

Ecotoxicology of chemicals using COPAS XL to analyze and sort *Daphnia magna* in an 'Acute Immobilization Test'

Objective

The purpose of this experiment was to test the feasibility of using the COPAS™ XL instrument to analyze and dispense *Daphnia magna* for the Acute Immobilization Test (OECD Guideline for Testing of Chemicals, (1984))¹. The experiment was performed in parallel using the standard procedure to determine if the COPAS XL affected the viability or ecotoxicology test results of a standard reference chemical.

Introduction

The COPAS family of research instrumentation comprises a collection of large particle cytometers utilizing at least 1 excitation laser and up to 8 channels of fluorescence collection. Unique to COPAS instrumentation is the Profiling feature which graphically plots the fluorescence intensity changes along the length of the object as it passes through the laser(s). Large objects up to 1.5mm in diameter can be analyzed for physical and fluorescence characteristics and gently dispensed into a multi-well plate or other collection container for further investigation or reuse. The COPAS Vision also has equipped a camera to take an image of the object inside the flow channel. This image accompanies the cytometry data and can be analyzed using Union Biometrica's FlowPilot software or other image analysis tools.

In this experiment the COPAS XL utilizing a 2000 micron flow channel was used to analyze and sort large objects (500-1600 microns) on the basis of the physical characteristics of size, density, and fluorescence signals. Objects are passed axially, one by one, through the focus of a laser beam. Relative size is determined by the time of flight (TOF) measurement. The optical density of the object is determined by the extinction (EXT) measurement. Objects can be dispensed into microtiter plates or stationary receptacles. Using a unique, pneumatic sorting mechanism, the COPAS is gentle enough to sort and dispense live organisms without affecting the viability. Applications for the XL include work with beads, seeds, Zebrafish embryos and early larvae, *Xenopus* blastula, third instar *Drosophila* larvae, and other like-sized model organisms, and large cells and cellular clusters. In this experiment we used the COPAS XL instrument to dispense *Daphnia magna* into wells of 6-well plates under standard conditions.

There are a number of standard tests prescribed by international organizations such as OECD, ISO and EN for evaluating both environmental and pure substances to determine whether they are harmful. In many countries, environmental safety studies are required by companies for the successful registration of their manufactured products. Ecotoxicology studies include investigations related to the effect of new active substances on the environment, and evaluation of their impact on the relative number and/or the biomass of elements of the ecosystem. One such standard test is the *Daphnia sp.*, Acute Immobilization Test. In the acute immobilization test, a specified number of healthy *Daphnia*, less than 24 hours old, are challenged with a range of concentrations of the investigated substance and then evaluated at a later time point. This is a broadly used test with many advantages over other similar standard tests (speed and cost).

One area of improvement for the *Daphnia sp.*, Acute Immobilization Test would be automation, to allow an increase in throughput. To this end, we have tested the COPAS XL for dispensing *Daphnia*. We show that the COPAS XL can provide an automated step for the setup of the *Daphnia sp.*, Acute Immobilization Test.

Materials and Methods

Test of viability:

As a test of the effect of the sorting and dispensing by the COPAS instrument on the viability of the *Daphnia*, the organisms were passed through the instrument and dispensed into wells of a 6-well plate. These wells contained 2 ml of culture water. In parallel, similar numbers of *Daphnia* were manually transferred to wells of a 6-well plate. The dispensed *Daphnia* were inspected immediately after sorting and again after 24 hours to confirm that the numbers of dispensed organisms were correct and that viability was not affected.

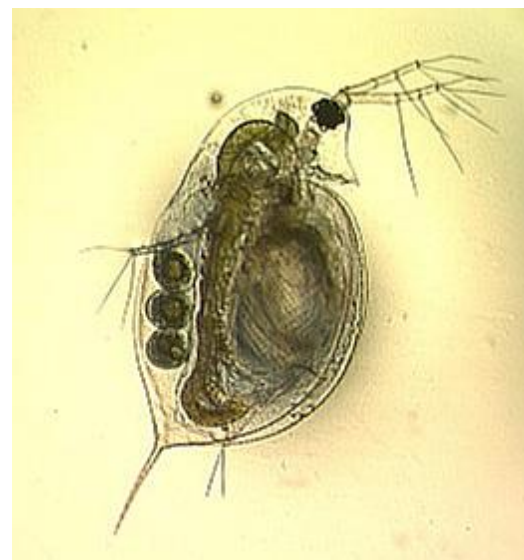


Figure 1. Brightfield image of *Daphnia magna*.
(courtesy of M. Vanpoucke)

Daphnia sp., Acute Immobilization Test:

The *Daphnia sp.*, Acute Immobilization Test was performed following the standard accepted procedure. Laboratory bred *Daphnia magna* that were less than 24 hours old at the beginning of the test were used. We used potassium bichromate as the reference compound for these tests. Potassium bichromate was tested at five different concentrations. The Acute Immobilization Test was set up in parallel. One set of tests used the COPAS XL to dispense the *Daphnia* to wells and the other set relied on manual dispensing of *Daphnia*. The *Daphnia* set up in these parallel tests were subsequently observed for effects on their swimming and movement capability. Effects of the reference compound, potassium bichromate, on these endpoints are compared with *Daphnia* tested in the absence of the test compound as a control. For the ecotoxicology test, each concentration of potassium bichromate and the no compound control is tested with 20 animals, divided into four groups of five animals. A volume of 2 ml of test solution at the different concentrations was provided for each animal during the test. The test temperature was 22°C, and held constant within $\pm 1^\circ\text{C}$. The effect of the compound can be expressed in a number of different ways. One of these is the Spearman-Kärber value (24h EC 50)², the concentration estimated to immobilize 50 percent of the *Daphnia* after 24 hours of exposure. Immobilization is defined in this test as: those animals not able to swim within 15 seconds after gentle agitation of the test container.

Results

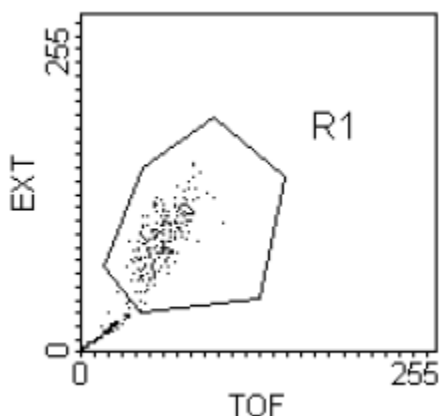


Figure 2. Dot plot of TOF vs EXT showing the region selected for sorting and dispensing *Daphnia* to wells for the Acute Immobilization Test.

In the test for viability, *Daphnia* were analyzed and sorted using TOF (size) and EXT (density) parameters (Figure 2, region labeled R1). *Daphnia* were dispensed five per well to three 6-well plates using the COPAS™ XL. Visual inspection with a binocular microscope verified that the correct numbers of *Daphnia* were dispensed to each well. The viability and motility after sorting was determined to be 100%, indicating that the XL instrument has no immediate effect on the condition of the organisms. Reanalysis after 24 hours showed 7 dead and 1 immobilized organism in the total population sorted. These results are similar to what occurs for manually transferred organisms.

The Acute Immobilization Test was performed with the reference compound potassium bichromate. *Daphnia magna* were dispensed using the COPAS™ XL to the control wells containing 2 ml of culture media and the test wells containing the five increasing concentrations of potassium bichromate in culture media. The results are presented below. The accuracy of dispensing and effect on motility were determined by observation using a binocular microscope.

Ecotoxicology test with potassium bichromate.

The Spearman-Kärber (24h EC 50) value was 1.18 and is in good agreement with the results obtained for potassium bichromate determined manually.

The normal value for this test determined manually for this compound is a range from 0.9 – 1.6. The range for our test was 1.05 to 1.34. This difference is considered acceptable and within the normal range of variation.

<u>Conc. of cmpd.</u>	<u># mobile</u>	<u>% immobile</u>
no compound	20 mobile	0%
0.62 mg/l	20 mobile	0%
0.80 mg/l	15 mobile	25%
1.00 mg/l	15 mobile	25%
1.30 mg/l	8 mobile	60%
1.70 mg/l	3 mobile	85%

Conclusions

This experiment demonstrates the use of the COPAS™ XL for dispensing of *Daphnia magna* for the Acute Mobilization Test. The COPAS™ XL does not affect the viability of *Daphnia magna* when it is used to dispense these organisms to multi-well plates while bringing automation to the test. Furthermore, results from the Acute Mobilization Test using a reference compound do not differ significantly when *Daphnia magna* are dispensed using the COPAS™ XL when compare with manual dispensing.

Work performed in 2004, QTN reissued 2024:

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References:

1. OECD Guidelines for testing of chemicals (1984).
2. Hamilton, M.A., R.C. Russo, and R.V. Thurston "Trimmed Spearman-Kärber Method for Estimating Media Lethal Concentrations in Toxicity Bioassays," Environ. Sci. Technol., 1977, 11(7), pp. 714-719; Correction:1978, 12(4), pp. 417