Environmental Science & Technology Letters

Letter

Cite This: Environ. Sci. Technol. Lett. 2019, 6, 571−577

pubs.acs.org/journal/estlcu

Quantitative Microbial Risk Assessment of Swimming in Sewage Impacted Waters Using CrAssphage and Pepper Mild Mottle Virus in a Customizable Model

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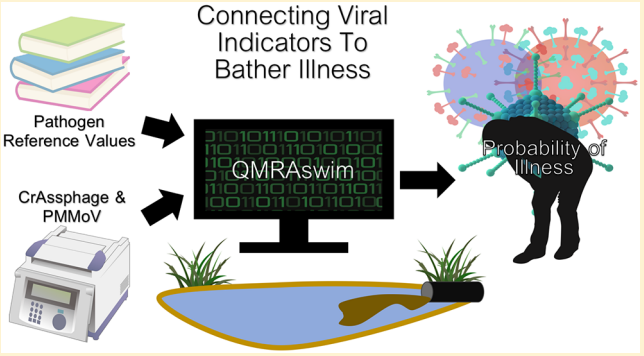
Supporting Information

ABSTRACT: Fecal indicator bacteria currently employed for microbial water quality management are poor representatives of viruses. Viral water quality indicators have recently been proposed based on the human gut bacteriophage crAssphage and the food virus pepper mild mottle virus (PMMoV) due to their high abundance in sewage and association to human waste. Here, we develop a model relating crAssphage and PMMoV abundance to the risk of swimmer illness in a recreational water contaminated with fresh, untreated domestic sewage. This model, entitled QMRAswim, is available via a Web-based user interface and is generalizable to any indicator or pathogen. The majority of predicted illnesses from exposure to untreated domestic sewage-contaminated water were attributable to viruses, primarily norovirus. The mean crAssphage and PMMoV concentrations correlating with 30 illnesses per 1000 bathers were 4648 GC/100 mL and 5054 GC/100 mL, respectively, approximately 50 times their standard detection limit. This study reaffirms the importance of monitoring viral water quality to adequately protect public health, suggests the high potential of both crAssphage and PMMoV for this application, and establishes a basis to relate viral indicator abundance with probability of illness due to viral pathogens.



INTRODUCTION

Current microbial water quality monitoring criteria based on fecal indicator bacteria (FIB) inadequately represents infectious risk from pathogenic viruses. For example, viral outbreaks have been documented following exposure to recreational waters with FIB at acceptable levels[1] or in the absence of FIB.[2,3] Human viral pathogens primarily enter the water environment through the release of untreated or inadequately treated wastewater. The majority of gastrointestinal infections resulting from exposure to sewage-impacted waters are predicted to be due to virus, primarily norovirus.[4,5] Improved methods to understand viral pathogens' presence and health impact in sewage impacted waters are critical to informing engineering and policy efforts to protect human health and improve microbial water quality.

Viral water quality monitoring approaches targeting culturable viruses, including both human pathogenic viruses and coliphages, have been previously developed. While these methods provide information on viral viability, they are generally challenged by low concentrations in sewage. An alternative approach is utilizing molecular (i.e., nucleic-acid based) assays. Two promising molecular viral water quality monitoring targets are based on the human gut bacteriophage crAssphage and the food virus pepper mild mottle virus (PMMoV). These targets are highly enriched in sewage and thus improve the ability to detect dilute concentrations of sewage in the environment. Here, we consider crAssphage and PMMoV due to their high enrichment in sewage; however, the approach is generalizable to any viral water quality indicator.

The bacteriophage crAssphage, short for "cross-assembly phage", was discovered in 2014 in human fecal metagenomes.[6] Notably, crAssphage was carried in a majority of surveyed metagenomes and was at least an order of magnitude more abundant than all other phages in the gut.[6] This first crAssphage is now recognized to be the prototypical member of a large family of crAss-like phages.[7] CrAssphage has recently been cultured confirming its host as *Bacteroides*[8] and is globally distributed.[9] CrAssphage was first evaluated as a source tracking marker using metagenomics to confirm its high abundance in sewage.[10] Subsequently, PCR assays were developed to detect crAssphage.[11,12] CrAssphage is highly

Received: July 30, 2019
Revised: August 26, 2019
Accepted: August 29, 2019
Published: August 29, 2019

DOI: 10.1021/acs.estlett.9b00468
Environ. Sci. Technol. Lett. 2019, 6, 571−577

Table 1. Viral Concentration and Dose-Response Data[a]

organism	$\log_{10}$ concentration range in sewage per liter	culturable fraction	P_{inf}	$P_{ill/inf}$	ref
crAssphage[b]	[7−9.5]	N/A			11
PMMoV	[6−10.3]	N/A			18, 28, 32
adenovirus	[5.5−8]	0.001	$P_{inf} = 1 - \exp(-0.4172\ \mu)$	0.5	5, 33, 34
norovirus	[3−6]	N/A[c]	$P_{inf} = 1 - {}_1F_1(0.04, 0.055, -\mu)$[d]	0.6	5, 32, 35
enterovirus	[4−6]	0.001	$P_{inf} = 1 - \exp(-0.0253\ \mu)$	0.5	5, 32, 36−39
rotavirus	[1−2.5]	N/A[e]	$P_{inf} = 1 - (1 + \mu/0.42)^{0.26}$	0.35	5, 37, 40

[a] P_{inf} corresponds to the probability of infection. "μ" corresponds to the dose of viable pathogens. $P_{ill/inf}$ corresponds to the probability of illness given infection. [b] Concentrations based on the CPQ56 assay. [c] Norovirus remains largely unculturable. RT-qPCR measurements were used to establish the dose−response relationship used here. [d] The norovirus dose−response model applied here was based on Norovirus GI[35] and is applied here for both Norovirus GI and GII[5] [e] Culturable rotavirus values were used to estimate the concentration range in sewage. Comparing these observed values with molecular measurements[41] would give an approximate culturable fraction of 0.0001

abundant in sewage, and independent studies have found average crAssphage wastewater concentrations of greater than 9 $\log_{10}$ genome copies (GC)/L.[11,13] CrAssphage appears to be human-associated; however, it has been detected in seagull, dog, duck, pig, and chicken fecal samples.[11,13,14] CrAssphage has also been successfully deployed to detect fecal pollution in environmental waters.[15−17]

PMMoV is a plant pathogen infecting a wide variety of pepper cultivars that was first proposed as a viral fecal pollution indicator[18] following the metagenomic observation of the abundance of PMMoV in feces.[19] PMMoV was previously found in 100% of tested wastewater samples from throughout the United States at concentrations of greater than 9 $\log_{10}$ GC/L.[18] PMMoV has been detected in sewage and impacted waters globally[20−26] and has also been proposed as a viral indicator for wastewater reuse.[27] PMMoV is human-associated but has been detected in chicken, geese, cow, pig, duck, and seagull fecal samples.[14,18,28] The potential of PMMoV in viral water quality monitoring was recently reviewed.[28−30]

Previous static quantitative microbial risk assessment models have been developed for bacterial fecal indicators, including the human fecal indicators HF183 and HumM2[4] and the seagull marker CAT.[31] In these previous studies, the concentration of fecal pollution markers were used to determine the fraction of source material (e.g., wastewater) in a contaminated water body, and infectious risk was estimated using concentration ranges of model pathogens in the source material. In this letter, we expanded previous work through construction of a customizable quantitative microbial risk assessment (QMRA) model, QMRAswim, to relate indicator abundance to the fraction of wastewater in an ambient water body and the probability of illness following swimming. QMRAswim is user-modifiable and can be adapted to any fecal pollution indicator and to include total infectious risk from any pathogen, including any reference pathogen type and combination of reference pathogens with an available dose response model. QMRAswim is available via either a Web platform or source code. Following development, QMRAswim was applied to viral fecal pollution indicators crAssphage and PMMoV to examine the association of these fecal pollution indicators with illness following swimming in sewage-contaminated waters.

MATERIALS AND METHODS

Initially, a comprehensive static QMRA was conducted using a suite of model wastewater pathogens shown in Table 1 and Table S1. Pathogens included in Table S1 are *Salmonella*, *Campylobacter*, *E. coli* O157:H7, *Cryptosporidium*, and *Giardia*. Details for the comprehensive static QMRA are available in the Supporting Information. Following this initial evaluation, the QMRA was reduced to model viruses (Table 1) for further evaluation and development.

QMRAswim was built using a similar approach to Boehm et al.[4] The model assumes a fresh untreated domestic wastewater contamination scenario from a single source (i.e., excludes other contamination sources) and is used to determine the disease burden attributable to water ingestion during swimming. The exposure scenario modeled here follows an adult bather during a 45 min swimming event occurring in water polluted by fresh sewage at the time of recreation. A cross-sectional approach was taken, and pathogen decay was not included. We note here for clarity that risk assumptions would change with multiple sources of contamination or with additional transport or aging of contamination. Throughout the letter we use the term "bather" to refer to individual swimmers, i.e., primary contact recreators.

A Monte Carlo approach with 10 000 samplings was performed at each of six assumed concentrations for both crAssphage and PMMoV, namely, 10^0, 10^1, 10^2, 10^3, 10^4, and 10^5 GC/100 mL. The assumed viral fecal indicator concentration in ambient water and the subsampled concentration ranges in wastewater were used to model the fraction of wastewater present in ambient water. For each sampling, the observed ambient viral marker (set to a fixed value) was normalized by the wastewater viral marker concentration taken from a distribution to determine the wastewater fraction in ambient water. Viral concentrations in wastewater vary geographically.[42] Norovirus, enterovirus, and rotavirus concentrations presented in Table 1 are from the United States, the adenovirus concentration is from Spain, and PMMoV values were pooled from multiple countries.

The total exposure volume (mL) for a bather ingested during a single 45 min long swimming event was modeled as a log-normal distribution with a mean of 2.92 with a standard deviation of 1.43 as described previously.[4,43] While alternative exposure models are available,[44,45] here we used the 2006 Dufour et. al study[43] to remain consistent with similar QMRAs. The amount of wastewater consumed for each sampling was the fraction of wastewater multiplied by total exposure volume. All pathogen and marker concentrations were modeled as a log-uniform distribution within the indicated ranges to account for variable occurrence and abundance.[46] The culturable fraction was estimated where appropriate to relate molecular viral quantifications with dose−response models based upon culturable units. Dose−response parameters and selected pathogens were based upon previous

DOI: 10.1021/acs.estlett.9b00468
Environ. Sci. Technol. Lett. 2019, 6, 571−577

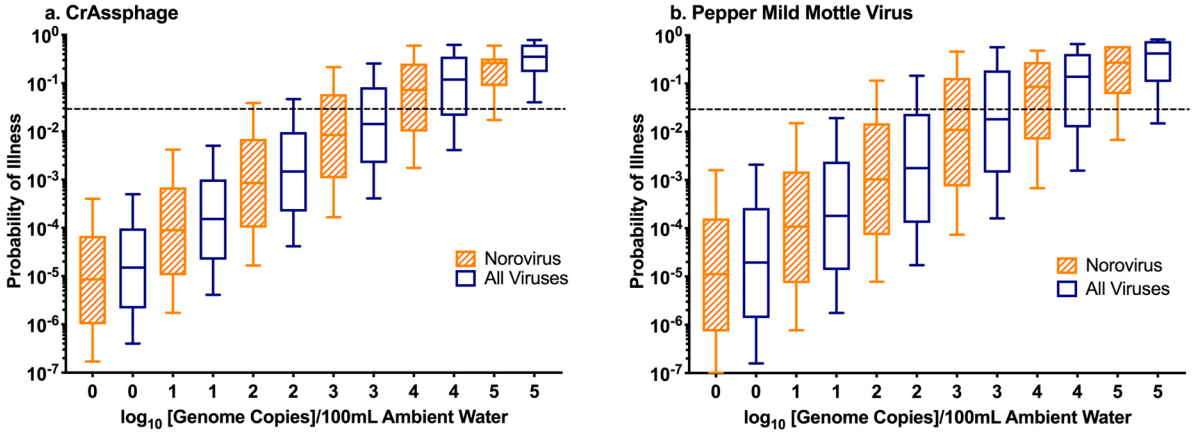


Figure 1. (a) Predicted probability of illness for all evaluated viruses and norovirus alone based on crAssphage concentration in ambient water. (b) Predicted probability of illness for all evaluated viruses and norovirus alone based on PMMoV concentration in ambient water. The dashed line represents 30 illnesses per 1000 bathers. The upper and lower bounds for the box and whisker plot represent the 10% and 90% bounds, and the box represents the first to third quartile.

QMRA assessments.[4,5] For enterovirus, the dose−response model for coxsackievirus A21 was applied as coxsackieviruses are the most common nonpolio enteroviruses.[38,39] For norovirus, the disaggregated hypergeometric 1F1 model was chosen as it has been applied previously for similar assessments[5,35] and provides a conservative risk estimate.[47] The probability of illness given infection was modeled as previously described.[5] The total probability of illness for n number of pathogens is calculated by QMRAswim as $P_{illtotal} = 1 - \Pi_n (1 - P_{illn})$, where P_{illn} is the probability of illness for an individual model pathogen. The reference water quality standard used here is 30 illnesses per 1000 bathers. This differs slightly from the 2012 U.S. EPA Recreational Water Quality Criteria (U.S. EPA RWQC) recommendation of 32−36 illness (NGI)/1000 bathers, and we use the lower value to allow a more direct comparison with prior QMRAs.[4] A Spearman's correlation coefficient sensitivity analysis was conducted for each viral pathogen and each viral indicator. The analysis identifies the parameters with the strongest influence on the calculated probability of illness. The culturable fraction was modeled as a range in the sensitivity analysis in order to determine the effect of the culturable fraction on the resulting probability of illness.

The QMRAswim model was built and implemented in RStudio version 1.1.456 (RStudio Inc., Massachusetts) using RShiny version 1.3.2. Additional data analysis and visualization was completed in Prism 7.0 (GraphPad, California) and in Excel version 16.25 (Microsoft Office, Washington). The model is available via a Web application at (https://qmraswim.shinyapps.io/qmraswim/) and the R source code is available at (https://github.com/kcrank1).

RESULTS AND DISCUSSION

Viruses Account for the Majority of Bather Gastrointestinal Illness. Initially, a comprehensive QMRA model was constructed using all model pathogens included in Table 1 and Table S1. This model was evaluated for a range of modeled wastewater exposure concentrations at both high and low pathogen exposure scenarios. Across all conditions, 77.5−87.0% of predicted illnesses were attributable to viruses (Table S2). Norovirus was the largest contributor to infectious risk, accounting for 68.4−78.6% of total predicted illnesses, followed by adenovirus. This is consistent with previous QMRA-based analyses that predict a majority of illnesses due to the norovirus.[4,48] As both crAssphage and PMMoV are being developed primarily to monitor viral water quality and viruses accounted for the majority of predicted infectious risk, nonviral model wastewater pathogens (pathogens listed in Table S1) were excluded from further analyses.

PMMoV and crAssphage in QMRAswim. QMRAswim was then implemented with the viruses in Table 1 with 10 000 samplings at each modeled crAssphage and PMMoV concentration (i.e., 10^0−10^5 GC/100 mL) (Figure 1). The range of probability of illness due solely to norovirus is also shown, as norovirus was predicted to be the largest contributor to observed infectious risk. CrAssphage and PMMoV performed similarly; the exception was the larger distribution for PMMoV due to a wider modeled concentration range in wastewater. The mean probability of illness for both crAssphage and PMMoV ranged from 10^{-5} at 1 GC/100 mL in ambient water to $>10^{-1}$ at 10^5 GC/100 mL in ambient water.

Indicator Concentrations Corresponding to Risk Threshold. A linear regression of the probability of illness versus indicator concentrations (Figure 2), calculated wastewater fractions (Figure S1), and calculated wastewater doses (Figure S2) was performed. Linear regression information as well as confidence intervals and prediction intervals are reported in Table S3. The mean crAssphage and PMMoV concentrations correlating with 30 illnesses per 1000 bathers were 4648 GC/100 mL and 5054 GC/100 mL, respectively. The 50% prediction intervals, indicating the area between the 25th and 75th percentile, were used to represent variability (Table S3). The linear regression slopes for indicator concentration versus probability of illness were 0.86 and 0.82 $\log_{10}$[probability of illness]/$\log_{10}$[GC]/100 mL ambient water for crAssphage and PMMoV, respectively. The difference between the two slopes is driven by the difference in the assumed crAssphage and PMMoV concentration ranges (Table 1), demonstrating how differences in concentration profiles may alter risk predictions and how improved concentration data would refine risk predictions.

Limit of Detectable Infectious Risk. The predicted crAssphage and PMMoV concentrations corresponding to 30

DOI: 10.1021/acs.estlett.9b00468
Environ. Sci. Technol. Lett. 2019, 6, 571−577

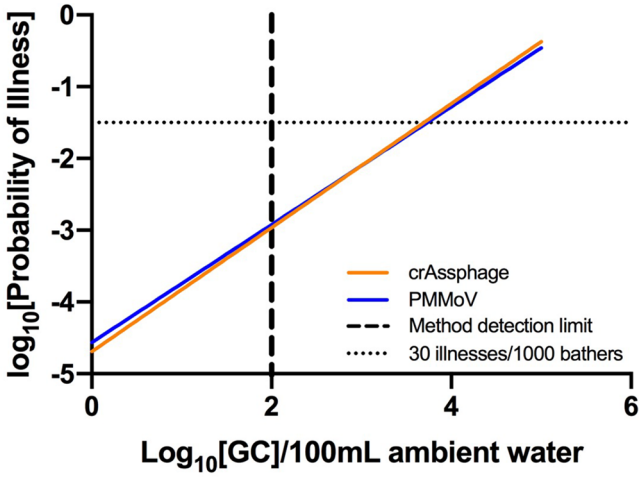


Figure 2. Linear regression of the probability of illness from evaluated viruses for different indicator concentrations. The dotted line represents 30 illnesses per 1000 bathers. The dashed lines represent the method detection limit of 100 GC/100 mL ambient water. Confidence intervals and prediction intervals are reported in Table S3.

illnesses per 1000 bathers[49] are within the standard process limit of detection. Assuming 500 mL of sample was used for viral concentration, sample DNA or RNA was eluted into 100 μL, of which 2 μL was used for each qPCR or digital PCR reaction and that the method detection limit is 10 GC per reaction, this method would result in an approximate process limit of detection of 100 GC per 100 mL of ambient water sample. The process limit of detection could potentially be lowered further by using enhanced viral recovery methods or eluting extracted nucleic acids in a smaller volume. We note that for simplicity, this calculation does not include losses due to reverse transcription and assumes 100% recovery efficiency. Applying the calculated regression, the detection limit of 100 GC per 100 mL of ambient water corresponds to a probability of illness of 1.09 and 1.20 out of 1000 bathers for crAssphage and PMMoV, respectively (Figure 2, prediction intervals shown in Table S3).

Sensitivity Analysis. A sensitivity analysis was performed using Spearman's rank correlation coefficient. Tornado plots for each individual pathogen scenario are shown in Figure S3. The main contributors to risk variability in all scenarios were the concentration of the indicator in wastewater and the concentration of the pathogen in wastewater. This agrees with similar QMRAs which found that the concentration of a pathogen in water is the main contributor to risk variability.[50,51] Since there is strong temporal and geographic variability in wastewater, a specific application of QMRAswim can reduce variability by using concentrations of indicators and pathogens from a known wastewater pollution source.

Contextualizing Results. A previous recreational water QMRA developed by Ahmed et al.[52] used an analytical approach to determine fecal indicator concentrations that would result in bather illness from norovirus and adenovirus. Water samples were seeded with known dilutions of sewage, and qPCR was performed on the water samples for multiple fecal pollution indicators, including PMMoV. Bather probability of illness was then calculated using literature concentrations of pathogens in wastewater from the known wastewater dilution. The mean predicted concentration of PMMoV in ambient water corresponding to a risk threshold of 36 illnesses per 1000 bathers was 544 GC/100 mL. This value is approximately an order of magnitude lower than the 5054 GC/100 mL predicted by this study at a risk threshold of 30 illnesses per 1000 bathers. Different assumptions between these two studies include variability of the fecal pollution indicator marker (i.e., fixed versus assumed concentration range), assumed norovirus concentration ranges in wastewater, and suites of pathogens evaluated. The differences between these studies emphasize the importance of assumptions in QMRA models and highlight the value of QMRAswim to rapidly evaluate multiple possible scenarios.

Boehm et al.[4] used the bacterial fecal indicators HF183 and HumM2 to determine the fraction of wastewater in ambient water and subsequently probability of bather illness from norovirus. HF183 and HumM2 concentrations corresponding to 30 illnesses per 1000 bathers were 4200 GC/100 mL and 2800 GC/100 mL, respectively. The detection limit calculated for HF183 and HumM2 qPCR methods used by Boehm et al. corresponded to median GI illness rates of 4 illnesses and 6 illnesses per 1000 bathers, respectively. The present study demonstrates the potential of crAssphage and PMMoV to reduce the detectable GI illness rate to below this previously reported threshold.

Study Limitations and Implications. The QMRAswim model has multiple limitations in its current iteration that suggest future research needs and developments. First, QMRAswim only accounts for a fresh wastewater pollution event from a single source. The differential decay of pathogens and markers present in wastewater may result in the under- or overestimation of infectious risk.[48] Future research should evaluate the persistence of both crAssphage and PMMoV under environmentally relevant conditions to update expected decay in the exposure model. In addition, previous research has shown that fecal pollution from multiple sources alters the expected risk profile.[51,53] Second, pathogen concentrations in wastewater are variable, both temporally and geographically.[42] This is accounted for in QMRAswim by using a distribution of the modeled pathogens and markers; however, additional information on the distribution of pathogen abundance in wastewater would reduce uncertainty in model predictions. A strength of QMRAswim is that as additional data becomes available it can be rapidly incorporated into a new risk assessment. Finally, QMRAswim is limited to known information on pathogen distributions in wastewater and available dose−response equations. Only a subset of pathogens that could cause bather infections have available dose−response models, and here we focus solely on gastrointestinal infections. QMRAswim uses a single dose−response model for each pathogen and therefore does not account for differences in host immunity or susceptibility to disease. Ultimately, additional exposure scenarios and pathogens must be considered to inform management decisions. Different scenarios, including those with child bathers, different geographic regions, and alternative pathogen sources would significantly alter the risk profile.

Current microbial water quality regulations, based nearly exclusively on FIB, are inadequate to accurately represent the likelihood of illness due to a virus. The majority of infectious risk from exposure to wastewater contaminated recreational waters is due to viruses. In this study, we first developed a QMRAswim model to relate indicator concentrations with probability of bather illness. Dose−response curves, pathogen distributions in sewage, and indicator distributions in sewage

DOI: 10.1021/acs.estlett.9b00468
Environ. Sci. Technol. Lett. 2019, 6, 571−577

are all customizable. Additionally, QMRAswim allows the user to input values for any fecal pollution indicator to determine risk of illness from any pathogen with available reference information. Outputs of QMRAswim can be downloaded for further data analysis. We then used the QMRAswim model to establish a relationship between observed crAssphage and PMMoV marker concentrations in sewage-polluted water and the probability of bather illness. It has not previously been possible to significantly reduce expected disease burden using FIB and existing epidemiological models. Detection limits for both crAssphage and PMMoV are approximately 50 times the concentrations predicted at the 30 illnesses per 1000 bathers risk threshold, offering the possibility to meaningfully reduce acceptable risk levels. Additional research on viral marker persistence and viral pathogen distribution, among other areas, would enhance model accuracy. The approach in this letter provides a basis to extend promising viral fecal pollution indicators to the probability of illness in fecal-polluted waters.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.9b00468.

Methodology for comprehensive QMRA; Table S1, model wastewater pathogen data for static QMRA; Table S2, probability of illness and percent of attributable illness due to virus and Norovirus under various wastewater exposure scenarios; Table S3, linear regression parameters and predicted target virus concentration corresponding to 30 illnesses per 1000 bathers; Figure S1, predicted probability of illness versus fraction of wastewater in ambient water; Figure S2, predicted probability of illness versus predicted wastewater dose; and Figure S3, tornado plots of sensitivity analysis results (PDF)

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Notes

The authors declare the following competing financial interest(s): K.B. is a co-inventor on a patent application entitled Cross-Assembly Phage DNA Sequences, Primers and Probes for PCR-based Identification of Human Fecal Pollution Sources (Application No. 62/386,532). The authors declare no other conflict of interest.

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■ ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of Lance Upton with the development of the Web application. This work was supported by National Science Foundation Grants 1748019 and 1818412 to K.B.

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