Analysis of RNA interference (RNAi) biopesticides: double-stranded RNA (dsRNA) extraction from agricultural soils and quantification by RT-qPCR

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Abstract

Double-stranded RNA (dsRNA) molecules are used as a novel class of biopesticides. To enable assessments of the ecological risk associated with their release to receiving environments, we developed an approach to quantify dsRNA in agricultural soils using quantitative reverse transcription polymerase chain reaction (RT-qPCR). To allow quantification of dsRNA adsorbed to particles, we also developed a protocol to transfer dsRNA from particles to the extraction buffer by changing particle surface charge and adding constituents to compete with dsRNA for adsorption sites. Our approach could quantify dsRNA amounts as low as 0.003 ng_{dsRNA}/g_{soil}. This approach is the first available field-applicable approach able to quantify dsRNA biopesticides down to environmentally relevant concentrations. We applied this approach to investigate dsRNA dissipation (including dilution, degradation, and adsorption) in two agricultural soils. When we applied a low amount of dsRNA (1 ng_{dsRNA}/g_{soil}) to the soils, we observed that a greater fraction of dsRNA was adsorbed to and extractable from soil particles in a silty clay loam soil than in a fine sandy loam soil. In both soils, dsRNA dissipated on the timescale of hours. Overall, these results demonstrate that our approach can be applied to assess the environmental fate of dsRNA biopesticides at concentrations relevant to their release to soils.

Introduction.

RNA interference (RNAi) has been used in emerging agricultural biotechnology to protect crops from pests.[1] RNAi is a cellular mechanism in which double-stranded RNA (dsRNA) directs the degradation of the homologous messenger RNA (mRNA), leading to gene silencing and preventing the synthesis of proteins.[2] When ingested or taken up by pests, dsRNA biopesticides trigger RNAi against specific mRNA inside of the pest cells, resulting in reduced growth and/or increased mortality of the pest organisms.[3-7] Dozens of different dsRNA biopesticides with various sequences have been designed to suppress the synthesis of specific essential proteins in several different target pests including insects, nematodes, viruses, and fungi.[4, 8-11] To use dsRNA biopesticides for pest control, two distinct delivery options are available. Firstly, genetically modified (GM) RNAi crops have been developed that expressed dsRNA biopesticides within their tissues.[1, 4, 12] Pests co-ingest the dsRNA biopesticides when feeding on the plant tissue. Secondly, dsRNA biopesticides have also been found to be effective when applied exogenously to the crop, similar to the application of traditional pesticides.[10, 13] Both delivery options are anticipated to result in the release of dsRNA biopesticides to receiving environments, in particular, agricultural soils, and thereby poses potential ecological risks (e.g., effects on nontarget organisms).[14-17] Therefore, dsRNA biopesticides must undergo ecological risk assessments,[18-20] which evaluates both the hazards posed by dsRNA biopesticides to specific organisms and the exposure to dsRNA biopesticides. The latter is directly linked to the dsRNA concentrations in receiving environments.

Currently, there is no publicly available field-applicable approach able to analyze dsRNA biopesticides down to environmentally relevant concentrations (Table S1). The release of dsRNA biopesticides from GM RNAi crops is estimated to result in initial dsRNA soil concentrations in the order of ~1 ng_{dsRNA}/g_{soil} (Supporting Information, Section 1). After release, dsRNA

concentrations in soils are expected to decrease as a result of parallel dissipation processes including dsRNA dilution, (bio)degradation, and adsorption to soil particles.[17] A recently published analytical approach used a radioactive labeling technique (i.e., phosphorus-32 radioactive label) to quantify dsRNA down to 1.5 ng/g_{soil},[21] which is close to the expected environmental dsRNA concentration. This technique was useful in studying dsRNA environmental fate because it enabled tracing of the total radioactive label and analysis of some labeled degradation products. Using this approach, dsRNA applied at 60 ng_{dsRNA}/g_{soil} to soils was found to undergo both adsorption to soil particles and biodegradation, which are both expected to be concentration-dependent processes. However, this technique is restricted to laboratory investigations under stringent safety requirements and regulations, making it less accessible than other techniques. More importantly, this technique is not field-applicable because it cannot quantify unlabeled dsRNA concentration in environmental media. Another published approach used a hybridization assay to measure dsRNA concentrations in solutions extracted from soils. They achieved a practical quantification limit of 25 ng_{dsRNA}/g_{soil},[22] which is higher than our estimated environmental dsRNA concentration. To perform dissipation experiments using this quantification approach, dsRNA was applied to soils at concentrations ranging from 300-40,000 ng_{dsRNA}/g_{soil}, with most experiments performed at initial dsRNA concentrations of 7,500 ng_{dsRNA}/g_{soil}.[22, 23] In these experiments, dsRNA concentrations typically remained close to the initial value for time periods up to one day before degradation occurred. One explanation for this observation is that surface sites and enzymes needed for dsRNA sorption to particles and degradation, respectively, may have been saturated by high concentrations of dsRNA. Therefore, an analytical approach capable of quantifying unlabeled dsRNA biopesticides at concentrations relevant to field conditions is needed to fill this methodological gap.

To address this need, we evaluated the use of quantitative reverse transcription polymerase chain reaction (RT-qPCR) to measure dsRNA concentrations in soils. RT-qPCR is a sensitive analytical technique that has been used to analyze single-stranded RNA (ssRNA) and dsRNA in water and food.[24-26] In soils, the quantification of dsRNA by RT-qPCR is challenged by two factors. Firstly, unlike in food and water, a significant fraction of the dsRNA may be adsorbed to soil particles,[21] requiring these dsRNA molecules to be extracted from the soil mineral particles prior to analysis by RT-qPCR. Extraction is complicated by the potential chemical instability of dsRNA. In particular, alkaline pH buffers which were proposed to increase dsRNA extraction efficiency[22] may also promote base-catalyzed dsRNA hydrolysis.[27, 28] For example, a prior study reported that increasing the pH values of buffers from 7 to 12 resulted in dsRNA concentrations decreasing by ~40%.[22] Secondly, organic matter in soils (as well as food[29]) is prone to be co-extracted with the dsRNA and subsequently may strongly inhibit enzymatic reactions in PCR[30] and RT-qPCR. Therefore, the co-extracted organic matter needs to be removed prior to RT-qPCR analysis. Consequently, unlocking the potential of RT-qPCR for dsRNA quantification in soils requires simultaneously optimizing protocols to extract dsRNA from particles without unintended dsRNA loss and to remove co-extracted organic matter.

This work aimed at developing an RT-qPCR based quantification approach for both dissolved and adsorbed dsRNA in soils and applying the approach to characterize dsRNA dissipation. Firstly, we evaluate methods to reduce inhibition of RT-qPCR by organic matter in soil solution containing dissolved dsRNA. Next, to quantify the adsorbed dsRNA, we develop methods to recover adsorbed dsRNA from soil particles without artefactual dsRNA degradation during extraction. To alleviate the inhibition of the co-extracted organic matter during the extraction of dsRNA from soil particles, we also develop a rigorous purification protocol to decrease the organic matter content

in samples extracted from soils. Finally, to evaluate the use of this approach in understanding the fate of dsRNA biopesticides in the environment, we apply our approach to measure dsRNA dissipation in agricultural soil samples.

Materials and Methods.

Selection & synthesis of nucleic acids.

Materials and supplies used in this study are described in the Supporting Information (Section 2, Table S2). To develop and validate our quantification approach, we synthesized two dsRNA molecules with length of 100 and 1000 base pairs (bp), similar to the size of developed dsRNA biopesticides (Table S3).[5, 10, 31] The synthesis protocol summarized here is fully described in the Supporting Information. We first amplified DNA templates using PCR with the T7 promoter sequence appended to the forward primers. Next, sense and antisense ssRNA molecules were transcribed *in vitro* from the DNA templates by T7 RNA polymerase and then annealed to produce dsRNA. The dsRNA molecules were confirmed to be the correct size (Figure S1). Selected experiments to test the impact of extraction conditions on dsRNA recovery were performed using the synthetic dsRNA analog, polyadenylic-polyuridylic acid (poly(A:U)) dsRNA supplied by InvivoGen. Poly(A:U) dsRNA is a mixture of dsRNA molecules of sizes ranging approximately from 100 to 1,000 bp.

Sampling & characterization of agricultural soils.

Two soil samples (a silty clay loam soil and a fine sandy loam soil) were collected from active agricultural fields in Carbondale, IL. The soils were air-dried at room temperature for 72 h, homogenized by sieving (2.36 mm), and then stored in the dark at 4 ºC until use. Physicochemical parameters of the soils are provided in Table S4. The dsRNA analytes were not present in the soils at measurable concentrations prior to their addition to the soils at the start of our experiments, as

confirmed using analysis of blank samples by RT-qPCR.

Soil incubation experiments.

We incubated 0.3 g of dry soils with 0.6 mL of buffer containing 3 mM 3-(4-morpholino)propane sulfonic acid (MOPS, adjusted to native soil pH), 10 mM NaCl and dsRNA in 2 mL Eppendorf Protein LoBind tubes. After vortexing for 10 s, we incubated the samples for 0-72 h on an overhead rotator at 24°C. At the end of the incubation period, we centrifuged the sample (21,100 g; 5 min) and separated the supernatant soil solution from the soil pellets by pipette transfer. After this process, 0.14 ± 0.01 mL and 0.11 ± 0.01 mL of soil solution that could not be pipette transferred remained in the tube with soil pellets of the silty clay loam and fine sandy loam soils, respectively. When applicable, we next added 0.875 mL of extraction buffer (after optimization detailed below: 12 mM orthophosphate, 10 mM NaCl, 200 mM tetraborate, pH 11) to the soil pellet and incubated the tubes using an overhead rotator for 4 h. The incubation was conducted at 4°C to prevent enzymatic degradation during the extraction process. We again centrifuged the sample (21,100 g; 5 min) and separated the supernatant soil extracts from the soil pellet. We quantified dsRNA in the soil solution by RT-qPCR after dilution and dsRNA in the soil extracts by RT-qPCR after sample preparation (Supporting Information, Sections 5 & 6).

We investigated RT-qPCR inhibition by organic matter by repeating the protocol without the addition of dsRNA in the initial soil sample to obtain dsRNA-free supernatant solutions. We subsequently reduced organic matter concentration in the supernatant solution by dilution and/or by column purification using RNeasy PowerClean Pro CleanUp Kit (QIAGEN, Hilden, Germany) prior to adding dsRNA to the final solution and performing RT-qPCR. In separate experiments to determine the recovery of dsRNA through the column purification process, we added dsRNA in the supernatants prior to column purification.

Optimization of dsRNA extraction buffer composition.

We evaluated dsRNA stability in buffers with increasing pH values by incubating 47 mg/L poly(A:U) dsRNA in buffers containing 18 mM NaCl and either 2.7 mM MOPS (pK_a = 7.2, for buffers with pH of 7 and 8) or 2.7 mM tetraborate (pK_a = 9.1, for buffers with pH = 9, 10, and 11). At the end of the incubation period, samples were analyzed by UV absorbance spectroscopy using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and gel electrophoresis (1% [w/w] agarose, containing 0.01% [v/v] SYBR Safe stain), run at 100 V for 25 min and imaged using a Digital Image System (UVP Inc, Upland, CA, USA). Unlike experiments conducted to quantify dsRNA at low concentrations (10^{-2} – 10^{2} ng/L) in soil solutions and extracts, UV absorbance spectroscopy and gel electrophoresis were able to be used to quantify dsRNA at the higher concentration in buffers without organic matter. The NanoDrop 2000c spectrophotometer has a reported nucleic acid detection limit of 2 mg/L.[32]

Next, we evaluated the effect of buffer composition on the extraction of dsRNA from a model mineral sorbent, goethite. We performed these initial tests with a model mineral sorbent because we could quantify dsRNA via UV light absorbance at 260 nm due to the lack of other light-absorbing constituents occurring in the mineral extract. We selected goethite as the model mineral phase because nucleic acids (i.e., DNA) readily adsorb to goethite at circumneutral pH values,[33, 34] allowing extraction conditions to be evaluated. We incubated 23 mg/L poly(A:U) dsRNA in the presence of 4 g/L goethite in 0.5 mL buffer (2.9 mM MOPS, pH 7, 14 mM NaCl) in 2 mL Protein LoBind tubes for 15 min while stirring at 200 rpm and 24°C using micro stir bars. During this time, dsRNA completely adsorbed to the goethite. We centrifuged the sample (6,200 g for 5 min) and separated the supernatant from the goethite pellet. Next, we applied 0.5 mL of an extraction buffer, designed to test a specific parameter, to the goethite pellet. After vortexing the sample for 10 s, the

samples were stirred at 24°C (unless otherwise stated) and 200 rpm for 2 h. We again centrifuged the sample (6,200 g for 5 min) and then measured the dsRNA concentration in the extracted supernatant using UV absorbance spectroscopy.

Statistical analysis.

Each sample was prepared independently in triplicate unless otherwise indicated. When evaluating dsRNA quantification by RT-qPCR, we compared the range among three measured values of the cycle threshold (Ct), which is inversely and linearly related to the logarithm of the dsRNA concentration. When we quantified dsRNA either in recovery experiments or in dissipation experiments, we reported the mean value of dsRNA concentration and its standard deviation (plotted as error bars). Statistical significance was evaluated using an unpaired Student's t-test performed using Excel (Version 1911). The confidence level was set as $p \leq 0.05$.

Results & Discussion.

Quantification of dissolved dsRNA in the presence of soil components.

We first investigated the impact of inhibitory substances in soil solutions on dsRNA quantification by RT-qPCR by comparing quantification metrics in the presence and absence of soil components. In the absence of any inhibitory compounds, we successfully quantified dsRNA at concentrations as low as 10^{-2} ng/L (the lowest dsRNA concentration tested) (Figure 1A), corresponding to 36 copies per reaction and consistent with values reported in other RT-qPCR studies (4-4000 copies/reaction).[35, 36] At this dsRNA concentration, the mean Ct was 38.2 (Figure 1A), below the typical upper limit of Ct (~40) for detection and quantification.[37, 38] Across all dsRNA concentrations tested, the maximum range of Ct (R_{Ct}) was 1.2 (Figure 1A), which is comparable with previous studies.[39, 40] We then assessed the inhibition of inhibitory substances in soil on RT-qPCR. Here we used soil solutions of fine sandy loam soil rather than that of silty clay

196 loam soil because the former soil solutions had higher UV absorbance at 230 nm (Figure S2),
197 suggesting higher organic matter content. For dsRNA spiked in soil solutions, we observed both
198 that the amplification efficiency decreased (indicated by the mean Ct increasing from 31.2 to 34.8)
199 and that the measurement variability increased (indicated by R_{Ct} increasing from 0.6 to 1.9) (Figure
200 1B).

201 Because the concentration of inhibitory substances was moderate (as indicated by increasing
202 mean Ct and R_{Ct}, while still maintaining Ct values below 40), we hypothesized that sample dilution
203 alone may be sufficient to reduce the concentration of organic matter and other inhibitory
204 substances to achieve reproducible measurements of dsRNA concentration. To test this hypothesis,
205 we diluted the soil solutions prior to adding dsRNA to the same concentration (0.25 ng/L) across
206 samples (Figure 1B). After being diluted 10 times, soil solutions still had large measurement
207 variability (R_{Ct} = 5.9). However, we found that a 100-fold dilution decreased the mean Ct from
208 34.8 to 32.2 and the R_{Ct} from 1.9 to 0.2 (Figure 1B). We further verified that, at the 100-fold
209 dilution level, the measurement was not affected by the organic matter concentration of soil
210 solutions (Figure S3), which was relatively low at 0 h and relatively high at 24 h (Figure S2). The
211 standard curves for dissolved dsRNA in the two soil solutions were similar (Figure 1C). We applied
212 these standard curves to measure dissolved dsRNA at different incubation times in soil microcosm
213 experiments described below.

214 Using the soil solutions from the silty clay loam and fine sandy loam soils, we generated
215 standard curves by applying dsRNA to soil solutions and then diluting by 100-fold (Figure 1C). In
216 all samples, Ct values were below 40. In both sets of data, the average R_{Ct} was ~0.3, and the
217 maximum R_{Ct} was ~0.6, matching RT-qPCR variability in the absence of inhibitory compounds
218 (Figure 1A). However, in both datasets, the lowest sample (10^{-2} ng/L *after* dilution, corresponding

to 1 ng/L in the undiluted sample) fell below the expected value based on a linear standard curve. Therefore, the concentration of our lowest quantifiable standard was 10^{-1} ng/L *after* dilution, which corresponded to a concentration of 10 ng/L in the undiluted soil solutions. As we collected the soil solutions from soil-buffer mixtures prepared at a ratio of 2 mL buffer per gram dry soil, 10 ng/L dsRNA corresponds to 0.02 ng_{dsRNA}/g_{soil}, approximately two orders of magnitude lower than expected environmental dsRNA biopesticide concentrations. Therefore, dilution alone was sufficient to reduce the concentration of inhibitory substances in soil solutions and enabled dissolved dsRNA quantification at the required level.

Extraction and quantification of adsorbed dsRNA.

In addition to being dissolved in soil solutions, dsRNA may also adsorb to soil particles.[21] To quantify this pool, we evaluated the effects of extraction buffer parameters (e.g., pH value,[21, 22] concentration, and type of (poly)phosphate ion[41, 42]) on dsRNA desorption from particle surfaces. We also investigated unintended degradation of dsRNA during the extraction process. The extraction buffer also liberates organic matter from the soil particle, requiring a buffer-specific sample preparation protocol prior to quantification of dsRNA in soil extracts by RT-qPCR. To independently evaluate key parameters determining dsRNA recovery prior to developing the sample preparation protocol, we first measured the effect of these parameters on the desorption of poly(A:U) dsRNA from organic matter-free goethite using UV light absorbance. Then, we applied the optimized buffer to extract 1000 bp dsRNA from soils, followed by sample preparation and quantification by RT-qPCR.

Extraction buffers with alkaline pH are expected to increase the desorption of dsRNA and other nucleic acids from environmental media.[22, 41] However, at sufficiently alkaline pH, dsRNA may denature to single-stranded RNA, which is expected to rapidly hydrolyze.[27, 28] This reaction

sequence may explain prior reports of substantial loss of detectable dsRNA concentrations (~50%) in control samples prepared in pH 12 extraction buffers.[22] To test the chemical stability of poly(A:U) dsRNA across the pH range we intended to use for extraction, we measured poly(A:U) dsRNA denaturation by observing increases in UV light absorbance resulting from denaturation.[43] We determined that poly(A:U) dsRNA denatures between pH 10 and 11 (Figure S4). In comparison, the 1000 bp synthesized dsRNA denatures at pH ~12 (Figure S5). Likely poly(A:U) dsRNA denatures at a lower pH due to the absence of strong GC bonds that are interspersed with weaker AU bonds in dsRNA molecules.[44] Gel analysis confirmed that the size distribution of poly(A:U) dsRNA did not change at pH 10 over a 24 h period (Figure S4). Therefore, we selected pH 10 as the maximum pH value to be used in experiments involving poly(A:U) dsRNA.

We next evaluated the effect of increasing extraction buffer pH from 7 to 10 on the recovery of poly(A:U) dsRNA from goethite. We measured the recovery of poly(A:U) dsRNA that was applied to achieve a mass loading of 5.8 $ng_{dsRNA}/\mu g_{goethite}$ on goethite, which had an adsorption capacity of 7.57±0.02 $ng_{dsRNA}/\mu g_{goethite}$ (Figure S6). The recovery of poly(A:U) dsRNA was only detected in the pH 10 buffer (Figure 2A). This is likely due to the surface charge of goethite changing from positive to negative at pH values above the point of zero charge (p.z.c. ≈ 8-9 [42, 45-47]) (Table S5), whereas dsRNA is negatively charged across this pH range. However, all the buffers within the pH range required to prevent dsRNA degradation resulted in low recoveries (< 25%) of poly(A:U) dsRNA. Because pH alone was insufficient to increase poly(A:U) dsRNA recovery, we next tested the ability of three different (poly)phosphates (orthophosphate: PO_4^{3-}, pyrophosphate: $P_2O_7^{4-}$, hexametaphosphate: $(PO_3)_6^{6-}$) to further increase dsRNA recovery. (Poly)phosphates have previously been included in nucleic acid extraction buffers[21, 41] because they are expected to compete with nucleic acids for adsorption sites.[42] The inclusion of (poly)phosphates did increase

poly(A:U) dsRNA recovery from goethite (Figure 2B). While the increase was specific to (poly)phosphates rather than a general ion effect (Figure S7A), the three different (poly)phosphates at the same concentration (6 mM) all similarly increased the recovery (from 13.5% to 15.8%). Because the different (poly)phosphates had the same effect when added at equimolar concentrations, we elected to use orthophosphate because, unlike the two polyphosphates, it can be autoclaved without risk of thermal decomposition.[48] We determined that poly(A:U) dsRNA recovery increased by < 15% when the orthophosphate concentration was increased from 3 to 48 mM (Figure S7B). The increase was slight probably because orthophosphate is already in high molar excess relative to the moles of phosphate in poly(A:U) dsRNA (~0.07 mM phosphate in 23 mg/L of poly(A:U) dsRNA). Therefore, we selected 12 mM orthophosphate to use for later experiments because dsRNA is expected to occur at even lower concentrations in environmental media.

Finally, we investigated the combined effects of the buffer pH and orthophosphate on poly(A:U) dsRNA extraction from goethite (Figure 2C). After 1 h, a buffer with a pH of 10 and 12 mM orthophosphate resulted in 56 ± 2% recovery of adsorbed poly(A:U) dsRNA, double that in either buffers selected to have alkaline pH (pH = 10, 25 ± 2%) or 12 mM orthophosphate (27 ± 2%) alone (Figure 2A, B). Prolonging the extraction time from 1 to 4 h increased the poly(A:U) dsRNA recovery to 76 ± 1%. Because low temperature might be used to prevent enzymatic degradation during extraction, we also tested the effects of temperature on extraction. We found low temperature (4°C) slightly reduced the recovery, resulting in 66 ± 1% at 4°C after 4 h. Besides buffer parameters, extraction time and temperature, we also investigated the effect of dsRNA size (100 bp and 1000 bp) on extraction recovery. We found the 100 bp dsRNA had a significantly higher extraction recovery than that of 1000 bp dsRNA ($p < 0.01$) (Figure S8). Due to its lower

recovery, we used the 1000 bp dsRNA in subsequent experiments as the more challenging of the two cases.

Overall, we found that both alkaline pH and inclusion of phosphate in the extraction buffer are needed to obtain high recoveries of adsorbed dsRNA from goethite. Next, we applied the extraction buffer to soils. We found that 200 mM of tetraborate in the extraction buffer was required to completely prevent changes in the pH of the solution (Figure S9). Using the higher concentration buffer salt, we collected soil extracts from silty clay loam and fine sandy loam soils and measured their light absorbance as a proxy for the concentration of inhibitory compounds. In comparison with the solutions tested in the above section, we found that the soil extracts of the silty clay loam soil had higher organic matter concentrations than those of the fine sandy loam soil (Figure S10). Therefore, we selected the silty clay loam soil for the development of the soil extract preparation protocol.

We evaluated the effect of preparation protocol on RT-qPCR inhibition by treating soil extracts and then adding 1000 bp dsRNA at a concentration of 1 ng/L in all samples. Due to the greater concentration of inhibitory substances in soil extracts relative to soil solutions (Figure S10), we first tested sample dilution after phenol:chloroform:isoamyl alcohol (PCI) purification and isopropanol precipitation to remove proteins and a fraction of the organic matter.[49-51] Even after PCI purification and isopropanol precipitation, we found > 10,000-fold dilution was needed to obtain Ct values < 40 and $R_{Ct} < 1.2$ (Figure 3A). However, diluting by ~10,000-fold dilution would increase the lowest quantifiable concentration to approximately 3 ng_{dsRNA}/g_{soil}, which was above the expected environmental concentration of dsRNA biopesticides. Therefore, a preparation protocol that did not dilute the sample to this extent was required. Therefore, we added column purification to our preparation protocol. Combining two-column purification steps in series with a

311 10-fold dilution reduced inhibition of the RT-qPCR, resulting in mean Ct = 34.94 and R_{Ct} = 0.5
312 (Figure 3B). We confirmed that 99±7% of the dsRNA (1.4 ng/L) added to the soil extracts was
313 recovered through the entire preparation sequence including (1) PCI purification and isopropanol
314 precipitation, (2) column purification twice, and (3) the 10-fold dilution (Figure S11). Using this
315 sequence of steps (Supporting Information, Section 5), we generated a standard curve spanning
316 the concentration range of 10^{-2} to 10^{2} ng/L *after* dilution (Figure 3C). While all samples resulted
317 in Ct values < 40, the linear region of the standard curve was confined from 1 to 10^{2} ng/L. Using
318 the approach developed herein, the lowest standard within the linear region corresponded to a
319 concentration of 0.003 ng_{dsRNA}/g_{soil}, indicating that the preparation protocol removed a sufficient
320 fraction of inhibitory substances to enable quantification at our target concentration. In prior
321 studies,[52, 53] bovine serum albumin (BSA) has been added to reduce the inhibition of organic matter
322 on RT-qPCR. The same dilution factor was required to reduce the inhibition of RT-qPCR by
323 organic matter when BSA was added at 5×10^{7} and 5×10^{8} μg/L as in the absence of BSA (Figure
324 S12).

325 ***Application of the approach in soil microcosm experiments.***

326 After validating the ability for our preparation methods to enable dsRNA quantification by
327 RT-qPCR in both dissolved phases and extracted from soils, we optimized and applied the
328 approach to measure the concentrations of 1000 bp dsRNA in these pools in soil microcosm
329 experiments. Due to the potential for adsorption to be a dominant process impacting dsRNA fate
330 in soils, we first optimized the extraction buffer pH further for these experiments. While the
331 extraction buffer pH in the previous experiments was limited by dsRNA stability due to the
332 denaturation and hydrolysis of the model poly(A:U) dsRNA at pH greater than 10 (Figure S4), the
333 1000 bp dsRNA used in these experiments was stable up to pH of 11 or higher (Figure S5). Using

an extraction buffer with a pH of 11, we developed two standard curves in soil extracts, one for each soil tested (Figure 4A). The two standard curves had similar slopes and slightly different intercepts (Figure 4A). Accurate analysis of dsRNA in extracts from additional soil types is therefore expected to require independent calibration. These standard curves yielded the same range of linearity (down to 1 ng/L dsRNA, corresponding to 0.003 ng_{dsRNA}/g_{soil}) as the standard curve developed previously for extraction buffers with a pH of 10 (Figure 3C). We subsequently determined the initial distribution of measurable dsRNA (initial mass = 0.3 ng) following the brief mixing of a dsRNA-containing buffer with soils (Figure 4B) and compared the results obtained from extraction buffers at pH 10 and 11. The distribution of dsRNA was very different between the two soils tested. In the silty clay loam soil, the dsRNA in soil extracts accounted for 87-92% of the total measurable mass, while, in the fine sandy loam soil, the dsRNA in soil extracts accounted for only 15-26% of the total measurable mass. The difference ($p < 0.0001$) in their dsRNA distribution may result from the smaller particle size and larger surface area in the silty clay loam soil than the fine sandy loam soil.[54, 55] In addition, previous studies suggest DNA and RNA readily adsorbed to clay[21, 56, 57] through possible mechanisms including cation bridging and ligand exchange.[58, 59] For both soils, increasing the extraction buffer pH from 10 to 11 resulted in increased dsRNA concentration ($p < 0.01$) in the extract, increasing the total recovery of spiked dsRNA from 39 ± 5 to 58 ± 7% from the silty clay loam soil and from 74 ± 12% to 86 ± 13% in fine sandy loam soil. Overall, this finding indicates that, to maximize recovery of measurable dsRNA, extraction buffers should be selected to have the highest pH value that does not promote dsRNA degradation.

We applied the pH 11 extraction buffer to monitor dsRNA dissipation overtime after addition to the silty clay loam (Figure 5A) and the fine sandy loam soils (Figure 5B). To facilitate

comparisons of the sizes of these dsRNA pools, the dsRNA concentration measured in the solution phase or in soil extract was converted to a mass basis by accounting for the volume of these phases. In the silty clay loam soil, the dsRNA rapidly moves from the dissolved phase to the particle-associated phase, such that the majority of the measurable dsRNA was associated with the particle phase at the first time point (Figure 5A). The amount of dsRNA remaining in the solution phase remained constant throughout the experiment (i.e., 0.015 ± 0.008 ng initially and 0.012 ± 0.007 ng at 72 h). In contrast, the amount of dsRNA measured in soil extracts decreased by a factor of 10 within 24 h and was below the quantification limit (0.003 ng_{dsRNA}/g_{soil}, corresponding to 0.0009 ng in our experiments) after 48 and 72 h. These results indicate that, in this microcosm experiment, extractable dsRNA decreased more quickly than dissolved dsRNA, contrary to previous studies reporting that adsorption of nucleic acids (i.e., DNA) to soil particles protected the nucleic acid from enzymatic and microbial degradation.[60-62] Our findings suggest that the adsorbed dsRNA may have become less extractable or that the adsorbed dsRNA is degraded by unanticipated pathways. In the fine sandy loam soil, the dsRNA distributed more evenly between the dissolved and extractable pools (Figure 5B). In both pools, the dsRNA concentration decreased by ~8-fold after 24 h and is not quantifiable after 72 h. Although the relative amounts of dsRNA in the dissolved and extractable pools differs between the two soils, both experiments indicate decreasing amounts of dsRNA in the extract from soils over the experiment duration.

Environmental Implications.

In this study, we developed a sensitive quantification approach for dsRNA in soils, which is critical to determining the environmental fate of dsRNA biopesticides when performing ecological risk assessment. We determined that efficient recovery of adsorbed dsRNA required extraction buffers incorporating both alkaline pH and phosphate species, including orthophosphate, that

compete with dsRNA for adsorption sites. Because phosphate promotes desorption of dsRNA, its application as a buffer in soil microcosm experiments[22, 23] (as well as potentially applied as phosphorus fertilizer) may inadvertently alter the distribution of dsRNA between dissolved and adsorbed phases. Because these extraction buffers also liberate organic matter from the soil particle, sample preparation using two-column purification steps and a 10-fold dilution step was required to apply RT-qPCR for dsRNA quantification. These substantive preparation steps allowed dsRNA quantification to be achieved at an environmentally relevant concentration (0.003 ng_{dsRNA}/g_{soil}). To quantify dsRNA in soil solutions, a 100-fold dilution was sufficient, resulting in a lowest quantifiable concentration of 0.02 ng_{dsRNA}/g_{soil}. On both a mass and a copy number basis, values are a least three orders of magnitude lower than prior hybridization approaches (applied to measure 968 nucleotide hairpin-structured dsRNA at 25 ng/g_{soil})[22] and two orders of magnitude lower than a radioisotopic labeling approach (applied to measure 261 bp dsRNA at 1.5 ng/g_{soil}).[21]

By applying this approach, we were able to analyze concentrations of dsRNA applied to soil microcosms at 1 ng_{dsRNA}/g_{soil}. Unlike prior studies that applied relatively high concentration (~7,500 ng_{dsRNA}/g_{soil}) in which no change in dsRNA concentration occurred for 12 – 36 h,[23] we did not observe an apparent lag phase before dsRNA concentrations decreased. This may indicate that the dsRNA degradation or irreversible adsorption accounts for a greater fraction of dsRNA rapid dissipation at lower concentrations than at higher concentrations, which may saturate surface sites or dsRNA-competent enzymes.[21] In addition to our quantification approach enabling experiments to be conducted at environmentally realistic concentrations, the approach allows separate measurements of dsRNA dissolved in solution and extractable from particles, whereas other approaches pooled dsRNA in these two phases.[22, 23] Due to this feature, we were able to determine that extractable dsRNA concentrations decreased over time scales of hours, suggesting that prior

403 studies reporting that adsorption to particles protects nucleic acids from loss processes[60-62] may
404 not apply to dsRNA.

405 Overall, our approach contributes to the ongoing ecological risk assessment of dsRNA
406 biopesticides by providing a means to quantify dsRNA concentrations in soils at environmentally
407 relevant levels. In future work, it may be coupled to other protocols for tissue sample preparation[63]
408 to enable dsRNA quantification in soils amended with RNAi plant tissue. This approach also
409 complements an existing approach using radioisotopic labeling that enables elucidation of specific
410 fate processes impacting dsRNA fate at low concentrations[21] by allowing quantification of
411 unlabeled molecules at even lower dsRNA concentrations. Unique among available approaches,
412 this technique will be able to detect dsRNA in environmental media after release from GM RNAi
413 crops or the application of dsRNA biopesticides in the field.

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422 **Supporting Information.** Supporting calculations, materials, and methods; sample
423 characterization; and figures.

424 **References.**

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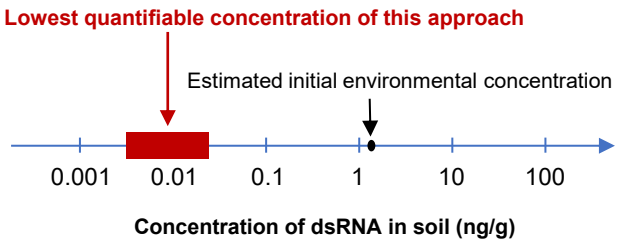

Lowest quantifiable concentration of this approach
Estimated initial environmental concentration
0.001
0.01
0.1
1
10
100
Concentration of dsRNA in soil (ng/g)

627

628 **TOC**

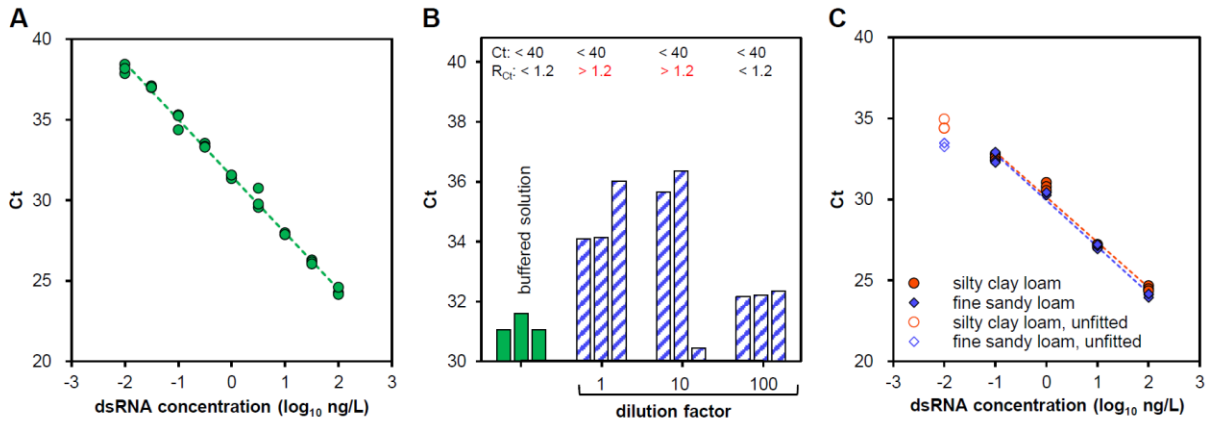
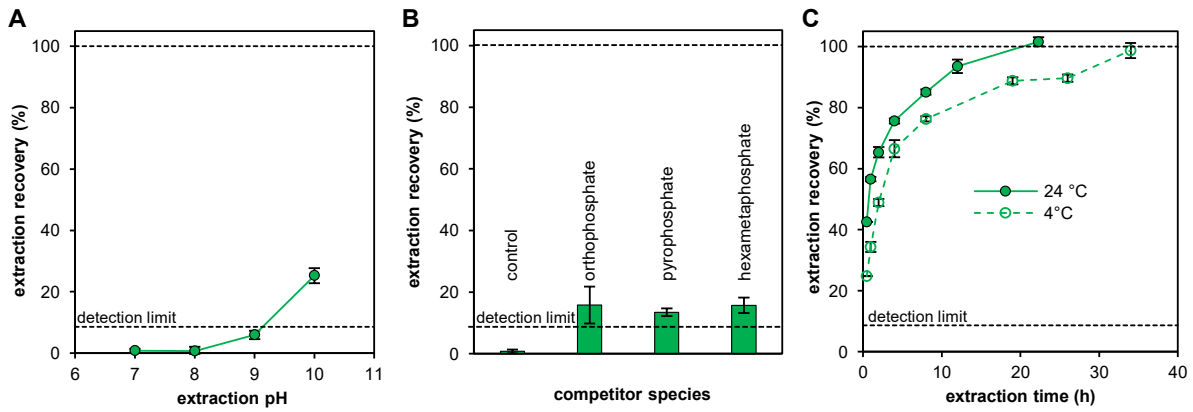


Figure 1. (A) Output of RT-qPCR (Ct) for dsRNA in buffered solution (3 mM tetraborate, 10 mM NaCl, 12 mM orthophosphate, pH 7). The standard curve is fitted with Ct = (-3.5 ± 0.1) $\log_{10}$[dsRNA] + (31.5 ± 0.1) with R^2 = 0.9951. The difference between the maximum and minimum Ct values at a given dsRNA concentration was used to calculate the range of Ct values. (B) Effect of dilution on Ct determined by RT-qPCR for dsRNA dissolved in solutions (3 mM MOPS (adjusted to native soil pH), 10 mM NaCl) that had previously been incubated with fine sandy loam soil. In the buffered solution control, dsRNA was spiked in a solution containing 3 mM tetraborate, 10 mM NaCl, and 12 mM orthophosphate at pH 7. The dsRNA concentration *after* dilution was the same (0.25 ng/L) in all groups. (C) Ct determined by RT-qPCR for dsRNA in a 100-fold diluted solution that had previously been incubated with fine sandy loam soil. The indicated dsRNA concentration is the concentration *after* dilution. The standard curve is fitted with Ct = (-2.8 ± 0.1) x + (30.1 ± 0.1), with R^2 = 0.9833 for the silty clay loam soil and y = (-2.9 ± 0.1) x + (29.9 ± 0.1), with R^2 = 0.9907 for the fine sandy loam soil. In both cases, the 10^{-2} ng/L dsRNA sample was excluded from the fit. Standard curves were developed by splitting single standards into triplicates prior to initiating RT-qPCR (A, C). In (B), samples were prepared in triplicate, and R_{Ct} represents the range of Ct.



647

648 **Figure 2.** Effect of extraction parameters on recoveries of adsorbed poly(A:U) dsRNA on goethite
649 (5.8 $ng_{dsRNA}/\mu g_{goethite}$; 4 $g_{goethite}/L$). Adsorption was carried out at 24°C for 15 min in a buffer (14
650 mM NaCl, 2.9 mM MOPS, pH 7). Extraction was carried out at 24°C for 1 h to test the effect of
651 extraction buffer pH (A) and phosphate species (all 6 mM) (B). Using an extraction buffer
652 containing 3 mM tetraborate, 10 mM NaCl, and 12 mM orthophosphate at pH 10, we evaluated
653 the effect of temperature and extraction time (C). Error bars represent the standard deviation of
654 measurements from samples prepared in triplicate. The indicated detection limit is the limit of the
655 spectrophotometer (2 mg/L) provided by the manufacturer, equivalent to a recovery of 8.6%.

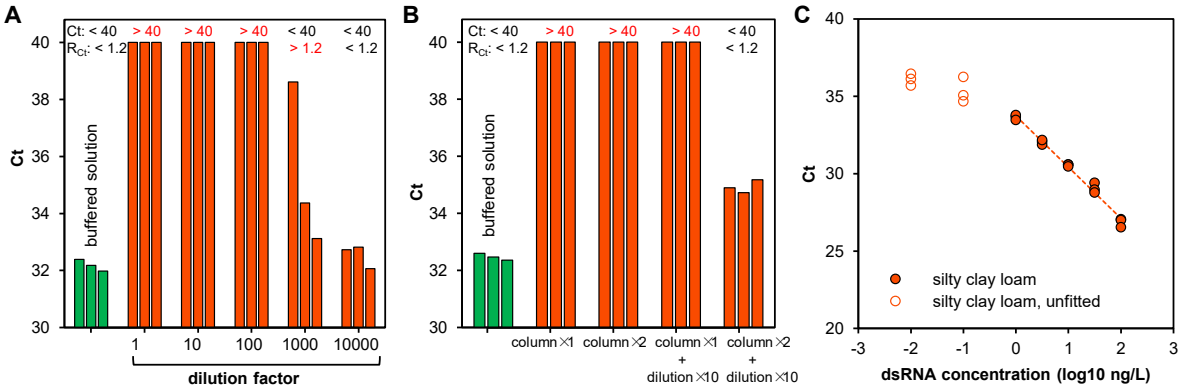
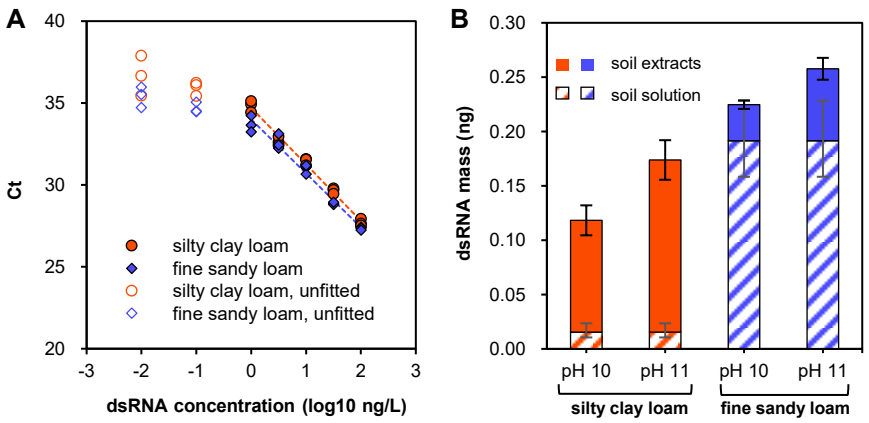
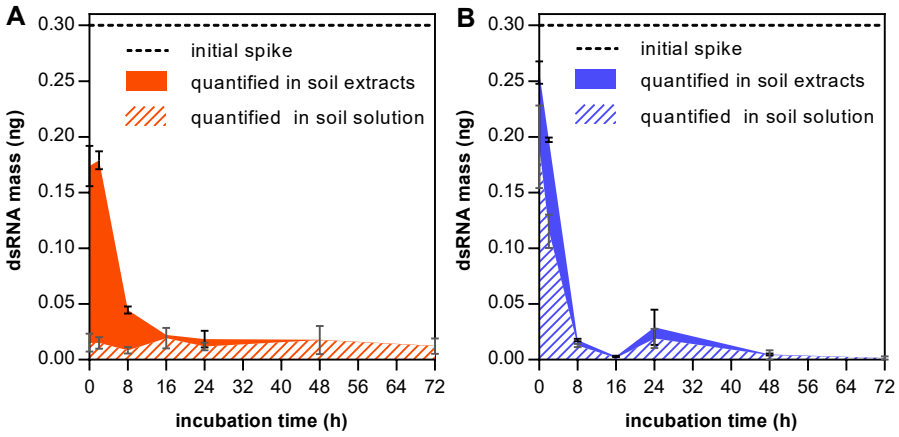


Figure 3. Effect of dilution alone (A) and dilution following column purification (B) on Ct determined by RT-qPCR for dsRNA spiked into purified and diluted extracts from silty clay loam soil. The dsRNA concentration *after* dilution was the same (1 ng/L) in all groups. The extracts were prepared from soil pellets (from 0.3 g of dry soil) first agitated with buffer (3 mM MOPS adjusted to native soil pH, 10 mM NaCl) for 2 h at 24°C (to replicate conditions applied in soil incubation experiments) followed by separation of the pellet and agitation with extraction buffer (10 mM NaCl, 12 mM phosphate, 200 mM tetraborate, pH 10) for 4 h at 4°C. In the buffered solution control, dsRNA was spiked in a solution containing 3 mM tetraborate, 10 mM NaCl, and 12 mM orthophosphate at pH 7. Ct > 40 is plotted as Ct = 40. R_{Ct} represents the range of Ct. (C) Standard curve of dsRNA in soil extracts prepared as in panel (B), followed by PCI purification, isopropanol precipitation, two sequential purification steps using columns, and 10-fold dilution. The indicated dsRNA concentration is the concentration *after* dilution. The standard curve is fitted with $Ct = (-3.3 \pm 0.1) \log_{10}[\text{dsRNA}] + (33.7 \pm 0.1)$ with $R^2 = 0.9883$ and excluded samples with dsRNA concentration of 10^{-1} and 10^{-2} ng/L. Samples were prepared in triplicate (A, B). The standard curve was developed by splitting single standards into triplicates prior to initiating RT-qPCR (C).



673

674 **Figure 4.** Effect of increasing extraction buffer pH value to 11. (A) Standard curve of dsRNA in
675 soil extracts prepared from soil pellets (from 0.3 g of dry soil) first agitated with buffer (3 mM
676 MOPS adjusted to native soil pH, 10 mM NaCl) for 2 h at 24°C (to replicate conditions applied in
677 soil incubation experiments) followed by separation of the pellet and agitation with extraction
678 buffer (10 mM NaCl, 12 mM phosphate, 200 mM tetraborate, pH 11) for 4 h at 4°C. The extracts
679 followed by PCI purification, isopropanol precipitation, two sequential purification steps using
680 columns, and 10-fold dilution. The indicated dsRNA concentration is the concentration *after*
681 dilution. The standard curves are fitted with Ct = (-3.5 ± 0.1) $\log_{10}$[dsRNA] + (34.7 ± 0.1), with
682 R^2 = 0.9874 for the silty clay loam soil and Ct = (-3.3 ± 0.1) $\log_{10}$[dsRNA] + (34.0 ± 0.2) with R^2
683 = 0.9773 for the fine sandy loam soil. Both curves excluded samples with dsRNA concentrations
684 of 10^{-1} and 10^{-2} ng/L. (B) Initial mass distribution of dsRNA (0.3 ng) applied to soil pellets (from
685 0.3 g of dry soil). Extraction buffer pH values of 10 and 11 were tested. Standard curves were
686 developed by splitting single standards into triplicates prior to initiating RT-qPCR (A). Error bars
687 represent the standard deviation of the mass measured in samples prepared in triplicate (B).



688

689 **Figure 5.** Mass of dsRNA measured in soil microcosms prepared with silty clay loam soil (A) and
690 fine sandy loam soil (B). Each sample was prepared by adding 0.3 ng dsRNA to 0.3 g dry soils
691 prior to incubation for 0 – 72 h. The solution and pellet were separated, followed by extraction of
692 adsorbed dsRNA in the pellet into the buffer (12 mM phosphate, 10 mM NaCl, 200 mM tetraborate,
693 pH 11) for 4 h at 4°C. The dsRNA concentration was below the lowest quantifiable mass for the
694 specified samples prepared using silty clay loam (extract: 48, 72 h) and fine sandy loam soils
695 (solution: 16, 48, 72 h; extract: 48, 72 h). Error bars represent the standard deviation of the mass
696 measured in samples prepared in triplicate.

697