Summary of Recommendations

Topic	Life stage	
	Post-settlement	Pre-settlement
Species identification	Select a collection location with one species. If mixed population, use morphological keys to identify and verify with DNA analysis. **Report** estimated percent of each species in the population and the method(s) used for identification.	Select a collection location with one species and verify species identification of the adult population at the site. DNA analysis can be used to verify species identification. If mixed population, examine a representative subsample with compound microscope and use morphological keys to identify. **Report** estimated percent of each species in the population and the method(s) used for identification.
Collection methods	Place artificial substrates 2-3 months before study initiation to obtain mussels of the same cohort and similar exposure history. Collect mussels from natural substrates when required to meet time frame and/or a range of size classes are tested. Remove mussels from substrates by cutting byssal thread attachment and avoid pulling on byssal organ. Rinse mussels with water to remove organic material before placement in transport container. **Report** water chemistry/ quality at the collection site (temperature, pH, alkalinity, hardness, conductivity at a minimum). **Report** the method of collection and handling methods for transport to the study site.	Laboratory culture: collect adult mussels in spawning condition and hold in the laboratory at < 15 °C until spawning is initiated. See Ram et al. 1993; Nichols 1993; Stoeckel et al. 2004 for spawning and culture methods. Field collection: Spawning occurs when water temperature is > 15 °C; monitor veliger development and density at least once a week during the spawning period to determine optimal time for test animal collection. Collect veligers with a 53 to 63 micron mesh plankton net or pump source water through a similar size mesh screen. Collect a representative subsample (minimum of 3 replicates) from the concentrated veliger stock and enumerate total number and number by life stage.

		Report water chemistry/ quality at the collection site (temperature, pH, alkalinity, hardness, conductivity at a minimum). **Report** the method of collection and density of the veliger stock.
Size/age class	Size at maturity varies among locations and can be determined by preparing gonadal squashes from a size range of animals. Once determined, juvenile and adult mussels can be separated by size to compare response to a test agent. Limit the size range (shell length) of mussels in a test group to 5 mm or less (e.g., 8 to 13 mm or 10 to 15 mm). **Report** shell length, average, standard deviation or error, minimum and maximum, number measured in test group (at a minimum)	Determine larval stage based on physical features, rather than shell size; refer to Nichols 1993 and Ackerman et al. 1994). When size is included with larval features, measure height and length of shell. Height is defined as maximum axis from hinge to ventral shell margin; length is defined as maximum anterior-posterior axis, parallel to hinge. **Report** method used to estimate and distinguish life stage.
Holding	Hold mussels for at least 1 week before testing to monitor mortality and to acclimate to test conditions. Provide substrate for attachment. Ensure adequate water exchange to maintain water quality, per ASTME279-23e1 (2023) guidelines. When mussels are held for > 1 week before testing, feed daily at 2.0 to 6.0 mg/L as dry weight with mixed algal die that includes *Isochrysis.* When holding mussels for > 2 weeks, reduce water temperature < 12 °C and reduce feeding rate to reduce metabolic and reproductive activity and prevent fouling of tanks. **Report** (at a minimum) temperature, pH, dissolved oxygen, mortality, feeding rate and ration (daily), alkalinity, hardness, ammonia, conductivity (weekly).	Do not hold veligers for > 48 h before testing. **Report** (at a minimum) temperature, pH, dissolved oxygen (daily), alkalinity, hardness, ammonia, conductivity (once in 48 h).

Monitoring condition	When mussels are held for an extended time > 2 weeks, measure condition every 2-4 week and before testing. Use 1) tissue: shell condition indices (e.g., Davenport and Chen 1987; Ciparis et al. 2019) or 2) glycogen concentration as indicators of condition. **Report** methods used to monitor condition and frequency of measurement.	Do not hold veligers for > 48 h before testing. See suitability for testing.
Acclimation	Acclimate mussels to test temperature and dilution water for a minimum of 48 per ASTM E729-23e1 (2023) guidelines. Acclimate to temperature at a maximum of 0.5 ˚C/h or 3 ˚C/12 h. Do not feed during acclimation. **Report** daily water temperature and water quality during the acclimation period. Monitor daily mortality and discard test organisms if mortality exceeds 0.2% per day during acclimation.	Acclimate to test temperature at no more than 3 ˚C/h. When the source and dilution water chemistry are similar, an acclimation period is not recommended. When source and dilution water chemistry differ, acclimate for 2 to 24 h by partial water exchanges to 100% dilution water. **Report** water temperature and water quality during the acclimation period.
Suitability for testing	Do not use for testing if mortality exceeds 0.2%/day for 3 consecutive days before test (ASTM E279-23e1, 2023). Use at least one additional criterion for evaluation including: 1. Select only mussels which are filtering and close when disturbed 2. Select mussels which resist opening when gentle pressure is applied to open the valves. Discard mussel that can be opened with minimal effort. 3. Cut byssal attachment one day before test initiation. Select only mussels that reattach within 24 h. **Report** the criteria used to determine suitability for testing. Optional: Include a reference toxicant with each toxicity trial to generate comparative data on sensitivity over time	Examine 3 to 4 representative subsamples of veliger stock and determine number dead and alive. Discard the test stock if the percent alive is < 90%. **Report** the method used to estimate veliger stock density and precent alive. Optional: Include a reference toxicant with each trial to generate comparative data on veliger sensitivity across trials and among source populations.

	(during the holding period) and among source populations.	
Sample size	Test 10-50 mussels per experimental unit; include a minimum of 3 replicates per treatment. Increase sample size and replicate number for test agents that induce prolonged valve closure. **Report** number of mussels per experimental unit, volume of experimental unit, number of replicates per treatment.	Test 100-500 veligers per experimental unit; include a minimum of 3 replicates per treatment. Increase sample size and the number of control units to account for natural mortality. **Report** number (or estimate) of veligers per experimental unit, volume of experimental unit, number of replicates per treatment, number of controls.
Mortality criteria	Detach mussels from substrate for mortality assessment. Mortality criteria are 1) closed valves do not resist opening when slight pressure is applied; 2) open valves do not close in response to probing. **Report** the process and criteria used for mortality assessment.	Conduct mortality assessment under a stereo- or compound (preferable) microscope. Stereomicroscope: Stain sample with fast green. Locate veligers with cross-polarized light and observe without polarizer. Live: motile, ciliary or internal organ movement, green "gut spot." Dead: no movement, green stain is not limited to gut, but is diffuse. Compound microscope: Stain can be used but is not essential. Place 1 mL sample on Sedgewick-Rafter counting cell and examine at 40 to 100. Live: ciliary, organ or locomotor movement. Dead: no ciliary or organ activity, extrusion or abnormal appearance of internal organs. **Report** the process and criteria used for mortality assessment. Adjustments for natural mortality: 1. Use statistical models to compare survival in control, or reference, and toxicant treatments. 2. Use Abbot's formula to correct for natural mortality.

		3. Calculate treatment-related mortality as a percent reduction from control mortality.
Attachment	When attachment is a response measure, place substrates in holding tanks for 3-7 days to promote biofilm formation. **Report** the type of substrate (material, size, texture used for mussel attachment. One or more of the following methods can be used to assess affects of a test agent on attachment: 1. Detach mussels before exposure to a test agent. Score reattachment during exposure as yes or no. 2. Detach mussels before exposure to a test agent. Count the number of byssal threads produced during exposure. 3. Expose attached mussels to a test agent. Count the number of mussels that fully detach during toxicant. 4. Expose attached mussels to a test agent. Calculate a byssogenesis index = number of foot extensions × number of byssus threads produced/time. **Report** the method used and criterion for evaluating attachment.	NA
Settlement	NA	Evaluate settlement in a flow-through system with adequate density of veligers in the source water (minimum 2-10 veligers/L). Estimate veliger density and viability at initiation, midpoint and end of a study (at a minimum). Provide uniform substrates for settlement (PVC plates, glass sides) and allow at least 2 weeks for settlement to occur. Settlement may be assessed with or without a stereomicroscope, depending on mussel size. **Report** method of assessment and number of mussels per area.

Exposure methods and reporting parameters	Use a test cohort that is collected from the same source, date and of similar size. Provide clean substrates and use only attached mussels, unless byssal attachment is an endpoint. The choice of dilution water will depend on study objectives. Avoid use of dechlorinated water. Measure temperature, dissolved oxygen, pH (daily), alkalinity, hardness, conductivity, ammonia (frequency depends on exposure duration) in at least one test vessel per treatment. **Report** source and date of collection, source water chemistry and temperature. **Report** source and chemistry of the dilution water. **Report** water quality/chemistry of test vessels during exposure. **Report** test cohort characteristics (shell length, mean, standard deviation, range, n).	Estimate the density, percent alive and life stage composition of the test population by examination at 40 to 100 ×. Discard if the percent alive is <90%. Use uncontaminated source water in control vessels and report water chemistry (temperature, pH, dissolved oxygen, alkalinity, hardness, conductivity). When the test water is not from the source of veliger collection, and differs in water chemistry, acclimate veligers to the test water for up to 24 h. Monitor mortality during the acclimation period and discard veligers if mortality exceeds 10% during the acclimation period. Avoid us of dechlorinated water. Use test vessels that allow for measurement of water quality and test chemical. Refer to Davis et al. 2016 for veliger immersion vessels. Limit exposure to 48 to 96 h. **Report** source and date of collection, source water chemistry and temperature. **Report** source and chemistry of the dilution water. **Report** water quality/chemistry of test vessels during exposure. **Report** veliger test population density, percent alive, number by life stage.
Post-exposure	Transfer mussels to untreated water for at least 48-96 h post-exposure. A longer post-exposure may be needed when water temperature is low or the test agent causes delayed mortality. **Report** water chemistry of holding vessels during the post-exposure period.	Limit the post-exposure period to ≤ 96 h. To minimize handling, use a veliger immersion vessel (Davis et al. 2016) to transfer veligers to untreated water. **Report** water chemistry of holding vessels during the post-exposure period.