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Early detection of invasive exotic insect infestations using eDNA from crop surfaces

Rafael E Valentin[1]*, Dina M Fonseca[1,2], Anne L Nielsen[2], Tracy C Leskey[3], and Julie L Lockwood[1]

The number of exotic species invasions has increased over recent decades, as have the ecological harm and economic burdens they impose. Rapid-response eradication of nascent exotic populations is a viable approach to minimizing damage, but implementation is limited by the difficulty of detecting such species during the early stages of infestation due to their small numbers. The use of environmental DNA (eDNA) has helped address this issue in aquatic ecosystems, but to the best of our knowledge has not been trialed for surveillance of exotic species in terrestrial systems. Using a high-resolution, real-time (quantitative) polymerase chain reaction assay, we developed a highly efficient protocol to survey agricultural fields for the invasive non-native brown marmorated stink bug (BMSB; *Halyomorpha halys*). We compared results using eDNA to those for conventional monitoring traps and documented substantially higher sensitivity and detection effectiveness. Our methodology is transferable to situations in which the DNA of terrestrial target species can be accumulated into a single substrate, suggesting that eDNA-based approaches could transform our ability to detect exotic insects in non-aquatic settings.

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Early detection of exotic populations, followed by rapid management responses, has resulted in successful eradication of several species known to cause ecological or economic harm (Mehta *et al.* 2007). Eradication requires lethal control measures, many of which have unwanted secondary effects (eg harm to non-target species leading to loss of ecosystem services). Delays in detection and eradication of exotic populations lead to increases in the magnitude and geographical extent of the invasion, and subsequently to escalation of the economic costs, while the probability of successful eradication declines substantially (Simberloff *et al.* 2013). Furthermore, when exotic populations are left unmanaged for long periods, efforts shift from eradication to protection of valued assets, which is often accomplished through the continual application of control methods (Simberloff *et al.* 2013). This general approach emphasizes the need for detection of unwanted and harmful exotic species when their presence is still very limited. However, this has proven extraordinarily difficult due to the low likelihood of detecting these small populations of individual invaders (Simberloff *et al.* 2013). In response, researchers have invested in improving survey design and statistical analysis, and in devising more sensitive surveillance tools (Mehta *et al.* 2007; Jerde *et al.* 2011). Here, we describe the use of environmental DNA (eDNA) to substantially improve the detection of agricultural insect pests, and in doing so provide a precedent for the use of eDNA for surveillance in other terrestrial invasion scenarios.

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Environmental DNA consists of freely available DNA or biological material containing DNA that has been shed or dropped by organisms as they move through the environment (eg skin flakes, hair, feathers, scales, setae [bristles], exuviae [molted exoskeletons], fecal matter) (Bohmann *et al.* 2014). This DNA can persist and accumulate within (or in terrestrial systems on the surface of) environmental materials or substrates, which can then be collected and tested using high-resolution processing techniques to detect trace amounts of DNA (Rees *et al.* 2014; Barnes and Turner 2016). Environmental DNA has been used successfully to surveil for invasive aquatic organisms (Jerde *et al.* 2011, 2013), and is considered a burgeoning field of investigation within invasion science (Ricciardi *et al.* 2017). However, as of this writing, the use of eDNA for exotic species surveillance in terrestrial ecosystems is comparatively rare. Extraction and analysis of DNA within soil is used extensively to characterize microbial and other communities, clearly indicating that the technical issues associated with using eDNA in terrestrial settings are minor. However, in the context of surveillance, eDNA approaches must be capable of detecting individuals of focal species when they are very rare. Due to the nature of water, aquatic systems can mix more readily and sampling approaches that filter large amounts of water facilitate the accumulation of DNA, making detection of exotic species more likely even when abundance is very low. However, the same conditions may not always be true for terrestrial systems, perhaps limiting the usefulness of eDNA approaches on land.

Nonetheless, terrestrial systems could benefit greatly from the use of eDNA techniques in terrestrial invasive species surveillance. Successful development of this methodology for exotic insects could translate into rapid-response eradications of species known to be harmful to

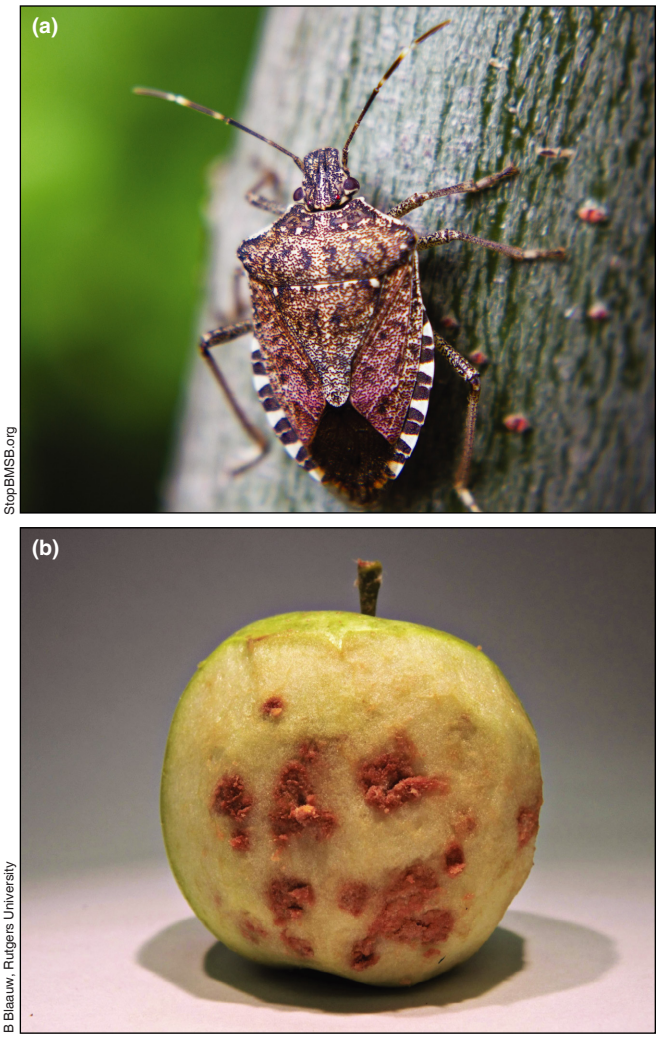

Figure 1. *Illustration of (a) the brown marmorated stink bug (BMSB) and (b) the damage it causes to fruits it has fed upon.*

crops and forests before infestations reach such an extent that tree removal programs, regulatory restrictions, and/or massive applications of chemical insecticides are required (McClure *et al.* 2001; Kovacs *et al.* 2010). The challenge to achieving this goal is addressing the inherent heterogeneity in terrestrial systems that prevent dispersed eDNA from being easily detected.

Our objective was to adapt current eDNA strategies to devise and test a highly sensitive surveillance framework for use in detecting an exotic terrestrial insect. We developed our approach in an agricultural system and focused on the brown marmorated stink bug (BMSB, *Halyomorpha halys*; Figure 1a), a rapidly spreading invasive insect. This species is native to northeast Asia, and was first detected in the US in Allentown, Pennsylvania, in 1996 (Hoebeke and Carter 2003). It has since been found in more than 40 US states, as well as parts of Canada and several European countries (Valentin *et al.* 2017), and has caused substantial damage to agricultural crops and ornamental plants (Figure 1b), resulting in economic losses totaling in the millions of dollars (Nielsen and Hamilton 2009; Leskey *et al.* 2012a). The range of crops BMSB attacks is extensive, making it a threat to farmers around the world; due to the severity of the damage caused, farmers have typically tried to control BMSB populations by increasing the frequency and intensity of broad-spectrum insecticide applications (Leskey *et al.* 2012b). Such applications are known to be disruptive to natural ecosystems, and undermine integrated pest management efforts (Leskey *et al.* 2012b).

Methods

Target eDNA collection

We used a genetic tool we had previously designed for BMSB; this tool is very sensitive to trace amounts of degraded DNA and exclusively targets BMSB (Valentin *et al.* 2016; Maslo *et al.* 2017). Because they are a sap-feeding species, BMSB individuals remain on a host plant for extended periods of time (Leskey *et al.* 2012a), potentially leaving detectable amounts of DNA as they feed, defecate, and/or molt. The crops they feed on are often harvested by farmers and transported to centralized locations for rinsing to remove soil and other detritus, and for boxing prior to sale. We posited that rinsing harvested crops in water, and then concentrating, extracting, and testing for DNA in the rinse water (Panel 1; Figure 2), could represent a potential surveillance technique.

To test whether BMSB DNA could be collected from water, we placed six individual samples of BMSB excreta and two individual BMSB exuvia each in a liter of deionized water, with two water-only samples acting as negative controls (for a total of 10 individual [one-liter] water samples). Following one methodology in the eDNA literature (eg Rees *et al.* 2014; Turner *et al.* 2014), we used a peristaltic pump (Pegasus Alexis, Pegasus Pump Company, Bradenton, FL) and a 10-µm polycarbonate track-etched (PCTE) filter membrane (GVS North America, Sanford, ME) combination to remove the DNA from the water. Once DNA collection was completed, we handled filter membranes with flame-sterilized tweezers, cut pieces approximately 14 mm^2 from the center of the filter with flame-sterilized scissors, and extracted DNA using an affordable and readily available HotSHOT extraction (Johnson *et al.* 2015) (Panel 1). To assess the presence of BMSB DNA in these samples, we used a TaqMan quantitative polymerase chain reaction (qPCR) assay designed specifically for BMSB (for details regarding assessments of specificity and sensitivity, see Valentin *et al.* 2016). Briefly, we used 20-µl reactions with 500 nanomolar (nM) concentration of each primer, 250 nM of the probe, 1X TaqMan® Environmental Master Mix 2.0, and 2 µl of DNA, following a reaction protocol with an initial denaturing step

Panel 1. Terrestrial eDNA surveillance

The use of eDNA in terrestrial systems differs from its use in aquatic systems primarily in that DNA from terrestrial species can remain localized. In contrast, DNA from aquatic species is commonly naturally dispersed, making surveillance a matter of concentrating water from a location and testing it for the presence of the target species' DNA.

To maximize probability of detection of eDNA in terrestrial systems, we argue that it is necessary to first assess likely sources and develop cost-effective strategies for bulk sampling (Figure 2). In the case of the brown marmorated stink bug, an agricultural pest, we made use of the aggregation of crops during harvest, in which harvested crops are brought to a centralized location for sorting, washing, and packaging. The washing process is where crop aggregation occurs, as all the material that was once on the surface of many individual crops is then transferred into a single bulk material (the water). From here, sampling, extraction, and testing can be carried out using standard eDNA strategies (Figure 2). A further benefit of this strategy is that, depending on expected levels of infestation, sampling can be stratified (analyses can be performed separately) at multiple scales (by crop, crop variety, farm, latitude, state) in order to gain insight into the presence and spatial distribution of the target species over many spatial scales to fit information needs. This strategy would allow for more focused management, wherein only infested locations would need treatment to control, or eradicate, nascent populations.

Although we found that sampling crop rinse water is a viable strategy for the detection of an agricultural pest, other approaches will certainly be needed for invasive species with different life histories and habitat preferences. Moreover, cost–benefit analyses comparing eDNA techniques to traditional surveillance methods are a necessary step toward real-world applications.

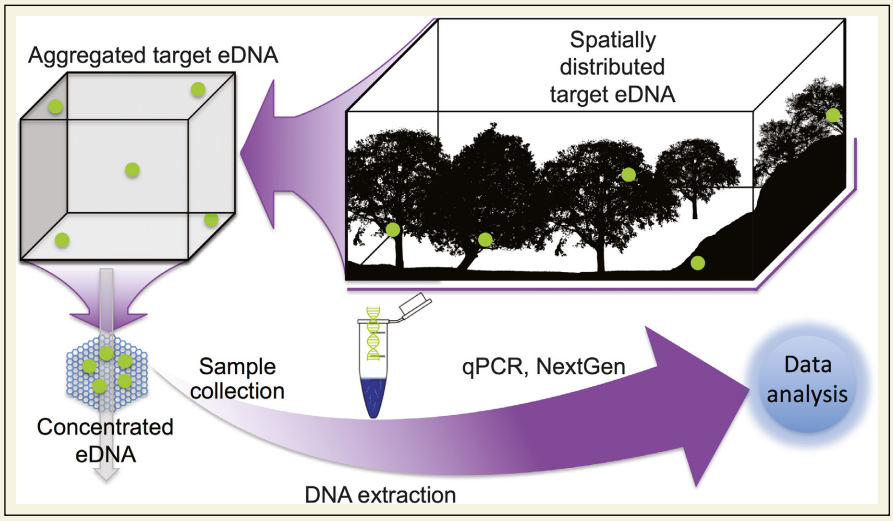

Figure 2. *Terrestrial surveillance efforts using eDNA require several steps: (i) assessment of likely sources of target DNA (fruit, bark, soil, etc); (ii) implementation of strategies to aggregate the target DNA (eg fruit washing); (iii) concentration of the target DNA (eg by using filtration); (iv) subsampling of the concentrated sample (when needed); (v) DNA extraction; (vi) testing for the presence or quantity of target DNA via qPCR, NextGen sequencing, or any other applicable method; and (vii) data analysis (eg occupancy modeling, spatial analyses).*

of 96°C for 10 min, followed by 45 cycles of denaturing for 15 s and annealing and extension at 60°C for 1 min. All reactions were run on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA) in replicates of two under a laminar flow hood. An ultraviolet light was used to sterilize the surface prior to qPCR setup to ensure a clean working environment.

BMSB DNA deposition rate

To document how much time an individual BMSB must be present and feeding on a piece of fruit before a detectable level of eDNA was deposited, we conducted a time-series experiment in which individual single BMSB adults (from a colony maintained at Rutgers University) were placed in small cages containing a single tomato. We allowed individual BMSB to feed on single tomatoes for a period of 2, 4, 6, or 8 hours, with four replicates of each treatment (ie a total of 16 BMSB and 16 tomatoes). Wearing nitrile gloves, we rinsed each tomato in a bucket containing a liter of deionized water (changing gloves before handling each tomato), pumped the water to collect the eDNA, then processed and tested the filters as described above. In addition, as controls, we rinsed and filtered water from tomatoes kept in cages without BMSB (two replicates) and from two tomatoes that were not placed in cages. Filter extraction and qPCR processing procedures were identical to those described in the previous section.

Development and testing of field protocol

To examine the efficiency of this protocol in locations with varying levels of BMSB infestation, we sampled crops from two farms. The first farm was located in New Jersey, where BMSB are prevalent. The second farm was in New Hampshire, where BMSB are not yet present in agricultural fields, but this site was very close to the edge of the species' current known range

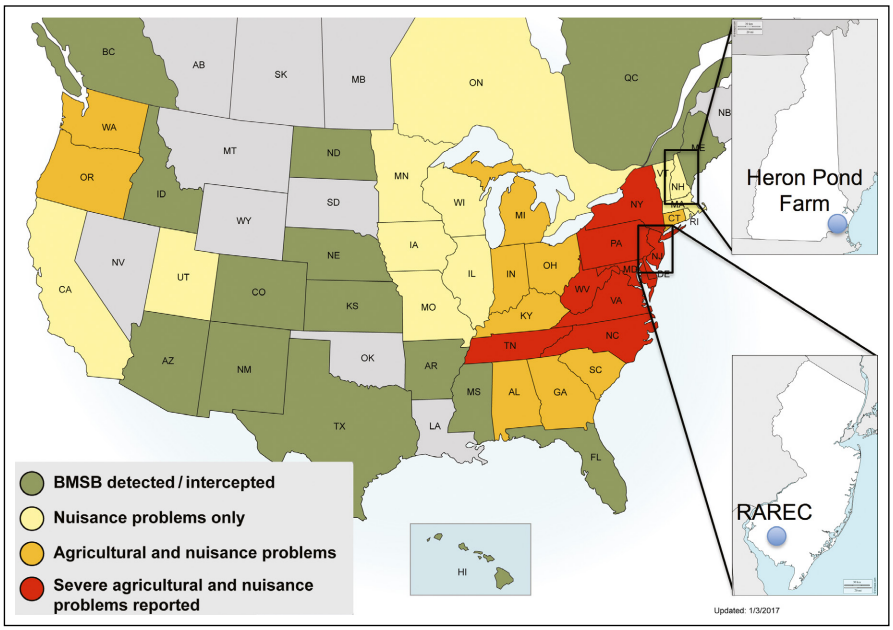

Figure 3. *Map of the distribution of the brown marmorated stink bug within the US, with both study locations shown. RAREC = Rutgers Agricultural Research and Extension Center. Range map provided by StopBMSB.org.*

(Figure 3). At both farms, we performed eDNA-based surveillance in conjunction with a blacklight trap (Old Boys Enterprises Inc, Oregon, WI) and four Dead-Inn 4-ft black pyramid traps (AgBio Inc, Westminster, CO) with Trécé PHEROCON® BMSB (low dose) pheromone lures (Trécé Inc, Adair, OK) (Weber *et al.* 2017) so that we could directly compare effectiveness at detecting BMSB. We trapped for BMSB and filtered one to two liters of rinse water at both sites in July and August (when BMSB are most abundant).

Field-testing at a high BMSB abundance site

The New Jersey site consisted of a peach orchard in the Rutgers Agricultural Research and Extension Center (RAREC) in Bridgeton, which is known to harbor large populations of BMSB (Figure 3). Wearing nitrile gloves, we collected five to seven peaches from four different peach trees and washed them in buckets with one liter of deionized water while still in the field. All peaches from each tree were washed in the same bucket, and each tree had a pyramid trap and pheromone lure directly next to it that had been in place since the start of the peach fruiting season (with lures being regularly replaced). Given that each tree had its own trap and was considered a separate location within the site, gloves were changed between trees to prevent cross contamination, and buckets associated with each tree were kept isolated from each other to assess positive or negative detections by location within each site. The water in each bucket was processed using the same pump and filter combination as in the laboratory experiments. Once filtration was completed, the filter membranes were removed from their housing and placed in 1.5-ml microcentrifuge tubes containing molecular grade 100% ethanol for storage and transport to the lab. Filters were handled using flame-sterilized tweezers and processed as described above immediately upon return to the lab. Trees were chosen by proximity to the four pheromone traps deployed at the site, which were >50 m from each other. The New Jersey site was visited twice, during the first and third weeks of July, and all four trees were tested once per visit.

Field-testing at an unknown BMSB abundance site

We further tested the performance of the eDNA field surveillance protocol against conventional monitoring methods (ie blacklight traps and pheromone traps) at Heron Pond Farm in New Hampshire, a diversified vegetable farm located near the expanding front of the BMSB geographical range but that was not known to be infested. We visited the New Hampshire farm twice, during the first and third weeks of August. We set one blacklight trap powered from a 120-v wall outlet, and four Dead-Inn 4-ft black pyramid traps with Trécé PHEROCON® BMSB (low dose) lures spread throughout four fields containing one to three different crop varieties each (cucurbits, chard, kale, arugula, tomatoes, and peppers) (WebTable 1). All traps were run continuously throughout the sampling period; blacklight traps were inspected each morning, whereas pheromone traps were inspected both in the morning and at various points throughout the harvesting period each day during each week. To ensure the farm's wash containers were not contaminated with BMSB DNA prior to contact with crops, after each container was filled with the farm's local water supply (river and well water) and readied for use, we filtered one liter of water and tested the filter paper for the presence of BMSB DNA, thereby ensuring that any positive identification of BMSB from using these containers was not due to contamination of BMSB DNA from anywhere else except the crops being washed that day. For each water container, after crops were harvested and thoroughly washed, approximately one to two liters of water (depending on the amount of suspended materials and subsequent filter saturation) were pumped through the filtration system and processed for eDNA collection. This resulted in seven to 13 filter samples per day, from nine different crops over 8 days (two 4-day sampling periods). Although some of the harvested and washed crops were grown directly

next to the pheromone trap in their respective fields, most were over 50 m away from the traps. Samples were processed in the field and lab in the same way as for the New Jersey experiments.

Detection probability calculation

We calculated detection probabilities using multi-method occupancy modeling for both eDNA surveillance and pheromone traps for the four fields surveyed at the New Hampshire farm. Multi-method occupancy modeling was used because it corrects for the fact that the two surveillance methods (eDNA and pheromone traps), whose sampling areas overlapped, were not independent of each other (Schmelzle and Kinziger 2016). We collapsed replicate filter samples for sampled containers, as well as experimental qPCR replicates, into a single detection event. We considered the container, and therefore the crops washed in that container, positive for BMSB DNA if at least one replicate produced a positive result. We binned the surveyed crops by field, in conjunction with the placement of the pheromone traps, and treated each day as a separate survey period (WebTable 1). All calculations were run using the program PRESENT v12.1 (Hines 2006).

Results

We found that all water samples spiked with BMSB were qPCR positive, and all negative controls were negative. The time-series experiment resulted in positive detections in the rinse water across all time ranges (ie 2, 4, 6, and 8 hours), indicating that detectable levels of BMSB DNA were deposited after only a few hours, at least under caged conditions.

Rinse water of peaches from the four trees on the New Jersey farm tested positive for BMSB DNA during both sampling periods (WebTable 1a). Pheromone traps located next to each of the trees were also positive for BMSB DNA, and on a few occasions we observed BMSB nymphs crawling on peaches before the fruit was collected for processing. All negative controls were negative for BMSB DNA.

We found that the eDNA strategy was effective in the field and more sensitive to smaller populations than both the blacklight and pheromone traps. At the New Hampshire farm, we found evidence of BMSB DNA on all 8 days sampled (WebTable 1b). Tests of the wash containers prior to washing harvested crops yielded no positive detections, indicating no pre-contamination. Several different insect species were collected in the blacklight traps, but not BMSB. A few native stink bug species were found in the pheromone traps throughout the sampling period (eg green stink bug [*Chinavia hilaris*]), but only one BMSB was caught, a nymph collected on the last day of sampling (WebTable 1b). Physical detection in the New Hampshire farm provided visual confirmation of the presence of BMSB. We note that this nymph was found near the end of August, after BMSB populations had the opportunity to grow throughout their reproductive season.

We found that, in contrast to the pheromone traps, our eDNA surveillance protocol detected the presence of BMSB across all sampling periods. Our multi-method occupancy model for the surveying efforts conducted on the New Hampshire farm yielded detection probabilities of 0.03 ± 0.038 and 1.0 ± 0.00 (mean ± standard error) for the pheromone traps and the eDNA method, respectively.

Discussion

We report here on the development and testing of a novel eDNA surveillance tool for the detection of a terrestrial exotic insect invader. The results of our experiments provided strong evidence that such an approach can be used successfully in an agricultural setting. Our eDNA-based approach exhibited much greater sensitivity to the presence or absence of BMSB than did the blacklight and pheromone-baited traps evaluated here. Although these traps were originally designed to monitor population abundance within established or spreading BMSB populations and not for surveillance (Nielsen *et al.* 2013; Short *et al.* 2017), they are currently the best option available for either purpose. The key to our eDNA approach is recognizing that individual BMSB naturally gather on fruit and vegetable crops, and regularly shed traces of their DNA as they feed. This DNA would still be difficult to sample effectively for use in surveillance protocols if there was no way to aggregate it and test for target species' presence. We solved this problem by noting that harvested crops are gathered before they are sold by farmers and thus can easily be sampled in bulk using rinse water. At the New Hampshire site – a fully operational vegetable farm – we showed that our eDNA surveillance protocol could be seamlessly incorporated into existing on-farm protocols by using their own wash containers and water sources. This demonstration was paramount for our method to be considered fully implementable on working farms.

The approach presented here has the potential to revolutionize agricultural pest surveillance, although additional research regarding the "ecology" of eDNA on working farms (Barnes and Turner 2016) and the cost effectiveness of using eDNA for surveillance is needed, as is a better understanding of when eDNA detection heralds an infestation, given that not all introductions result in establishment (Blackburn *et al.* 2011). Nevertheless, the growing number of exotic insects that are known to be harmful to agricultural crops makes such research investments worthwhile. In particular, once optimized, stratified sampling of eDNA in agricultural settings to pinpoint infested areas has the potential to substantially reduce the need to apply chemical insecticides over an entire landscape (Panel 1), thus diminishing their impacts

on native species and ecosystems (Kremen *et al.* 2002). Stratifying sampling across farms or discrete fields may also be useful from a biosecurity perspective if it allows nascent infestations to be tightly spatially delineated, and appropriate quarantine or control methods strategically applied so that they minimize disruption to normal farm operations.

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Supporting Information

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