered Z-Fix or ~10% seawater buffered formalin.
- 3 25G needles, 3, new 1ml syringes, 3, 3ml cryovials pre-filled with 2 ml RNALater (in **Bag #1** if kit is provided)
- 3, 3ml cryovials pre-filled with 2 ml DNA/RNA Shield (or RNALater) (in **Bag #2** if kit is provided)
- 3, 0.5oz plastic cosmetic jars pre-filled with 3 ml of RNALater (in **Bag #3** if kit is provided)
- 6, 1.2ml cryovials pre-filled with 1.2 ml RNALater (in **Bag #4** if kit is provided)
- 9, 1.2ml cryovials pre-filled with 1 ml Trump's TEM fixative (in **Bag #5** if kit is provided)
- Pencil, or a chemically resistant, permanent marker pen (not a Sharpie®)
- Sharpies/marker pens (provided)
- Ziploc Bags

Processing the 3 Bagged, Apparently Healthy *Diadema.* Process first**.** Start with one urchin. When finished, repeat in turn with the other two.

1. To avoid puncture wounds while handling, using the gloves and eye protection, take *Diadema* with tongs or a hand rake and put it on the cutting board.
2. Very carefully trim the urchin's spines with the ENT shears or heavy scissors, being careful since spines can cause injury, leaving only 2 -3 cm at their bases.
3. Divide the *Diadema* vertically in two *(i.e.*, dividing left and right hemispheres) to reveal its internal organs (Fig. 7). Start by inserting the sharp-ended scissors into the peristomal membrane (the fleshy, soft ring around the lantern on the underside of the animal) at one side, cutting toward the top of the animal and while avoiding the anus then down again to the peristomal membrane on the other side. Try to leave most of the Aristotle's lantern and peristomal membrane on whichever is the 'slightly larger' part.
4. Place the two sections on a cutting board with the test sides down (Fig. 8) and take a picture of its internal tissues (Fig. 8), along with one of its identification labels.

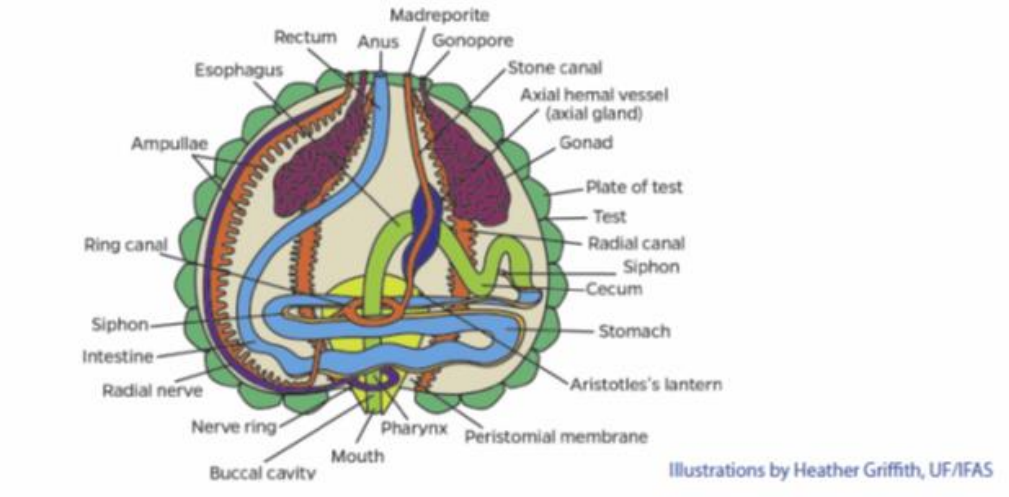

Fig. 7. Diagram of sea urchin anatomy. From Francis-Floyd et al. (2020).

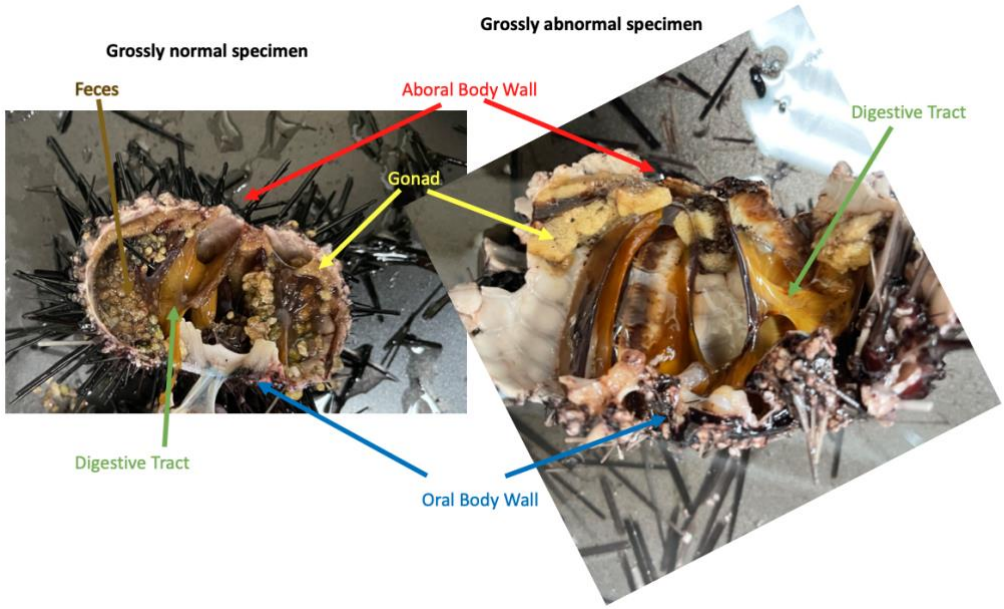

Fig. 8. Vertically sliced sections from a grossly normal *(i.e.*, healthy or healthy looking) *Diadema* (left) and a grossly abnormal *(i.e.*, early-stage diseased) *Diadema* (right). Photos © Ian Hewson.

Light Microscopy

1. Place the 'slightly larger' section in jar or a sealable container with its cut side up. Slowly add 10% seawater formalin or Z-Fix to the jar to keep from disturbing the organs by gently filling at first from one side, then into the middle of the urchin, and finally by filling to the top. IMPORTANT: Make sure to use plenty of fixative (volume of tissue to fixative ratio should be **1:10**). Cap the jar and use the **pencil or chemically resistant marker pen,** (but not a Sharpie) to label with its unique specimen identification number and its condition (as healthy looking), date collected, site name, and type of fixative used.
2. Store at **room temperature** for **at least 48 hours** before shipping. **Do not refrigerate or freeze.**

Environmental Contaminants

1. Cover the entire 'slightly smaller' section with aluminum foil, place in a marked Ziploc bag and **store in a freezer at -20 ºC until shipped.**

Process the 3 Bagged, Early-stage Diseased, *Diadema*. Start with one urchin. When finished, repeat in turn with the other two. Be careful that each animal retains the unique diseased number to which it was originally assigned in the field.

1. To avoid puncture wounds while handling, using the gloves and eye protection, take *Diadema* with tongs, large fork or small rake and put it on the cutting board.
2. (If provided, use the materials in **Bag # 1**.) Attach a new 25 G needle to a new 1ml syringe. One person should maintain the urchin upside down, with its ventral surface uppermost, on a small rake, large fork or equivalent tool, using one hand if needed to steady the urchin. While pointing away from the midline to avoid Aristotle's lantern and, being sure to keep the penetration depth shallow to avoid puncturing the animal's digestive tract, a second person carefully inserts the needle through the test near the mouth at an angle of about 70^{o} to the urchin's vertical axis. Then the plunger is gently withdrawn to allow the collection of 0.5 ml of the coelomic fluid from its body cavity and the needle is removed. The fluid is transferred to a 3ml cryovial containing 2ml of RNALater, capped, and labeled with its unique urchin number. (See demonstration in Williams 2022).
3. (If provided, use the materials in **Bag # 2**.) While the Diadema is still lying upside down on the tool, pull upwards on one of its short, ventral spines to detach it along with some of the tissues around the base. Place in a 3ml cryovial prefilled with 3 ml of DNA/RNA Shield (or RNALater). Similarly remove another 2 spines and tissue bases, transfer them to the same cryovial, cap and label with its unique urchin number. (Use the pliers included in **Bag #2** if necessary to dislodge a spine that resists separation, or substitute with another spine that proves to be more easily removed.)
4. Very carefully trim the remaining spines with the ENT shears or heavy scissors, leaving only 2 -3 cm at their bases and avoiding personal injury from the spines.
5. Divide the *Diadema* vertically in two *(i.e.*, separating its left and right hemispheres) to reveal the internal organs (Fig. 7). Start by inserting the sharp-ended scissors into the peristomal membrane (the fleshy, soft ring around the lantern on the underside of the animal) at one side, cutting toward the top of the animal and while avoiding the anus then down again to the peristomal membrane on the other side. Try to leave most of the Aristotle's lantern and peristomal membrane on whichever is the 'slightly larger' part.
6. Place the two sections on a cutting board with the test sides down (Fig. 8) and take a picture of their internal tissues (Fig. 8), along with one of its identification labels.

Light microscopy

1. Place the 'slightly larger' section in jar or a sealable container with its cut side up. Slowly add 5% Z-Fix or 10% seawater formalin to the jar or container to keep from disturbing the organs by gently filling at first from one side, then into the middle of the urchin, and finally by filling to the top. IMPORTANT: Make sure to use plenty of fixative (volume of tissue to fixative ratio should be **1:10**). Cap the jar lid tight or seal the container with its lid and use the **pencil or chemically resistant marker pen,** (but not a Sharpie) to add a label with its unique urchin number and its condition (as early-stage disease), date collected, site name, and type of fixative used.
2. Store at **room temperature** for **at least 48 hours** before shipping. **Do not refrigerate or freeze.**

Microbial and molecular. Target tissues to be dissected from the 'slightly smaller' section are the external epidermis (with test, ampullae, tube feet), plus the internal digestive tract (stomach and/or intestine), and gonads (see Figs. 7, 8).

1. (If provided, use the materials in **Bag # 3**.) For the epidermis, remove ~0.5 cm^2 pieces of the dorsal (aboral) and ventral (oral) surfaces of the specimen, using clean scissors or shears. Put these tissues in a labelled 0.5oz plastic cosmetic jar containing 0.3 ml of RNALater, cap and label with its unique urchin number (=labelled cryovial). The volume of RNA Later should be at least 3X the volume of the sample.

2. (If provided, use the materials in **Bag # 4**.) Using the sharp-ended scissors, remove ~0.5cm^2 of the digestive tract and put in a labelled cryovial containing 2 ml of RNALater. Similarly remove ~0.5cm^2 of a gonad and put in a labelled cryovial with 2 ml of RNALater.
3. Put all samples in a marked Ziploc bag and **store in a freezer at -20 ºC until shipped.**

TEM. Target tissues to be dissected from the 'slightly smaller' section are any lesions on the test that is visible, the test with tube feet and digestive tract (see Figs. 7, 8).

1. (If provided, use the materials in **Bag # 5**.) Remove a small (~ 5 mm^2) portion of each of the following: any visible lesion on the test, the test with tube feet, and a section of the digestive tract. Place each in a labelled cryovial containing 2 ml of Trump's fixative.
2. Put all samples in a marked Ziploc bag and **refrigerate**. **Do not freeze.**

Processing the Environmental Samples

1. Very carefully trim the spines from 1of the 3 unbagged *Diadema* as described above.
2. Cover the entire urchin with aluminum foil, place in a marked Ziploc bag and **store in a freezer at -20 ºC until shipped.**
3. Repeat with each of the other 2 unbagged *Diadema*.
4. Put all algal samples, the field blank, sediment and any other benthic samples in marked Ziploc bags and **store in a freezer at -20 ºC until shipped.**

Disinfection. After processing, disinfect all field gear and materials with a 10-minute, 5% bleach soak before any reuse

Metadata. Be sure to keep careful records for all collected samples. Create a backup and store in a secure location.

Shipping instructions will be provided at a later date.

References

Francis-Floyd, R., Landsberg, J., Yanong, R., Kiryu, Y., Baker, S., Pouder, D., Sharp, W., Delgado, G., Stacy, N., Waltzek, T., Walden, H., Smolowitz, R., Beck, G., 2020. Diagnostic methods for the comprehensive health assessment of the long-spined sea urchin, *Diadema antillarum*. EDIS 2020 (3). Available for download at https://journals.flvc.org/edis/article/view/107904

Williams, SM, 2022 Coelomic fluid collection. Mp4. 11.3 MB. If you are interested, please contact info@agrra.org for more information.