

Journal of Visualized Experiments

Streamlined Sampling and Cultivation of the Pelagic Cosmopolitan Larvacean, *Oikopleura dioica*

--Manuscript Draft--

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|--|---|
| Article Type: | Methods Article - Author Produced Video |
| Manuscript Number: | JoVE61279R1 |
| Full Title: | Streamlined Sampling and Cultivation of the Pelagic Cosmopolitan Larvacean, <i>Oikopleura dioica</i> |
| Section/Category: | JoVE Environment |
| Keywords: | Oikopleura, Appendicularian, Larvacean, Plankton, Zooplankton, Culture, Sampling, Tunicate, Algae, Marine, Growth |
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| Additional Information: | |
| Question | Response |
| Please indicate whether this article will be Standard Access or Open Access. | Open Access (US\$3000) |

TITLE:

Streamlined Sampling and Cultivation of the Pelagic Cosmopolitan Larvacean, *Oikopleura dioica*

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KEYWORDS:

Oikopleura, Appendicularian, Larvacean, Plankton, Zooplankton, Culture, Sampling, Tunicate, Algae, Marine, Growth

SUMMARY:

Oikopleura dioica is a tunicate model organism in various fields of biology. We describe sampling methods, species identification, culturing setup, and culturing protocols for the animals and algal feed. We highlight key factors that helped strengthen the culture system and discuss the possible problems and resolutions.

ABSTRACT:

Oikopleura dioica is a planktonic chordate with exceptional filter-feeding ability, rapid generation time, conserved early development, and a compact genome. For these reasons, it is considered a useful model organism for marine ecological studies, evolutionary developmental biology, and genomics. As research often requires a steady supply of animal resources, it is useful to establish a reliable, low-maintenance culture system. Here we describe a step-by-step method for establishing an *O. dioica* culture. We describe how to select potential sampling sites, collection methods, target animal identification, and the set-up of the culturing system. We provide troubleshooting advice based on our own experiences. We also highlight critical factors that help sustain a robust culture system. Although the culture protocol provided here is optimized for *O. dioica*, we hope our sampling technique and culture setup will inspire new ideas for maintaining other fragile pelagic invertebrates.

INTRODUCTION:

Model organisms have been instrumental in addressing many biological questions including those relating to development, genetics, and physiology. Moreover, additional model organisms facilitate new discoveries and therefore are crucial to achieve a greater understanding of nature^{1,2}. Marine zooplankton are diverse groups of organisms that play an important role in ocean ecosystems³⁻⁶. Despite their abundance and ecological importance, gelatinous organisms such as planktonic tunicates are often under-represented in plankton biodiversity studies because their transparency and fragility make field collection and identification challenging^{7,8}. Adapted sampling techniques and laboratory culturing allow closer observation of the animals in vitro, which has furthered the knowledge in the biology of planktonic tunicates⁹⁻¹².

Larvaceans (Appendicularians) are a class of free-swimming marine tunicates comprising around 70 described species worldwide^{8,13}. As they are one of the most abundant groups within zooplankton communities¹⁴⁻¹⁷, larvaceans represent a primary food source for larger planktonic organisms such as fish larvae^{18,19}. Unlike ascidians-the sessile tunicates-larvaceans retain a tadpole-like morphology and remain planktonic throughout their lives²⁰. Each animal lives inside a self-built, intricate filter-feeding structure known as a house. They accumulate particulates in their houses by creating water currents through the undulating motion of their tails²¹. Clogged houses are discarded throughout the day, some of which form carbon aggregates and eventually sink to the seabed²²; thus, larvaceans play a major role in global carbon flux²³. Most species are reported to live in the pelagic zone within the upper 100 m of the water column¹³; however, the giant larvacean *Bathochordaeus* is known to inhabit the depths of 300 m²⁴. A study on *Bathochordaeus* in Monterey Bay, California revealed that the animals also serve as a biological vector of microplastics, suggesting a potential importance in understanding the role of appendicularians in the vertical transport and distribution of microplastics in the oceans²⁵.

Oikopleura dioica, a species of larvacean, has attracted attention in recent years as a model organism due to several remarkable characteristics. It is commonly reported throughout the world's oceans. It is especially abundant in coastal waters²⁶, which allows easy sampling from the shore. Long-term, stable culturing is possible with both natural and artificial seawater²⁷⁻²⁹. Temperature dependent generation times are as short as 4-9 days in laboratory conditions. It has high fecundity with each female capable of producing >300 eggs throughout the year. As a urochordate, it occupies an important phylogenetic position for understanding chordate evolution^{30,31}. At 70 Mb, *O. dioica* has the smallest identified genome amongst all chordates³². Among larvaceans, *O. dioica* is the only described non-hermaphroditic species thus far³³.

The first successful *O. dioica* culture with laboratory grown microalgae was reported by Paffenhöfer³⁴. The original culture protocol using synchronous motors and paddles was developed by Fenaux and Gorsky³⁵ and later adopted by multiple laboratories. More recently, Fujii et al.³⁶ reported *O. dioica* culturing in artificial seawater, a robust culture system and field collection were described by Bouquet et al.²⁷ and an optimized protocol for a simplified, affordable system was reported by Marti-Solans et al.²⁹. Aside from the traditional *Oikopleura* culture system, a newly reported design with a double tube rearing tank also has the potential to culture *Oikopleura sp.*³⁷.

We present a detailed protocol for initiating an *O. dioica* monoculture based on a combination of protocols developed by major *Oikopleura* research groups at the Sars International Centre for Marine Molecular Biology²⁷, the University of Barcelona²⁹, Osaka University²⁸, and our own observations. In previously published culture protocols, detailed information regarding the composition of algal media, shore sampling techniques, and *Oikopleura* identification were only roughly described, leaving a lot of ambiguity. Here, with the aid of visual information in the video protocol, we have assembled all the critical information needed to set up an *O. dioica* culture from the ground up in a straightforward, step-by-step manner. We describe how to distinguish *O. dioica* from another commonly reported species, *O. longicauda*, which is one of the most challenging steps. Although the existing culture systems are applicable for the cultivation of *O. dioica* worldwide, we highlight the importance of protocol adjustment based on local environmental conditions. The presented information combines widely published data as well as knowledge gained through experience. The current protocol is ideally suited for researchers interested in establishing a culture from scratch.

PROTOCOL:

1. *O. dioica* culture facility

1.1. Water filter system (Figure 1)

1.1.1. Collect natural seawater from a harbor at 2-3 m depth. Pass the seawater through a sand filter (pore size 1.4 mm) and transport to a shared reservoir tank in the laboratory. Use a canister filter to circulate the water in order to maintain the water quality in the shared reservoir tank.

1.1.2. In a culture room, set up a multi-step filter system consisting of a 100 L reservoir tank with a magnetic drive pump, 5 µm and 1 µm polypropylene wound cartridge filters, and a UV sterilizer (100 V) (Figure 1).

1.1.3. Transfer the seawater from the shared reservoir tank to the culture room reservoir tank. Pass the seawater through a 25 µm filter unit (Figure 1A,B) before entering the culture room reservoir tank. Circulate the seawater through 5 and 1 µm filters overnight to thoroughly remove particles that could potentially hinder the development of animals.

NOTE: An additional filter with larger mesh size (25-50 µm) is useful to prevent larger particles from clogging the cartridge filters with smaller mesh sizes. The filtered seawater (fSW) is ready for use the following morning.

1.2. *Oikopleura* culturing unit (Figure 2)

1.2.1. Maintain animals in 5 or 10 L round, transparent plastic beakers.

1.2.2. Place culture beakers on a steady, two-level stainless-steel shelving unit (L x W x H = 150 cm x 45 cm x 90 cm) with a 5 mm thick, transparent acrylic surface board.

1.2.3. Position white fluorescent lights underneath the acrylic surface to illuminate the animals from the bottom of the beakers.

1.2.4. Place a black plastic sheet behind the beakers. The black sheet creates contrast and enhances visualization of the transparent animals.

1.2.5. Connect synchronous electric motors to acrylic paddles (L x H = 8 cm x 27 cm) (**Supplemental File 2**). Suspend the paddles in the culture beakers from parallel rails running along the length of the shelving unit (**Figure 2A**).

1.2.6. Switch on the motors to generate a gentle circular motion in the beakers at 15 RPM.

NOTE: Animals in their cellulose houses are neutrally buoyant; however, water circulation helps to keep eggs, larvae, and algal food to be suspended and evenly distributed in the culture beakers.

1.3. Automatic dosing pump (Optional)

NOTE: An automatic feeding unit reduces staffing requirements, especially during the weekends.

1.3.1. Calibrate the volume of dispensing liquid from an automatic dosing pump according to the manufacturer's instructions.

1.3.2. Use 50 mL tubes as algal reservoirs.

1.3.3. Drill two 5 mm holes on the caps of 50 mL tubes to pass through airline tubing. Connect one tube to a standard aquarium air pump to introduce air bubbles, and the other tube to the inlet port of dosing pump (**Figure 2B**).

NOTE: Introducing a thin stream of air bubbles helps to prevent algae from settling on the bottom of the tubes.

1.3.4. Program the time and volume of algal feed to be dispensed on a given day.

1.4. Algae station

1.4.1. Use a shelving unit (L x W x H = 90 cm x 46 cm x 115 cm) to place four 1 L round bottom flasks containing algal working cultures (See step 2.1).

1.4.2. Back-illuminate the working cultures by placing fluorescent lights behind the flasks.

1.4.3. Seal flasks with two-hole rubber stoppers.

1.4.4. Pass a 1 mL disposable pipette through the rubber stopper. Use airline tubing to connect the pipette to an aquarium air pump. Introduce a stream of air bubbles in the flask.

2. Microalgal food

2.1. Initiating algal cultures

NOTE: Maintain three sets of cultures (stock, sub-, and working cultures) for three microalgal species, *Chaetoceros calcitrans*, *Isochrysis* sp., *Rhinomonas reticulata*, and one species of cyanobacteria, *Synechococcus* sp.. Stock and sub-cultures are used as back-ups. The working culture is used for daily feeding.

2.1.1 Prepare reagents necessary for the cultivation of microalgae and cyanobacteria (**Table 1**).

2.1.2. To initiate stock culture, autoclave (121 °C, 25 min) 60 – 80 mL of fSW in a 100 mL Erlenmeyer flask. Aseptically inoculate specified amount of modified Conway medium²⁷ and microalgae (**Table 2**). For example, to inoculate a stock culture of *C. calcitrans*, autoclave 60 mL of seawater, aseptically inoculate 30 µL each of the vitamin and solution A, 15 µL of sodium silicate, 60 µL of streptomycin, and 30 µL of *C. calcitrans* from the previous stock culture.

NOTE: *R. reticulata* turns from reddish-pink to orangish-brown when exposed to too much light. Move them away from light once they have started to turn from clear to light pink.

2.1.3. Maintain the stock culture in an incubator set at 17 °C with continuous lighting. After about 10 days, the culture changes color to indicate algal growth (**Figure 3**). Once the colors appear, move them to 4 °C for long-term storage for up to 1 month.

2.1.4. On a clean bench, aseptically inoculate a sub-culture from the stock culture (**Table 2**). Incubate at 17 °C with continuous lighting. After algal colors appear, continue to store them in the incubator up to 2 weeks.

2.1.5. Inoculate working culture from sub-culture (**Table 2**). Seal the flask with a rubber cap and insert 1 mL disposable pipette. Move the flask to the algae station and maintain at room temperature with a 8 h photoperiod. Supply with constant aeration. Renew the working culture every 4 days.

2.1.6. Stir stock and sub-cultures twice a day by swirling.

NOTE: Long-term storage of algal culture on solid media and cryopreservation are possible up to 3 months and 1 year, respectively²⁹.

2.2. Creating algal growth curves (Optional)

NOTE: Accurate assessment of feeding quantity is important to maintain a stable culture of *O. dioica*. We created growth curves for two primary algal food species, *Chaetoceros calcitrans* and *Isochrysis* sp.

2.2.1. Prepare *C. calcitrans* and *Isochrysis* sp. working cultures (**Table 2**).

2.2.2. For each species of working culture, sample three separate times and measure absorbances at 660 nm using a spectrophotometer. Take the average measurements of the triplicates from each working culture.

2.2.3. Following the manufacturer's instructions for an automated cell counter, prepare algal samples for counting. Count each sample three times. Take the average of three counts to determine the total number of cells present in each sample.

2.2.4. Continue to count daily until approximately 50 average measurements are recorded.

2.2.5. Create growth curves for both algal species (**Figure 4**).

3. Field collection of wild *Oikopleura* spp.

3.1. Modified plankton net (Figure 5**)**

NOTE: The key to successful sampling of *Oikopleura* spp. is the slow towing of a plankton net with a weighted, non-filtering cod-end. **Figure 5** shows a schematic diagram of a modified plankton net.

3.1.1. Replace the cod-end of a hand-held plankton net with a modified 500 mL screw-top wash bottle.

3.1.2. Drill a 3 cm diameter hole in the 4 cm diameter screw-top of the wash bottle to allow water and animals to enter the cod-end.

3.1.3. Fit the bottle cap at the end of plankton net. Wrap it tightly with electric tape. Secure the cap further with a stainless-steel hose clamp.

3.1.4. Attach a 70 g weight to the outside of the modified cod-end with zip ties.

3.1.5. Attach safety leash to further secure the cod-end.

3.2. Selecting collection sites (Figure 6**)**

NOTE: All sample collections were approved by the OIST Fieldwork Safety Committee. There might be seasonal variation in the presence of *Oikopleura* spp. depending on the location (**Figure 6**). Avoid sampling immediately after extreme weather events such as severe rainstorms.

3.2.1. Use the satellite view on a map website to identify potential sampling sites. We focused on harbors and fishing piers that are easily accessible by car and located inside bays or near ocean drop-offs where plankton tend to accumulate: Ishikawa harbor in Kin Bay, Okinawa, Japan (GPS: 26°25'39.3"N 127°49'56.6"E).

3.2.2. Visit potential sampling locations to assess shore accessibility and safety of each site. Obtain collection permit from local authorities as needed.

3.3. Sampling procedure

3.3.1. Cast the plankton net into the sea and allow the cod-end to sink 1-2 m below the water's surface.

3.3.2. Tow the net horizontally by hand at 50-100 cm s⁻¹. Continue towing by walking back and forth for 2-5 minutes. Adjust towing time according to the abundance of phytoplankton in the harbor, with shorter tow when there are more phytoplankton.

NOTE: Larvaceans are fragile animals. Fast towing or repeated casting of the net could damage animals trapped in the cod-end.

3.3.3. Gently lift the net. Slowly transfer the contents of the cod-end into a 500 mL round glass bottle. Completely fill the sample bottle with seawater to avoid air bubbles.

NOTE: The presence of *Oikopleura* spp. can be confirmed by viewing sample bottles against a black background. Most animals abandon their houses while being collected. Therefore, microscopic observation is needed for species-level identification.

3.3.4. Repeat sampling until three 500 mL bottles are collected.

3.3.5. Measure salinity, temperature, and chlorophyll a using a CTD profiler to record the range of physical parameters where animals naturally exist.

3.3.6. Collect 10-15 L of surface seawater in a bucket to acclimatise animals in the laboratory setting.

4. Animal isolation and identification (Figure 7, Figure 8)

4.1. *Oikopleura* spp. identification

NOTE: Other planktonic organisms that may resemble *Oikopleura* spp. at first glance include chaetognaths, *Fritillaria* spp., nematodes, fish larvae with yolk-sacs, and *Ciona* spp. larvae.

4.1.1. To acclimatize animals to laboratory conditions, transfer each 500 mL sample to a 10 L beaker containing 1:1 ratio of surface seawater from the sampling location and filtered seawater (fSW) maintained in the lab (**Figure 7A,B**). Adjust the volume of the beaker to 5-10 L depending on the concentration of plankton sample.

NOTE: If the plankton sample contains unwanted debris, run through a coarse filter (mesh size ~600 µm) before transferring to a 10 L beaker.

4.1.2. Use a paddle attached to a synchronous electric motor (15 RPM) and keep the plankton in suspension overnight (step 1.2.5).

4.1.3. Identify *Oikopleura* spp. by looking for 1-2 mm long, tadpole-shaped animals undulating their tails inside a spherical, translucent house. Some animals may be temporarily free-swimming without the houses. Gently transfer ~5 animals to an empty Petri dish using a blunt-end pipette.

4.1.4. For genus identification, evict animals from their houses by gently poking the house with a transfer pipette.

4.1.5. Observe houseless animals under a 20-40x dark-field microscope and confirm *Oikopleura* spp (**Figure 8**).

4.2. *O. dioica* identification

NOTE: *O. dioica* can be visually identified by the presence of fully mature males and females or two large subchordal cells located on the distal half of their tails. The distance between two subchordal cells may vary between individuals.

4.2.1. Next, check if there is a fully matured *Oikopleura* with a gonad filled with eggs (**Figure 8A**) or sperm (**Figure 8B**). If the animal only possesses eggs or sperm, skip to step 4.2.3 as it is *O. dioica*, the only described non-hermaphroditic species.

4.2.2. If the animal is immature (**Figure 8C**), look for two subchordal cells at the end of its tail (**Figure 8D**).

4.2.3. Once the species is confirmed, transfer it to a new Petri dish. Repeat steps 4.1.3-4.2.2 until 10-20 individuals are confirmed at species-level.

NOTE: For easier identification, anesthetize animals in a Petri dish containing 0.015% tricaine methanesulfonate (MS222) in fSW.

4.2.4. If no *O. dioica* are found, keep the beakers suspended for an extra day or two. There might be immature *O. dioica* that will continue growing and become easier to be detected. If none appear after a week, discard the sample, and try sampling again.

5. Cultivation protocol for *O. dioica*

5.1. Initiating an *O. dioica* monoculture from a field collected sample (Figure 7)

NOTE: Algal food is prepared daily from working cultures and each monoculture beaker is fed three times a day at 9 AM, 12 PM, and 5 PM, respectively (See step 5.2). The animals are maintained at 23 °C. Under these conditions, the Okinawa *O. dioica* lifecycle is 4 days (Figure 7C).

5.1.1. To initiate a monoculture of *O. dioica*, isolate 120 animals and transfer to a new beaker containing 5 L of fresh fSW (Figure 7B, C).

5.1.2. The following morning, look for fully mature males with yellow gonads and females with eggs that appear as golden spheres (Figure 8A, B).

5.1.3. Make a spawning beaker by gently transferring 15 males and 30 females to a new beaker containing 2.5 L of fresh fSW with a 5 mL blunt-end pipette.

NOTE: If there are not enough males and females, transfer as many adults as possible to a beaker containing 1 L of fSW and let them spawn naturally. To minimize physical stress to animals during manual transfer, they should be slowly siphoned and released under the water surface.

5.1.4. Let the animals spawn naturally to initiate the next generation. Tailed larvae should appear approximately 3 hours after fertilization.

NOTE: Spawning is performed by fully matured animals abandoning their houses, swimming towards the surface water, and releasing their gametes. Successful fertilization can be confirmed by extracting 5-10 mL of seawater from the bottom of the spawning beaker and identifying eggs with cleavages under a microscope.

5.1.5. On the first morning post-spawning (Day 1), a new generation of animals with inflated houses should appear in the beaker. Use a 500 mL hand-held beaker to gently transfer the contents of the spawning beaker into a new beaker containing 7.5 L of fresh fSW (making a total of 10 L). Pour at an angle to avoid a splashing motion.

5.1.6. On the second morning (Day 2), manually transfer 150 animals to a new beaker containing 5 L of fresh fSW.

5.1.7. On the third morning (Day 3), manually transfer 120 animals to a new beaker with 5 L of fresh fSW.

NOTE: In order to synchronize the development of animals, it is important to select individuals with similar sizes during the manual transfer on day 2 and 3. A maximum of 10 animals can be siphoned in a single transfer.

5.1.8. On the fourth morning (Day 4), fully matured animals should appear. Repeat step 5.1.3 to close the lifecycle.

NOTE: An automated feeding pump can be set to feed the animals at 5 PM on weekends without the presence of culturing staff.

5.2. Daily preparation of algal food from working culture

5.2.1. Measure the absorbance of the working culture at 660 nm.

5.2.2. Based on the Daily Feeding Chart, find out how many algal cells need to be fed for the animals of specific size (**Table 3**).

5.2.3. Using the algal growth curves (**Figure 4**), solve the equations below to calculate the volume of algal food (mL) required on a given day.

5.2.3.1. To calculate the volume of a particular algae needed for a specific day and feeding time, use the following equation:

$$Y_A = c + mx$$

$$A = V_s \frac{K}{Y_A}$$

$$Y_A = \text{algal conc.} \left[\frac{\text{cells}}{\text{mL}} \right]$$

$$x = \text{absorb. of algae [nm]}$$

$$A = \text{volume of algal species needed per day [mL]}$$

$$V_s = \text{volume of seawater in each beaker [mL]}$$

$$K = \text{algal conc. per feeding} \left[\frac{\text{cells}}{\text{mL}} \right]$$

Where Y_A is the algal concentration on a given day and A is the volume of algae needed per feeding. Furthermore, the linear relationship between Y_A to x , the values for intercept (c) and slope (m) are shown in Figure 4. Refer to Table 3 for K values.

5.2.3.2. For example, to calculate the volume of *Isochrysis sp.* needed at a 9 AM feeding of Day 3 animals maintained in a 5 L culture and with the algal absorbance of 0.234 (measured at 660 nm), the following was computed:

$$Y_{iso} = 233526 + (1.9e + 07) \times 0.234 = 4679526$$
$$\text{Isochrysis vol. [mL]} = 5000 \text{ mL} \times \frac{4000 \text{ cells/mL}}{Y_{iso}} = 3.2 \text{ mL}$$

NOTE: Store these equations in a spreadsheet so the daily feeding amount is automatically calculated based on absorbance measurements, the size of the animals, and the volume of culture seawater (**Supplemental File 1**).

5.2.4. Transfer the calculated volume of algae to 50 mL tubes, centrifuge at 5000 x *g* for 5 min at 20 °C.

5.2.5. Remove the supernatant. Fill the tubes back up to the original volume with fresh fSW, replacing old algal media.

5.2.6. Store prepared food in fridge until ready to be used for the next feed. Discard the old algal food after new food is prepared the next morning.

5.3. Activated charcoal (Optional)

NOTE: 10 g of activated charcoal is added to each culture beaker to maintain water quality. The charcoal can be reused up to four times. Open the charcoal bag slowly to avoid charcoal dust from entering the culture beakers.

5.3.1. Transfer ~700 g of activated charcoal in a container. Soak in fresh water (FW) for 48 hours and allow them to settle on the bottom.

5.3.2. Rinse with FW to remove residual charcoal dust.

5.3.3. Boil charcoal in FW for 15-20 min. Remove from heat and allow to cool.

5.3.4. Rinse until most charcoal dust is removed, and the water becomes clear.

5.3.5. Store clean charcoal in 2 L beaker containing fSW. Cover the beaker to prevent dust from entering.

5.3.6. Add charcoal to each new beaker before transferring the animals.

REPRESENTATIVE RESULTS:

Oikopleura can be collected from a boat or from a harbor by slow, gentle towing of a 100 µm mesh plankton net with a non-filtering cod-end (**Figure 5**). Due to the fragile nature of the animals, it is important to avoid any movement that could cause physical stress, such as rough handling of the net or splashing due to a trapped air pocket in the sample jar.

It is important to understand the seasonal pattern of local *Oikopleura* populations as well as the accompanying fluctuations in the physical characteristics of the water at a sampling site. Sampling between 2015 and 2019 revealed consistent seasonal variation in the presence of *O. dioica* in Ishikawa and Kin harbors in Okinawa (**Figure 6**). Surface seawater temperature appears to be a major factor. *O. dioica* was the dominant species when surface seawater reached ≥28 °C,

and *O. longicauda* coexisted with *O. dioica* at temperatures between 24 °C and 27 °C; however, *O. longicauda* dominated below 23 °C (**Figure 6A**). Gradual change in salinity after several consecutive days of heavy rain did not correlate with the abundance of *O. dioica* (**Figure 6B**).

Using the sampling procedures described above, most *O. dioica* we recovered were between day 2 and 3 of their 4-day lifecycle (**Figure 7C**). Mature males were recognized by the yellow coloration of gonads whereas female gonads shimmered gold from eggs that were 70-80 µm in diameter (**Figure 8A, B**). Immature *O. dioica* were confirmed by two subchordal cells on their tails (**Figure 8D**). Another dominant species in the local waters, *O. longicauda*, were similar in size and morphology. We used the following criteria to distinguish *O. longicauda* from *O. dioica*³⁸⁻⁴⁰: a lack of subchordal cells in the tail, the presence of velum in the trunk, and the presence of a hermaphrodite gonad (**Figure 8E, F**). The differing tail morphologies are also useful for distinguishing *O. longicauda* from *O. dioica*. When an intact naked animal without the house was oriented laterally, the tail of *O. longicauda* was more straight with less curvature, giving it a “stiffer” appearance compared to that of *O. dioica*.

The three most important factors for establishing a stable *Oikopleura* culture system are (i) maintaining high water quality, (ii) identifying the optimal feeding regime, and (iii) setting up a spawning beaker with sufficient numbers of males and females. The introduction of a multi-step filter system (**Figure 1**) improved the water quality and stability of the culture. A filtration system is not necessary for artificial seawater; however, the cost, availability, and convenience of natural seawater makes it a better option for labs situated near the coast. To establish the feeding regime, we recommend measuring algal growth curves that apply to individual laboratory settings, since temperature and light conditions vary greatly. We combined the growth curves with previously published feeding schedules to optimize algal feed concentrations and compositions²⁷ (**Figure 4**). We also follow a strict algal inoculation schedule to maintain a fresh supply of algal food (**Table 2**). The automated feeding system allows us to maintain a consistent daily feeding schedule without the presence of culturing staff (**Figure 2B**).

Once optimal seawater and feeding conditions are achieved, it is important to initiate new generations by creating a spawning beaker with 15 males and 30 females in 2.5 L of fSW. This ensures a good concentration of Day 1 animals the following morning, which is sufficient to isolate 150 animals on Day 2, 120 on Day 3 and 45 mature adults on Day 4 for spawning. If there are not enough males and females on Day 4, collect and transfer as many mature individuals as possible to 1 L of fSW and let them spawn naturally in the hope that there will be enough larvae to carry onto the next generation. Following the provided protocol, the lifecycle of *O. dioica* is 4 days at 23 °C (**Figure 7C**). We have reliably established six independent wild populations of *O. dioica*, all of which lasted more than 20 generations.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of seawater filter system (A and B) Seawater is initially filtered through a 25 µm filter unit before entering the reservoir tank (**C**) A magnetic drive pump is used to draw seawater from the reservoir tank. The seawater is then pushed through two polypropylene filters and a UV sterilizer before returning to the reservoir tank.

Figure 2: Culture system for *O. dioica* (A) Overview of the culture system (B) Close-up view of synchronous motor and algae reservoir for the automated dosing pump. Inner diameters of silicon tube A and B are 2 mm and 4 mm, respectively.

Figure 3: Stock cultures for *O. dioica*. From left- *C. calcitrans*, *Isochrysis* sp., *Synechococcus* sp., and *R. reticulata* after being grown at 17 °C under continuous light for ~10 days.

Figure 4: Algal growth curve for two of the major food species, *C. calcitrans* and *Isochrysis* sp.. Scatter plots of optical density (OD) at 660 nm and total cell concentrations for (A) *C. calcitrans* and (B) *Isochrysis* sp.. Each point represents the average of three measurements. A cell counter was used to determine the percentage of viable cells and total cell concentrations (cells/mL). Measurements were recorded for 20 days (n = 47).

Figure 5: Modified plankton net for *Oikopleura* sampling. The cod-end of a hand-held plankton net (100 µm mesh) is replaced with a 500 mL wash-bottle. A 70 g weight is attached to the cod-end. Approximately 5 m of rope is attached to the key ring. A safety leash is attached to further secure the cod-end.

Figure 6: Seasonality of *O. dioica* in Okinawa. Presence and absence of *O. dioica* and *O. longicauda* in relation to seasonal changes in (A) temperature and (B) salinity at harbors in Ishikawa (26°25'39.3"N 127°49'56.6"E) and Kin (26°26'40.2"N 127°55'00.3"E) between 2015-2019. Each species was recorded as present if more than 50 animals were manually counted. Temperature and salinity measurements of surface water were recorded.

Figure 7: Flow chart for initiating *O. dioica* monoculture (A) Three, 500 mL plankton samples are collected from a sampling site (B) Each sample jar is diluted and *O. dioica* is isolated from the rest of plankton (C) A monoculture of *O. dioica* is initiated by manually transferring 120 Day 3 animals to a new beaker containing 5 L of fresh filtered seawater (fSW). Set up a spawning beaker containing 30 females, 15 males and 2.5 L of fresh fSW. The first morning post-spawning (Day1), carefully empty the spawning beaker with the new generation of animals into a beaker containing 7.5 L of fresh fSW. On the second day post-spawning (Day 2), transfer 150 animals into a beaker containing 5 L fresh fSW. On the third day post-spawning (Day 3), transfer 120 animals into a beaker containing 5 L fresh fSW. On the final day (Day 4), set up a new spawning beaker containing 30 females, 15 males and 2.5 L fresh fSW in preparation of the next generation. The animals have a 4-day lifecycle at 23 °C.

Figure 8: Identification of *Oikopleura* spp. (A-D: *O. dioica*, E and F: *O. longicauda*) (A) Female *O. dioica* with eggs (B) Male *O. dioica* with sperm (C) Lateral view of immature *O. dioica* (D) Ventral view of immature *O. dioica* with two subchordal cells indicated with white arrows (E) Ventral view of mature *O. longicauda* carrying eggs (arrow 1) and sperm (arrow 2) (F) Lateral view of *O. longicauda* showing velum (arrow 3).

Table 1: Recipe of reagents necessary for the maintenance of algal food. After dissolving all the chemical listed for solution B, HCl is added until the solution becomes clear with no turbidity. All the reagents are sterilized by either autoclaving (120 °C, 25 min) or by use of a 0.22 µm filter. All the reagents except for the vitamin stocks are sterilized after the addition of specified chemical. For the vitamin stocks, autoclave the water first, and then dissolve the listed chemical. Storage temperatures for stock and opened reagents are listed.

Table 2: Instruction for the maintenance of three algal culture types. Add the specified amount of supplements to flasks containing autoclaved seawater. Inoculate each flask with specified amount of algal culture. Incubate and store algal cultures at specified temperatures. Inoculate new stock culture and sub-culture from the previous stock culture, and new working culture from the previous sub-culture. Inoculate new stock culture, sub-culture, and working culture every two weeks, one week, and four days, respectively. This schedule provides enough food for approximately 10 beakers of *O. dioica* culture. Maintain 2 – 3 sets of each algal culture type as back-ups. RM – room temperature.

Table 3: Algal concentration per feeding- modified from Bouquet et al.²⁷. Algal concentrations (cells mL⁻¹) and algal species used for daily feeding during the 4-day lifecycle of Okinawa *O. dioica*.

Supplemental File 1: Daily feeding chart. Daily feeding amounts for each culture beaker are automatically calculated after entering daily algal absorbance measurements (OD), the size of the animals (Day), and the volume of seawater (SW vol.) in each culture beaker. Growth curves of *R. reticulata* and *Synechococcus* sp. were adapted from Bouquet et al.²⁷.

Supplemental File 2: How to connect synchronous motor to acrylic paddle. Tightly screw on the paddle to the motor using a hexagon wrench.

DISCUSSION

To facilitate flexibility in the establishment of *O. dioica* culture, it is important to understand the animals' natural habitat. Seasonal data provides information about the ranges of physical parameters, which can be used to guide laboratory culturing conditions. It also helps in understanding seasonal fluctuations in the abundance of animals. In Okinawa, *O. dioica* is most reliably found from June to October. However, in Tokyo bay, populations peak in February and October⁴¹. Although culturing of *O. dioica* is often reported at 20 °C or lower²⁷⁻²⁹, Okinawan *O. dioica* shows better survival at temperatures above 20 °C; this might be explained by the fact the minimum surface seawater temperature in Okinawa is ~20 °C (**Figure 6**). The abundance of *O. dioica* might also be influenced by phytoplankton blooms⁴² and predator abundance^{43,44}. Regardless of where *O. dioica* are collected, understanding the seasonality of local populations maximizes the chance of sampling and culturing success.

Given the appropriate season and location, net sampling is an effective way of collecting large numbers of *Oikopleura* with minimal effort. Plankton nets with smaller mesh size (60-70 µm) may also be used to collect all stages of the animals. Fully mature animals are rarely found in the net, perhaps due to their fragility at the end of the lifecycle. Therefore, species identification followed

by sampling is achieved by microscopic observation of subchordal cells. Mature individuals usually appear one or two days post-sampling as animals continue to grow in the laboratory. Although net sampling is efficient, alternative sampling methods might be necessary in different circumstances. For example, net sampling near urban areas can collect large numbers of phytoplankton, making it difficult to isolate *Oikopleura*. In such cases, simple bucket sampling to collect surface seawater or boat sampling from areas beyond the harbor are recommended. The results showed that the gradual change in salinity due to consecutive days of rain did not affect the abundance of *O. dioica*; however, shore sampling immediately after extreme weather events such as tropical cyclones should be avoided. These events cause sudden and drastic biogeochemical changes in a sheltered body of water^{45,46}. The stormwater runoff may carry pollutants, sediments, and excess nutrients, which increase turbidity and lower water quality⁴⁷. Filter-feeding plankton, such as *Oikopleura*, may be especially susceptible to these changes due to their mode of feeding and limited mobility. In such a circumstance, we recommend postponing sampling for a few days until the local conditions return to normal.

The introduction of a multi-step filter system is essential to maintain small, filter-feeding organisms such as *O. dioica*. Using poorly filtered seawater (for example, a 25 µm mesh in the previous culture system), the culture was often unstable especially during summer, potentially due to the higher abundance of phytoplankton. Although some phytoplankton are beneficial to *O. dioica* growth, others produce biotoxins that can cause abnormal development of *O. dioica* embryos⁴⁸. In addition, a high concentration of diatoms such as *Chaetoceros* spp. are potentially harmful to *O. dioica* growth as they can possess long setae which can clog the house and prevent efficient feeding⁴⁹. We frequently observed houses of small animals being clogged by *C. calcitrans* setae; therefore, we now feed *C. calcitrans* only to animals at Day 2 and older (**Table 3**).

Although it was not a problem here, small scale long-term culturing of *O. dioica* can experience sudden drops in population size due to a genetic bottleneck; in such cases, Martí-Solans et al.²⁹ recommend adding new wild individuals to the culture every 20 generations.

The *Oikopleura* culture system is flexible. A stable culture can be established within a week. Long-term culturing of *O. dioica* is possible on a modest budget with non-specialist equipment. The daily effort required for the maintenance of 5-10 beakers of *Oikopleura* is generally less than 2 hours with 2 people. *O. dioica* can also be maintained in artificial seawater, which is beneficial to those without access to natural seawater²⁸. Long-term storage of algal food is possible using solid-culture and cryopreservation²⁹. Moreover, *O. dioica* sperm can be cryopreserved, and remain viable for more than a year⁵⁰. All of these factors mean that cultures can be easily re-established. Finally, past experience with accidental culturing of *Pleurobrachia* sp. may suggest that the culturing system developed for *Oikopleura* could potentially be extended to a broader community of fragile pelagic organisms.

O. dioica continues to provide powerful insights into various biological fields. An understanding of local seasonality, a meticulous culture system, and a few dedicated individuals allow effective culture to be established with little effort. *Oikopleura* culture system provides the baseline

resources to investigate a wide range of biological fields relating to ecology, development, genomics, and evolution of this unique marine chordate.

ACKNOWLEDGMENTS:

We are grateful to Garth Ilsley for his support in establishing the culture system. We acknowledge Ritsuko Suyama's and Sylvain Guillot's contributions to early sampling and species identification efforts. Special thanks are due to Hiroki Nishida, Takeshi Onuma, and Tatsuya Omotezako for their generous support and guidance throughout, including initial establishment of the local culturing system and sharing animals and microalgal culture. We also thank Daniel Chourrout, Jean-Marie Bouquet, Anne Aasjord, Cristian Cañestro, and Alfonso Ferrández-Roldán for sharing their expertise on sampling and culturing. Jai Denton, Charles Plessy, and Jeffrey Jolly provided invaluable feedback on the manuscript. Charlotte West formulated a generalized equation for algal calculation. Finally, we thank OIST for funding, Mary Collins and the OIST Fieldwork Safety Committee for advice on safe sampling procedures, the staff of OIST machine shop for the construction of culturing and sampling equipment, and Koichi Toda for delivering seawater.

DISCLOSURES:

The author has nothing to declare.

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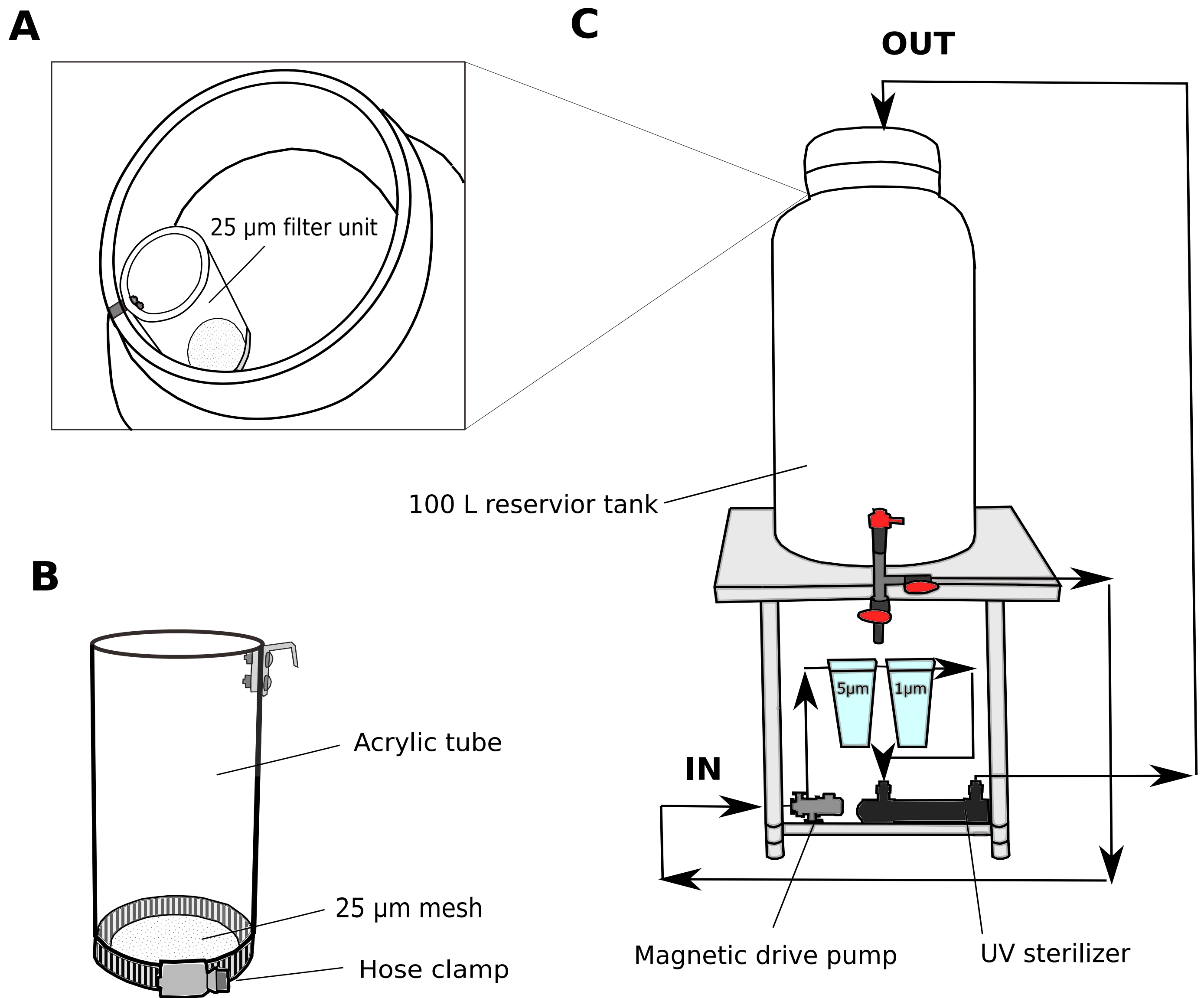
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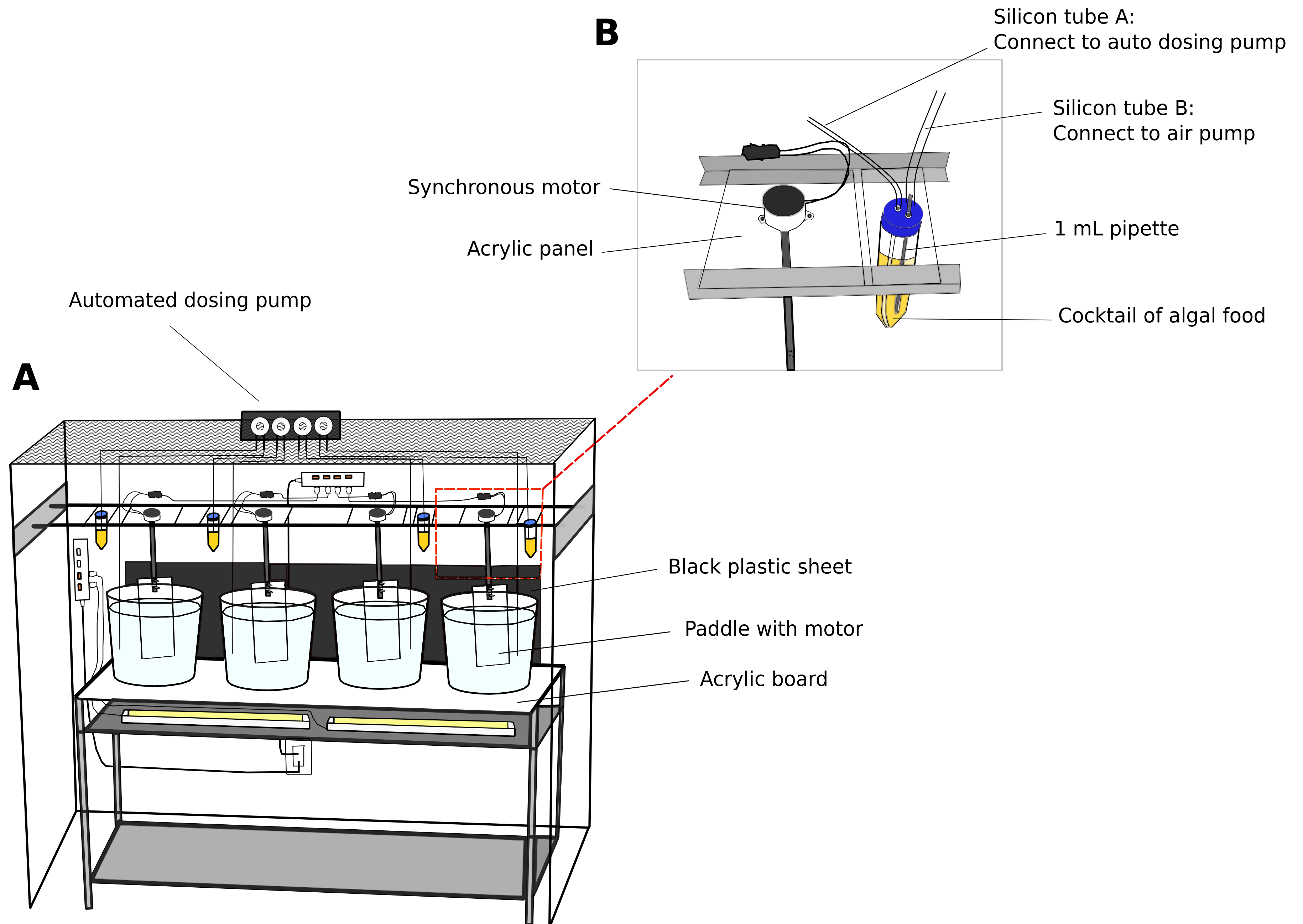
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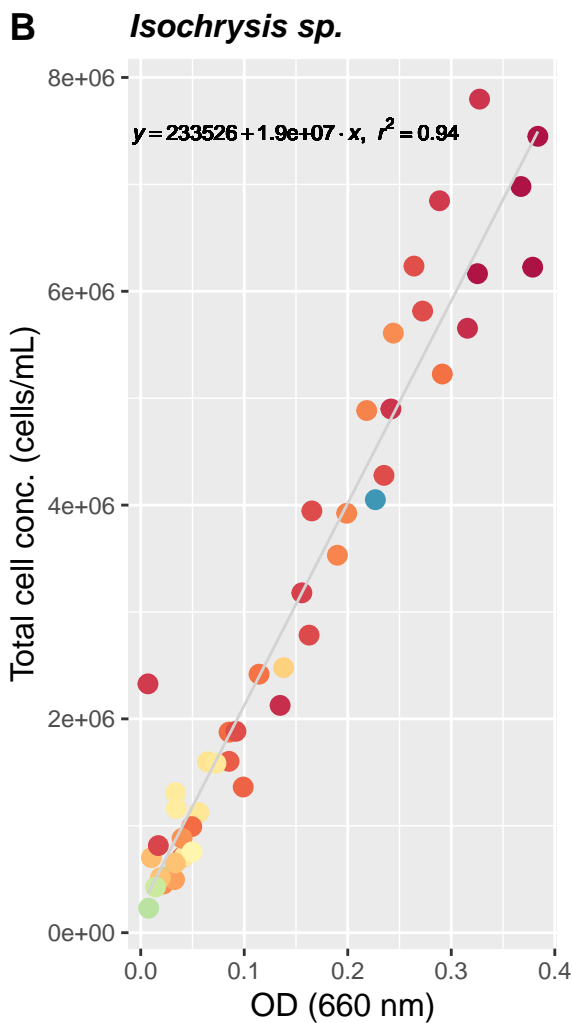
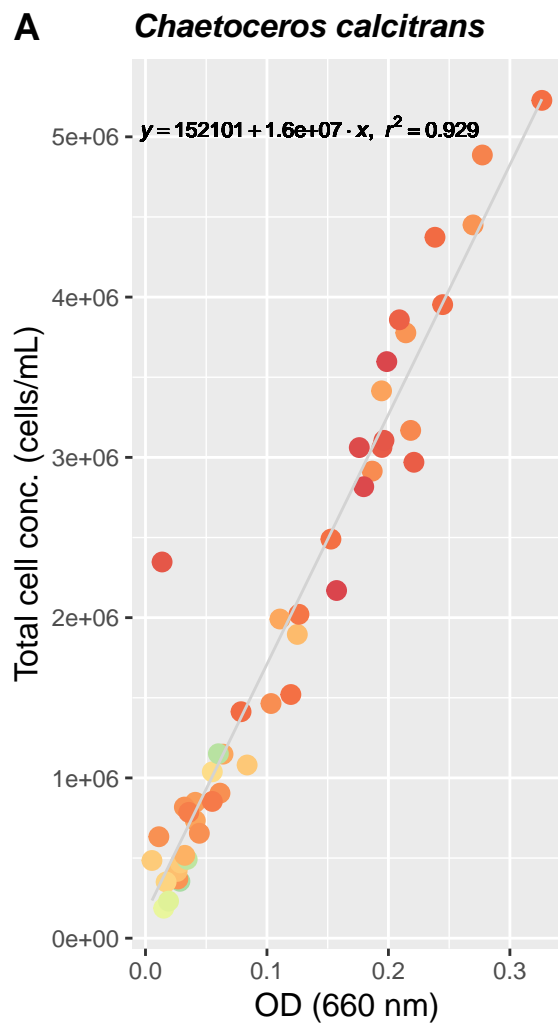
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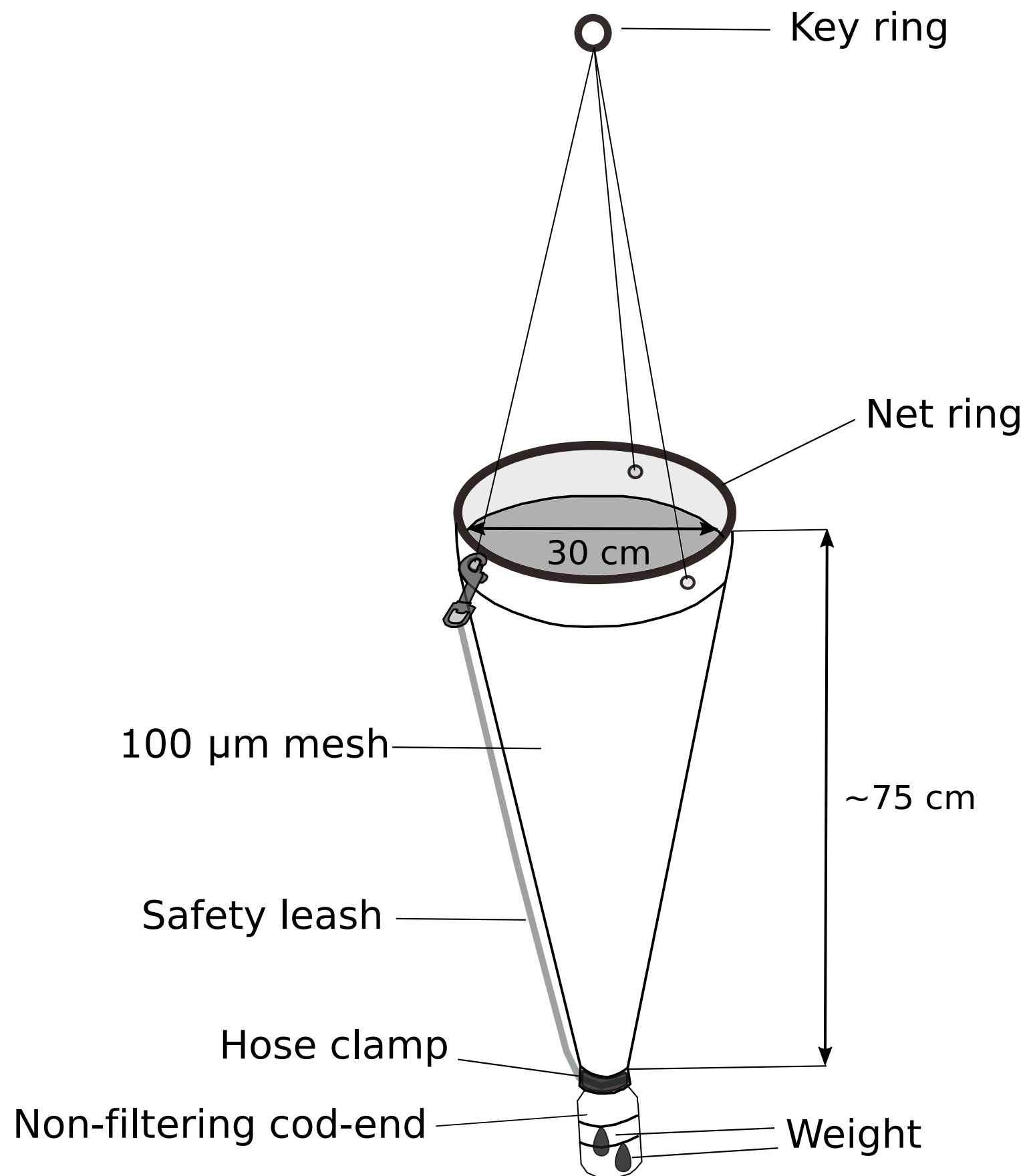
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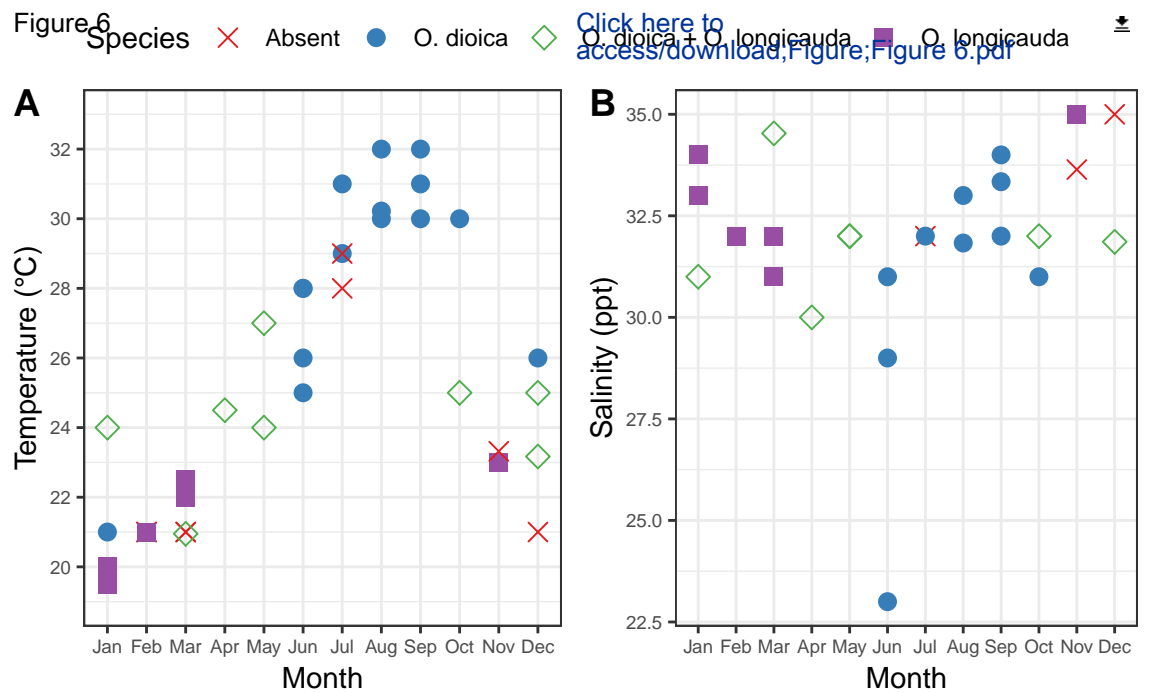


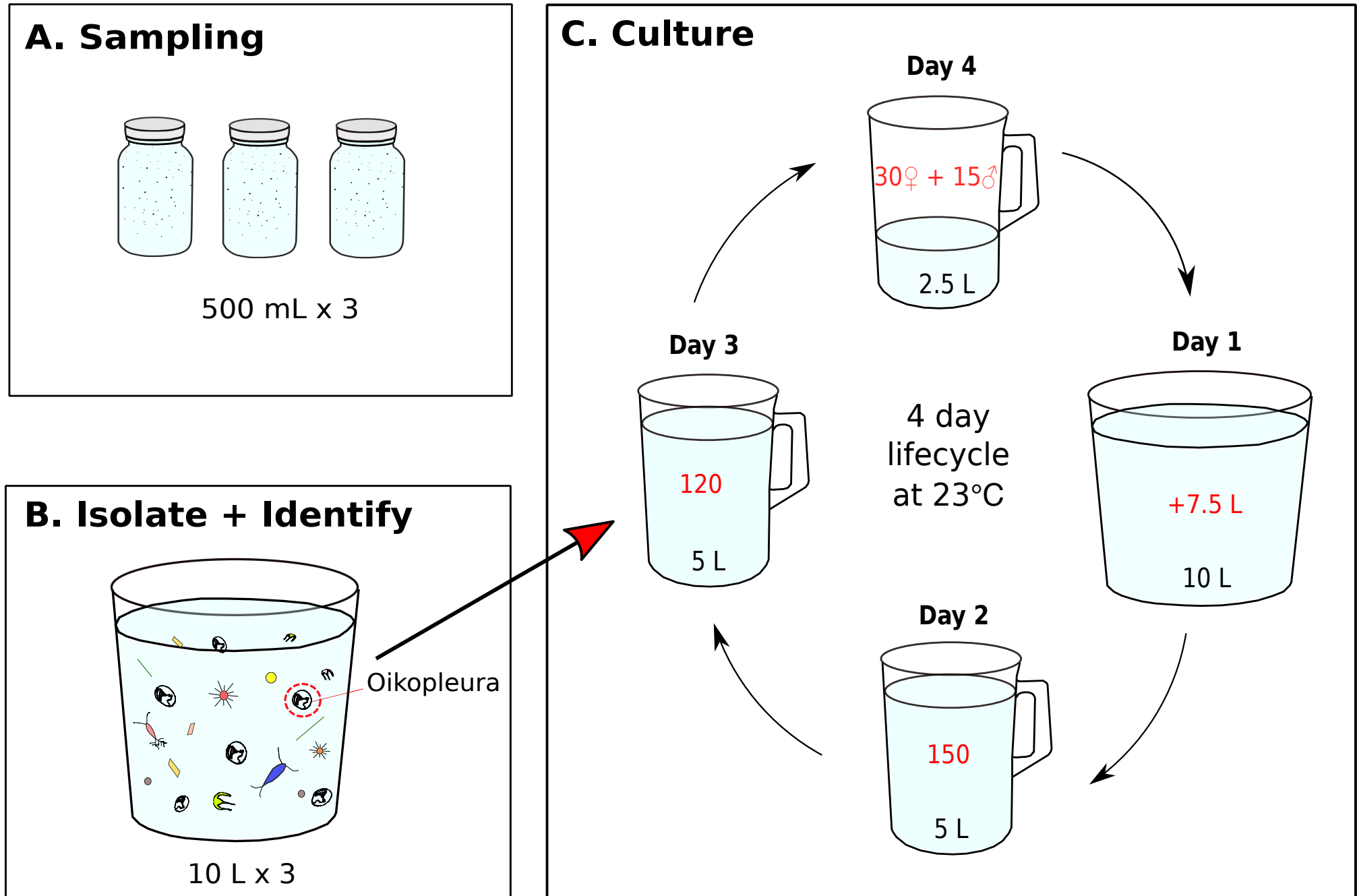




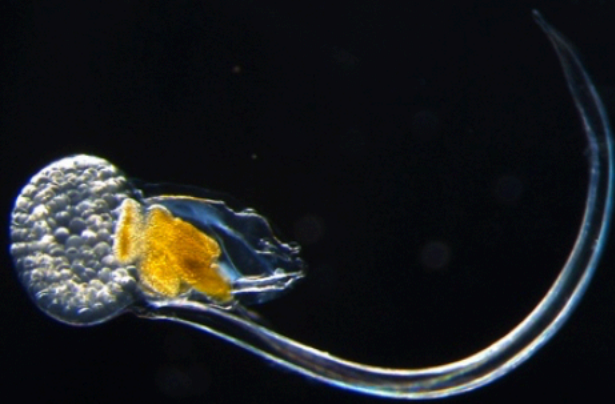








A



B



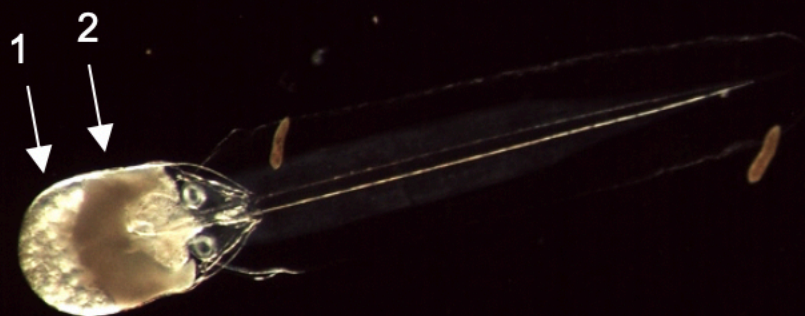
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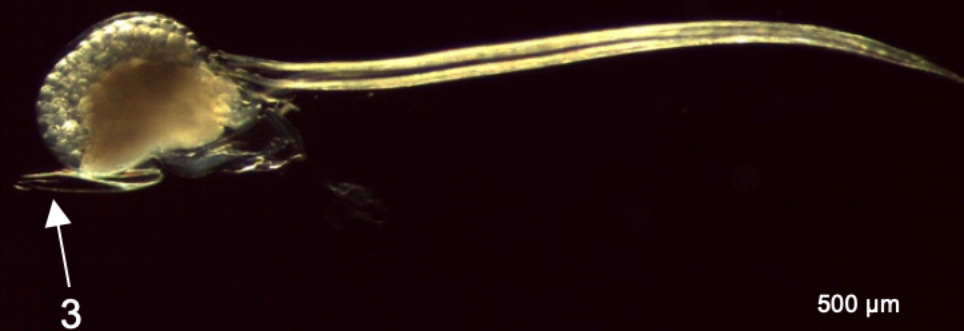
D



E



F

500 μ m

| Reagents | Chemical products | Amount | Final vol. (mL) | Sterilization | Stock / Opened |
|-----------------|--|----------|-----------------|----------------|-----------------|
| Solution A | Na ₂ EDTA | 45 g | 1000 | Autoclave | -20 °C / 4 °C |
| | NaNO ₃ | 100 g | | | |
| | H ₃ BO ₃ | 33.6 g | | | |
| | NaH ₂ PO ₄ | 20 g | | | |
| | MnCl ₂ ·4H ₂ O | 0.36 g | | | |
| | FeCl ₃ ·6H ₂ O | 1.3 g | | | |
| | Solution B | 1.0 mL | | | |
| Solution B | ZnCl ₂ | 2.1 g | 1000 | Autoclave | 4 °C / 4 °C |
| | CoCl ₂ ·6H ₂ O | 2.0 g | | | |
| | (NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O | 0.9 g | | | |
| | CuSO ₄ ·5H ₂ O | 2.0 g | | | |
| | *HCl | -- mL | | | |
| Vitamin | Thiamin (B ₁) ·HCl | 200 mg | 1000 | Autoclave | -20 °C / 4 °C |
| | Biotin | 1 mg | | | |
| | Cobalamin (B ₁₂) | 1 mg | | | |
| Sodium silicate | Na ₂ SiO ₃ | 5% | 1000 | 0.22 µm filter | 4 °C / 4 °C |
| Streptomycin | C ₂₁ H ₃₉ N ₇ O ₁₂ | 25 mg/mL | 50 | 0.22 µm filter | -20 °C / -20 °C |

Table 2

| Culture type | Algal spp. | ASW (mL) | Vitamin | Solution A | Sodium silicate | Streptomycin | Algae (mL) / Culture type | Incubate / Store | Frequency |
|-----------------|---------------|----------|---------|------------|---------------------------------|--|---------------------------|------------------|--------------|
| Stock culture | <i>Chaeto</i> | 60 | 1/2000 | 1/2000 | 1/4000 (<i>Chaeto</i> only) | 1/1000 (All except for <i>Syn</i>) | 0.03 / stock | 17°C / 4°C | Biweekly |
| | <i>Iso</i> | 60 | | | | | 0.03 / stock | | |
| | <i>Rhino</i> | 80 | | | | | 0.06 / stock | | |
| | <i>Syn</i> | 60 | | | | | 0.03 / stock | | |
| Sub-culture | <i>Chaeto</i> | 500 | 1/2000 | 1/2000 | 1/4000 (<i>Chaeto</i> only) | 1/1000 (All except for <i>Syn</i>) | 10 / stock | 17°C / 17°C | Weekly |
| | <i>Iso</i> | 500 | | | | | 10 / stock | | |
| | <i>Rhino</i> | 500 | | | | | 20 / stock | | |
| | <i>Syn</i> | 500 | | | | | 10 / stock | | |
| Working culture | <i>Chaeto</i> | 400 | 1/2000 | 1/2000 | 1/4000 (<i>Chaeto</i> only) | 1/1000 (All except for <i>Syn</i>) | 100 / sub | RM / RM | Every 4 days |
| | <i>Iso</i> | 400 | | | | | 100 / sub | | |
| | <i>Rhino</i> | 400 | | | | | 150 / sub | | |
| | <i>Syn</i> | 400 | | | | | 100 / sub | | |

| Day | Algal spp. | 9AM and 5PM | 12PM |
|-----|---------------|-------------|--------|
| 1 | <i>Chaeto</i> | — | — |
| | <i>Iso</i> | 1000 | 2000 |
| | <i>Syn</i> | 20,000 | 40,000 |
| 2 | <i>Chaeto</i> | 1000 | 2000 |
| | <i>Iso</i> | 2000 | 2000 |
| | <i>Rhino</i> | 1000 | 1000 |
| 3 | <i>Chaeto</i> | 3000 | 4000 |
| | <i>Iso</i> | 3000 | 4000 |
| | <i>Rhino</i> | 1500 | 1500 |
| 4 | <i>Chaeto</i> | 1000 | 2000 |
| | <i>Iso</i> | 1000 | 2000 |
| | <i>Rhino</i> | 1000 | 1000 |

| Name of Material/Equipment | Company | Catalog Number |
|--------------------------------------|------------------|----------------|
| Activated charcoal | Sigma | C2764-2.5KG |
| Alluminum pulley | Rainbow Products | 10604-10607 |
| Biotin | Sigma | B4501-100MG |
| Boric acid | Wako | 021-02195 |
| Cobalamin (B12) | Sigma | V2876-100MG |
| Cobalt(II) chloride hexahydrate | Wako | 036-03682 |
| Copper(II) sulfate pentahydrate | Wako | 039-04412 |
| Disodium edetate hydrate | Wako | 044-29525 |
| tetrahydrate | Wako | 019-03212 |
| Hexagon wrench | Anex | No.6600 |
| Hydrochloric acid | Wako | 080-01066 |
| Iron(III) chloride hexahydrate | Wako | 091-00872 |
| Jebao programmable auto dosing pump | Jebao | DP-4 |
| Magnet pump | REI-SEA | RMD-201 |
| Manganese(II) chloride tetrahydrate | Wako | 134-15302 |
| Polypropylene wound cartridge filter | Advantec | TCW-10N-PPS |
| | | TCW-5N-PPS |
| | | TCW-1N-PPS |
| Screwless terminal block | SATO PARTS | SL4500 |
| Simple plankton net | RIGO, Japan | 5512-C |
| Sodium metasilicate | Sigma | 307815-1KG |
| Sodium nitrate | Wako | 195-02545 |
| Sodium phosphate monobasic anhydrous | MP Biomedicals | 194740 |
| Streptomycin sulfate salt | Sigma | S6501-25G |
| Synchronous electric motor | Servo | D5N6Z15M |
| Thiamin hydrochloride | Wako | 201-00852 |
| UV sterilizer | Iwaki | UVF-1000 |
| Zinc chloride | MP Biomedicals | 194858 |

Editorial and production comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. As some authors are affiliated with UK institutions, please print and sign the attached Author License Agreement - UK. Please also check whether open access is required by your funding agencies.

Authors' response:

Open access is required by all author institutions.

Authors' actions

We attach a signed copy of the Author License Agreement - UK.

3. 1.1.1: Please specify the pore sizes of the filters used.

Authors' response:

There are two filters. The pore size for the sand filter is 1.4 mm. The canister filter is used to circulate water rather than removing particles from the reservoir tank, so the pore size isn't important.

Authors' actions. Line 114

"1.1.1. Collect natural seawater from a harbor at 2 - 3 m depth. Pass the seawater through a sand filter (pore size 1.4 mm) and transport to a shared reservoir tank in the laboratory. Use a canister filter to circulate the water in order to maintain the water quality in the shared reservoir tank."

4. 2.2.4: Do you mean 50 average measurements? Then how many samples (150?) are taken?

Authors' response:

Yes, we counted each sample in triplicate and reported the average (ie, 150 measurements and 50 average measurements).

Author's actions. Line 239

"2.2.4. Continue to count daily until approximately 50 average measurements are recorded."

5. Figure 5 and Table 1: Please use the micro symbol μ instead of u (i.e., μm).

Authors' actions:

We have replaced all instances of u with μ .

6. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol (e.g., vitamin, streptomycin, etc.). Please sort the materials alphabetically by material name.

Authors' actions:

We have corrected the table.

Changes to be made by the author(s) regarding the video:

1. All information in the video should be in the written protocol; please add the details mentioned in the video (2:05-2:25, 4:15-4:20, 7:24-7:33, 7:42-7:48, 8:35-9:00, etc.) to the written protocol.

Authors' actions:

Please refer to the following steps in the manuscript.

Video 2:05 - 2:25, Line 210 and 213

2.1.4. "On a clean bench, aseptically inoculate a sub-culture from the stock culture (**Table 2**).

Incubate at 17 °C with continuous lighting. After algal colors appear, continue to store them in the incubator up to 2 weeks."

2.1.5. "Inoculate working culture from sub-culture (**Table 2**). Seal the flask with a rubber cap and insert 1 mL disposable pipette. Move the flask to the algae station and maintain at room temperature with a 8h photoperiod. Supply with constant aeration. Renew the working culture every 4 days."

Video 4:15 - 4:20, Line 314

4.1.1. “To acclimatise animals to laboratory conditions, transfer each 500 mL sample to a 10 L beaker containing 1:1 ratio of surface seawater (sSW) from the sampling location and filtered seawater (fSW) maintained in the lab (**Figure 7A, B**). The volume of the beaker should be adjusted to 5 - 10 L depending on the concentration of plankton sample.”

Video 7:24 - 7:33, Line 403

5.1.8. “On the fourth morning (Day 4), fully matured animals should appear. Repeat step 5.1.3. to close the lifecycle.”

Video 7:42 - 7:48, Line 362

NOTE: Algal food is prepared daily from working cultures and each monoculture beaker is fed three times a day at 9 AM, 12 PM, and 5 PM, respectively (See step 5.2.).

Video 8:35 - 9:00, Line 406

“NOTE: an automated feeding pump can be set to feed the animals at 5 PM on weekends without the presence of culturing staff.”

2. Please remove commercial language (8:14 Falcon, 8:34 Excel) from the narrative.

Authors’ actions:

We have edited the video to remove commercial language.

3. Please use the same protocol section title in the video as in the written protocol if possible; this will help guide the viewers. These should be separate title cards, so that our chaptering system can recognize the chapters.

Authors’ actions:

We have edited the video to use the same section titles as in the written protocol.

Please upload a revised high-resolution video here:

<https://www.dropbox.com/request/8sXcFGkWYZOSRI8gfUhC?oref=e>

Reviewer #1 comments:

Manuscript Summary:

The appendicularians are very fragile organisms which are difficult to cultivate. However they are very abundant in coastal ecosystems worldwide. Thus any protocol to improve cultivation of them are very useful for the scientists and I valorate very much this work.

The video is welldown with information for the collection of larvaceans in the field (eg. net sampling protocol and the cares with the filtering house system) and also the details for the food aliment cultures are very well described. The isolation of *Oikopleura dioica* is appropriate as it is the only species with separate sexes, males and females.

Authors' response:

We thank the referee for their positive review of the manuscript.

Major Concerns:

For the identification of immature *O. dioica* organisms I propose to add more information to distinguish *O. dioica* from *O. longicauda*. The identification of the later species would be better using the morphology of the tail which is more stylized, shining and refractory. Thus these characters would be a practical way to identificate both species. Looking at the seasonal cycle of both species, it appears that *O. dioica* is present all year around but *O. longicauda* is detected only during November-March period. So the methodology to identificate and collect each one would be more explained in detail.

Authors' response:

We thank the reviewer for this comment. We agree that the tail morphology is another distinguishing factor for *O. longicauda*. We originally excluded this observation since it has not been documented in literature describing Appendicularian morphology (Fenaux 1993, Shiga 1997). However, we have now added the sentences below.

Authors' action. Line 495

"We used the following criteria to distinguish *O. longicauda* from *O. dioica*³⁸⁻⁴⁰; a lack of subchordal cells in the tail, the presence of velum in the trunk, and the presence of a hermaphrodite gonad (**Figure 8E, F**). The differing tail morphologies are also useful for distinguishing *O. longicauda* from *O. dioica*. When an intact naked animal without the house was oriented laterally, the tail of *O. longicauda* was more straight with less curvature, giving it a "stiffer" appearance compared to that of *O. dioica*"

Minor Concerns:

It would be important to mention that a net of 62/67 pore micras is also very appropriate to catch all the stages of the population, mainly the immatures stages. Many studies use those pore nets to describe seasonal cycles.

Authors' response:

We thank the reviewer for this comment. We added the sentence below.

Authors' action. Line 615

"Plankton nets with smaller mesh size (60 - 70 μ m) may also be used to collect all stages of the animals."

In the introduction section I recommend to remove or reformulate some ideas:

- "This protocol will be beneficial for anyone wishing to start an *O. dioica* culture, as well as researchers who maintain other fragile, planktonic marine organisms such as jellyfish and ctenophores that live in suspension."

In this regards, I think that the protocol for jellyfish and ctenophores would be very different... these are macrozooplankters without filtering houses such as appendicularians and probably they have others problems to cultivate.

Authors' response:

We thank the referee for this comment. We have previously accidentally cultured small bycatch plankton such as jellyfish and ctenophores (*Pleurobrachia* sp.) using this system; therefore we believe that it could be adapted for the maintenance of other small gelatinous plankton without too much difficulty. We have clarified this point now.

Authors' action. Line 41

*"Although the culture protocol provided here is optimized for *O. dioica*, we hope our sampling technique and culture setup will inspire new ideas for maintaining other fragile pelagic invertebrates."*

Line 655

“Finally, our past experience with accidental culturing of Pleurobrachia sp. may suggest Oikopleura culture system has its potential expansion of applicability to a broader community of fragile pelagic organisms.”

- “A study on Bathochordaeus in Monterey Bay, California revealed that the animals also serve as a biological vector of microplastics, suggesting a vital role in the vertical transport and distribution of microplastics in the oceans.”
I don't understand this idea at all. The presence and dispersion of microplastics in the oceans are actually a contamination vector, one of the more important ones, that would affect negatively the ocean health. I propose to remove it.

Authors' response:

We thank the referee for this comment. We have revised the sentence here which we hope clarifies the point we tried to communicate.

Authors' action. Line 68

“A study on Bathochordaeus in Monterey Bay, California revealed that the animals also serve as a biological vector of microplastics, suggesting a potential importance in understanding the role of appendicularians in the vertical transport and distribution of microplastics in the oceans²⁵.”

Reviewer #2 comments:

Manuscript Summary:

This paper provides a step-by-step protocol for establishing an *O. dioica* culture. A culture protocol for *O. dioica* has been previously published by Bouquet et al (2009), based on a system originally developed by Fenaux and Gorsky (1985). The authors should do a better job of explaining what is new or streamlined in their protocol relative to prior studies. The figures/schematics are well done. Section 3 of the manuscript presents a useful, simple summary of how to go about collecting appendicularians from the field.

Authors' response:

We thank the reviewer for the constructive feedback on the manuscript. We have worked hard to incorporate the recommendations, which have helped to improve the study.

Major Concerns:

My major comment is that the introduction of the manuscript can be strengthened. Much of it is overly general and should be focused to provide a better description of the previous literature on culturing appendicularians, and articulating the purpose/contribution of the current study relative to what has already been done. An attempt at this is made in lines 82-87, but it should be greatly elaborated.

Authors' response:

We thank the referee for this comment. We have added a new paragraph summarizing the history of *O. dioica* culturing. We have also extended the description of the current study.

Authors' action. Line 84

*"The first successful *O. dioica* culture with laboratory grown microalgae was reported by Paffenhöfer³⁴. The original culture protocol using synchronous motors and paddles was developed by Fenaux and Gorsky³⁵ and later adopted by multiple laboratories. More recently, Fujii et al.³⁶ reported *O. dioica* culturing in artificial seawater, a robust culture system and field collection were described by Bouquet et al.²⁷ and an optimized protocol for a simplified, affordable system was reported by Marti-Solans et al.²⁹. Aside from the traditional *Oikopleura* culture system, a newly reported design with a double tube rearing tank also has the potential to culture *Oikopleura* sp.³⁷."*

Line 96

*"In previously published culture protocols, detailed information regarding the composition of algal media, shore sampling techniques, and *Oikopleura* identification were only roughly described, leaving a lot of ambiguity. Here, with the aid of visual information in the video protocol, we have assembled all the critical information needed to set up an *O. dioica* culture from the ground up in a straightforward, step-by-step manner. We describe how to distinguish *O. dioica* from another commonly reported species, *O. longicauda*, which is one of the most challenging steps. Although the existing culture systems are applicable for the cultivation of *O. dioica* worldwide, we highlight the importance of protocol adjustment based on local environmental conditions. The presented information combines widely published data as well as knowledge gained through experience. The current protocol is ideally suited for researchers interested in establishing a culture from scratch."*

Lines 51-55 are very general and overused. Quite a lot is actually known about the general biology of appendicularians and their filtration mechanics.

Authors' response:

We thank the referee for this comment; these lines were very much presented from the perspective of molecular and genomic investigations and we agree that there is a great deal known about the general biology. We have therefore removed the sentence "little is known about the general biology"

Authors' action. Line 50

"Despite their abundance and ecological importance, gelatinous organisms such as planktonic tunicates are often under-represented in plankton biodiversity studies because their transparency and fragility make field collection and identification challenging^{7,8}. Adapted sampling techniques and laboratory culturing allow closer observation of the animals in vitro, which has furthered our knowledge in the biology of planktonic tunicates⁹⁻¹²."

Line 65: Not all houses eventually sink to the seabed. There are different fates for the aggregates. The discussion of microplastics seems tangential and does not flow well.

Authors' response:

We thank the referee for this comment. We have now revised the sentence to clarify. We have also modified the sentence regarding microplastics.

Authors' action. Line 64

"Clogged houses are discarded throughout the day, some of which form carbon aggregates and eventually sink to the seabed²²; thus, larvaceans play a major role in global carbon flux²³."

Line 394: V_a is not defined. The term "A" on the left-hand side of the first equation is unclear. What are the volumetric units? c and mx in the next equation are also not defined. The subsequent example in lines 405-406 helps answer these questions, but the equations still need to be made more clear.

Authors' response:

We thank the referee for this comment. All the terms for the equations are now defined correctly.

Authors' action. Line 419

To calculate the volume of a particular algae needed for a specific day and feeding time, the following equation was used:

$$Y_A = c + mx$$

$$A = V_s \frac{K}{Y_A}$$

$$Y_A = \text{algal conc.} \left[\frac{\text{cells}}{\text{mL}} \right]$$

$$x = \text{absorb. of algae} [\text{nm}]$$

$$A = \text{volume of algal species needed per day} [\text{mL}]$$

$$V_s = \text{volume of seawater in each beaker} [\text{mL}]$$

$$K = \text{algal conc. per feeding} \left[\frac{\text{cells}}{\text{mL}} \right]$$

Where Y_A is the algal concentration on a given day and A is the volume of algae needed per feeding. Furthermore, the linear relationship between Y_A to x , the values for intercept (c) and slope (m) are shown in **Figure 4**. Refer to **Table 3** for K values.

For example, to calculate the volume of *Isochrysis* sp. needed at a 9 AM feeding of Day 3 animals maintained in a 5 L culture and with the algal absorbance of 0.234 (measured at 660 nm), the following was computed:

$$Y_{iso} = 233526 + (1.9e + 07) \times 0.234 = 4679526$$
$$\text{Isochrysis vol. [mL]} = 5000 \text{ mL} \times \frac{4000 \text{ cells/mL}}{Y_{iso}} = 3.2 \text{ mL}$$

Minor Concerns:

Line 33: The abstract states that "Marine zooplankton serve as the base of marine food web." Although I understand what the authors are saying, it seems odd to refer to zooplankton as the "base" of the foodweb rather than phytoplankton. I suggest re-wording this sentence.

Authors' action.

We have removed this sentence.

1.2.5: Provide more detail on how to connect synchronous electric motors to acrylic paddles (e.g. tubing size?).

Authors' action:

We added Supplemental File 2 describing how to connect synchronous motors to paddles. Please refer to Figure 2 for tubing sizes.

1.4.2: Do the dimensions of the shelving unit really matter here?

Authors' response:

We think it's useful to provide approximate dimensions of the shelving unit for those who are starting out from scratch. Therefore, we decided to keep the dimensions.

2.2.1: De-italicize "sp."

Authors' actions:

We have replaced all instances of "sp." with "sp."

Line 246-247: It would be helpful to articulate the rationale for why it is good to avoid sampling *O. dioica* immediately after rainstorms. Also, this seems contrary to what is stated in the representative results (lines 453-454).

Authors' response:

The animals tend to survive gradual changes in salinity following several days of rain. However, we advise against shore sampling after extreme weather events such as typhoons or hurricanes as they can cause sudden and extreme changes to habitats. Stormwater runoff may carry pollutants and excess nutrients, which increase turbidity and lowers water quality; *Oikopleura* may be especially susceptible to increased turbidity as their filter-feeding capabilities will be affected.

Authors' actions. Line 624

*"Our results showed that the gradual change in salinity due to consecutive days of rain did not affect the abundance of *O. dioica*; however, shore sampling immediately after extreme weather events such as tropical cyclones should be avoided. These events cause sudden and drastic biogeochemical changes in a sheltered body of water^{45,46}. The stormwater runoff may carry pollutants, sediments, and excess nutrients, which increase turbidity and lower water quality⁴⁷."*

Filter-feeding plankton, such as Oikopleura, may be especially susceptible to these changes due to their mode of feeding and limited mobility. In such a circumstance, we recommend postponing sampling for a few days until the local conditions return to normal."

Lines 273-274: I would be cautious with this statement. In my own experience, visually checking sample bottles for *O. dioica* without a microscope has yielded mixed results. It depends on how many individuals are present relative to the background plankton concentrations, whether animals abandoned their houses in the tow, etc. I would suggest always confirming presence with microscopy. Recognize that many animals will likely abandon their houses during the transfer process from cod end to sample bottle.

Authors' response:

We agree. However, visually checking for *Oikopleura* (in the houses or not) in the field helps us determine whether to continue sampling from the same location or move on to another potential location. This is a useful screen measure when looking for a reliable shore sampling location without immediate access to a microscope. The microscopic observation is necessary to confirm species identification and relative concentration of the animals.

Authors' actions. Line 294

"NOTE: The presence of Oikopleura spp. can be confirmed by viewing sample bottles against a black background. Most animals abandon their houses while being collected. Therefore, microscopic observation is needed for species-level identification."

Lines 4.1.1: Is the surface seawater always raw? Sometimes harbors can have a lot of "gunk" in surface seawater, and in my experience it can be helpful to run it through a coarse filter.

Authors' response:

We thank the referee for this comment. We haven't experienced this problem, but we think it is a good idea.

Authors' actions. Line 318

"NOTE: If the plankton sample contains unwanted debris, it is useful to run through a coarse filter (mesh size ~600 μ m) before transferring to a 10 L beaker."

Lines 4.1.3: Not all readers may be familiar with what the "translucent house" looks like, so it may be helpful to provide some additional search features (e.g. spherical, size range). It's also possible that some animals may also be "in between" houses (i.e., inflating house rudiment or free-swimming).

Authors' actions. Line 324

*"4.1.3. Identify *Oikopleura* spp. by looking for 1 - 2 mm long, tadpole-shaped animals undulating their tails inside a spherical, translucent house. Some animals may be temporarily free-swimming without the houses. Gently transfer ~5 animals to an empty petri-dish using a blunt-end pipette."*

Line 302: This line is unclear. Suggest revising to: "For genus identification, evict animals from their houses by poking the house with a transfer pipette."

Authors' actions. Line 329

"4.1.4. For genus identification, evict animals from their houses by gently poking the house with a transfer pipette."

5.1.4: For broader applicability, it would be helpful to reframe these as hours as opposed to time of day (e.g. Microscopic larvae should appear approximately 6 hours after combining mature individuals).

Authors' actions. Line 380

"5.1.4. Let the animals spawn naturally to initiate the next generation. Tailed larvae should appear approximately 3 hours after fertilization."

Line 456: Use past tense consistently throughout this paragraph.

Authors' actions. Line 490

*"Using the sampling procedures described above, most *O. dioica* we recovered were day 2-3 of their 4-day lifecycle (Figure 7C). Mature males were recognized by the yellow coloration of gonads whereas female gonads shimmer gold from eggs that were 70-80 μ m in diameter (Figure 8A, B). Immature *O. dioica* were confirmed by two subchordal cells on their tails (Figure 8D). Another dominant species in our local waters, *O. longicauda*, were similar in size and morphology."*

Line 574: Change "minimum" to "minimal."

Authors' actions. Line 615

We replaced “minimum” with “minimal.”

Lines 578-582: I appreciate the authors' inclusion of this statement. It largely addresses my previous comment about Lines 4.1.1.

Authors' response:

We thank the referee for this comment.

The headers for the algal absorbance table are cut off. The table of materials includes a "comments/description" column that is empty. Some rows under the "name of material/equipment" are cut off.

Authors' actions.

We have corrected the table.

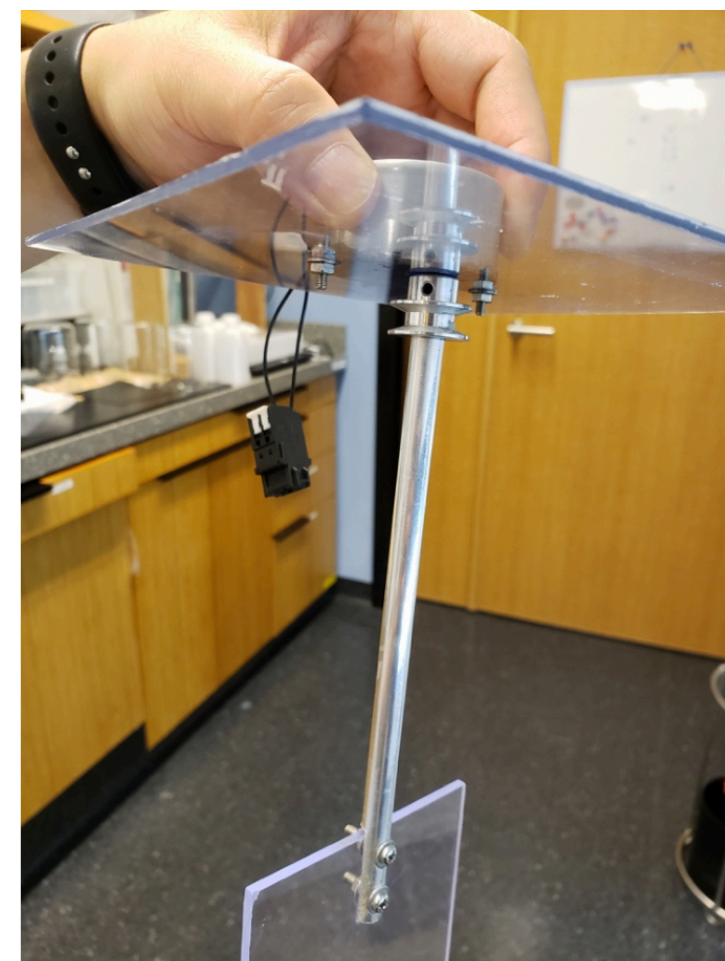
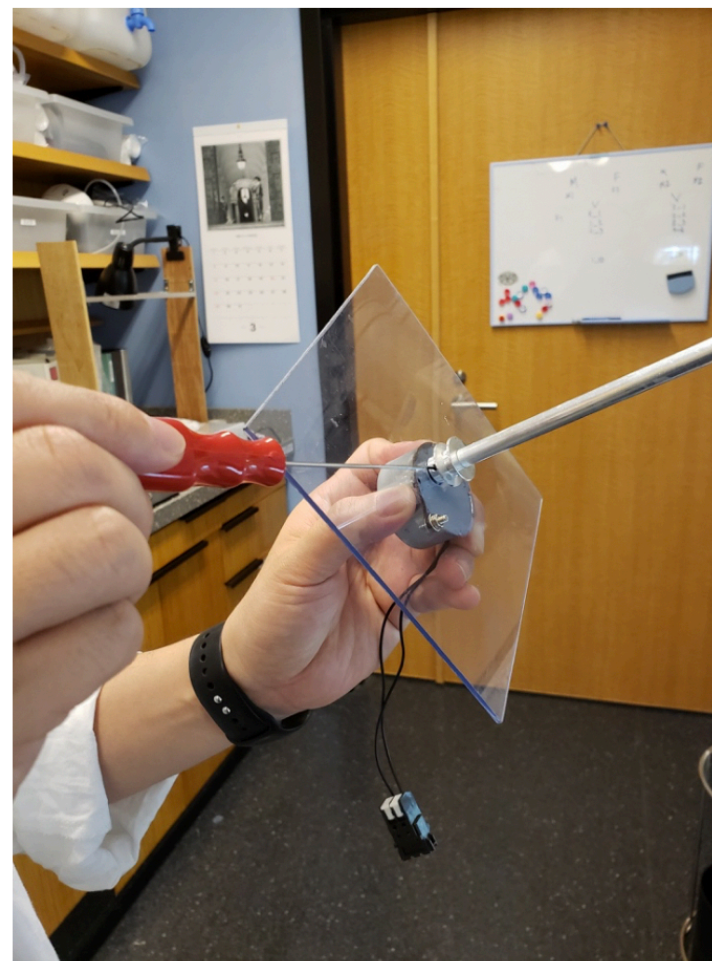
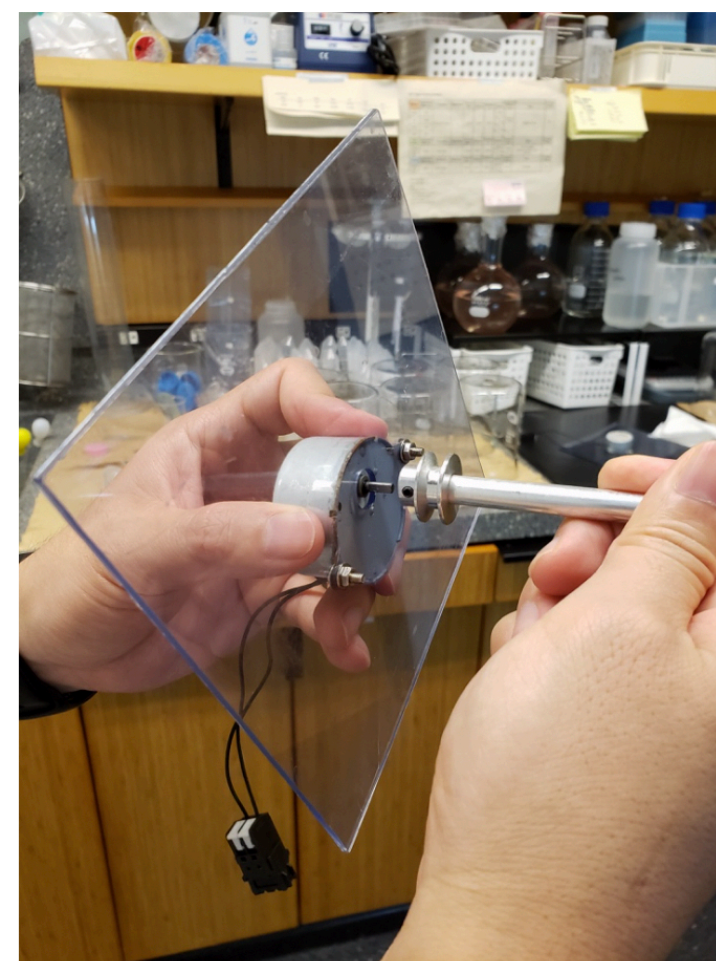
Algal absorbance

| Algal spp. | OD (660nm) | # cells/mL |
|---------------|------------|------------|
| <i>Chaeto</i> | 0.182 | 3792437 |
| <i>Iso</i> | 0.234 | 4914999 |
| <i>Rhino</i> | 0.145 | 1033162 |
| <i>Syn</i> | 0.259 | 77700000 |

Algal volume per feeding

Date:

| | | | 9PM + 5PM (mL / feeding) | | | | 12PM (mL / feeding) | | | |
|--------------|-----|--------------|--------------------------|------------|--------------|------------|---------------------|------------|--------------|------------|
| Beaker | Day | SW vol. (mL) | <i>Chaeto</i> | <i>Iso</i> | <i>Rhino</i> | <i>Syn</i> | <i>Chaeto</i> | <i>Iso</i> | <i>Rhino</i> | <i>Syn</i> |
| 1 | 1 | 10000 | 0 | 2.0 | 0 | 2.6 | 0 | 4.1 | 0 | 5.1 |
| 2 | 2 | 10000 | 2.6 | 4.1 | 9.7 | 0 | 5.3 | 4.1 | 9.7 | 0 |
| 3 | 3 | 5000 | 4.0 | 3.1 | 7.3 | 0 | 5.3 | 4.1 | 7.3 | 0 |
| 4 | 4 | 5000 | 1.3 | 1.0 | 4.8 | 0 | 2.6 | 2.0 | 4.8 | 0 |
| 5 | 1 | 10000 | 0 | 2.0 | 0 | 2.6 | 0 | 4.1 | 0 | 5.1 |
| 6 | 2 | 10000 | 2.6 | 4.1 | 9.7 | 0 | 5.3 | 4.1 | 9.7 | 0 |
| 7 | 3 | 5000 | 4.0 | 3.1 | 7.3 | 0 | 5.3 | 4.1 | 7.3 | 0 |
| 8 | 4 | 5000 | 1.3 | 1.0 | 4.8 | 0 | 2.6 | 2.0 | 4.8 | 0 |
| 9 | 1 | 10000 | 0 | 2.0 | 0 | 2.6 | 0 | 4.1 | 0 | 5.1 |
| 10 | 2 | 10000 | 2.6 | 4.1 | 9.7 | 0 | 5.3 | 4.1 | 9.7 | 0 |
| Total (mL) = | | | 98.6 | 119.5 | 169.7 | 50.9 | | | | |



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Author(s):

Aki Masunaga, Andrew W. Liu, Yongkai Tan, Andrew Scott, Nicholas M. Luscombe

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