

### Notes:

Welcome to this presentation on the United States Environmental Protection Agency's, hereafter USEPA, Testing Methods for Whole Effluent Toxicity, or WET. This presentation is part of a Web-based training series on Whole Effluent Toxicity sponsored by the USEPA Office of Wastewater Management's Water Permits Division.

You can review this stand-alone presentation, or, if you have not already done so, you might also be interested in viewing the other presentations in the series, which cover the use of Whole Effluent Toxicity in the NPDES permits program.

Before we get started with this presentation, I'll make some introductions and cover two important housekeeping items.



### Notes:

First, the introductions.

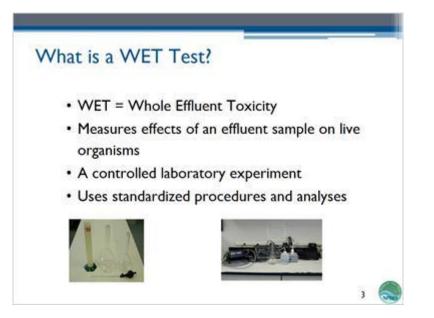
Your speakers for this presentation are, me, Laura Phillips, USEPA's National WET Coordinator with the Water Permits Division within the Office of Wastewater Management at the USEPA in Washington D.C., and Marcus Bowersox, USEPA HQ's contractor and an aquatic toxicologist with Tetra Tech, Incorporated in Owings Mills, Maryland. Second, now for those housekeeping items.

You should be aware that all the materials used in this presentation have been reviewed by USEPA staff for technical and programmatic accuracy; however, the views of the speakers are their own and do not necessarily reflect those of USEPA. The NPDES permits program, which includes the use of Whole Effluent Toxicity testing, is governed by the existing requirements of the Clean Water Act and USEPA's NPDES permit implementation regulations. These statutory and regulatory provisions contain legally binding requirements. However, the information in this presentation is not binding. Furthermore, it supplements, and does not modify, existing USEPA policy and guidance on Whole Effluent Toxicity under the NPDES permits program. USEPA may revise and/or update this presentation in the future. Also, this module was developed based on the live USEPA HQ's NPDES WET course that the Water Permits Division of the Office of Wastewater Management has been teaching to USEPA regions and states for several

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years. This course, where possible, has been developed with both the nonscientist and scientist in mind, and while not necessary, it is recommended that a basic knowledge of biological principles and Whole Effluent Toxicity will be helpful to the viewer. Prior to this course, a review of the USEPA's Permit Writer's online course, which is also available at USEPA's NPDES website, is recommended.

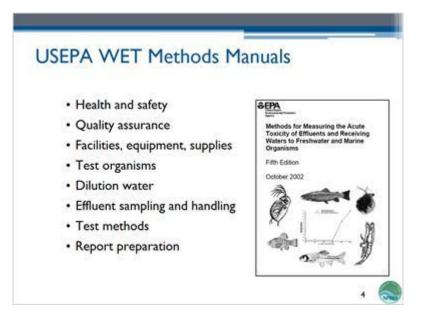
When appropriate a blue button will appear on a slide. By clicking this button, additional slides will present information regarding either freshwater or marine USEPA WET test methods. When these additional slides are finished, you will be automatically returned to the module slide where you left off. The blue button on this slide provides the references for USEPA's WET test methods that will be presented throughout this module. Alright. Marcus will guide us through the USEPA WET test methods used to conduct Whole Effluent Toxicity testing for the NPDES permits program.



#### Notes:

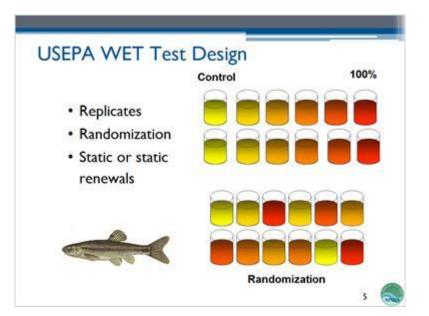
First, let's define WET, or Whole Effluent Toxicity. WET tests are controlled laboratory experiments using standardized procedures and analyses to measure effects of a permitted effluent sample on live WET test aquatic organisms. Depending on the test endpoint being measured (lethality versus sublethal effects), WET tests are considered to be acute or chronic. In acute tests, the exposure periods are considered short-term and therefore are no longer than 96 hours measuring only lethality, defined as the ability to survive after exposure to an effluent. Chronic tests assess possible impacts from effluent exposure to aquatic test organisms for critical lifecycle measurements and therefore may be longer than 96 hours and are usually conducted for 7 days, or in some cases up to 8 days maximum. Chronic tests measure both lethality and immobility and sublethal endpoints such as growth, development, and reproduction.

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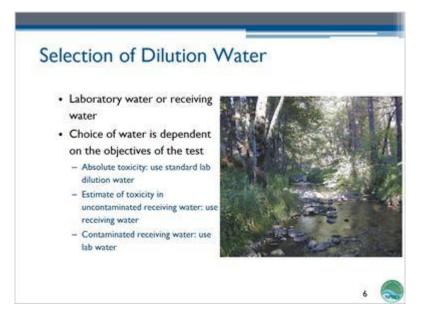
The USEPA has standardized WET test methods for freshwater and marine test organisms. The current USEPA WET test methods manuals were released in 2002 for freshwater and East Coast marine species, whereas the current USEPA West Coast marine WET test manual was released in 1995. These documents define many important aspects of WET test methods for each USEPA approved test species, including, but not limited to, test organism culturing and handling, health and safety, quality assurance, facilities, equipment and supplies, dilution water, effluent sampling and handling, and report preparation. Many of these subjects are discussed in more detail later in this module.



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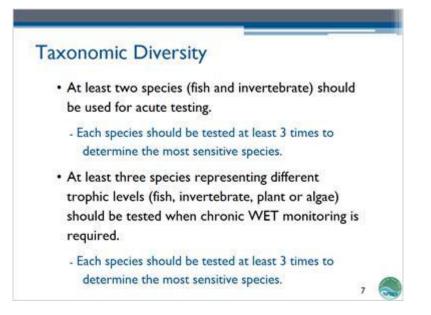
The test set-up or test design for each of the WET test methods is specific to the test organisms and may include different requirements, such as: the number of replicates, randomization of test chambers, and whether to conduct tests using either a static, static-renewal, or flow-through mode. A static test is one in which the test solution that organisms are exposed to is the same throughout the exposure, whereas in a static-renewal test, the solution in the test chambers is replaced with fresh solutions after some predetermined amount of time, typically 24 or 48-hours. In many instances, a static-renewal test will incorporate more than one effluent sample to account for possible effluent variability over time. For the promulgated USEPA WET test methods, all WET tests used for NPDES permitting must consist of a control and five serial dilutions of the effluent. Dilutions of effluent should be made with one of two types of water, as described in the next slide.

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### Notes:

USEPA's WET testing methods indicate that WET tests should be conducted using one of two choices of dilution water: receiving water or laboratory water. The choice of dilution water is dependent on the objectives of the WET test. If the objective is to determine the absolute toxicity of the effluent alone, then standard laboratory water would be used. If an estimate of the toxicity in receiving water is the objective, then receiving water that is known to be non-toxic to the WET test species would be used. Generally, receiving water upstream or outside of the area affected by an effluent discharge should be used in this case. If the receiving water is known or suspected to be contaminated, then standard laboratory water should be used.



### Notes:

According to USEPA's 1991 Technical Support Document for Water Qualitybased Toxics Control, or TSD, it is recommended that at least two USEPA approved WET test species, representing different trophic levels, such as a fish and an invertebrate, should be used in acute WET tests. For chronic WET tests, USEPA recommends testing with at least three USEPA approved WET test species, including a fish, an invertebrate, and a plant or algae. Under both acute and chronic WET testing, each test species should be assessed against the permitted effluent at least 3 times to determine the most sensitive test species to be used for determining whether there is or may be an excursion of a state's water quality standard and/or for monitoring to determine compliance with NPDES WET permit limits. USEPA has developed test methods for acute and chronic WET testing using standardized or surrogate WET test species for each of the three trophic levels.



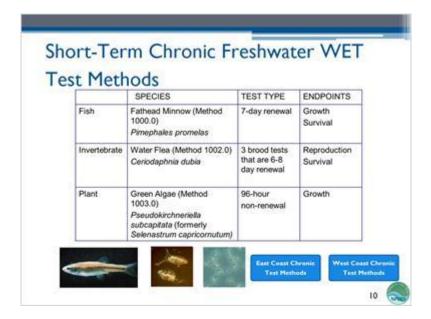
### Notes:

USEPA has standard WET test species for both acute and chronic tests. The pictures presented here represent some of the freshwater test species, including *Ceriodaphnia dubia* (an invertebrate - water flea), *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum* - an algae), *Daphnia magna* (another invertebrate - water flea), *Pimephales promelas* (vertebrate - fathead minnow), and *Oncorhynchus mykiss* (vertebrate - rainbow trout).

	SPECIES	TEST TYPE	ENDPOINTS
Fish	Fathead Minnow Pimephales promelas Rainbow Trout Oncorhynchus mykiss Brook Trout Salvelinus fontinalis	24-, 48-, or 96-h static, renewal, or flowthrough	Survival
Invertebrate	Water Flea Ceriodaphnia dubia Daphnia magna Daphnia pulex	24-, 48-, or 96-h static, renewal, or flowthrough	Survival

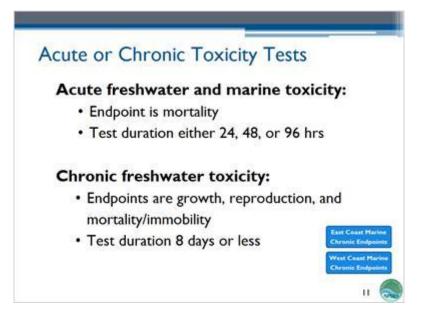
### Notes:

Standard acute fish WET test methods include those for fathead minnows, rainbow trout, and brook trout. Fathead minnows are the standard WET test species for warm-water receiving waters, while rainbow trout or brook trout can be used as test species in those instances where the receiving water may be classified for cold water aquatic life. Standard acute invertebrate WET tests methods include those for the water fleas: *Ceriodaphnia dubia, Daphnia magna,* and *Daphnia pulex.* Acute WET tests are typically less than 96-hours long and can be as short as 24-hours. Acute WET tests are typically conducted in static- or static-renewal mode, but could be conducted as a flow-through test if the potential toxicants are volatile. The only endpoints measured in an acute WET test is lethality.



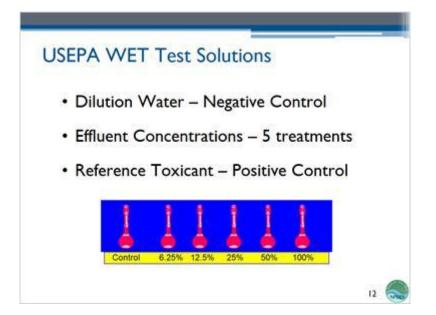
### Notes:

USEPA has three standard species for conducing chronic freshwater WET tests, including: *Pimephales promelas* (the fathead minnow), *Ceriodaphnia dubia* (a water flea), and *Pseudokirchneriella subcapitata* (the green algae). Fathead minnow chronic tests are seven (7) days in length, and the endpoints include mortality/immobilization and growth. *Ceriodaphnia dubia* chronic WET tests are three brood tests which are six (6) to eight (8) days in length depending on the control response, and the endpoints are mortality/immobilization and reproduction. The green algae WET test using *Pseudokirchneriella subcapitata* is ninety-six (96) hours in length, and the endpoint measured is growth in terms of cell density.



### Notes:

This slide summarizes the test endpoints and durations of acute freshwater and marine and chronic freshwater WET tests. Acute WET tests can be 24-, 48-, or 96-hours in length. Typically, acute tests that are over 48-hours in length are conducted in static-renewal mode to prevent overall water quality, especially dissolved oxygen, from changing during the test. For chronic toxicity, the test endpoints include both lethal endpoints, measured as lethality or immobility, as well as sublethal endpoints, measured as growth or reproduction. For freshwater chronic tests, test duration is no longer than 8 days.

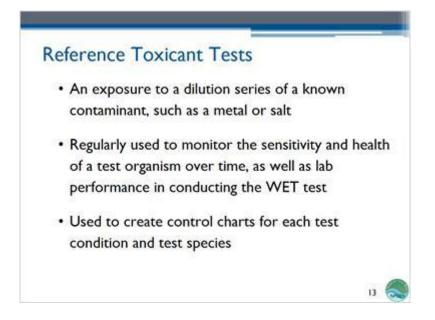


### Notes:

Two test controls are typically employed for WET testing, a negative control and a positive control. The negative control consists of 100% dilution water. The dilution water could be either receiving water or laboratory water as noted previously, but the responses of the organisms in the negative control must meet USEPA's WET test methods' Test Acceptability Criteria, or TACs, for each WET test species in order for the WET test to produce valid data for NPDES permit compliance purposes. USEPA's TACs for different WET test methods are discussed later in this module.

WET tests used in the NPDES permits program must consist of five effluent dilutions plus a control in order to determine the effects of the effluent at different effluent concentrations. These effluent concentrations should include the In-stream Waste Concentration, or IWC, and other effluent concentrations that bracket the IWC (i.e., some less and some greater) to allow for an effective evaluation of the concentration-response pattern observed in the test. In addition to the negative control, a positive control is generally conducted using a separate reference toxicant test that may or may not be run concurrently with the effluent test. Reference toxicant testing will be discussed more in the next slide.

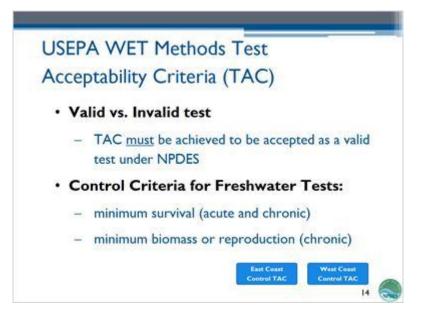
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### Notes:

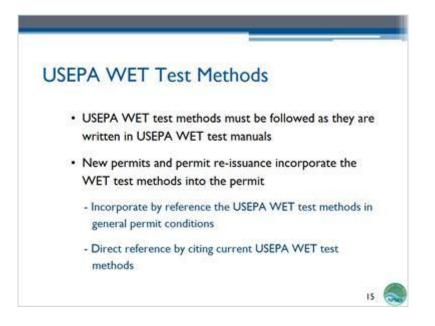
The 2002 USEPA WET test methods define a reference toxicant tests as an exposure to a dilution series of a known contaminant, such as a metal or salt for which the WET test organism response is well established. The choice of reference toxicants is typically lab and species specific, however, they should be fairly stable chemicals that are soluble in water at concentrations that are toxic to the WET test species. Potassium chloride, sodium chloride, and copper sulfate, for example, are all suitable reference toxicants. A reference toxicant testing program is used as part of a laboratory Quality Assurance (QA) program to demonstrate the sensitivity and health of test organisms used in WET tests over time, as well as an approach for determining a lab's performance in conducting the WET tests. Since the test organism's response to a reference toxicant should be similar every time it is conducted, a control chart for each test condition and test species can be created. USEPA recommends that the most recent twenty (20) endpoints be displayed and evaluated in the control chart. Each endpoint needs to be within plus or minus 2 standard deviations of the running average endpoint value (e.g., IC<sub>25</sub>). Reference toxicant tests, control charts, and other Quality Assurance/Quality Control aspects of WET testing are evaluated in detail in the Reviewing WET Tests and WET QA/QC module.

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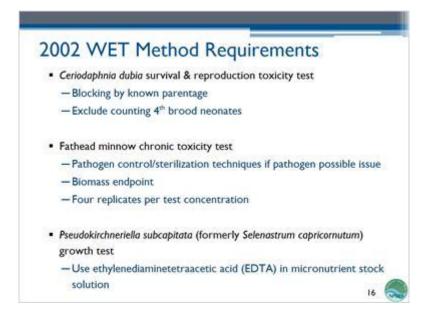
### Notes:

All USEPA WET tests must meet method-specific Test Acceptability Criteria, or TACs, in order for the WET tests to be considered valid. The TACs for freshwater tests consist of minimum survival, growth and reproduction that is considered acceptable in the controls of the test. For acute WET testing, the TAC is that the controls are to have no more than 10% mortality (or greater than or equal to 90% survival) at the end of the test. Chronic tests include TACs for both survival and biomass or reproduction (sublethal endpoints) depending on the WET test method.



### Notes:

The USEPA WET test methods must be followed as written. NPDES permit writers should incorporate the WET test methods into new permits and permit re-issuance by incorporation by reference to the USEPA WET test methods in the permit's general permit conditions section or as a direct reference by citing the current USEPA WET test methods.

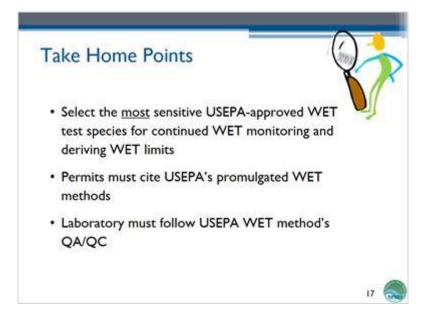


### Notes:

Some of the requirements in the USEPA WET test methods as published in the 2002 USEPA WET test methods include those to the *Ceriodaphnia* survival and reproduction toxicity test. These requirements included the use of blocking by known parentage and the exclusion of fourth broods to determine the reproduction endpoint result.

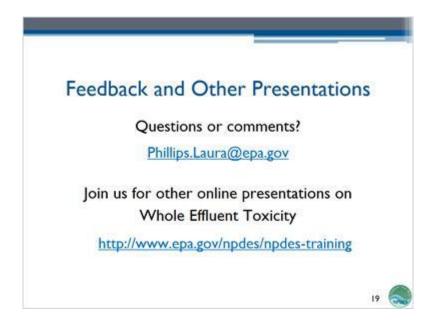
Requirements included in the fathead minnow chronic toxicity test include the ability to control for potential pathogen (microorganism) interference using sterilization techniques, the use of the biomass endpoint, and the use of a minimum of four replicates per test concentration.

The green alga test requires the use of EDTA in micronutrient stock solutions.



### Notes:

In conclusion, some of the points that we hope you learned in this module were that the most sensitive USEPA-approved WET test species should be used for continued WET monitoring and for the potential development of NPDES WET permit limits. New NPDES permits and re-issued permits need to cite the most recent USEPA WET test methods either by a direct cite or by incorporation by reference. WET testing laboratories must follow USEPA WET test methods Quality Assurance/Quality Control (QA/QC), which includes the use of negative controls (dilution water) and reference toxicant tests.



### Notes:

Thank you for joining us for this USEPA's NPDES Whole Effluent Toxicity training presentation. We hope that you have enjoyed it! If you have questions or comments on this or any part of the USEPA's NPDES WET online training curriculum, click on the email address given on this slide to send a message to Laura Phillips, USEPA HQ National WET Coordinator. Remember, you will find all of the USEPA's NPDES WET online training presentations, under the USEPA's NPDES training section found on the Office of Wastewater Management's NPDES website. See you next time!



### Notes:

The pictures presented here represent some of the USEPA WET test species used in acute and chronic marine WET testing on the East Coast, including *Arbacia punctulata* (a sea urchin), *Menidia beryllina* (the inland silverside), *Cyprinodon variegatus* (the sheepshead minnow), *Americamysis bahia* (formerly *Mysidopsis bahia*, a mysid shrimp), and *Champia parvula* (the red macro-algae).

	SPECIES	TEST TYPE	ENDPOINTS
Fish	Sheepshead Minnow Cyprinodon varigatus Silversides Menidia beryllina Menidia menidia Menidia peninsulae	24-, 48-, or 96-h static, renewal, or flowthrough	Survival
Invertebrate	Americamysis bahia (formerly Mysidopsis bahia)	24-, 48-, or 96-h static, renewal, or flowthrough	Survival

### Notes:

Standard acute marine WET test species include sheepshead minnow and three different species of silversides, as well as the mysid shrimp, *Americamysis bahia* (formerly *Mysidopsis bahia*). As indicated for freshwater acute tests, test length can be 24-, 48-, or 96-hours and can be conducted in a static, static-renewal, or flow-through mode. The only endpoint measured in an acute WET test is lethality.

VET T	Test Methods		
	SPECIES	TEST TYPE	ENDPOINTS
Fish	Sheepshead Minnow Cyprinodon variegatus	7-day or 9-day renewal	Growth Survival Teratogenicity
	Inland Silverside Menidia beryilina	7-day renewal	Growth Survival
Invertebrate	Mysid shrimp Americamysis bahia (formerly Mysidopsis bahia)	7-day renewal	Growth Survival Egg Development
	Sea urchin Arbacia punctulata	1 h and 20 min static	Fertilization
Plant	Red Macroalga Champia parvula	7-day to 9-day static non-renewal	Reduction in cytocarp production

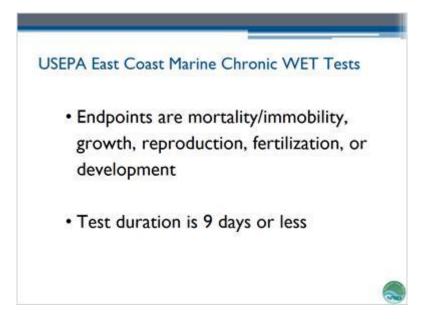
### Notes:

USEPA has multiple fish and invertebrates for conducting chronic East Coast marine WET tests, including the sheepshead minnow and inland silverside, the mysid shrimp, and the sea urchin. The plant or algae WET test method is limited to the red macroalga. Durations for most chronic marine East Coast tests are 7 days, but the sea urchin fertilization test is a 1 hour and 20 minute exposure. WET test endpoints for chronic marine East Coast WET tests include survival, growth, egg development, fertilization, and a reduction in cytocarp production (algae reproduction).

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WE	T Test Methods		
_	SPECIES	TEST TYPE	ENDPOINTS
Fish	Topsmelt (Atherinops affiinis)	7-day renewal	Growth, Survival
Invertebrate	Mysid shrimp (Holmesimysis costata)	7-day renewal	Growth, Survival
	Pacific Oyster (Crassostrea gigas) Mussel (Mytilus sp.)	48-hour static	Shell Development, Survival
	Red Abalone (Haliotus rufescens)	48-hour static	Shell Development
	Purple Urchin (Strongylocentrotus purpuratus) Sand Dollar (Dendraster excentricus)	72-hour static	Larval Development Survival
	Purple Urchin (Strongylocentratus purpuratus) Sand Dollar (Dendraster excentricus)	40-minutes static	Egg Fertilization
Plant	Giant Kelp (Macrocystis pynifera)	48-hour static	Germination, Length

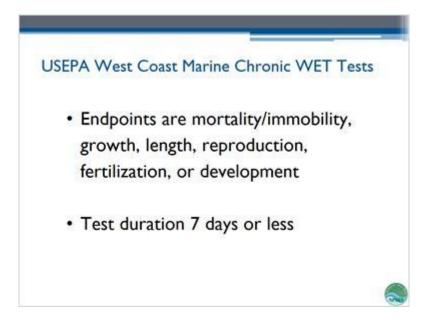
### Notes:

USEPA has standard WET test methods for conducting short-term chronic marine test with species that are more indicative of those on the West Coast, including topsmelt, mysid shrimp, Pacific oyster, mussel, red abalone, purple urchin, sand dollar, and giant kelp. Durations for chronic marine USEPA West Coast WET tests methods vary from 40-minutes for the purple urchin or sand dollar fertilization test, to 48-hours for the Pacific oyster and mussel shell development test, to 7 days for the mysid and topsmelt survival and growth test. WET test endpoints for the USEPA West Coast chronic marine WET test methods include growth, survival, shell development, larval development, egg fertilization, and germination.



### Notes:

For USEPA East Coast chronic WET tests, the test endpoints include lethal endpoints measured as mortality or immobility, as well as sublethal endpoints measured as growth, reproduction, fertilization, or development. For USEPA East Coast marine chronic WET tests, the test duration is no longer than 9 days.



### Notes:

For USEPA West Coast chronic tests, the WET test endpoints include lethal endpoints measured as mortality or immobility, as well as sublethal endpoints measured as growth, length, reproduction, fertilization, or development. For USEPA West Coast marine chronic WET tests, the test duration is no longer than 7 days.



### Notes:

The pictures presented here represent some of the USEPA WET test species used in chronic marine WET tests on the West Coast of the United States, including *Atherinops affinis* (the topsmelt), *Holmesmysis costata* (a mysid shrimp), *Crassostrea gigas* (the Pacific oyster), *Mytilus* sp. (a blue mussel), *Stronglyocentrotus purpuratus* (the purple sea urchin), *Macrocystis pyrifera* (the giant kelp), and *Dendraster excentricus* (a sand-dollar).



### Notes:

For acute USEPA East Coast marine WET testing, the TAC is that the test controls have no more than 10% mortality or no less than 90% survival at the end of the WET test. Chronic tests include TACs on both survival (lethal endpoints) and on growth, reproduction, fertilization, or development (sublethal endpoints) depending on the WET test method.



### Notes:

Chronic USEPA West Coast marine WET tests include TACs for both survival (lethal endpoints) and for growth, length, reproduction, fertilization, or development (sublethal endpoints) depending on the WET test method.