**Appendix A. Supplementary information**

**Sources and Prevalence of *Cyclospora cayetanensis* in Southeastern U.S. Growing Environments**

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**Table S1**

Performance of standard curves used for the qPCR assays for *C. cayetanensis* and the human fecal markers.

|  |  |  |
| --- | --- | --- |
| **Assay (gene target)** | **Standard** | **Standard curve slope, intercept (R2)** |
| *Bacteroides* HF183 (16S rRNA)a | Linearized synthetic DNA plasmid | -3.33, 36.58 (0.987) |
| crAssphage (Genomic region 14712–14860)b | Synthetic gBlocks™ gene fragment | -3.21, 42.02 (0.992) |
| *C. cayetanensis* (18S rRNA gene)c | Synthetic gBlocks™ gene fragment (HMgBlock135m) | -3.40, 38.07 (0.993) |

a(U.S. Environmental Protection Agency, 2019)

b(Stachler et al., 2017)

c(Qvarnstrom et al., 2018)

**Table S2**

Median measured water quality parameters (range) for irrigation pond (N=27) and packing house dump tank (N=23) samples.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Grower** | **Location** | **Temperature (°C)** | **Conductivity (µS/cm)** | **Turbidity (NTU)a** | **Dissolved Oxygen (mg/L)** | **pH** | **ORP (mV)** |
| **A** | **Pond 1** | 21 (13–32) | 217 (107–424) | 4.3 (0.29–27) | 7.9 (3.2–13) | 8.0 (7.0–11) | NT |
| **A** | **Pond 2** | 23 (14–33) | 238 (117–324) | 7.9 (0.13–270) | 8.9 (2.6–13) | 8.3 (7.0–10) | NT |
| **A** | **Pond 3** | 22 (11–31) | 211 (102–311) | 13 (0.86–420) | 4.6 (0.66–15) | 7.9 (6.6–9.6) | NT |
| **A** | **Pond 4** | 24 (15–36) | 238 (183–325) | 14 (1.7–530) | 8.1 (1.5–12) | 8.2 (6.9–10) | NT |
| **A** | **Packing house** | 15 (12–21) | 382 (110–621) | 23 (0.11–310)b | 9.2 (5.9–11) | 8.2 (7.7–9.6)c | 518 (145–695)c |
| **B** | **Pond 1** | 22 (14–32) | 237 (96–302) | 19 (0.27–380) | 11 (4.1–17) | 9.1 (7.6–11) | NT |
| **B** | **Pond 2d** | 21 (13–30) | 117 (16–171) | 4.9 (0.15–87)c | 8.7 (2.5–11) | 8.5 (7.5–14) | NT |
| **B** | **Pond 3** | 21 (11–29) | 256 (7–673) | 13 (2.3–290) | 9.2 (4.2–17) | 8.4 (7.0–11) | NT |
| **B** | **Pond 4** | 21 (14–30) | 247 (83–299) | 11 (0.29–220) | 8.3 (4.0–14) | 8.4 (7.5–11) | NT |
| **B** | **Packing house** | 21 (12–24) | 358 (115–680) | 23 (1.2–190)c | 8.6 (7.4–11) | 8.4 (8.0–9.2) | 613 (248–715) |

a Maximum turbidity for ponds may be artificially high due to water coloration from algal blooms.

b Measurements excluded for four samples in which instrument error occurred.

c Measurements excluded for one sample in which instrument error occurred.

d N=28 for Pond B-2.

NT: Not tested.

Chart, scatter chart

Description automatically generated

**Figure S1: Grower A – Pond 1.** Log10gene target concentrations ofthe*C. cayetanensis* 18S rRNA gene (black squares), HF183 (blue circles), and crAssphage (pink triangles) from irrigation pond water samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample (Definition 1: duplicate Cqs <40). Detection limit varied based on final concentrate volume and proportion of concentrate that was extracted for PCR testing.

Chart, scatter chart

Description automatically generated

**Figure S2: Grower A – Pond 2.** Log10gene target concentrations ofthe*C. cayetanensis* 18S rRNA gene (Definition 1, black squares), HF183 (blue circles), and crAssphage (pink triangles) from irrigation pond water samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample (Definition 1: duplicate Cqs <40). Detection limit varied based on final concentrate volume and proportion of concentrate that was extracted for PCR testing.

Chart, scatter chart

Description automatically generated

**Figure S3: Grower A – Pond 3.** Log10gene target concentrations ofthe*C. cayetanensis* 18S rRNA gene (Definition 1, black squares), HF183 (blue circles), and crAssphage (pink triangles) from irrigation pond water samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample (Definition 1: duplicate Cqs <40). Detection limit varied based on final concentrate volume and proportion of concentrate that was extracted for PCR testing.

Chart, scatter chart, box and whisker chart

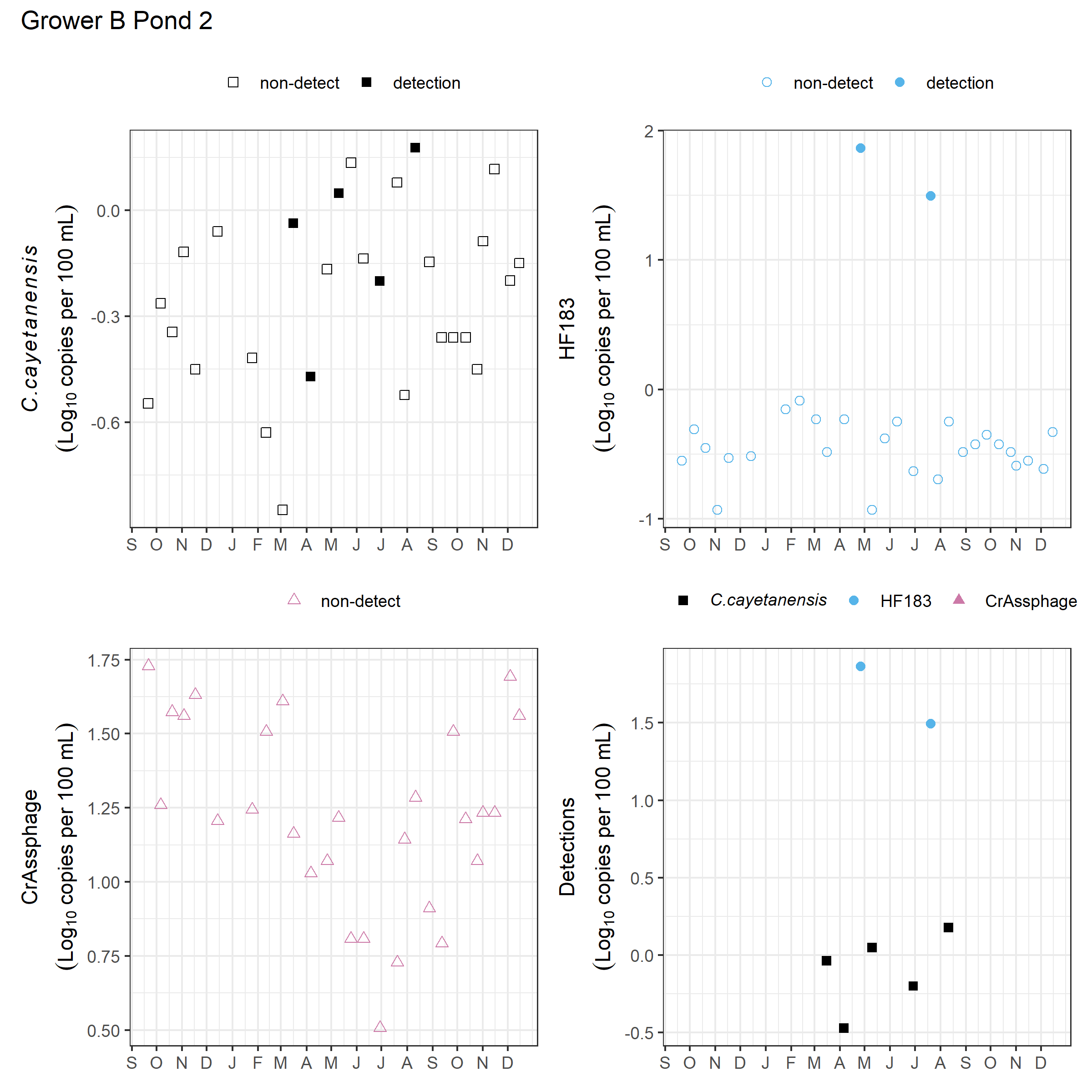
Description automatically generated

**Figure S4: Grower A – Pond 4.** Log10gene target concentrations ofthe*C. cayetanensis* 18S rRNA gene (Definition 1, black squares), HF183 (blue circles), and crAssphage (pink triangles) from irrigation pond water samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample (Definition 1: duplicate Cqs <40). Detection limit varied based on final concentrate volume and proportion of concentrate that was extracted for PCR testing.

**Chart

Description automatically generated**

**Figure S5: Grower B – Pond 1.** Log10gene target concentrations ofthe*C. cayetanensis* 18S rRNA gene (Definition 1, black squares), HF183 (blue circles), and crAssphage (pink triangles) from irrigation pond water samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample (Definition 1: duplicate Cqs <40). Detection limit varied based on final concentrate volume and proportion of concentrate that was extracted for PCR testing.

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**Figure S6: Grower B – Pond 2.** Log10gene target concentrations ofthe*C. cayetanensis* 18S rRNA gene (Definition 1, black squares), HF183 (blue circles), and crAssphage (pink triangles) from irrigation pond water samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample (Definition 1: duplicate Cqs <40). Detection limit varied based on final concentrate volume and proportion of concentrate that was extracted for PCR testing.

**Chart, scatter chart

Description automatically generated**

**Figure S7: Grower B – Pond 3.** Log10gene target concentrations ofthe*C. cayetanensis* 18S rRNA gene (Definition 1, black squares), HF183 (blue circles), and crAssphage (pink triangles) from irrigation pond water samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample (Definition 1: duplicate Cqs <40). Detection limit varied based on final concentrate volume and proportion of concentrate that was extracted for PCR testing.

**Chart, box and whisker chart

Description automatically generated**

**Figure S8: Grower B – Pond 4.** Log10gene target concentrations ofthe*C. cayetanensis* 18S rRNA gene (Definition 1, black squares), HF183 (blue circles), and crAssphage (pink triangles) from irrigation pond water samples collected from September 2020 through December 2021. qPCR detections are plotted as solid shapes and non-detects are plotted as open shapes at the detection limit for each sample (Definition 1: duplicate Cqs <40). Detection limit varied based on final concentrate volume and proportion of concentrate that was extracted for PCR testing.

**Figure S9.** Neighbor joining tree of closely related coccidia species with isolates from irrigation pond water samples in green, sludge samples in pink, and clinical *C. cayetanensis* in yellow.

**Table S3**

Molecular typing results for sludge samples with amplification of the eight typing markers.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sludge** | **Date collected** | **Number and type of amplified markers in Sample** | **Marker haplotypes** | **Genotyping result** | **Sample result interpretation** |
| REC | 8/5/20 | 1 nuclear | *C. cayetanensis* | NAa | Confirmed  *C. cayetanensis* |
| RAS | 8/5/20 | 2 mitochondrial  3 nuclear | *C. cayetanensis* | Clinical *C. cayetanensis*b | Confirmed  *C. cayetanensis* |
| RAS | 8/18/20 | 1 nuclear | *C. cayetanensis* | NA | Confirmed  *C. cayetanensis* |
| REC | 9/1/20 | 3 nuclear | *C. cayetanensis* | NA | Confirmed  *C. cayetanensis* |
| RAS | 9/1/20 | 1 nuclear | *C. cayetanensis* | NA | Confirmed  *C. cayetanensis* |
| RAS | 9/14/20 | 1 nuclear | *C. cayetanensis* | NA | Confirmed  *C. cayetanensis* |
| RAS | 7/20/21 | 2 mitochondrial | *C. cayetanensis* | NA | Confirmed  *C. cayetanensis* |
| RAS | 8/10/21 | 1 mitochondrial | Non–  *C. cayetanensis* | NA | Suspected cross-reaction |

a NA: Sample did not meet inclusion criteria for genotyping.

b Genetic cluster includes clinical specimens from 2018–2021.

**References**

Qvarnstrom, Y., Benedict, T., Marcet, P. L., Wiegand, R. E., Herwaldt, B. L., & da Silva, A. J. (2018). Molecular detection of *Cyclospora cayetanensis* in human stool specimens using UNEX-based DNA extraction and real-time PCR. *Parasitology*, *145*(7), 865–870. https://doi.org/10.1017/S0031182017001925

Stachler, E., Kelty, C., Sivaganesan, M., Li, X., Bibby, K., & Shanks, O. C. (2017). Quantitative crAssphage PCR assays for human fecal pollution measurement. *Environmental Science & Technology*, *51*(16), 9146–9154. https://doi.org/10.1021/acs.est.7b02703

U.S. Environmental Protection Agency. (2019). *Method 1696: Characterization of Human Fecal Pollution in Water by HF183/BacR287 TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay*. Office of Water.