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Suitability of High-Hardness COMBO Medium for Ecotoxicity Testing Using Algae, Daphnids, and Fish

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A variety of factors can influence the results in ecotoxicity testing using freshwater organisms. These factors include biological variability inherent between individuals as well as physical conditions such as the source and quality of dilution water. Examples of dilution water used in culturing and testing aquatic species are diluted mineral water, reconstituted synthetic water, ground water, surface water, or dechlorinated tap water. All of these waters contribute to the variability of testing results. For example, intra- and inter-laboratory precision values ranging from 12 to 167% (expressed as coefficient of variation) have been observed (USEPA 1991).

Many agencies are recommending the use of a fully defined medium in order to improve standardization between laboratories. However, most synthetic media have been found to be inadequate to meet animal requirements (Keating et al. 1989), while other media such as Elendt M7 (Elendt and Bias 1990) and High-Hardness (H-H) COMBO (Baer and Goulden 1998) have been reported to provide acceptable results in the culturing and testing of *Daphnia magna*. COMBO, a low hardness medium, was initially developed for the culturing of both algae and zooplankton, specifically *D. pulicaria,* a soft water species (S. Kilham and C. Goulden, personal communication). The COMBO medium was modified by increasing the total hardness and was found to be suitable for long-term culturing of *D. magna,* a hard water species (Baer and Goulden 1998). This medium is also closer in total hardness to natural water found in aquatic ecosystems as compared to the Elendt medium (~112 mg/L as $CaCO_3$ for H-H COMBO and ~250 mg/L as $CaCO_3$ for Elendt medium). However, none of these media have been employed in bioassay testing with species such as algae and fish. In addition, the suitability of synthetic medium in the toxicity evaluation of mixtures and effluents containing metals is in question (Guilhermino et al. 1997).

The purpose of the present study was to expand the use of H-H COMBO medium in ecotoxicity testing to include fathead minnows (*Pimephales promelas*) and green algae (*Selenastrum capricornutum* and *Ankistrodesmus falcatus*). In addition, the medium was evaluated for testing heavy metals such as cadmium in the presence and absence of a complexing agent, EDTA. Results are compared to reconstituted water currently recommended by regulatory agencies.

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MATERIALS AND METHODS

D. magna Straus cultures (clone 5 obtained from Academy of Natural Sciences of Philadelphia) were individually held in 150 mL glass beakers containing 100 mL, of H-H COMBO medium. The medium was prepared using analytical grade chemicals and Barnstead® Nanopure ultrapure water (Table 1; Baer and Goulden 1998). Medium was stored in the dark at 4°C and warmed in ambient room temperature with vigorous aeration approximately 12 hr prior to use. Cultures were fed live green algae, *A. falcatus,* at a concentration of 75,000 to 100,000 cells/ml three times weekly. All culturing and testing were performed in a constant temperature bath at 20 ± 2°C with a 16:8-hr light:dark photoperiod. Fathead minnows (*P. promelas*) juvenile and embryos/larvae were purchased from Aquatic Biosystems, Inc., Fort Collins, CO and acclimated in dechlorinated tap water for ~14 days or used immediately. Algal cultures were grown in semi-continuous cultures of H-H COMBO or aerated static cultures in ASM medium. Cells were harvested and culture solution replenished to maintain cells in log growth phase.

Cadmium chloride and potassium chloride were purchased from Fisher Scientific. All test solutions were prepared in H-H COMBO and used immediately in toxicity tests. Cadmium was measured at selected time periods using an air/acetylene-flame atomic absorption Spectrophotometer (Perkin Elmer, Model 3030B). The detection limit for this method is approximately 0.050 mg/L. Recoveries of cadmium in test solutions above the detection limit ranged from 85 to 106%.

All ecotoxicity tests were performed using standard U.S. EPA guidelines. Tests were performed at Northeast Louisiana University and SmithKline Beecham, Environmental Research Laboratory. In the daphnids tests, randomly selected neonates from laboratory cultures (<24 hr old, ≥ third brood, 5 daphnids/replicate, 2 or 4 replicates/concentration) were used. In the fathead minnow 48- and 96-hr tests, 5 fish per replicate, 2 replicates/concentration were used. Solutions were not renewed and animals were not fed during all daphnid and fathead minnow acute tests. In the fathead minnow 7-d larvae study, 3 replicates containing 10 larvae each (<24 hr old) were used. Solutions were renewed daily and larvae were fed live brine shrimp. In all studies, dissolved oxygen, pH, temperature, total alkalinity, total hardness and conductivity were measured at the beginning and end of the study, or daily for the 7-d larvae study.

Algal tests were conducted in 250 mL flasks containing 100 mL of medium. Illumination was ~4,000 lux and all solutions were continuously shaken at 100 rpm. Flasks were inoculated with 10,000 cells/ml to start the study. Temperature was maintained at 24 ± 2°C. *S. capricornutum* and *A. falcatus* were utilized in screening studies and NOECs were estimated.

The LC_{50} or EC_{50}s were determined by either EPA statistics program or GraphPad Prism statistics program using the following equation;

Table 1. Chemical composition of H-H COMBO Medium[a].

Constituents	Final concentrations (mg/L)
Major Stocks	
$CaCl_2 2H_2O$	110.28
$MgSO_4$	55.45
K_2HPO_4	1.742
$NaNO_3$	17.0
$NaHCO_3$	126.0
$Na_2SiO_3 9H_2O$	28.42
H_3BO_3	24.0
KCl	5.96
Animal Trace Elements (ANIMATE)	
LiCl	0.31
RbCl	0.07
$SrCl_2 6H_2O$	0.15
NaBr	0.016
KI	0.0033
Algal Trace Elements (ATE)	
$Na_2EDTA 2H_2O$	4.36
$FeCl_3 6H_2O$	1.0
$MnCl_2 4H_2O$	0.18
$CuSO_4 5H_2O$	0.001
$ZnSO_4 7H_2O$	0.022
$CoCl_2 6H_2O$	0.010
$NaMoO_4 2H_2O$	0.022
H_2SeO_3	0.0016
Na_3VO_4	0.0018
Vitamins	
B_{12} (Cyanocobalamine)	0.00055
Biotin (d-Biotin)	0.0005
Thiamin (HCl)	0.1

[a]Baer and Goulden (1998).

$(Y=((0-100)/(1+(x/LC_{50})^b))+100$. NOECs were determined by one-way analysis of variance (ANOVA) followed by Duncan's or Dunnett's multiple comparison tests.

RESULTS AND DISCUSSION

All water quality parameters were within acceptable limits (Table 2). Total hardness of the H-H COMBO medium ranged from 94 to 124 mg/L as $CaCO_3$ and total alkalinity ranged from 40 to 68 mg/L as $CaCO_3$. A sumrnary of toxicity tests employed in this study is presented in Table 3. The daphnid 48-hr EC_{50} values for

Table 2. Summary of water quality characteristics.

	Water quality measurement					
	Temperature (°C)	D.O. (mg/L)	pH	Hardness[a]	Alkalinity[a]	Conductivity (µmhos)
Daphnia magna						
Mean[b]	22.2	8.5	7.7	118	57	424
SD	1.3	0.5	0.14	4	4	26
Range	19.1-24.0	7.4-9.3	7.2-8.2	94-123	40-63	382-450
Pimephales promelas						
Mean[b]	24.9	5.2	7.2	118	65	435
SD	0.09	0.3	0.03	6	4	15
Range	20.3-25.1	4.6-8.5	7.2-8.0	96-124	48-68	400-450

[a]Total hardness or alkalinity (mg/L as $CaCO_3$). [b]Mean values (± SD) measured weekly in selected replicates (Northeast Louisiana University only).

cadmium in the presence of EDTA ranged from 0.656 to 1.11 mg/L. The laboratory precision based on the coefficient of variation was 18% (mean and standard deviation were 0.856 and 0.154 mg/L, respectively). A control chart showing cumulative means and upper and lower limits is presented in Figure 1. The toxicity of cadmium in the absence of EDTA was approximately 13 times higher than in medium containing EDTA. The toxicity of potassium chloride in medium containing EDTA ranged from 728 to 1149 mg/L (the coefficient of variation of was also 18%).

The influence of EDTA on metal toxicity is not surprising. Other investigators have reported significant differences in metal toxicity between media with and without EDTA (OECD 1995; Sorvari and Sillanpää 1996). Guilhermino *et al.* (1997) evaluated Elendt M7, ASTM and EEC reconstituted media and observed that the chelator EDTA (in Elendt M7 only) reduced the toxicity of some metals to *D. magna.* Acute testing results using waters from different sources are extremely variable. For example, 48-hr EC50s for daphnids following cadmium exposure are as follows: 0.332 mg/L in Elendt M7 (Guilhermino *et al.* 1997); 0.0095 and 0.0098 mg/L in ASTM and EEC reconstituted media, respectively (Guilhermino *et al.* 1997); 0.038 mg/L in hard reconstituted water (180 to 200 mg/L as $CaCO_3$) (USEPA 1991); 0.058 mg/L in moderately hard tap water (130 mg/L as $CaCO_3$) (Attar and Maly 1982); 1.88 mg/L in hard well water (240 mg/L as $CaCO_3$) (Khangarot and Ray 1987); 0.166 mg/L in St. Louis River water (hardness 55-79 mg/L as $CaCO_3$) (Spehar and Carlson 1984). Several of these dilution waters were undefined in terms of the presence or absence of chelators.

Table 3. Summary of reference toxicant tests with H-H COMBO Medium.

Test	LC_{50}[a] (mg/L)	95% Confidence Limits (lower - upper, mg/L)	NOEC (mg/L)
Cadmium			
Daphnia magna (48-hr)	0.734	0.658-811	0.633
Daphnia magna (48-hr)	0.918	0.811-1.040	0.560
Daphnia magna (48-hr)	0.748	--	0.560
Daphnia magna (48-hr)	0.964	--	0.686
Daphnia magna (48-hr)	1.11	1.057-1.160	0.750
Daphnia magna (48-hr)	0.656	0.518-0.794	0.481
Daphnia magna (48-hr)	0.872	0.816-0.929	0.450
Daphnia magna (48-hr) No EDTA	0.066	0.061-0.070	0.039
Daphnia magna (48-hr) No EDTA	0.069	0.035-0.104	0.050
Pimephales promelas (96-hr; 30-d old)	6.64	6.15-7.13	3.05
Pimephales promelas (96-hr; 3-d old)	0.86	0.301-1.43	0.109
Pimephales promelas (7-d; embryo/larvae)	0.40	0.323-0.479	0.041
Pimephales promelas (7-d; embryo/larvae)	0.35	0.167-0.540	0.020
Selenastrum capricornutum			0.2
Selenastrum capricornutum No EDTA			0.005
Ankistrodesmus falcatus			1.0
Ankistrodesmus falcatus No EDTA			0.01
Potassium chloride			
Daphnia magna (48-hr)	728	634-836	500
Daphnia magna (48-hr)	933	753-1157	500
Daphnia magna (48-hr)	1149	997-1324	500
Daphnia magna (48-hr)	980	882-1089	667
Pimephales promelas (48-hr; 1-3 d old larvae)	841	735-962	500

[a]For daphnids LC_{50} is synonymous with EC_{50} (immobility).

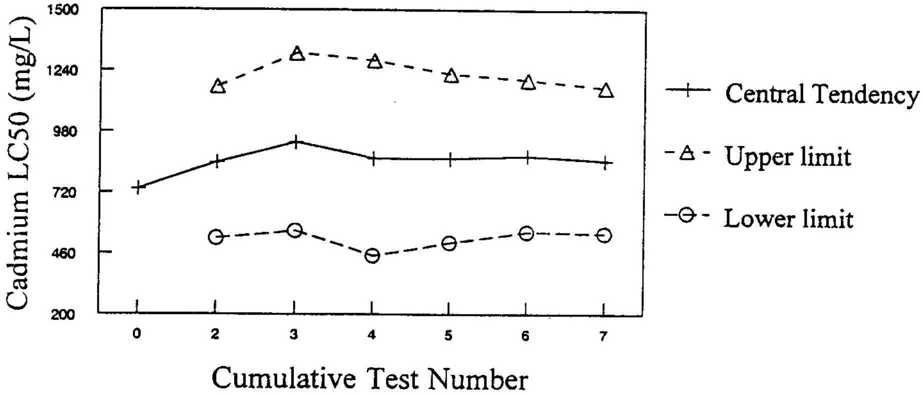

Figure 1. Control chart for 48-hr daphnid tests using cadmium chloride.

These results indicate that using medium containing chelating compounds may be a cause for concern when testing mixtures or effluents containing heavy metals. Employing medium free of chelators may be desirable for improved inter-laboratory repeatability of test results with metal containing substances. However, the presence of EDTA or other chelating compounds appears to be an important requirement for long-term maintenance of daphnid and algal cultures. EDTA is used to improve solubility of trace elements required for daphnids and algae. There may be an advantage in using a fully defined medium with a known EDTA concentration compared to using an undefined medium. A correction factor could be applied to the test results if the complexing capacity of a fully defined medium is known. However, removing EDTA from H-H COMBO medium appears to be acceptable for the short-term testing metals based on acceptable control survival.

Fathead minnow 7-d larvae NOECs (survival and growth) using cadmium were 0.40 and 0.35 mg/L and the 96-hr static, acute LC_{50}s were 6.64 (30-d old) and 0.86 mg/L (3-d old). In the 7-d study, fathead minnow larvae control growth exceeded the acceptability criteria of 0.25 mg average dry weight (USEPA 1994). The 48-hr LC_{50} using potassium chloride was 841 mg/L. Results using fathead minnows conducted in H-H COMBO are in agreement with published values. Fathead minnow 96-hr LC_{50}s using KC1 ranged from 944 to 832 mg/L (USEPA 1991). Values for cadmium ranged from 0.15 mg/L to 1.05 mg/L in soft water (20 to 48 mg/L as $CaCO_3$)(USEPA 1991; Pickering and Henderson 1966) and 3390 mg/L in water with a hardness of 55 to 79 mg/L as $CaCO_3$ (Spehar and Carlson 1984).

Cadmium screening studies using *S. capricornutum* in medium with and without EDTA resulted in NOECs of 0.2 and 0.005 mg/L, respectively. Estimated NOECs for *A. falcatus* in medium with and without EDTA were 1.0 and 0.01 mg/L, respectively. The growth in the controls after 96 hrs met the EPA criteria of 1,000,000 cells/mL and 200,000 cells/mL for medium with and without EDTA, respectively. For example, 96-hr control *growth* for S. *capricornutum* in H-H COMBO containing EDTA averaged 3,200,000 ± 403,000 cells/ml (coefficient of variation of 12.5%). Other investigators have reported LOEC values of 0.05 mg/L for several species of algae (Hörnström 1990).

Results from the present study indicate that H-H COMBO medium can be employed in the long-term culturing of algae and macroinvertebrates as well as in ecotoxicity testing using three important, representative species; *Daphnia magna*, fathead minnows (*Pimephales promelas*) and green algae (*Selenastrum capricornutum* and *Ankistrodesmus falcatus*). In addition, this medium may be modified by removing EDTA and employed in testing the acute toxicity of mixtures or effluents containing heavy metals.

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