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Silencing genes of a pest with double-stranded RNA (dsRNA) is a promising new pest management technology. As part of the environmental risk assessment for dsRNA-based products, the environmental fate and the potential for adverse effects to on-target organisms should be characterized. In the present study, a nonbioactive dsRNA was spiked into the water column of a water and sediment microcosm to mimic drift from a spray application run off of unbound dsRNA or transport of plant tissues. Dissipation of dsRNA in the water column and partitioning into sediment was determined. The dsRNA rapidly dissipated in the water column and was below the limit of detection after 96 h. The levels detected in the sediment were not significant and may indicate rapid degradation in the water column prior to partitioning to sediment.

Keywords
dsRNA, Double-stranded RNA, Environmental fate, Aquatic microcosms

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DISSIPATION OF DOUBLE-STRANDED RNA IN AQUATIC MICROCOSMS

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Abstract: Silencing genes of a pest with double-stranded RNA (dsRNA) is a promising new pest management technology. As part of the environmental risk assessment for dsRNA-based products, the environmental fate and the potential for adverse effects to on-target organisms should be characterized. In the present study, a nonbioactive dsRNA was spiked into the water column of a water and sediment microcosm to mimic drift from a spray application run off of unbound dsRNA or transport of plant tissues. Dissipation of dsRNA in the water column and partitioning into sediment was determined. The dsRNA rapidly dissipated in the water column and was below the limit of detection after 96 h. The levels detected in the sediment were not significant and may indicate rapid degradation in the water column prior to partitioning to sediment. *Environ Toxicol Chem* 2017;36:1249–1253. © 2016 SETAC

Keywords: dsRNA Double-stranded RNA Environmental fate Aquatic microcosms

INTRODUCTION

The use of RNA interference as a potential pest management strategy is a new and rapidly expanding field of study. The RNA interference process was first described 15 yr ago by Fire et al. [1] who observed that introducing exogenous double-stranded RNA (dsRNA) in the nematode *Caenorhabditis elegans* resulted in silencing of homologous mRNA. Since its discovery, RNA interference has been employed in many research areas including genomics, cancer research, and more recently, for use in controlling agricultural insect pests [2]. The RNA interference process is initiated when dsRNA is taken up by a cell and cleaved into short interfering RNAs. This sets off a sequence of events that leads to the silencing of a protein target that can lead to the eventual death of an organism. (For further discussion of RNA interference mechanism of action, see [2–5]).

Numerous dsRNAs specific for some of the most destructive agricultural insect pests, including western corn rootworm, Colorado potato beetle, and cotton bollworm, have been synthesized and shown to have activity against these pests by inducing RNA interference through feeding of the dsRNA at very low concentrations [6–9]. In addition to exhibiting activity against other coleopteran and lepidopteran pests, dsRNAs also have been synthesized and shown to have activity against insects from the orders including Diptera, Hemiptera, Hymenoptera, Isoptera, and Orthoptera [10–15]. Although most of these studies used laboratory-synthesized dsRNA incorporated or overlaid onto an artificial diet, a few studies expressed dsRNA in plant tissue and then performed bioassays using that plant tissue. In all of these studies, the dsRNA significantly reduced damage to the plants. Finally, the spectrum of activity of dsRNA can be very narrow with the potential to act as species-specific insecticides [12]. For example, a dsRNA designed to target the western corn rootworm only displayed activity against species closely related to the corn rootworm, which greatly limits the potential for effects to nontarget organisms [16,17]. In addition, some hemipteran and lepidopteran pests exhibit a lack of sensitivity to environmental RNA that further allays concerns for effects on nontarget organisms [18].

`Although knowledge of pests susceptible to dsRNAs and methods of dsRNA delivery to pest insects is increasing at a rapid pace, there is currently very little published research on the fate of these nucleic acids in various environmental matrices. Little information exists on the degradation RNA in the environment, but the available studies indicate that rapid degradation occurs, with most of the RNA degraded after 4 d to 30 d [19,20]. Research on the environmental fate of DNA may offer a better comparison for predicting the fate of dsRNA, because both are double-stranded nucleic acids. Pure plasmid DNA has a half-life of 9 h to 28 h depending on soil type, whereas tomato and soybean DNA have a reported half-life of approximately 1.5 d in soil [21–23]. Half-lives of DNA in water are slightly shorter with maize and soybean DNA reported to have half-lives of <2 h and 4 h, respectively [24].

Rapid degradation of RNA and DNA in the environment is expected due to the presence of nuclease enzymes that rapidly degrade these molecules. However, despite the presence of these enzymes, extracellular DNA has been reported to persist in some aquatic and terrestrial ecosystems for a few months to years [25,26]. Therefore, determining the environmental fate of dsRNA is crucial before insecticidal sprays or transgenic plants containing these molecules can be approved for commercial use. Currently, there are only 3 known studies on the environmental fate of dsRNA. Dubelman et al. [27] investigated the environmental fate of transgenic maize containing an insecticidal dsRNA molecule in 3 agricultural soils. They determined that the dsRNA was rapidly degraded (half-lives of 15–28 h) and that it was unlikely to persist in soil [27]. A second paper studied the fate of an insecticidal dsRNA and a non-insecticidal dsRNA surrogate molecule in soil. Both the insecticidal dsRNA and the non-insecticidal dsRNA surrogate were undetectable after 32 h [28]. Finally, Fischer et al. [29] studied the fate of an insecticidal dsRNA in aquatic microcosm.

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The authors estimated the half-life of the dsRNA at less than 3 d, and the time to 90% dissipation at approximately 4 d [29]. However, there is very little published research on whether or not dsRNA will persist in aquatic ecosystems, or if partitioning will occur between the water and sediment phases.

The objective of the present study was to determine the dissipation of pure dsRNA in aquatic systems. Currently, dsRNA technologies are being developed for use in formulated sprays and transgenic plants to confer protection against pest insects [6,30]. Thus, dsRNA may enter aquatic systems directly (via overspray) or indirectly (via surface water runoff, transport of plant tissues, etc.). Sterile and nonsterile aquatic microcosms were set up to determine the role biotic and abiotic factors have on the rate of dsRNA dissipation. The dsRNA was applied to the water phase of each microcosm to simulate entry into an aquatic ecosystem (via overspray or transport of plant tissues) and to determine if partitioning from the water phase to the sediment phase is a major factor in dissipation. A nonbioactive dsRNA was used as a surrogate for future insecticidal dsRNAs.

METHODS AND MATERIALS

dsRNA

The dsRNA used in the present study was designed, produced, and supplied by Monsanto. It is a 100 base pair dsRNA designed to have no biological activity against known sequences. The dsRNA was produced using the T7 method for dsRNA preparation in the MEGAscript® RNA interference kit (ThermoFisher Scientific). The dsRNA was received in solution and was stored at –80 °C prior to use. (For the full dsRNA sequence, see Fischer et al. [28]).

Sample preparation and treatment

Water and sediment were collected from the pond located on the Iowa State University Horticulture Farm near Ames, Iowa, USA and was stored under aerobic conditions for approximately 2 mo. The water was filtered through glass wool to remove large organic matter. The sediment was homogenized and sieved through a 2.86 mm mesh to remove rocks and large debris. The sediment and water were stored at room temperature prior to use.

Three different aquatic microcosms were tested: 1) laboratory water over sterilized sediment, 2) sterilized pond water over sterilized sediment, and 3) active pond water over active sediment. Laboratory water was obtained from a NANOpure® ultrapure water system (Barnstead/Thermolyne). Portions of the field-collected water and sediment were sterilized by autoclaving for 1 h at 121 °C on 3 consecutive days; this procedure has previously shown to significantly reduce microbial populations [31]. At the start of the present study, 1 g of sediment was weighed into a 50-mL conical tube and 5 mL of water was carefully pipetted on top of the sediment. The water portion of each replicate was spiked with a total of 7.5 μg dsRNA, for a final concentration of 1.5 μg/mL in the water. All treatments were placed in an incubator at 25 °C (+2 °C) under constant light. All vials were vented every 24 h to prevent development of anaerobic conditions. Samples were collected at 0 h, 1 h, 4 h, 8 h, 12 h, 24 h, 48 h, 96 h, 168 h, 240 h, and 336 h. At each sampling point, 3 replicates were sacrificed by carefully pipetting off the water portion of the microcosm into a separate vial. All water and sediment samples were flash frozen in liquid nitrogen and stored at –80 °C until analysis.

Sample extraction and processing

Extraction procedures were optimized through the use of spike and recovery samples. Sediment samples were extracted prior to processing. Briefly, 25 6.5-mm steel ball bearings (McMaster-Carr) and 25 mL phosphate-buffered saline with Tween buffer (pH 7) were added to the sediment sample in the 50-mL conical vial. Samples were agitated in a Multi-Wrist® shaker (Lab-Line Instruments) for 10 min and then passed through a 0.22 μm vacuum filter. The sediment extracts and water samples were then processed by a proteinase K digestion modified from the QuantiGene® Bacterial Homogenate Preparation protocol. Briefly, 303 μL of each sample was combined with 300 μL of homogenizing solution (Affymetrix) and 3 μL Proteinase K (Affymetrix). The solutions were vortexed for 1 min and then digested at 65 °C for 15 min. Digested sediment samples were analyzed directly; water samples were diluted with sample diluent to fall within the standard curve.

QuantiGene analysis

A QuantiGene 2.0 Singleplex assay kit (Affymetrix) was used to determine dsRNA concentrations in the samples. This method has been proven to be capable of detecting low concentrations of dsRNA in soil regardless of sequence, molecular weight, or structure [28]. For this procedure, 1.2 μL of a custom QuantiGene probe set designed by the manufacturer to hybridize to the dsRNA used in the present study was combined with 80 μL of each sample in a disposable PCR plate. The plate was sealed with a foil seal, heated at 95 °C for 5 min and then heated at 45.5 °C for 30 min. Samples were plated on the QuantiGene plate in triplicate by combining 20 μL of each sample with 80 μL of working solution in each well. Each sample was analyzed in triplicate. The plate was sealed with foil and incubated at 45.5 °C for 16 h to 20 h. Plates were washed 3 times with 300 μL of wash buffer, then 100 μL of preamplifier solution was added to each well, plates were sealed with foil and incubated at 45.5 °C for 1 h. The previous step was repeated for the amplifier solution and the label probe solution. Following incubation with the label probe solution, the plates were washed 3 times and 100 μL of substrate was added to each well. Plates were sealed with foil and incubated at room temperature for 5 min. Luminescence of each well was read by a Fusion-Alpha HT Universal Microplate Reader (PerkinElmer) with a read time of 2 s per well. A 4 parameter fit curve was used to determine the concentration of dsRNA in the samples and the mean concentration of each sample was determined from the triplicate analysis. Table S1 in the Supplemental Data contains instructions on preparation of the various solutions used in the assay.

RESULTS AND DISCUSSION

The environmental fate of a nonbioactive dsRNA was investigated in 3 different aquatic model systems to determine if biotic or abiotic factors were responsible for the dissipation of dsRNA. The chemical and physical properties of the field-collected sediment and pond water utilized in the present study were characterized before the start of the study and can be found in Table S2 in the Supplemental Data.

Prior to the start of the experiment, spike and recovery tests were performed to ensure the extraction and sample processing procedures were adequate to achieve high recovery of the dsRNA from the tested matrices. Recovery rates of 106%, 89%, and 88% were achieved in the laboratory water, sterilized pond water, and active pond water, respectively. In sediment, 107%

and 79% of the dsRNA was recovered from sterilized sediment and active sediment, respectively. Due to the inherent variability in biological systems, recovery rates of 70% to 120% are considered acceptable; all the recovery values generated in the present study fall within this acceptable range.

In all 3 microcosms, the dsRNA persisted for 48 h, and then rapidly dissipated to the extent that the dsRNA was undetectable at 96 h (Figures 1–3). The half-life of dsRNA (time needed for 50% of the dsRNA to dissipate) was calculated for each of the 3 microcosms, and these values were similar for all 3 microcosms, 63 h, 72 h, and 56 h in laboratory water, sterilized pond water, and active pond water, respectively. One possible explanation is that abiotic factors were responsible for most of the degradation of the dsRNA, because there is little difference between the laboratory water microcosm (biotic factors absent) and the active pond water microcosm (biotic factors present; Figure 4). However, incomplete sterility of the microcosms cannot be ruled out because the microcosms were not checked for colony-forming units. In addition, some ribonucleases (RNases), like RNase A, become deactivated by autoclaving, but can be slowly reactivated [32].

The dissipation of dsRNA reported in the present study compares favorably with previously reported dissipation rates for nucleic acids in environmental matrices. Although single-stranded RNA has been reported to persist in the environment for up to 30 d, other nucleic acids have been shown to dissipate rapidly [19,20]. For example, DNA, which is also double-stranded, has been reported to have a half-life of less than 4 h in water [24]. The only known study on the environmental fate of a biological active dsRNA reported half-lives of 15 h to 28 h in various soil types [27]. These results, combined with the data presented in the present study, suggest that dsRNA is not anticipated to persist in environmental matrices.

An interesting aspect observed in both the present study and published research [27–29], is that dissipation of dsRNA does not appear to follow normal dissipation kinetics. Dissipation of most conventional chemicals follows a first-order reaction; rapid degradation of the chemical occurs early, followed by a slower degradation rate later. This is true for agricultural chemicals, personal care products, pharmaceuticals, and plant-incorporated protectants, such as Cry proteins [33–38]. In Dubelman et al. [27], the concentration of dsRNA remains relatively unchanged of the first 12 h to 24 h, followed by rapid dissipation over a 12 h period. In the present study, a similar phenomenon was observed. The dsRNA concentrations in all 3 microcosms was unchanged, or decreased slightly, over the first 48 h (Figure 4). The dsRNA then rapidly dissipated over the next 48 h and was at or below the limit of quantification after 96 h. The other 2 published studies show similar degradation patterns [28,29]. It is difficult to draw any definite conclusions on the dissipation kinetics of dsRNA based on only 4 studies, and it may need to be revisited in the future after more data is generated.

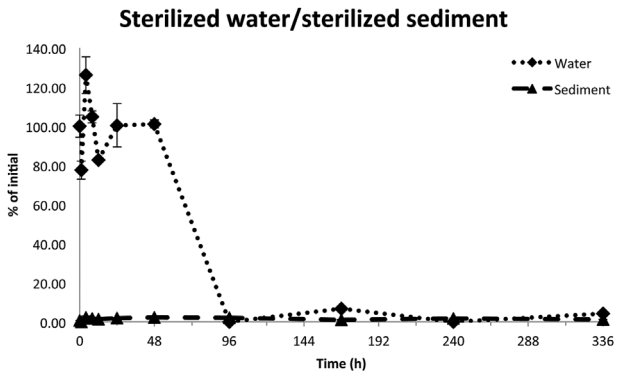

Figure 2. Rapid dissipation of a nonbioactive double-stranded RNA (dsRNA) occurs in a sterilized water/sterilized sediment microcosm. Partitioning of the dsRNA into the sediment phase does not occur. Error bars represent 1 standard error of the mean ($n = 3$).

The second aspect of the present study was to determine if dsRNA could partition from the water phase of the aquatic microcosm into the sediment phase. Double-stranded RNA was detected at almost all time points in the sterilized water/sterilized sediment and active pond water/active sediment microcosms, and at only 1 time point in the laboratory water/sterilized sediment microcosm (Figures 1–3). However, the total amount of dsRNA in all sediment samples never represented more than 3% of the applied dsRNA, suggesting that partitioning to sediment is not a major factor in the dissipation from the water phase. Partitioning into sediment might have been prevented by the rapid dissipation of dsRNA in water (likely the result of degradation). Further, the sediment used in the present study had a high sand content (80%). Greater partitioning may be observed in sediments with higher clay contents (i.e., more binding sites). Fischer et al. [29] also measured dsRNA concentrations in sediment after the dsRNA was applied to the water phase of aquatic microcosms. The authors found that approximately 75% of the applied dsRNA

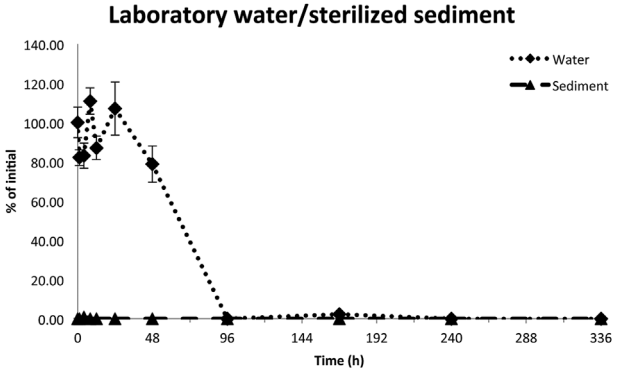

Figure 1. Rapid dissipation of a nonbioactive double-stranded RNA (dsRNA) occurs in a laboratory water/sterilized sediment microcosm. Partitioning of the dsRNA into the sediment phase does not occur. Error bars represent 1 standard error of the mean ($n = 3$).

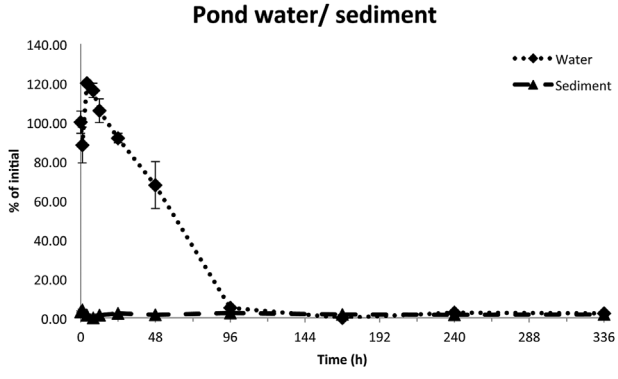

Figure 3. Rapid dissipation of a nonbioactive double-stranded RNA (dsRNA) occurs in a pond water/active sediment microcosm. Partitioning of the dsRNA into the sediment phase does not occur. Error bars represent 1 standard error of the mean ($n = 3$).

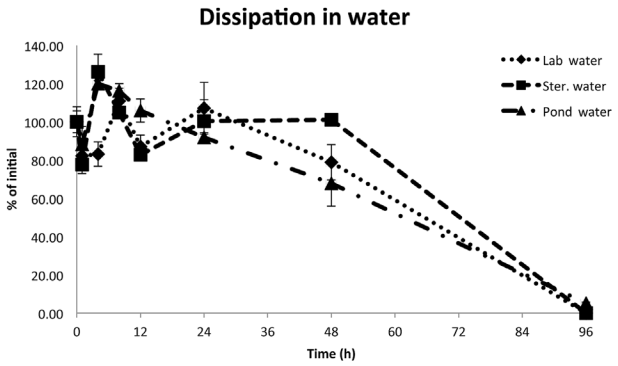

Figure 4. The presence of biotic factors in a microcosm has no effect on the dissipation rate of double-stranded RNA. Error bars represent 1 standard error of the mean ($n = 3$). Lab = laboratory; Ster. = sterilized.

was detected in the sediment phase of microcosms that were disturbed, but no dsRNA was found in undisturbed sediment, suggesting that the probability of dsRNA partitioning into sediment from water is low.

In conclusion, the environmental fate of a nonbioactive dsRNA, representative of future insecticidal dsRNAs, was determined in aquatic microcosms. The dsRNA was rapidly degraded in all 3 microcosms, and was undetectable after 96 h. In addition, partitioning of the dsRNA was not a major factor in the dissipation of dsRNA from water. Therefore, it can be expected that dsRNA will not persist in aquatic environments, and its use in agricultural settings should have little long-term environmental impact.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3648.

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Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (jcoats@iastate.edu).

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