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SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 18, No. 4, pp. 679–684, 1999
Printed in the USA
0730-7268/99 $9.00 + .00

NEW TESTING APPARATUS FOR ASSESSING INTERACTIVE EFFECTS OF SUSPENDED SOLIDS AND CHEMICAL STRESSORS ON PLANKTON INVERTEBRATES

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(*Received* 10 *December* 1997; *Accepted* 15 *June* 1998)

Abstract—To better predict and interpret the responses of aquatic organisms to environmentally relevant chemical exposures, it is necessary to investigate the combined effects of physical (e.g., suspended solids) and chemical stressors. One of the limitations in investigating suspended solids–chemical interactions has been the lack of an appropriate testing system. The specific objective of the current study was to develop and assess a suspended solids testing apparatus (SSTA) for studies on the combined effects of suspended solids and chemicals on aquatic invertebrates. The SSTA was designed to permit the assessment of varying suspended solids concentrations on *Daphnia magna* at a constant freely dissolved concentration of a compound. The system was also designed to facilitate the control of exposure variables without the need for large numbers of replicates and chemical analyses. The experiments reported here demonstrate that the SSTA is effective for assessing the combined or interactive effects of suspended solids and a chemical stressor on aquatic organisms.

Keywords—Suspended solids Carbofuran *Daphnia magna* Acute toxicity Mixture effects

INTRODUCTION

Toxicity testing with fish and aquatic invertebrates has traditionally addressed the effects of individual chemicals on organisms. However, in natural systems organisms are exposed to a myriad of combined chemical and physical stresses. Exposure to these mixtures in the field can affect the responses of organisms in a manner that may not be readily apparent from assessments of the stressors in isolation. Multiple chemicals and physical stressors in the environment can affect bioavailability and toxicokinetic and toxicodynamic processes. Chemical mixtures can affect uptake and depuration rates of individual components, and thereby affect a compound's toxicokinetics [1,2]. Different components in a chemical mixture can also selectively increase stress on an organism, thereby affecting its susceptibility to other toxicants in the mixture [3]. Toxic responses in these instances are primarily due to changes in toxicodynamic sensitivity.

In considering the interactions of physical and chemical stressors, the addition of solids to an aquatic system is generally assumed to decrease the toxicity of hydrophobic organic toxicants through a reduction in bioavailability [4–6] caused primarily by sorption to organic carbon [7]. If an organic toxicant is not hydrophobic, bioavailability and toxicokinetics with or without suspended solids are assumed not to be significantly influenced. However, numerous studies have indicated that aquatic organisms can be affected by exposure to suspended solids alone. For example, inverse relationships have been reported between suspended solids concentrations and daphnid fecundity, growth, and juvenile survival [8–11]. Therefore, it is reasonable to suspect that suspended solids could increase the stress on an organism and affect the toxicodynamic response(s) to a chemical toxicant, even if the compound's bioavailability or toxicokinetics are not significantly altered.

One of the limitations confronting detailed investigations of suspended solids–chemical interactions has been the lack of an appropriate testing system. Hypothesis testing in a system where two variables are under investigation is facilitated if the variables can be controlled. However, currently available experimental apparatus, with unshared water and discrete chambers, limit the ability to control chemical exposure in a suspended solids environment. The specific objective of this study was to develop and assess a suspended solids testing apparatus (SSTA) as a versatile tool for future studies on the combined effect of suspended solids and toxicants on aquatic invertebrates. The apparatus was designed to allow diffusion of dissolved chemicals between a main tank and multiple rotating chambers containing suspended solids and test organisms. This capability assures that test organisms exposed to different suspended solids concentrations are exposed to equivalent freely dissolved chemical concentrations throughout an experiment. Therefore, differences in toxicity to organisms in an experiment are the result of varied suspended solids exposures.

Initial experiments to characterize some of the operating characteristics of the SSTA consisted of acute toxicity studies using *Daphnia magna* as a model organism and carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate) as a model organic pesticide. Experiments were conducted across an ecologically relevant range of suspended solids concentrations. Although *D. magna* is not indigenous to most of North America, water fleas (*Daphnia*) are present in most lentic water bodies throughout the world. The relatively

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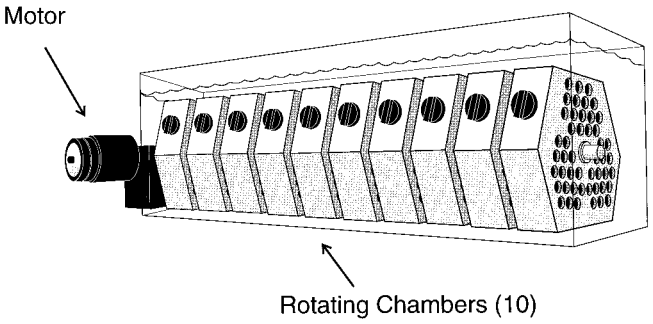

Fig. 1. Diagram of the suspended solids testing apparatus (SSTA) showing motor and rotating chambers in tank.

large size of *D. magna* and its history as a laboratory test organism make it a good subject for use in these experiments. Carbofuran, a carbamate nematicide and insecticide, was chosen because it is a nonionic organic pesticide with low hydrophobicity (log K_{ow} = 1.63–2.32) [12,13]. As a nonhydrophobic chemical, it is expected to partition into test organisms almost exclusively from the freely dissolved phase, and therefore its bioavailability should not be significantly affected by the addition of suspended solids. This compound attribute was deliberately chosen to minimize the influence of toxicokinetics and bioavailability on subsequent studies (C. Herbrandson et al., submitted). Finally, experiments were also conducted that compared the combined effects of carbofuran and suspended, settling, or sedimented solids on survival of *D. magna*.

MATERIALS AND METHODS

The SSTA (Fig. 1) [14] is a 37.75-L polycarbonate tank containing 10 rotating, 12-sided, 1-L chambers, and a 1-L static chamber. Two sides of each chamber have polycarbonate-reinforced walls of 0.1-μm nylon membrane (MSI, Westboro, MA, USA) to restrict the movement of sediment. Each rotating chamber also has two 2.25-inch (5.7-cm) threaded plugs made of polypropylene and a stainless steel bleeder screw for removal of air. The chambers are connected by stainless steel pinned, polycarbonate shafts and sleeves. A polycarbonate shaft, which passes through a water seal at the end of the tank, connects the chambers to a variable speed dc gearmotor (Dayton 4Z725A, Dayton, Chicago, IL, USA). Chambers were rotated at 5 rpm.

Suspended solids were a mixture of subsoil and decomposed peat that were collected in August 1993. The subsoil was 0.46% organic carbon (OC) and was obtained from a site in Roseville, Minnesota, USA, that has not been cultivated for more than 20 years. The decomposed peat was 34.9% OC and was collected from the Carlos Avery Wildlife Refuge, Forest Lake, Minnesota, USA. Both soils were dried in a forced draft Leekow furnace at 35°C. The decomposed peat was ground in a Wiley mill and filtered through a 250-μm stainless steel sieve. The subsoil was ground in a Wisconsin mill and sieved to 43 μm. The soils were stored in sealed containers at 4°C.

Mixtures of decomposed peat and subsoil were added to each chamber 4 h before the addition of carbofuran and 24 h before the addition of *D. magna*. Experiments were conducted with solids mixed to 3% OC. For experiments 1 and 2 in this report (see *Experimental design*, below), rotating chambers contained suspended solids at 0, 10, 50, 100, 500, 1,000, 5,000, and 10,000 mg/L. These concentrations are representative of the range of suspended concentrations found in a wide variety of ecosystems. Replicate chambers of 100 and 1,000 mg/L were also included in these experiments. For experiments 3 and 4 all chambers, except nonsuspended solids controls, contained suspended solids at 1,000 mg/L.

Technical grade carbofuran (99.2%) was provided by FMC (Philadelphia, PA, USA). All carbofuran additions to the SSTA were made from a stock solution of carbofuran dissolved in acetone. Carbofuran stock was added directly into the main tank 20 h before the addition of *D. magna*. The maximum concentration of acetone in any experiment, at the time of carbofuran addition, was 27 μl/L. Acetone controls at this concentration showed no effect on *D. magna* (data not shown).

Quantitative analysis of carbofuran concentrations in water samples from every experiment was conducted with an enzyme-linked immunosorbent assay (ELISA; RaPID Assay, Ohmicron, Newton, PA, USA). The assay is reported by the manufacturer to be 21 times more sensitive to carbofuran than to any carbofuran degradation product. One milliliter of tank water was sampled at 0, 24, and 48 h. Rotating chambers were sampled at 0 and 48 h. Samples were frozen at −20°C until analyzed. Before chemical analysis, samples were thawed and centrifuged (Eppendorf 5415 C, Brinkman Instruments, Westbury, NY, USA) at 10,000 *g* for 30 min. From each sample, 20 to 100 μl was removed and diluted to bring concentrations within the range of the ELISA (0.1–5.0 μg/L). Samples and replicates analyzed are noted in the Results and Discussion section. Concentrations of freely dissolved carbofuran (i.e., the 10,000 *g* supernatant) used in toxicity analyses were based on the mean of three ELISA replicates of the 48-h tank water samples. Excel 5.0 (Microsoft, Redmond, WA, USA) was used for ANOVA analysis of freely dissolved carbofuran concentration data. The coefficient of variability (CV) of ELISA controls was 8.2% between assays and 6.1% within assays.

Daphnia magna cultures were maintained in small aquaria at room temperature (19–21°C) on a 16:8 h light:dark cycle. Culture and test water was reconstituted NANOpure II water (Barnstead, Newton, MA, USA), following U.S. Environmental Protection Agency (U.S. EPA) methods for moderately hard water [15]. The pH was adjusted daily to 7.15 to 7.35. The *D. magna* were 72 to 96 h old for each experiment and all came from the same age parentage (17–22 d old).

The *D. magna* were fed a mixture of yeast–alfalfa–trout chow (YAT) and Tetra Conditioning Food (TetraWerke, Melle, Germany) in a 75:25 ratio (YAT:Tetra) throughout the studies. Except as noted, *D. magna* in test chambers were provided food at 2.5 mg/L at the beginning of all experiments. Reference toxicity tests were performed on approximately a weekly basis to monitor the health of the *D. magna* cultures in the laboratory.

Unless noted, water and suspended solids were added to the apparatus at −24 h, carbofuran was added at −20 h, and 50 *D. magna* were added to each chamber at 0 h. Chambers were rotated for 24 h and then stopped to allow the suspended solids to settle. This procedure was designed to simulate a storm event in a wetland. Experiments were completed at 48 h and toxic response of test organisms was rated. The *D. magna* were removed from chambers by passing the chamber contents through a 425-μm nylon filter. The organisms were then transferred to counting pans. No differences in effects were observed for organisms processed in this manner and culture control subjects that were not filtered.

Surviving *D. magna* were scored individually in a counting pan. Organisms that quivered or moved along the bottom of

the pan were considered affected by the treatments. All surviving *D. magna* that moved vertically in the counting pan water were considered normal even though there could be subjective behavioral differences, such as lethargy or ''pinwheeling,'' when compared to the control organisms. Only *D. magna* that were rated ''normal'' were considered to be unaffected by the experimental treatments. Maximum-likelihood probit analysis (ToxCalc 5.0, Tidepool Scientific Software, McKinleyville, CA, USA) was used to determine suspended solids EC50s for *D. magna* that were exposed simultaneously to a constant carbofuran concentration.

Experimental design

Four experiments were performed to assess the SSTA. Experiment 1 was designed to determine the time period over which carbofuran concentrations equilibrate throughout the apparatus. Experiment 2 was initially used to assess the capability of the apparatus and the protocol to discern suspended solids effects and is representative of experiments described in Herbrandson [14] and Herbrandson et al. (submitted for publication) where *D. magna* were exposed to a series of carbofuran concentrations across different concentrations of suspended solids. Experiments 3 and 4 were designed to assess whether or not toxic effects were caused by solids in suspension, or solids that had settled from suspension. Variations of each experiment from standard protocols are described in the Results and Discussion section.

For purposes of this study, the experiments presented were not replicated and are provided as examples of the utility and applicability of the SSTA. Our application of this apparatus has demonstrated excellent repeatability [14]. It should be noted that conditions in individual chambers can be replicated within a given experiment, which eliminates uncontrolled uncertainty due to different generations of test organisms.

RESULTS AND DISCUSSION

Understanding the interactions between suspended solids and chemicals is important for developing and validating models of chemical fate and toxicity. Without experimental methods that can permit the isolation of treatment variables and reduce the amount of chemical analysis necessary to perform combined studies, these studies will remain difficult to interpret, expensive, and time-consuming. The SSTA is an apparatus that allows the use of a single tank for concurrent exposures of test organisms to multiple concentrations of suspended solids and a single equivalent freely dissolved concentration of a chemical toxicant. The data collected in the present study were designed to determine some of the performance parameters of the SSTA by assessing the stability of aqueous concentrations of carbofuran across a range of suspended solids of 0 to 10,000 mg/L, as well as the associated survival of *D. magna*.

Chemical concentration equilibration

The SSTA was designed to differentiate relative effects between chambers. Therefore, as long as the measured freely dissolved concentrations of the tested chemical have equilibrated between chambers, differences in effects can be presumed to be caused by suspended solids or chemicals associated with them.

Experiment 1 was performed in the SSTA without test organisms, but following all other protocols. Suspended solids concentrations were 0, 10, 50, 100 (×2), 500, 1,000 (×2), 5,000, and 10,000 mg/L. This experiment tested the hypothesis that the concentration of freely dissolved carbofuran reaches equilibrium between all chambers and the tank before test organisms are added to the system. The target freely dissolved concentration of carbofuran was approximately 60 μg/L. For this experiment polypropylene tubes were connected to the bleeder holes of each chamber to allow the collection of chamber water samples with minimal disturbance. Triplicate samples (200 μl) were taken by syringe from all chambers every 3 h, from 5 min after the addition of carbofuran to the tank through 18 h. Triplicate samples were also taken at 24 and 30 h. Samples from the chambers with suspended solids at 0 and 5,000 mg/L, and the tank itself, were analyzed by ELISA to assess the freely dissolved carbofuran concentrations.

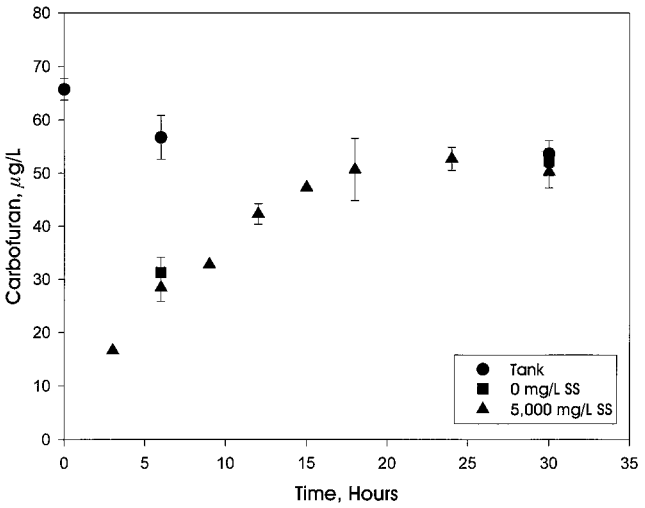

Fig. 2. Aqueous concentrations of freely dissolved carbofuran in samples taken from the tank and the chambers with suspended solids at 0 and 5,000 mg/L from 0 through 30 h. Carbofuran concentrations were determined by ELISA. Error bars denote standard deviations for three analyses; lack of error bars indicates those times where a single sample analysis was performed.

The results of experiment 1 indicated that carbofuran equilibriated in the SSTA chambers within 15 to 20 h (see Fig. 2). Carbofuran was added to the tank at 0 h and readily diffused into the chambers with suspended solids at 5,000 mg/L, with concentrations in the chambers increasing from 17 μg/L at 3 h to 48 μg/L at 15 h. Between 18 and 30 h, carbofuran concentrations in the chambers with suspended solids at 5,000 mg/L averaged 52.5 μg/L. A similar trend was noted for the chamber with suspended solids at 0 mg/L (data not shown). Carbofuran concentrations at 30 h were 52.3 ± 2.3 and 50.3 ± 3.0 μg/L in the chambers with suspended solids at 0 and 5,000 mg/L, respectively. The carbofuran concentration in the tank decreased from an initial value of 65.7 ± 1.9 μg/L at 10 min to 53.7 ± 2.5 μg/L at 30 h postaddition. The freely dissolved carbofuran concentrations at 30 h were equivalent in the tank and in the chambers with suspended solids at 0 and 5,000 mg/L ($p > 0.05$, two-tailed t test; see Fig. 2). Studies by Achik et al. [16] and Sharom et al. [17] showed that carbofuran sorption to soil takes place over a time period of less than 6 h. Therefore, results showing similar times to equilibration for freely dissolved carbofuran in chambers with and without suspended solids are expected.

Data from experiment 2 were used to monitor changes in freely dissolved carbofuran concentrations over the 48-h *D. magna* exposure period in the presence of suspended solids.

Concentrations in the tank and the chambers with suspended solids at 0 and 5,000 mg/L at 0 and 48 h were not statistically different ($p > 0.05$, ANOVA). Measured concentrations ranged from 77.5 ± 4.8 μg/L at 0 h to 74.5 ± 4.0 μg/L at 48 h. These data demonstrate that concentrations of carbofuran in different chambers of the SSTA do not vary significantly over the course of a 48-h experiment. Nonmicrobial degradation of carbofuran is strongly affected by pH [18,19]. In the present experiments, pH was controlled to 7.25 ± 0.1 and therefore degradation of carbofuran was not expected to be significant.

Although these data show that a compound with a low K_{ow}, such as carbofuran, will equilibrate between the chambers of the SSTA over a relatively short time, a hydrophobic chemical would be expected to behave somewhat differently. Diffusion of hydrophobic chemicals through the nylon membranes would be driven by molecular size and the concentration gradient, but the sorption kinetics of freely dissolved chemical to organic carbon in the system would determine the time to equilibration. Although most carbofuran would be expected to remain in the freely dissolved phase, a significant fraction of a hydrophobic compound would sorb to the organic carbon in the suspended solids [17,20]. A significant amount of a hydrophobic chemical would also be expected to sorb to the polycarbonate in the SSTA. The initial sorption of hydrophobic compounds to organic carbon in the tank and the suspended solids would be rapid, but equilibrium could take months [21,22]. Because diffusion is driven by a concentration gradient, equivalent freely dissolved chemical concentrations across the SSTA chambers cannot be achieved until equilibrium partitioning is reached between the freely dissolved and sorbed phases, which in the case of hydrophobic compounds would certainly be greater than that observed for carbofuran and would likely be a function of log K_{ow}.

Toxicity of suspended sediments to D. magna

Experiment 2 was also undertaken to assess the capability of the SSTA to provide sound suspended solids dose–response curves during a simultaneous exposure to carbofuran. In experiment 2, *D. magna* were tested in eight different concentrations of suspended solids (including 0 mg/L). Over the duration of the experiment, the carbofuran concentration ranged from 77.5 to 79.5 μg/L in the tank and sample chambers. At 0 h, 50 *D. magna* were added to each chamber and 1-ml water samples were taken from the tank, as well as the chambers with suspended solids at 0 and 5,000 mg/L. At 24 h the chamber rotation was stopped. When the test organisms were removed at 48 h, water samples were again taken from the tank and the chambers with suspended solids at 0 and 5,000 mg/L. Three replicates of each sample were quantified by ELISA to determine freely dissolved carbofuran concentrations. Toxicity to *D. magna* was recorded based on the criteria described previously and the suspended solids concentration–response curve was analyzed.

The calculated EC50 to *D. magna* in this experiment was suspended solids at 181 mg/L (134–238 mg/L, 95% confidence limits). A plot of the fraction of *D. magna* affected as a function of suspended solids concentration is provided in Figure 3. Ten percent of the organisms were affected at suspended solids concentrations between 0 and 10 mg/L, whereas approximately 75 and 98% were affected at suspended solids concentrations of 500 and 10,000 mg/L, respectively. In contrast, other experiments following protocols reported here with

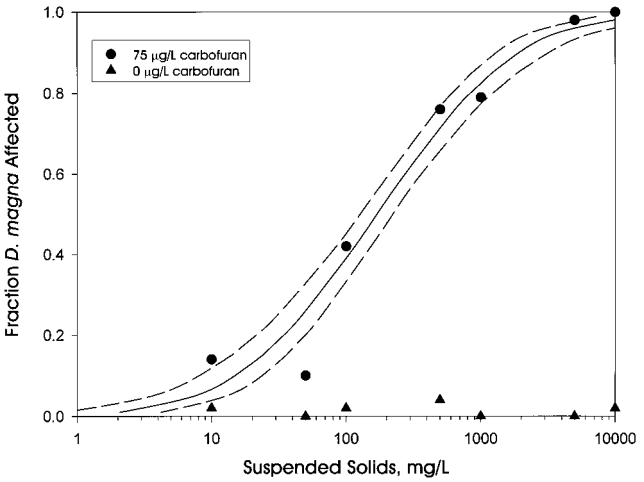

Fig. 3. Fraction of *Daphnia magna* affected as a function of exposure to varying suspended solids at a constant concentration of freely dissolved carbofuran of 75 μg/L (♦) and 0 mg/L (*). Maximum-likelihood probit curve with 95% confidence limits is presented. Experiments with carbofuran up to 10,000 mg/L and no carbofuran exhibited no significant toxicity to exposed *D. magna*.

suspended solids at concentrations up to 10,000 mg/L and no carbofuran have demonstrated no adverse effects to exposed *D. magna* [14; Herbrandson et al., submitted for publication]. When reviewed in conjunction with the analytical data from experiments 1 and 2, these data demonstrate the dose-dependent effect of suspended solids or particulate-adsorbed carbofuran on *D. magna* simultaneously exposured to carbofuran at 75 mg/L.

Although no previously published studies were found in which *D. magna* were exposed simultaneously to suspended solids and a toxicant, reported carbofuran EC50 values [14,23] and chronic suspended solids sensitivity of other daphnid species [8,9,11] suggest that the SSTA and associated protocols provide comparable results. Experiments exposing *D. magna* to no suspended solids and a range of carbofuran concentrations in the SSTA have produced a dose–response curve with a calculated EC50 for carbofuran of 92 mg/L (81–116 mg/L, 95% confidence limits). Static reference toxicity studies conducted in this laboratory with 72- to 96-h-old *D. magna* have resulted in 48-h EC50s for carbofuran of 35 μg/L for unfed organisms and 76 μg/L for organisms fed 2.5 mg/L YAT:Tetra. Johnson [23] reported a 48-h EC50 for carbofuran of 48 μg/L for *D. magna* neonates exposed under standard protocols. Chronic exposures of *Daphnia pulex* and *Daphnia ambigua* to 100 and 50 mg/L of clay, respectively, have resulted in low survivability [8,9]. The stable concentration–response curve resulting from this experiment demonstrates that the SSTA and the associated protocol are sufficiently sensitive to document combined or interactive toxicologic effects of suspended solids and carbofuran.

Influence of settling solids on toxicity

Test organisms in experiment 2 previously described (Fig. 3) were not exposed to solids in suspension during the entire experiment (i.e., a settling period of 24 h without chamber rotation follows the 24-h period of chamber rotation and solids suspension). Experiments 3 and 4 were designed to assess the influence of settling solids on toxicity.

In experiments 3 and 4 chambers containing 3% organic carbon suspended solids at 1,000 mg/L were rotated for dif-

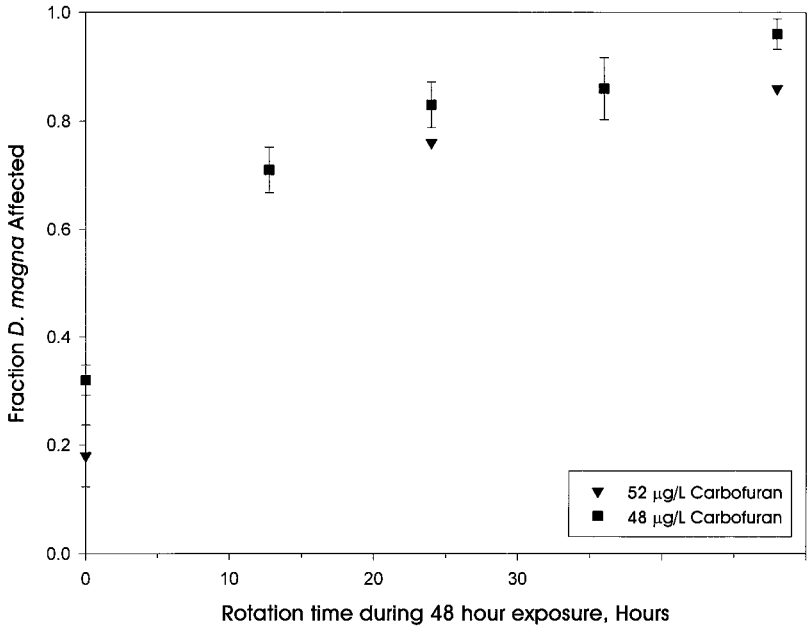

Fig. 4. Mean fraction of *Daphnia magna* affected when exposed to suspended solids at 1,000 mg/L (3% organic carbon) and freely dissolved carbofuran at 48 or 52 mg/L as a function of rotation time from 0 to 48 h. Error bars denote the standard deviation for duplicate chambers.

ferent lengths of time during a 48-h exposure period. Experiment 3 had a measured freely dissolved carbofuran concentration of 48 µg/L. Rotation of duplicate chambers was stopped at 0, 12, 24, 36, and 48 h. In experiment 4, 2 of the 10 chambers were controls and contained no suspended solids. Rotation was stopped 24 h before the addition of test organisms in these controls and two chambers containing suspended solids. The other chambers were stopped, two at a time, at 0, 24, and 48 h. Experiment 4 had a measured freely dissolved carbofuran concentration of 52 µg/L, but differed from the standard protocol in that the addition of carbofuran took place 25 h, rather than 20 h, before test organisms were added to the chambers.

The four chambers in experiment 4 that were stopped at −24 h never reached freely dissolved carbofuran equilibrium with the rest of the chambers because of the lack of turbulence at their membrane surfaces. Their freely dissolved carbofuran concentrations were 67% of the tank concentration at 0 h and 82% at 48 h. Mortality in these chambers would be expected to be much lower than in rotating chambers as a result of decreased carbofuran concentrations. Consequently, data from chambers stopped before 0 h were not considered further.

The results from these two variable rotation time experiments (Fig. 4) show a direct relationship between increasing rotation time and increasing fraction of *D. magna* affected. In these experiments, rotation time is a conservative estimate of the exposure to suspended solids, because additional exposure to small suspended solids occurs during the time the suspended solids are settling. If *D. magna*, under the influence of carbofuran, fall to the bottom of the chamber after rotation stops, the dose–response relationship observed could be a function of the amount of settling solids necessary to smother a disabled organism. If smothering resulted in increased numbers of affected organisms, the chambers that rotated for 4–8 h would be expected to have fewer affected *D. magna* than chambers that stopped before 48 h. However, the observed increase in mortality with increasing rotation time strongly suggests that the overall effect of solids on *D. magna* in the standard protocol is caused by the suspended particles during the first 24 h and not by particles that settled during the second 24 h of the bioassay.

CONCLUSIONS

Using the SSTA for toxicity testing where solids are present allows for the control of the freely dissolved portion of the chemical. The apparatus design also allows control of variables without large numbers of replicates and chemical analyses. Thus, the flexibility of the SSTA allows it to be used effectively in investigating factors affecting the combined toxic responses of chemical compounds and suspended solids. For example, the initial studies reported here demonstrate that the SSTA is an effective tool for investigating relationships between the combined stresses of carbofuran and suspended solids. Although characteristics of the SSTA have been determined for its application to carbufuran effects, further study of the SSTA's operational characteristics regarding its utility in experiments involving hydrophobic chemicals is necessary.

Acknowledgement—This research was supported by the National Biological Service Cooperative, Fish and Wildlife Research Unit of the University of Minnesota, directed by D.E. Andersen, in part through an Interagency Agreement with the U.S. EPA. We thank M.S. Henry for the initial project design, N. Detenbeck and C. Eulis for their support, E. Wattenberg for laboratory space and stimulating discussion, D. Jaschke for technical assistance, and K. Brademeyer for expert preparation of the manuscript.

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