Appendix C:

Additional reference materials

<table>
<thead>
<tr>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Questions and answers on the Duckweed Toxicity Test</td>
<td>C-1</td>
</tr>
<tr>
<td>Recipe for Cabbage Indicator Solution</td>
<td>C-14</td>
</tr>
<tr>
<td>Soil Nitrogen Test using the LaMotte Test Kit</td>
<td>C-15</td>
</tr>
<tr>
<td>Petri plate grid templates</td>
<td>C-16</td>
</tr>
</tbody>
</table>
Questions and Answers on
the Duckweed Toxicity Test

Wuncheng Wang
USGS, WRD
PO Box 1230
Iowa City, IA 52240

Since the publication of the short article “Toxicity Testing and Biomonitoring Using Aquatic Plants” in Aquaphyta newsletters in fall 1990 (Wang, 1990a), I have received several calls from the U. S. Environmental Protection Agency, consulting laboratories, colleges, and overseas regarding the duckweed toxicity test. In addition to request for literature, some callers asked for specific information on duckweed cultures, culturing and testing conditions, and related questions. These requests arose because the test is not widely known compared to the more conventional tests using algae, daphnids, and fish, the “so-called “three base-set tests” adopted by regulatory agencies. Based on correspondence I suspect that inexperience and lack of information could dampen some researchers’ interest in exploring the test — which would be unfortunate.

The duckweed toxicity test has been described as simple, rapid and cost-effective (Wang and Williams, 1988). Further studies are encouraged to shed more light on the test. For this reason, I am presenting the following basic information in the format of questions and answers, hoping that the discussion may stimulate interest in this area, especially for its application in Toxic Substances Hydrology and NAWQA programs of the U. S. Geologic Survey.
Q. What is the rationale for conducting the duckweed toxicity test?

A. Duckweed is a common aquatic vascular plant, is easy to culture, and requires low maintenance. It is small so that large laboratory space is not required for culturing and testing, yet it is large enough so that toxic effects can be observed visually. The duckweed toxicity test requires a short start-up time. Once the culturing facilities are operational and under optimum conditions, duckweed multiplies readily, making available inexhaustible test species at all times.

The duckweed toxicity test has a relatively long history of studies and a large data base among phytotoxicology or plant toxicology (Hillman, 1961; Hillman and Culley, 1978; Landolt and Kandeler, 1987; Wang, 1990b; Lewis, 1991; Walsh, in press). The test has been used for toxicity assessment of river water, ground water, industrial and municipal effluents, leachates from industrial and municipals solid landfill, sediment and soil.

The duckweed test is especially sensitive to herbicides toxicity, to which no other test species currently in the literature is more appropriate. This is highly significant because herbicide application rates in the United States and many parts of the world have steadily increased over the past twenty years, and as a consequence, herbicide contamination has been widespread in rivers, reservoirs, lakes and ground water. (Goolsby and others, 1991; Wang, 1991).

Some reports have shown that herbicides have very low toxicity to fish and other faunal species (Gersich and Mayer, 1986). Faunal species, however, are a part of the ecosystem, but not the whole system. Herbicides, when reach a non-target area, will cause unacceptable damages directly to plant species and eventually to other species (Boutin and Freemark, pending).

The author recently completed a manuscript entitled "The role of ecotoxicology in water-quality studies" (Wang, pending). Some issues presented in the manuscript, particu-
larly the comparison of toxicity tests and chemical analysis for assessment of toxic substances or water samples and, the status of phytotoxicology, are equally applicable in the current article. These two papers, one pertaining general discussion of ecotoxicology and the other, specifically the duckweed toxicity test, are mutually supporting each other.

Q. What is the status of the duckweed toxicity tests?

A. The duckweed toxicity test has had its share of early naysayers. Industrial scientists tried to discourage the use of the test on issue of sensitivity and ecological relevancy (Kenaga and Moorlennaar, 1979; Bishop and Perry, 1981). The methods they used and, conclusions they reached, however, were questioned (Wang, 1984; Wang and Freemark, pending).

The inclusions of the duckweed test for chemical hazard assessment as part of the Toxic Substance Control Act (Federal Register, 1985) and the Federal Insecticide, Fungicide, and Rodenticide Act (Holst and Ellwanger, 1982), and its requirement by the Organization for Economic Cooperation and Development (currently under development) symbolize the test as a legitimate part of ecotoxicology. The duckweed toxicity test recently has received more attention since scientist form the U.S. Environmental Protection Agency, consulting laboratories, and research institutions are focusing on this test as a part of effluent biomonitoring required under National Pollutant Discharge Elimination System permitting. The major advantage of the duckweed toxicity test over the algal toxicity test is that the duckweed test can be preformed using either renewable flow-through method, as pre-requisite for effluent biomonitoring, while the algal test can not. An extensive literature review on the test methodology and results have been reported (Wang, 1990b).

A consensus duckweed test protocol has been published by the American Society for Testing and Materials (1991), while another protocol has also been published as Section 82111
Q. Which duckweed culture is recommended for toxicity testing?

A. I believe in a two-pronged approached. For regulatory purposes, the use of a standardized test culture under specified testing conditions is a pre-requisite for acceptance. Currently the U. S. Environmental Protection Agency favors *Lemna gibba* G3 as the test species (Holst and Ellwanger, 1982). The same species is also adopted by the American Society for Testing and Materials (1991). The agency, however, may accept results using other species. Common duckweed, *Lemna minor*, is another strong candidate because it is more widely distributed. The test results of *L. minor* are thus considered relevant to far wider areas. In addition, the data base of *L. minor* is larger than that of *L. gibba* G3 (Wang, 1990b). Both *L. gibba* and *L. minor* are available from sources of culture collections.

The field of duckweed toxicity test is relatively young, and many discoveries remain to be made. Researchers are encouraged to explore other culture(s) so that the test can benefit from new discoveries and be refined further.

Q. What difference does it make to use different cultures?

A. Two pieces of information suggests that different cultures have a relatively minor effect on toxicity test results. First, in a five month continuous study of duckweed tests for chromium (VI) toxicity, the frond increase on the control samples ranges from 61 to 99 counts, a variation...
of 62 percent. During these tests, the 50 percent inhibitory effect concentration (IC50) values of chromium (VI) ranged from 12 to 14 mg/L, a variation of 14 percent (Wang, 1987). Secondly, industrial and municipal effluent samples (23) were tested twice using cultures I and II (Wang, 1990c). Although the increase frond counts of the control samples were 60 to 93 counts, respectively, the regressive analysis of test results between these two cultures indicated that toxic effects were highly correlated, r-0.914, n-23. These results suggest that if test conditions were comparable, toxic effects would be close, regardless of species and conditions of the test specimens used for testing.

Let me hasten to add that interlaboratory variation may account for major error in toxicity test results. At this time, I am not aware of any round-robin study of the duckweed toxicity test, which I believe is urgently needed.

Q. Is axenic culture of duckweed required?

A. Axenic duckweed culture has been used for toxicity testing (Hughes and others, 1988; Cowgill and Milazzo, 1989; Clement and Bouvet, 1992). For this method to be meaningful, duckweed culture, test sample integrity is comprised, whether through filtering or autoclaving.

For this reason, I have been conducting all of my duckweed toxicity tests in nonaxenic conditions and the results were published in a series of scientific papers (13). The studies encompass method development, toxicity assessment of inorganic and organic compounds, industrial and municipal effluents, toxicity reduction evaluation, sediments, and surface and ground water. Other researchers also used nonaxenic conditions for duckweed culturing and testing (King and Coley, 1985; Taraldsen and Norberg-King, 1990).

Algal contamination, especially blue-green algae, can cause problems with duckweed
Duckweed roots are the major site of algal contamination. One approach to minimize algal contamination is to cut off all the roots of duckweed test specimens (Taraldsen and Norberg-King, 1990). The no-root specimens can be used for testing just as they have roots; duckweed specimens regrow new roots rapidly and the plants multiply normally. This approach is especially useful if the duckweed test is maintained for seven days or longer, but it appears unnecessary if the test is held for either 96 or 120 hours.

Some algal bloom, especially blue-green algae, can kill off duckweed culture, possibly through allelopathy and/or nutrient competition. If algal contamination is a problem, a researcher can select one or a combination of the following approaches: consider the use of axenic culturing and testing, start a new culture, or maintain the culture in several (not interconnected) vessels.

Q. Which duckweed nutrition solution do you recommend?

A. At least ten duckweed nutrient solutions are reported in the literature, although there is no comparative study of their strengths and weaknesses. Because algal culturing and testing have been widely used, I thought it would be ideal to use the same nutrient base for both algae and duckweed (Wang, 1986).

There are two main differences between algal and duckweed nutrient solutions. First, instead of seven individual stock solutions for the algal test (American Public Health Association and others, 1992), there are three stock solutions for the duckweed test (American Public Health Association and others, 1992). This is a considerable saving of labor and laboratory space. Second, the nutrient concentration was increased to either 20-fold (American Society for Testing and Materials, 1991) or 10-fold (American Public Health Association and others. 1992) in the duckweed nutrient solution comparing with the algal nutrient solution.
If toxic metals are tested, the chelating agent (typically ethylenediaminetetraacetic acid) must not be included in the nutrient stock solutions. To prevent precipitation from occurring in the stock solutions, these solutions can be acidified to pH 2. Before the duckweed test is initiated, the pH value of the working nutrient solution should be adjusted to 7.5.

Sediment and soil extract have been used as duckweed nutrient supplement (Wang, 1986; Taraldsen and Norberg-King, 1990). The approach, however, is no longer recommended as a standardized protocol for regulatory purpose, because it is difficult to standardize the soil or sediment sample(s) to be used.

Q. Do you recommend using tap water as the dilution water?

A. NO. All my experiments have been conducted with the same amounts of nutrients in both control and test samples with various dilutions. This is the recommended method by both the American Society for Testing and Materials (1991) and the American Public Health Association and others (1992). If a researcher is interested in diluting effluents or toxicants with tap water, then it is required that the control sample be diluted with tap water in the same manner. One can than compare the results of diluted samples and diluted controls, both with the same dilution steps (5-6 dilutions).

Q. What is the optimal temperature for duckweed culturing and testing?

A. Common duckweed can tolerate temperatures from 5 to 30 degrees C and will die above 30 C. For long-term culturing, temperature can be held at 25-29 C. For duckweed testing, the temperature should be held at 26-28 C. Ideally, conditions for culturing and testing should be the same.
Q. What are advantages to culturing and testing duckweed with continuous lighting?

A. All my experiments have been conducted with continuous lighting. Although this method is not as natural as light-dark cycle, it has two advantages. First, duckweed growth is faster and frond count is larger. Second, conditions in the environmental chamber for culturing and testing are more consistent and thus easier to control. While the advantage of light-dark cycle is a larger biomass, if biomass is used as the test indicator. The important factor to remember is that the test results are expressed as relative to the control sample. In other words, the same conditions must be held true for the test and control samples, and the same condition must be held from one experiment to the other, if results are to be compared.

Q. Why only two-frond colonies are selected for testing?

A. Duckweed multiplies rapidly, mostly be asexual reproduction. Mother and daughter fronds tend to form a colony, in two, three, or more fronds. There are two reasons for selecting two frond colonies for testing. One is consistency (using the same age group) of specimens for testing. The other is practicality. At the beginning of and end of test, the task of frond counting is easier if two-frond colonies are used. For example, if four-frond colonies were used, then at the end of the test, the frond counting becomes time consuming because duckweed fronds aggregate and overlap, and small fronds may be hidden and overlooked.

To obtain two-frond colonies, I grabbed a batch of culture and placed in a 20 x 30 x 5 cm steel vessel containing approximately 1 L tap water. The culture spread out and I used a lighted magnifying glass to select test specimens. After repeating a few times, I often obtained sufficient specimens for one ore more tests. Each test typically consists of six dilutions and one control, three replications of each, and 16 fronds in each vessel, thus a total of 336 fronds (168
colonies) are required.

The selection of the test specimens requires one to two hours for each test. Prior to placing the selected specimens into test vessels, screen the specimens again to remove duckweed plant which develop new frond. Make sure the specimens are without blemish, such as necrosis (partial dead tissue), discoloration, chlorosis, and imperfect frond development.

Q. Which test vessels are recommended?

A. Dishes, beakers, sups, jars, and flasks all have been used (Wang, 1990b). Any vessels, such as polystyrene materials, to which duckweed specimens adhere strongly, are not recommended. Use only one kind of vessel in one test.

Test vessels should be covered during the test to prevent excessive evaporation and to prevent dust or insects from falling into the test solution. Flasks can be covered with a glass beaker or a foam plug. Beakers, cups, or jars can be covered with watch glasses. The use of cover will reduce light energy duckweed specimens receive. Use only one kind of cover for one test.

If the known test compounds are organic substances, glass vessels should be used. For unknown or complex mixtures, glass vessels are recommended.

The test vessel can be as small as a 60 x 15 mm petri dish containing 15 ml of test solution and 16 duckweed fronds. The method offers a small-volume, convenient, and economic approach for duckweed toxicity test. In addition, the test results (using frond increase as the test end point) can be obtained using a small vessel (15 ml) were found comparable to those using a larger vessel (100 ml) of liquid (Wang, 1990b).
Q. What is the acceptable duckweed test result (s) for quality control?

A. In the case of L. minor, the test result is acceptable if the duckweed control produces twice (2x) or more of new fronds in 96 hours. In the two-year study mentioned previously, all control samples of 59 tests showed growth more then 2x (Wang, 1987). In other studies, the control sample did not meet this requirement. They were repeated using new culture (Wang, 1990c).

Q. How do you express test results?

A. Duckweed test results can be expressed using increased frond count versus concentration, similar to the approach adopted by Walsh and others (1991). A null hypothesis can be used to determine whether a significant difference existed between a test and a control sample.

Alternatively, a 50 percent inhibitory concentration (IC50) can be calculated using the moving average method (Weber and others, 1990). In this method test results are expressed as “relative growth inhibition,” comparing with the control sample.

References


Boutin, C. and Freemark, K., pending, Impacts of agricultural herbicide use on terrestrial wildlife—A review.


Kenaga, E.E., and Moolenaar, R.J., 1979, Fish and Daphnia toxicity as surrogates for aquatic vascular plants and algae: Environmental Science and Technology 13, 1479-1480.


Appendix C / 11


Wang, W., 1984, Editorial—Use of aquatic plants in ecotoxicology: Environmental International 10, i-iii.

Wang, W., 1986, Toxicity tests of aquatic pollutants by using common duckweed: Environmental Pollution (Series B) 11, 1-14.

Wang, W., 1987, Chromate ion as a reference toxicant for aquatic phytotoxicity tests: Environmental Toxicology and Chemistry 6, 953-9690.

Wang, W., 1990a, Toxicity testing and biomonitoring using aquatic plants: Aquaphyte Newsletter, University of Florida, Gainsville, FL, 10. 1.

Wang, W., 1990b, Literature review on duckweed toxicity testing: Environmental Research 51, 7-22.

Wang, W., 1990 c, Toxicity assessment of pretreated industrial wastewaters using higher plants: Research Journal of Water Pollution Control Federation 62, 853-860.

Wang, W., 1991, Literature review on higher plants for toxicity testing: Water, Air, Soil Pollution 59, 381-400.

Wang, W., pending, The role of ecotoxicology in water-quality studies.

Wang, W., and Freemark, K., pending, Plant toxicology—Addendum to four-part series.


The author is chairman of the Joint Task Group (8211) of the Standard Method Committee, prepared the duckweed toxicity test as a part of Standard Methods for Examination of Water and Wastewater since 1989. He was chairman (198301987) of the Duckweed Task Group of the American Society for Testing and Materials, Committee E-47 on Biological Effects and Environmental Fate.
Cabbage Indicator Solution

Directions for preparation:

1. Cut up 1/2 of a red cabbage into 1-inch pieces with a knife and a cutting board.

2. Place the pieces in a 1000 ml beaker or flask.

3. Add water, preferably distilled, until the cabbage is covered.

4. Place the beaker on a hot plate and heat to boiling until the red color is released from the leaves and colors the leaves the solution. The cabbage pieces will then appear colorless.

5. Strain out the cabbage, store the liquid in a 1-liter plastic bottle and refrigerate. For long term storage, dilute 50% with a 70% isopropyl alcohol or ethanol solution.
Soil Nitrogen Test using the LaMotte Test Kit

The LaMotte kit is a groundwater test kit but can be used to test soil nitrogen with minor modifications in the procedure. These procedures apply only to soils or potting mix (such as Jiffy) with low nitrogen concentration and low clay content. Clay particles will interfere with colorometric results. High nitrogen concentration can be adjusted for by increasing the proportion of water used to dissolve nitrogen from the soil.

Procedure:

1. Dissolve nitrogen from the soil by mixing a 10:1 water to dry soil mixture and letting it stand for 15 minutes.

2. Pass mixture through a filter and collect mixture. A minimum of 3 mls is required for the LaMotte test. Keep this and the absorbancy of the filter paper in mind when deciding on the amount of water and soil to collect in the experiment.

3. Make sure that the water sample is clear as any suspended particles will interfere with the interpretation of the results.

4. Follow instructions in the LaMotte test kit.

5. When interpreting the results, remember to multiply them by the dilution factor. For example, if the soil sample was diluted 10:1 with water, a colorometric reading of .25ppm would mean a soil concentration of 2.5ppm.