Evolution of Ceriodaphnia dubia
An Inquiry Based Learning Lesson Plan

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Abstract

Students were challenged to learn about various aspects of evolution, especially natural selection, through the process of inquiry. Students were provided asexually reproducing cultures of *Ceriodaphnia dubia* (water fleas indigenous to Ohio streams, ponds, and other natural waters). Multiple broods in short periods of time make *C. dubia* ideally suited to learn about their morphology and evolution through the process of inquiry. The inquiry portion of the lesson plan followed the Dragonfly QUEST model. Using a guided inquiry, students were encouraged to question and observe, uncover comparative questions, explore predictions, start an action plan, and think hard and share findings. The actual lesson plan scaffolded learning with the aspects of the inquiry model supported with instruction. Ideally, the project results in open inquiries continued throughout the school year. The overall goal of the project was for students to learn about *C. dubia* and evolution through the process of inquiry. The plan was well executed by Connie Lott in her 8th grade class at Lima South School. The students exhibited good scientific technique, collaborated in groups, made timely observations, learned much about the morphology and life cycle of *C. dubia.* By comparing reproduction from asexually reproducing *C. dubia* in various water sources, students were able to formulate comparative questions regarding their life cycles and how they evolve that could lead to new inquiries. The students presented their data well. The level of thought that went into the observations and questions was quite impressive for this young group of students. And, certainly not least they had fun. Science should be fun and interesting.

Type of lesson: An inquiry-based learning lesson through observation and investigation.

Background Science

Ceriodaphnia dubia are a member of the Cladocera family. They are filter feeders and via their movement through water they feed upon algae and other particulates. During the normal part of their life cycle they reproduce asexually. Diploid eggs develop into larvae that grow in a brood chamber. Young are typically released after about three days. Throughout their life cycle *C. dubia* adapt to extreme environmental conditions including over population, cold weather, lack of food, and toxic environments. Under these conditions *C. dubia* reproduce sexually and fertilize eggs can remain dormant for extended periods of time until environmental conditions improve (Stollewerk, 2010). This is important because sexual reproduction allows for evolution of the species maintaining some sense of gene flow. *Daphnia* therefore have an interesting process of mutating to adapt to environmental conditions and then passing genes on to future generations. This combination affords remarkable durability of the species in its quest to survive.

An asexually reproducing female *C. dubia* can have three broods in as little as six days. Because of this short life cycle, mutations can occur quickly, and the species has a remarkable ability to adapt. One of the course themes in evolutionary biology is how species adapt to their environment. *Daphnia* are particularly suitable to provide insight into evolution because they reproduce quickly, they are easily observed, and adaptations are often relatively quick and in direct response to their environment. One adaptation to extreme environmental conditions is the ability to reproduce sexually.

The evolutionary time-line for *daphnia* is somewhat controversial. However, fossils found in various sites in Mongolia that are very similar in morphology to today's genus Daphnidae reveal the species probably originated about 145 Mya (Kotov and Taylor, 2011). Fossil records in limestone have also been discovered at other site in Europe and in the western portion of North America. A subgenus Ctenodaphnia has also been found in Eurasia (Kotov and Taylor, 2011). Additional research using nuclear and mtDNA evidence has revealed multiple *daphnia* lineages found in the Palearctic ecozone (Kotov, et. al. 2006). The four lineages include *D. curvirostris, D. tanakai, D. sinevi sp.nov. and D. morsei.* Some of the morphological characteristics are similar to today's *D. pulex* and *D. dentifera* (Kotov, et.al. 2006). It is not clear at what point the species

Ceriodaphnia dubia emerged, however there are many lineages documented from genetic information and fossils that would indicate many speciations in the *daphnia* family.
A number of genetic traits in *C. dubia* are evident and observable. These traits include mobility, size, age, morphology, behavior, predator response, and their immune system (Stollewerk, A. 2010).

Predators can also induce traits that result in adaptations. For example, in the presence of predatory fish, daphnia will respond with smaller offspring; conversely in the presence of midges they will often have larger offspring (Stollewerk, A. 2010). Kairomones are the chemicals present in the predators that induce changes in the *Daphnia* that result in a change in the size of offspring (Stollewerk, 2010). Fossils of daphnia have been found along with fossils of dipteran (Chaoboridae), a predator of daphnia. These two species have been co-evolving for at least 145 Mya (Kotov and Taylor, 2011)

Parasites can be highly virulent to daphnia. A yeast *Metschnikowia bicuspidate* is highly virulent to *Daphnia dentifera* (Duffy and Hall, 2008). Parasites are a major ecological concern and of evolutionary importance because of their virulence and impact on host populations (Harvell et. al. 2002). Although parasites are prevalent in nature, and their virulence well established, massive reduction in hosts are typically the exception and not the norm (Moller 2005). There are two possible reasons for this: predator behavior and rapid evolution by the host to develop resistance to the parasite (Duffy and Hall, 2008). Both reasons are important from an ecology and evolutionary prospective. Because the daphnia population contains more genetic diversity than the parasite, is reasonable to expect the host to evolve and not the host and parasite together (Duffy and Hall 2008). This may in part be one of the reasons daphnia fossils and genetic material predate daphnia to >145 Mya. The answer might be found in the fact that daphnia can evolve and respond to varying environmental conditions that include parasites very quickly. Parasites and hosts are often in somewhat of a evolutionary race and this race can occur very quickly, especially in a species with a short life cycle like daphnia (Zbinden, et.al. 2008). A field study was conducted to demonstrate this. Populations of *Daphnia magna* were infected with the parasite *Octosporea bayeri.* The control group of *D. magna* was uninfected. After about 15 generations (two summers) the genetic make-up of the infected group differed from the control group. The evolutionary effect

was the infected group had lower mortality to spore exposure than the control group, and the conclusion to the study was that *daphnia* can adapt very rapidly (Zbinden, et.al. 2008) and a likely reason they have been around for a long time. Interestingly enough, evolution allowing the host to tolerate the parasite benefits both the host and parasite (Zbinden, et.al. 2008). If the hosts were completely destroyed, the parasites would be unable to persist.

Given this race to evolve between the host and the parasite, the evolutionary question arises as to which genes adapt faster. For a species to adapt, the question becomes do the genes that are involved with parasitic immunity able to adapt faster than non-immune genes? This was the focus of research using *Daphnia pulex*. The science is somewhat beyond me, but McTaggart et.al. (2012) concluded by using estimations of nucleotide sites that immune genes do undergo more adaptive evolution than the non-immune genes.

Daphnia has the ability to adapt to a broad range of environmental conditions; this is known as ecological generalization. Narrow environmental tolerance is referred to as ecological specialization. *Daphnia* are able to adapt to increasing levels of salinity. Two challenges for an organism in a saline environment are dealing with osmosis and toxicity from the sodium and chloride (Latta, et.al. 2012). Usually, generalization results in some cost but *daphnia* populations that have a broad range of tolerance do better with increasing levels of salinity than do populations with narrow environmental tolerance. There does not seem to be an obvious cost to the *daphnia* (Latta, et.al. 2012) that lends itself to a remarkable ability to survive and evolve.

Daphnia are one of the most studied species. It is an important species because it is widespread and important to the ecology. This zooplankton spans over much of the northern hemisphere, and was thought to have spread as a result of glacier activity. The genus is endemic to some regions including Japan, but not to others (i.e., Russia) and so it is difficult to pin point the exact area of speciation (Kotov et.al. 2006). Many studies have been conducted on evolutionary issues such as gene flow, hybridization, adaptation, and inbreeding. All of this helps link evolutionary adaptations with environment and understand all of the genetic aspects that go along with this (Stollewerk, 2010).

Instructional Context

My company, Alloway, is a partner with four local schools benefiting from an Ohio Environmental Education Fund (OEFF) grant that created outdoor learning centers (OLCs) at each school, and provided materials to support inquiry-based learning (IBL). The grant mandates ten professional development (PD) programs. There are five primary objectives of this grant. This lesson plan addresses the second objective that is to provide students with the opportunity to perform hands-on scientific and environmental investigations and studies. Junior high students at Lima South will participate in the program and the lead teacher for Lima South, Connie Lott, will provide in-class facilitation.

IBL is a process of learning. It is a process that is question driven and it stimulates curiosity in student-lead explorations (Spronken et. al. 2008). The Dragonfly Leader's Guide (1988) uses the "QUEST" methodology to guide investigators. The Dragonfly vision promotes learning via methods of inquiry and helps children develop as researchers (Myers and Myers, 2009). There are various forms of inquiry, and inquiry can either be structured, guided or open in nature (Liang and Richardson, 2009). This lesson plan follows a guided inquiry format that might lead to some ongoing open inquiries.

The science involved in this IBL lesson plan uses *Ceriodaphnia dubia* cultures to initiate observation and questioning. The instructional plan will be scaffolded in such a way as to support learning and to open the door for other inquiry projects using *C. dubia*. After students have had an opportunity to observe the *daphnia,* a comparative question will be presented. The action plan will involve monitoring of brood sizes in three different waters from natural environments from a single genetic replicate of *C. dubia*.

Since *C. dubia* reproduce quickly, generating up to three broods in as little as one week, data can be readily obtained comparing offspring numbers and traits. This will enable the teacher to transition the discussion to evolution and how species adapt to their environment.

Overview

This is an inquiry-based learning lesson in which students learn about *C. dubia* and their habitat. Students will participate in hands-on activities to culture C.dubia and set-up an experiment to answer a comparative question regarding reproduction under various environmental conditions. Finally, the project will link IBL with the biology involved in evolution and how species adapt. Hopefully, more questions will emerge to continue scientific investigations.

Lesson Plan

Grade Level: 6 – 8

Class: Lima South Middle School

Teacher: Connie Lott

Time Required/Duration: About 5-10 minutes per day over two weeks

Objectives:

1. Students will learn about life cycles, morphology, and habitats.
2. Students will learn about the life cycle, morphology, and habitat of *C. dubia*.
3. Students will learn some ways that species adapt to their environment and specifically *C. dubia*.
4. Differences in adaptation will lead to a comparitive question that the teacher will provide to the class.
5. Students will work together in groups to maintain stock cultures of *C. dubia* and complete an action plan to explore various adaptations to different environments.
6. Students will learn how to collect, tabulate and present scientific data.
7. Students will uncover additional questions.

Educational Standards (Ohio Science Academic Content Standards) :

1. Scientific inquiry – Students develop scientific habits of mind as they use the processes of scientific inquiry to ask valid questions, and to gather and analyze information. They understand how to develop hypothesis and make predictions. They are able to reflect on scientific practices as they develop plans of action to create and evaluate a variety of conclusions. Students are also able to demonstrate the ability to communicate their findings to others.
2. Life Science (Topic: Species and Reproduction)
    a. Reproduction is necessary for the continuation of every species
    b. Every organism alive today comes from a long line of ancestors who reproduced successfully every generation.
    c. Reproduction is the transfer of genetic information from one generation to the next. It can occur with mixing of genes from two individuals (sexual reproduction). It can

occur with the transfer of genes from one individual to the next generation (asexual reproduction).

d. The ability to reproduce defines living things.

Materials:

1. *C. dubia* stock cultures in moderately hard synthetic rearing water.
2. Eyedroppers.
3. Cheesecloth.
4. Disposable 30 ml cups.
5. *C. dubia* food supply: YCT (yeast, Cerophyll and trout chow) and unicellular green algae (*Selenastrum capricornutum*).
6. Magnifying glass or stereoscope.
7. Three sources of naturally occurring water (i.e., river, pond, lake, stream, ditch, etc.)

Note: The above materials to be provided by Alloway.

Anticipatory Set

The teacher will:

1. Sketch a *daphnia* on the board and briefly discuss the morphology of *C.dubia*.
2. Initiate a discussion of the ecology, life history, and life cycle of *C. dubia*.
3. Provide adequate information to bridge the scientific background with the hands-on part of the lesson.
4. Explain the difference between sexual and asexual reproduction
5. Explain the lesson will use asexually reproducing organisms that are genetically identical (clones).
6. Discuss how species are able to adapt to their environment and evolve.
7. Let students know they will be working in groups.
8. Introduce a comparative question. For example, will average brood sizes as measured by the total number of young in three broods be larger in more nutrient rich waters (like agricultural runoff)?

Teaching – Direct Instruction

1. The teacher will provide the background information with a lecture.
2. The teacher will introduce the students to the materials provided and discuss the logistics and dynamics of the hands-on learning and make assignments.
3. The teacher will present a comparative question.
4. Students will observe the *daphnia* and sketch one. They will proceed with the actual experiment (Appendix 1).
5. Students will collect data on the provided data sheets (Appendix 2).
6. The teacher may play the video provided regarding *C.dubia* and acute toxicity if time allows.

Checking for Understanding

1. The teacher will engage the students in a discussion after the experiment.
2. The teacher will assess understanding by evaluating both the responses to the questions asked by the teacher and the questions asked by the students. The students will provide feedback (Appendix 3)
3. Wrap it up by uncovering more questions and clarifying points that seem vague to the class. Ask specific questions about the life cycle, ecology, adaptations, and the data collected that brings everything together.
4. The teacher will provide feedback (Appendix 4).

Note: reference material regarding the distribution, life cycle, taxonomy, and culture methods for *C. dubia* is found in Appendix 5 (Appendix A of USEPA Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms)

Reflections

The project went exceptionally well. The only change in plan was a result of my not having participated in classroom activities; there was insufficient time for the required background check that would have enabled me to participate on the first and last day of the experiment in the classroom as I had originally planned. However, my participation was not particularly necessary as the teacher, Connie Lott, and her students did an admirable job executing the lesson plan. Therefore, the only significant change would have been in the advanced planning to get the background checks completed in time in the event my participation was necessary.

An improvement might be made in the area of "assessment". The plan should be amended to include more specific questions as to exactly what was learned. Nevertheless, the student feedback revealed the students learned much in this brief experiment. Their responses to the questions were impressive and thoughtful. Based upon their observations, they came up with predictions and explanations. They collaborated and groups and compiled their own data. They learned about the morphology and life cycle of the water fleas. Based upon the questions the students raised, they obviously connected the life cycle of the water fleas with adaptation.

Two things are most impressive about the 8th graders. One is the nature of their questions and the fact they had so many that were either comparative in nature or could easily be turned to comparative questions. The other is how enthused the kids were about the experiment. They really enjoyed it and thought it was a "cool" experiment. Science should be fun, and young people will be more likely to purse careers in STEM careers if it is made to be fun and interesting.

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Appendix 1: Experiment

1. Remove a single *C. dubia* from the culture provided and place in a shallow dish with the aid of a magnifying glass or a stereoscope if available observe the *daphnia* and make a sketch of it. Everyday make sure to feed the culture with six or seven drops of the food provided.
2. Write down the comparative question that your teacher provided.
3. Regarding the comparative question, what do you (not the group, but you) think the answer to the comparative question will be.
4. Pour some of the rearing water provided into three disposable cups. With the dropper, transfer one *daphnia* to each cup. Add one drop of the food to each cup and do so daily.
5. As the individual *daphnia* grows, note changes over the next two or three days and make an updated sketch. Question: What differences did you observe from day one to day three? Day two to day three?
6. After two to three days each *daphnia* will have a few young.
7. Transfer the mom to a fresh cup filled with rearing water and continue the daily feeding. After two more days the *daphnia* will have more young. Once again, transfer the mom to a cup with fresh rearing water.
8. After about two more days the mom should have its third and largest brood. You will use these genetically identical clones for the experiment. Transfer one of the babies to each of three cups containing natural water from three different sources (nine total cups – make sure you have nine babies to start with).
9. Feed each cup daily with one drop of food. After seven days count the total *daphnia* in each cup. Complete the data sheet (Appendix 2).

Procedure provided to the students:

Procedure (Each Group)

Monday: Take one of the cups with the "mom" and her brood. The mom will be the large one and the babies will be very small. Observe under bright light and magnification and sketch the mom on the bottom of the data sheet. The mom is reproducing asexually. The babies are all "clones". That is, they are genetically identical.

Take 4 of the small clear, plastic cups and label them with your group number and "A", "B" "C" and "D". Add the appropriate sample to each cup, filling about ¾ of the way. Using an eyedropper, bright light, and a magnifying glass if needed, transfer a baby to each cup. Add one drop of food. Place your 4 cups under the fluorescent light with the timer set to 16 hours on (daylight) and 8 hours off (night time). We will be counting the total number of young produced over the next week from these babies (soon to become moms). Note: We are now finished with the original cup containing the mom and her brood.

Tuesday: Add one drop of food to each cup.

Wednesday: In each cup, you should now see a mom and many babies. Take 4 brand new cups and label them with your group number and "A" "B" "C" and "D". Pour new solution into each cup from the fours samples provided. Using the eyedropper, bright light and magnifying glass if needed, carefully transfer the mom to the new solution. Add one drop of food. Now as best as you can, count the babies in the cup left behind and record the data on the data sheet. Note: We are finished with that cup.

Thursday: Add one drop of food to each cup.

Friday: Same as Wednesday, but add 2 drops of food to last through the weekend. Make sure to record your data.

Monday: Count the remaining babies from the weekend and record your data.

Appendix 2 – Data Sheet

Data Sheet

Group # ____
Student Names: _____
_____
_____
_____

Sample "A": **Laboratory Rearing Water**
Sample "B" **Aquarium Water**
Sample "C" **Farm Pond Water**
Sample "D" **Runoff Water**

	Number of "babies" in cup			
	A	**B**	**C**	**D**
Monday 11/12	**0**	**0**	**0**	**0**
Wednesday 11/14	___	___	___	___
Friday 11/16	___	___	___	___
Monday 11/20	___	___	___	___
Total:	___	___	___	___

Typical Data Sheet

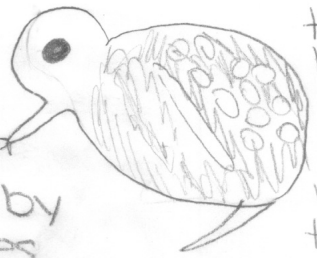
Data Sheet

Group # 7
Student Names: Grace Kellum, Rhaven Rodgers

Sample "A": Laboratory Rearing Water
Sample "B" Aquarium Water
Sample "C" Farm Pond Water
Sample "D" Runoff Water

	Number of "babies" in cup			
	A	**B**	**C**	**D**
Monday 11/12	0	0	0	0
Wednesday 11/14	0	2	3	0
Friday 11/16	12	2	8	3
Monday 11/20	2	2	20	3
Total:	14	6	31	6

Question; Is the reproduction rate of the water flea affected by different types of water



Answer; It seems that they reproduce better and faster in pond water(c) because thats where we ended up having the most babies, then any other cup.

Appendix 3 – Student Feedback
Responses to Questions

1. **Did the initial *daphnia* in all 4 cups with different waters have the same number of babies?**
   a) *No because some of the waters was too healthy.*
   b) *No because some types didn't make babies and some didn't have babies.*
   c) *No, because the aquarium water had more than the others.*
   d) *No, sample D had twice as many as any of the other samples.*
   e) *No they didn't. Because they were in different waters so they adapted differently.*
   f) *No, they all had a different amount of babies in each cup because of the different type of water.*
   g) *No, even in the first generation the pond water had more babies.*
   h) *No.*

2. **If you repeated the test using babies from Monday, would you expect similar results, or might some adapt to the environment and do better?**
   a) *Yes, because they need to have a healthy environment.*
   b) *No because based on little data and information collected over days and observations of the previous babies I cannot infer about their adaptation to their surrounding.*
   c) *They might adapt and do better because now they have gotten used to it.*
   d) *I'm not sure. But if I had to choose I think that you wouldn't expect the same results and that they wouldn't do better.*
   e) *Yes, because its getting use to being in same type of environment. Then it reproduces which means it is asexual.*
   f) *Yes, I think so because they will get more comfortable with their surroundings and produce more.*
   g) *I think the results would be the same.*

3. **What did you learn from this?**
   a) *Water fleas reproduce rapidly.*
   b) *I learned about water fleas responses and reactions to experimental changes to them and I learned how they could reproduce.*
   c) *Water fleas can reproduce asexually.*
   d) *How asexual animals reproduce without a partner or mother or father.*
   e) *I learned about how water fleas adapted to the different types of water and how the asexual way works with water fleas.*
   f) *I learned that different types of water show different types of results. Because Cup C made more babies with farm pond water.*
   g) *That different types of water get different results.*

4. **What additional questions come to mind after having done the experiment?**
   a) *Why do they reproduce so fast?*
   b) *If you change the temperature to 90 degrees would they reproduce at all?*

c) *If you change their food how fast would they reproduce? 3 responses*
d) *If you added more would they reproduce more babies?*
e) *If there was a bigger area would the fleas produce more?*
f) *What would happen if you put one on a diet and feed the rest regular food?*
g) *If you put 2 babies in a new environment, who becomes the "queen" or "mom"?*
h) *How many babies can a flea actually have?*
i) *What do they have to change water all the time?*
j) *Do they live in cold or warm water?*
k) *What would happen if we have a bigger volume of water?*
l) *Would we have had better results if we did this multiple times, with more fleas?*
m) *What would happen if they were given more light? Would they produce more?*
n) *If we give them a different amount of food how would that effect them?*

5. Was it fun and/or interesting?

a) *Yes because I got to work with my friend.*
b) *Yes, it was fun because we don't get to do experiments in science like this that often so it was nice to take a break from book work and do something physical and active. But it was really interesting.*
c) *I thought it was fun trying to find the flea babies, because they are so tiny.*
d) *Yes, it was a lot of fun! It was interesting learning about water fleas.*
e) *It was interesting I guess.*
f) *It was very interesting seeing how the fleas increased by day and it was fun because it's a cool experiment.*
g) *Seeing how fast they have babies.*
h) *Yes, because working with fleas and helping them reproduce is kind of cool.*

Appendix 4: Teacher Feedback (Connie Lott's Responses)

What evidence suggests students grasped the major themes of the experiment (i.e., life cycle of C. dubia, culturing techniques, reproduction of C. dubia, IBL, evolution, etc.)?

Connie Lott: As I was observing the students, they were using correct lab techniques. They were asking questions about how the fleas could produce asexually and sexually. They wanted to know how it could happen so fast. How they could go from a baby to an adult that reproduces so quickly (life cycle and reproduction). They had many questions that could lead to other investigations.

Do you anticipate other guided or open inquiry projects arising from this project? What questions did the students ask that suggest understanding and interest in the subject?

There were many other investigations that could come from this one experiment. The students had many questions that could lead to other investigations such as:

If we fed them different food would it affect how much they reproduce?
How would different degrees of light affect them?
If the fleas had a larger space to reproduce would they reproduce more offspring?

To what extent did this project fit into your curriculum and teaching agenda?

This project fit very well into my curriculum because we do teach life cycle, reproduction, lab techniques, questioning, correct scientific process, etc.

Would you consider doing this again?

ABSOLUTELY! This was a great inquiry lab that the kids enjoyed and learned from.

What would improve the experience?

The only thing I would change if I could, would be to have more time. At the moment I only have 40 minutes for science but I am trying to get the administration to change that for next year so I have more time to do labs.

Appendix 5 – Appendix A of Methods for Measuring the Acute Toxicity of Effluents (USEPA)

APPENDIX A

DISTRIBUTION, LIFE CYCLE, TAXONOMY, AND CULTURE METHODS

A.1. *CERIODAPHNIA DUBIA*

1. **SYSTEMATICS**

1.1 MORPHOLOGY AND TAXONOMY

1.1.1 *Ceriodaphnia* are closely related and morphologically similar to *Daphnia*, but are smaller and have a shorter generation time (USEPA, 1986). They are generally more rotund, lack the prominent rostral projection typical of *Daphnia*, and do not develop the dorsal helmets and long posterior spines often observed in *Daphnia*.

1.1.2 With *Ceriodaphnia dubia*, the female has a heavy, setulated pecten on the postabdominal claw (Figure 1A), and the male was long antennules (Figure 1C), in contrast to the closely related *C. reticulata*, where the female has heavy, triangular denticles in the pecten of the postabdominal claw (Figure 2A), and the male has very short antennules (Figure 2C). Some clones having intermediate characters may be hybrids or phenotypic variants of *C. dubia* (USEPA, 1986). Detailed descriptions of the males and females of both species and the variant were given by USEPA (1986).

1.1.3 Although males are very similar to females, they can be recognized by their rapid, erratic swimming habit, smaller size, denser coloration, extended antennules and claspers, and rostrum morphology.

2. **ECOLOGY AND LIFE HISTORY**

2.1 DISTRIBUTION

2.1.1 *C. dubia*, has been reported from littoral areas of lakes, ponds, and marshes throughout most of the world, but it is difficult to ascertain its true distribution because it has been reported in the literature under several other names (*C. affinis, C. quadrangula*, and *C. reticulata)*. It has also been suggested that reports of *C. dubia* in New Zealand and parts of Asia may be yet another unnamed species (Berner, personal communication).

2.2 ECOLOGY

2.2.1 *Ceriodaphnia* ecology and life history are very similar to those of other daphnids. Specific information on the ecology and life history of *Ceriodaphnia dubia* is either not available or is widely scattered throughout the literature. However, it is known to be a pond and lake dwelling species that is usually common among the vegetation in littoral areas (Fairchild, 1981). In the Lake of Velence, Hungary, *C. dubia* was most common in regions where "grey" and "dark brown" waters merged (Pal, 1980). In Par Pond (Savannah River Plant, Aiken, SC) the *Ceriodaphnia* were much more abundant in the heated water (effluent from the nuclear reactor) than in the ambient area (Vigerstad and Tilly, 1977), and in a reservoir in Russia, animals from the heated water were larger and heavier than those living under normal water temperatures (Kititsyna and Sergeeva, 1976). In Iran they are

common in warmer, montane, oligotrophic lakes (Smagowicz, 1976).

2.2.2 In Lake Kinneret, Israel, *Ceriodaphnia reticulata* are abundant only between March and June, with a peak in May when the temperature ranges between 20 and 22°C. When summer temperatures reached 27-28°C, the *Ceriodaphnia* were reduced in size and egg production became significantly less, leading to a progressive decline of the population (Gophen, 1976). In Lake Parvin, France, the period of development was from June to September (Devaux, 1980).

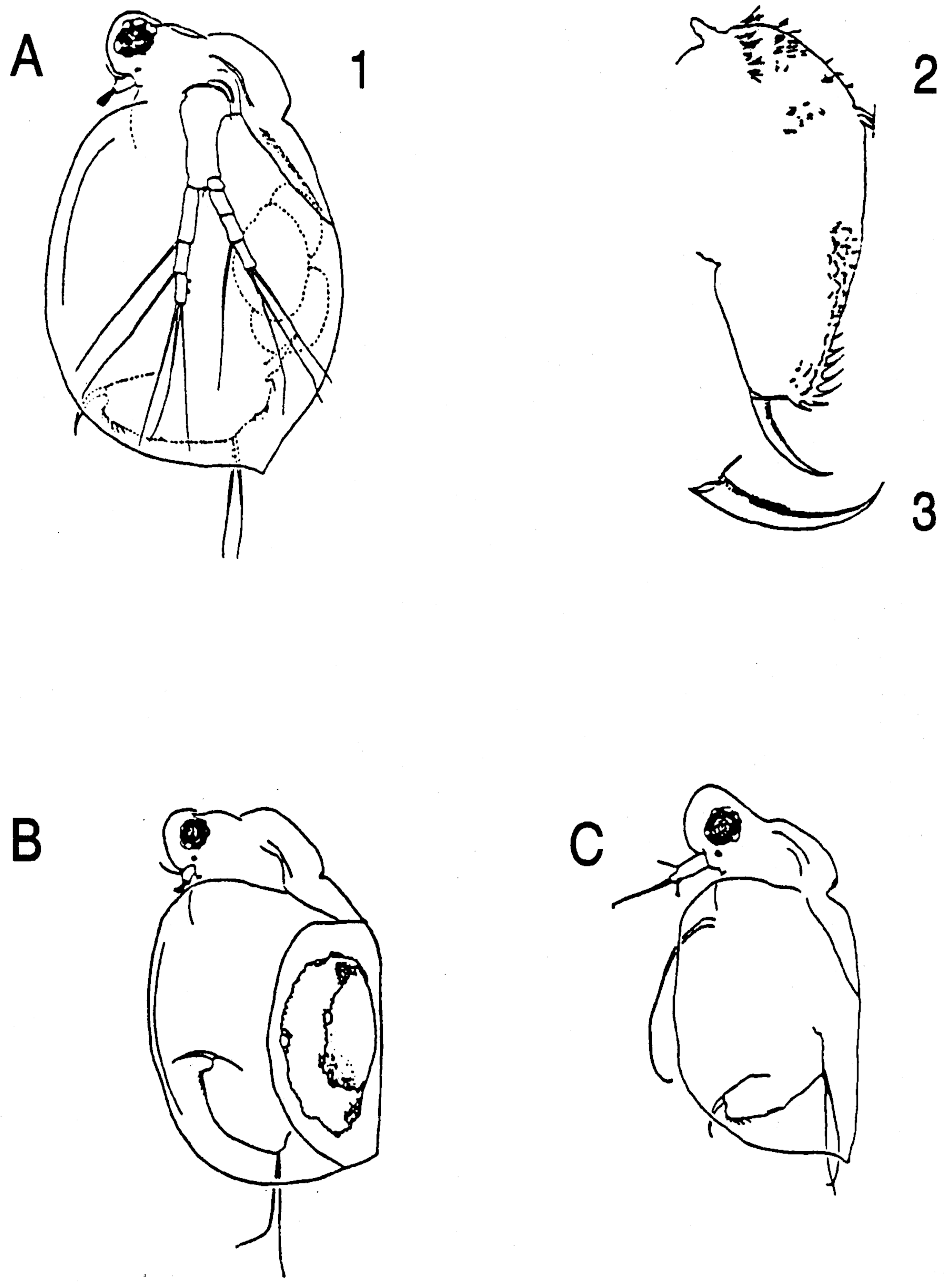


Figure 1. *Ceriodaphnia dubia*. A. (1) parthenogenetic female, (2) postabdomen, and (3) claw; B. ephippial female; C. Male. (From USEPA, 1986)

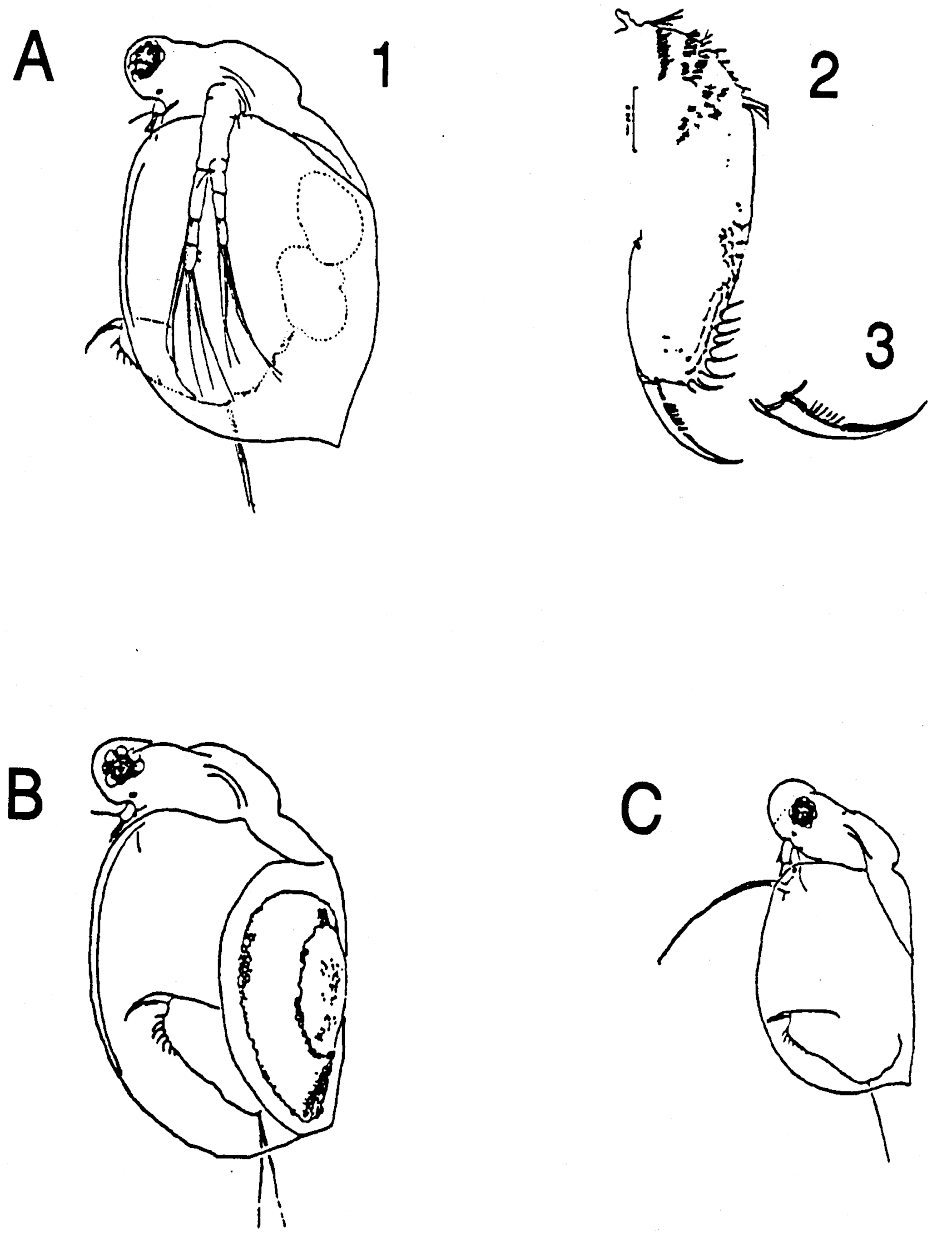


Figure 2. *Ceriodaphnia reticulata*. A. (1) parthenogenetic female, (2) postabdomen, (3) and claw; B. ephippial female; C. Male. (From USEPA, 1986)

2.2.3 *Ceriodaphnia* typically swim with an erratic, jerking motion for a period of time, and hang motionless in the water between swimming bouts. This swimming behavior results in a mean speed of 1.5-2.5 mm/s. When approached by a predator, however, it flees by swimming away quickly along a straight path (Wong, 1981).

2.2.4 During most of the year, populations of *Ceriodaphnia* consist almost entirely of females; the males appearing principally in autumn. Production of males appears to be induced primarily by low water temperatures, high population densities, and/or a decrease in available food. As far as is presently known, *C. dubia* reproduce only by cyclic parthenogenesis in which the males contribute to the genetic makeup of the young during the sexual stage of reproduction.

2.2.5 The females tend to aggregate during sexual reproductive activity, when ephippia are produced (Brandl and Fernando, 1971). Ephippia are embryos encased in a tough covering, and are resistent to drying. They can be stored for long periods and shipped through the mail in envelopes, like seeds. When placed in water at the proper temperature, ephippia hatch in a few days producing a new parthenogenetic population.

2.2.6 *Ceriodaphnia* have many predators, including fish, the mysid *Mysis relicta, Chaoborus* larvae, and copepods. As with *Daphnia*, it also reacts to intense predation with defensive strategies. *Ceriodaphnia reticulata* (possibly *C.dubia*) in a Minnesota lake, reacted to the copepod, *Cyclops vernalis*, by producing large offspring and growing to a large size at the expense of early reproduction (Lynch, 1979). They reacted to fish predators by producing smaller offspring in larger numbers.

2.3 FOOD AND FEEDING

2.3.1 Cladocera are polyphagous feeders and find their food in the seston. Daphnids, including the *Ceriodaphnia*, are classified as fine mesh filter feeders by Geller and Mueller (1981). These fine mesh filter feeders are most abundant in eutrophic lakes during summer phytoplankton blooms when suspended bacteria are available as food only for filter-feeding species with fine mesh.

2.3.2 Lynch (1978) examined the gut contents of *Ceriodaphnia reticulata* (possibly *C. dubia*) from a Minnesota pond and found bacteria, detritus and partially digested algae. In this pond, *Ceriodaphnia* and *Daphnia pulex* shared the same resource base and had very similar diets, but the *Ceriodaphnia* fed more intensively on diatoms. The *Ceriodaphnia* were considered to be less sensitive to low food levels than *Daphnia*, because of their high rate of population growth during periods of low food levels in late summer.

2.4 LIFE CYCLE

2.4.1 Four distinct periods may be recognized in the life cycle of *Ceriodaphnia*: (1) egg, (2) juvenile, (3) adolescent, and (4) adult. The life span of *Ceriodaphnia*, from the release of the egg into the brood chamber until the death of the adult, is highly variable depending on the temperature and other environmental conditions. Generally the life span increases as temperature decreases, due to lowered metabolic activity. For example, the average life span of *Ceriodaphnia dubia* is about 30 days at 25°C, and 50 days at 20°C. One female was reported to have lived 125 days and produced 29 broods at 20°C (Cowgill et al., 1985).

2.4.2 Typically, a clutch of 4 to 10 eggs is released into the brood chamber, but clutches with as many as 20 eggs are common. The eggs hatch in the brood chamber and the juveniles, which are already similar in form to the adults, are released in approximately 38 h, when the female molts (casts off her exoskeleton or carapace). The total number of young produced per female varies with temperature and other environmental conditions. The most young are produced in the range of 18-25°C (124 young per female in a 28-day life span at 24°C) (113 young per female in a 77-day life span at 18°C) but production falls off sharply below 18°C (13 young per female in a 24-day life span at 12°C) (McNaught and Mount, 1985).

2.4.3 The time required to reach maturity (produce their first offspring) in *C.dubia* varies from three to five days and appears to be dependent on body size and environmental conditions. A study of the growth and development of parthenogenetic eggs by Shuba and Costa (1972) revealed that at 24°C the embryos matured to free-swimming juveniles in approximately 38 h. The eggs that did not develop fully usually were aborted after 12 hours.

2.4.4 The growth rate of the organism is greatest during its juvenile stages (early instars), and the body size may double during each of these stages. Each instar stage is terminated by a molt. Growth occurs immediately after each molt while the new carapace is still elastic.

2.4.5 Following the juvenile stages, the adolescent period is very short, and consists of a single instar. It is during the adolescent instar that the first clutch of eggs reaches full development in the ovary. Generally, eggs are deposited in the brood chamber within minutes after molting, and the young which develop are released just before the next molt.

2.4.6 In general, the duration of instars increases with age, but also depends on environmental conditions. A given instar usually lasts approximately 24 h under favorable conditions. However, when conditions are unfavorable, it may last as long as a week. Four events take place in a matter of a few minutes at the end of each adult instar: (1) release of young from the brood chamber to the outside, (2) molting, (3) increase in size, and (4) release of a new clutch of eggs into the brood chamber. The number of young per brood is highly variable, depending primarily on food availability and environmental conditions. *C. dubia* may produce as many as 25 young in a single brood, but more commonly the number is six to ten. The number of young released during the adult instars reaches a maximum at about the fourth instar, after which there is a gradual decrease.

3. **CULTURING METHODS**

3.1 *Ceriodaphnia* are available from commercial biological supply houses. Guidance on the source of culture animals to be used by a permittee for self-monitoring effluent toxicity tests should be obtained from the permitting authority. Only a small number of organisms (20-30) are needed to start a culture. Before test organisms are taken from a culture, the culture should be maintained for at least two generations using the same food, water, and temperature as will be used in the toxicity tests.

3.2 Cultures of test organisms should be started at least three weeks before the brood animals are needed, to ensure an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolific reproduction.

3.3 Starter animals may be obtained from an outside source by shipping in polyethylene bottles. Approximately 20-30 animals and 3 mL of food (see below) are placed in a l-L bottle filled full with culture water. Animals received from an outside source should be transferred to new culture media gradually over a period of 1-2 days to avoid mass mortality.

3.4 It is best to start the cultures with one animal, which is sacrificed after producing young, embedded, and retained as a permanent microscope slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by a taxonomic authority. The following procedure is recommended for making slide mounts of *Ceriodaphnia* (Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.
3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip).
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended mounting medium is CMCP-9/9AF Medium, prepared by mixing two parts of CMCP-9 with

one part of CMCP-9AF. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used.

5. Using a forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing CMC-10 around the edges of the coverslip.
9. Identify to species (see Pennak, 1989, and USEPA, 1986).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

3.5 CULTURE MEDIA

3.5.1 Although *Ceriodaphnia* stock cultures can be successfully maintained in some tap waters, well waters, and surface waters, use of synthetic water as the culture medium is recommended because (1) it is easily prepared, (2) it is of known quality, (3) it yields reproducible results, and (4) allows adequate growth and reproduction. Culturing may be successfully done in hard, moderately hard or soft reconstituted water, depending on the hardness of the water in which the test will be conducted. The quality of the dilution water is extremely important in *Ceriodaphnia* culture. The use of MILLIPORE MILLI-Q® or SUPER-Q®, or equivalent, to prepare reconstituted water is highly recommended. The use of diluted mineral water (DMW) for culturing and testing is widespread due to the ease of preparation.

3.5.2 The chemicals used and instructions for preparation of reconstituted water are given in Section 7, Dilution Water. The compounds are dissolved in distilled or deionized water and the media are vigorously aerated for several hours before using. The initial pH of the media is between 7.0 and 8.0, but it will rise as much as 0.5 unit after the test is underway.

3.6 MASS CULTURE

3.6.1 Mass cultures are used only as a "backup" reservoir of organisms. Neonates from mass cultures are not to be used directly in toxicity tests.

3.6.2 One-liter or 2L glass beakers, crystallization dishes, "battery jars," or aquaria may be used as culture vessels. Vessels are commonly filled to three-fourths capacity. Cultures are fed daily. Four or more cultures are maintained in separate vessels and with overlapping ages to serve as back-up in case one culture is lost due to accident or other unanticipated problems, such as low DO concentrations or poor quality of food or laboratory water.

3.6.3 Mass cultures which will serve as a source of brood organisms for individual culture should be maintained in good condition by frequent renewal of the medium and brood organisms. Cultures are started by adding 40-50 neonates per liter of medium. The stocked organisms should be transferred to new culture medium at least twice a week for two weeks. After two weeks, the culture is discarded and re-started with neonates in fresh medium. Using this schedule, 1-L cultures will produce 500 to 1000 neonate *Ceriodaphnia* each week.

3.6.6 Reserve cultures also may be maintained in large (80-L) aquaria or other large tanks.

3.7 INDIVIDUAL CULTURE

3.7.1 Individual cultures are used as the immediate source of neonates for toxicity tests.

3.7.2 Individual organisms are cultured in 15 mL of culture medium in 30-mL (1 oz) plastic cups or 30-mL glass beakers. One neonate is placed in each cup. It is convenient to place the cups in the same type of board used for toxicity tests.

3.7.3 Organisms are fed daily and are transferred to fresh medium a minimum of three times a week, typically on Monday, Wednesday, and Friday. On the transfer days, food is added to the new medium immediately before or after the organisms are transferred.

3.7.4 To provide cultures of overlapping ages, new boards are started weekly, using neonates from adults which produce at least eight young in their third or fourth brood. These adults can be used as sources of neonates until 14 days of age. A minimum of two boards are maintained concurrently to provide backup supplies of organisms in case of problems.

3.7.5 Cultures which are properly maintained should produce at least 20 young per adult in three broods (seven days or less at 25°C). Typically, 60 adult females (one board) will produce more than the minimum number of neonates (120) required for two tests.

3.7.6 Records should be maintained on the survival of brood organisms and number of offspring at each renewal. Greater than 20% mortality of adults or less than an average of 20 young per adult on a board at 25°C during a one-week period would indicate problems, such as poor quality of culture media or food. Organisms on that board should not be used as a source of test organisms.

3.8 CULTURE MEDIUM

3.8.1 Moderately hard synthetic water prepared using MILLIPORE MILLI-Q® or equivalent deionized water and reagent grade chemicals or 20% DMW is recommended as a standard culture medium (see Section 7, Dilution Water).

3.9 CULTURE CONDITIONS

3.9.1 *Ceriodaphnia* should be cultured at the temperature at which they will be used in the toxicity tests (20°C or 25°C $\pm$ 2°C).

3.9.2 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A 16-h/8-h day/night cycle is recommended.

3.9.3 Clear, double-strength safety glass or 6 mm plastic panels are placed on the culture vessels to exclude dust and dirt, and reduce evaporation.

3.9.4 The organisms are delicate and should be handled as carefully and as little as possible so that they are not unnecessarily stressed. They are transferred with a pipet of approximately 2-mm bore, taking care to release the animals under the surface of the water. Any organism that is injured during handling should be discarded.

3.10 FOOD PREPARATION AND FEEDING

3.10.1 Feeding the proper amount of the right food is extremely important in *Ceriodaphnia* culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may reduce the toxicity of the test solutions, clog the animal's filtering apparatus, or greatly decrease the DO concentration and increase mortality. A combination of Yeast, CEROPHYLL®, and Trout chow (YCT) or flake food, along with the unicellular green alga, *Selenastrum capricornutum*, will provide suitable nutrition if fed daily.

3.10.2 The YCT and algae are prepared as follows:

3.10.2.1 Digested trout chow (or flake food):

1. Preparation of trout chow requires one week. Use starter or No. 1 pellets prepared according to

current U.S. Fish and Wildlife Service specifications, or flake food.

2. Add 5.0 g of trout chow pellets or flake food to 1 L of MILLI-Q® water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation is replaced during digestion. Because of the offensive odor usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.

3. At the end of digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (i.e., NITEX® 110 mesh). Combine with equal volumes of supernatant from CEROPHYLL® and yeast preparations (below). The supernatant can be used fresh, or frozen until use. Discard the sediment.

3.10.2.2 Yeast:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S® to 1 L of MILLI-Q ® water.
2. Stir with a magnetic stirrer, shake vigorously by hand, or mix with a blender at low speed, until the yeast is well dispersed.
3. Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow (above) and CEROPHYLL® preparations (below). Discard excess material.

3.10.2.3 CEROPHYLL® (Dried, Powdered, Cereal Leaves):

1. Place 5.0 g of dried, powdered, cereal leaves in a blender. Dried, powdered, alfalfa leaves obtained from health food stores have been found to be a satisfactory substitute for cereal leaves.
2. Add 1 L of MILLI-Q® water.
3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.
4. If a blender is used to suspend the material, place in a refrigerator overnight to settle. If a magnetic stirrer is used, allow to settle for 1 h. Decant the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard excess material.

3.10.2.4 Combined YCT Food:

1. Mix equal (approximately 300 mL) volumes of the three foods as described above.
2. Place aliquots of the mixture in small (50-mL to 100-mL) screw-cap plastic bottles and freeze until needed.
3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks.
4. It is advisable to measure the dry weight of solids (dry 24 h at 105°C) in each batch of YCT before use. The food should contain 1.7 - 1.9 g solids/L. Cultures or test solutions should contain 12-13 mg solids/L.

3.10.3 Algal (*Selenastrum*) Food

3.10.3.1 Algal Culture Medium

1. Prepare (five) stock nutrient solutions using reagent grade chemicals as described in Table 1.
2. Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of MILLI-Q® water. Mix well after the addition of each solution. Dilute to 1 L and mix well. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES

STOCK SOLUTION	COMPOUND	AMOUNT DISSOLVED IN 500 ML MILLI-Q® WATER
1. MACRONUTRIENTS		
A.	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	$NaNO_3$	12.75 g
B.	$MgSO_4 \cdot 7H_2O$	7.35 g
C.	K_2HPO_4	0.522 g
D.	$NaHCO_3$	7.50 g
2. MICRONUTRIENTS:		
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg[a]
	$ZnCl_2$	1.64 mg
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg[b]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[c][d]
	$CuCl_2 \cdot 2H_2O$	0.006 mg
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
	Na_2SeO	1.196 mg[e]

[a] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #2.

[b] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #2.

[c] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock #2.

[d] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock #2.

[e] Na_2SeO_4 - Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #2.

TABLE 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

MACRONUTRIENT	CONCENTRATION (MG/L)	ELEMENT	CONCENTRATION (MG/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

MICRONUTRIENT	CONCENTRATION (µG/L)	ELEMENT	CONCENTRATION (µG/L)
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	--	----
Na_2SeO_4	2.39	Se	1.00

3. Immediately filter the medium through a 0.45 µm pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter with 500 mL deionized water prior to use.
4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.
5. Unused sterile medium should not be stored more than one week prior to use, because there may be substantial loss of water by evaporation.

3.10.3.2 Algal Cultures

3.10.3.2.1 Two types of algal cultures are maintained: (1) stock cultures, and, (2) "food" cultures.

3.10.3.2.2 Establishing and Maintaining Stock Cultures of Algae

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring one milliliter to each of several 250-mL culture flasks containing 100 mL algal culture medium (prepared as described above). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.
2. The stock cultures are used as a source of algae to initiate "food" cultures for *Ceriodaphnia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *Ceriodaphnia* cultures and tests. Stock culture volume may be rapidly "scaled up" to several liters, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.
3. Culture temperature is not critical. Stock cultures may be maintained in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).
4. Cultures are mixed twice daily by hand or stirred continuously.
5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three milliliters of 7-day old algal stock culture, containing approximately 1.5 X 10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10,000-30,000 cells/mL in the new stock cultures. Aseptic techniques should be used in maintaining the stock algal cultures, and care should be exercised to avoid contamination by other microorganisms.
6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6-12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms every four to six months.

3.10.3.2.3 Establishing and Maintaining "Food" Cultures of Algae

1. "Food" cultures are started seven days prior to use for *Ceriodaphnia* cultures and tests. Approximately 20 mL of 7-day-old algal stock culture (described in the previous paragraph), containing 1.5 X 10^6 cells/mL, are added to each liter of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The inoculum should provide an initial cell density of approximately 30,000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms. However, sterility of food cultures is not as critical as in stock cultures because the food cultures are terminated in 7-10 days. A one-month supply of algal food can be grown at one time, and the excess stored in the refrigerator.
2. Food cultures may be maintained at 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent

lighting of approximately 86 ± 8.6 $\mu E/m^2/s$, or 400 ft-c).

3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees. Caution should be exercised to prevent the culture temperature from rising more than 2-3°C.

3.10.3.3 Preparing Algal Concentrate for Use as *Ceriodaphnia* Food

1. An algal concentrate containing 3.0 to 3.5 X 10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for approximately two-to-three weeks and siphoning off the supernatant.
2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer, or spectrophotometer, and used to determine the concentration required to achieve a final cell count of 3.0 to 3.5 X 10^7 cells/mL.
3. Assuming a cell density of approximately 1.5 X 10^6 cells/mL in the algal food cultures at 7 days, and 100% recovery in the concentration process, a 3-L, 7-10 day culture will provide 4.5 X 10^9 algal cells. This number of cells would provide approximately 150 mL of algal cell concentrate (1500 feedings at 0.1 mL/feeding) for use as food. This would be enough algal food for four *Ceriodaphnia* tests.
4. Algal concentrate may be stored in the refrigerator for one month.

3.11 FEEDING

3.11.1 Cultures should be fed daily to maintain the organisms in optimum condition so as to provide maximum reproduction. Stock cultures which are stressed because they are not adequately fed may produce low numbers of young, large number of males, and ephippial females. Also, their offspring may produce few young when used in toxicity tests.

1. If YCT is frozen, remove a bottle of food from the freezer 1 h before feeding time, and allow to thaw.
2. Mass cultures are fed daily at the rate of 7 mL YCT and 7 mL algae concentrate/L culture.
3. Individual cultures are fed at the rate of 0.1 mL YCT and 0.1 mL algae concentrate per 15 mL culture.
4. YCT and algal concentrate should be thoroughly mixed by shaking before dispensing.
5. Return unused YCT food mixture and algae concentrate to the refrigerator. Do not re-freeze YCT. Discard unused portion after one week.

3.12 FOOD QUALITY

3.12.1 The quality of food prepared with newly acquired supplies of yeast, trout chow, dried cereal leaves, or algae, should be determined in side-by-side comparisons of *Ceriodaphnia* survival and reproduction, using the new food and food of known, acceptable quality, over a seven-day period in control medium.

4. **TEST ORGANISMS**

4.1 Neonates, or first instar *Ceriodaphnia* less than 24 hours old, taken from the 3rd or 4th brood, are used in toxicity tests. To obtain the necessary number of young for an acute toxicity test, it is recommended that the animals be cultured in individual 30 mL beakers or plastic cups for seven days prior to the beginning of the test. Neonates are used from broods of at least eight young. Fifty adults in individual cultures will usually supply enough neonates for one toxicity test.

4.2 Use a disposable, widemouth pipette to transfer *Ceriodaphnia*. The diameter of the opening should be approximately 4 mm. The tip of the pipette should be kept under the surface of the water when the *Ceriodaphnia* are released to prevent air from being trapped under the carapace. Liquid containing adult *Ceriodaphnia* can be poured from one container to another without risk of injuring the animals.

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John Hoffman Issues in Evolution: Lesson Plan December 2, 2012