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DISCLAIMER

METHODS FOR COLLECTING, CULTURING AND PERFORMING TOXICITY TESTS WITH *Daphnia ambigua*

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Background

Toxicity tests conducted on water collected from impacted locations in SRS streams often failed chronic toxicity tests and sometimes failed acute toxicity tests (Specht 1995). These findings prompted SRS to determine the cause of the failures. Some SRS NPDES outfalls were also failing chronic toxicity tests, even though no toxicant could be identified and when TIEs were performed, none of the TIE treatments removed the toxicity. Ultimately, it was determined that the failures were due to the low hardness of SRS surface waters, rather than to the presence of a toxicant. The species of cladoceran that the EPA recommends for toxicity testing, *Ceriodaphnia dubia*, is stressed by the very low hardness of SRS waters. SRS developed an alternate species toxicity test that is similar to the EPA test, but uses an indigenous cladoceran, *Daphnia ambigua* (Specht and Harmon, 1997; Harmon et al., 2003). In 2001, SCDHEC approved the use of *D. ambigua* for toxicity testing at SRS, contingent upon approval by EPA Region 4. In 2002, EPA Region 4 approved the use of this species for compliance toxicity testing at SRS. Ultimately, the use of this species demonstrated that SRS effluents were not toxic, and most toxicity testing requirements were removed from the NPDES permit that was issued in December 2003, with the exception of one round of chronic definitive testing on outfalls A-01, A-11, and G-10 just before the next NPDES permit application is submitted to SCDHEC.

Although the alternate species test was developed at SRS (1996 – 1998), the culture was transferred to a contract toxicity testing lab (ETT Environmental) located in Greer, SC in 1998. ETT Environmental became certified by SCDHEC to perform toxicity tests using *D. ambigua* in 2002, and at this time is the only laboratory certified by SCDHEC to perform tests with this species.

Because of the expense associated with maintaining the *D. ambigua* culture for several years when no toxicity testing is required, SRS decided to suspend financial support associated with maintaining the cultures until testing is needed. The purpose of this document is to provide guidance on how to establish a laboratory culture of *D. ambigua* so that a culture can be restarted when needed.

Source of Organisms

To date, all of the *Daphnia ambigua* maintained in culture at SRS and at ETT Environmental have been collected from the same source, a privately owned pond located in north-central Aiken County just south of I-20. The pond is called Brooks Pond, and it is owned by Dr. Barbara Taylor (SREL) and her husband, Dr. Mark Brooks. Dr. Taylor has been very cooperative in either collecting organisms or provided us with the equipment needed to collect *D. ambigua* needed to start a laboratory culture. Contact information for Dr. Taylor is: 725-9609 (office at SREL) or 725-7455 (lab at SREL) or btaylor@srel.edu.

Collection Method

D. ambigua is typically a lentic species that inhabits blackwater ponds. The organisms are often found just above the surface of the sediment, or in the bottom-most half meter of water in a pond. They are collected using a plankton net that is allowed to sink to

near the bottom and then is obliquely towed toward the surface (using a row boat) or pulled slowly through the water. Water from the plankton bottle at the tip of the plankton net should be carefully poured into a larger collection bottle. Multiple tows should be performed to ensure that sufficient organisms are collected. The organisms should be transported at ambient temperature (do not chill). Dr. Taylor has indicated that *D. ambigua* can probably be collected at anytime of the year, but it is probably easiest to collect them in late spring or early summer. In the wild, by late summer they are typically in poor condition, due to insufficient food.

Identification

D. ambigua is the smallest species of the genus *Daphnia*. It generally is less than 1 mm in length, and never greater than 1.3 mm. It is sometimes confused with *Daphnia parvula*. Distinguishing characteristics for the two species are listed in Table 1. A photograph is D. ambigua is shown in Figure 1.

Table 1. Distinguishing Characteristics of *Daphnia ambigua* and *Daphnia parvula*

Daphnia ambigua	***Daphnia parvula***
Proximal, middle, and distal pecten of postabdominal claw all the same size	Middle pecten long and thin in comparison to proximal and distal pectin
Ocellus present	Ocellus absent
Swimming hair at the base of the second segment of the three-segmented ramus extends beyond tip of ramus.	Anterior margin of head broadly rounded (sheep head)
Swimming hairs of antennae do not extend beyond posterior margins of valves.	Posterior spine less than ¼ valve length
Small head and valves, no more than 1.3 mm in length	Head and valves no more than 1.2 mm in length

Figure 1. Photo of *Daphnia ambigua*

D. ambigua can be most easily differentiated from D. *parvula* by the presence of an ocellus in *D. ambigua*. Dr. Barbara Taylor at SREL is probably the best source for confirming that the organisms collected are *D*. *ambigua*, rather than *D. parvula*.

Culture Methods

Culture Water - *D. ambigua* prefers a water hardness of around 10 mg/l. The synthetic water that has been used to successfully maintain this species is a modified version of the EPA's formulation for very soft water (U.S. EPA 2002), that has 3 mg/l of NaCl added (Table 2). The EPA method of preparing the water should be followed. This involves adding all of the chemicals except calcium sulfate to nanopure water and aerating overnight. Then calcium sulfate is dissolved in an appropriate volume of nanopure water, stirred on a magnetic stirrer until dissolved and added to the synthetic water. The final mixture is then aerated vigorously for at least 24 hours. It is also possible to use a synthetic version of Fire Pond water (see Specht, 2000 for details). When using synthetic waters, ETT Environmental found that the quality of the nanopure water used to prepare the synthetic water is very important. The water must flow through the nanopure filter at a very slow rate. They also found that the synthetic water must be aged for at least a week before it is used. *D. ambigua* performs poorly in freshly prepared synthetic water. SRS surface waters that have been used to maintain *D. ambigua* cultures include Fire Pond (an impoundment located west of Road F) and the A-01 wetland.

Table 2. Modified EPA Very Soft Synthetic Water (target hardness = 9 to 10 mg/l)

Chemical	Chemical Added (mg/l)
$NaHCO_3$	12
$CaSO_4.2H_2O$	7.5
$MgSO_4$	7.5
NaCl	3
KCl	0.5

Resulting Concentrations (measured)

Ion	Measured Concentration (mg/l)
Na	4.5
Ca	1.7
Mg	1.5
K	0.3
SO_4	10.2
HCO_3	8.7
Cl	2.1
Hardness	9.6

Culture/Testing Conditions.

Feeding - In general, the EPA methods for culturing and testing using *Ceriodaphnia dubia* (U.S. EPA 2002), are followed. However, *D. ambigua* does better when fed twice as much *Selanastrum* as is used for *C. dubia* (0.2 ml, rather than 0.1 ml), but the normal amount of YCT (0.1 ml).

Temperature - The EPA recommends a temperature of 25° C for culture maintenance and testing of *C. dubia* (U.S. EPA 2002). Specht and Harmon (1997) found that *D. ambigua* did better at a temperature of 21° C, but required 10 days to produce 3 broods. However, ETT Environmental successfully maintained cultures at a temperature of 25° C. If possible, a temperature of 25° should be used, since tests can be completed in less time (7 or 8 days) at the higher temperature..

Test Conditions -. *D. ambigua* are weaker swimmers that *C. dubia* and are more prone to getting caught in the surface tension of the water. ETT Environmental found that the organisms were more likely to get caught in the surface film when plastic containers were used that with glass containers. They also found that for test containers, narrow glass vials (tall, narrow 30 dram vials) seem to work much better than disposable plastic cups. During tests, the vials should be checked several times each day, and any organisms caught in the surface film should be gently removed from the film using a dropper and repositioned deeper in the water.

Length of Test - Following EPA protocol (U.S. EPA 2002), a chronic toxicity test is terminated when 80% of the control organisms complete their third brood. With *C. dubia*, tests almost always finish on the seventh day. ETT Environmental found that *D. ambigua* tests often require 8 days, rather than 7 days, to produce 3 broods.

Number of Young Produced - EPA protocol (U.S. EPA 2002), specifies that a minimum of 15 young must be produced in three broods. The same criterion is used for *D. ambigua*. In healthy cultures, the number of young produced in three broods typically ranges from the low 20's to around 30.

Issues Related to Compliance Toxicity Testing

All toxicity testing performed for NPDES compliance must be performed by a SCDHEC certified laboratory. At present, ETT Environmental is the only laboratory certified by SCDHEC with D. ambigua. If ETT is not available to perform the required testing, possible alternatives include Clemson University (initial contact would be Dr. John Rodgers (864-646-2960), or possibly Dr. Michele Harmon (adjunct faculty at USC Aiken and USC Columbia).

SCDHEC currently requires five rounds of testing with a reference toxicant (generally either sodium chloride or cupric chloride) to document that organisms in the culture exhibit the appropriate sensitivity to a chemical toxicant.

The current contact at SCDHEC for toxicity testing is David Graves 803-898-4398; for Certification of Environmental Laboratories, R. Wayne Davis, 803-896-0970

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