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American Petroleum Institute

STEP Strategies for Today's Environmental Partnership

Reducing Uncertainty in Laboratory Sediment Toxicity Tests

Health and Environmental Sciences Department
Publication Number 4632
September 1995

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Strategies for Today's Environmental Partnership

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Reducing Uncertainty in Laboratory Sediment Toxicity Tests

Health and Environmental Sciences Department

API PUBLICATION NUMBER 4632

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ABSTRACT

Many contaminants entering aquatic systems may accumulate in sufficient quantities in sediments to adversely impact benthic organisms. Laboratory sediment toxicity tests may be the most accurate means of determining whether or not these sediments are toxic to benthic communities. However, concerns have been expressed regarding the ability of existing laboratory sediment toxicity testing methods to accurately assess sediment toxicity. This report presents methods for improving laboratory sediment toxicity tests. A formulated reference sediment was developed that is suitable for survival, growth, and reproduction of commonly used sediment testing species. Copper sulfate was evaluated and found to be a suitable reference toxicant for sediment toxicity tests. Tolerances of *Hyalella azteca* and *Chironomus tentans* were determined for various particle size classes and organic matter content of sediments. This information can be used to reduce the likelihood of erroneously concluding that a sediment is toxic simply because the sediment is incompatible with test organisms. An evaluation of the relative sensitivities of commonly used sediment testing organisms and sublethal endpoints using a copper-contaminated sediment indicated that test duration, species selection, and test endpoint can influence test results. These data provide a means for increasing the accuracy of sediment toxicity test results.

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EXECUTIVE SUMMARY

Many consolidated sediments of aquatic systems near urban or industrial areas contain elevated concentrations of materials such as metals, organics, and pesticides that could adversely affect organisms associated with bottom sediments. Historically, most of the attention on materials in aquatic systems has focused on limnetic organisms. However, sediments are an important habitat for benthic organisms that may spend nearly their entire life cycle in intimate contact with bottom sediments and have important roles in aquatic systems where they occur. Clearly, sediments are vital components of aquatic environments, providing habitat, feeding and breeding areas for many aquatic organisms. Thus, it would be useful to be able to accurately assess the toxicity of contaminants in sediments.

Currently, there are a number of sources of information that may provide presumptive evidence of sediment toxicity and bioavailability. These include: 1) a list of contaminants in sediments with their associated concentrations, 2) survey of the total number of benthic organisms and species present, and 3) laboratory experiments. However, there are uncertainties and ambiguities associated with each of these sources of information. The presence of a material or contaminant in sediment does not definitively indicate that the sediment is toxic. Presence or absence of benthic species inhabiting a particular site is only presumptive evidence of sediment potency because such studies do not take into account physical characteristics of sediments or environmental perturbations that may render the sediment inhospitable to benthic species. Therefore, the use of laboratory experiments to assess the potency of sediments may provide the most direct evidence of sediment quality. Unfortunately, current approaches for conducting whole sediment experiments for predicting potency or bioavailability of materials in sediments have weaknesses that limit their ability to accurately assess sediment quality.

Since the potential for contaminated sediments to serve as sources or sinks for contaminants has not been sufficiently evaluated, and sediments serve as vital habitat for many organisms in aquatic systems, this research focused on evaluating critical components of laboratory experiments that may contribute to more accurate assessments of sediment quality.

The concerns and uncertainties related to laboratory experiments and sediments led to the experiments described in this report. The intent of these experiments was to help clarify questions regarding the accuracy of laboratory experiments and the ability of these experiments to predict adverse effects of sediment-associated contaminants. These results should help provide increased accuracy for laboratory experiments in predicting adverse effects of sediment-associated contaminants.

Formulated sediment was prepared to match representative natural sediments with respect to various sediment characteristics and provided a suitable substrate for toxicity testing organisms during life cycle exposures. Constituents used to prepare formulated sediment were commercially available, thus permitting widespread use of this sediment as a standard reference sediment.

In definitive sediment toxicity tests, formulated sediment, when used to dilute a copper contaminated sediment, provided predictable exposure-response curves for all organisms at all test durations. Formulated sediment provided a suitable reference and control sediment for the copper contaminated sediment, thus providing a means of accurately assessing sediment potency.

Formulated sediment was also used to successfully determine *Hyalella azteca* and *Chironomus tentans* tolerances of particle size regimes and organic matter content of sediments. *H. azteca* and *C. tentans* were tolerant of a wide range of particle size regimes and organic matter content of bottom sediments, thus making them suitable test organisms to assess sediment quality in most situations. Formulated sediments should be suitable for evaluating tolerances of other benthic toxicity testing organisms for sediment characteristics, thus reducing somewhat the risk of drawing erroneous conclusions regarding sediment toxicity.

These experiments and development efforts have provided clear evidence of the tremendous utility and potential for formulated sediments in advancing sediment quality evaluations.

Results from water-only and sediment copper toxicity experiments with test durations ranging from 48 hours to 14 days indicated differences in survival between toxicity testing organisms, with organisms listed in order of decreased sensitivity to copper: *Ceriodaphnia* *dubia*, *Daphnia magna*, *Pimephales promelas*, *H. azteca*, and *C. tentans*. A threshold was observed for *C. dubia* and *D. magna* survival after 96 hours of exposure to aqueous and sediment-bound copper. For *H. azteca*, *C. tentans*, and *P. promelas*, exposure durations of at least 10 - 14 days may be required for these organisms to correctly assess copper toxicity in whole sediment and water-only exposures.

Examinations of sublethal endpoints indicated that 14 days of exposure were required to elicit a reduction in *C. dubia* reproduction, which was not observed after 7 and 10 days of exposure. Growth was not a sensitive endpoint for *P. promelas* when exposed to a copper contaminated sediment in 10 and 14 day exposures. *C. tentans* growth was more sensitive than survival during a 10 day exposure. *C. tentans* growth was considerably more sensitive than survival when exposed to copper contaminated sediment. Results from this study with a copper contaminated sediment indicated that test duration was critical in eliciting sublethal endpoints for *C. dubia* and *C. tentans*. These results helped clarify some sources of uncertainty regarding test duration and endpoints for these test organisms exposed to copper.

This study provided evidence that *C. dubia*, *D. magna*, *H. azteca*, *C. tentans*, and *P. promelas* are suitable as toxicity testing organisms in assessing sediment copper toxicity. Although *H. azteca* and *C. tentans* were generally less sensitive to copper in sediment than *C. dubia* and *D. magna*, they may be more sensitive to other sediment-sorbed contaminants. Another consideration is the degree in which an organism is associated with sediment. For example, daphnids are generally considered water column organisms, although *D. magna* graze on sediment surfaces. *H. azteca* and *C. tentans* are both considered benthic organisms, although their association with sediment differs. *H. azteca* is an epibenthic detritavore, feeding on organic material on the sediment surface and on rooted aquatic vegetation, thus is in contact with sediment and the overlying water. *C. tentans*, however, is a benthic infaunal organism, which during its larval stage, constructs a case from organic material in sediment and thus is in intimate contact with sediments. By using several organisms that represent a variety of niches, the likelihood of making an erroneous conclusion regarding sediment toxicity can be reduced.

INTRODUCTION

The use of laboratory experiments to assess the potency of sediments may provide the most direct evidence of sediment quality. Unfortunately, current approaches for conducting whole sediment experiments for predicting potency or bioavailability of materials in sediments have weaknesses that limit their ability to accurately assess sediment quality. To address these uncertainties, a series of experiments were performed: 1) development and evaluation of a formulated reference sediment; 2) examination of the tolerances of commonly used freshwater sediment testing species to sediment characteristics; 3) evaluation of copper sulfate as a sediment reference toxicant; and 4) determination of the relative sensitivities of freshwater testing organisms to a copper contaminated sediment and an evaluation of potential sublethal endpoints that can be used to evaluate sediment potency.

To develop and evaluate formulated reference sediments for use in definitive sediment toxicity testing, the rationale behind developing formulated reference sediment was reviewed and potential formulated sediment constituents and their physical and chemical properties examined. The formulated reference sediment evaluation included: 1) matching formulated reference sediment with representative freshwater sediments from the University of Mississippi Biological Field Station (UMBFS) and estuarine sediments from Horn Island, MS; 2) life-cycle experiments examining formulated reference sediment as a suitable substrate for survival and reproduction of the amphipod, *Hyalella azteca* Saussure, midge, *Chironomus tentans* Fabricius, and waterflea, *Daphnia magna* Straus; and 3) fourteen-day experiments examining formulated reference sediment as a suitable substrate for survival of *H. azteca*, *C. tentans*, *D. magna*, the waterflea, *Ceriodaphnia dubia* Richard, and fathead minnow, *Pimephales promelas* Rafinesque.

To determine responses of commonly used freshwater sediment test species (*H. azteca* and *C. tentans*) to sediment characteristics, formulated and natural freshwater sediments were used to evaluate *H. azteca* and *C. tentans* tolerances of particle size classes and organic matter content of sediments encompassing the range of values encountered in bottom sediments throughout the U.S.

To determine whether copper sulfate is suitable as a reference toxicant for use in whole sediment toxicity tests, *H. azteca* (Saussure) was exposed for 10 days to two field collected sediments amended with copper sulfate. The rationale for selecting copper sulfate as a sediment reference toxicant and proposed selection criteria for sediment reference toxicants was also developed and discussed.

To address concerns regarding the impact of experimental conditions on test results, several experiments were conducted utilizing the organisms *C. tentans*, *H. azteca*, *D. magna*, *C. dubia*, and *P. promelas*. The objectives were to: 1) determine the relative sensitivities of these organisms to copper in water-only exposures and a field collected copper-contaminated sediment; 2) determine the duration of exposure required to elicit mortality or sublethal biological effect (e.g., reduced growth or reproduction) from a copper-contaminated sediment; 3) determine whether or not sublethal test endpoints are more sensitive than survival of test organisms; and 4) illustrate the ability of formulated reference sediment to match selected sediment characteristics and to act as dilution for the copper contaminated sediment, thus providing an accurate means of assessing sediment potency.

This report is divided into four separate sections. Each section represents a separate research task that was part of a more comprehensive research study examining the uncertainty of laboratory sediment toxicity tests in accurately assessing sediment potency. The individual research tasks follow a logical pattern, with results from initial studies influencing subsequent tasks. In Section I, the development and evaluation of formulated reference sediments is presented. Upon successful completion, the results were used to examine benthic organism tolerances of sediment characteristics in Section II, and then used as a reference and dilution sediment in Section III. Results of Section I have been published elsewhere as follows:

> Suedel, B.C. and J.H. Rodgers, Jr. 1994. Development of formulated reference sediments for use in freshwater and estuarine sediment tests. *Environmental Toxicology and Chemistry* 13:1163-1175.

Results from Section II have been published elsewhere as follows:

> Suedel, B.C. and J.H. Rodgers, Jr. 1994. Responses of *Hyalella azteca* and *Chironomus tentans* to particle-size distribution and organic matter content of formulated and natural freshwater sediments. *Environmental Toxicology and Chemistry* 13:1639-1648.

Material in the current report (Sections I and II) is used with the permission of the Society of Environmental Toxicology and Chemistry (SETAC).

Results from Section III have not been published. Results from Section IV have been published as follows:

Suedel, B.C., E. Deaver and J.H. Rodgers, Jr. 1996. Experimental factors that may affect toxicity of aqueous and sediment-bound copper to freshwater organisms. *Archives of Environmental Contamination and Toxicology*, 30(1): 40-46.

Suedel, B.C., E. Deaver and J.H. Rodgers, Jr. 1996. Formulated sediment as a reference and dilution sediment in definitive toxicity tests. *Archives of Environmental Contamination and Toxicology*, 30(1): 47-52.

Section 1

DEVELOPMENT OF FORMULATED REFERENCE SEDIMENTS FOR FRESHWATER AND ESTUARINE SEDIMENT TESTING

Recently, several concerns have arisen regarding locating and collecting representative, uncontaminated and nontoxic reference and dilution sediments in freshwater and estuarine systems. Frequently, reference sediments possessing sediment characteristics similar to the test sediment are not readily available at nearby localities for use in sediment toxicity tests (Suedel and Rodgers, 1991). In addition, naturally occurring reference sediments may be contaminated, toxic, or contain extant flora or fauna that may inhibit growth or survival of test organisms, thus making the sediment undesirable for use as a reference sediment. A source of representative, uncontaminated and nontoxic sediments is essential for definitive sediment toxicity testing (Suedel and Rodgers, 1995a). Prompted by the requirement for nontoxic reference sediments and unambiguous dilution sediments, formulated reference sediments to be used in a comprehensive sediment toxicity testing or quality evaluation programs were developed. Such sediments have many uses in sediment toxicity testing. These include:

1) Answering the question, "How toxic is a sediment?" (i.e., definitive sediment toxicity testing)

2) Use as reference and dilution sediments for diverse sediment types in definitive sediment toxicity testing and for use as a control and reference sediment in screening tests addressing the question, "Is a sediment toxic?"

3) Determining organism tolerance of sediment characteristics such as particle size distribution, organic carbon content, etc.

4) Use as a standard for evaluating the fate and effects of contaminants or specific test compounds in sediments.

"Reference sediment" as used in this document refers to a sediment that is used 1) to dilute a toxic sediment in a definitive sediment test or 2) for evaluating the potency of a test chemical in an amended sediment toxicity test. In these tests, formulated reference sediment would also serve as a control sediment to 1) evaluate unexposed test organism responses and serve as a statistical basis for comparisons of responses of exposed test organisms, and 2) verify that formulated reference sediment is a suitable substrate for test organism survival, growth, or reproduction. Thus, it is crucial that formulated reference sediments yield unambiguous responses by the sediment testing species.

Characteristics of the formulated reference sediment should be amenable to manipulation so the formulated reference sediment can closely approximate important characteristics of the test sediment. Formulated reference sediments were developed and evaluated to match physical and chemical characteristics of field-collected sediments spanning a range of sediment characteristics from throughout the U.S. (Suedel and Rodgers, 1991; Weaver, 1989).

Development and evaluation of formulated reference sediment for use in freshwater and estuarine toxicity testing was initiated at the U.S. EPA Gulf Breeze (ERL) in Gulf Breeze, FL (Walsh *et al.* 1991a,b) where "synthetic" sediments were developed to be used in toxicity tests with wetland plants. "Synthetic" sediment development for use in marine toxicity testing is an active research activity in progress at the Gulf Coast Research Laboratory (GCRL) in Ocean Springs, MS jointly with the U.S. Fish and Wildlife Service (P. Roscigno, personal communication).

The objective of this research was to develop and evaluate formulated reference sediments to be used in definitive sediment toxicity testing. In the following sections, discussion of the rationale behind developing formulated reference sediment is included, and potential formulated sediment constituents and their physical and chemical properties are examined. Formulated reference sediment evaluation includes: 1) matching formulated reference sediment with representative freshwater sediments from the University of Mississippi Biological Field Station (UMBFS) and estuarine sediments from Horn Island, MS; 2) life-cycle experiments examining formulated reference sediment as a suitable substrate for survival and reproduction of the amphipod, *Hyalella azteca* Saussure, midge, *Chironomus* *tentans* Fabricius, and waterflea, *Daphnia magna* Straus; and 3) fourteen-day experiments examining formulated reference sediment as a suitable substrate for survival of *H. azteca*, *C. tentans*, *D. magna*, the waterflea, *Ceriodaphnia dubia* Richard, and fathead minnow, *Pimephales promelas* Rafinesque.

MATERIALS AND METHODS

Formulated Reference Sediment Development

Formulated reference sediment constituents were chosen to encompass the range of characteristics of representative freshwater and estuarine bottom sediments occurring throughout the U.S. (Suedel and Rodgers, 1991). Selection criteria for formulated reference sediment constituents included the following:

1) Must contain mineral constituents commonly occurring in a variety of soils and sediments throughout the country.
2) Must be commercially available or readily collected from a homogeneous (well characterized) deposit in the field.
3) Constituents selected should be capable of spanning the range of characteristics encountered in bottom sediments.
4) Must be suitable for test organism growth, reproduction and survival in definitive sediment toxicity testing.
5) A consistent uncontaminated source must be available.

Clay, silt, sand, and organic constituents were obtained from commercial sources for preparing formulated reference sediments (Table 1-1). Kaolinitic clay, represented by ASP 600® and ASP 900®, is the most ubiquitous aluminosilicate mineral found in soils and bedrock in warm, moist regions of the world, including eastern and southeastern regions of the U.S. (Moore and Reynolds 1989). Kaolinites lack internal surfaces and thus tend to have a relatively low cation exchange capacity (CEC) (1-20 meq/100g) (Weaver, 1989; Grim, 1968). Kaolinite minerals (ASP 400®, ASP 600®, ASP 900®) were obtained from Engelhard Corporation, Pigments and Additives Division, Menlo Park, CN28, Edison, NJ, 08818.

Table 1-1. Composition of sands, kaolin based silt and clay, and montmorillonite based clay minerals used to prepare formulated reference sediments (composition according to manufacturers' specifications).

Sands (as oxides)		Kaolin Silt and Clay*		Montmorillonite Clay**	
SiO_2	97.5%	SiO_2	45.2%	SiO_2	60.4%
Fe_2O_3	0.07%	Fe_2O_3	0.3-0.9%	Fe_2O_3	1.4%
Al_2O_3	1.50%	Al_2O_3	38.8%	Al_2O_3	17.6%
Na_2O	0.03%	Na_2O	0.05-0.3%	P_2O_5	0.02%
TiO_2	0.04%	TiO_2	0.6-1.7%	TiO_2	0.24%
CaO	0.08%	CaO	0.02%	CaO	2.8%
MgO	0.06%	MgO	0.03%	MgO	6.5%
K_2O	0.51%	K_2O	0.05-0.2%	K_2O	0.2%
Other***	0.21%		12.85-14.95%	10.84%	

*Components are combined as complex aluminum silicate rather than free oxides.

**From Van Olphen and Fripiat (1979).

***"Other" refers to constituents that were not a part of the mineralogical analyses.

Montmorillonite (smectite) clay from Apache County, Arizona was evaluated and is well characterized. It is one of the "reference clays" chosen by the Clay Minerals Society representing typical clay minerals throughout the U.S. (Van Olphen and Fripiat, 1979; Kerr *et al.*, 1950). Montmorillonite clays are typically derived from volcanic rocks and occur as major constituents in soils of temperate zones (Weaver, 1989). Montmorillonite clays are also abundant in sedimentary rocks and occur in many tropical, subtropical, and arid soils throughout the world (Weaver, 1989). This aluminosilicate clay has a relatively high CEC (120 meq/100g) (Van Olphen and Fripiat, 1979) and can be used to match field-collected sediments with a CEC of >3 meq/100g. Montmorillonite was used in this study because of its presence in soils throughout Mississippi. Montmorillonite clay was obtained from Dr. W.D. Johns, Source Clays, Dept. of Geology, University of Missouri, Columbia, MO, 65211. Although not used in this study, illite is a commonly occurring clay mineral found in sediments (Van Olphen and Fripiat, 1979; Weaver, 1967). If illite predominates in a sediment sample, then illite may be a more appropriate clay constituent than montmorillonite.

The silt fraction of sediments typically consists of very fine sand particles, particulate organic material and agglomerated clay particles. ASP 400® was used to represent the silt fraction of sediments. Although ASP 400® is a kaolinite clay mineral, it is ground to 0.0048 mm diameter and does not separate into clay sized particles upon treatment with dispersant (sodium hexametaphosphate). The silt fraction of sediments refers to particles ranging in size from 0.05 mm to 0.002 mm in diameter (USDA classification system).

Sieved Mystic White® #18 and #90 sands were used to represent coarse (2.0-0.5 mm), medium (0.5-0.25 mm) and fine (0.25-0.05 mm) sand particles of sediments. Mystic White® sands are silica-based minerals that span the range of coarse, medium, and fine sand particles occurring in bottom sediments. Silica sands are found in a variety of freshwater and estuarine sediments where their size distribution is largely controlled by physical processes (Moore 1989). Sands were obtained from New England Silica, Inc., 1370 John Fitch Blvd., South Windsor, CT, 06074.

Organic matter, organic carbon, pH, and CEC are common chemical characteristics that may affect the bioavailability and toxicity of materials in sediments. In this study, humus represented a source of particulate organic matter in formulated reference sediments.

The humus consisted of decaying plant material and manure, which is more representative of naturally occurring organic matter than peat moss. Peat moss has a somewhat restricted distribution and consists of only plant material (Crum, 1988). In some situations where peat moss is present in sediments, it may be a more appropriate source of organic matter than humus. Other sources of humus such as ground plant material typically have a high CEC, ranging from 200 to 400 meq/100g (Bailey and White, 1964), and would be of limited utility due to excessive contribution to CEC content of formulated reference sediment. Humus was obtained from Sims Bark Co. Inc., P.O. Box 896, Tuscumbia, AL, 35674.
Organic matter content of formulated reference sediment was emphasized because organic matter represents a more realistic food source for toxicity testing organisms than organic carbon. Elemental forms of organic carbon are likely not utilized as a food source by benthic organisms. Organic matter is the organic fraction of sediment that includes both fresh and decaying plant, animal, and microbial residues at all stages of decomposition, as well as humus and highly carbonized compounds such as charcoal, graphite, and coal (elemental forms) (Black, 1986). Total organic carbon is contained within the organic matter fraction of sediments and can be estimated as the difference between total carbon and inorganic carbon (Black, 1986).

Dolomite from Wayne County, New York represented a natural bicarbonate buffer occurring in soils and sediments. This source of dolomite is well characterized and is one of the reference minerals chosen by the Clay Minerals Society representing typical minerals occurring in the U.S. (Van Olphen and Fripiat, 1979; Kerr *et al.*, 1950). Dolomite was obtained from Ward's Natural Science Establishment, Inc., P.O. Box 92912, Rochester, New York, 14692. Although not used in this study, calcite is a commonly occurring bicarbonate buffer found in freshwater and marine sediments (Krumbein and Garrels, 1952). Calcite may be a more appropriate bicarbonate buffer than dolomite, depending on location. Formulated reference sediment constituents were characterized with respect to particle size distribution, CEC, solids, organic matter, and pH (Table 1-2).

Table 1-2. Physical and chemical properties of formulated reference sediment constituents after conditioning*.

Constituent	Mineral	Particle Size (µm)	CEC (meq/100g)	Solids (%)	Organic Matter (%)	pH**
ASP 600	clay	0.6	1.59	52.12	13.6	3.5
ASP 900	clay	1.5	2.53	56.21	13.2	3.4
Montmorillonite	clay	<2.0	120	46.59	8.79	7.4
ASP 400	silt	4.8	2.87	62.61	13.2	3.4
Mystic White #18 coarse sand	silica	500-850	-***	88.76	1.0	6.8
Mystic White #90 medium sand	silica	250-500	-	75.30	0.02	7.0
Mystic White #90 fine sand	silica	50-250	-	61.45	1.8	6.8
Humus	organic matter	<2.0 mm	17.3	30.06	86.9	5.9
Dolomite (buffer)	carbonate	<0.05 mm	0.27	84.91	1.31	8.5

*See Table 1-3 for sediment parameters and corresponding analysis.
**pH measurements taken after conditioning each constituent for 7 days.
***Not measured.

Sediment Matching

The proposed formulated reference sediment must be amenable to formulation to match diverse characteristics found in bottom sediments (Suedel and Rodgers, 1991). The objective of the matching phase of formulated reference sediment evaluation was to prepare these sediments to match naturally occurring sediments with respect to particle size distribution, organic matter, CEC, pH, redox potential, and solids (Table 1-3). The accuracy of matching these characteristics was also evaluated. In addition to these parameters, ability to match organic carbon in field-collected sediments was also evaluated (Table 1-3).

The formulated reference sediment constituents, ASP 400® and ASP 600®, were used as representative silt and clay sediment fractions, respectively (Table 1-2). Mystic White® #18 and #90 silica sands were dry sieved into coarse, medium and fine sand fractions on a SoilTest sand shaker. Sand particles correspond to particle size categories following the USDA soil classification system (coarse sand 2.0 - 0.5 mm; medium sand 0.5 - 0.25 mm; fine sand 0.25 - 0.05 mm) (Black, 1986). Dried organic humus was used as a source of organic matter. Humus was dried at 70°C and milled to 2.0 mm in a Wiley Mill before use. Due to the high organic matter content (1.3-13%) of ASP 400® silt, ASP 600® and ASP 900® clays, dolomite, and montmorillonite clay (Table 1-2), these constituents were ashed at 550 °C for 1 hour in a muffle furnace to remove organic matter before use. For buffering capacity, dolomite was added as 1.0% of the total amount of silt required to match a given sediment. In preliminary experiments, it was determined that 1.0% dolomite provided optimal buffering of formulated reference sediments. Four sediments from the UMBFS (Field Sediments 1 - 4) were selected for matching because of their availability in large quantities from pristine areas and because they span the range of sediment characteristics encountered in representative bottom sediments from throughout the U.S. (Suedel and Rodgers, 1991). Two sediments from Horn Island on the Mississippi Gulf Coast, a pristine area with 10-15 0/00 salinity, were used as representative estuarine sediments for matching (Field Sediments 5 - 6).

Formulated reference sediment constituents were mixed dry and hydrated with UMBFS pond water (see Table 1-6 for water chemistry analysis) or synthetic seawater (reconstituted using Instant Ocean per label instructions) prior to analyses. Formulated Reference Sediments 1 - 4 were conditioned for 7 days in cement raceways in flowing (2-3 volume additions per day) UMBFS pond water. Formulated Reference Sediments 5 - 6 were conditioned under static conditions for 7 days. Parameters presented in Table 1-4 were measured after conditioning.

Table 1-3. Sediment Parameters, Corresponding Analysis, and Range of Values Reported for Each Sediment Parameter (from Suedel and Rodgers 1991) except where indicated).

Parameter	Analytical Method	Analysis	Range
Percent Solids	Drying at 104 °C	(a)	11.9-91.7%
Organic Matter	Ashing at 550 °C	(a)	0.3-45% (c,d)
Organic Carbon	High Temp. Induction Furnace (LECO CR-12)	(a)	0.02-11.8%
Cation Exchange Capacity (CEC)	Displacement After Washing	(b)	0.02-71.0 meq/100g
Redox Potential*	Orion Redox Probe	(b)	-409 - +379
pH*	Orion pH Probe	(b)	5.8-9.0 (d,e)
Particle Size Distribution	Hydrometric	(a)	sand = 2-100% silt = 0-95% clay = 0-60%

*Redox and pH measurements were taken at a depth of 1 to 3 cm in all sediment samples.
(a)=Black 1986;(b)=Plumb 1981;(c)=Reynoldson and Hamilton 1982;(d)=Barko and Smart 1986;(e)=Krumbein and Garrels 1952.

Table 1-4. Comparison of field sediments (FS) 1-6 and matching formulated reference sediments (FRS) 1-6 for selected sediment characteristics (mean±SD; N=3).

Sediment Number	Solids (%) FS	Solids (%) FRS	Organic Matter (%) FS	Organic Matter (%) FRS	Organic Carbon (%) FS	Organic Carbon (%) FRS	CEC (meq/100g) FS	CEC (meq/100g) FRS
1	72.95 ±1.24	79.13 ±0.21	1.7 ±0.64	1.1 ±0.04	0.38 ±0.005	0.37 ±0.04	1.50 ±0.25	0.74 ±0.09
2	59.98 ±5.76	59.23 ±0.25	6.8 ±0.25	6.6 ±0.26	1.4 ±0.04	3.0 ±0.05	4.18 ±0.25	4.17 ±0.10
3	76.35 ±0.28	77.45 ±0.77	0.30 ±0.087	0.17 ±0.089	0.021 ±0.020	0.014 ±0.015	0.19 ±0.044	0.08 ±0.01
4	41.51 ±0.29	58.85 ±0.06	7.8 ±0.16	8.4 ±0.19	2.21 ±0.03	2.76 ±0.05	7.8 ±2.61	3.1 ±0.38
5	78.62 ±0.75	77.74 ±0.24	0.33 ±0.010	0.27 ±0.026	0.05 ±0.002	0.02 ±0.011	0.12 ±0.03	0.11 ±0.03
6	75.73 ±0.45	66.25 ±12.1	7.64 ±0.35	6.18 ±0.17	1.82 ±0.27	2.73 ±0.03	5.1 ±1.10	2.2 ±0.06
Mean Difference(±SE)	2.26±3.65		0.32±0.28		0.478±0.26		1.41±0.80	

Table 1-4 (Cont'd). Comparison of field sediments (FS) 1-6 and matching formulated reference sediments (FRS) 1-6 for selected sediment characteristics (mean±SD; N=3).

	Sediment Characteristic							
Sediment	Redox (mv)		pH		Coarse Sand (%)		Medium Sand (%)	
Number	FS	FRS	FS	FRS	FS	FRS	FS	FRS
1	+57	+238	6.0	6.9	8.4	9.2	31.7	23.0
	±21	±9	±0.06	±0.06	±3.41	±0.85	±1.13	±0.16
2	-168	+217	6.9	6.5	2.5	2.9	5.5	6.0
	±6	±4	±0.06	±0.06	±1.59	±0.12	±0.64	±0.10
3	+252	+235	6.5	7.5	11.3	6.5	61.9	36.0
	±16	±3	±0.12	±0.06	±1.79	±0.29	±0.70	±0.98
4	-140	+231	6.5	6.8	0.2	0.6	1.0	1.2
	±17	±1	±0.15	±0.06	±0.06	±0.03	±0.15	±0.03
5	-100	+223	6.6	7.5	9.7	6.5	64.0	36.0
	±25	±4	±0.06	±0.06	±1.65	±0.29	±2.90	±0.98
6	-110	+239	6.9	6.3	0.8	1.9	8.1	11.0
	±9	±6	±0.17	±0.10	±0.84	±0.09	±1.89	±0.07
Mean Difference(±SE)	267±64*		0.37±0.30		0.88±1.01		9.86±5.66	

Table 1-4 (Cont'd). Comparison of field sediments (FS) 1-6 and matching formulated reference sediments (FRS) 1-6 for selected sediment characteristics (mean±SD; N=3).

Sediment Number	Fine Sand (%) FS	Fine Sand (%) FRS	Total Sand (%) FS	Total Sand (%) FRS	Silt (%) FS	Silt (%) FRS	Clay (%) FS	Clay (%) FRS
1	35.8 ±4.56	40.7 ±0.97	75.9 ±1.79	72.8 ±0.55	21.4 ±4.10	27.2 ±0.55	2.7 ±2.31	0.0 ±0.0
2	8.8 ±1.42	13.1 ±0.20	16.8 ±3.25	22.0 ±0.25	82.6 ±4.51	78.0 ±0.42	1.0 ±0.89	0.0 ±0.0
3	19.7 ±1.13	46.7 ±1.15	93.1 ±0.51	92.0 ±2.21	6.9 ±0.51	8.0 ±2.27	0.0 ±0.0	0.0 ±0.0
4	2.5 ±0.21	5.3 ±0.03	3.8 ±0.36	7.1 ±0.75	93.8 ±3.79	92.9 ±0.75	2.4 ±4.10	0.0 ±0.0
5	19.8 ±4.49	46.7 ±1.15	93.5 ±1.19	89.2 ±0.55	6.5 ±1.19	10.8 ±0.55	0.0 ±0.0	0.0 ±0.0
6	65.3 ±2.75	56.5 ±0.00	74.2 ±1.00	69.4 ±0.23	20.2 ±1.20	27.2 ±0.95	5.6 ±0.20	3.5 ±0.98
Mean Difference(±SE)	9.5±5.87		0.83±1.71		0.76±1.88		1.36±0.49*	

*Indicates significant difference at the 0.05 level.

Organism Tolerance of Formulated Reference Sediment

The suitability of formulated reference sediment as a reference or control sediment depends on its ability to provide test organisms sufficient habitat so these organisms can survive and reproduce with no adverse effects. In addition, "normal" test organism behavior such as burrowing, case building, grazing, etc. should occur. Experiments for determining organism performance in formulated reference sediment should include experiments spanning test organisms' entire life-cycles. These experiments were performed by culturing organisms in formulated reference sediment, which is more rigorous than conducting experiments of 10-14 days duration where only a fraction of an organism's life history is taken into account. *D. magna* (water column), *H. azteca* (epibenthic) and *C. tentans* (infaunal) were selected for experimentation because they occupy different niches in freshwater aquatic habitats and are sensitive to a wide variety of toxic materials occurring in aquatic systems. These organisms were cultured using formulated reference sediment and Field Sediment 1 (control) as substrates to determine the effect of these sediments on organism survival or reproduction. Field Sediment 1 was selected as a control sediment because it was used successfully in previous sediment toxicity testing programs with the organisms used in this study (Suedel and Rodgers 1994a). Previous organic and inorganic analyses of Field Sediment 1 indicated below detection limits for organics and near detection limits for metals except iron and aluminum (associated with smectite clays).

When hydrated without conditioning, formulated reference sediment constituents were not suitable for *H. azteca* survival in preliminary experiments (see Table 1-5 and related discussion). Therefore, a conditioning period was required before conducting additional experiments. Conditioning involved placing each constituent (ASP 400®, ASP 600®, ASP 900®, sands, humus) separately in plastic tubs (60 x 40 x 22 cm) and hydrating with UMBFS pond water. Tubs containing hydrated constituents were placed in flow-through (2-3 volume additions per day) cement raceways at the UMBFS until colonization by various invertebrate species was noted on the sediments. The presence of various oligochaetes, midges, isopods and algae was noted on the surfaces of constituents after 7 days of conditioning. The presence of indigenous invertebrate species on these constituents initially signaled the end of the conditioning period for test organisms and for formulations of reference sediments.

Table 1-5. Exposure of *H. azteca* to unconditioned formulated reference sediment constituents for 10 days.

Constituent	Number Surviving/Total	Percent Survival
100% coarse sand	2/10	20%
60% coarse sand, 40% medium sand	3/10	30%
60% medium sand, 40% ASP 400 silt	0/10	0%
60% medium sand, 40% ASP 600 clay	0/10	0%
40% medium sand, 60% ASP 600 clay	0/10	0%

After the conditioning period and before mixing, invertebrates on the sediment surface and standing water were removed. The sediment constituents were returned to the laboratory and stored wet at room temperature (22±2 °C) up to 30 days before testing.

In initial non-replicated *D. magna*, *H. azteca*, and *C. tentans* culture experiments, aquaria were maintained under ambient laboratory conditions for temperature (25±1 °C) and light (16 h light/8 h dark photoperiod). For each organism, the experiment was conducted in two 39 L glass aquaria containing either 2.0 kg of Field Sediment 1 (as a control) or 2.0 kg of formulated reference sediment, with approximately 15 L of filtered UMBFS pond water overlying each sediment. In these experiments, formulated reference sediment was prepared to match Field Sediment 1 with respect to particle size distribution (i.e., 8% coarse sand, 32% medium sand, 36% fine sand, 21% silt, and 3% clay). Humus was also added, achieving a concentration of 0.1% organic matter. All formulated reference sediment constituents were reference sediment (Table 1-2) and were previously conditioned for 7 days. ASP 600® and ASP 900® each represented 50% of the clay fraction on a dry weight basis. After adding 30 L of filtered UMBFS pond water to both sediment treatments, contents of the culture aquaria were allowed to equilibrate and suspended particles to settle for 24 hours before introducing test organisms.

The experiment with *D. magna* was initiated by adding 50, <48 hours old neonates to each aquarium with a 6 mm ID glass pipet and maintained for 28 days. This exposure was sufficient for exposure of juvenile, adolescent, and adult life stages producing several broods of *D. magna* neonates. Only the egg stage was not exposed to formulated reference sediment. *D. magna* were fed 5 ml (approximately $5x10^6$ cells/ml) of *Selenastrum capricornutum* every other day. The aquaria were not aerated. After 28 days, *D. magna* in each aquarium were collected and counted.

The experiment with *C. tentans* was initiated by adding two egg masses to each aquarium. Each egg mass consisted of approximately 200 to 500 eggs. Aquaria were vigorously aerated and *C. tentans* were fed 2-3 ml of a Tetra conditioning food (Tetrawerke, Germany) suspension daily. This feeding rate has been used to successfully culture *C. tentans* at our laboratory for 2 years (see below). *C. tentans* were cultured until all larvae had pupated and emerged as adult midges (60 days). Although not technically a full life-cycle exposure, larval, pupae, and adult life stages were exposed. Only the *C. tentans* eggs were not exposed.

The *H. azteca* experiment was initiated by adding 100 adults (2 - 3 cm length) to each aquarium. *H. azteca* were fed ground maple leaves (*Acer rubrum*) once per week as needed. Both aquaria were gently aerated (2 bubbles/sec). *H. azteca* were cultured for 2 months, sufficient time for one complete life cycle at room temperature (24-25 °C) (de March 1981). At the end of two months, *H. azteca* in each aquarium were collected and counted.

For a more rigorous evaluation of formulated reference sediment, replicated experiments were also conducted with *D. magna* and *C. tentans*. The experiment with *D. magna* (28 days duration) was initiated by placing two <24 hour-old *D. magna* neonates per 250 ml beaker (N=5 replicates/sediment) containing 160 ml of alkalinity and hardness adjusted (see culture procedures) UMBFS pond water (alkalinity and hardness of unadjusted UMBFS pond water is outside the tolerance range of *D. magna*) and either 40 ml of Field Sediment 1 (control) or formulated reference sediment. *D. magna* were fed *S. capricornutum* daily. The overlying water from each beaker was partially renewed (80-100 ml) weekly by gently pouring water on a watch glass to minimize disturbance. Neonates were removed from each beaker and counted every 2-3 days. The water was not aerated since dissolved oxygen concentrations did not fall below 40% saturation.

The replicated experiment with *C. tentans* (40 days duration) was initiated by placing from 5-8 <24 hour-old first instar *C. tentans* larvae to each 250 ml beaker (N=5 replicates/sediment) containing 160 ml UMBFS pond water and either 40 ml of Field Sediment 1 or formulated reference sediment. *C. tentans* were fed 0.05 ml of a cerophyll suspension every other day, and 80-100 ml of the overlying water was renewed weekly. *C. tentans* survival, growth, and development (qualitative) was monitored throughout the experiment. The water was not aerated since dissolved oxygen concentrations did not fall below 40% saturation.

Formulated reference sediment used in replicated culture experiments contained all of the constituents listed in Table 1-2 and was prepared as follows: percent solids - 64.0%; organic matter - 7.6%; redox potential - +228 mv; CEC - 3.61 meq/100g; pH - 6.2; coarse sand - 3.1%; medium sand - 5.1%; fine sand - 14.6%; silt - 77.2%; clay - 0.0%. This formulated sediment was prepared separately and was not designed to match field collected sediments listed in Table 1-4.

In addition to conducting chronic experiments, static 14 day whole sediment exposures were conducted with *C. tentans* (10 days old, 2nd instar), *H. azteca* (2-3 weeks old), *D. magna* (24-48 h old), *C. dubia* (<24 h old), and *P. promelas* (3-4 d old) using formulated reference sediments. These experiments (except for *C. dubia*) were conducted in 250 ml beakers with 40 ml formulated reference sediment and 160 ml UMBFS pond water. There were 4 replicate beakers for each organism, with 8 organisms per beaker (6 for *C. tentans*). The *C. dubia* experiment was conducted in 50 ml beakers with 8 ml formulated reference sediment and 32 ml UMBFS pond water. *C. dubia* experiments were initiated with 10 replicate beakers, each containing one <24 hour-old neonate. Characteristics of formulated reference sediment used in 14 d whole sediment exposures were the same as those used in replicated exposures (see previous paragraph). Test organisms were fed as described previously. *C. dubia* were fed 0.5 ml (approximately $5x10^6$ cells/ml) *S. capricornutum* daily and *P. promelas* were fed newly hatched *Artemia* nauplii daily.

Sediment Test Organism Culture Procedures

All test organisms were cultured at the UMBFS. *D. magna* and *C. dubia* culturing procedures followed the methods of U.S. EPA (U.S. EPA 1985a, 1989). *D. magna* and *C. dubia* cultures were maintained in UMBFS pond water in light (16 h light/8 h dark photoperiod) and temperature (21$\pm$1 °C) controlled incubators. UMBFS pond water is characteristically soft, with low levels of dissolved ions. Hardness and alkalinity were adjusted with (0.1 g/L) $NaHCO_3$ and $CaCl_2$ to a total hardness of 80 mg/L as $CaCO_3$ and an alkalinity of 60 mg/L as $CaCO_3$. *D. magna* and *C. dubia* cultures were fed *S. capricornutum* daily.

H. azteca and *C. tentans* cultures were maintained under ambient temperature conditions (22$\pm$2 °C) in 39 L glass aquaria containing filtered, dechlorinated tap water. *H. azteca* culturing procedures followed the methods of de March (1981). *H. azteca* were cultured using maple leaves (*Acer rubrum*) as a substrate and were fed ground rabbit chow pellets (Clover Brand, Madison, MS) twice each week. Amphipods used for testing were gently poured through a series of stainless steel sieves. Those individuals passing through a 1000 um mesh sieve but retained by a 600 um mesh sieve were used for testing. *C. tentans* culture methods followed those of Townsend *et al.* (1981). *C. tentans* culture substrate consisted of shredded brown paper towels (#ML96 Georgia Pacific, Battleboro, VT) to a depth of three cm. A suspension (0.1 g food/1 ml water) of Tetra conditioning food

(Tetrawerke, Germany) and a cerophyll (Ward's Nat. Sci., Rochester, NY) suspension (0.025 g cerophyll/1 ml water) was introduced to the cultures daily.

P. promelas culture methods followed those of U.S. EPA (1985a). *P. promelas* cultures were maintained under ambient temperature (25±2 °C) and light (16 h light/8 h dark photoperiod) conditions. Fish were cultured in 60 L glass aquaria under flow through conditions with filtered, dechlorinated tap water. Fish were fed a daily diet consisting of Tetra staple food (Tetrawerke, Germany) and frozen brine shrimp (San Francisco Bay Brand, Newark, CA).

Statistical Analysis

Mean differences of formulated reference sediment characteristics were statistically compared with those of natural sediments using paired t-tests (Zar 1984; Sokal and Rohlf 1981). The mean difference within each pair (field sediment vs. formulated reference sediment) for each sediment characteristic was compared for all six sediments matched (N=6; i.e., the number of pairs of data). Respectively, univariate and t-test procedures were used to determine normality and differences in means from replicated life-cycle experiments with *C. tentans* and *D. magna* cultured on UMBFS sediment 1 or formulated reference sediment (SAS 1989). Analysis of variance and Tukey's test (SAS 1989) were used to determine differences between means of sediment organic matter required for *C. tentans* survival in 10 day tests. The 5% alpha level was used in all statistical tests.

RESULTS AND DISCUSSION

Formulated Reference Sediment Development

Formulated reference sediment constituents spanned the range of particle sizes encountered from fine clay (0.6 um) to coarse sand (850 um) [Table 1-3]. Cation exchange capacity (CEC) for clay and silt constituents ranged from 1.6 to 2.9 meq/100g, which would encompass only 50% of the CEC values encountered in freshwater and marine sediments from throughout the U.S. (Table 1-3). Sediments with a CEC of >2.9 meq/100g require an additional clay constituent such as montmorillonite to match sediments with CECs >2.9 meq/100g. Solids concentrations varied from near 46% for montmorillonite clay to near 90%

for coarse sand, encompassing the range of solids concentrations found in bottom sediments (Table 1-3). Organic matter content of formulated reference sediment constituents ranged from 0.02% for medium sand to 89.6% for humus. Organic matter content of silt and clay constituents ranged from 8.8% to 13.6%, and these constituents may require ashing at 550 °C to remove organic matter to permit matching sediments with organic matter <10%. Addition of humus (organic matter content = 86.9%) to formulated reference sediment will allow matching virtually all concentrations of organic carbon and organic matter that may be encountered in field collected sediments.

Although sediments were matched quantitatively with respect to organic matter, qualitative differences between sediments may still exist, due to the various forms of organic carbon present in sediments (e.g. mineral forms such as coal, dissolved and particulate forms). The humus used in this study was not characterized further than shown in Table 1-2. Complete characterization between batches of organic matter and organic carbon sources is essential to monitor the effects of source, degree of decomposition, and potential for contamination. Sources of organic matter and organic carbon are not typically exposed to rigorous quality assurance and quality control procedures as are the other constituents used (sands, clays), as sand and clay constituents are used in various products designated for human use.

As illustrated above, mineral constituents of formulated reference sediments are commonly found in soils and sediments throughout the U.S. and are commercially available. Preparation of sand, silt and clay constituents in appropriate quantities should permit formulation of sediments that span the range of sediment characteristics encountered in practice (Table 1-3). Because the quantities of sand, silt, clay, and humus are supplied in substantial amounts (19-37 kg), several experiments or studies can be conducted with a single batch, minimizing the potential for inconsistencies between batches. In addition, many of these constituents (clay, silt, dolomite) are considered reference materials or are used extensively for human use and thus are subject to rigorous Quality Assurance/Quality Control procedures to ensure consistency between batches. Sources of organic matter or organic carbon (e.g., potting soil, humus) are typically not subjected to rigorous QA/QC procedures, as they are not used in applications subject to strict requirements. Further evidence demonstrating formulated reference sediments' ability to encompass sediment characteristics and suitability of these constituents for organism survival, growth and reproduction are discussed in the following sections.

Sediment Matching

Table 1-4 presents the results of an effort to match formulated reference sediments with freshwater sediments (Field Sediments 1-4) and estuarine sediments (Field Sediments 5 and 6). Sediment solids values of formulated reference sediments were similar to all field sediments. Solids concentrations of formulated reference sediments were within 6% of all field sediments except for a 17% difference (41.5% vs. 58.9%) between Field Sediment 4 and Formulated Reference Sediment 4 (Table 1-4). These data indicate that formulated reference sediment can match a wide range of solids concentrations, with the exception of field collected sediments with silt composition >90% (e.g., Field Sediment 4). Solids concentration of sediments is negatively correlated with silt content of sediments, i.e., as silt content increases, solids concentration decreases (Suedel and Rodgers, 1991). Although sediments with >90% silt content are relatively uncommon (Suedel and Rodgers, 1991), these sediments may be difficult to match with formulated reference sediment with respect to solids concentration. Differences between the composition of silt fractions of field collected and formulated reference sediment may significantly affect solids concentration when present in sufficient quantities (e.g. >90%).

Organic matter content of formulated reference sediments was not significantly different from field collected sediments studied (Table 1-4). Organic matter content of formulated reference sediment was within a factor of two for all field sediments, indicating that field sediments with varying organic matter content can be matched by formulated reference sediments.

Organic carbon values of formulated reference sediments were not statistically different from field sediments characterized, even though no attempt was made to match sediments with respect to organic carbon. Except for Field Sediments 2 and 5, organic carbon values varied by a factor of two or less. Matching for organic carbon was less successful than organic matter because the organic carbon fraction of organic matter is not quantitatively consistent between sediments. From field sediments used in this study, the organic carbon fraction of organic matter ranged from 10 to 50%. Therefore, matching for organic matter will not necessarily result in matching organic carbon. Nevertheless, these data indicate that sediments can be matched for organic carbon content.

CEC values of formulated reference sediments were not statistically different from CEC values of field sediments (Table 1-4). All CEC values of formulated reference sediments were within a factor of 2.5 of field collected sediments. CEC is dependent upon a number of factors such as pH and cations present in water (Black, 1986), thus the formulated reference sediment CEC data in this study reflect UMBFS pond water characteristics used to hydrate these sediments. CEC values obtained for formulated reference sediment will be influenced in part by the source water used to condition formulated reference sediment as well as the overlying water used in whole sediment toxicity tests.

Redox potentials for all formulated reference sediments were oxidized, with measured Eh values between +210 to +240 mv, considerably higher than Eh values measured for field collected sediments (except Field Sediment 3). Formulated reference sediment as prepared herein did not match sediments exhibiting slightly reduced (+100 to -100 mv) or reduced conditions (less than -100 mv) without further manipulation. Matching redox potential of sediments may be important in situations where formulated reference sediment is used for diluting metal-contaminated sediments or when amending formulated reference sediment with metals. Redox has been hypothesized to influence metal bioavailability (Gambrell and Patrick, 1988). Redox potential of sediments has been controlled in previous studies by supplying appropriate quantities of oxygen, nitrogen, or helium gas in an incubated system (Hambrick *et al.* 1980).

Formulated reference sediment pH values were similar to field collected sediments (Table 1-4). Formulated reference sediment pH values were within one pH unit of all field collected sediments, indicating some success in matching pH values. As noted in Table 1-3, pH values for clay, silt and humus constituents of formulated reference sediment were below 6.0. The buffering capacity of dolomite added to each sediment was sufficient to increase the pH of each formulated reference sediment to near neutral values (6.3 to 7.5). However, a period of up to 7 days was required to achieve stable pH readings in formulated reference sediments (Figure 1-1).

There was considerable success in matching particle sizes of field collected sediments, as significant differences were not observed for coarse, medium, fine, total sand, and silt (Table 1-4). However, clay content of formulated reference sediments was significantly different from field sediments. This reflected the lack of variability (0.0% in many cases) between clay samples. Coarse and total sand particles of formulated reference sediment matched within 5% of all field collected sediments examined. However, medium and fine sand fractions of Formulated Reference Sediments 3 and 5 were considerably different than Field Sediments 3 and 5. These differences between medium and fine sand particles were likely due to the use of different sieves, one sieve series for preliminary separation of sand particles (SoilTest shaker), and another sieve series used for particle size determinations. This underscores the importance of using identical sieves for all particle size determinations and constituent preparation.

Formulated reference sediments can be prepared to match naturally occurring sediments with respect to particle size distribution, solids, OC, CEC, pH, and organic matter content. It is recommended that silt, clay and dolomite constituents be ashed before use to remove excess dissolved organic matter, especially when matching sediments with low organic matter content. It is not assumed that factors influencing sorption and bioavailability remain unaltered, although the suitability of test organism survival, growth, and reproduction of formulated reference sediments containing ashed constituents may remain unaltered. This would need to be verified on a case by case basis.

Organism Tolerance of Formulated Reference Sediment

Conditioning Formulated Reference Sediments

When hydrated without conditioning, formulated reference sediment constituents were not suitable for *H. azteca* survival in preliminary experiments (Table 1-5) and therefore a conditioning period prior to conducting experiments was initiated. The conditioning period was designed to add an aquatic biological component to formulated reference sediment which was initially absent in unconditioned formulated reference sediment. Addition of microbial species (e.g., bacteria, algae) to formulated reference sediment may provide test organisms a

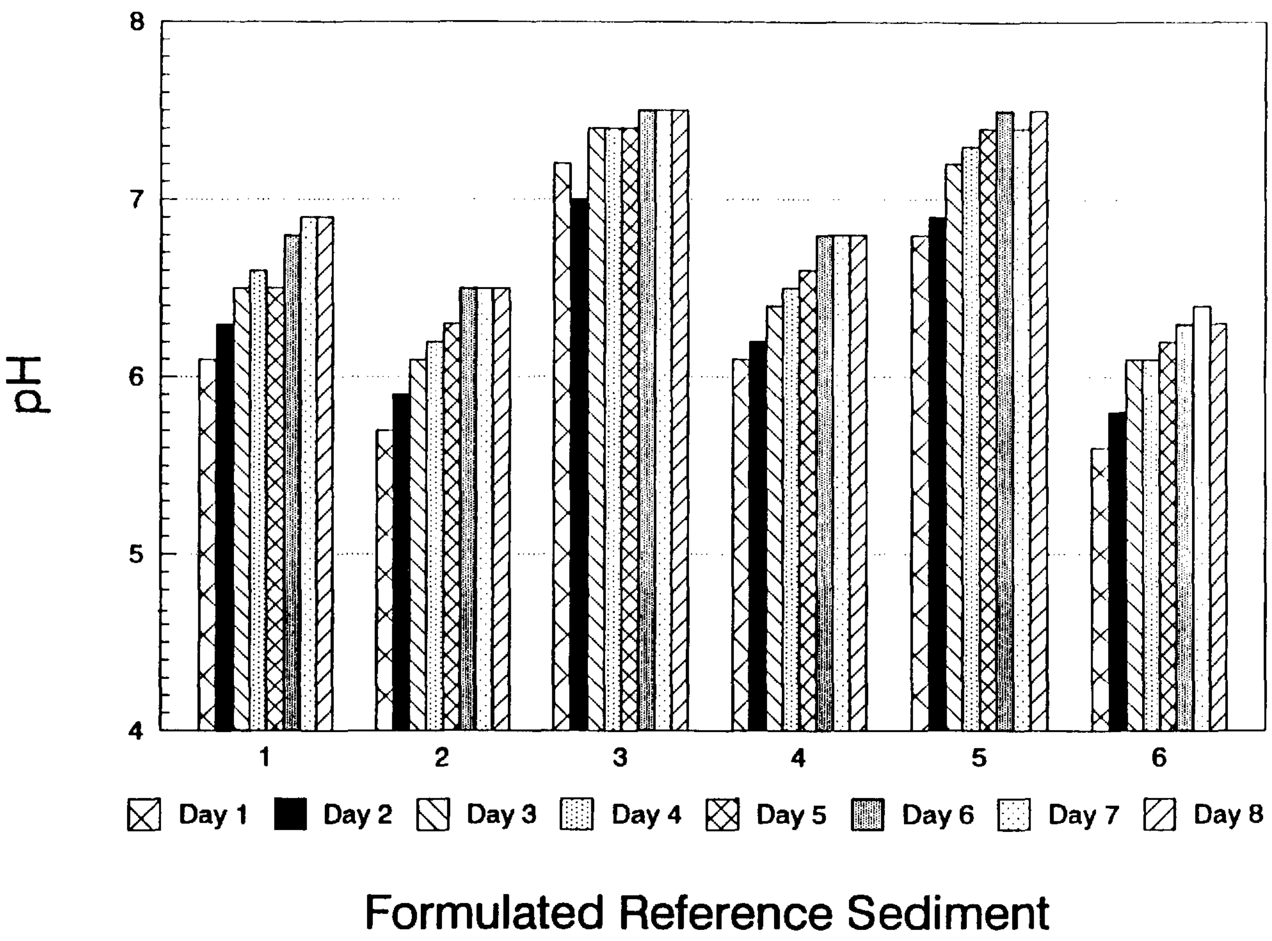

Figure 1-1. Time (days after initial mixing) required for stabilization of pH values of formulated reference sediments prepared to match field collected sediments in this study.

more suitable substrate in which to survive and reproduce, and may also serve as a food source as well. Conditioning formulated reference sediment with UMBFS pond water resulted in a biological component in formulated reference sediment that is unique to the UMBFS and will vary, depending on the source of the water used to condition formulated reference sediment. Although colonization of formulated reference sediment by indigenous oligochaete and midge species occurred in this study, in practice, formulated reference sediment should be conditioned in a natural water source (i.e., flow-through, static, or static-renewal) that includes various microbial species but not indigenous macroinvertebrate species such as oligochaetes and midges, which can interfere with test organism performance. Toxicity observed with unhydrated formulated reference sediment should not be viewed as unusual since experiments conducted with dried (70 °C) Field Sediment 1 also resulted in toxicity (>50%) to *H. azteca* and *C. tentans*.

D. magna Experiments

In the non-replicated experiment exposing *D. magna* to Field Sediment 1, 100% mortality was observed within 14 days (Table 1-6). Although eggs were observed in the brood chamber of some adults, no release of live offspring from these adults was observed. This was not surprising on account of the low levels of dissolved salts present in the water and the fact that *D. magna* can not be successfully cultured in unaltered UMBFS pond water. Naturally occurring populations of *D. magna* are generally restricted to waters exceeding hardness values of UMBFS pond water (6-24 mg/L as $CaCO_3$; U.S. EPA, 1985a). *D. magna* cultured on formulated reference sediment, however, demonstrated rapid growth and reproduction during the exposure period resulting in 2234 *D. magna* present in the culture after 28 days (Table 1-6). Characteristics of UMBFS pond water were considerably altered when overlying formulated reference sediment and thus were different than pond water overlying Field Sediment 1. Increased pH, conductivity, alkalinity and hardness values resulting from placement of UMBFS pond water on this sediment likely contributed to the successful culture of *D. magna* on formulated reference sediment. *D. magna* were also observed vigorously foraging on formulated reference sediment at the sediment-water interface.

These results illustrate that sediments can affect the properties of water placed over sediment in sediment toxicity testing. Water placed on a sediment will reflect the general physical and chemical characteristics of that sediment, regardless of the water source. UMBFS pond water contains low levels of dissolved salts when overlying UMBFS sediments, but when UMBFS pond water is placed on another sediment (e.g., formulated reference sediment), it is altered by the physical and chemical properties of that sediment. Other sources of water placed on formulated reference sediment will likely be altered by formulated sediment, regardless of the water source because water removed from its original sediment and placed on formulated reference sediment will change with respect to chemical properties (e.g. pH, hardness, alkalinity, conductivity). Likewise, reconstituted water placed on bottom sediments will be chemically altered by the sediment and altered differently by dissimilar sediments. A viable area of future research could be to investigate the variability and degree of influence of overlying water on sediments and vice versa.

The results from the replicated 28 day experiment with *D. magna* showed 100% survival when exposed to both sediments with adjusted UMBFS pond water (Table 1-6). The mean number of offspring per replicate ($\pm$S.D.) was 95.2$\pm$5.26 and 91.5$\pm$7.33 for Field Sediment 1 (control) and formulated reference sediment, respectively, and were not significantly different from each other. These data provide additional evidence that formulated reference sediment can provide suitable habitat for *D. magna* through 28 d of exposure.

C. tentans Experiments

The results from the unreplicated experiment exposing *C. tentans* to Field sediment 1 showed no larvae achieving the adult life-stage after 60 days (Table 1-7). The presence of numerous other indigenous organisms such as oligochaetes and other species of midges observed in Field Sediment 1 may have contributed to the mortality of *C. tentans*. Other species of midges and oligochaetes were observed in abundance and likely preyed upon or outcompeted *C. tentans* for food and space. No third or fourth instar *C. tentans* larvae or

Table 1-6. Results of water chemistry analysis and reproductive success of *D. magna* cultured on Field Sediment 1 and formulated reference sediment for 28 days.

Parameter	Non-replicated Experiment*** Field Sediment 1	Non-replicated Experiment*** Formulated Sediment	Replicated Experiment*** Field Sediment 1	Replicated Experiment*** Formulated Sediment
Temperature	25-26°C	25-26°C	20.1-22.6°C	20.0-22.6°C
Dissolved Oxygen (mg/L)	7.2-8.0	8.0-9.8	7.5-8.4	7.4-8.6
pH	6.2-6.5	7.6-7.9	7.1-7.7	7.8-7.9
Conductivity (umhos/cm)	40-60	140-155	230-290	320-350
Alkalinity (mg/L as $CaCO_3$)	5	48	12-28	56-68
Hardness (mg/L as $CaCO_3$)	12	56	8-12	12-16
D. magna survival	0*	2234*	(10/10)=100%	(8/8)=100%
D. magna reproduction			95.2±5.26**	91.5±7.33**

*Total number of *D. magna* present at test termination (initial N=50).

**Mean (±S.D.) number of neonates produced per replicate during the 28 d exposure.

***Unaltered UMBFS pond water was used for the non-replicated experiment and altered UMBFS pond water was used in the replicated experiment.

cases were observed, with a majority of the mortality likely occurring during the marked increase of other midge and oligochaete species in the aquarium. This is in sharp contrast with results from experiments conducted for 14 days *C. tentans* exposed for 14 days to Field Sediment 1 resulted in 88% survival (Table 1-8). This demonstrates one of the weaknesses of using naturally occurring sediments as reference or dilution sediment in sediment toxicity testing. The sediment may be biologically "toxic" due to the presence of indigenous organisms (e.g., other species of midges and oligochaetes) that may render the sediment unsuitable for test organisms to survive and reproduce. Sieving sediment through a 2.0 mm mesh sieve (as in this study) is frequently used to remove rooted aquatic plants, large debris and some indigenous organisms before using sediment in toxicity tests. However, sieving is not sufficient to remove many plant seeds, daphnid resting eggs, and oligochaete and midge species that may subsequently colonize and adversely affect test organism performance during a chronic study (e.g. >10 days). Sieving sediment with a smaller mesh such as a 0.05 mm or 1.0 mm mesh sieve will remove most, if not all, of the coarse sand fraction of sediments, thereby removing the potential habitat for many benthic species.

C. tentans exposed to formulated reference sediment in the non-replicated experiment resulted in 20 midges reaching the adult life-stage (Table 1-7). The presence of another species of midge (*Cladotanytarsus* spp.) observed in the aquarium (from the initial conditioning period) and limited carrying capacity may have contributed to the relative lack of *C. tentans* success in reaching the adult life-stage. *C. tentans* larvae forage for food in an area surrounding its case roughly equal to its body length (4th instar larvae = 2.5 cm length = 5.0 cm^2 area). This area is the minimum required to support one 4th instar midge. In a 39 L aquarium, there is only enough surface area (20 cm x 40 cm = 800 cm^2) to support approximately 160 4th instar midges under ideal conditions. In addition, the formulated reference sediment mixture contained only 0.1% organic matter in the form of humus, which later proved to be insufficient for midge survival at organism densities (6 midges/40 ml sediment; 250 ml beaker) in 10 d tests (Table 1-9). Effects of sediment organic matter content on *C. tentans* survival are further examined in Suedel and Rodgers (1994a).

Table 1-7. Results of water chemistry analysis and reproductive success of *C. tentans* cultured on Field Sediment 1 and formulated reference sediment.

Parameter	Non-replicated Experiment (60 d)		Replicated Experiment (40 d)	
	Field Sediment 1	Formulated Sediment	Field Sediment 1	Formulated Sediment
Temperature	25-26°C	25-26°C	20.3- 22.2°C	20.7-22.1°C
Dissolved Oxygen (mg/L)	7.6	7.8	4.5-7.4	4.5-7.4
pH	7.4	7.6	5.5-6.7	7.0-7.4
Conductivity (umhos/cm)	125	172	30-75	110-215
Alkalinity (mg/L as $CaCO_3$)	10	40	4-12	56-69
Hardness (mg/L as $CaCO_3$)	24	85	4-8	8-16
Number of *C. tentans*	0*	20*	70.4+8.8%**	62+10.9%**

*Total number of emerging adults at test termination (initial N approximately 400-1000).

**Mean percent emergence (±S.D.) per replicate during the 40 d exposure.

Table 1-8. Percent survival of selected freshwater toxicity testing species exposed to formulated reference sediment or Field Sediment 1 for 14 days.

	Sediment	
Organism	Formulated	UMBFS 1
H. azteca	88%	100%
C. tentans	88%	89%
D. magna	100%	93%
C. dubia	90%	-
P. promelas	94%	-

Results from the replicated experiment with *C. tentans* exposed to formulated reference sediment (7.6% organic matter) and Field Sediment 1 (control) resulted in a mean (±S.D.) adult emergence of 62±11% and 70±9%, respectively, and were not significantly different from each other (Table 1-7). *C. tentans* was observed successfully building and maintaining cases in both sediments throughout the experiment. *C. tentans* mortality was observed in both sediments predominantly during the metamorphosis from larvae-to-pupae and from pupae-to-adult life stages. No mortality of first, second, or third instar larvae was observed on either sediment. In this experiment, percent survival of *C. tentans* from <24 h old larvae to adult life stages was greater than expected based on data from Townsend *et al.* (1981), reporting 49+16% (mean±S.D.) emergence of *C. tentans*. These data provide additional evidence that formulated reference sediment can provide a suitable habitat for *C. tentans* throughout most of its life-cycle.

<u>*H. azteca* Experiment</u>

H. azteca cultured on Field Sediment 1 in the initial non-replicated experiment resulted in 425 individuals at the end of the 56 day period (Table 1-10). The presence of numerous daphnids and oligochaetes may have contributed to the reduced number of *H. azteca* present relative to formulated reference sediment, again demonstrating a disadvantage of using naturally occurring sediments as reference or dilution sediments in chronic (e.g. >10 days) sediment toxicity testing. Had daphnids been used as a test organism in this case,valid test results would not have been obtained.

H. azteca cultured on formulated reference sediment in the non-replicated experiment yielded 600 individuals at the end of the 56 day period (Table 1-10). *H. azteca* were observed on numerous occasions foraging on the formulated reference sediment substrate. Although increased numbers of *H. azteca* (600) were observed in the aquarium containing formulated reference sediment compared to the aquarium containing Field Sediment 1 (425), this difference is probably not biologically significant, with considerable population growth of *H. azteca* occurring in each sediment.

Additional experiments conducted exposing *H. azteca*, *C. tentans*, *D. magna*, *C. dubia*, and *P. promelas* to conditioned formulated reference sediment and unaltered UMBFS pond water for 14 days resulted in survival ranging from 88 to 100% (Table 1-8). These results provide further evidence of the suitability of formulated reference sediment as habitat for these organisms for up to 14 days of exposure.

Advantages and Disadvantages of Using Formulated and Natural Sediments

The results from this study illustrated some potential disadvantages in using naturally occurring sediments as reference and dilution sediments in sediment toxicity testing. The presence of physical, chemical, or biological properties of field collected sediments may adversely affect test organism performance. Other potential disadvantages of using field collected sediments noted in this study or by Walsh *et al.* (1991c) include; 1) inconsistent composition (heterogeneity) among sample collections or locations collected at different times; 2) cannot be formulated as desired; 3) may change physically or chemically (redox) upon removal from field location (e.g. loss of fine particles from dredge upon removal; 4) storage problems, i.e., freezing or drying may alter sediment characteristics or toxicity; 5) may be contaminated or toxic; 6) may contain extant organisms (e.g., invertebrates, plant seeds that can confound results; 7) removal of organisms may alter sediment characteristics; 8) deep water sediments may be difficult and expensive to obtain; and 9) instability of collection site.

Some inherent advantages of using naturally occurring sediments include; 1) natural sediments contain a variety of constituents found in natural systems (e.g. particulate, dissolved, nutrients, etc.); 2) are usually inexpensive (except for initial characterization); and 3) preparation may not be time-consuming.

Table 1-9. Effects of particulate organic matter (as added humus) on *C. tentans* survival exposed to formulated reference sediment for 10 days.

Organic Matter*	Percent Survival
0.0%	33%
0.5%	53%
1.0%	73%
2.0%	80%
3.0%	87%**
4.0%	87%**
5.0%	87%**

*Organic matter expressed as percent particulate organic matter added as humus.

**Statistically significantly greater than all other treatment means (parametric analysis of variance, Tukey multiple range test, N=3, F value=3.72, $p \leq 0.05$).

Table 1-10. Results of water chemistry analysis and reproductive success of *H. azteca* cultured on Field Sediment 1 and formulated reference sediment for 56 days (non-replicated).

Parameter	Field Sediment 1	Formulated Sediment
Temperature	25-26°C	25-26°C
Dissolved Oxygen (mg/L)	7.3-7.6	7.4-7.7
pH	6.6-6.9	8.1-8.2
Conductivity (µmhos/cm)	80-85	240-250
Alkalinity (mg/L as $CaCO_3$)	10	40
Hardness (mg/L as $CaCO_3$)	24	85
Total Number of *H. azteca* at Expt. Termination (56 d) Initial N=100	425	600

Some disadvantages of using formulated reference sediments in sediment toxicity testing as noted in this study or by Walsh *et al.* (1991c) include; 1) the difficulty of duplicating exactly the physical and chemical properties of field collected sediments; 2) obtaining a consistent source of organic matter; 3) components must be purchased or collected; 4) preparation of formulated reference sediments may be more time consuming than that of naturally occurring sediments; and 5) formulated reference sediment constituents must be conditioned before use. Although organic matter and organic carbon content may be matched quantitatively, there is no guarantee that the sediment is matched qualitatively with respect to these characteristics. This may affect the fate and kinetics of sediment-sorbed materials and subsequent availability to test organisms.

Advantages of using formulated reference sediments include; 1) support desirable survival and growth rates of plants and animals (This study; Walsh *et al.* 1991c); 2) formulated reference sediments can be prepared as desired for particle size, organic matter, etc.; 3) can be prepared as needed, with little difference in particle size distribution and chemical composition between sample collections; 4) convenient storage (room temperature, wet); 5) pH can be stabilized or increased by addition of dolomite; and 6) when conditioned properly, does not contain naturally occurring organisms that may interfere with test organisms.

As seen in this study, sediment characteristics such as percent solids, pH, organic matter, organic carbon, particle size distribution, and cation exchange capacity were controlled as necessary to match a variety of natural sediments. However, redox potential of field sediments was not matched in this study without further manipulation. Other mineral constituents could also be developed (e.g. illite and calcite) to match locally occurring sediments. However, further experimentation is required if additional constituents are used in these cases. Control of these characteristics of sediments and possibly others will permit examination of organism tolerances to these characteristics. Development of formulated reference sediments may also provide a source of reference and dilution sediments that are of more consistent and reliable quality for use in sediment toxicity testing without the inherent disadvantages of using field collected sediments.

Other studies evaluating the suitability of formulated or synthetic sediments for use in sediment testing have been scarce. The initial development of formulated sediments was undertaken by Walsh *et al.* at the U.S. EPA Gulf Breeze Environmental Research Laboratory, Florida (Walsh *et al.*, 1991a, 1991b, 1991c, 1991d), examining the performance of marsh plants in formulated reference sediments. Walsh *et al.* (1991b) prepared formulated sediments that supported productive plant growth and permitted toxicological comparisons between a variety of marsh plants. Other studies demonstrating the utility of formulated reference sediments in assessing organism tolerances to sediment characteristics, as dilution sediments in sediment toxicity testing, and for use in sediment reference toxicity testing have been recently completed (Suedel and Rodgers, 1994a, 1995a).

SUMMARY

Formulated reference sediment constituents spanned the range of characteristics encountered in representative bottom sediments of the U.S. with respect to particle size distribution, organic matter, pH, solids and cation exchange capacity, thus allowing preparation of formulated reference sediments to match these characteristics. Matching other characteristics such as redox potential may require more effort. A conditioning period of 7 days were necessary for pH stabilization and *H. azteca*, *C. tentans*, *D. magna*, *C. dubia*, and *P. promelas* survival in 14 day formulated reference sediment exposures. Chronic culture experiments indicated that formulated reference sediment provided a suitable habitat for *D. magna*, *H. azteca* and *C. tentans* survival and/or reproduction. Naturally occurring sediments may possess physical, chemical or biological properties unsuitable for organism survival and reproduction, thus introducing uncertainty in definitive and other toxicity testing situations. These initial experiments and development efforts have provided evidence of the utility and potential for formulated reference sediments in advancing sediment quality evaluations as well as a variety of other research endeavors.

Section 2

RESPONSES OF *Hyalella azteca* AND *Chironomus tentans* TO PARTICLE SIZE DISTRIBUTION AND ORGANIC MATTER CONTENT OF FORMULATED AND NATURAL FRESHWATER SEDIMENTS

Two concerns may arise when using laboratory tests for determining the extent of contamination and potential toxicity of sediments. The first is whether or not the sediment is toxic. This concern can be resolved with screening level sediment toxicity testing. If sediment toxicity is observed in initial testing, the second concern is the degree of sediment toxicity. If adverse effects are observed for a contaminated sediment in screening level testing, the test sediment is then diluted with a clean, uncontaminated (reference) sediment to determine potency. Sand has been proposed for use as a reference sediment (OECD, 1981). However, reference or dilution sediments ideally should be similar to the contaminated sediment with respect to physical, chemical, and biological characteristics. If the test sediment and the reference or dilution sediment are not similar, then effects observed in definitive testing may not be due to chemical contamination, but rather to physical or biological characteristics of the reference or dilution sediment mixture.

The responses of test organisms to modifications of sediment physical and chemical characteristics must be known to accurately assess sediment quality. This may be valuable in evaluating results from benthic field surveys. In some instances, the absence of benthic organisms in bottom sediments may be due to physical factors such as storm events (which may resuspend clay and silt particles, thus altering habitat), or sediment characteristics such as particle size distribution and organic matter content of sediments that may be outside the preferred range for benthic organisms, rather than chemical contamination (Nichols, 1979). For example, the burrowing mayfly, *Hexagenia*, prefers adhesive muds or fine sandy muds with high organic matter and tend to avoid gravel, clay and sandy clay sediments (Wright and Mattice 1981; Thornley, 1985). Survival of *Rhepoxynius abronius* (marine amphipod) decreased in fine grained sediments and sediments high in organic matter (Dewitt *et al.* 1988).

Since sediment toxicity may be due to physical and biological characteristics as well as chemical contamination of sediments, the ability to distinguish between these effects would reduce the probability of concluding a sediment is chemically toxic when it is not. Responses of benthic species commonly used for freshwater sediment testing (e.g., *Hyalella azteca* (Saussure) and *Chironomus tentans* (Fabricius)) to sediment characteristics such as particle size distribution and organic matter have not been thoroughly investigated.

Reference sediments that possess similar characteristics as the test sediment are often not readily available. Naturally occurring reference sediments may also be contaminated, toxic, or contain extant fauna and flora that may inhibit growth, reproduction, or survival of test organisms, thus making the sediment undesirable for use as a reference or control sediment.

In response to these difficulties, formulated sediments were developed for use as reference and dilution sediments in definitive toxicity testing (Suedel and Rodgers, 1994b). Formulated sediments were developed to: 1) match field collected sediments with respect to both physical and chemical characteristics, and 2) to provide a suitable habitat for survival and reproduction of invertebrate species such as *H. azteca* and *C. tentans*. The ability to prepare formulated sediments representing a broad range of characteristics of naturally occurring sediments permits evaluation of benthic organism tolerances of a wide variety of sediment characteristics, including particle size distribution and organic matter content.

The purpose of this research was to determine responses of *H. azteca* and *C. tentans* to particle size regimes and organic matter content of natural freshwater and formulated sediments spanning the range of characteristics found in bottom sediments throughout the U.S. (Suedel and Rodgers, 1991). Formulated sediments were used for evaluating *H. azteca* and *C. tentans* tolerances of particle size classes and organic matter content of sediments further demonstrating the utility of using formulated sediments for this purpose.

MATERIALS AND METHODS

Culture Procedures

The amphipod, *H. azteca*, was selected as a representative freshwater epibenthic detritivore. *H. azteca* is relatively sensitive to a variety of organic and inorganic compounds (Borgmann *et al.* 1989; Ingersoll and Nelson, 1990), burrow into surficial sediments, and are in intimate contact with sediments (Nebeker *et al.*, 1984). The midge, *C. tentans*, was selected as a representative freshwater sediment infaunal organism. *C. tentans* directly contacts sediments by burrowing and constructing cases. *C. tentans* is a sensitive indicator of contaminants associated with sediments (Giesy *et al.* 1988).

H. azteca and *C. tentans* cultures were maintained at the University of Mississippi Biological Field Station (UMBFS) laboratory. *H. azteca* culturing procedures followed the methods of de March (1981). Amphipods were cultured under ambient laboratory temperature conditions (22$\pm$2° C) in 19 L glass aquaria covered with 1 mm mesh nylon screen. Amphipods were cultured in 15 L of filtered, dechlorinated tap water with vigorous aeration. Water in each aquarium was changed weekly. Amphipod substrate was fallen maple (*Acer rubrum*) leaves that had been previously soaked in reverse osmosis water for 7 to 10 days. Daily water changes allowed for removal of tannic acids from the leaves before placing in culture aquaria. Additional leaves were added to culture aquaria as needed. Amphipods were also fed a ground rabbit chow pellet suspension (Clover Brand, Madison, MS) twice each week (1.0 g food/3.0 ml water).

Amphipods used for testing were gathered from culture aquaria by removing a portion of the leaves from a culture aquarium. The leaves were placed on a 1.00 mm mesh stainless steel sieve and culture water was gently poured over the leaves through the 1.0 mm mesh sieve onto a 0.6 mm mesh sieve. Organisms (2-3 weeks old) that passed through the 1.0 mm sieve but were retained by the 0.6 mm sieve were collected and used for testing.

C. tentans culture methods followed those of Townsend *et al.* (1981). Midges were cultured under ambient laboratory temperature conditions (22$\pm$2 °C) in 39 L glass aquaria covered with 1 mm mesh nylon screen. Midges were maintained in 30 L of filtered (Whatman 340AH), dechlorinated tap water with vigorous aeration. Water in each aquarium was changed weekly. Culture substrate consisted of shredded brown paper towels (#ML96 Georgia Pacific, Battleboro, VT) to a depth of 3 cm. Midges were fed a 2.0 ml suspension (0.1 g food/1.0 ml water) of Tetra conditioning food (Tetrawerke, Germany) daily and 2.0 ml of a Cerophyll (Ward's Natural Science Establishment, Inc., Rochester, NY) suspension (0.025 g cerophyll/ 1.0 ml water) weekly. Midges used for testing were second instar (8 days old post-hatch).

Sediment Characterization

Particle size distribution of sediments was determined using a hydrometric method (Gee and Bauder, 1986). Percent organic matter (percent volatile solids) was determined as percent of initial dry weight of sample remaining after ashing in a muffle furnace at 550 °C for 1 hour (Plumb, 1981). Cation exchange capacity followed the displacement-after-washing method of Plumb (1981). Sediment pH was measured with an Orion pH probe according to the method of Plumb (1981). Percent sediment solids was calculated as percent of initial wet weight of sample remaining after drying at 104 °C for 24 hours (Millar *et al.*, 1965).

Formulated Sediments

Silica sands (Mystic White® #18 and #90; New England Silica, Inc., South Windsor, CT) were dry sieved into coarse (2.0-0.5 mm), medium (0.5-0.25 mm), and fine (0.25-0.05 mm) sand fractions using a SoilTest sand shaker before use. ASP600® and ASP900® (kaolinite clay minerals), Attagel 50® and Attasorb LVM® (attapulgite clay minerals) were used for the clay fraction of formulated sediments. ASP400® and Attacote LVM2® were used as the silt fraction. Kaolinite and attapulgite minerals were purchased from Engelhard Corp., Pigments and Additives Division, Edison, NJ. ASP600®, ASP900®, Attagel 50®, and Attasorb LVM® were combined in equal quantities on a dry weight basis. This mixture combined with montmorillonite in appropriate quantities represented the clay fraction. ASP400® and Attacote LVM2® were combined in equal quantities to represent the silt fraction. "Organic Humus" from Sims Bark Co. Inc., Tuscumbia, AL, was used as recalcitrant particulate organic matter in sediments. Humus was dried at 70 °C and milled to <2.0 mm (2.00-0.05 mm = 98%, <0.05 mm = 2%) in a Wiley Mill before use. Dolomite was obtained from Ward's Natural Science Establishment, Inc. and was used to represent bicarbonate buffers occurring in soils and sediments. Montmorillonite (smectite) clay from Apache County, AZ (Prof. W.D. Johns, Source Clays, Univ. of MO, Columbia, MO) was used as a typical clay mineral occurring in sediments throughout the U.S. (Weaver 1989). Because of the relatively high CEC of smectite, it was selected for use in formulated sediments to match field collected sediments with high CECs (Suedel and Rodgers, 1994b). All constituents were conditioned separately in plastic tubs in a cement raceway at the

UMBFS under flow through conditions for 7 days to permit colonization by various microorganisms such as algae, diatoms, and bacteria. Physical and chemical properties of formulated sediment constituents are given in Table 2-1. Additional information regarding formulated sediment development is given in Suedel and Rodgers, 1994b).

Particle size distribution and organic matter content of sediments were selected for evaluation in this study based on:

1) The habitat preference of *C. tentans* for silty sediments (Acton and Scudder, 1971; Topping, 1971) and use of detrital sediment organic matter as a food source (Oliver, 1971);
2) The observed occurrence of *H. azteca* in a wide variety of sediment types (Strong, 1972) and use of sediment organic matter and detritus as a food source (Cooper, 1965; Hargrave, 1970);
3) Previous studies reporting particle grain size and organic matter content of sediments affecting substrate selection by freshwater and marine invertebrate species (Wright and Mattice, 1981; DeWitt *et al.*, 1988; Oakden, 1984; Meadows, 1964).

Selection of particle size regimes in sediment tolerance tests was based on the USDA textural triangle (Millar *et al.*, 1965) (Figure 2-1) and encompassed the range of particle sizes found in bottom sediments throughout the U.S. (Suedel and Rodgers, 1991; Weaver, 1989). Based on such data, clay content in bottom sediments of both freshwater and marine systems seldom achieve 60%, therefore sediments containing >60% clay were not examined. Other sediment characteristics chosen for evaluation cover the extremes of particle sizes encountered in bottom sediments. Thus they represent the entire range of expected cases in terms of test organism exposure to these particle regimes as well as sediments representing intermediate mixtures of all three particle size classes (sands, silts, and clays). Selection of organic matter content in sediment tolerance tests was based on Ritchie's (1989) data showing organic matter content of sediments in freshwater reservoirs across the U.S. ranging from 0.3 to 10%.

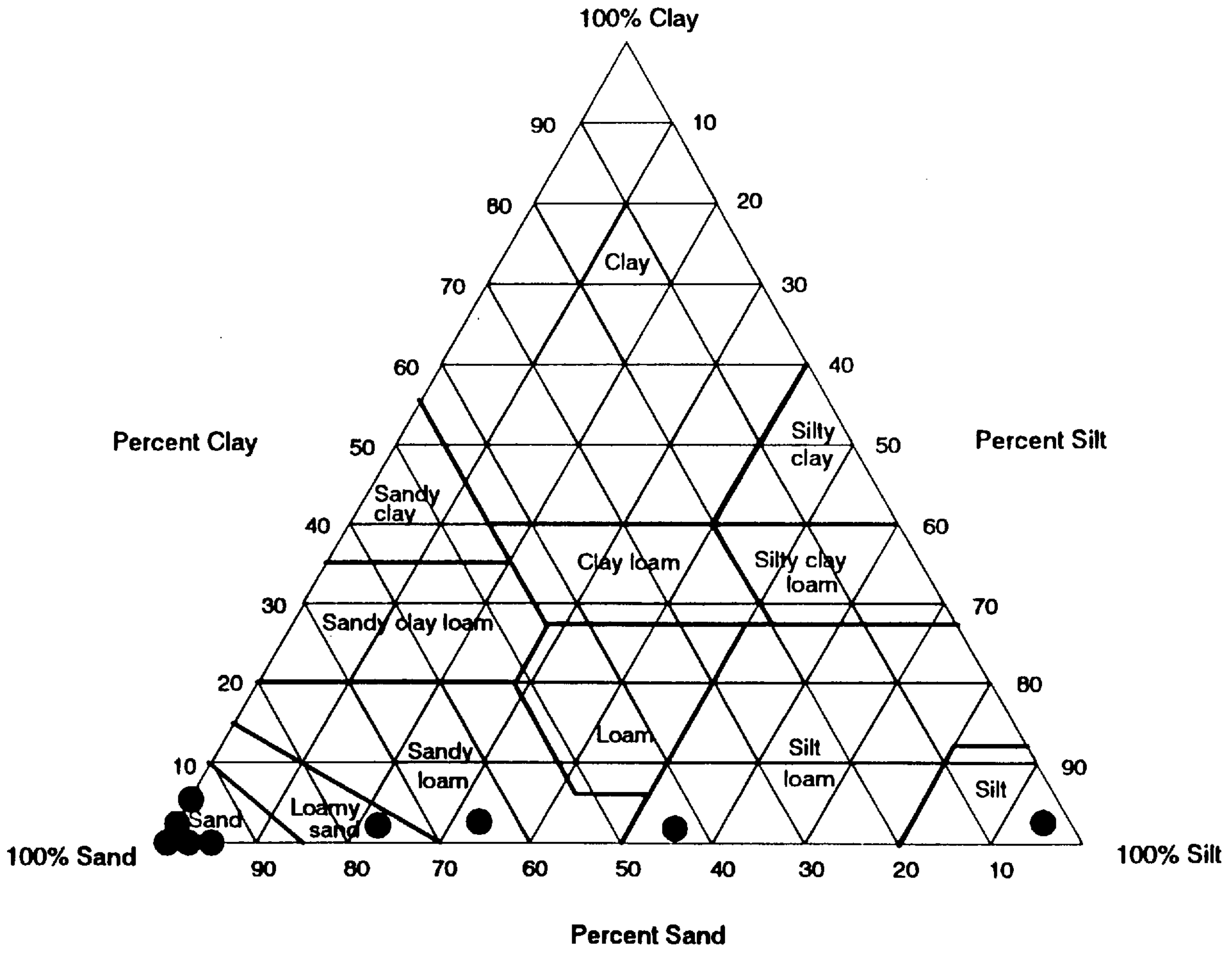

Figure 2-1. USDA textural triangle. Circles represent particle size distribution of one or more field collected sediments used in this study.

Table 2-1. Physical and chemical properties of formulated sediment constituents after conditioning.

Constituent	Mineral	Particle Size (um)	CEC (me/100g)	Solids (%)	Organic Matter (%)	pH*
ASP 600	clay	0.6	1.59	52.12	13.6	3.5
ASP 900	clay	1.5	2.53	56.21	13.2	3.4
Montmorillonite (smectite)	clay	<2.0	120	46.59	8.79	7.4
Attasorb LVM	clay	2.0	24.4	35.28	10.3	-
Attagel 50	clay	0.1	22.1	21.25	16.5	-
ASP 400	silt	4.8	2.87	62.61	13.2	3.4
Attacote LVM2	silt	40	12.1	36.31	10.7	-
Mystic White #18 coarse sand	silica	500-850	-	88.76	1.0	6.8
Mystic White #90 medium sand	silica	250-500	-	75.30	0.02	7.0
Mystic White #90 fine sand	silica	50-250	-	61.45	1.8	6.8
Humus	organic matter	<2.0 mm	17.3	30.06	86.9	5.9
Dolomite	buffer	<0.05 mm	0.27	84.91	1.31	8.5

*pH measurements taken 7 days after preparing formulated sediments.

Field Collected Sediments

Sixteen natural freshwater sediments were used to further evaluate *C. tentans* and *H. azteca* responses to particle sizes and organic matter content of formulated sediments and to validate the use of formulated reference sediments in predicting responses of test organisms to natural sediments. Four sediment samples were collected from relatively pristine areas of both the Pascagoula (sediments P1-P4) and Escatawpa Rivers (sediments E1-E4) near the Mississippi Gulf coast in Jackson County, Mississippi. Three sediments were collected from the UMBFS, Oxford, Mississippi. UMBFS1 sediment was collected from a stream, while UMBFS 2, UMBFS 3 (control), and UMBFS 4 sediments were collected from spring-fed ponds at the UMBFS. Other sediments used in organism tolerance tests were collected from Lake Holbrook (HOL) in Wood County, Texas, Eagle Mountain Lake (EML) in Tarrant County, Texas, and the Water Research Field Station (WRFS) in Denton County, Texas. Flat Rock (FR) sediment (Flat Rock Bagging, Flat Rock, MI) is a coarse silica sand soil that has been used previously as a reference sediment (M. Nelson, U.S. FWS, Columbia, MO, personal communication). Characterization results of these sediments are presented in Table 2-2. Modifications of particulate fractions of field collected sediments were performed by wet sieving sediments into coarse, medium, and fine sand fractions and mixing appropriate quantities of each to achieve the desired particle size distribution. Except for Flat Rock sediment, all sediments were collected using a grab sampler and were prepared according to established procedures (Plumb, 1981).

Sediment Experiments

All experiments were conducted in light (16 h light/8 h dark photoperiod) and temperature (21$\pm$1°C) controlled incubators. *H. azteca* and *C. tentans* were exposed to either mixtures of preconditioned formulated sediment or field collected sediments separately in 250 ml beakers for 10 days without aeration. Each beaker contained 40 ml of sediment and 160 ml UMBFS pond water (1:4 sediment to water ratio). Sediment/water mixtures

Table 2-2. Characteristics of field collected sediments used to identify organism tolerances. All values reported as percentages.

Sediment[*]	Solids	Organic Matter	Clay	Silt	Total Sand	Coarse Sand	Medium Sand	Fine Sand
UMBFS 1	76.35	0.30	0.0	0.0	100	46.0	53.0	1.0
UMBFS 2	79.31	0.31	0.0	0.6	99.4	38.7	30.9	25.3
UMBFS 3(control)	72.95	1.68	2.7	21.4	75.9	8.4	31.7	35.8
UMBFS 4	41.51	7.80	2.4	93.8	3.8	-	-	-
E1	76.67	0.15	1.0	0.8	98.0	2.3	78.6	17.1
E2	76.92	0.16	0.0	1.9	98.1	2.0	78.8	17.3
E3	76.75	0.12	0.0	0.8	99.2	2.6	83.2	13.4
E4	77.13	0.13	1.9	0.0	98.1	2.4	80.7	15.0
P1	76.55	0.19	1.8	0.0	98.2	0.1	29.4	68.7
P2	76.93	0.16	4.7	0.0	95.3	0.1	19.3	75.9
P3	77.58	0.17	0.0	4.8	95.2	0.0	13.3	81.9
P4	75.95	0.13	0.0	1.9	98.1	0.0	14.9	83.2
EML	60.30	5.48	-	-	-	-	-	-
HOL	76.39	0.91	0.5	32.1	67.4	-	-	-
WRFS	68.17	4.08	1.3	56.0	42.7	-	-	-
FR	-	0.42	0.0	0.0	100	78.8	18.5	2.7

[*]UMBFS = University of Mississippi Biological Field Station, E1-E4 = Escatawpa River, P1-P4 = Pascagoula River, EML = Eagle Mountain Lake, HOL = Lake Holbrook, WRFS = Water Research Field Station, and FR = Flat Rock Bagging sediments, respectively.

were allowed an overnight contact period before adding organisms. Experiments were started by adding either 10 amphipods or 6 midges to each of four replicate beakers per treatment (three replicates for formulated sediment tests). *H. azteca* were fed approximately 0.5 g ground maple leaves (organic matter content = 92.6%; prepared as described in culture procedures) on the initial day of each amphipod test and *C. tentans* were fed 2 drops of Tetra conditioning food suspension daily. Test organisms were fed because of the inability of some sediments to provide sufficient food for >80% test organism survival in 10 day exposures; therefore, *H. azteca* and *C. tentans* were fed in all experiments. The organic matter content of both food sources was not accounted for in the estimation of organic matter content of sediments.

Water quality characteristics of the initial UMBFS pond water and the overlying water during formulated and natural sediment tests are presented in Table 2-3. UMBFS pond water has no observable toxicity in tests with a variety of limnetic and benthic invertebrate species including *H. azteca* and *C. tentans* in 10 day exposures in the absence of sediments.

Data Analysis

Statistical analyses were performed to determine the extent of *H. azteca* and *C. tentans* tolerance of organic matter and particle size distribution of formulated and natural sediments. The criterion for acceptability (tolerance) of sediments with varying particle sizes was >80% survival, i.e., the level of test acceptance in aqueous and sediment toxicity testing (ASTM, 1990; U.S. EPA, 1989). The test endpoint was mortality, defined as no visible signs of organism movement after gentle prodding. SAS (1989) univariate procedure was used to determine whether or not data were normally distributed. Analysis of variance (ANOVA) was used to detect statistically significant differences between means ($p \leq 0.05$) and Dunnett's test was used to locate differences between treatment means (Gulley *et al.*, 1989). Nonparametric Spearman correlation coefficients (SAS, 1989) were used to measure the intensity of the relationships between field sediment characteristics and *C. tentans* survival.

Table 2-3. Ranges of water quality characteristics measured during field sediment tests, formulated sediment tests, and for initial UMBFS pond water*.

Parameter	UMBFS Pond Water	Formulated Sediment Tests	Field Sediment Tests
Dissolved Oxygen (mg/L)	7.0-9.0	6.3-8.8	5.6-8.5
pH	6.2-7.2	5.7-8.0	6.0-7.2
Hardness (mg/L as $CaCO_3$)	6-12	10-50	6-32
Alkalinity (mg/L as $CaCO_3$)	8-20	30-50	9-36
Conductivity (umhos/cm)	5-30	20-550	25-125

*Water quality measurements were made at the start, end, and throughout each experiment.

RESULTS AND DISCUSSION

H. azteca response to particle size regimes

Experiments examining *H. azteca* responses to particle size regimes of formulated sediments conditioned for 7 days indicated amphipods were tolerant of nearly all sediment particle regimes examined (Figure 2-2). *H. azteca* survival was ≥84% except for formulated sediments containing 100% medium sand. These results indicate that the sediment particle regimes tested, with the exception of 100% formulated sand, are suitable substrates for *H. azteca* survival (>80%) in 10 day experiments.

An experiment was then conducted with silica sand particles of formulated sediments to determine *H. azteca* responses to 100% coarse, medium, and fine sands that had been conditioned for 14 days (n=5 replicates for each sand treatment). *H. azteca* exposed to sand particles of formulated sediments that had been conditioned for 14 days resulted in amphipod survival of 97% for coarse sand, 96% for medium sand, and 90% for fine sand. The intolerance of *H. azteca* to 100% sand particles was apparently due to an insufficient conditioning period of formulated sediments rather than *H. azteca* intolerance of sand particles. To verify these results, experiments were conducted with field collected sediments.

To verify *H. azteca* tolerance of sand particles, UMBFS1, UMBFS2, and Flat Rock sediments were modified by wet sieving such that the resulting sand particle regimes would be expected to elicit toxicity of *H. azteca* based on initial tests (Figure 2-2) using formulated sand constituents (e.g. ≥90% medium or fine sand). *H. azteca* exposure to modified particle size regimes of sediments resulted in sufficient survival in all cases: 1) UMBFS1 - 100% medium sand - 92% survival; 2) UMBFS2 - 100% medium sand - 88% survival; 3) UMBFS2 - 100% fine sand - 88% survival; and 4) FR - 90% medium/10% fine sand - 92% survival. These results further demonstrate that *H. azteca* is tolerant of sandy sediments with predominantly medium and fine sand fractions.

Additional experiments were conducted using field collected sediments to verify *H. azteca* tolerances of a wide variety of particle size regimes. *H. azteca* exposed for 10 days to

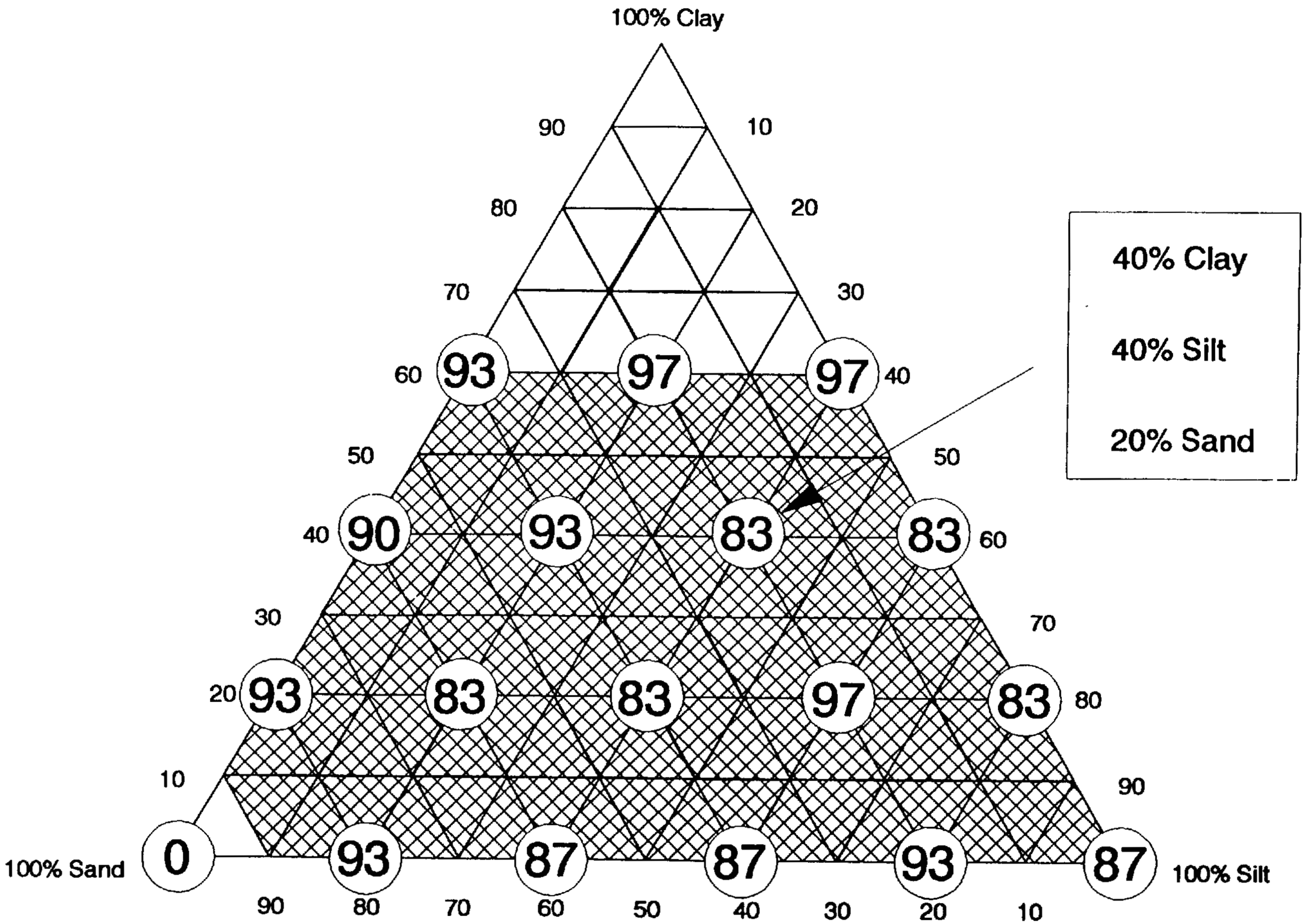

Figure 2-2. *H. azteca* tolerance of various particle regimes of formulated sediments. Numbers within circles indicate percent survival of *H. azteca* exposed to each corresponding particle size regime. Hatched area indicates >80% *H. azteca* survival.

field collected sediments resulted in >80% amphipod survival in all sediments (Figure 2-3). *H. azteca* survival ranged from 84 to 100% for all field sediments examined, with particle sizes ranging from 4-100% sand, 0-94% silt, and 0-5% clay.

H. azteca Response to Organic Matter

There was no statistically significant reduction in survival of *H. azteca* in the control sediment (UMBFS3) compared to field collected sediments with organic matter contents ranging from 0.12 to 7.80% (Figure 2-3), indicating that *H. azteca* survival was not adversely affected by organic matter content of sediments ranging from 0.12 to 7.80% in 10 day exposures. These results are not unexpected, based on observations of *H. azteca* utilizing organic debris and decayed plant and animal material in substrates associated with rooted aquatic vegetation (Cooper, 1965).

Results from formulated and field collected sediment experiments indicate *H. azteca* is tolerant (>80% survival) of sediment particle regimes ranging from 100% sand and 100% silt to 60% clay content, which represent the range of particle sizes of sediments encountered throughout the U.S. (Suedel and Rodgers, 1991). These findings are in agreement with field studies describing *H. azteca* habitat (substrate) preferences. *H. azteca* has been observed in lake substrates with a wide variety of particle sizes and organic matter content ranging from sand and gravel to fine silts (Strong, 1972; Hargrave, 1970). Amphipod mortality observed with formulated medium and fine sands was initially due to an insufficient conditioning period (7 days) of these constituents. Conditioning formulated sand constituents for 14 days resulted in *H. azteca* survival ranging from 90-97%.

By initially using formulated sediments to assess organism tolerances of particle sizes, time and effort are saved in locating and collecting a wide variety of uncontaminated field sediments that may or may not be available. If intolerances are observed using formulated sediments, then field collected sediments can be used to verify results from formulated sediment tests. In this capacity, formulated sediments may serve as a useful tool in determining adverse effects of particle size regimes on benthic organisms. Acquired knowledge of *H. azteca* tolerance of sediment particle sizes will help decrease the probability of making erroneous conclusions regarding sediment toxicity. *H. azteca* tolerance of a wide variety of particle size regimes and organic matter content of sediments (0.12 to 7.8%

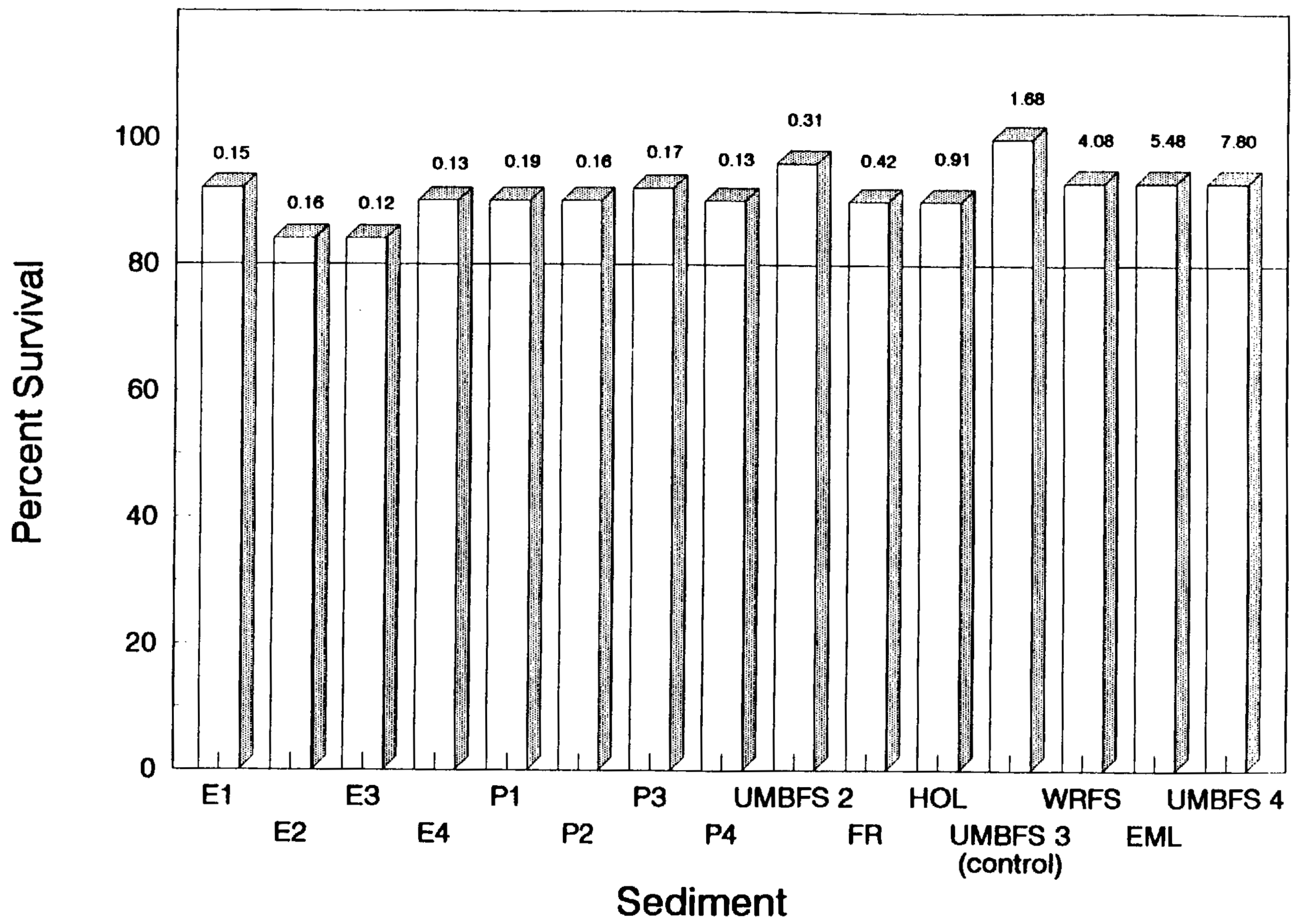

Figure 2-3. *H. azteca* exposed to field collected sediments for 10 days. Numbers above bars indicate percent organic matter content of each sediment. Percent survival was not significantly different between sediments ($p<0.05$).

organic matter) makes *H. azteca* a suitable benthic species for sediment toxicity testing in determining sediment quality.

C. tentans Response to Particle Size Regimes

C. tentans response to varying particle size regimes of formulated sediments resulted in midge survival ranging from 6 to 83%, indicating a distinct intolerance of nearly all particle regimes examined except for formulated sediments with 80-100% silt content (Figure 2-4). The observed intolerance was likely due to sand, silt, and clay constituents used to prepare formulated sediments containing no visible signs of particulate organic matter (no humus was added) that *C. tentans* utilizes as a food source and for case building (Oliver, 1971). This also indicates that *C. tentans* requires some minimum particulate organic matter concentration for acceptable survival.

A second experiment was then conducted with formulated sediments in which particulate organic matter was added (2.5% organic matter as humus) on a dry weight basis to all formulated sediment particle regimes examined (Figure 2-5). When particulate organic matter was present in sufficient quantities in sediment (i.e., 2.5%), adequate survival ($\geq$80%) of *C. tentans* for all particle regimes tested was achieved varying concentrations of humus ranging from 0.14 to 1.34% organic matter. In this experiment, all silt and clay constituents were ashed at 550 °C to remove organic matter present in these constituents so that the humus fraction was the only constituent contributing to the organic matter content of these sediments.

C. tentans Response to Organic Matter

To better quantify *C. tentans* intolerance of low sediment organic matter content, midges were exposed to natural sediments with organic matter content ranging from 0.12 to 7.8% (Figure 2-6). *C. tentans* exposed to UMBFS2 sediment, Pascagoula River sediments P1-P4, and Escatawpa River sediments E1-E4 for 10 days resulted in significant midge mortality (Figure 2-6). *C. tentans* survival in these sediments ranged from 7 to 46%, indicating significant intolerance of sediments with $\leq$0.3% organic matter content. When exposed to HOL, EML, WRFS, and UMBFS 3 and 4 sediments with organic matter content ranging from 0.91 to 7.8%, *C. tentans* survival ranged from 84 to 100%. These data indicate

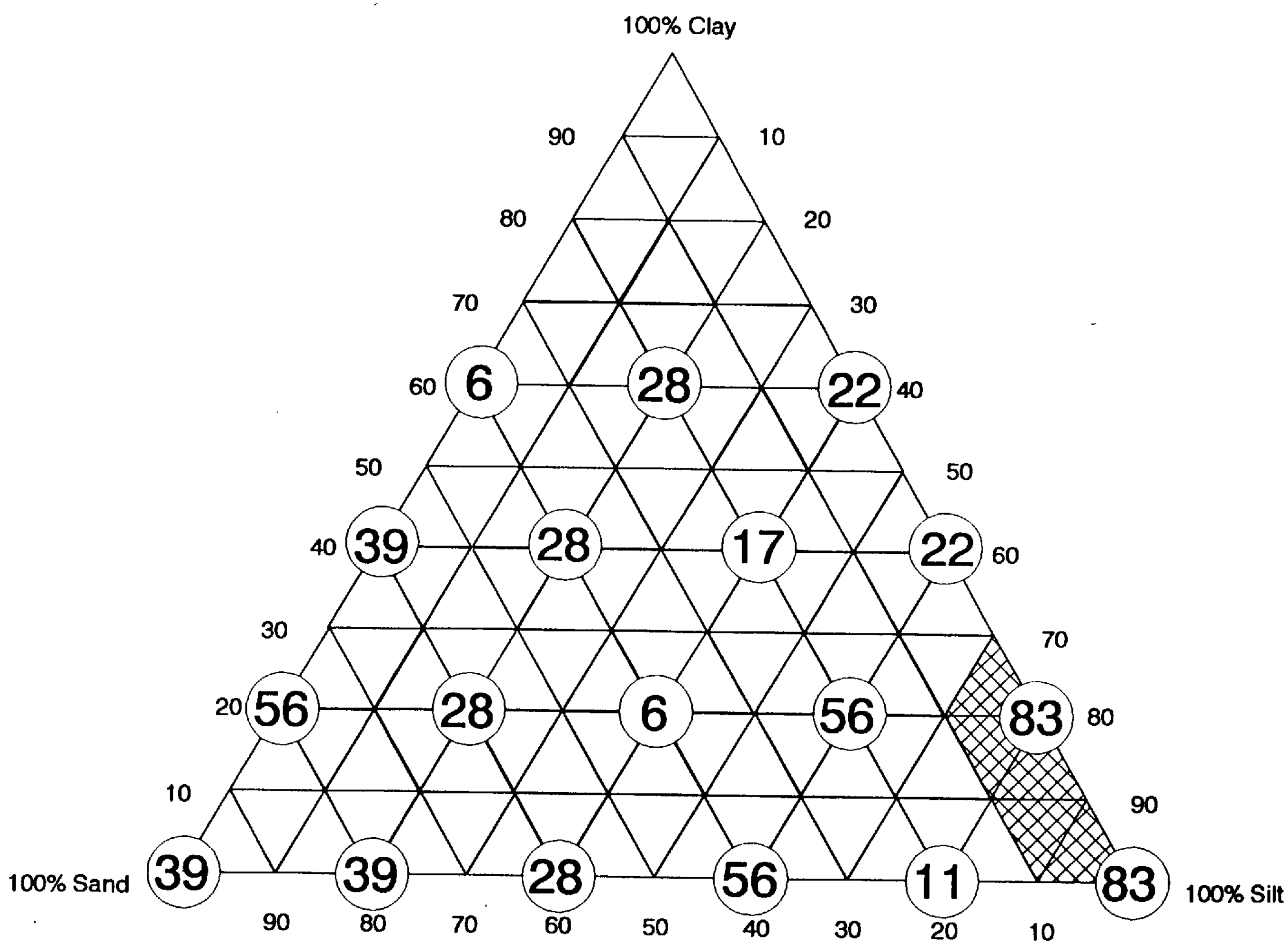

Figure 2-4. *C. tentans* tolerance of various particle regimes of formulated sediments (0% particulate organic matter content). Numbers within circles indicate percent survival of *C. tentans* exposed to each corresponding particle size regime. Hatched area indicates >80% *C. tentans* survival.

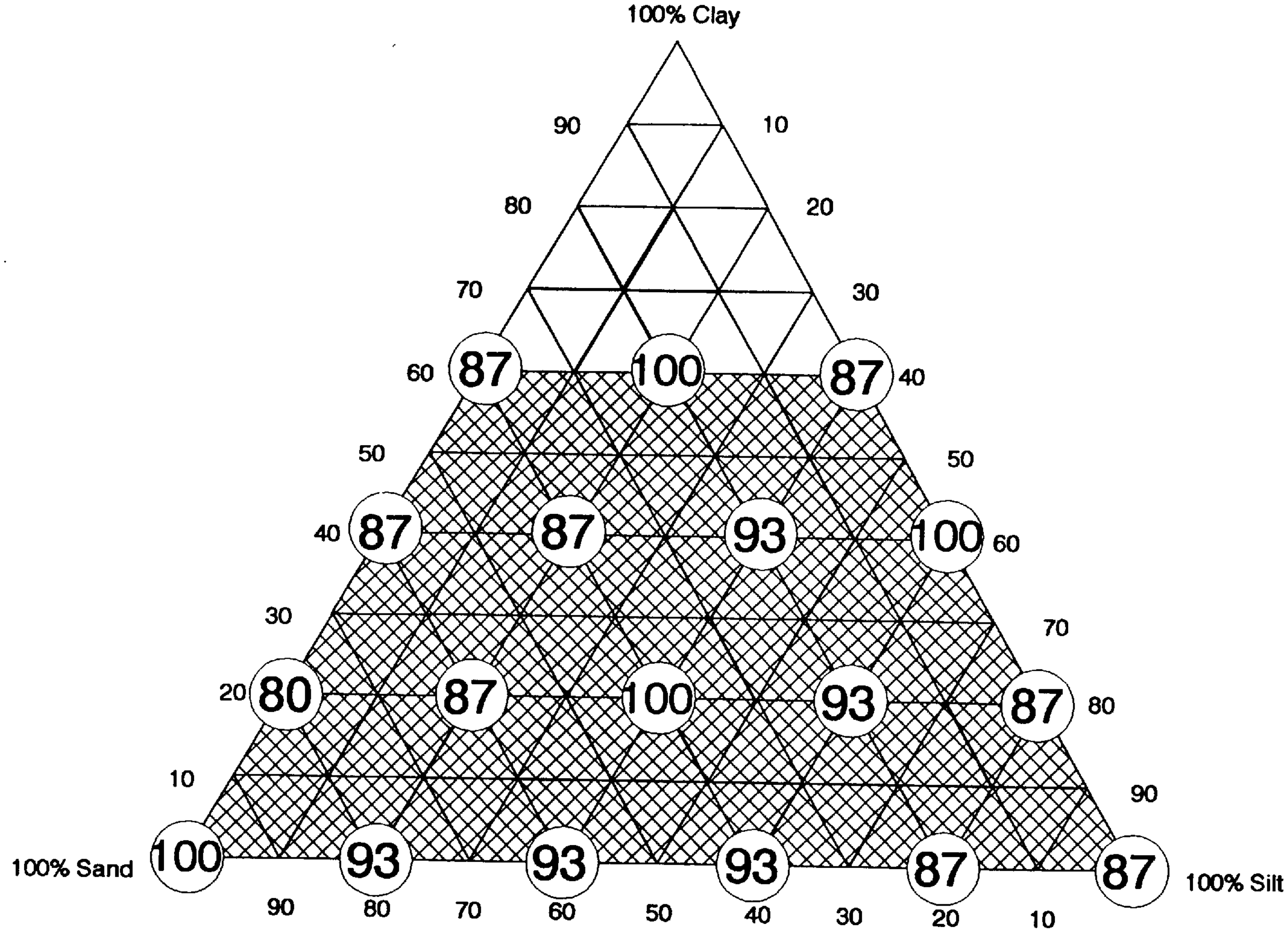

Figure 2-5. *C. tentans* tolerance of various particle regimes of formulated sediments with 2.5% particulate organic matter added as humus. Numbers within circles indicate percent survival of *C. tentans* exposed to each corresponding particle size regime. Hatched area indicates >80% *C. tentans* survival.

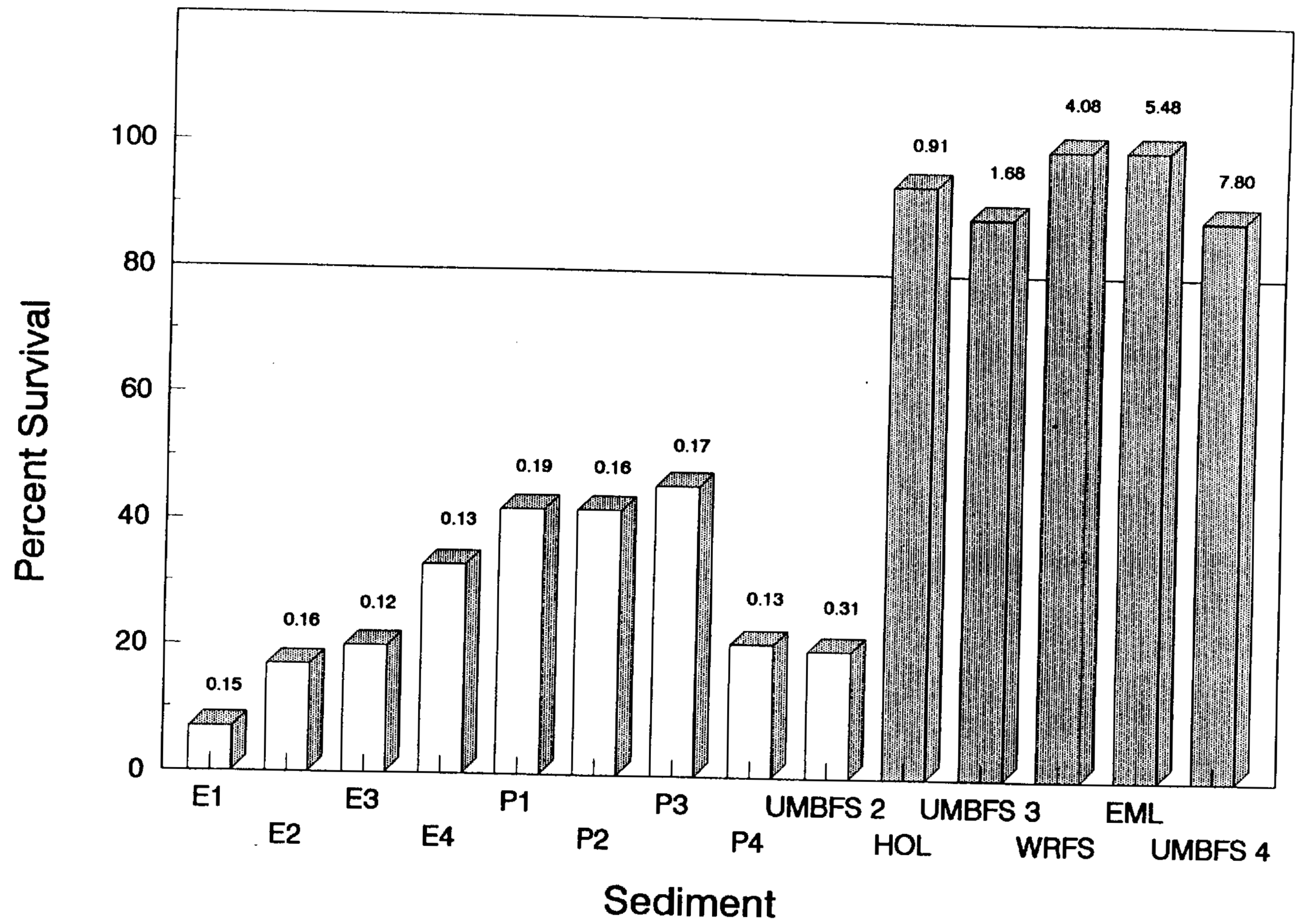

Figure 2-6. *C. tentans* exposed to field collected sediments for 10 days. Numbers above bars indicate percent organic matter content of each sediment. Bars of the same color are significantly different from each other ($p<0.05$).

that ≥0.9% organic matter content of these sediments was suitable for *C. tentans* survival in 10 day exposures.

To demonstrate formulated sediment utility as a viable method for determining *C. tentans* tolerance of organic matter content of sediment and to more accurately describe the tolerance range of *C. tentans* for organic matter, an additional experiment was conducted. For this experiment, all formulated sediment constituents listed in Table 2-1 were prepared with varying concentrations of humus ranging from 0.14 to 1.34% organic matter.

In this experiment, all silt and clay constituents were ashed at 550 ° C to remove orgnic matter present in these constituents so that the humus fraction was the only constituent contributing to the organic matter content of these sediments.

As Figure 2-7 illustrates, humus content of formulated sediments had a significant effect on *C. tentans* survival in 10 day whole sediment exposures. *C. tentans* survival ranged from 13 to 83%, with statistically significant reductions in survival (below the acceptable level of 80%) occurring in sediments with organic matter content ≤0.76%. The results from this experiment were similar to the data presented in Figure 2-6 showing *C. tentans* survival of >80% in formulated sediments with organic matter content ≥0.91%.

Data from experiments examining *C. tentans* intolerance of low levels of organic matter using formulated sediments agree with *C. tentans* intolerance to low levels of organic matter in field collected sediments. Survival of *C. tentans* was statistically significantly reduced when exposed to sediments with low organic matter content. Midge survival was noticeably below the acceptable level (≥80%) when formulated sediment contained ≤0.91% organic matter. These results are not unexpected based on studies examining *C. tentans* habitat preferences, where *C. tentans* is commonly associated with silty sediments and sediments high in organic matter (Acton and Scudder, 1971; Topping, 1971).

Based on the formulated and field collected sediments used in this study, a threshold organic matter content between 0.76 and 0.91% exists for *C. tentans*. The results also indicate that the presence of particulate organic matter in bottom sediments plays a major role in *C. tentans* tolerance of bottom sediment characteristics. The midges were observed feeding on and collecting humus to build cases in all experiments so that all humus visible on the sediment surface had been used as a food source and for case building by the end of an experiment.

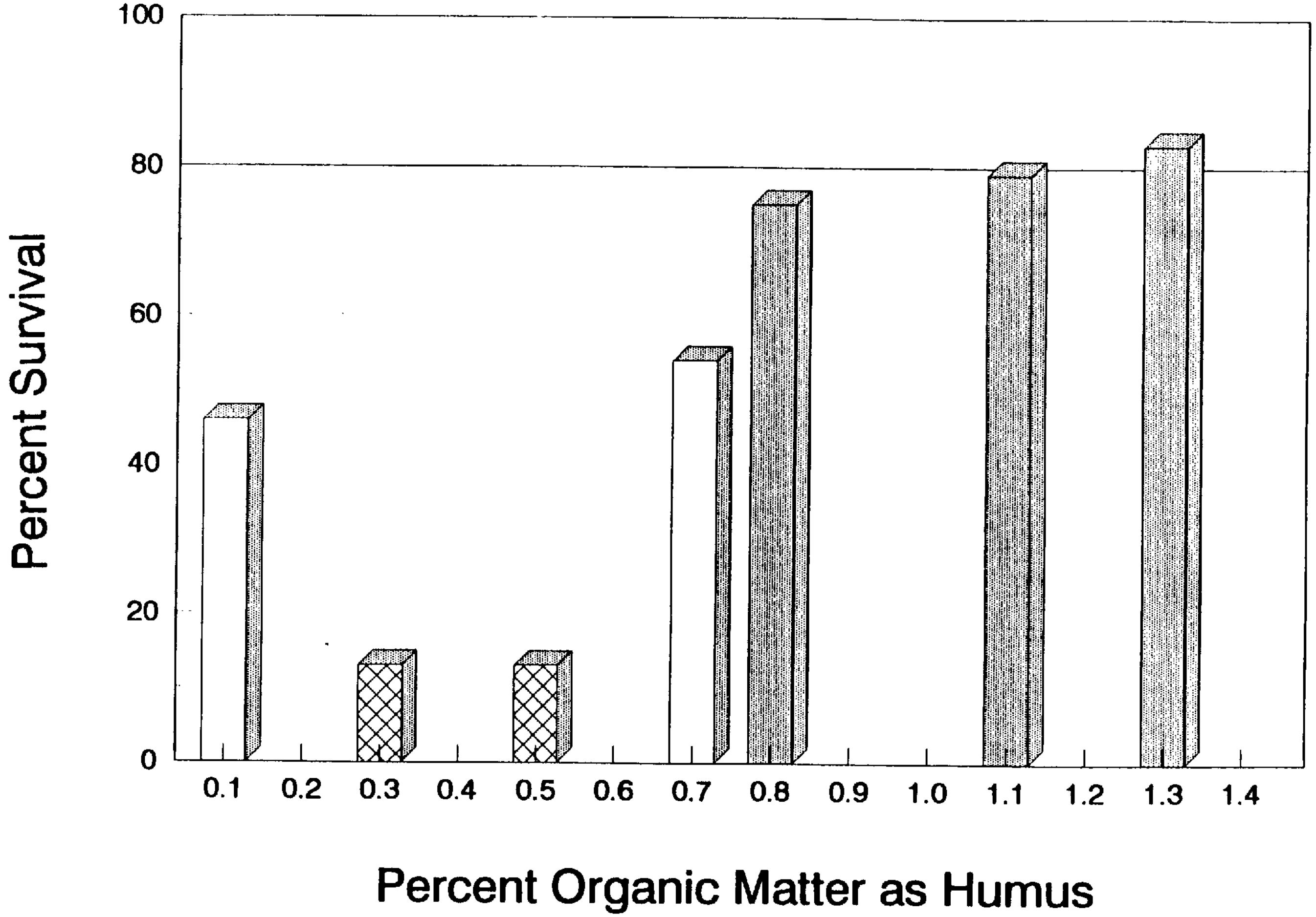

Figure 2-7. Survival of *C. tentans* exposed for 10 days to formulated sediments containing varying amounts of humus. Bars of the same color are not significantly different from each other ($p<0.05$).

Spearman correlation coefficients were calculated for the natural sediment test results to determine the relationships between measured sediment characteristics and *C. tentans* survival (Table 2-4). As expected, *C. tentans* survival was positively correlated with percent silt and organic matter, and negatively correlated with percent sand and solids. Correlations between these sediment characteristics are not surprising because many of these characteristics covary (Table 2-4). For example, organic matter is negatively correlated with percent sand and solids, and positively correlated with percent silt. These data are in agreement with the correlations found in Suedel and Rodgers (1991) between organic carbon and percent sand, silt, clay, and solids of freshwater and marine sediments from throughout the U.S.

In this study, formulated sediments served as a useful tool for determining adverse effects of particle sizes and organic matter content on *C. tentans* performance in 10 day whole sediment experiments. Acquired knowledge of *C. tentans* tolerance of particle size regimes and organic matter content of formulated sediments will help decrease the probability of making erroneous conclusions regarding sediment toxicity. Tolerances of a wide variety of particle size regimes (0-100% sand, 0-100% silt, and 0-60% clay) and organic matter content (0.91% to 7.8%) of sediments makes *C. tentans* a suitable benthic species for sediment toxicity testing for determining sediment quality. However, *C. tentans* exposed to sediments with organic matter content 0.91% may result in reduced survival due to physical characteristics rather than chemical contamination.

CONCLUSIONS

When formulated sediment constituents were sufficiently conditioned, *H. azteca* survival was >80% when exposed to all particle size regimes of formulated sediments. *H. azteca* exposure to field collected sediments resulted in amphipod survival of >80% in all sand particle regimes examined. *H. azteca* was tolerant (>80% survival) of formulated and field collected sediments with organic matter content ranging from 0.12 to 7.8%. *C. tentans* was not tolerant (survival ranging from 6 to 56%) of nearly all formulated sediment particle regimes (except for 80 to 100% silt) without a source of particulate organic matter (e.g., humus) although dissolved organic matter was present up to 13%. Formulated and field collected sediments containing sufficient concentrations of organic matter (0.91 to 7.8%) resulted in >80% survival for *C. tentans* in 10 day whole sediment exposures. *C. tentans* survival was also >80% in all formulated sediment particle sizes when sufficient particulate organic matter was present (2.5%). Formulated sediments were suitable for determining *H. azteca* and *C. tentans* tolerance of organic matter and particle size distribution of sediments. Based on these results, *H. azteca* and *C. tentans* are tolerant of a wide range of particle size regimes and organic matter content of bottom sediments, thus making them suitable test organisms to assess sediment quality in most situations. Formulated sediments should be suitable for evaluating tolerances of other benthic toxicity testing organisms for sediment characteristics, thus reducing the risk of drawing erroneous conclusions regarding sediment toxicity.

Table 2-4. Spearman correlation coefficients, associated probabilities, and number of replicates (N) for each coefficient listed for field collected sediment characteristics and *C. tentans* survival. Asterisks mark coefficients that are significant at $p \leq 0.05$.

	Sand	Silt	Clay	Organic Matter	Solids
Silt	-0.76* 0.001 (15)				
Clay	-0.61* 0.016 (15)	0.04 0.88 (15)			
Organic Matter	-0.50 0.056 (15)	0.59* 0.021 (15)	0.17 0.55 (15)		
Solids	0.48 0.084 (14)	-0.54* 0.048 (14)	-0.25 0.39 (14)	-0.55* 0.032 (14)	
C. tentans Survival	-0.87* 0.0001 (13)	0.60* 0.029 (13)	0.45 0.12 (13)	0.73* 0.033 (14)	-0.59* 0.027 (14)

Section 3

COPPER SULFATE AS A REFERENCE TOXICANT FOR USE IN SEDIMENT TOXICITY TESTS

The lack of a suitable reference toxicant to assess the condition or health of benthic test organism populations is a hindrance to the precision and accuracy in definitive whole sediment toxicity tests. Although reference tests are commonly conducted with aqueous tests (Lee, 1980), whole sediment reference tests are seldom conducted in conjunction with sediment toxicity tests.

Standard protocols for sediment toxicity tests (U.S. EPA/COE, 1991) recommend the use of reference toxicant tests. These protocols suggest performing reference toxicant tests in the absence of sediment, even for those benthic test organisms that spend much of their life burrowing in sediment. Benthic animals tested without a burrowing substrate may be stressed, resulting in increased test organism mortality (Dorn *et al.*, 1987; Wright and Mattice, 1981; Environment Canada, 1990). Reference tests using benthic organisms should be conducted using a sediment substrate to reduce organism stress, provided the particle size tolerance and other habitat requirements of the test organism are known. The presence of sediment may affect the fate or persistence of the reference toxicant by sorption and biotransformation, thus altering toxicant bioavailability and toxicity. Therefore, development of a suitable reference sediment is required in conjunction with development of an appropriate sediment reference toxicant.

Some reference toxicants utilized previously in aqueous reference testing such as copper sulfate (Carlson *et al.*, 1986; U.S. EPA, 1989) may also be appropriate for use as a sediment reference toxicant. A suitable sediment reference toxicant should elicit a response from benthic organisms at a fraction of its aqueous solubility and not have a propensity to sorb to sediments, or at least the extent and rate of sorption should be readily predictable.

Development of a reference toxicant for whole sediment toxicity testing would provide: 1) a means of assessing the health and sensitivity of test organisms, 2) a mechanism for monitoring intra-laboratory precision of test results over time, and 3) a common "yardstick" through which independent laboratories could evaluate results on the same test substances through time.

The objective of this study was to determine whether copper sulfate is suitable as a reference toxicant for use in whole sediment toxicity tests. Also discussed is the rationale for selecting copper sulfate as a sediment reference toxicant and proposed selection criteria for sediment reference toxicants.

In combination with the identification of a satisfactory reference toxicant, development of a reference sediment for toxicity testing will provide a starting point for standardization of sediment toxicity tests. Without suitable sediment reference tests to measure the relative health of field-collected and laboratory-reared benthic species, errors in assessment of sediment toxicity or potency are likely.

MATERIALS AND METHODS

Culture Procedures

H. azteca (Saussure), an epibenthic amphipod, was selected for experimentation because it is sensitive to a wide variety of toxic materials occurring in aquatic systems (Ingersoll and Nelson 1990). *H. azteca* cultures were maintained at the University of Mississippi Biological Field Station (UMBFS) laboratory. *H. azteca* culturing procedures followed the methods of de March (1981). Amphipods were cultured under ambient laboratory temperature conditions (22$\pm$2 °C) in 19 L glass aquaria covered with 1 mm mesh nylon screen. Amphipods were cultured in 15 L of filtered, dechlorinated tap water with moderate aeration. Amphipod culture substrate was fallen maple (*Acer rubrum*) leaves that had been previously soaked in ultra purified water for 7 to 10 days. Additional leaves were added to culture aquaria as needed. Amphipods were also fed ground rabbit chow pellets (Clover Brand, Madison, MS) twice each week.

Amphipods were gathered for testing by removing a portion of the leaves from a culture aquarium. The leaves were placed on a 1.0 mm mesh stainless steel sieve and culture water gently poured over the leaves through the 1.0 mm mesh sieve onto a 0.6 mm mesh sieve. Organisms (2-3 weeks old) that passed through the 1.0 mm sieve but were retained by the 0.6 mm sieve were collected and used for testing.

Water

The water used in this study was UMBFS pond water, which has no observable toxicity in tests with a variety of limnetic and benthic invertebrate species including *H. azteca* in 14 day exposures in the absence of sediments.

Sediments

UMBFS and Brown's Lake (Warren County, Mississippi) sediments were selected because they are characteristically dissimilar with respect to organic matter and organic carbon, are nontoxic, well characterized, and have been used successfully in previous sediment toxicity testing programs (Suedel and Rodgers, 1994ab, Barko and Smart, 1986). Both sediments were collected using a grab sampler and were prepared according to established procedures (Plumb, 1981). Particle size distribution of sediments was determined using a hydrometric method (Gee and Bauder, 1986). Percent organic matter (percent volatile solids) was determined as percent of initial dry weight of sample remaining after ashing in a muffle furnace at 550°C for 1 h (Plumb, 1981). Cation exchange capacity followed the displacement-after-washing method of Plumb (1981). Sediment pH was measured with an Orion pH probe according to the method of Plumb (1981). Percent sediment solids was calculated as percent of initial wet weight of sample remaining after drying at 104 °C for 24 hours (Black 1986). Redox potential of sediments was measured using an Orion redox probe according to the method of Plumb (1981). Total organic carbon was measured by the dry combustion method using a high temperature induction furnace (Leco Model CR-12) (Nelson and Sommers, 1986). Characteristics of these sediments are presented in Table 3-1.

Toxicity Tests

Hyalella azteca (Saussure) was exposed for 10 days to two field collected sediments amended with copper sulfate. Experiments were conducted in light (16 h light/8 h dark photoperiod) and temperature (21 ± 1 °C) controlled incubators. In sediment tests, amphipods were exposed to copper sulfate-amended sediments in 250 ml glass beakers, with each beaker containing 40 ml of sediment and 160 ml UMBFS pond water (1:4 sediment to water ratio). Sediment/water mixtures were allowed an overnight contact period before adding organisms.

The water-only test was conducted in 250 ml glass beakers with 200 ml UMBFS pond water. Untreated UMBFS pond water and UMBFS and Brown's Lake sediments were used as controls. Experiments were started by adding 10 amphipods to each of four replper treatment. *H. azteca* were fed ground maple leaves on the initial day of each test. All tests were 10 days in duration and were not aerated. Results of water quality monitoring are presented in Table 3-2. The test endpoint was mortality, defined as no visible signs of organism movement after gentle prodding.

Sediment Amending Procedure

To prepare each sediment treatment, sediment was amended with microliter quantities of reagent grade $CuSO_4*5H_20$ (Fisher Scientific, Norcross, GA) dissolved in Milli-Q (MQ) water. After adding measured amounts of copper sulfate, sediment for each treatment was icate beakers stirred by hand for approximately two minutes with a stainless steel spatula and then distributed into replicate beakers. UMBFS pond water was then gently poured over the sediment and the sediment/water mixture allowed to equilibrate for 24 hours before adding organisms.

Analytical Procedures

Stock solutions were prepared by dissolving reagent grade cupric sulfate ($CuSO_4*5H_20$) in MQ water. Water samples for analytical verification were obtained from sacrificial beakers (one replicate per concentration) at the start of each test. Water samples were filtered through a 0.45 μm filter and acidified with redistilled nitric acid (Aldrich Chemical Co., Milwaukee, WI) to a pH of 1-2 before analysis. After the overlying water was removed for analysis, approximately 4.0 g of wet sediment from each sacrificial beaker (one replicate per copper concentration) was placed in an aluminum weighing boat and dried at 80 °C for 24 h. After drying, 2.0 g of dry sediment was acid digested with redistilled nitric acid (Aldrich Chemical, St, Louis, MO) by the nitric acid digestion procedure described in Plumb (1981). Samples were brought to a slow boil on a hot plate at approximately 100 °C until the solution approached dryness (4-5 h). After cooling to room temperature, digestate was then filtered (0.45 μm) and diluted to 25 ml with MQ water before analysis. Pore water

Table 3-1. Properties of University of Mississippi Biological Field Station (UMBFS) sediment and Brown's Lake sediment.

Parameter	UMBFS sediment	Brown's Lake sediment[a]
Solids (%)	72.95	56.00
Organic Matter (%)	2.4	6.0
Organic Carbon (%)	0.38	0.9
Cation Exchange Capacity (meq/100g)	1.50	-[b]
Redox Potential (mv)	+57	-[b]
pH	6.0	6.9
%Coarse Sand	8.4	-[b]
%Medium Sand	31.7	-[b]
%Fine Sand	35.8	-[b]
%Total Sand	75.9	5.0
%Silt	21.4	77.0
%Clay	2.7	18.0

[a]From Barko and Smart (1986).

[b]Not analyzed.

copper samples were extracted from the remaining sediment (approximately 50 g wet wt.) by centrifuging at 12,000 x g for 10 minutes. After centrifuging, pore water was removed by pipet, filtered through a 0.45 μm filter, and acidified to pH 1-2 before analysis.

Copper concentrations of <30 μg Cu/L were concentrated by boiling on a hot plate at 100 °C for 4-5 hour. Copper concentrations of >30 μg Cu/L were not concentrated for analysis. All water and sediment copper samples were determined on a Buck model 200-A flame atomic absorption spectrophotometer.

Statistical Analyses

Lethal concentration values (LC_{50}) and 95% confidence intervals (C.I.) were calculated on raw data using the moving average method of Stephan (1977). Tests for normality and homogeneity of variance of data were performed using the Shapiro-Wilk's test and Bartlett's test, respectively (Gulley *et al.*, 1989). Analysis of variance (ANOVA) was used to test the null hypothesis that all treatment means are equal. Dunnett's test was used to compare each of the treatment means to the control mean and determines if the two are significantly different (Gulley *et al.*, 1989). Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Toxicity Tests

H. azteca exposed to copper sulfate in UMBFS pond water resulted in a 10 day LC_{50} value of 67.2 μg Cu/L (95% C.I. = 58.80-90.29) and a 10 day no observed effects concentration (NOEC) of 51.0 μg Cu/L (Table 3-3). Survival of *H. azteca* was significantly reduced in copper concentrations above 51 μg Cu/L. *H. azteca* exposed to copper-amended UMBFS sediment resulted in 10 days LC_{50} and NOEC values of 62.6 μg Cu/L and 49.7 μg Cu/L, respectively (Table 3-4). *H. azteca* exposed to copper sulfate-amended Brown's Lake sediment resulted in 10 days LC_{50} and NOEC values of 76.8 μg Cu/L and >29.3 μg Cu/L, respectively (Table 3-5).

LC_{50} and NOEC values for both sediment tests based on pore water copper concentrations were considerably greater than values obtained from the overlying water. Pore water LC_{50} and NOEC values in the UMBFS sediment test were 6801 μg Cu/L and 3665

Table 3-2. Water quality characteristics measured during UMBFS pond water, UMBFS sediment, and Brown's Lake sediment experiments.

	Experiments		
Parameter	UMBFS Pond Water	UMBFS Sediment	Brown's Lake Sediment
Temperature (°C)	20.0-22.6	19.8-20.0	19.5-19.9
Dissolved Oxygen (mg/L)	7.9-8.4	7.0-7.7	7.2-7.7
pH	6.2-6.6	6.0-7.2	7.2-7.9
Conductivity (μS/cm)	25-35	30-260	110-725
Alkalinity (mg/L as $CaCO_3$)	10	30-32	95
Hardness (mg/L as $CaCO_3$)	8	12	20-28

μg Cu/L, respectively, and LC_{50} and NOEC values in the Brown's Lake sediment test were 229 μg Cu/L and >75.4 μg Cu/L, respectively. These results indicate that toxicity of copper to *H. azteca* in 10-day sediment tests was dependent on the concentration of copper in the overlying water, rather than copper concentrations in pore water.

The variability of pore water LC_{50} and NOEC values between sediment tests was likely due to differences in characteristics between Brown's Lake and UMBFS sediments (Table 3-1), as there are distinct differences between sediments with respect to organic carbon and organic matter (Brown's Lake - organic carbon [0.9%], organic matter [6.0%]; UMBFS - organic carbon [0.38%], organic matter [2.4%]). These data indicate that sediment organic matter and organic carbon content are driving copper toxicity, as increased organic carbon and organic matter content resulted in decreased toxicity. These results are consistent with those of Malueg *et al.* (1986) who observed that the addition of peat moss (organic carbon) to sediments significantly reduced the toxicity of copper-amended sediment to *Daphnia magna.* Davies-Colley *et al.* (1984) also observed that copper binding in estuarine sediments is dependent on humic substances (organic matter) and iron oxides and that clays such as montmorillonite (smectite) were not significant sinks for copper. Conversely, acid volatile sulfide (AVS) has not been shown to be a useful predictor of copper toxicity in freshwater sediments (Ankley *et al.* 1993). Both the UMBFS and Brown's Lake sediments produced similar LC_{50} values (based on overlying water) even with widely different concentrations of organic carbon content. Yet the Brown's Lake sediment was amended with five times more copper sulfate than the UMBFS sediment to achieve the same level of copper toxicity. The characteristics of a particular sediment (e.g., particle size, organic carbon content, etc.) must be known for appropriate dilution concentrations to be prepared when amending sediments with copper.

In addition to sediment organic carbon/organic matter content, pH is an additional factor that affects the bioavailability and toxicity of copper. Copper in water with pH <7 exists predominantly in the bioavailable Cu^{+2} form, whereas copper in water with pH >7 exists predominantly in the relatively non-toxic $CuCO_3$ form (Figure 3-1; Leckie and Davis 1979). As shown in Table 3-2, pH values in the UMBFS sediment test ranged from 6.0-7.2, within the range of pH values where copper occurs predominantly as Cu^{+2}. Values for pH in

Table 3-3. Static water-only test results for *Hyalella azteca* exposed to copper sulfate in UMBFS pond water for 10 days.

Concentration			
Nominal (μg Cu/L)	Measured (μg Cu/L)	Organisms Exposed	Percent Survival
0	6.3	32	97%
10	16.0	32	94%
20	18.0	32	97%
40	28.0	32	84%
60	51.0	32	78%
80	60.0	32	53%*
100	78.0	32	44%*

(LC_{50} = 67.2, 95% C.I. = 58.88-90.29)

*Significantly different from the control ($p \leq 0.05$).

Table 3-4. Static whole sediment test results for *Hyalella azteca* exposed to copper-amended UMBFS sediment for 10 days.

	Measured Copper Concentrations				
Nominal Cu Sediment Concn. (mg Cu/kg)	Overlying Water (µg Cu/L)	Pore Water (µg Cu/L)	Sediment (mg Cu/kg)	Organisms Exposed	Percent Survival
0	1.5	21.1	0.6	32	97%
70	8.9	5501	15.8	32	100%
100	17.9	5778	71.0	32	94%
130	49.7	3665	82.5	32	88%
170	152.0	8859	117.2	32	3%*
200	318.1	10780	131.8	32	0%*
10-d LC_{50}	62.6	6801	80.8		
(95% C.I.)	(51.73-76.22)	(6447-7191)	(75.00-86.83)		

*Significantly different from the control ($p \leq 0.05$).

Table 3-5. Static whole sediment test results for *Hyalella azteca* exposed to copper-amended Brown's Lake sediment for 10 days.

	Measured Copper Concentrations				
Nominal Cu Sediment Concn. (mg Cu/kg)	Overlying Water (µg Cu/L)	Pore Water (µg Cu/L)	Sediment (mg Cu/kg)	Organisms Exposed	Percent Survival
0	10.6	15.0	23.9	32	100%
200	10.9	55.3	124.7	32	100%
400	11.7	64.4	360.0	32	94%
600	16.7	35.2	540.7	32	97%
800	21.0	85.5	562.5	32	97%
1000	29.3	75.4	624.9	32	82%
10-d LC_{50}* (95% C.I.)	76.78 (39.9-58813)	229 (119-∞)	1218 (777-∞)		

*LC_{50} and 95% confidence intervals (C.I.) for this test was calculated using the probit method of Stephan [16].

the Brown's Lake sediment test ranged from 7.2-7.9, within the range of pH values where copper predominates as $CuCO_3$. Regardless of the predominant copper species present, copper was still toxic to *H. azteca* in both tests, even though copper concentrations in sediment varied by a factor of five between tests. The Brown's Lake sediment test had a slightly higher LC_{50} (76.8 µg Cu/L) than the UMBFS sediment test (62.6 µg Cu/L). These data indicate that copper behavior and toxicity in sediment tests, with pH ranging from 6 to 8, are relatively predictable. Copper toxicity should be assessed, however, in sediments where pH is >8 to ensure that copper sulfate is also a suitable reference toxicant for these sediments.

<u>Proposed Criteria for Selection of Reference Toxicants for Use in Sediment Tests</u>

Criteria for reference toxicant selection in aqueous phase tests have been discussed by several authors, and usually pertain to the nature of the toxicant and the effects of the toxicant on biological systems (Lee, 1980; Dorn *et al.*, 1987; Environment Canada, 1990; Fogels and Sprague, 1977). As expected, it is difficult, if not impossible, for a reference toxicant to meet all the criteria listed. However, these criteria (and possibly others) should be considered when selecting a suitable reference toxicant for use in sediment toxicity tests. It is unlikely that any single toxic substance will meet all the criteria proposed for reference toxicants in aqueous tests (Lee, 1980; Dorn *et al.*, 1987; Fogels and Sprague, 1977). Further, criteria used for selecting reference toxicants for aqueous tests may not be appropriate for selecting a sediment reference toxicant. The criteria listed below are proposed as the most important criteria for selecting a reference toxicant for use in sediment testing. Following each criterion is 1) the rationale for selecting the criterion, and 2) evidence supporting copper sulfate as a reference toxicant.

1. Should be consistently toxic to aquatic organisms at a fraction of its aqueous solubility (give predictable response curve).

Rationale: A suitable sediment reference toxicant should elicit a toxic response to benthic organisms at a fraction of its aqueous solubility, thus eliminating or reducing the need for using carrier solvents or extraordinary means for introducing the toxicant. A predictable response curve is crucial for evaluation of organism health through time. If the response curve is not predictable or consistent, then organism health cannot be determined and comparisons between laboratories is not possible. The aqueous solubility for copper sulfate is 316 g Cu/L (Weast 1967). Data from this study and that of Suedel *et al.* (1995b) demonstrated that toxicity of copper to freshwater organisms such as *H. azteca*, *Chironomus tentans*, *Daphnia magna*, *Ceriodaphnia dubia*, and *Pimephales promelas* occurs at a fraction of its aqueous solubility, thus eliminating the need for a carrier solvent, as required for hydrophobic organic compounds. Exposure response curves of *H. azteca* exposed to copper sulfate-amended sediment in this study were predictable, with copper toxicity decreasing with increasing pH, organic matter, and organic carbon content of sediments.

2. Should be a stable compound which is persistent for the entire test duration in both water and sediment.

Rationale: It is critical that the sediment reference toxicant be present at a constant concentration for the duration of the test in order to ensure constant exposure to test organisms. This also aids in analytical verification. Based upon oxidation states and speciation, copper partitioning is predictable in aquatic systems (Reinert and Rodgers, 1987). There is no evidence for biotransformation of copper, and photolysis and volatilization processes are not known to be important mechanisms for determining copper fate (U.S. EPA, 1979).

3. Minimal change in toxicity (fully dissociated) within the range of water quality (pH, hardness, alkalinity) and sediment characteristics (e.g., organic carbon, particle size distribution, cation exchange capacity) observed in naturally occurring environments.

Rationale: Many substances such as chromium speciate and are reduced to less available or less toxic forms when water or sediment quality are changed. An ideal reference toxicant would be unaffected by changes in water and sediment characteristics. Copper speciation is dependent on pH, with the more toxic Cu^{+2} form prevalent at pH <7 while the less toxic

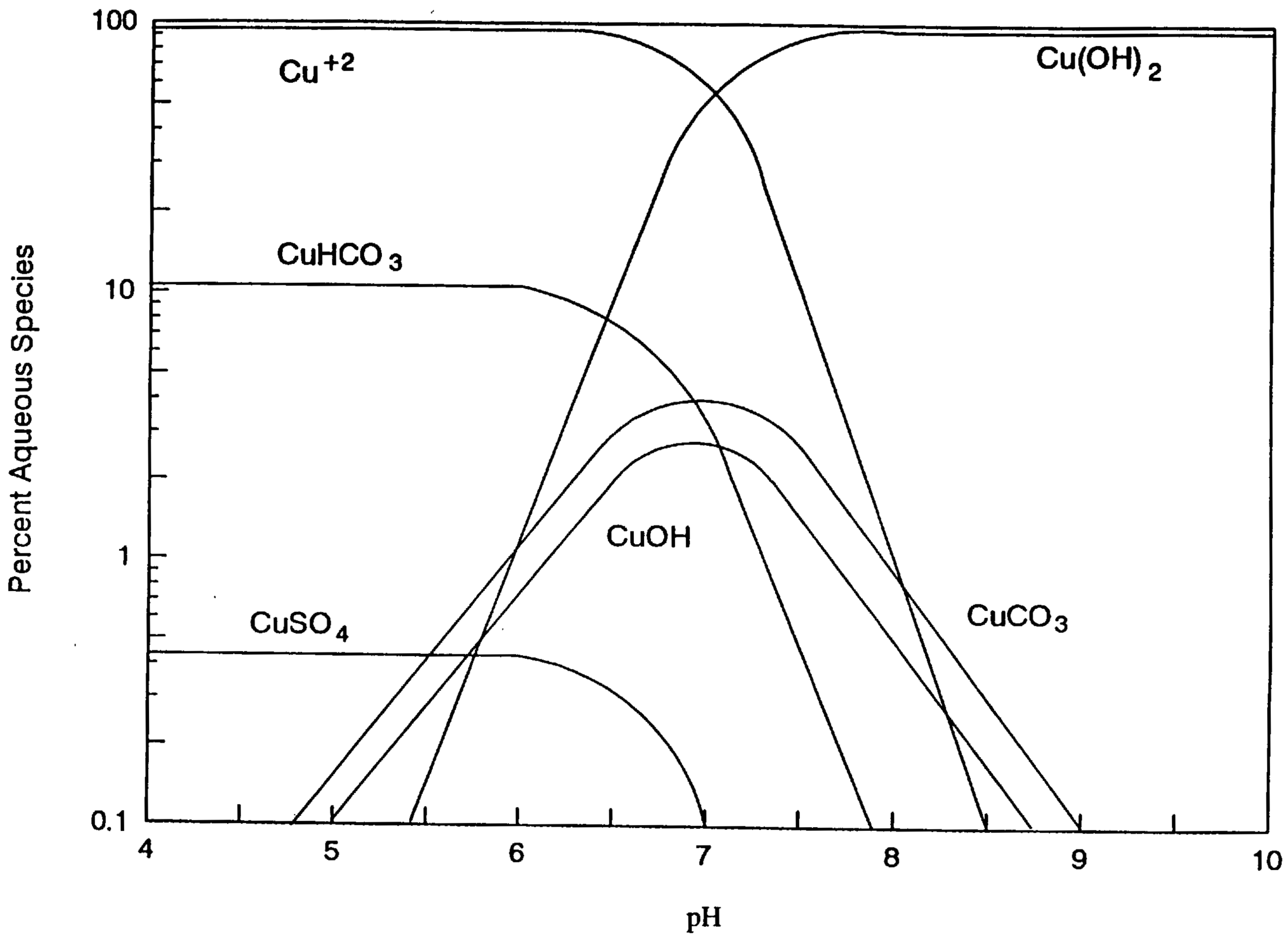

Figure 3-1. Chemical speciation of Cu(II) in unpolluted, organic-free freshwater aquatic systems with total copper concentrations $\leq$10 ppm.

species $CuCO_3$ predominates at pH >7 (Leckie and Davis, 1979; Bodek *et al.*, 1988). As seen in this study and others (Malueg *et al.*, 1986; Davies-Colley *et al.*, 1984; Ramamoorthy and Rust, 1978), copper bioavailability is also affected by the quantity and quality of sediment organic matter and organic carbon content.

In aqueous solution, copper is present as Cu^{+2}. Although most cupric salts are not considered to be readily water-soluble, there are several exceptions, including cupric chloride ($CuCl_2$), cupric nitrate ($Cu(NO_3)_2$) and cupric sulfate ($CuSO_4$).

Copper has a pronounced tendency to form complexes with both organic and inorganic ligands. Stiff (1971a & b) found that, at pH values and inorganic carbon concentrations characteristic of natural waters, most of the copper in solution is present as complexes of cupric carbonate rather than as the (hydrated) divalent cupric ion (Cu^{+2}).

Sylva (1976) examined the speciation of Cu^{+2} in fresh water with respect to inorganic and organic complexation, adsorption, precipitation, and found that these processes are capable of reducing the level of soluble copper to very low values even in the presence of high levels of total copper. Hydrolysis and precipitation dominate the chemistry of Cu^{+2} at pH values expected in most natural water systems wherever there is a limited amount of organic complexing agents. The speciation of Cu^{+2} can vary considerably from one natural water system to another and also within a given system over a period of time. In most surface waters, organic materials prevail over inorganic ions in complexing copper. The strong tendency of copper to form complexes has important ramifications in its precipitation and sorption behavior and is an important process for considering the aquatic fate of copper.

4. Compound behavior (partitioning) predictable in sediment and water.

Rationale: Although a compound may speciate or strongly interact with sediments, if the compound's behavior in sediment is predictable or the toxic species is measurable, then the compound may be appropriate for use as a sediment reference toxicant. Even though copper toxicity is apparently affected by pH and organic matter/organic carbon content of sediment, behavior of copper was predictable and measurable in this study, with increased organic matter/organic carbon content and increased pH resulting in decreased copper toxicity. Protocols for sediment reference toxicity tests should include the use of a single reference sediment over time, which once characterized could then be amended with copper to produce predictable organism responses. Characteristics of field-collected sediments, however, may vary even between sample collections. Therefore, development of a standardized formulated sediment may reduce the inherent variability associated with collecting natural sediments. Used with formulated reference sediment, copper sulfate behavior would be predictable and repeatable.

5. Relatively high water solubility (mg Cu/L range) and low partition coeficient (Kp<10).

Rationale: A compound with a relatively high water solubility and low Kp is less likely to be affected by changes in sediment characteristics. Copper sulfate is highly soluble in water (solubility = 316 g Cu/L) (Weast, 1967). The Kp criterion, however, would apply primarily to organic reference toxicants, rather than metals. Calculation of a single Kp value for copper in sediments is not appropriate as there are numerous factors that regulate binding of copper (e.g., pH, organic carbon, redox). These factors may vary depending upon the sediment type amended.

6. Should be easily analyzed in sediment and water.

Rationale: Analytical verification of nominal concentrations is essential to verify experimental concentrations, as well as to illustrate toxicant stability in water and sediment. Rapid analytical verification reduces costs related to training personnel, extraction procedures and analytical equipment. Total copper is easily analyzed using AA flame or graphite furnace and standard methods exist for determining copper in both sediment and water (APHA 1992). In addition, techniques for measurement of the labile copper species (i.e., Cu^{+2}) are also available. Methods such as ion selective electrodes (ISE) and anodic stripping voltammetry (ASV), may offer lower detection limits (as low as 1 µg Cu/L for ASV) than conventional AA techniques which typically have a lower detection limit in the mg Cu/L range for copper (Laitinen, 1975; Morrison, 1986).

7. Should pose minimal human health risk and be available commercially in purified form (>98%).

Rationale: To ensure health of workers, the compound should not be toxic to humans (e.g., carcinogenic) at concentrations used in testing. If the compound is not available in purified form, then other components in the mixture may be toxic to target organisms (1% impurity = 10,000 ppm). Commercial availability is imperative for widespread use as an interlaboratory reference toxicant. Copper sulfate is relatively nontoxic to humans as compared to the other reference toxicants listed in Table 3-6, and is not a known carcinogen (U.S. EPA, 1979).

8. Should be easily applied and mixed uniformly within the substrate.

Rationale: A chemical that can be directly applied to the sediment without the use of a carrier solvent is desirable. Copper sulfate has been successfully amended to sediments as a concentrated solution of copper sulfate mixed by hand (as in this study), or can be amended to sediments by the rolling method described by Ditsworth *et al.*, (1990).

In addition to the eight criteria listed above, copper sulfate also possesses other properties that make it a suitable sediment reference toxicant. For example, it has been used previously as a reference toxicant (Environment Canada, 1990; U.S. EPA, 1989), it is recognized as an environmental contaminant, i.e., it is one of EPA's priority pollutants (U.S. EPA, 1979), and it is nonselective (toxic) to both freshwater and marine organisms (Hodson et al. 1979; U.S. EPA, 1985b).

Several organic and inorganic chemicals have been used as reference toxicants in water-only testing programs (Table 3-6). However, most of these chemicals do not meet the criteria recommended above for sediment reference toxicants. For example, hexavalent chromium, cadmium chloride, and sodium pentachlorophenate are known carcinogens and pose human health risks (Environment Canada, 1990). Sodium chloride is inappropriate for use with marine organisms and may not be sufficiently toxic to indicate stressed organism populations (Environment Canada, 1990). Potassium chloride lacks a sufficient database (Environment Canada, 1990) and may not be sufficiently toxic to indicate stressed organism populations.

Extensive research has been conducted on the toxic effects of copper on aquatic biota. Data are presented for seventy different invertebrates and ten species of fish in Figure 3-2. Sensitivity to copper varies from 1 μg Cu/L for *Daphnia* (Suedel *et al.*, 1995b) to as high as 10,000 μg Cu/L for stoneflies (Hodson *et al.*, 1979). Although the data in Figure 3-2 indicate that there are considerable differences in organism sensitivity to acute copper exposures, experimental conditions likely contributed to the observed variability. Differing levels of pH and dissolved organic carbon in test water, and use of unmeasured copper concentrations and differing copper compounds (e.g., copper sulfate, copper nitrate, copper chloride) are known to affect copper toxicity and bioavailability (Hodson *et al.*, 1979; Meador, 1991).

Although few plant sediment bioassays have been developed, copper sulfate may also be a suitable reference toxicant for plants. Copper sulfate is highly toxic to both vascular and nonvascular plants (Reinert and Rodgers, 1987) and is a registered aquatic herbicide in the U.S. (WSSA, 1983). Copper sulfate also possesses algicidal, fungicidal and bactericidal properties (WSSA, 1983; Windholz, 1976). This evidence suggests that a kingdom-specific toxicant may not be necessary since toxicological properties of copper sulfate have been demonstrated for animals, plants, fungi, and bacteria.

CONCLUSIONS

Copper sulfate has been proposed as a reference toxicant for sediment toxicity testing for aquatic and benthic animals. It possesses many distinct properties that make it suitable for use as a sediment reference toxicant. The experiments described herein with *H. azteca* and field-collected sediments demonstrate the potential of copper sulfate as a reference toxicant in sediment toxicity tests. Sediment reference toxicant tests conducted with characteristically dissimilar reference sediments amended with copper sulfate yielded predictable exposure response relationships. Copper toxicity was related to the organic carbon and organic matter content of sediments. The successful use of copper sulfate as a reference toxicant in sediment reference toxicity testing should eliminate some of the concerns with sediment reference toxicant tests to measure the health of benthic species. Sediment reference toxicity tests using *H. azteca* and *C. tentans* with copper sulfate-amended formulated sediments are currently in progress.

Table 3-6. Compounds previously used as reference toxicants in aqueous phase tests.

Organic		
1.	Antimycin	Lee 1980
2.	4-Chlorophenol	Environment Canada 1990
3.	Dehydroabietic acid	Lee 1980
4.	Sodium lauryl sulfate	Lee 1980, Environment Canada 1990; Cowgill *et al.* 1990; U.S. EPA 1989
5.	Phenol	Lee 1980, Environment Canada 1990; Fogels and Sprague 1977
6.	Sodium azide	Klaverkamp *et al.* 1975
7.	Sodium pentachlorophenate (NaPCP)	Lee 1980, Environment Canada 1990
Inorganic		
1.	Cadmium chloride	Environment Canada 1990; U.S. EPA 1989
2.	Chromium (hexavalent)	Environment Canada 1990; Dorn *et al.* 1987
3.	Copper sulfate	Environment Canada 1990; U.S. EPA 1989
4.	Potassium chloride	Environment Canada 1990
5.	Silver nitrate	Environment Canada 1990
6.	Sodium chloride	Environment Canada 1990; U.S. EPA 1989
7.	Zinc	Environment Canada 1990

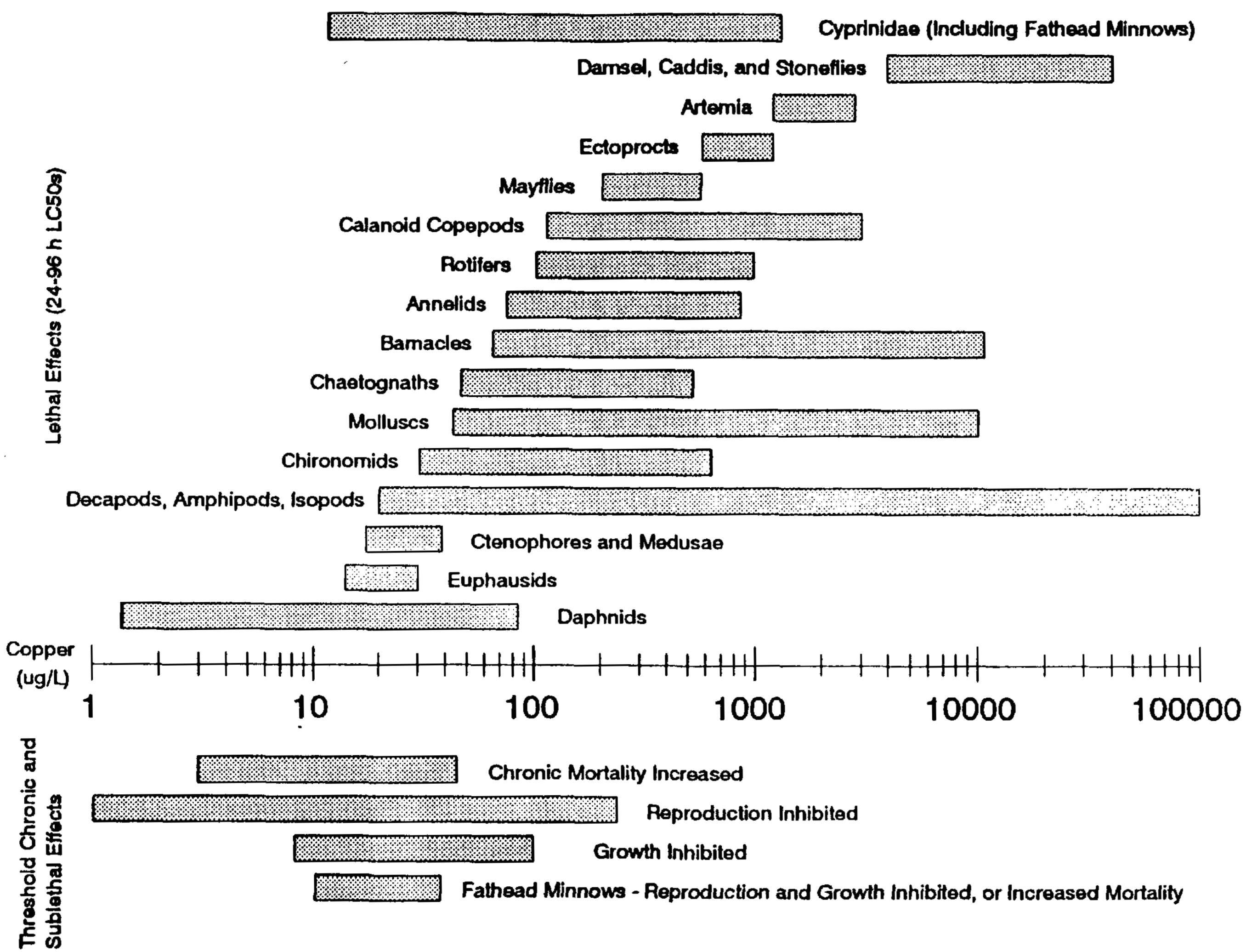

Figure 3-2. Comparisons of lethal and sublethal effects of copper on aquatic organisms. Letters represent 96-h LC_{50} or lowest observed effects concentrations. Data combined from Hodson *et al.* (1979) and Suedel *et al.* (in review). Figure modified from Hodson *et al.* (1979).

Section 4

EXPERIMENTAL FACTORS THAT MAY AFFECT TOXICITY OF AQUEOUS AND SEDIMENT-BOUND COPPER TO FRESHWATER ORGANISMS

Due to recent concerns regarding sediments as sources and sinks for contaminants in aquatic systems, attention has focused on the ability to predict potential adverse effects of sediment-bound materials such as copper (Ankley *et al.* 1993). Copper is an essential element in organisms and is also considered a priority pollutant by U.S. EPA (U.S. EPA, 1980). Because of the widespread use of copper as an algicide, aquatic herbicide, fungicide, and bactericide (WSSA, 1989), as well as discharges from smelting, refining and other copper producing industries, copper may accumulate sufficiently in sediments to produce adverse effects on aquatic organisms. Copper sulfate was chosen as a model chemical in part because of the recent interest in the role sediment characteristics such as acid volatile sulfide (AVS) have in regulating the toxicity of metals in sediments (Ankley *et al.* 1993; Di Toro *et al.* 1992).

Copper occurs in particulate, colloidal, dissolved, organic, and inorganic chemical forms (Leckie and Davis, 1979). Organic ligands and pH are the most important factors affecting copper speciation, as well as transport and deposition, in aquatic environments (Lewis, 1992). Divalent copper (Cu^{2+}) is the predominant oxidation state in soluble aqueous complexes. Hydrolysis of copper is a function of both the concentration of copper and the solution pH. In general, the predominant species of copper at pH ≤ 6 is Cu^{2+}. Between pH of 6 and 8 $CuCO_3$ is the dominant species, and above pH 8, $Cu(OH)_2$ predominates (Leckie and Davis, 1979). The chemical form of copper is critical to its behavior in biological processes, its bioavailability, and its toxicity to aquatic organisms from sediments.

Sediment characteristics observed to affect copper toxicity include organic carbon (Malueg *et al.*, 1986) organic matter (Davies-Colley *et al.*, 1984), pH (Leckie and Davis, 1979) and organic ligands (Lewis, 1992). Di Toro *et al.* (1992) hypothesized that acid volatile sulfide (AVS) concentrations in sediments could be used to predict copper toxicity.

However, Ankley *et al.* (1993) recently demonstrated that AVS alone is not an appropriate partitioning phase for predicting copper toxicity in freshwater sediments.

Assessments of effects of sediment associated materials such as copper on aquatic organisms frequently involve conducting laboratory sediment experiments. The ability of these laboratory experiments to detect adverse effects of sediment sorbed materials depends on several factors, including exposure duration, species selection, and test endpoints. Previous studies conducted with contaminated field collected sediments (Burton *et al.*, 1989), contaminated dredged sediments (LeBlanc and Suprenant, 1985), and sediments amended with fluoranthene (Suedel and Rodgers, 1993) found that sufficient test duration was crucial to accurately assess contaminated sediments. In this study, test durations of 48 hours, 96 hours, 7 days, 10 days, and 14 days were used to determine test duration required to observe adverse effects on freshwater toxicity testing organisms. These test durations were selected because of their previous use in sediment toxicity testing (Burton, 1991; ASTM, 1990). Determining an exposure duration to elicit adverse effects eliminates concerns regarding the adequacy of exposure duration of laboratory experiments to correctly assess sediment potency.

Accurate laboratory assessments of the potency of sediment associated materials also require selection of appropriate experimental organisms. For example, organisms such as oligochaetes (i.e., *Lumbriculus*) are tolerant of contaminants in sediments (Rosiu *et al.*, 1989; Keilty and Landrum, 1990). Midge species such as *Chironomus tentans* and *Chironomus riparius* have been perceived to be insensitive to various metals (i.e., copper) and inorganic compounds (Ingersoll *et al.*, 1990; Gauss *et al.*, 1985). An organism's sensitivity to materials in sediments may depend on the organism's feeding habits, contact with sediment (habitat), and physiology. Chironomids accumulate materials from both the sediment and water due to their collecting and filtering feeding habits (Sadler, 1935; Pennak, 1978; Muir *et al.* 1983). Freshwater amphipod species, such as *Hyalella azteca*, forage at the sediment surface as well as feed on debris covering aquatic plants in the water column, thus reducing their exposure to sediment. *Daphnia magna*, although considered a water column organism, also forages on sediments and is exposed to sediment sorbed materials (Suedel and Rodgers, 1993). *Ceriodaphnia dubia* is often considered a water column organism, but has also been observed in this study to feed on sediments by rapidly striking the sediment surface, thus suspending sediment surface particles. Variation in exposure regimes and organism physiology are key factors that influence the sensitivity of test organisms to sediment-bound materials such as copper. For example, some chironomid larvae apparently can regulate the accumulation of various metals (i.e., zinc, nickel, and copper) in their tissues (Krantzburg and Stokes, 1989). These differences may affect survival, growth, and reproduction rates.

Prior knowledge of an organism's feeding behavior, contact with sediment, and physiology will help to reduce the uncertainty regarding selection of appropriate organisms to determine the potency of sediment sorbed materials. These experiments examine the relative sensitivities of selected aquatic and benthic organisms to a copper contaminated sediment and further our knowledge as to which test species and test endpoints are sensitive to copper contaminated sediments.

Sensitive test endpoints are also a necessary component of sediment toxicity tests in verifying the toxicity of sediments (Burton, 1991). Tests results have indicated a wide range of sensitivity of test endpoints to aqueous and sediment-bound contamination. Changes in *H. azteca* body length has been shown to be less sensitive than percent survival, and Chironomus riparius survival more sensitive than head capsule width and percent emergence when exposed to metal contaminated sediments (Ingersoll and Nelson, 1990). Test endpoint sensitivity has also been shown to be compound and species specific, with body length and total number of young produced per female being the most sensitive endpoint for *D. magna* and *C. dubia*, respectively, when exposed to aqueous cadmium (Winner, 1988). As such, selection of appropriate test endpoints are essential for accurately assessing sediment toxicity.

Reference and control sediments are needed to accurately determine the potency of toxic sediments. To conduct definitive sediment toxicity tests, appropriate sediments for dilution of toxic field collected test sediments are required. These "dilution" or reference sediments need to be inherently nontoxic; i.e., uncontaminated by toxic chemicals and free of biota (e.g., predators) that may confound test results, and they must possess similar sediment characteristics as the test sediment. Such sediments are often not available at field sampling sites. Since potential field reference sediments may have unknown chemical and physical constituents and may contain extant organisms that cannot be readily removed, reference sediments are needed that can be formulated for matching diverse freshwater and estuarine sediments that may be encountered. Formulated reference sediment for test endpoints of organism survival, growth, and reproduction during chronic experiments was prepared to match characteristics of a variety of field collected sediments (Suedel and Rodgers, 1994).

Utilizing the organisms *Chironomus tentans* Fabricius, *Hyalella azteca* Saussure, *Daphnia magna* Straus, *Ceriodaphnia dubia* Richard, and *Pimephales promelas* Rafinesque, the objectives of this research were to: 1) determine the relative sensitivities of these organisms to copper in water-only exposures and a field collected copper-contaminated sediment; 2) determine the duration of exposure required to elicit mortality or sublethal biological effect (e.g., reduced growth or reproduction) by a copper-contaminated sediment; 3) determine whether or not sublethal test endpoints are more sensitive than survival of test organisms used in this study; and 4) illustrate the ability of formulated reference sediment to match selected characteristics and dilute a copper contaminated sediment, yielding decreases in toxicity with concomitant increases in test sediment dilution. Comparisons were made between test organism mortality in water-only copper tests with concentrations of copper in overlying water, pore water, and sediment in sediment tests. These experiments will further our understanding of factors that affect the toxicity of aqueous and sediment-bound copper to common freshwater sediment toxicity testing organisms in laboratory tests.

MATERIALS AND METHODS

Test Organism Culture Procedures

All test organisms were cultured at the University of Mississippi Biological Field Station (UMBFS) laboratory. *H. azteca* culturing procedures followed the methods of de March (1981). Amphipods were cultured under ambient laboratory temperature conditions (22±2 °C) in 19 L glass aquaria covered with 1 mm mesh nylon screen. Amphipods were cultured in 15 L of filtered, dechlorinated tap water with vigorous aeration. Water in each aquarium was changed weekly. Amphipod substrate was fallen maple (*Acer rubrum*) leaves that had been previously soaked in reverse osmosis water for 7 to 10 days. The leaves were rinsed with water daily before placing in culture aquaria. Additional leaves were added to culture aquaria as needed. Amphipods were also fed a suspension of ground rabbit chow pellets (Clover Brand, Madison, MS) twice each week.

To prepare for testing, amphipods were collected by removing a portion of the leaves containing amphipods from culture aquaria. Leaves containing amphipods were placed on a 1.00 mm mesh stainless steel sieve and gently washed with culture water. Water and organisms were washed through the 1.0 mm mesh sieve and onto a 0.6 mm mesh sieve. Organisms that passed through the 1.0 mm sieve but were retained by the 0.6 mm sieve (approximately 2-3 weeks old) were collected and used for testing.

C. tentans culture methods followed those of Townsend *et al.* (1981). Midges were cultured under ambient laboratory temperature conditions (22$\pm$2 °C) in 39 L glass aquaria covered with 1 mm mesh nylon screen. Midges were maintained in 30 L of filtered, dechlorinated tap water with vigorous aeration. Water in each aquarium was changed weekly. Culture substrate consisted of shredded brown paper towels (#ML96 Georgia Pacific, Battleboro, VT) to a depth of 3 cm. Midges were fed a 2.0 ml suspension of Tetra conditioning food (Tetrawerke, Germany) daily and 2.0 ml of a Cerophyll (Ward's Natural Science Establishment, Inc., Rochester, NY) suspension weekly. Midges used for testing were second instar larvae (10 days old).

D. magna and *C. dubia* culturing procedures followed the methods of Peltier and Weber (1985). *D. magna* and *C. dubia* cultures were maintained in UMBFS pond water in light (16 h light/8 h dark photoperiod) and temperature controlled (21$\pm$1 °C) incubators. Hardness and alkalinity of the UMBFS pond water were adjusted with (0.1 g/L) $NaHCO_3$ and $CaCl_2$ to a total hardness of 80 mg/L as $CaCO_3$ and alkalinity of 60 mg/L as $CaCO_3$. Both daphnid species were fed a combination of *Selenastrum capricornutum* and a formulated (baker's yeast, catfish chow, alfalfa) diet daily.

P. promelas culturing procedures followed the methods of Peltier and Weber (1985). Fish were cultured under temperature controlled conditions (25$\pm$2 °C) in 39 L glass aquaria with 30 L of filtered, dechlorinated tap water with moderate aeration. The culture consisted of a flow-through recirculating system with a biological filter consisting of crushed coral and gravel. Fish were fed a daily combination of Tetra staple food (Tetrawerke, Germany) and frozen brine shrimp (San Francisco Bay Brand, Newark, CA). Fatheads used in testing were 2-4 days old.

Water-Only Experiments

All water-only experiments were conducted in light and temperature controlled incubators at 20±1 °C under a 16 hour light/ 8 hour dark photoperiod. Tests were conducted in 250 ml borosilicate glass beakers with 200 ml of UMBFS pond water, except for *C. dubia* tests, which were conducted in 50 ml beakers with 40 ml of UMBFS pond water. Experiments were started by adding eight *H. azteca*, six *C. tentans*, eight *D. magna*, or eight *P. promelas* to each of four replicate beakers. Experiments with *C. dubia* were started by adding one neonate to each of 10 replicate beakers per treatment. UMBFS pond water was used as a control, except for *D. magna* tests, where adjusted UMBFS pond water (see culture procedures) was used. Glass beads (150-212 μm, Sigma Chemical Co., St. Louis, MO) were used as a substrate in *C. tentans* tests to allow for tube building and to reduce stress (Suedel and Rodgers, 1993).

All organisms were fed in all tests. Feeding regimes for each organism were: *D. magna* and *C. dubia* - 0.5 ml and 0.05 ml, respectively, of *S. capricornutum* algae daily; *H. azteca* - leached and ground maple leaves at test initiation; *C. tentans* - 0.1 ml cerophyll suspension at test initiation and every other day thereafter; *P. promelas* - 10-20 newly hatched *Artemia* nauplii per fish daily. Dissolved oxygen concentrations did not drop below 40% of saturation in any test, therefore aeration was not required.

Sediments - Field Collected

Copper contaminated sediment was collected from a creek site downstream from a copper smelting operation. Copper in sediment was believed to be from the smelter's discharge since this was the only nearby source of copper. Sediment was collected by a grab sampler, transported on ice to the UMBFS laboratory and stored at 4 °C until use. Characteristics of this sediment are given in Table 4-1. Total copper concentration in this sediment was 20,365 mg/kg. This sediment was also analyzed for barium and lead, but these metals were below analytical detection limit (<5 mg/kg).

Table 4-1. Properties of copper contaminated sediment and matching formulated sediment.

Parameter	Copper contaminated sediment	Formulated sediment
Solids (%)	63.33	63.97
Organic Matter (%)	5.52	7.59
Cation Exchange Capacity (meq/100g)	3.91	3.61
Redox Potential (mv)	+4	+228
pH	6.6	6.2
%Coarse Sand	1.8	3.1
%Medium Sand	3.0	5.1
%Fine Sand	15.2	14.6
%Total Sand	20.0	22.8
%Silt	80.0	77.2
%Clay	0.0	0.0

Sediments - Formulated

Several inorganic and organic materials from commercial sources were used as sand, silt, clay, and organic constituents for preparing formulated sediment (Table 4-2). Silica sands ("Mystic White" #18 and #90 sands; New England Silica, Inc., South Windsor, CT) were sieved into coarse (2.0-0.5 mm), medium (0.5-0.25 mm), and fine (0.25-0.05 mm) sand fractions using a SoilTest sand shaker. ASP®600 and ASP®400 (kaolinite clay minerals) were used to represent the clay and silt fractions of formulated sediments. Kaolinite minerals were purchased from Engelhard Corp., Pigments and Additives Division, Edison, NJ. Montmorillonite (smectite) clay from Apache County, AZ (Prof. W.D. Johns, Source Clays, Univ. of MO, Columbia, MO) was also used, representing a typical clay mineral found in sediments throughout the U.S. (Weaver, 1989). Organic humus from Sims Bark Co. Inc., Tuscumbia, AL, was used as recalcitrant particulate organic matter in sediments. Humus was dried at 70 °C and milled to <2.0 mm (2.00-0.05 mm = 98%, <0.05 mm = 2%) in a Wiley Mill before use. Dolomite was obtained from Ward's Natural Science Establishment, Inc. and represented bicarbonate buffer found in soils and sediments. Each constituent was added in quantities sufficient to match the test sediment. Dry constituents were hydrated with UMBFS pond water and conditioned in a plastic tub in a cement raceway at the UMBFS under flow-through (2-3 volume additions/day) conditions for 14 days. The conditioning period was hypothesized to permit colonization of the formulated sediment by various microorganisms such as algae and bacteria. Additional information regarding formulated sediment preparation and use is given in Suedel and Rodgers, 1994b).

Sediment Characterization

Particle size distribution of sediments was determined using a hydrometric method (Gee and Bauder, 1986). Percent coarse (2.00-0.5 mm), medium (0.5-0.25 mm), and fine (0.25-0.05 mm) sands were determined by dry sieving. Percent organic matter (volatile solids) was determined as percent of initial dry weight of sample remaining after ashing in a muffle furnace at 550 °C for 1 hour (Plumb, 1981). Cation exchange capacity followed the displacement-after-washing method of Plumb (1981). Sediment pH was measured with an

Table 4-2. Physical and chemical properties of formulated sediment constituents after conditioning*.

Constituent	Mineral or Component	Particle Size (um)	CEC (meq/100g)	Solids (%)	Organic Matter(%)	pH**
ASP 600	clay	0.6	1.59	52.12	13.6	3.5
ASP 900	clay	1.5	2.53	56.21	13.2	3.4
Montmorillonite	clay	<2.0	120	46.59	8.79	7.4
ASP 400	silt	4.8	2.87	62.61	13.2	3.4
Mystic White #18 coarse sand	silica	500-850	-	88.76	1.0	6.8
Mystic White #90 medium sand	silica	250-500	-	75.30	0.02	7.0
Mystic White #90 fine sand	silica	50-250	-	61.45	1.8	6.8
Humus	organic matter	<2.0 mm	17.3	30.06	86.9	5.9
Dolomite	buffer	<0.05 mm	0.27	84.91	1.31	8.5

*See Table 4-1 for sediment parameters and corresponding analysis ("conditioning" is explained in the text).

**pH measurements taken after conditioning each constituent for 7 days.

Orion pH probe (Plumb, 1981). Percent sediment solids was calculated as percent of initial wet weight of sample remaining after drying at 104 °C for 24 hours (Black, 1986). Field collected test sediment was matched by formulated sediment with respect to these sediment characteristics (Table 4-2).

Sediment Experiments

All sediment experiments were conducted in light (16 h light/8 h dark photoperiod) and temperature controlled (21±1 °C) incubators. For definitive sediment tests, test sediment and formulated sediment were mixed on a wet weight basis at test sediment dilutions (i.e., 0.1-100%) that produced mortality in range-finding tests. Organisms were exposed to sediments in 250 ml glass beakers, with each beaker containing 40 ml of sediment and 160 ml UMBFS pond water, except for *C. dubia* tests, which were conducted in 50 ml beakers with 8 ml of sediment and 32 ml UMBFS pond water (1:4 sediment to water ratio). The number of organisms per replicate and number of replicates per sediment concentration were the same as for the water-only experiments. Formulated sediment (100%) was used as a control sediment in all experiments. UMBFS pond water was used as overlying water in all experiments except for *D. magna*, where adjusted UMBFS pond water was used (see culture methods). Sediment/water mixtures were allowed an overnight contact period before adding organisms. Feeding regimes were the same as for the water-only experiments. Since some nontoxic naturally occurring sediments do not provide sufficient food for >80% test organism survival in 10 day exposures (Suedel and Rodgers, 1994), organisms were fed in all experiments.

Water

The water used in this study was obtained from the University of Mississippi Biological Field Station (UMBFS). In 14 day water-only exposures, this water had no observable toxicity (mortality) to the organisms used in this study. Although not generally recommended, the test sediment used in this study was decoupled from its overlying water. Overlying water quality is affected by sediment characteristics; likewise, sediments can be affected by overlying water, especially if sediment and overlying water are from chemically divergent sources (Suedel and Rodgers, 1994). In the present study, the sediment and water were decoupled for experimental purposes only. Water quality characteristics measured in water-only tests and sediment tests are presented in Table 4-3.

Analytical Procedures

Stock solutions used for water-only tests were prepared by dissolving reagent grade cupric sulfate ($CuSO_4$*$5H_20$) in Milli-Q water. Samples for water-only copper analyses were obtained from sacrificial beakers (one replicate per concentration) at the start of each test. Water samples from the water only experiments were filtered through a 0.45 um filter and acidified with redistilled nitric acid (Aldrich Chemical Co., Milwaukee, WI) to a pH of 1-2 before analysis. For the sediment experiments, overlying water samples were prepared and analyzed in the same manner as the water-only analyses. After the overlying water was removed, approximately 4.0 g of wet sediment from each sacrificial beaker (one replicate per copper concentration) was placed in an aluminum weighing boat and dried at 80 °C for 24 hours. After drying, 2.0 g of dry sediment was acid digested for 5 h at 100 °C. Samples were then filtered (0.45 um) and diluted to 25 ml with Milli-Q water before analysis. Pore water copper samples were extracted from the remaining sediment (approximately 50 g wet wt.) by centrifugation at 12,000 x g for 10 minutes. After centrifuging, pore water was removed by pipet, filtered through a 0.45 um filter, and acidified to pH 1-2 before analysis.

Copper samples of <30 μg/L were concentrated by boiling while copper samples of >30μg/L were not concentrated prior to analysis. Copper concentrations in all water and sediment samples were determined using a Buck model 200-A flame atomic absorption spectrophotometer. Mean ($\pm$S.D.) copper recovery in both water-only and sediment tests was 96.5$\pm$8.15%.

Statistical Analyses

Lethal concentration values (LC_{50}) and 95% confidence intervals (C.I.) for all tests were calculated using the moving average method of Stephan (1977). Tests for normality and homogeneity of variance of data were performed using Shapiro-Wilk's test and Bartlett's test, respectively (Gulley *et al.*, 1989). Analysis of variance (ANOVA) and Dunnett's multiple

Table 4-3. Water characteristics measured in water-only and sediment experiments.

Parameter	Water-Only Experiments	Sediment Experiments
Temperature (°C)	19.7-24.1	19.5-22.3
pH	6.9-8.0	6.7-7.8
D.O. (mg/L)	6.2-8.5	5.2-7.7
Alkalinity* (mg/L as $CaCO_3$)	9-21	34-56
Hardness* (mg/L as CaCO3)	6-10	8-120
Conductivity* (µS/cm)	20-50	50-180

*Data from *D. magna* tests were not included because adjusted UMBFS pond water was used, resulting in higher values for alkalinity, hardness, and conductivity (water-only tests: alkalinity=68-70, hardness=72-80, conductivity=280-305; sediment tests: alkalinity=67-71, hardness=136-148, conductivity=320-360).

range test were used to detect differences between control and treatment survival means (Gulley *et al.* 1989). Bonferroni's T-Test was used to detect differences between control and treatment means of growth and reproductive endpoints (Gulley *et al.*, 1989). Regression analysis (SAS, 1989) was used to determine the magnitude of the relationships between sediment copper concentrations and pore water and overlying water copper concentrations. The 5% alpha level was used in all statistical tests.

RESULTS AND DISCUSSION

Water-only Experiments

Control survival in all water-only experiments ranged from 80-100%, indicating control water (UMBFS pond water) was within the tolerance range of all test organisms. LC_{50} and NOEC values were based on copper concentrations measured at the start of each experiment. Copper concentrations measured at experiment termination were, on average, 83±16.8% of initial copper concentrations. As shown in Table 4-3, low hardness values (6-10 mg/L as $CaCO_3$) may have resulted in a "worst case" copper exposure, as increasing water hardness and associated carbonate alkalinity is thought to reduce the acute toxicity of copper (U.S. EPA, 1985b; Gauss *et al.*, 1985). In the pH range measured in these experiments (6.9-8.0), the predominant copper species present was likely $CuCO_3$ (Leckie and Davis, 1979).

Ceriodaphnia dubia.

In water-only experiments, *C. dubia* was the most sensitive organism tested, followed in decreasing sensitivity by *P. promelas*, *D. magna*, *H. azteca* and *C. tentans* (Figure 4-1). *C. dubia* LC_{50} values ranged from 4.18 µg/L at 10 and 14 days to 1.16 µg/L at 7 days (Figure 4-1). LC_{50} values for *C. dubia* exposed to copper in water-only experiments decreased over time for test durations of 48 hours, 96 hours, and 7 days (with LC_{50} values of 2.72, 1.46, and 1.16 µg/L, respectively), but then slightly increased in the longer duration tests of 10 days and 14 days (LC_{50} = 4.18 µg/L in both tests). In these experiments, a mortality threshold within 7 days of exposure was observed for *C. dubia* exposed to aqueous copper, with little additional mortality occurring after 96 hours (Figure 4-1).

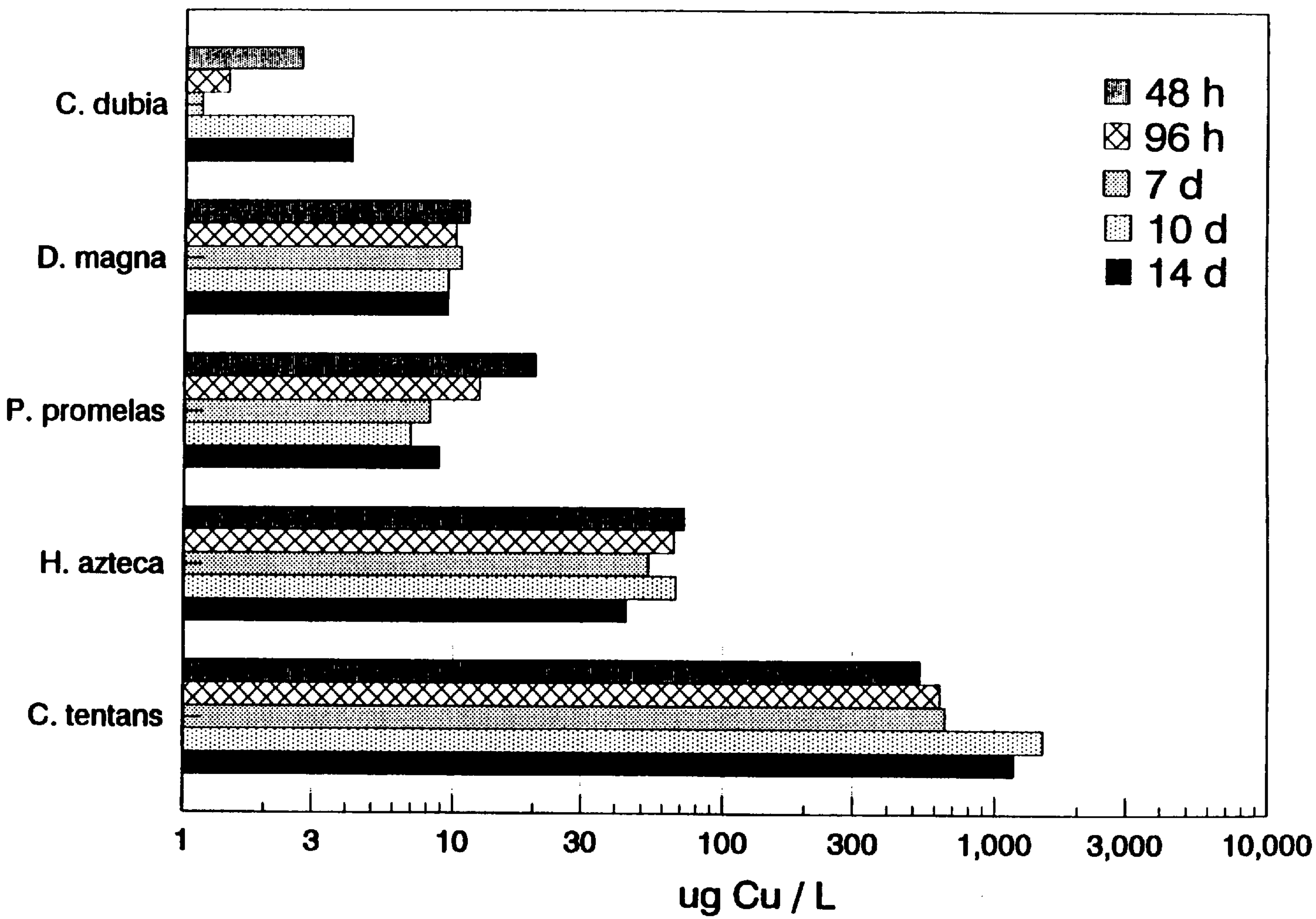

Figure 4-1. LC_{50} values for test organisms exposed to copper in water-only exposures.

Daphnia magna.

D. magna LC_{50} values were similar to those for *P. promelas*, and ranged from 11.32 µg/L at 48 h to 9.52 µg/L at 14 days (Figure 4-1). *D. magna* responded to aqueous copper predominantly within the first 96 hours of exposure, resulting in a mortality threshold at 96 hours. In these experiments, no appreciable increase in *D. magna* mortality was observed after 96 hours of aqueous copper exposure.

Pimephales promelas.

P. promelas was sensitive to copper in water-only experiments, with LC_{50} values ranging from 20.2 µg/L at 48 hours to 6.96 µg/L after 10 days of exposure (Figure 4-1). *P. promelas* survival decreased over time from the 48 hour test to the 10 day test but increased slightly at 14 days. These data indicate that the majority of *P. promelas* mortality is manifested after 7 to 10 days of exposure to aqueous copper.

Hyalella azteca.

LC_{50} values for *H. azteca* exposed to aqueous copper were 5 to 8 times higher than *P. promelas* and *D. magna* LC_{50} values (Figure 4-1). *H. azteca* mortality increased through 14 days of exposure, with LC_{50} values decreasing from 72.2 µg/L at 48 h to 44.1 µg/L at 14 d. The 10 day LC_{50} value obtained in this study (67.2 µg/L) was similar to the 10 day LC_{50} value reported for *H. azteca* by Cairns *et al.* (1984) of 59 µg/L. *H. azteca* did not achieve a mortality threshold even after 14 days of exposure, indicating that continued exposure to copper would likely yield increased mortality of *H. azteca.*

Chironomus tentans.

C. tentans LC_{50} values were 10 times greater than *H. azteca* LC_{50} values, ranging from 529 µg/L at 48 h to 1502 µg/L at 10 d (Figure 4-1). *C. tentans* survival in water-only copper exposures increased (rather than decreased) through time, illustrating the ability of this organism to sequester copper and reduce copper toxicity.

Sediment Experiments

Control survival in all sediment experiments ranged from 85-100%, indicating the characteristics of the formulated control sediment and overlying water were within the tolerance ranges of the species examined. LC_{50} and NOEC values were based on copper concentrations measured in overlying water, pore water, and sediment at the start of each experiment. Copper concentrations measured at experiment termination in sediment tests were, on average, 107±22% of initial measured copper concentrations. The effects of the test sediment on water quality of the control water (UMBFS pond water) are illustrated in Table 4-3, resulting in increased alkalinity, hardness, and conductivity of the overlying water compared to the water-only experiments.

Ceriodaphnia dubia.

C. dubia responded to copper contaminated sediment predominantly within the first 96 h of exposure. LC_{50} values for overlying water increased slightly in the 96 h test as compared to the 48 hour test, but otherwise remained constant at 6.5 µg/L in the 7, 10, and 14 day tests (Figure 4-2). This trend was also observed with pore water LC_{50} values, which ranged from 84 µg/L in the 48 hour test to 122 µg/L in the 96 hour test (Figure 4-3). LC_{50} values based on sediment concentrations decreased ten times from the 48 hour test (129 mg/kg) to the 96 h test (36 mg/kg), but remained relatively constant in the 7, 10, and 14 day tests (32 mg/kg, Figure 4-4).

C. dubia reproduction, expressed as the total number of offspring per female, was also measured in the 7, 10, and 14 day tests (Table 4-4). Based on no observed effects concentrations (NOECs), reproduction was less sensitive than survival in the 7 day and 10 day tests. In the 14 day test, reproduction was slightly more sensitive than survival. Similar to the water-only tests, sediment test data showed that a 96 hour exposure duration was sufficient to elicit mortality in this species when exposed to the copper. *C. dubia* required 14 days of exposure to a copper contaminated sediment to elicit significant reproductive impairment.

Table 4-4. Comparison of No Observed Effects Concentrations (NOECs) for lethal and sublethal endpoints of test organisms exposed to copper contaminated sediment.

			No Observed Effects Concentration (NOEC)		
Organism	Test Duration	Endpoint	Water (µg Cu/L)	Pore Water (µg Cu/L)	Sediment (mg Cu/kg dry wt)
C. dubia	7d	Survival	3.7	79.9	18.1
		Reproduction	14.1	163	52.5
	10d	Survival	3.7	79.9	18.1
		Reproduction	9.6	132	45.9
	14d	Survival	3.7	79.9	18.1
		Reproduction	3.2	48.9	11.9
P. promelas	10d	Survival	8.6	42.8	136.9
		Growth (dry wt)	8.6	42.8	136.9
	14d	Survival	15.1	20.2	129.3
		Growth (dry wt)	>32	>52	>461
C. tentans	10d	Survival	22.9	36.1	>>216
		Growth (dry wt)	<21.6	<16.3	<216

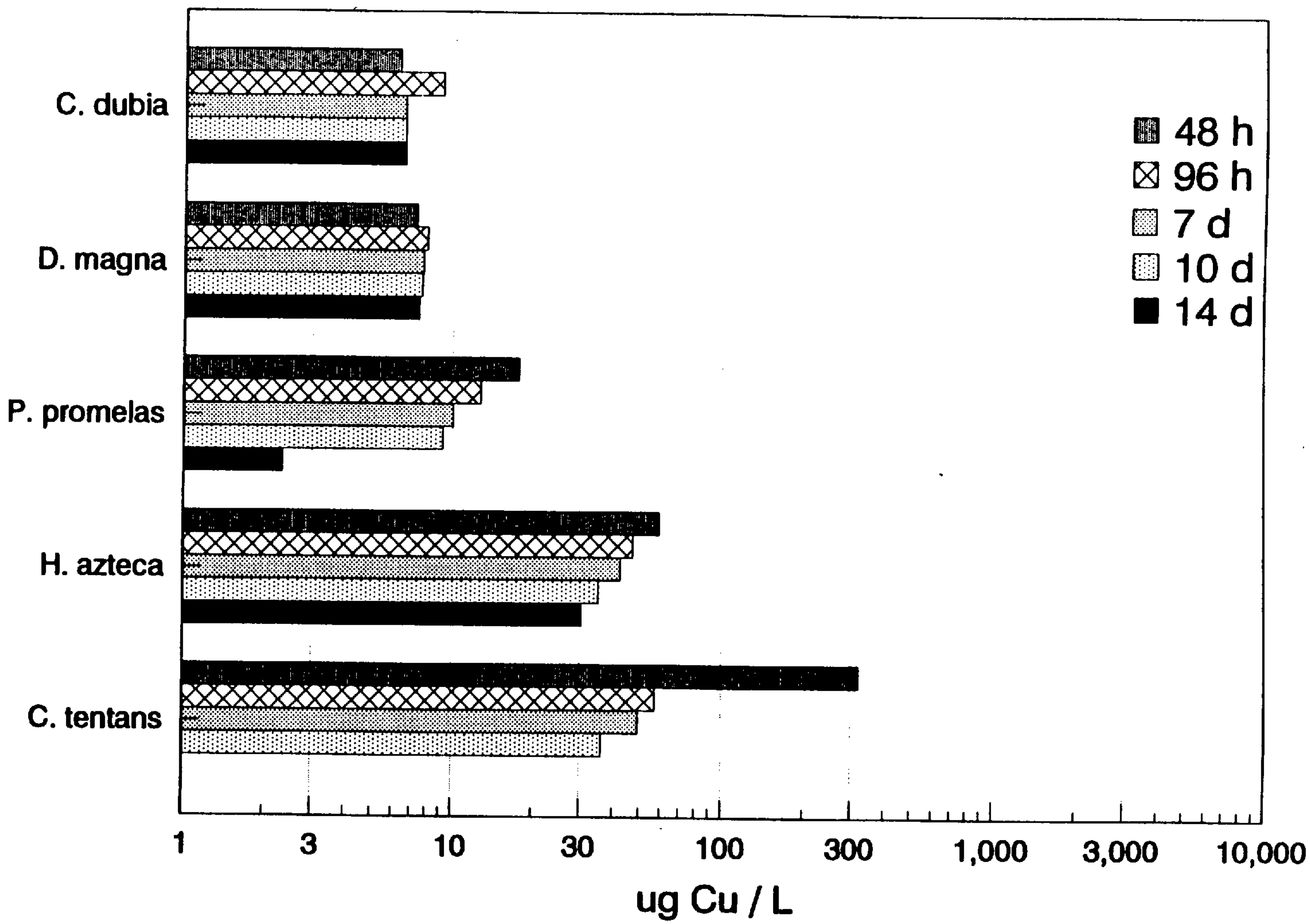

Figure 4-2. LC_{50} values for test organisms exposed to copper contaminated sediment based on copper concentrations in overlying water.

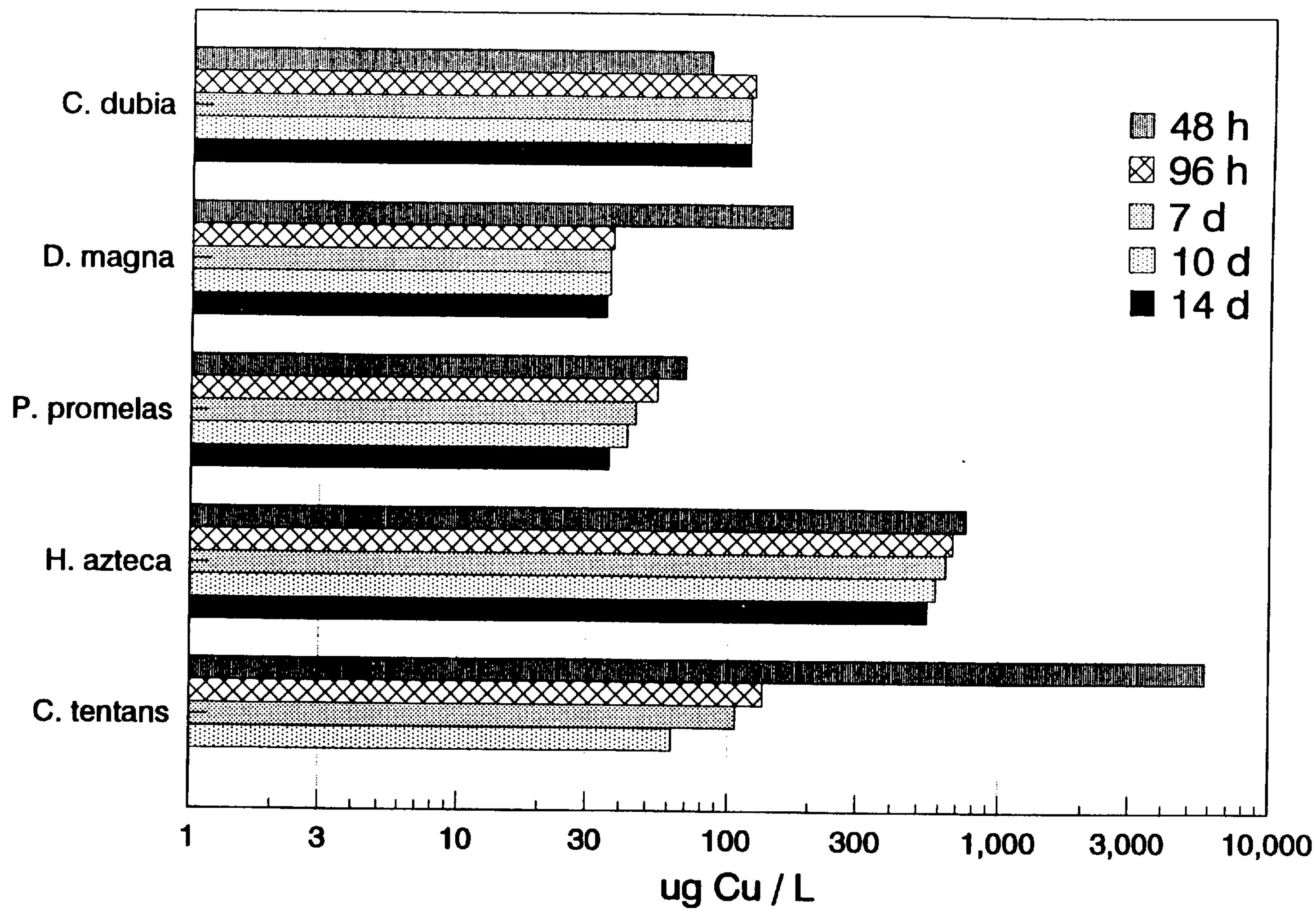

Figure 4-3. LC_{50} values for test organisms exposed to copper contaminated sediment based on copper concentrations in pore water.

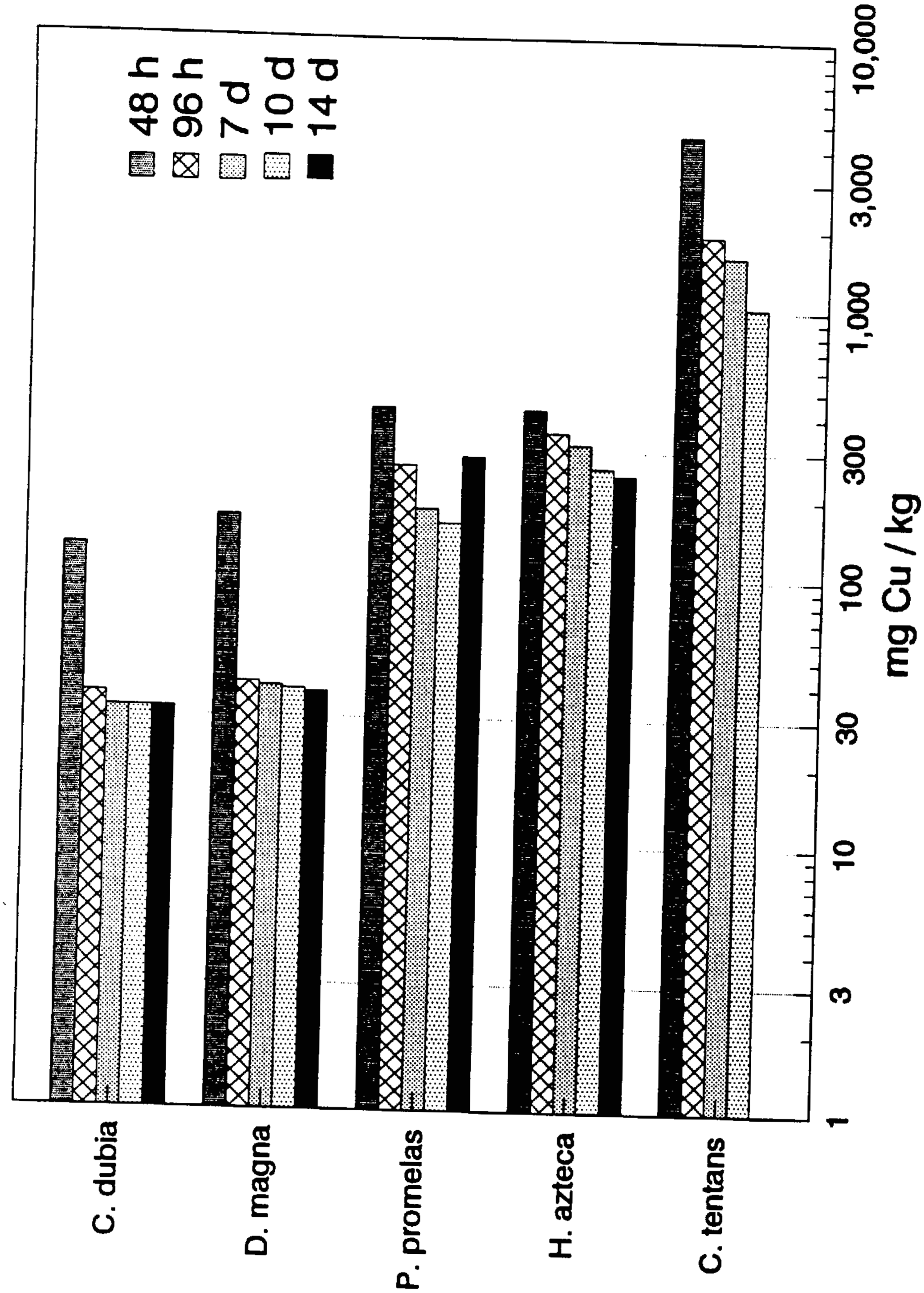

Figure 4-4. LC_{50} values for test organisms exposed to copper contaminated sediment based on copper concentrations in sediment.

Daphnia magna.

Test duration (48 hours - 14 days) had little effect on survival of *D. magna* exposed to copper contaminated sediment (Figures 4-2 through 4-4). LC_{50} values for overlying water were similar in all tests, and ranged from 7.25 µg/L at 48 hours to 7.96 µg/L at 96 hours (Figure 4-2). LC_{50} values based on pore water and sediment copper concentrations decreased four times from the 48 hour test to the 96 hour test (170 to 37 µg/L, and 170 to 40 mg/kg for pore water and sediment, respectively; Figures 4-3 through 4-4). However, pore water and sediment LC_{50} values did not decrease at the 7 day, 10 day, and 14 day test durations, ranging from 35 to 37 µg/L for pore water, and from 37 to 40 mg/kg for sediment, respectively. As for the water-only tests, these data showed that *D. magna* achieved a mortality threshold at 96 hours of exposure and that further exposure to this sediment (up to 14 days) did not result in a decrease in *D. magna* survival. The 48 hour LC_{50} values for overlying water (7.25 µg/L) and sediment (170 mg/kg) determined in this study were four times lower than 48 hour LC_{50} values of 30 µg/L in overlying water and 681 mg/kg in sediment reported for *D. magna* exposed to a copper-amended sediment (Cairns *et al.* 1984).

Pimephales promelas.

P. promelas response to copper contaminated sediment showed a general decrease in survival with increasing exposure duration (Figures 4-2 through 4-4). LC_{50} values based on overlying water decreased from 17.7 µg/L at 48 hours to 9.2 µg/L at 10 days, but increased to 22.3 µg/L at 14 days (Figure 4-2). LC_{50} values based on sediment concentrations showed a similar trend, with sediment LC_{50} values decreasing from 428 mg/kg at 48 hours to 162 mg/kg at 10 days, then increasing to 286 mg/kg at 14 days (Figure 4-4). Pore water LC_{50} values, however, decreased through time, from 69 µg/L at 48 hours to 36 µg/L at 14 days (Figure 4-3).

P. promelas growth (expressed as dry weight) was equally sensitive as survival in the 10 d test, with NOEC values of 8.6 µg/L, 42.8 µg/L, and 136.9 mg/kg for overlying water, pore water, and sediment, respectively (Table 4-4). However, in the 14 day test, growth was less sensitive than survival, with NOEC values for growth at least two times greater than NOEC values for survival.

<u>*Hyalella azteca*</u>.

H. azteca response to copper contaminated sediment resulted in decreasing survival with increasing test duration through 14 days of exposure (Figures 4-2 through 4-4). LC_{50} values for overlying water, pore water, and sediment decreased from 59 µg/L to 31 µg/L, from 754 to 545 µg/L, and from 424 to 247 mg/kg, respectively, in 48 hours to 14 day tests. No threshold for mortality was achieved for *H. azteca* exposed to copper contaminated sediment through 14 days of exposure. Test durations of at least 14 days may be required to accurately assess the toxicity of sediment-sorbed copper to *H. azteca*. The 10-day LC_{50} value reported in this study, 35 µg/L in overlying water for *H. azteca* was similar to 10-day LC_{50} value reported by Cairns *et al.* (1984) of 39 µg/L with copper-amended sediments. LC_{50} values based on sediment copper concentrations, however, were considerably different, ranging from 262 mg/kg in this study to 1078 mg/kg by Cairns *et al.* (1984). These data indicate that copper concentrations in the overlying water were likely the source of copper toxicity to *H. azteca*. The variability in LC_{50} values based on sediment concentrations reflects the influence of sediment characteristics on copper bioavailability.

<u>*Chironomus tentans*</u>.

C. tentans survival decreased through time when exposed to copper contaminated sediment (Figures 4-2 through 4-4). LC_{50} values for overlying water, pore water, and sediment decreased from 323 µg/L to 36 µg/L, from 5820 to 62 µg/L, and from 4522 to 1026 mg/kg, respectively in 48 hours to 14 day tests. *C. tentans* growth, expressed as dry weight, was considerably more sensitive than survival in the 10 day test (Table 4-4). In this study, no temporal threshold for mortality or growth was achieved for *C. tentans* exposed to copper contaminated sediment, up to 14 days of exposure. *C. tentans* survival in sediment tests decreased through time whereas survival in water-only tests increased through time. These data suggest that the toxic responses exhibited by *C. tentans* correspond to exposures via sediment or pore water rather than overlying water. Test durations of at least 10 days may be required to accurately assess the toxicity of sediment-sorbed copper to *C. tentans*. Conducting laboratory evaluations with *C. tentans* with an insufficient test duration (i.e., $\leq$10 days) or insensitive endpoint (i.e., survival) may lead to the erroneous conclusion that a sediment is not toxic.

The 10 day LC_{50} values reported here of 36 µg/L and 1026 mg/kg (overlying water and sediment, respectively) for *C. tentans* exposed to a copper contaminated sediment were similar to 10 day LC_{50} values of 38 µg/L and 857 mg/kg reported for *C. tentans* exposed to a copper-amended sediment (Cairns *et al.*, 1984). Cairns *et al.* (1984) concluded that the route of exposure was via the overlying water, but based this conclusion on water-only data for *H. azteca* and not *C. tentans* data.

Sediment Experiments - Relative Sensitivities

Relative sensitivities of the organisms examined in this study when exposed to a copper contaminated sediment were dependent upon test duration (Figures 4-2 through 4-4) and test endpoint (Tables 4-4 and 4-5). In general, *C. dubia* was the most sensitive organism examined, with NOEC values for reproduction for overlying water copper concentrations as low as 3.2 µg/L. *D. magna* was two times less sensitive than *C. dubia* with reductions in survival occurring in overlying water concentrations as low as 8.7 µg/L. *P. promelas* was slightly less sensitive than *D. magna* with NOEC values in overlying water concentrations as low as 8.6 µg/L. Survival of *H. azteca* was similar to that of *P. promelas*, with reductions in survival occurring in overlying water concentrations as low as 16 µg/L. *C. tentans* was less sensitive than the other organisms to sediment-sorbed copper based on data from 48 hours, 96 hours, and 7 day tests. However, in the 10 day test with *C. tentans*, considerable mortality occurred at overlying water concentrations of 48 µg/L, and appreciable growth effects occurred at 22 µg/L. If exposure duration and test endpoint are properly selected (i.e., 10 day duration or greater), *C. tentans* sensitivity to this copper contaminated sediment approaches that of the other organisms examined. Based on copper concentrations measured in sediment, *H. azteca* and *C. tentans* are more sensitive to copper in sediment than *P. promelas*, with significant effects on survival and growth observed for *H. azteca* at 193 mg/kg (14 d), for *C. tentans* at 216 mg/kg (10 d), and for *P. promelas* at 259 mg/kg (14 d). As in the water-only experiments, *C. dubia* was again the most sensitive organism based on sediment concentrations, with *C. dubia* being adversely impacted at concentrations as low as 18.1 mg/kg (Table 4-4).

Table 4-5. Experimental conditions and species with their associated resolutions examined in this study.

Experimental Condition	Test Organism	Resolution
Reference Sediment (Toxic or Otherwise Unavailable)	*Daphnia magna*	Formulated Reference Sediment
	Ceriodaphnia dubia	
	Chironomus tentans	
	Hyalella azteca	
	Pimephales promelas	
Relative Sensitivity of Test Organisms	*Daphnia magna*	Screening Tests (96 h)
	Ceriodaphnia dubia	Screening Tests (96 h)
	Chironomus tentans	Exposure Dependent
	Hyalella azteca	Exposure Dependent
	Pimephales promelas	Exposure Dependent
Test Endpoint	*Daphnia magna*	Survival (96 h)
	Ceriodaphnia dubia	Reproduction (14 d)
	Chironomus tentans	Growth as Dry Weight (>10 d)
	Hyalella azteca	Survival (>10 d)
	Pimephales promelas	Survival (>10 d)

Organism physiology plays an important role in an organism's sensitivity to copper in sediment. Some chironomids, for example, are resistant to metals such as zinc, chromium, and cadmium in sediments (Wentsel *et al.*, 1977). Chironomid larvae regulate accumulation of copper, nickel and zinc in their tissues when exposed to these metals in sediments (Krantzberg and Stokes, 1989). Chironomid larvae apparently can detoxify or regulate the absorbed, or internal portion of their metal body burdens. Metal-binding proteins (e.g., metallothioneins) act as sinks or sequester metals such as Zn, Cu, Cd, and Hg in organism tissues (Petering and Fowler, 1986; Fowler, 1987; Olsson and Haux, 1986). Calcium accumulation in tissues of these midges has been hypothesized to interact with metals and reduce metal toxicity in chironomids (Krantzberg and Stokes, 1989). Although tissue levels of copper were not measured, *C. tentans* was apparently able to regulate copper in its tissues during 48 and 96 hour exposures. However, increased exposure to copper (7-14 days) resulted in increased mortality and reduced growth, with no lower threshold observed, indicating that a minimum exposure of 7 days to copper is required to manifest an adverse response in *C. tentans*.

Sediment Experiments - Test Duration and Endpoints

The effect of test duration and endpoint on test results was species dependent (Tables 4-4 and 4-5). In general, the cladocerans *C. dubia* and *D. magna* responded to the copper contaminated sediment within 96 hours of exposure, demonstrating a mortality threshold at 96 hours of exposure. Test durations longer than 96 hours (7, and 10 days) resulted in no further decreases in survival or reproduction for either organism (Table 4-4). However, in the 14 day exposure, *C. dubia* reproduction was more sensitive than survival, indicating that 14 day exposure to copper contaminated sediment was required to manifest reduced reproduction. *P. promelas* survival in copper contaminated sediment decreased through time with the exception of the 14 day test, indicating a mortality threshold at 10 day exposure duration. *P. promelas* growth in 10 and 14 day tests was equal to or less sensitive than survival, even though tests were started with 2-4 day old fry. *H. azteca* and *C. tentans* survival both decreased through time, with no mortality threshold being achieved through 14 days of exposure for either

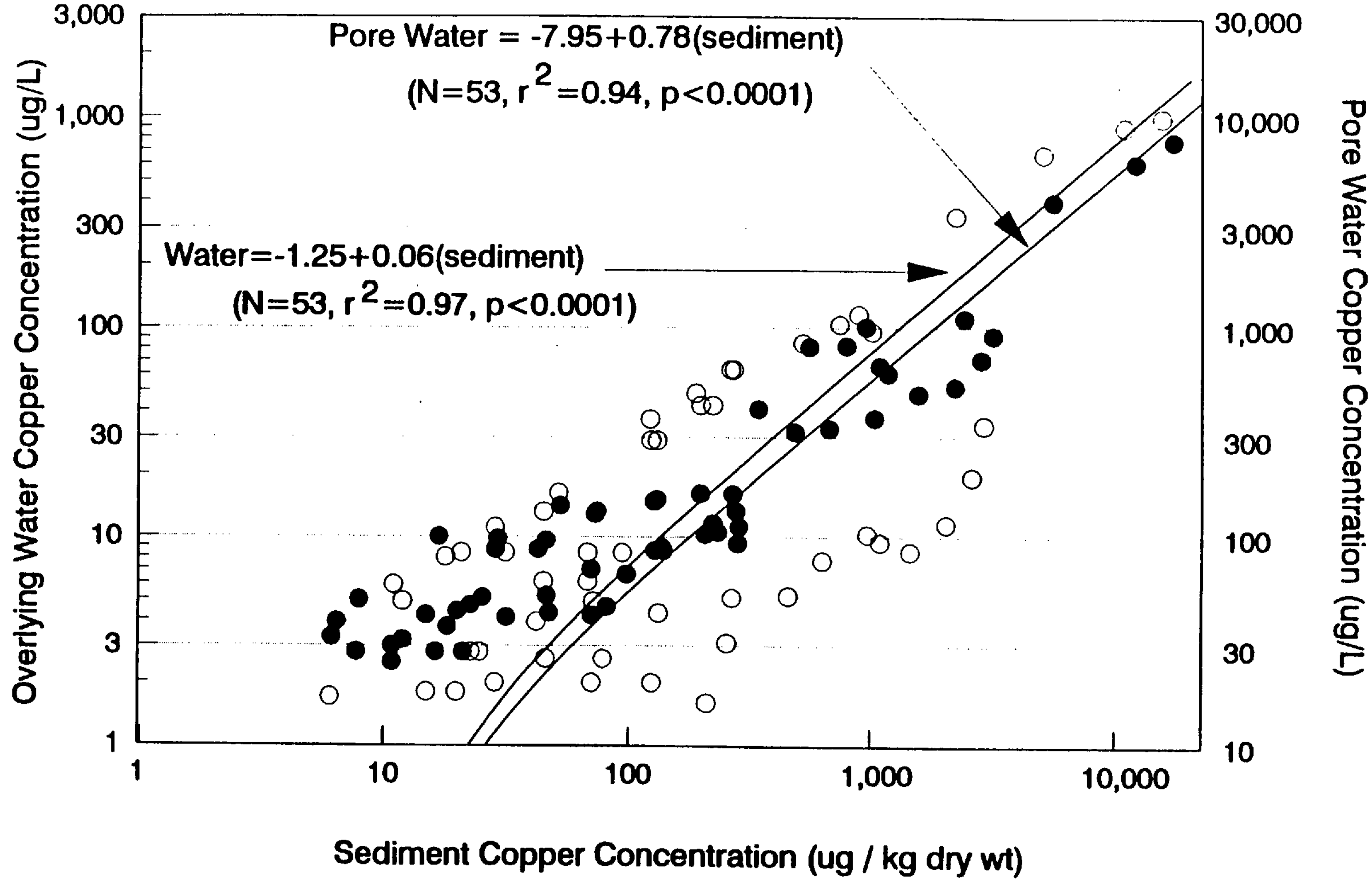

Figure 4-5. Relationship between concentrations of copper in sediment and concentrations of copper in pore water (open circles) and overlying water (closed circles) in sediment tests.

organism. These results indicated that laboratory sediment tests of at least 10-14 day duration were required to accurately assess the potency of copper contaminated sediments to *H. azteca* and *C. tentans*. In the 10 day *C. tentans* test, growth as dry weight was more sensitive than survival, illustrating that growth was more sensitive than survival for this organism when exposed to copper.

Regression analysis was performed to evaluate the relationship between copper concentrations in sediment and copper concentrations in pore water and overlying water in sediment tests (Figure 4-5). Both pore water and overlying water copper concentrations were highly dependent on the concentration of copper in sediment (r^2=0.94 and 0.97, respectively). For a given sediment copper concentration, pore water concentrations of copper varied up to 30 times whereas overlying water concentrations varied less than three times. In these experiments the relationship between copper in sediment and overlying water was more predictable than the relationship between copper in sediment and pore water. Even though highly significant r^2 values were obtained for the relationships between copper concentrations measured in sediments and those measured in either overlying water or pore water, the factor of three to 30 difference in variability for a given sediment concentration is sufficient to encompass from 100% survival to 100% mortality for the organisms examined in this study. Thus, measuring copper concentrations in sediment or pore water would only provide presumptive evidence of sediment toxicity.

To determine the source of copper toxicity, percent mortality of each organism was plotted against concentrations of copper in water-only tests, and concentrations of copper in overlying water, pore water, and sediment in sediment tests (Figures 4-6 through 4-9). For *C. dubia*, *D. magna*, *P. promelas*, and *H. azteca*, toxicity observed in copper contaminated sediment tests was associated with copper in the overlying water. Exposure-response curves for overlying water in sediment tests were similar to exposure-response curves from water-only tests with copper. This trend was also observed when LC_{50} values from water-only and sediment tests were compared (Figures 4-1 and 4-2). In these experiments, little, if any toxicity was caused by sediment-bound copper. In water-only tests with *C. tentans*, percent mortality was, in general, less than overlying water and pore water copper concentrations in sediment test (Figure 4-10). These results reflect the physiological differences as well as

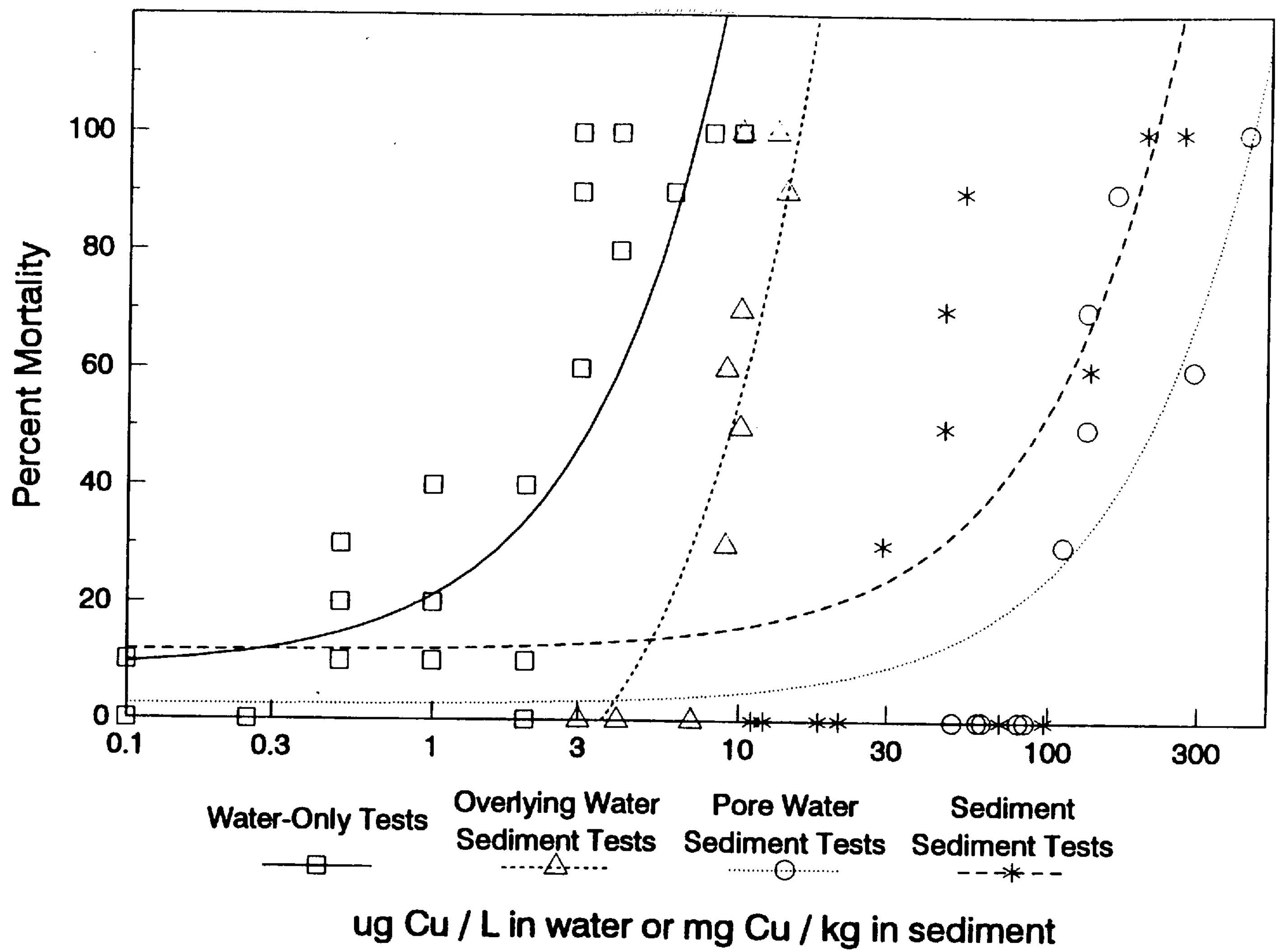

Figure 4-6. Toxicity in copper to *C. dubia* in water-only exposures (squares) vs. contaminated sediment tests in overlying water (triangles), pore water (circles), and sediment (asterisks).

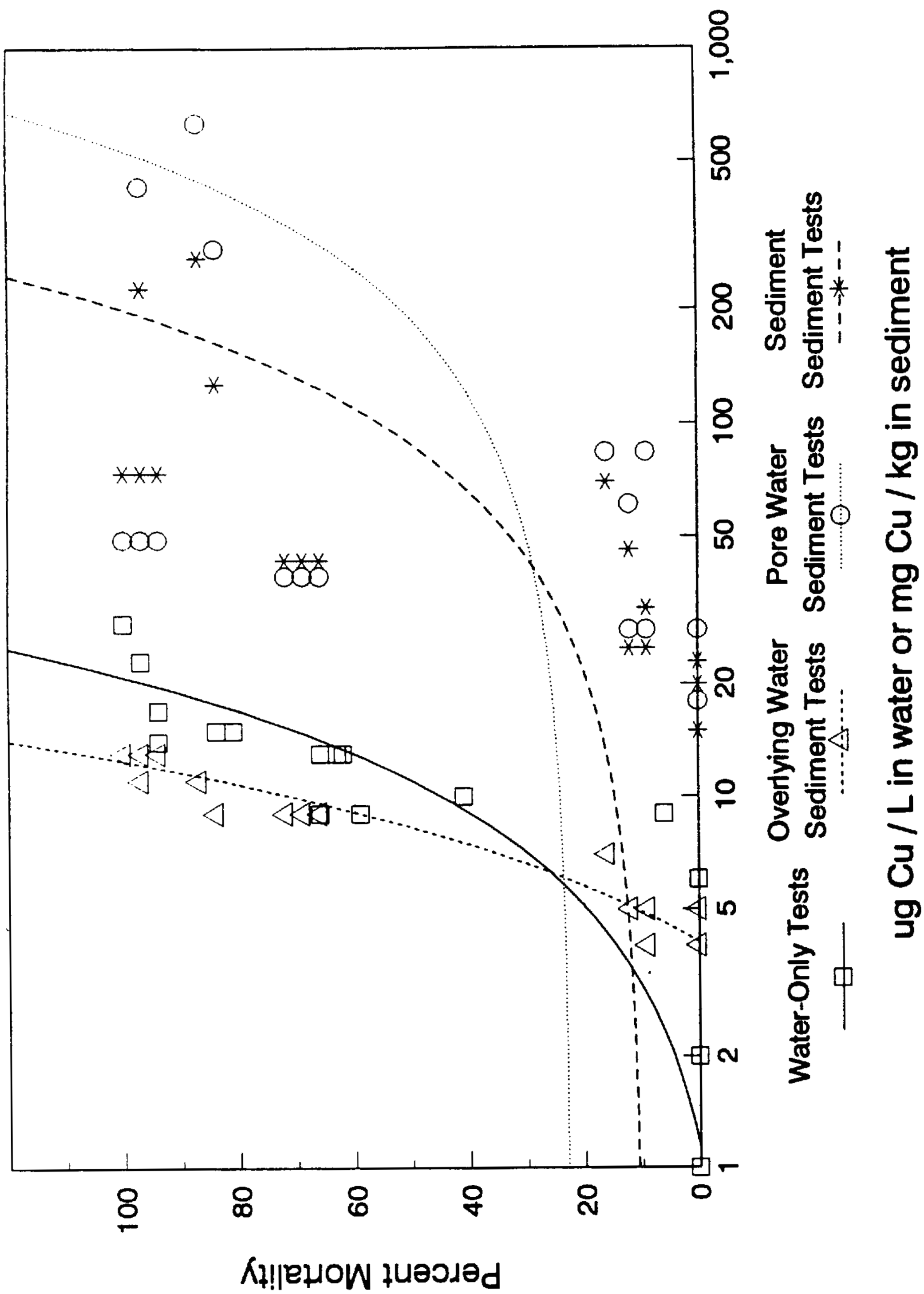

Figure 4-7. Toxicity of copper to *D. magna* in water-only exposures (squares) vs. contaminated sediment tests in overlying water (triangles), pore water (circles), and sediment (asterisks).

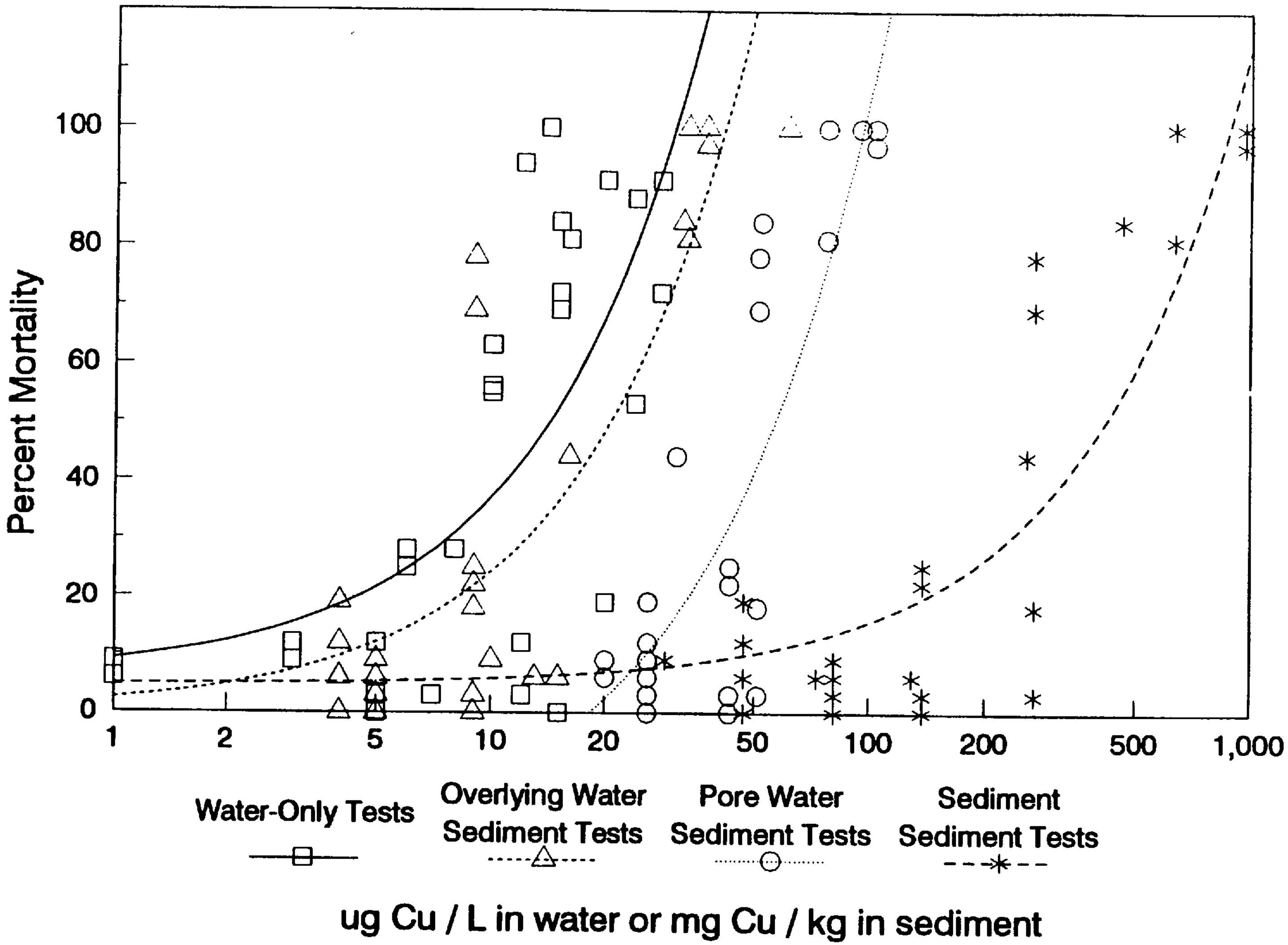

Figure 4-8. Toxicity of copper to *P. promelas* in water-only exposures (squares) vs. contaminated sediment tests in overlying water (triangles), pore water (circles), and sediment (asterisks).

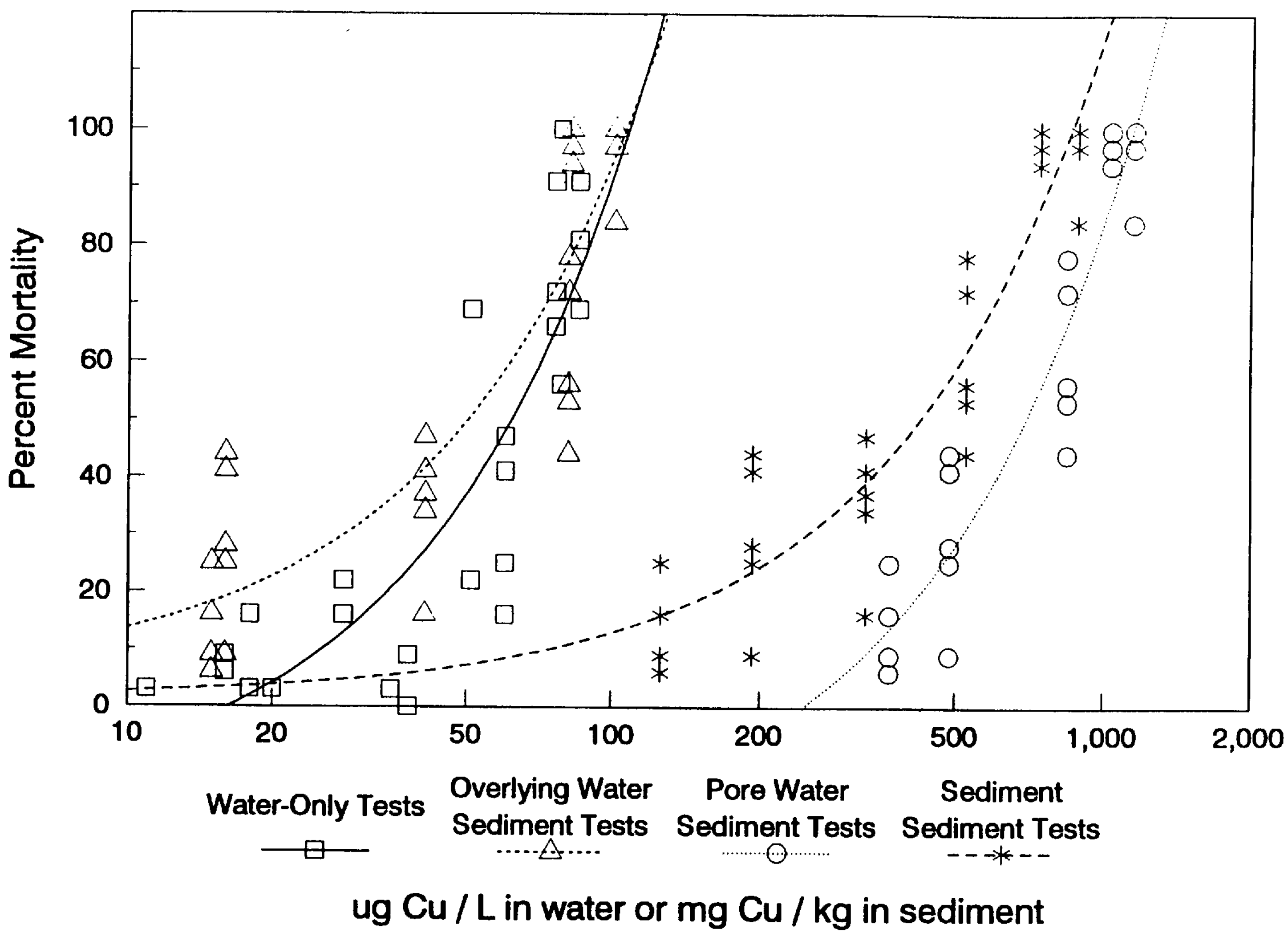

Figure 4-9. Toxicity of copper to *H. azteca* in water-only exposures (squares) vs. contaminated sediment tests in overlying water (triangles), pore water (circles), and sediment (asterisks).

differing routes of exposure between test organisms examined in this study. These results also reflect a "worst case" copper exposure due to low hardness values measured in these experiments (6-10 mg/L as $CaCO_3$). Increasing water hardness and associated carbonate alkalinity is thought to reduce the acute toxicity of copper (U.S. EPA, 1985; Gauss *et al.*, 1985). Both the U.S. EPA Ambient Water Quality Criteria for Copper and Canadian water criteria documents include expressions of the copper criteria as a function of hardness to allow adjustments for these water parameter effects (U.S. EPA, 1985b; Gauss *et al.*, 1985).

Formulated Sediment as a Reference and Dilution Sediment

To determine the degree of sediment toxicity, the test sediment is diluted with an appropriate reference sediment. The copper contaminated sediment used in this study was toxic (100% mortality) to *H. azteca* and *C. tentans* in 10 day screening tests (data not shown). Because a suitable natural sediment from the field was not available, a formulated reference sediment was used to dilute the test sediment. Diluting a test sediment with a reference sediment that is characteristically dissimilar may result in a U-shaped rather than a sigmoidal-shaped exposure-response curve, making assessments of the degree of sediment toxicity difficult, if not impossible (Nelson *et al.*, 1993).

A suitable reference sediment in definitive toxicity tests possesses similar characteristics as the test sediment, is inherently nontoxic, and when used as a dilution sediment, should provide decreases in sediment toxicity with concomitant increases in sediment dilution, thus generating predictable exposure response relationships for test organisms. As shown in Table 4-2, formulated sediment was prepared so that it possessed similar sediment characteristics as the test sediment. The only deviation was redox potential, which was slightly reduced (+4 mv) in the test sediment and oxidized (+228 mv) in the formulated sediment. Control survival for all test organisms was consistently above 80% (typical minimum survival required for a valid test; ASTM, 1990), ranging from 85 to 100% in all tests. These data illustrated that formulated sediment served as a suitable control sediment as a nontoxic substrate for the evaluation of the condition of the test organism populations subject to laboratory procedures and for statistical comparisons with the test

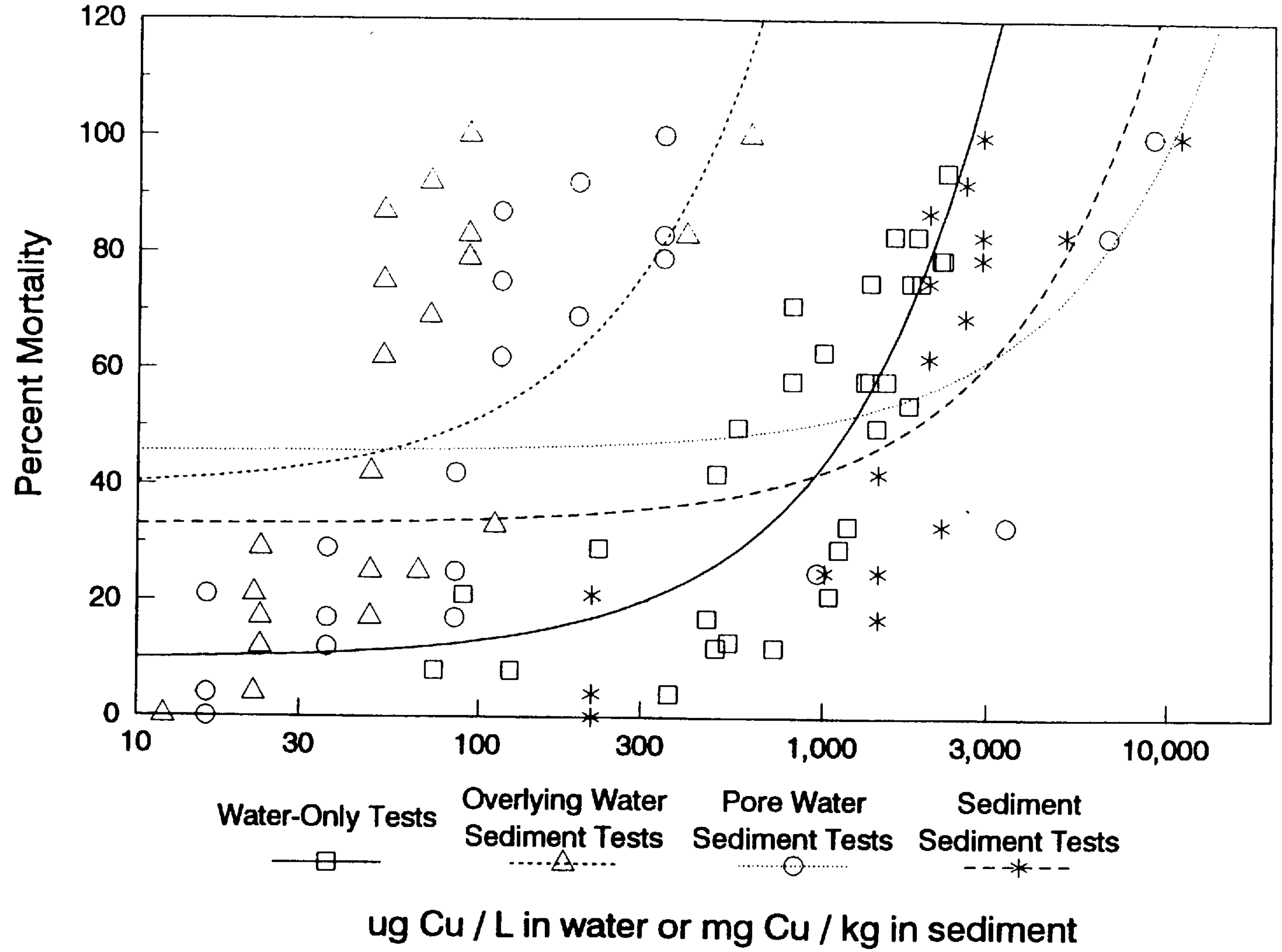

Figure 4-10. Toxicity of copper to *C. tentans* in water-only exposures (squares) vs. contaminated sediment tests in overlying water (triangles), pore water (circles), and sediment (asterisks).

sediment. Since formulated sediment possessed characteristics similar to the test sediment (e.g., particle size) and control survival of all organisms was between 85 and 100%, formulated sediment provided confirmation that the characteristics of the copper contaminated sediment did not contribute to test organism mortality in this study.

Formulated sediment, when used to dilute the copper contaminated sediment, resulted in decreased concentrations of copper in sediment, pore water, and overlying water with increases in test sediment dilution (Figure 4-11). Decreases in copper concentrations measured in water, pore water, and sediment resulted in a concomitant decrease in organism mortality in all tests, and provided predictable exposure-response curves for all organisms at all test durations. Formulated sediment provided a suitable reference and control sediment for the copper contaminated sediment exposures for all organisms examined in this study, thus permitting an accurate assessment of sediment potency. Without the use of a reference sediment, there is no mechanism for determining the potency or degree of sediment toxicity if 100% mortality of toxicity test organisms occurs. Two sediments may result in 100% test organism mortality, yet when diluted with a reference sediment may produce dissimilar results, one with concomitantly reduced toxicity with sediment dilution and the other sediment displaying toxicity when diluted as much as 75%. The determination of the divergent potency of sediments therefore requires conducting a definitive sediment toxicity test using reference sediment.

Sediment Experiments - Risk Characterization

Laboratory toxicity tests are frequently used to determine the presence of toxicity (screening tests) and degree of toxicity (definitive tests) of sediments. Based on data from the experiments conducted in this study, 96 hours *D. magna* and *C. dubia* tests would be suitable as screening level tests due to the immediate response (96 hours) of these organisms to copper exposure in sediment and water, but would not provide information regarding the degree of toxicity (Tables 4-4 and 4-5). *H. azteca* and *C. tentans* were not sensitive to copper during 48 and 96 hour tests and would not be suitable as screening level tests. However, *H. azteca* survival and *C. tentans* growth in 10 and 14 day tests were suitable for determining the degree of copper toxicity in water and sediment definitive tests, thus determining the relative

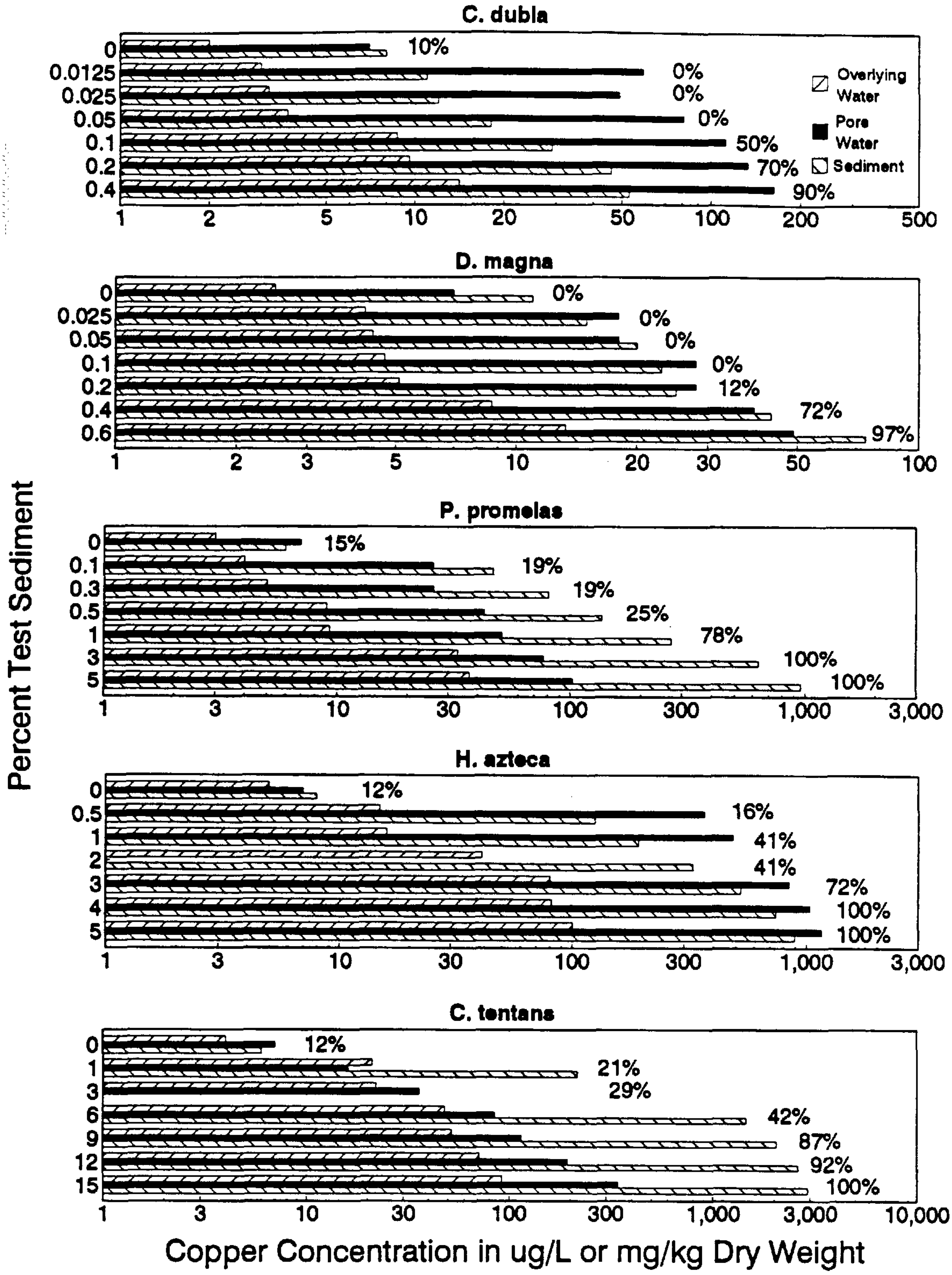

Figure 4-11. Effects of test sediment dilution on test organism survival in sediment tests based on measured copper concentrations in water, pore water, and sediment. Numbers within figures are percent mortality of test organisms.

potency of this sediment (Table 4-5). In this study, the 10 and 14 day *C. tentans* growth tests were particularly useful for assessing sediment potency because they provided a more continuous response than mortality, which provided only a quantal, or all-or-nothing response. The exposure-response relationship developed from *C. tentans* growth tests could be used to estimate to what degree a sediment may be above or below the threshold of toxicity. The *C. dubia* 14 day reproduction test also provided a continuous response and was useful for determining copper potency in sediments and water.

Other factors influencing the selection of organisms to assess sediment potency in laboratory tests include the ability to culture test organisms, the species- and toxicant-specific differences in toxicity, and the degree of association of the organisms with sediment (Cairns *et al.*, 1984). Culture of test organisms is crucial to provide individuals of known age and health and to eliminate the risk of collecting organisms from the field that have become resistant to low levels of sediment-sorbed contaminants. Studies have shown that field collected organisms such as *C. tentans* can become resistant to a variety of contaminants, including copper (Krantzberg and Stokes, 1989; Wentzel *et al.*, 1978). Using tolerant field collected organisms to assess sediment toxicity could lead to erroneously concluding that a toxic sediment is nontoxic. Benthic organisms from relatively uncontaminated areas attempting to colonize the contaminated area would likely be unable to fully colonize the contaminated area. In addition, culturing test organisms permits observation during all life stages of development, thus providing a standard for evaluation of adverse sublethal behavioral effects observed in toxicity tests. Established culture protocols exist for all organisms used in this study.

This study provided evidence that these test species were useful for assessing copper toxicity in sediment. Although *H. azteca* and *C. tentans* were generally less sensitive to copper in sediment than *C. dubia* and *D. magna*, they may be more sensitive to other sediment-sorbed contaminants. For example, Suedel and Rodgers (1993) found that *H. azteca* and *C. tentans* were more sensitive than *D. magna* when exposed to fluoranthene-amended sediment. Another consideration is the degree of association of an organism with sediment. For example, daphnids are generally considered water column organisms, but *D. magna* $\geq$48 hour old graze on sediment surfaces. *H. azteca* and *C. tentans* are both considered benthic organisms, although their association with sediment differs in potentially important ways. *H. azteca* is an epibenthic detritivore, which feeds on organic material on the sediment surface and on epiphytes of rooted aquatic vegetation, thus is in contact with sediment and the overlying water. *C. tentans*, however, is a benthic infaunal organism, which during its larval stage, constructs a case from organic material in sediment and thus is in intimate contact with sediments. By using several organisms that represent a variety of niches, the likelihood of making an erroneous conclusion regarding sediment toxicity can be reduced. When this is not possible, one should use caution in the extrapolation of laboratory results to field situations.

SUMMARY

Effects of experimental conditions including exposure duration, test organism selection, and test endpoint on the observed toxicity of aqueous phase copper and a copper contaminated freshwater sediment were evaluated. Relative sensitivities of test organisms exposed to copper in water and copper contaminated sediment varied with test duration and test endpoint. In general, *C. dubia* was the most sensitive organism tested, followed in decreasing sensitivity by *D. magna*, *P. promelas*, *H. azteca*, and *C. tentans*. Effects of test duration on copper toxicity were most pronounced for *H. azteca* and *C. tentans*, with mortality and growth effects becoming increasingly sensitive with increasing test duration. Formulated sediment served as a suitable control, reference, and dilution sediment in this study, matching characteristics (except redox potential) of the test sediment.

Section 5
GENERAL SUMMARY

This research focused on evaluating the critical components of laboratory experiments that may contribute to more accurate assessments of sediment quality. The concerns and uncertainties related to laboratory experiments and sediments led to the experiments described in this report. The intent of these experiments was to help clarify questions regarding the accuracy of laboratory experiments and the ability of these experiments to predict adverse effects of sediment associated contaminants. A series of experiments were performed to address these uncertainties, with the results summarized below.

Formulated sediment was prepared to match representative natural sediments with respect to various sediment characteristics and served as a suitable substrate for toxicity testing organisms during life cycle exposures. Constituents used to prepare formulated sediment were commercially available, thus permitting widespread use of this sediment as a standard reference sediment. In definitive sediment toxicity tests, formulated sediment, when used to dilute a copper contaminated sediment, provided predictable exposure-response curves for all organisms at all test durations. Formulated sediment provided a suitable reference and control sediment for the copper contaminated sediment, thus providing a means of accurately assessing sediment potency. Formulated sediment was also used to successfully determine the tolerance of *H. azteca* and *C. tentans* to particle size regimes and organic matter content of sediments. *H. azteca* and *C. tentans* were tolerant of a wide range of particle size regimes and organic matter content of bottom sediments, thus making them suitable test organisms to assess sediment quality in most situations. Formulated sediments should be suitable for evaluating tolerances of other benthic toxicity testing organisms for sediment characteristics, thus reducing somewhat the risk of drawing erroneous conclusions regarding sediment toxicity.

When formulated sediment constituents were sufficiently conditioned, *H. azteca* survival was >80% in all particle size regimes of formulated and field-collected sediments. *H. azteca* was tolerant (>80% survival) of formulated and field-collected sediments with organic matter content ranging from 0.12 to 7.8%. *C. tentans* was not tolerant (survival ranging from 6 to 56%) of most formulated sediment particle regimes (except for 80 to 100% silt) without a source of particulate organic matter (e.g., humus) even though dissolved organic matter was present up to 13%. Formulated and field collected sediments containing sufficient concentrations of organic matter (0.91 to 7.8%) resulted in >80% survival for *C. tentans* in 10-day whole sediment exposures. *C. tentans* survival was also >80% in all formulated sediment particle sizes when sufficient particulate organic matter was present (2.5%). Formulated sediments were suitable for determining *H. azteca* and *C. tentans* tolerance of organic matter and particle size distribution of sediments. *H. azteca* and *C. tentans* were tolerant of a wide range of particle size regimes and organic matter content of bottom sediments, thus making them suitable test organisms to assess sediment quality in most situations.

The experiments conducted with *H. azteca* and field collected sediments demonstrated the potential for use of copper sulfate as a reference toxicant in sediment toxicity tests. Sediment reference toxicant tests conducted with characteristically dissimilar reference sediments amended with copper sulfate yielded predictable exposure response relationships. Copper toxicity was related to the organic carbon and organic matter content of sediments. The successful use of copper sulfate as a reference toxicant in sediment reference toxicity testing should eliminate some of the concerns with sediment reference toxicant tests to measure the health of benthic species.

Effects of experimental conditions on toxicity testing results indicated that the relative sensitivities of test organisms exposed to copper in water and copper contaminated sediment varied with test duration and test endpoint. In general, *C. dubia* was the most sensitive organism tested, followed in decreasing sensitivity by *D. magna*, *P. promelas*, *H. azteca*, and *C. tentans*. Effects of test duration on copper toxicity were most pronounced for *H. azteca* and *C. tentans*, with mortality and growth effects becoming increasingly sensitive with increasing test duration. Formulated sediment served as a suitable control, reference, and dilution sediment in these experiments, matching characteristics (except redox potential) of the test sediment.

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