# A semi-automated counting method for improved accuracy in invertebrate whole effluent toxicity testing

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## Abstract

Fresh and salt-water aquatic toxicity tests can be conducted with fish, algae or invertebrates. The invertebrates have several advantages for these tests, including low cost, small size, rapid turnaround due to a short life cycle, ease of culturing, and low volume of test solution (and thus potential toxicants) to be used and discarded. However, since the invertebrates used in these tests are typically less than 1-mm long and are highly motile, assessment of the population is confounded by miscounting during unassisted visual observations. This miscounting causes wider dispersion in the data than would be expected given the organism sample sizes used. Of the many factors leading to variability in these tests (data acquisition errors, analysis errors, variability of organisms, variability of dilution water, variation in effluent composition and concentration), improvement in the accuracy of the counting techniques may provide substantial improvements in data quality. Reduction in the cost and time taken to conduct the tests may be a by-product of automation of the counting process.

I will review the legal and technical background that stimulated the development of whole effluent toxicity testing, outline the methods used in this technique, and point out some difficulties that lead to variability in these experiments. Finally, I will demonstrate a prototype method to aid in counting of zooplankton consisting of an image capture system, close-focusing lens and custom image analysis software. This system can rapidly analyze small volumes of test liquid for motile organisms. The system takes advantage of the widespread availability of low-cost, high powered video capture hardware and software that has appeared in the last five years. I will show examples of counting performed with this system and discuss advantages and limitations of the automated system over more traditional manual methods, using the widely available salt-water brine shrimp, *Artemia salina*.

# 1. Legal Motivation: The Clean Water Act and National Pollution Elimination System

Much of the research and development in environmental toxicity testing has been motivated by public policy as defined in national legislation. The laws established by the United States, Canada, Europe and Japan require toxicity testing to assess the influence of discharges to the air and public waters.

The 1977 amendment (33 CFR sections 1251 to 1376) to the Federal Water Pollution Control Act of 1972, commonly referred to as the Clean Water Act (CWA) set the structure for regulating discharges of pollutants to waters of the United States. The purpose of this landmark set of laws is to restore and maintain the integrity of the waters, protect public health, and assure that every facility treats wastewater.

Under Section 402 of the CWA, the Environmental Protection Authority (EPA) has the authority to set effluent standards for discharges and receiving waters on an industry-by-industry basis. The EPA also sets overall standards for contaminants in surface waters. The CWA makes it unlawful for any person to discharge any pollutant into surface waters unless a permit is obtained. These permits are negotiable between EPA and the discharger, and are part of the National Pollution Discharge Elimination System (NPDES). The permits are site specific and contain specific effluent limits and compliance monitoring and reporting requirements. Regulated pollutants are classified in one of the following categories:

1. *Conventional pollutants* contained in the sanitary wastes from households, business and industry. The two most important conventional pollutants are fecal coliform bacteria, which can indicate the presence of pathogenic organisms, and oils and grease, which may produce sludge solids that are difficult to process.
2. *Toxic pollutants*, loosely grouped into organics (pesticides, solvents, polychlorinated biphenyls (PCB), dioxin) and inorganics (lead, silver mercury, copper chromium, zinc, nickel and cadmium). Toxic pollutants are directly harmful to animal or plant life.
3. *Nonconventional pollutants* including materials that are not toxic or conventional, but whose introduction into the ecosystem may have deleterious effects. Examples of nonconventional pollutants are nitrogen and phosphorous compounds.

There are several exceptions to this permit requirement including return flows from irrigated agricultural lands, runoff from agricultural crops and cultivated forests, and certain small volume discharges of storm water. Larger storm water discharges, including those associated with industrial activity and construction disturbing more than five acres, are regulated under the NPDES permit program.

NPDES point source permits specify that discharges must meet water *quality* provisions defined in Section 301 of the CWA. This means that the discharge may not contain toxins in levels that would cause the receiving body of water to fail to meet EPA-defined water quality standards. Additionally, the discharge must meet a *technologically* defined standard for particular pollutants regardless of whether or not that level of control is required to protect the receiving water body. These technologically defined standards assure a minimum level of control for certain pollutants, no matter what the receiving water body.

NPDES permitted dischargers are required to monitor and report compliance to EPA or the appropriate state agency. Failure to meet permit conditions constitutes violation of the CWA and the EPA and state agencies may bring to bear a range of enforcement actions including fines and suspension of operation of the discharger. Under Section 505 of CWA, private citizens may also sue the violator.

### *EPA NPDES Discharger Categories*

The EPA currently defines three broad NPDES discharger categories based on the type of facility, processes and discharges. These categories are:

1. Municipal Wastewater Treatment Plant Discharge Permits, including the Biosolids Program: These permits are issued to towns, cities counties, etc. that operate publicly owned treatment works (POTW). Municipal wastewater contains primarily biodegradable organic material that is treated by settling and biodegradation. Pollutants limited and monitored in permits issued to POTW include suspended solids, biochemical oxygen demand, fecal coliform bacteria, substances that alter the pH, and oil and grease. The Biosolids Program regulates generators of sewage sludge such as POTWs. Sludge is disposed of through application to land, surface disposal or incineration. Biosolids are considered a recyclable bio-resource, and reuse or reclamation of sludge is encouraged by the EPA.

2. Storm Water Discharge Permits: Discharges from municipal separate storm sewer systems (MS4s) and from construction sites disturbing more than 5 acres require a NPDES permit. The permit is required to encourage municipalities to reduce pollutant loadings to the maximum extent practicable. Industrial storm water discharge are covered by a similar permitting scheme. Erosion control and storm water management during construction phases of projects also require permits. It should be noted that in most localities states issue storm water discharge permits (State Storm Water Discharge Permit - SSWDP) even when industrial and wastewater permitting is performed by the EPA.
3. Industrial Discharge Permits: Industrial facilities generating wastewater as a result of various processes (including chemical manufacturing, steam electric power generation, mining, smelting facilities, pulp and paper mills, etc.) are required to treat wastewater and obtain an NPDES permit if they discharge directly to a receiving body of water. EPA as established guidelines and criteria for more than 50 different industries, which are called 'categorical standards'. A typical NPDES permit contains numeric limits on the quantity and concentration of discharge, effluent monitoring requirements, biomonitoring requirements (usually including whole effluent toxicity (WET) testing), reporting requirements, and a schedule for compliance.

In 1987 the CWA was reauthorized, continuing action on toxic substances. The power of the Act was extended to authorize citizen suits against polluters and to fund sewage treatment plants (POTW's). In so-called compliance states (states that have sufficient expertise and manpower to administer the required permits), administrative, enforcement and permitting may be performed by the state, with the understanding that EPA still retains final responsibility and jurisdiction. New York is such an EPA 'delegated' state so that the New York State Department of Environmental Conservation (NYSDEC) issues permits (State Pollution Discharge Elimination System, SPDES) and monitors compliance with relevant EPA codes.

## *Verifying Permit Compliance with Biological Testing*

Since discharge into public waters was to be regulated under the CWA, regulations were promulgated to establish specific test methods that were acceptable proof of compliance with the discharger's permit. These NPDES permit compliance tests were defined by Federal code 40 CFR part 136. These tests can be used determine the toxicity of individual components of the discharge, or of the entire discharge stream. These nationally approved tests must be performed for the initial permit application, for discharge monitoring reports and for state certification. Tests have been approved for analysis of 262 different parameters covering the broad areas of bacteriological, inorganic (metals, minerals, nutrient, and residue) non-pesticide organic, pesticides, a radioactive materials. The acceptable biological testing methods listed under this code is included in Table 1. 40 CFR part 136 was modified in October 1995 to include WET testing methods.

Because of the difficulty in determining the overall toxic effect of a effluent wastewater by chemical of physical means, WET testing methods were developed. These tests measure the effect of the effluent on indicator or surrogate organisms. The effect can be lethality, impaired growth or compromised reproductive ability. In the remainder of this paper, I will limit the discussion of NPDES permitting and testing to industrial generators, and testing methods to WET testing.

| Measured Parameter | Method | EPA Method | Standard Method | USGS Method |
|---|---|---|---|---|
| **Bacteria:** | | | | |
| Fecal Coliform, # per 100-ml | Most probable number (5 tube), 3 dilution<br>Membrane filter, single step | 1 | 5 | 6 |
| Fecal Coliform in presence of Chlorine, # per 100-ml | Most probable number (5 tube), 3 dilution<br>Membrane filter, single step | 1 | 5 | 6 |
| Total Coliform, # per 100-ml | Most probable number (5 tube), 3 dilution<br>Membrane filter, single step or two step | 1 | 5 | 6 |
| Total Coliform in presence of chlorine, # per 100-ml | Most probable number (5 tube), 3 dilution<br>Membrane filter, single step | 1 | 5 | 6 |
| Fecal streptococci, # per 100-ml | Most probable number (5 tube), 3 dilution<br>Membrane filter, single step<br>Plate count | 1 | 5 | 6 |
| **Aquatic Toxicity:** | | | | |
| Acute toxicity to freshwater organisms (LC50) in % effluent | Daphnia, ceriodaphnia, fathead minnow, rainbow trout, brook trout or bannerfish shiner mortality | 2 | - | - |
| Acute toxicity to marine organisms, (LC50) in % effluent | Mysid, sheepshead minnow or medidia mortality | 2 | - | - |
| Chronic toxicity to fresh water organisms, (NOEC or IC25) in % effluent | Fathead minnow larval survival and growth<br>Fathead minnow embryo-larval survival and teratogenicity<br>Ceriodaphnia survival and reproduction<br>Selenastrum growth | 3 | - | - |
| Chronic toxicity to marine organisms (NOEC or IC25) in % effluent | Sheepshead minnow larval survival and growth<br>Sheepshead minnow embryo-larval survival and teratogenicity<br>Menidia beryllina larval survival and growth<br>Mysidopssis bahia larval survival, growth and fecundity<br>Arbacia punctulata fertilization<br>Champia parvula reproduction | 4 | - | - |

**Table 1 - Acceptable biological test methods for determining NPDES compliance as defined in 40 CFR 136.3. The approved test methods are listed by number in Table 2.**

| Reference # | Approved Reference |
|---|---|
| **1** | USEPA, 1978, Microbiological Methods for Monitoring the Environment, Water and Wastes, U.S. Envirnomental Protection Agency, Cincinnati, Ohio, EPA/600/8-78/017 |
| **2** | USEPA, 1993, Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms, Fourth Edition, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio, August 1993, EPA/600/4-90/027F |
| **3** | USEPA, 1994, Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Wastes to Freshwater Organisms, Third Edition, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, EPA/600/4-91/002 |
| **4** | USEPA, 1994, Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Wastes to Marine and Estuarine Organisms, Second Edition, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, EPA/600/4-91/003 |
| **5** | APHA, 1992, Standard Methods for Examination of Water and Wastewater, American Public Health Association, 18$^{th}$ Edition, Amer. Publ. Hlth. Assoc., Washington D.C. |
| **6** | USGS, 1989, U.S. Geological Survey Techniques for Water Resources Investigations, Book 5, Laboratory Analysis, Chapter A4, Methods of Collection and Analysis of Aquatic Biological and Microbiological Samples, U.S. Geological Survey, U.S.. Department of the Interior, Reston Virginia |

**Table 2 - Approved Biological Test Method Proceedures and References**

## 2. General Methods of Aquatic Toxicity Testing

The goal of any environmental toxicity test is to provide a measure of the harmful effects of a substance on the system under observation[1 2]. The system can be an individual, a collection of individuals, part of a food web, or an entire ecosystem. This measure can be expressed by any one of a number of end points (death, loss of fecundity, etc.) using any number of statistically-derived metrics (LC50, EC50, IC50, etc.) If a sufficient number of organisms are used, over a range of exposures wide enough to encompass no effect and 100% lethality, a concentration-response curve may be generated, which may allows estimation of a maximum allowable toxicant concentration (MATC) for release to the environment. The cost and relevancy of the tests (with regard to environmental effects) increase as we progress from individual species to communities to ecosystems and from individual components of an effluent to the whole effluent stream. In practice, very few ecosystem tests are actually performed because of the complexity, expense and difficultly in interpretation of the results. In multi-species toxicity tests, bio-accumulation and other secondary effects may be observed, especially if the species represent components of a food chain.

Aquatic toxicity tests that are repeated over a period of time from the same location in the body of water can be used to track changes or trends. In the case of industrial discharge, these changes in toxicity may be due to failure of remediation systems, seasonal variations in production, or unrelated environmental changes. Environmental changes that can affect the toxicity of the effluent stream could include upstream changes like runoff from agricultural operations, roads and parking facilities, or seasonal, natural effects like changes in pH or dissolved oxygen. These environmental changes can alter the toxicity of the discharge stream through a multiplicative or symbiotic effect, and add to the variability of a test.

Tests using whole effluent streams are especially important in water pollution evaluation because chemical and physical tests cannot adequately predict detrimental effects of multi-component discharge streams in low concentrations on aquatic biota. Ideally, the organism or organisms tested will be one indigenous to the effected body of water, and will be a member of an ecologically import niche. The test can take on even more significance in measuring overall environmental effects if the organism is in a food chain leading to man or other important species, or if several members of a food chain can be tested in a microcosm.

The data from aquatic toxicity tests may be used to predict the environmental effects of chemical waste stream, to compare toxicants, or as a regulatory tool for control of

industrial and municipal discharges. As mentioned above, standard tests are recommended or required by regulatory agencies to verify compliance with NPDES and SPDES discharge permits. Test data can be used to determine the sensitivity of various organisms, the scope (effected zone) of the effect, and the effects of accidental releases. Beyond regulatory compliance, data from these tests can be used as an industrial management tool to monitor the effectiveness of waste treatment methods, and thus total amount of waste that is treatable economically and practically.

### *Acute Aquatic Toxicity Testing*

Acute toxicity tests are a relatively short-term test where death or some other easily measured biological response that occurs within a time period that is short compared to the life span of the test species. These tests can be used as a screen for toxicity ('range-finding'), or as a method to establish a concentration-response for the discharge ('definitive effluent toxicity test'). Since the end point for all these acute methods is usually death or some other catastrophic physical observable, the sensitivity of these tests may be inadequate to judge the effects of small concentrations of effluent to the receiving water. For short-lived invertebrates like *Daphnia magna* or *Ceriodaphnia dubia*, the acute test lasts 24 to 96 hours. The acute tests are useful in setting LC50 concentrations and establishing ranges for further study in chronic tests. Acute tests can also give an indication of maximum allowable concentrations for episodic high level exposures like waste spills.

Establishment of an accurate LC50 requires that a wide enough concentration range be used to test a large enough number of animals so that a statistically significant does-response curve can be generated. LC50 acute testing results are compromised when the toxicant is of very low or very high toxicity, resulting in either no mortality or total mortality. If the test species response to the toxicant is variable because of genetic reasons, the slope of the dose response curve will have slope that is too low to make an accurate determination of LC50 (see Figure 1). For this reason, acute tests are usually used only as screening for more sensitive chronic toxicity tests.

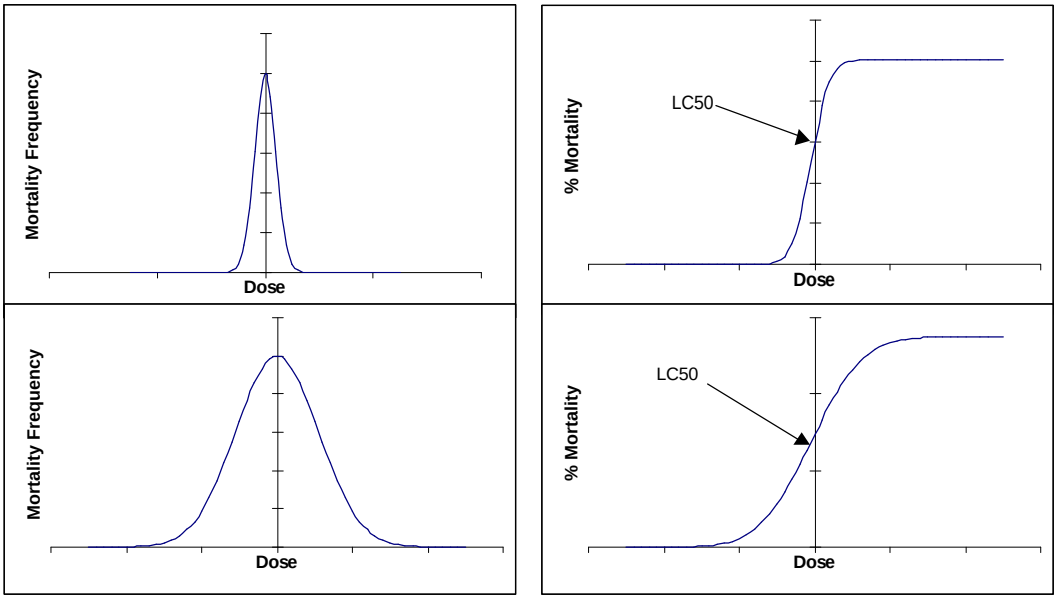


**Figure 1 - The effect of the dose-response curve slope steepness on LC50 determination. The upper left shows a well-defined response to a toxicant, the upper right the mortality frequency for this response. The lower left shows the response where the response is less pronounced the test organisms susceptible over a wide range of concentrations. The % mortality curve on the lower left has a shallow slope, thus making determination of LC50 subject to 'noise' around the 50% mortality point.**

## *Chronic Aquatic Toxicity Testing*

Chronic toxicity tests observe longer term biological changes that are displayed in subtle effects like changes in appetite, growth, metabolism, reproduction, fecundity, or the occurrence of mutations. Since chronic tests have sensitivity to second-order effects (other than death) they can probe lower concentration levels than would be possible using acute methods. Chronic testing is often used for determining MATC, no observable effect concentration, and evaluating regulatory compliance. The disadvantage of chronic testing is the long duration of the tests when mature fish are used as test subjects. Tests using short lived crustaceans like *Ceriodaphnia dubia* and larval stage fish have reduced the time required while maintaining sensitivity to toxicants.

## *Aquatic Testing Exposure Scenarios*

In aquatic testing, organisms may be exposed to the toxicant using one of the following methods:

a. Static - test solution is not replaced during the test.

b. Static-Renewal - test solution is replaced after a specific time period.

c. Recirculating - filtration used to remove waste products and keep dissolved oxygen levels adequate in the test solution.

d. Continuous or intermittent flow - proportional dilution of waste stream used with continuous replenishment of test solution.

These experiment designs have particular advantages and disadvantages. Static tests have the benefit of simplicity and low cost. These tests are good as screens for more detailed studies, but are poor for compounds that are volatile or degrade rapidly. Static-renewal tests provide a more consistent chemical concentration at the expense of increased handling of the test specimens. This handling may cause stress, the effects of which can be difficult to distinguish from the toxic effects of the effluent. Recirculation and filtration methods may be used to maintain water quality for the test specimens, but it must be determined that the aeration and filtration that takes place does not alter the available concentration or toxicity of the test solution. Aeration can force volatile toxicants out of solution, and filtration, especially using activated carbon, can remove many organic compounds. Flow-through tests are seldom used for effluent tests because of the complexity of the apparatus (which includes flow metering, pumps and mixing chambers) and the large quantities of test solution required, but provide the best estimate of toxicity while minimizing animal handling. Flow-through methods are the only way to test chemicals with very high oxygen demand. Because of the complexity and expense of flow through testing, static and static-renewal test methods are the only methods routinely used for invertebrate testing of effluents.

### *Whole Effluent Toxicity Testing*

In order to replicate to the greatest extent the effect of a complex, multi-component effluent stream on the environment, whole effluent toxicity tests were developed by the EPA and other organizations starting in the mid-1980s, using fish, protozoans, algae and bacteria. WET testing does not require identification of specific components of the effluent stream, which would not only be expensive, but would not include the effects of interaction among the toxicants. Such interactions can lead to simple additive responses, synergistic interactions (where the toxic effect of the mixture is much greater than the sum of the effects of the

components) or even of antagonism or inactivation (where the interaction among the toxicants in the stream produce a less toxic mixture).

Much of the work in development of aquatic toxicity tests has been centered on the lethality of a discharge to fish. Fish are easy to culture, are well understood in the environment, and have a discernible value as perceived by most people. Tests that include all age groups of test fish (including larva and young) are an even more sensitive indicator, because young specimens are usually more sensitive to toxicants than adults. Freshwater fish species that are amenable to culturing and use in acute toxicity tests include the fathead minnow, *Pemephales promelas*, rainbow trout, *Onchorynchus mykiss*, and the brook trout, *Salvelinus fontinalis*.

The base of the food chain in an aquatic ecosystem, the protozoa, algae and bacteria, also make suitable test species for toxicity tests. Ciliated protozoa are the most numerous zooplankton in the food chain, and have been shown to concentrate certain persistent chemicals and translocate them to higher members of the chain[3 4 5]. Among the protozoans, the cladocerans (water fleas) are easily cultured and have been used in toxicity studies for over a century. They convert phytoplankton and bacteria into animal protein that is then nutritionally available to larger species.

The EPA has developed WET testing methods for both freshwater and marine test species. In the case of the daphnid *Ceriodaphnia dubia*, the acute testing methods use mortality as an end point in a static or static-renewal test and take place over a period of 96 hours or less. Chronic EPA WET tests using *Ceriodaphnia* expose the animal in a static-renewal method over a period of 7 days, observing growth, reproduction, and mortality. Depending on the nature of the discharge and the arrangements between the EPA and the discharger, both acute and chronic tests using invertebrate, vertebrate and plant species may be required to identify the most affected species for NPDES permit limits.

## 3. WET Test Methods for NPDES Permit Compliance Testing

The EPA has developed acute and chronic (short-term) WET test methods for use in NPDES compliance evaluations. The ultimate goal of these tests is to establish the safe (no observed adverse effect concentration - NOAEC) concentration for the whole effluent. These tests may also be used for self-monitoring and periodic sampling, both for regulatory compliance and to assess the efficacy of the discharger's remediation methods. I will limit the discussion of the tests to those developed for freshwater invertebrates, and for *Ceriodaphnia dubia* in particular.

### *Acute Testing Methods*[6]

From the mid-70's to the mid-80's, several groups reported on standard tests using the cladocerans *Daphnia magna*, *Daphnia pulex*, *Ceriodaphnia reticulata*, and *Ceriodaphnia dubia*.[7 8 9] The goal of this research was to develop simple test methods that would provide a rapid method to assess toxicity of effluents when sample volume and time was limited. It was hoped that these methods would replace tests traditionally performed with fish and other higher forms of life. Toxicity tests using fish typically take a longer time span to complete and require a large laboratory with relatively expensive aquarium apparatus.

Of the cladocerans used in these early papers, the Ceriodaphnia were shown to be easier to culture and easier to feed than the Daphnia species. More importantly, Ceriodaphnia are native to the waters of North America and have a wide distribution. With further research and testing to insure adequate sensitivity to toxicants and repeatability, these tests later developed into procedures recognized by EPA to be suitable for CWA and other regulatory compliance. Central to the acceptance of these tests by the EPA was the development of:

a. A reliable method to culture the animals

b. Proof of repeatability between laboratories and among separate tests within a laboratory (Section 4)

c. Sensitivity of the test organisms to a wide range of toxicants

d. Ease of application of the tests

e. Low cost.

I will discuss these factors as they apply to the development of standardized acute and chronic toxicity tests using cladocerans, and discuss methods that may be used to reduce the variability of the experiments.

## Ceriodaphnia culturing

The fish culture industry is well established. Fish are cultured for use as baitfish ('minnows') and for food and stocking (trout). Volumes devoted to the breeding, feeding and rearing of fish are available, and control of the aquatic environment for fish rearing is provided commercially by aquarium supply companies. The opposite situation exists for cladocerans, where rearing and feeding methods, and even species identification, is poorly known outside of a small academic community. Maintaining suitable cultures over long periods of time can be difficult. Obtaining stable, healthy cultures is absolutely critical to toxicity tests, as the use of unhealthy or weak animals would compromise the outcome of the toxicity measurements.

A group at EPA's Duluth Environmental Research Laboratory (and others) developed a simple culture media and technique that enables production of stable cultures of *Ceriodaphnia*[7]. Feeding tests were conducted for 10 generations, to determine food adequacy, reproduction rate and efficacy, and general animal health, resulting in stable cultures that were maintained for as long as 18 months. The animals were fed a diet consisting of yeast, CEROPHYLL (cereal leaves) and digested trout chow (YCT) that provided adequate nutrition for stable populations and high asexual reproduction rates. Constant temperature and pH were shown to be critical to rearing. Although the physical characteristic of the species has been described[18], the diet and feeding behavior of Ceriodaphnia was not well known prior to the work of the EPA group.

For toxicity testing it is absolutely essential to be able to recognize an unhealthy or weak culture, as the use of such compromised animals in a toxicity test would lead to an overestimate of toxicity. Unlike other species that exhibit population crashes and incidence of disease when stressed, these animals do not appear to exhibit these easily observed effects, so other signs must be observed to insure that the stock culture is in good health prior to their use in tests. *Ceriodaphnia* normally reproduce parthenogenetically producing only female young. When under stress, however, the organisms are seen to produce males and reproduce sexually, with smaller broods. This switch from asexual to sexual reproduction in stressed populations is fairly common among invertebrates and signals the species attempt to cope with the stress through increasing genetic diversity[18]. Similar behavior is observed in the natural seasonal cycle that occurs in these animals in the spring in most bodies of water, when crowding, a decrease in available food, cold water temperatures or low light intensities

stresses the population. The establishment of a culturing, rearing and observation schedule for the animals was seminal to the success of this test method.

## Sensitivity to toxicants

Daphnia have been used in toxicity tests since 1929[9]. Various species have been used to test the toxicity of crude oil[10 11], insecticides[12 13], refinery wastes[14], herbicides[15], metals[16], and 'cocktails' of industrial wastes[17]. These tests included acute toxicity tests, and chronic tests which included reproductive impairment, as well as death, as an end point. In all cases daphnia showed qualitatively similar response to the toxicants as higher animals.

## Biology of *Ceridodaphnia dubia*[18]

It is important to be able to accurately identify and understand the test specimens. It is possible for a culture source can actually change species over a period of time, either from introduction of water containing other cladocerans, or from eggs present in airborne dust. It is also necessary to understand the behavior of the animals to assess their health and fitness for the test.

*Ceriodaphnia dubia* is a member of the family Cladocera, the water fleas. These interesting, easily cultured animals have been studied by biologists since the invention of the microscope. It spite of its widepsread distribution in the littoral regions of ponds and lakes through North America, little is known of the exact habits of Ceriodaphnia, including the exact nature of their food in the wild. *Ceriodaphnia dubia* females are up to 0.09-cm. in length, the males as large as 0.07-cm., with unsegmented flattened bodies of oval, angular shape (See Figure 2). There is a small spine at the posterior end of the shell, and the ventral ends edges of the shell are lined by setae.

The head, which is set off from the body by a cervical notch, contains a large compound eye that is clearly visible. The eye has several lenses as is moved constantly by three pairs of muscles. There are two sets of antennae, a very small set near the 'mouth' and a much larger set attached in back of the compound eye. The posterior antennae are moved by a powerful set of muscles that originate in the region in back of the cervical notch.

Small mouthparts are located near the junction of the head and body, above the cervical notch. The animal has sets of toothed or ridged jaws that are opposed.

There are five or six pairs of legs along the front of the abdomen. In most Claodcerans, these legs are used for maneuvering or grasping, the primary locomotion coming

from the antennae. The animals move about more or less continuously, in short hops produced by powerful strokes of the antennae. The mean speed when swimming is 0.15 - 0.2 cm./s, along an erratic path.

During most of the year, the population consists mostly of females, the males being abundant in the fall and spring. Males are produced when the population is stressed in times of low water temperatures, high population densities and limited food supply. When sexual reproduction takes place, ephippia (embryos encased in a tough covering) are produced, which are resistant to drying. The ephippia may become active and hatch when conditions permit.

*Ceriodaphnia dubia* live for up to 30 days in ideal conditions. Three to five days after birth, the female is mature, and given adequate conditions, releases a clutch of four to ten eggs into her brood chamber. The eggs hatch in the brood chamber and are released into the environment as juveniles within about 40 hours, after the female molts. The ability to produce several broods in a week under good conditions makes the animal attractive for experiments where reproductive effectiveness and multi-generational effects can provide sensitive indicators of toxicity.

**Figure 2 - Physical appearance of *Ceriodaphnia dubia*. The parthenogenetic female is under 0.1 - cm long when mature.**

## The Acute Test Protocol

*Daphnia*-based acute toxicity tests are simple (in theory). A temperature controlled laboratory with normal laboratory illumination and method to control the light/dark time is required, as all cladocerans are sensitive to rapid temperature and light variations. A source of dilution water, and simple test containers complete the apparatus. The sample cup recommended for the EPA 600 acute tests is a 30-ml beaker with 15-ml of test solution. This size allows moderately easy observation of the specimens. The surface area-to-volume ratio of this container also insures that dissolved oxygen will remain adequate for survival and reproduction without external aeration, if the loading of animals in the sample container is kept as suggested.

The life span of the animals, and their reproductive and development times all work out fortuitously to produce a test which can be completed within a seven day period. The test

duration is defined for one, two or four days, with a day for preparation of the samples and culling of test animals and a day for data analysis.

Five effluent concentrations and a control are used, with a minimum of five young (less than 24 hours old) organisms in each sample cup. A minimum of four sample cups at each concentration (plus control) is used to provide adequate statistics for the test. Grab (one-shot, for intermittent discharges) or composite (sampled periodically over a 24 hour period, for continuous discharges) sampling of the effluent stream is employed. Death of the animals is the endpoint of the acute test.

## *Chronic Testing Methods*[19]

Acute testing with mortality as an endpoint is useful in determining toxicity (LC50) at high concentrations, but cannot be expected to observe subtle effects like changes in growth or reproductive rate. Since the acute testing protocol lasts only one to four days and takes place in a single generation, the test may not accurately assess the lowest concentration allowable in the environment for a 'no-effect' condition.

From 1967 to the present, studies were performed to examine the issue of determining the 'safe' concentration of toxicants when observed in full life cycle test [20] [21] [22]. These tests were conducted with higher forms of aquatic life that have several developmental stages (fish). These tests showed that partial life cycle tests that include only the young and larval stages of fish development could provide an accurate assessment of chronic toxicity when compared to full life cycle tests.

The driving force behind these efforts was to develop a meaningful test that meets the goal of accurately determining a NOAEC while not taking an excessively long time or being overly expensive. Continuing efforts shortened the test cycle further and introduced new sensitive species and new protocols [23] [24] [25] [26] [27] [7]. In all cases the predictive ability of these short-term tests was shown to be equal (to within experimental errors) to that of long term, full life cycle tests.

Short term chronic testing is essential for compliance testing and permit verification under NPDES. A chronic fish testing protocol that takes 6 months to complete is useless to verify compliance on a quarterly basis.

The details of the protocol are provided in Appendix A.

## The Chronic Testing Protocol[28]

The equipment used in the acute test is very similar to that used in the chronic test protocol. In the chronic protocol, however, a single specimen of per sample cup of

*Ceriodaphnia dubia* is exposed in a static renewal dosing system (daily renewal) at several effluent concentrations. A control group is also maintained. A minimum of ten identical sample cups are maintained at each concentration to provide sufficient statistical basis for the test. The cultures are maintained until 0% of the control organisms have had three broods of offspring. Daily feeding is provided of the 'standard' YCT suspension.

If the control group is healthy, the food correct, and the control water pure, 15 or more offspring should be produced in three broods. Eight days are allowed for three broods to be produced. If three broods and 15 (average) offspring have not been produced in 8 days in the control group, then some interfering factor has reduced the fecundity, survivability, or growth of the group, and the test must be repeated.

The details of the protocol are provided in Appendix B.

# 4. Sources of Variability in WET Aquatic Toxicity Testing

The implementation of any experimental model requires compromises in order to reduce cost, ease analysis and reduce the need for highly trained personnel to conduct the experiment. The experimental protocol must still produce a result that is both easy to interpret and meaningful in the context of the model. These compromises can reduce the value of the experiment by introducing additional uncontrolled variables, causing correlations between unrelated factors in the experiment, and generally reducing the repeatability of the experiment given the same initial conditions.

## *Precision, Repeatability and Reproducibility*

To define the quality of an experimental observation we use the term precision[29]. Precision refers to the agreement of a measurement with a (usually unknown or unavailable) perfect measurement of the para meter. The precision of an experiment is affected by both internal sources (systematic errors) and random errors inherent due to limited sample size or other unidentified external, uncontrolled sources.

Repeatability is the ability to reproduce the measurement from one experiment to another under identical conditions, while reproducibility is the ability to produce the same results under differing conditions at (perhaps) different laboratories. There are several types of repeatability - intra-experiment (within the same test), inter-experiment (at one laboratory, with a given set of workers, equipment, etc. for repeated tests) and inter-laboratory (for the same experiments performed at different laboratories). The design goal of any experiment, of course, is high absolute accuracy, along with repeatability. Precision and repeatability are especially important in effluent toxicity testing, where under or over estimates of toxicity can lead to hazardous environmental releases or unnecessary industrial restraint, respectively.

Variation in experiments can result from poor initial design, or poor control or ignorance of sensitive elements in the experiment. These causes of variation are not mutually exclusive. A good test team can often get high quality results from a protocol because they understand and compensate for the subtleties and 'unwritten laws' of the experiment.

In biological experiments, control of all confounding elements may be nearly impossible. For example, the effects of small variations in feeding of the test organisms at critical times during their development may result in organisms that are particularly

susceptible to a toxicant, skewing the results of experiments with this organism-toxicant protocol. The skewing has been produced by a factor that is quite 'upstream' from the actual test and may not be recognized. The situation is in general even more complicated than this in WET tests that attempt to gauge the toxicity of a highly variable and complex waste stream. (Synergistic effects can exist between elements of the waste stream, the receiving water, and test organisms.)

I will discuss the sources of variability as applied specifically to the EPA-prescribed WET testing methods[6 19] using *Ceriodaphnia dubia* and similar invertebrates. An important point is that the sources and amount of variability produced by different factors in these protocols using *Ceriodaphnia dubia* is representative of the EPA tests for other animals, and, in fact, is representative of all aquatic toxicity tests.

### *Intrinsic Variables*

Intrinsic variables are those which arise from ranges or choices in the test protocol. Because of the cost and difficulty of precisely controlling physical and genetic parameters, ranges are allowed in temperature, lighting, dilution water chemical properties and organism diet genotype and age.

#### Sampling Methods and Sample Handling

The EPA Acute and Chronic WET test methods allow both 'grab' (single sample) and composite (time-averaged continuous) sampling of the waste stream. The main benefit of composite sampling - namely time averaging - may be impossible to meet if the waste stream is discontinuous or variable in nature.

Holding/storage time and temperature are specified in the standard. The samples are to be kept cold until used, to minimize boil-off of volatile components, but the act of heating the sample to the test temperature before use may drive off these volatile components. The maximum holding time is specified as 36 hours, but for waste streams that are either reactive or very volatile, variation in the actual use time (within the 36 hour limit) from one test run to another can cause significant variation in composition and/or bio-availability of toxicants.

#### Biological Variations of the Test Species

The test organism's response to toxicants can be expected to be variable among different genotypes of the same species. At least one study has been performed to investigate the genotype of the species used for laboratory testing (compared to the distribution of genotypes in the wild) for *Ceriodaphnia dubia* [30]. For this test species, it was found that the specimens from a number of laboratories were derived from a single genotype. *Ceriodaphnia dubia* from wild populations exhibited much larger genetic variability. Thus the laboratory specimens, from a single genotype, would be expected to produce a narrower distribution of responses for the same toxicant concentration, but whether this result is representative of the response of the species in the environment (which is the overall goal of these test methods) is not clear. Using nine different genotypes of *Daphnia magna*, it has been shown[31] that the response to a variety of toxicants was variable, but not large. Further research need to be performed with the other allowable test species to determine whether this is true for other species, or in fact for other strains of the same species. In general it is believed that the effect on toxicant sensitivity of small differences in genotype is less for asexually reproducing organisms like *Ceriodaphnia dubia* than for sexually reproducing organisms [32].

As a measure of the suitability of cladocerans for toxicity testing, we should look at similar experiments using similar toxicants, from other aquatic species (e.g. fish).

One study[33] observed variation in lethal endpoints for fish (fat minnows and rainbow trout) and several invertebrates. It was found that, by several estimates (steepness of dose-response curve, scatter in LC50 concentrations) that variability of test data was lower using invertebrates. The data was especially good for the daphnids tested, *Daphnia pulex* and *Daphnia magna*.

Another study[34] looked at the effect of a herbicide on bluegills and their food organisms. These food organisms consisted of benthic organisms, daphnia and small forage fish (minnows and young fish of several species). Once again it was found that the scatter in LC50 data was significantly smaller in simpler organisms than in fish. This may be expected from the physiology of fish, which includes many enzyme detoxification systems.

It thus appears that in addition to simplicity of the test system and the short term required for testing, animals from the lower orders can provide more accurate data than more complex types.

Perhaps more important than genetic makeup is the care and conditioning that the test organisms have had prior to testing. Proper feeding and water purity is essential to producing organisms that are not over-sensitive to toxicants because of compromised health. If, on the

other hand, the water used for rearing has chemical or physical contaminants, the animals could become physiologically adapted to these contaminants and produce under-sensitivity in toxicity tests.

Another factor increasing variability in WET testing is the age of the test specimens. In the acute test regimen, the age is specified as 'less than 24 hours', while in the chronic protocol, the animals must be less than 24 hours old, but all released within an 8-hour period. This is a significant time span for animals with a life span of around 30 days. The age of specimens would be expected to affect the sensitivity to toxicants, with younger organisms being more sensitive in general. Narrowing the range of acceptable ages for test organisms might decrease the variability of the tests, but would require considerable additional effort in selection of specimens and require a large number organisms to be kept so that organisms of the correct age could be obtained in quantity.

## Dilution Water

There have been several published studies investigating the effect of the quality of dilution water on WET tests using *Ceriodaphnia dubia* [35] [36]. These papers have shown that the hardness and pH of the dilution water can have an effect on the toxicity of metals, ammonia, cyanide compounds, phenols and surfactants. The change in toxicity is the result of pH or water hardness effects on both bio-availability and effects of the toxicant. This is particulary true for the case of metallic ions in the effluent. Even when the control sample dilution water produces acceptable results (< 10% mortality for the duration of the test) dilution water changes can affect toxicity, especially when the sample concentration is near the threshold of toxicity. When dilution water is obtained from the commercial water supply or from the receiving waters, seasonal variations in physical and chemical factors may be a source of variability.

## Testing Variables

The acute and chronic test protocols allow wide ranges in several physical and test parameters to minimize the cost and difficulty of performing these tests. The ranges in

representative test parameters allowed by the Acute and Chronic test standards are outline in Table 3 and Table 4 .

| Test Parameter | Effect on experiment outcome |
|---|---|
| Test duration (1,2 or 4 days) | Large - long exposure can increase toxicity |
| Test chamber size (> 30-ml) | Large - sampling issues and container-sample interactions |
| Age of test organisms (< 24-h) | Large - 1 h organism more sensitive than 24-h |
| Dilution water (non-specific) | Large - variation can cause differences in bio-availability |
| Test chamber composition | Large - effluent may react with chamber |
| No. of test organisms (total > 20) | Large - significantly better statistics obtained for low-toxicity effluents with more samples |

**Table 3 - Testing Variables for the EPA Acute WET test method (*C.dubia*).**

| Test Parameter | Effect on experiment outcome |
|---|---|
| Feeding regimen (YCT once daily) | Medium - daily does not specify every 24 hours |
| Test chamber size (> 30-ml) | Large - sampling issues and container-sample interactions |
| Age of test organisms (< 24-h all within 8 hour period) | Medium - narrows specimen age better than acute test |
| Dilution water (non-specific) | Large - variation can cause differences in bio-availability |
| Test chamber composition | Large - effluent may react with chamber |
| Feed (YCT) | Large - no easy way to assay nutritive value of feed |

**Table 4 - Testing Variables for EPA Chronic WET Test Method (*C. dubia*)**

## *Extrinsic Variables*

The extrinsic variables include those factors that are outside the specifications of the standard tests, including failure to follow the standard test procedures. The methods outlined in the standard procedures are simple, but may be violated inadvertently by even a conscientious laboratory staff. For example, given a concentrated effluent with high inherent toxicity, small variations in mixing and measuring of the diluted samples can lead to large differences in measured toxicity, resulting in scatter or skew of the dose response curve. Another example of introduction of an extrinsic variable would be the use test specimens that are slightly out of 'spec' for age because of a lack of suitable test specimens and a rigid schedule. A final example would be miscounting of either the initial population or final population because of the small size and rapid movement of the test organisms.

### Analyst Variability

In general, the effect of extrinsic variables on test data is reduced as the training and expertise of the analysts and laboratory personnel increases. To this end, the EPA cooperating state agencies have recently begun a program to verify the skill and education of laboratory staff who conduct testing under NPDES and SPDES permit programs. This program is called the Performance Audit Inspection (PAI) and started in 1993 as part of the NPDES Compliance Inspection Program. In initial performance monitoring, it was found that analysts at many facilities were untrained in biology and testing in general. This results in increased variability of tests from these facilities because the staff cannot recognize weak test organism populations, cannot perform the necessary statistical analysis, and in general are not aware of the subtleties of the test procedure.

## *Decreasing Variability of WET Tests*

Analyst training, experience and judgment has been cited as one of the most significant sources of variability in WET testing[37]. The EPA manual for acute WET toxicity testing[6] contains strong language suggesting that:

...each analyst must generate the ability to generate acceptable test results with these methods using the procedures described in this methods manual….

The implication of this is that the procedures in the EPA WET testing are sufficiently accurate that, when administered by skilled personnel, produce results with 'acceptable' accuracy for the purpose intended (regulatory compliance verification).

Given that skilled laboratory personnel are always difficult to find and that compliance tests with poor quality data can either damage the environment (toxicant not identified) or cause financial damage to the discharger (over estimate of toxicant activity resulting in extraordinary measures to reduce toxicity) it seems reasonable to attempt to automate and remove the human element from as much of the test procedure as possible.

One area where significant statistical improvement can be obtained is in counting the populations of test organisms. Especially for the smaller test specimens like *Ceriodaphnia dubia*, the difficulties in determining populations and can lead to large variations in the data. In the next section I provide a method to automatically count macro invertebrates, and show how improved counting can lead to reduced variation in a simulated test scenario.

# 5. Automation of macro-invertebrate counting

## *Observing live macro-invertebrates*

*Ceriodaphnia dubia* and other macro-invertebrates used in NPDES compliance testing are small organisms that move with an erratic, unpredictable motion. The standard 30-ml sample cup (containing 15-ml of water) suggested by the EPA test procedure, has a volume about 10,000 times that of an adult *Ceriodaphnia* (one of the smaller invertebrates). The test cups depth of almost 3-cm, provides an added third dimension which further confounds the counting problem. The inability to accurately assess the living and dead populations during the test, combined with the relatively few individuals present in a sample, can result in large dispersion in the data. If the sample-to-sample scatter is large enough, the test will be invalid and will have to be repeated. Additionally, the effort and fatigue induced by counting large number of samples causes the procedure to be labor intensive and therefore expensive, especially when frequent discharge monitoring is required by the permit.

Most laboratories use the unaided eye, or at best, a low power, wide field, dissection microscope to view the test specimens. Observing the specimen container with the unaided eye at close range, while tracking and differentiating the living and dead animals, while at the same time attempting to maintain a count the animals, is fatigue and eye-strain inducing. Similar problems have arisen in other fields of biology (cell counting), in astrophysics (observation of relative motions of stars and planetary objects) and in high energy physics (observation of particle tracks in film emulsions and bubble and cloud chambers). In all of these cases, however, optical, mechanical and computer aids have evolved to assist in data analysis and remove the operator from the visual observation 'loop'. It was hoped that a similar machine vision system might be developed to aid in analysis of zooplankton based toxicity data, reducing the time required for the observations and increasing the counting accuracy.

The standard 20 power, wide field stereo dissection microscope aids greatly in observation of individual specimens. The use of a dissection microscope is suggested in several papers describing the tests[6 7 8]. However, the dissecting microscope is hampered by a small depth of field and field of view (about 0.1-cm and 0.9-cm, respectively, at 20X for the representative Edmund Model R81-285). The depth of field and field of view of a dissecting microscope limit the useful observation volume to about 0.2-ml. The required field size in the

standard 15-ml sample cups is about 3.0-cm, with a depth of field of 1.5-cm. Thus the specimen container must be moved many times with accurate registration in order to avoid missing or multiply counting volumes of the specimen container.

The dissecting microscope itself can also be a source of fatigue, both physical and visual. In practical use, with more than a few specimens in the test cup, it is difficult for most observers to move the microscope from one field to another without losing track of which specimens were in the field and which had entered it during the move. Slosh of fluid in the cup during movement mixes the animals throughout the volume and can cause dead specimens to be counted as live. Calibrated mechanical stages or motorized stages that move in a predetermined pattern can eliminate miscounting in *area* of the specimen container. Additionally, the vertical motion of the specimens within the *volume* of the specimen container combined with the small depth of field of the dissection microscope, forces constant refocusing, again confounding the counting process. It is therefore desirable to be able to observe the whole volume of the sample cup for any measurement, with minimum movement of the cup and readjustment of the optical system.

A better solution is an optical system with field of view and depth of field matched to the observation volume. With proper optical design both the area (field of view) and volume (depth of field) requirements for counting micro invertebrates during toxicity testing can be met. Such a wide field microscope can be constructed at relatively low cost. I will show that by suitable choice of lenses and lighting, optical systems can be developed which allow observation of specimens as large as fish (brook trout) and as small as the smaller macro invertebrates (Ceriodaphnia dubia).

Many laboratories have found that operator fatigue is reduced when fixed-position microscopes are replaced by video-microscopes with observations performed on a large television screen, with no other changes in equipment[38]. The transition from a fixed, rigid position during observations and the ability to use binocular vision accounts for this fatigue reduction [38].

### *Equipment to improve accuracy of observations of plankton*

If a wide field microscope is now attached to a video camera, motion pictures of the specimens in their specimen container may be recorded. Reviewing frames from a prototype camera/microscope system recorded on a video cassette recorder, it was found that the motion of the live plankton[39] was irregular throughout the volume of the container, while the dead

plankton remained stationery near the bottom of the container. It was reasoned that if two video frames could be captured, with a suitable time elapsed between the frames, and the two frames subtracted from each other, the dead, stationary plankton would be subtracted out of the image, and what would remain would be an image of only the live animals (see Figure 3). Using a stop-frame video recorder which allowed time analysis of the movie generated by the video microscope, it was found that a period of 3 to 5 seconds between exposures allowed ample time for the living plankton to move without significant motion of the immobile, dead plankton occurring due to turbulence and vibration.

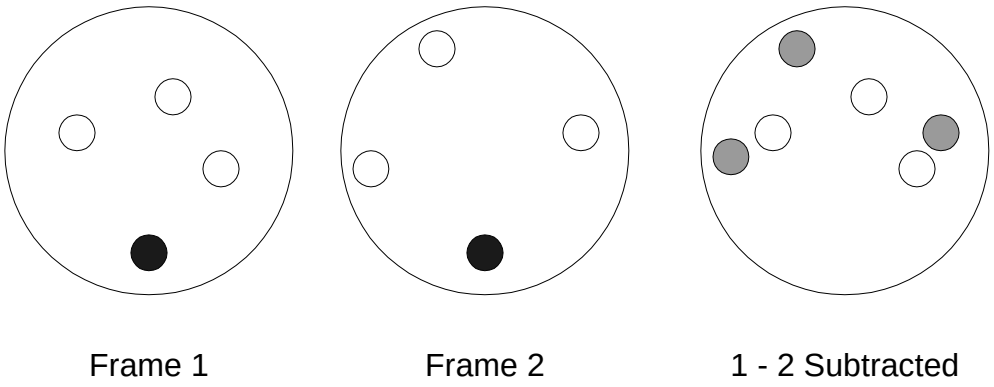


**Figure 3 - Example of subtraction of video frames to eliminate observation of dead plankton. Note that the live plankton appear twice, the live plankton in the subtracted frame appearing 'negative'.**

Note in Figure 3 that the live plankton that have moved appear 'negative'. This is due to the fact that subtraction of one frame from the other causes 'wraparound' of the pixel values in the image, caused by modulo 255 arithmetic being used for calculation in the frame. By discarding such 'negative' pixel values, we can eliminate the double counting caused by the subtraction operation. This is shown in the example in Figure 4.

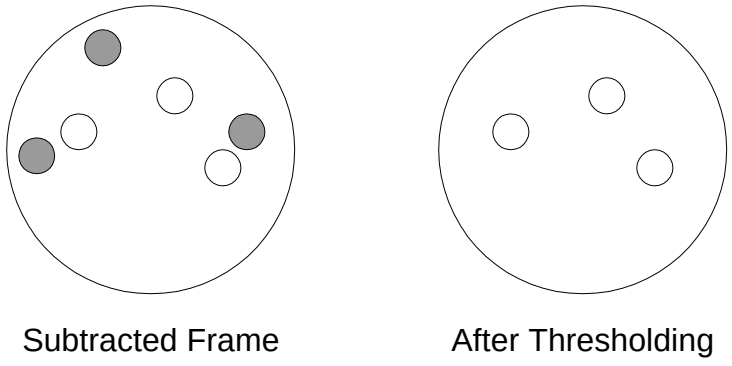

**Figure 4 - Eliminating negative pixels (negative pixel thresholding) eliminates double counting of live plankton.**

A system with the capabilities described will consist of a wide field video microscope (covering the entire field and volume of interest), frame capture electronics (to record individual frames for processing), image processing software (to eliminate observing the dead plankton and count the live plankton) and a suitable illumination system (to provide adequate contrast and brightness for the video system). I will discuss two variations of these components and their integration into a plankton observation system.

## *Wide Field Video Microscope*

A wide field video microscope can be constructed from a conventional video camera, long focal length video lens, and spacing rings. The spacing rings allow the lens to be positioned further away from the image sensor in the video camera, so that objects that are relatively close to the lens (1 - 5-cm) remain in focus. With this system, the macro invertebrates can be viewed conveniently on a large (17" or greater) television monitor, which, even without further enhancements, provides a great measure of strain relief for laboratory personnel. This combination is readily available commercially, is commonly called a 'video microscope', and is available commercially from several vendors[40]. The system is shown schematically in Figure 5. With a suitable choice of lens, spacer rings, and television monitor, daphnids or other macro-invertebrates may be seen throughout the entire volume of the specimen container in a single field. A bright 2-axis illuminator allows the lighting to be adjusted for best contrast on the semi-transparent plankton.

The optimum lens for a particular video camera is determined by the required field of view (FOV), depth of field (DOF) and the resolution and dimensions (in the image plane) of the imaging device (sensor in the video camera). As free parameters, we have the working distance to the object and the focal length of the lens. Current generation television cameras have an active imaging area that is between ¼" and ½ diagonally. The resolution of these cameras in the sensor plane is about 10-μm. Applying the lens equation[41] and referring to Figure 6, we find the so-called depth of focus (DOF) equation:

$$DOF := \frac{\left(2 \cdot o^2 \cdot f \cdot pitch\right)}{fl^2}$$

where fl is the focal length of the lens, f is the focal ratio of the lens (ratio of focal length to lens diameter), o is the distance from the lens to the object (sample cup) and pitch is the center-to-center spacing of the picture elements (pixels) in the image sensor. Taking as an example the TP-505D/3 video camera manufactured by Topica (retail price of $80, 2000), we can calculate the pitch from the size of the sensor and number of pixels in each direction. Along the 'long' direction of the sensor, there are 510 pixels in a 0.48-cm dimension, resulting in a pixel pitch of 9.4-μm.

The distance from the specimen cup to the front of the camera is set by th focal length of the lens and the required field of view. Using a lens of 0.60-cm focal length (Edmund Scientific K53-222, $47 (2000)), we calculate the FOV (from geometry) as 4-cm when the camera is at a distance of 5-cm from the specimen cup. The depth of field (DOF) at a lens opening of f/8 is calculated (from the above equation) to be:

$$DOF = 2.1 \text{ cm},$$

which is adequate for the sample cup depth of around 1.5-cm.

The only additional information required for this design is the resolution in the object plane of the system. Calculation of the resolution will determine whether the camera can capture sufficient detail to observe the (relatively small) test organisms. Assuming that the FOV of the camera from above (4-cm) each pixel of the sensor covers about 75 μm (4-cm / 510 pixels) in the object plane. This is adequate to resolve the 300 - 500 μm micro-invertebrates used in these tests.

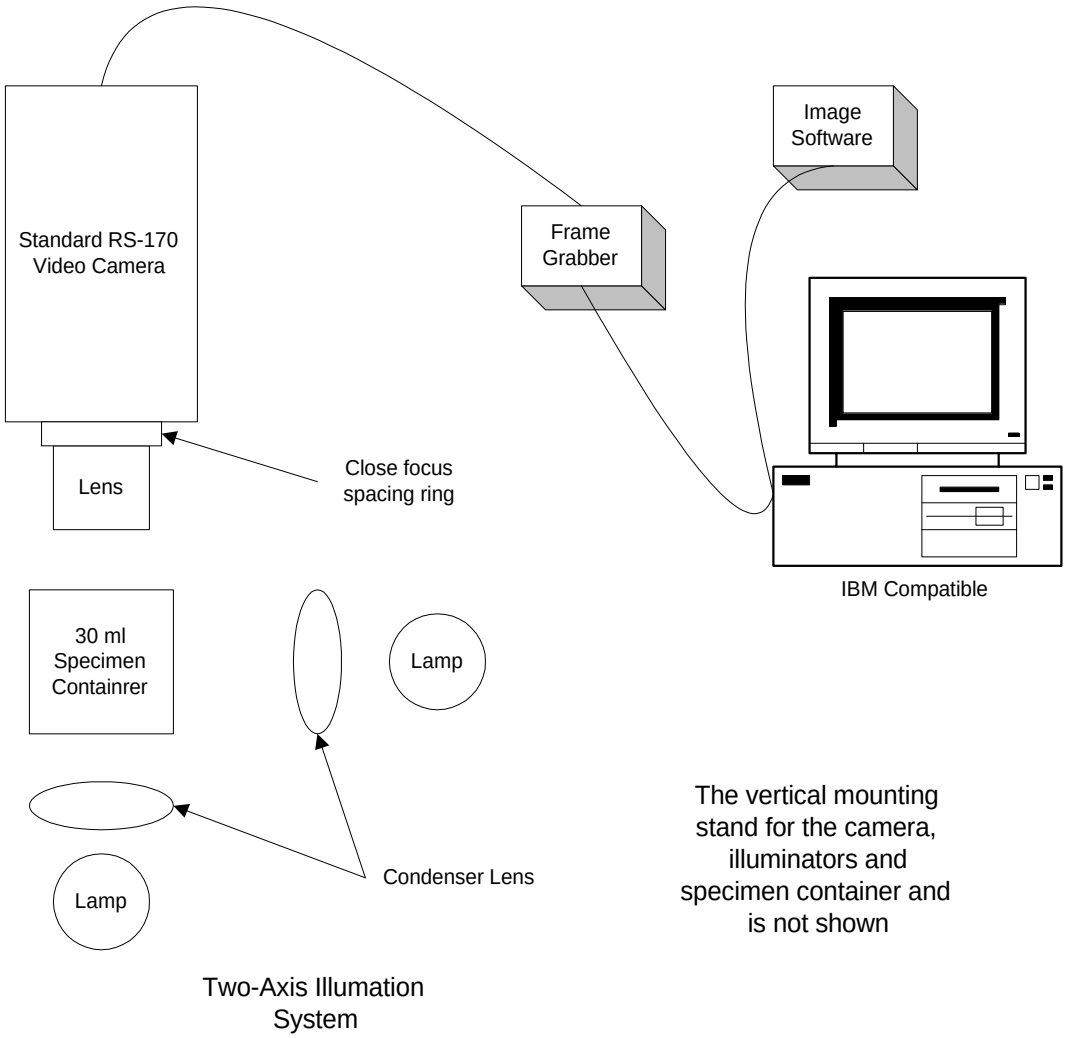


**Figure 5 - A conventional video microscope suitable for counting of plankton.**

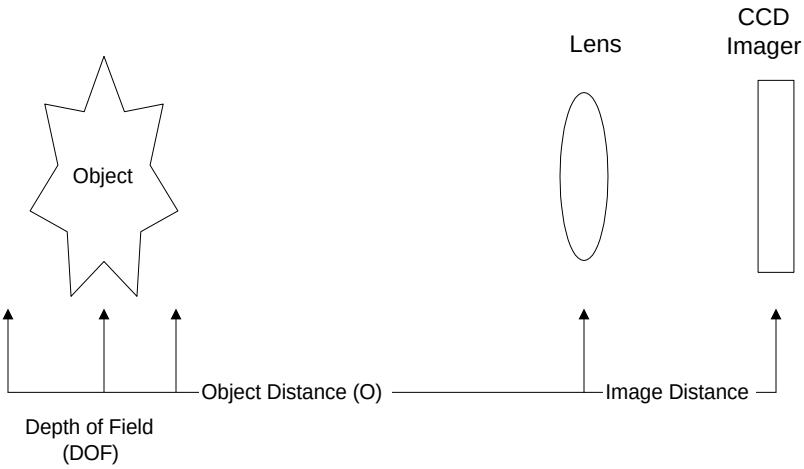


**Figure 6 - Schematic of camera optical system for calculation of depth of field.**

The imaging system for the daphnid counting system is thus seen to be easily attainable using inexpensive, off-the shelf components.

In order to capture the image from the video camera, a computer peripheral known as a frame grabber is required. This device digitizes the incoming video stream and converts the analog signal to a stored digital frame. The stored frame can then be displayed on the computer screen and manipulated. Because of the relatively low volume of production, frame grabbers tend to be expensive computer peripherals, starting at around $500 (2000).

Recently, self-contained, tethered color cameras have become available as computer peripherals. These units typical have 640 X 480 pixel resolution, and read directly to the attached computer along a dedicated bus like USB (Universal Serial Bus). The cameras are typically priced less than $100 (2000) and come complete with software. I investigated the use of one such camera, the Kodak DVC323, for use as a video microscope (see Figure 7). The camera is supplied with a 0.62-cm focal length lens that can focus to a distance of 13-cm. With the addition of an auxiliary close-up lens, the close focus distance drops to 3 - 4-cm, and we obtain a field of view and depth of field similar to the video microscope described above, in a simplified system (see Figure 8). The resolution has increased because of the increase in the number of pixels in this camera, to about 65 μm, so that even better imaging performance is obtained. The cost of the system has been reduced from $650 to under $100, without sacrificing imaging performance.

**Figure 7 - The Kodak DVC323 Tethered Video Camera**

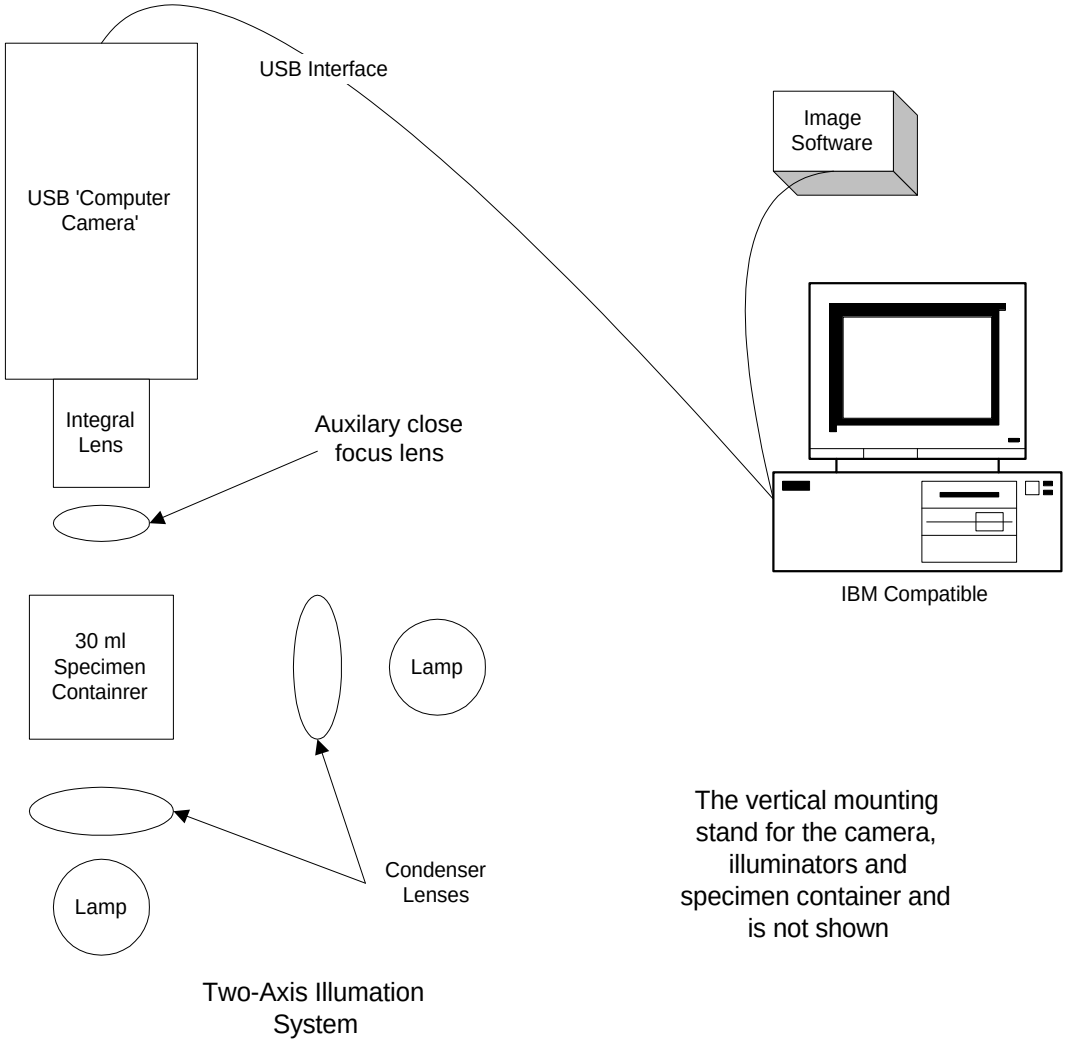


**Figure 8 - Adaptation of a tethered computer camera for use as a video microscope.**

### *Image Processing Software for Automated Analysis*

With the increase in the computing power of desktop computers, commercial image analysis software has become inexpensively available. These software packages allow image enhancement (to bring out hidden features in the image) and analysis (measurement and quantification) without extensive programming experience. Electronically captured images from the CCD camera can be processed numerically, enhancing the quality of the image or reducing the information content of the image to a few numbers[42]. Examples of commercially available image analysis software packages are *Matlab* (from Mathworks, Inc.) *IDL* (from Research Systems, Inc.) and *Mathcad* (from Mathsoft, Inc.). There are also several public domain analysis packages available, the most notable being *ImageTool* developed at the University of Texas, Austin.

The EPA WET toxicity tests require identification and counting of the living macro invertebrates at several different times during the test. The method described above ("Equipment to improve accuracy of observations of plankton") has been implemented using the software *Mathcad*, with the Image Processing toolbox. In the next section I will describe the results of tests with small objects (glass spheres) in a static test of the method.

### *Results of static target simulations*

A static target test was performed to simulate image acquisition, field subtraction, image analysis, and counting. Ten clear glass beads 600 - 1000 μm in diameter were placed at random into 15-ml of water in the 30-ml sample cup. A collimated microscope illuminator is used to illuminate the spheres from above. The beads have low contrast (like the macro invertebrates) but are larger than newborn *Ceriodaphnia dubia* and more regularly shaped than most macro-invertebrates. The test objects also sit in the same plane on the bottom the sample cup, which does not provide a fair test of the ability of the system to simultaneously focus objects at the top and bottom of the liquid layer in the sample cup. In spite of these shortcomings, this test was considered useful to develop software and imaging techniques under controlled conditions without the complications of using living specimens.

The results of these experiments are shown in Figure 9 through Figure 13. The first two frames show the initial capture and a second capture with 5 beads displaced from their original positions. This simulates the movement of living macro invertebrates. The third and fourth frames show the results of subtraction and addition of the first two frames, respectively. Notice that during the subtraction operation the stationary beads have been

effectively removed from the image, while the new positions of the 'living' beads show as 'negative'. The increase in noise in the subtracted frame results from pixel values in the background of two frames being nearly equal. Comparing Figure 12 with Figure 9 and Figure 10, we can see that five beads have moved from their original positions. Figure 13 shows the result of a 'threshold' operation followed by a 'scale' or 'contrast stretch'. The threshold operation sets pixels with a value less than a fixed amount (in this case 30) equal to zero, the remaining pixel values being unaffected. Thresholding is used here to remove the confusing and objectionable noise that was a result of the subtraction operation. The scale or contrast stretch operation expands the now noise-free data so that the features are easier to see. By using additional image processing operations like sharpening and edge detection on the 'raw' (first and second) frames even better comparative results are obtained. This additional image processing is helpful when the sample water is cloudy or stained and direct observation of the specimens is poor.

With the non-moving specimens removed from the final image, noise removed and contrast increased, the field may now be counted manually, or automatically using standard blob or cell recognition software. Since the processed frames are stored in the computer, they may be counted at leisure, and recounted if required. The stored frames also provide a record of the tests for permitting agencies.

I have included a listing of the easy-to-read *Mathcad* code used to process these images as Appendix A.

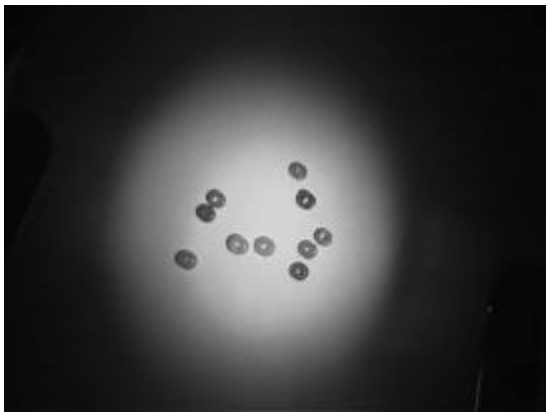

**Figure 9 - Initial frame capture.**

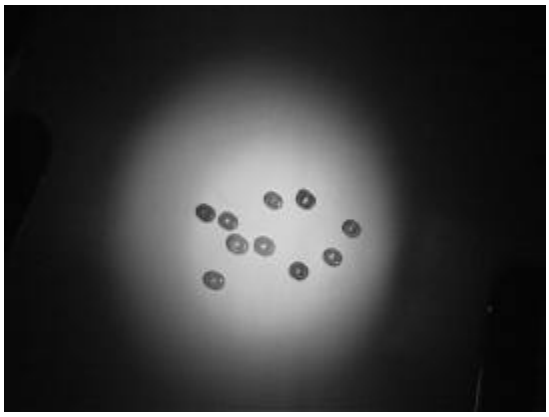

**Figure 10 - Second frame capture. 5 beads have moved relative to the first frame.**

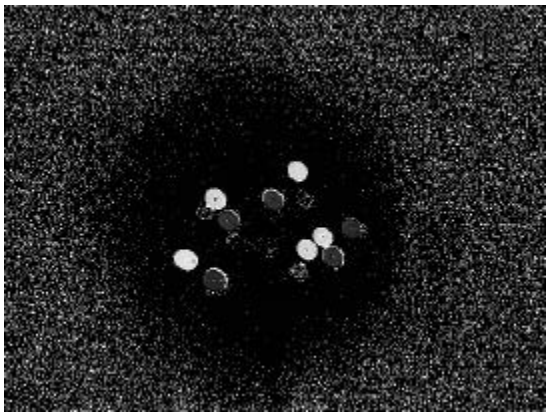

**Figure 11 - The frame resulting from subtraction of the two above images.**

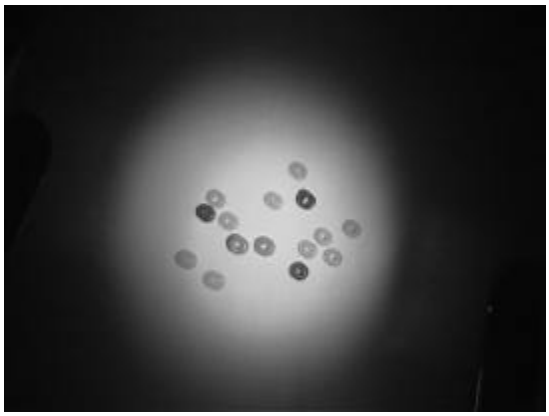

**Figure 12 - Result when adding the two frames above.**

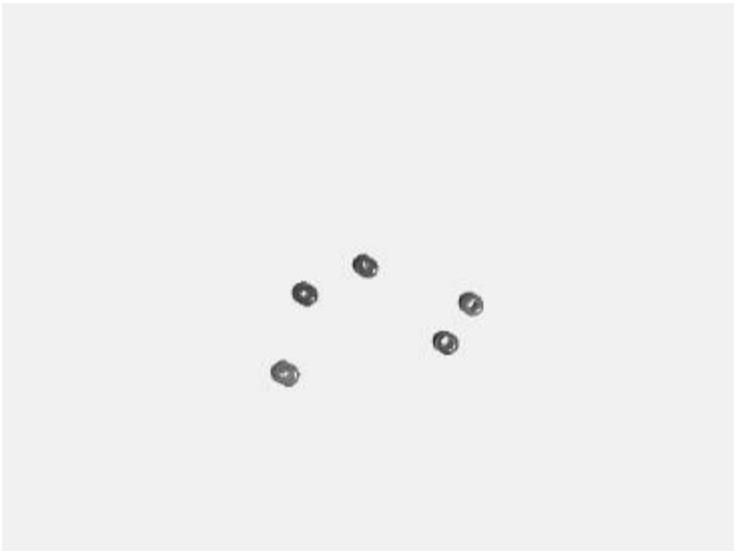

**Figure 13 - Thresholded and scaled image (fully processed). Notice that only the 5 beads that moved are visible in this processed image.**

### *Results with live macro-invertebrates vs. manual counting*

Having demonstrated the video microscope system, capture techniques and image processing algorithms with stationary objects, I proceeded to culture macro-invertebrates for use in a 'live' test. Some initial tests were performed with *Ceriodaphnia dubia* from cultures maintained at the Eastman Kodak Company's Environmental and Health Laboratory. When this supply became unavailable, I looked to culture animals locally for testing. Cultures of *Ceriodaphnia dubia* and other freshwater macro invertebrates were not generally available at low cost, so it was decided to use the commonly available *Artemia salina*, the brine shrimp, as a test specimen. *Artemia*, being a salt water organism not approved for NPDES permit tests, was used in this case only because of similar size, motility and ease of culture. *Artemia* is widely used as a fish food by aquarists.

Supplies of *Artemia* cysts are available at low cost from aquarium supply stores, and the animals are exceptionally easy to culture and maintain for a period of time long enough to be useful in our tests. I obtained cysts from San Francisco Bay Brand, Newark California.

From previous casual observation, it appeared that the speed of motion of *Ceriodaphnia dubia*, is similar to that of *Artemia* (0.1 - 0.2-cm/s). The nature of the motion of the two species, is quite different, however the *Artemia* moving in smooth continuous fashion propelled by its 11 pairs of thoracopods, the *Ceriodaphnia* moving with a powerful but jerky motion.

The size (long dimension) of a juvenile *Ceriodaphnia* is about 350 μm, whereas the newborn *Artemia* is slightly under 500μm. The *Ceriodaphnia* grows rapidly and reaches sexual maturity and a size of just under 0.1-mm in a few days when well fed, whereas the *Artemia* will reach 1.3-cm in about 3 weeks if nutritional conditions are optimal[43].

The procedures outlined in Appendix A[44] of the EPA Acute toxicity test and in the instructions provided by supplier of the cysts were followed when culturing the *Artemia*. A rearing container was made from an inverted 2-liter plastic drink bottle with the bottom removed and the top cap drilled to receive a plastic tube which had been glued in place. An aquarium aerator pump was attached to an 'air stone' that was placed in the bottom of the provided mixing and aeration. The container was kept warm (~27$^{o}$ C) by direct heat from a 60 watt incandescent light bulb (see Figure 14). The culture media was prepared by dissolving 50-g of non-iodized table salt in 1.8-liters of water and agitating until dissolved. The nauplii (as newly hatched, yolk-feeding *Artemia* are called) hatched in about 24 hours at 25$^{o}$ C and

generally remained viable for 48-72 hours without feeding, during which time the organisms increased in size from 400-μm to 500 - 600-μm. Organisms for testing were drained from the tube at the bottom of the rearing container.

One set of results from these tests is shown in . The organisms were individually transferred from a Petri dish into clear, 20-ml 12-well sample plate by pipette to insure an accurate count. The well plate (Costar polycarbonate well plate) was used to contain the specimens because of the exceptional clarity and uniformity of the plastic from which it is cast. The cup was counted with the unaided eye several times to insure that all specimens were transferred successfully.

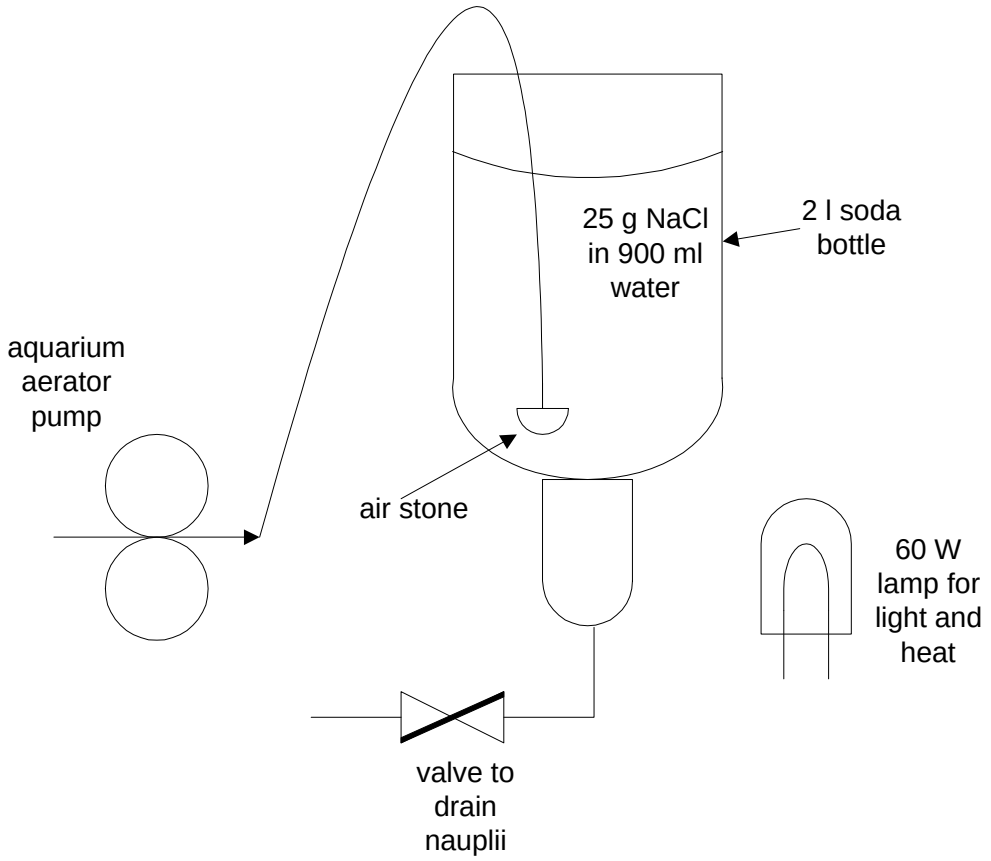


**Figure 14 - Hatching and rearing setup for Artemia salina.**

## Appendix A - EPA Acute Test Protocol using *Ceriodaphnia dubia*

## Appendix B - EPA Chronic Test Protocol (Survival and Reproduction) using *Ceriodaphnia dubia*

# Appendix C - Mathcad Source Code for Image Processing and Evaluation

Read and display raw data
a := READBMP( "first.bmp" )
View the image:
VIEW( "First" ) := a
b := READBMP( "second.bmp" )
---

Subtract second frame from first and normalize

$$c := \frac{(a - b)}{2}$$

WRITE_BMP( "diff.bmp" ) := c

View the differenced frame:
VIEW( "Diff" ) := c

---

$$g := \frac{(a + b)}{2}$$

Also get the sum (normalized to 8 bit pixels)
VIEW( "Sum" ) := g
WRITE_BMP( "sum.bmp" ) := g

---

Set a threshold of 4 (discards all pixel values < 3 to eliminate 'bit' noise)
Then scale this image to stretch pixel values from 10 to 180 (contrast stretch)
The threshold was determined experimentally by visually observing the processed image for shadow and other artifacts.

d := invert( scale( threshold ( c , 30) , 15, 250) )
VIEW( "Fixed" ) := d

Write the frame for later use:
WRITE_BMP( "fixed.bmp" ) := d

## Glossary

Bioaccumulation - increase in concentration of a substance in tissue of a specimen, compared to the concentration in the environment.

CCD Sensor - Charge Coupled Device sensor, in which charge produced by incident light is swept out of a segmented array of active sites row by row, and column by column in a 'bucket-brigade' fashion. Found in digital cameras, video camcorders, surveillance cameras, etc.

CWA - Clean Water Act, 33 USC 1251 et seq. (1977) The Clean Water Act of 1977, an amendment to the Water Pollution Control Act of 1972.

Depth of Field (DOF) - The range of distances in the object plane that are in focus in the image (detector) plane. The DOF increases with smaller lens openings (larger f/numbers) and shorter lens focal lengths.

Dissection microscope - A low power, wide field microscope, usually binocular, to aid with routine dissections, metallurgy, mechanical parts inspection and metrology. Typically, this instrument has a magnification of 10-60, field of view (FOV) of 0.8 - 1-cm and depth of focus (DOF) of less than 0.1-cm.

Dose-Response Curve - A curve generated by plotting a biological effect (e.g. death, brood size, loss of reproductive ability, etc.) versus the dose of a toxicant.

EC50 - The concentration that has an effect on 50% of the organisms tested. The effect is usually not death, but some other well defined end-point.

EPA - Environmental Protection Agency

Field of View (FOV) - At a fixed distance from the imaging lens, the size of the area in the object plane that is imaged in the image plane

IC50 - Inhibitory response that reduces the normal response of an organism by 50%. Often used for growth rates of algae, bacteria or other similar organisms.

Image plane - The imaginary plane at which a lens system forms an image of the object. Typically, a detector (eye, photographic film or electronic image sensor) is placed in the image plane.

LC50 - The concentration that causes mortality in 50% of the organisms tested

MATC- Maximum allowable toxicant concentration

NOAEC- No Observed Adverse Effect Concentration

NPDES -National Pollution Discharge Elimination System.

Object plane - The plane on the other side of the lens from the image plane.

Setae - bristles

TSCA - Toxic Substances Control Act CFR 15 2601 et. seq.

YCT - The recommended feed for *Ceriodaphnia dubia* and other cladocerans, consisting of yeast (Y) dried, powdered cereal leaves (C), digested trout chow (T).

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