# **Chapter 3 – Results dataset**

All data has been electronically stored in the Department of Plant and Soil Sciences.

# **Confirmation of *Cryptosporidium* Occurrence in Irrigation Water, Soil, and Fresh Produce through Spiking Experiments**

### **3.3.1 Phase 1: Determination of the best DNA extraction method from inactivated oocysts for each sample type**

The Qubit HighSensitivity measurements showed very low detectable nucleic acid concentration for genomic DNA extracted from spiked *C. parvum* oocysts using all methods (Table 3.1). Despite the methods tested (Table 3.1) in this study displaying varying low detectable DNA concentrations, *Cryptosporidium* was detected from some of the spiked samples (Table 3.3). In water samples, the DNeasy Blood and Tissue kit was the best extraction method, extracting DNA from as low as 1250 oocysts (Cq 46.25). For spinach and soil samples, the PowerLyzer DA isolation kit was chosen as the best extraction method since it showed better positive results compared to the other extraction methods tested. Therefore, the DNeasy Blood and Tissue kit method was chosen as the best-performing method for further experiments in water samples, and the PowerLyzer DNA isolation kit for fresh produce and soil samples.

**Table 3.3:** Performances of chosen DNA extraction protocol determined with real-time PCR

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Sample type** | **Extraction protocol** | **Proportion of positive samples based on Cq values** | | | | | | |
| **1,250,000 oocyst/µl** | **125000 oocyst/µl** | **12500 oocyst/µl** | **1250 oocyst/µl** | **125 oocyst/µl** | **12.5 oocyst/µl** | **1.25 oocyst/µl** |
| **PBS** | 1 | 40.23 | 45.91 | 44.04 | 46.25 | - | - | - |
| 2 | 35.81 | - | - | - | - | - | - |
| 3 | - | - | - | - | - | - | - |
| **Spinach** | 1 | - | - | - | - | - | - | - |
| 2 | 37.15 | 35.13 | - | - | - | - | - |
| 3 | - | 46.24 | - | - | - | - | - |
| **Soil** | 2 |  | 35.18 | - | - | - | - | - |
| 3 | - | - | - | - | - | - | - |

1) DNeasy Blood and Tissue Kit, 2) PowerLyzer DA Isolation Kit, 3) Genesig Easy DNA/RNA Extraction Kit, and “-” indicates undetected

**3.3.2 Phase 2: Optimisation of ddPCR for the detection of *Cryptosporidium* based on positive reference DNA**

The ddPCR assay was successfully optimised for detecting and quantifying *C. parvum* using the *Cryptosporidium* mix primers/probes from the PrimerDesign™ Genesig Standard Kit for *Cryptosporidium* (Crypto) Genomes. The assay had clear separation of positive and negative droplets and no evidence of inhibitors or nonspecific binding. Diluting the sample at a 1:10 dilution factor revealed a decrease in droplet concentration (Figure A1, Appendix A). Based on the obtained results (Table 3.4), the limit of detection for the ddPCR assay targeting *C. parvum* is 0.14 copies in a 26 μl reaction. Further dilutions led to "no-call" or undetectable copies.

**Table 3.4:** Limit of detection for the droplet digital PCR assay using reference DNA

|  |  |  |
| --- | --- | --- |
| Dilution | Copies detected in ddPCR (26 μl reaction volume) | Detection rate (X detected/Y (2) replicates) |
| 10–1 | 1000000 | 2/2 |
| 10–2 | 4365.5 | 2/2 |
| 10–3 | 393 | 2/2 |
| 10–4 | 44.3 | 2/2 |
| 10–5 | 4.2 | 2/2 |
| 10–6 | 0.77 | 2/2 |
| 10–7 | 0.14 | 2/2 |
| 10–8 | 0 | 0/2 |

**3.3.3 Phase 3: Determination of a lower detection limit (LOD) of the proposed extraction protocol for *C. parvum* oocysts**

Variable levels of detection were observed depending on the oocyst load and sample type. For 1L distilled water samples, the limit of detection (LOD) assessment using real-time PCR detected up to 1250 oocysts/L, while ddPCR showed higher sensitivity, detecting up to 500 oocysts/L. For environmental water samples, the LOD assessment detected no oocysts on Farm 1 and up to 12500 oocysts/200 mL on Farm 2. In contrast, ddPCR demonstrated greater sensitivity, detecting up to 1250 oocysts/200 mL on both Farms 1 and 2. In spinach, real-time PCR detected up to 1250 oocysts/30g, whereas ddPCR detected up to 125 oocysts/30g. For soil samples, real-time PCR detected up to 500 oocysts/0.25g, while ddPCR showed higher sensitivity, detecting up to 50 oocysts/0.25g. Interestingly, variations in detection limits for different sample types were observed. For instance, ddPCR was able to detect up to 1250 oocysts/30g in lettuce, while real-time PCR showed higher sensitivity, detecting up to 500 oocysts/30g. Overall, ddPCR showed a higher sensitivity for each of the samples compared to real-time PCR. No natural occurrence of *Cryptosporidium* oocyst contamination was detected in any of the spiked samples, as all non-spiked leaves were negative. Additionally, it is worth noting that instances of positivity were observed even when the criterion of three out of five positive replicates was not met. For example, oocysts were detected at five oocysts for 1L water and spinach with real-time PCR and five oocysts for soil and spinach with ddPCR (Table 3.5).

**Table 3.5:** Performances of the chosen DNA extraction protocol were determined with real-time PCR and ddPCR (1L water, lettuce, spinach, and soil done in replicates of five and environmental water done in triplicate)

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Sample type** | **Extraction protocol** | **Molecular detection protocol** | **Proportion of positive samples at each concentration (%)** | | | | | | | **Overall proportion of positive samples** | |  |
| **12500 oocysts** | **1250 oocysts** | **500 oocysts** | **125 oocysts** | **50 oocysts** | **12.5 oocyst** | **5 oocysts** | |  |
| **1L Water** | DNeasy Blood and Tissue kit | Real-time PCR | 80 | 80 | 20 | 0 | 20 | 20 | 40 | | 37.1 |
| ddPCR | 100 | 80 | 60 | 0 | 40 | 40 | 0 | | 45.7 |
| **Environmental spiked water: Farm 1** | Real-time PCR |  |  |  |  | 33.33 | 33.33 |  | | 9.5 |
| ddPCR | 100 | 66.66 | 33.33 | 33.33 |  |  | 33.33 | | 38.1 |
| **Environmental spiked water: Farm 2** | Real-time PCR | 66.66 |  |  | 33.33 |  |  |  | | 14.3 |
| ddPCR | 100 | 100 |  |  |  |  | 33.33 | | 33.3 |
| **Lettuce** | PowerLyzer DA isolation kit | Real-time PCR | 100 | 100 | 60 | 20 | 0 | 0 | 0 | | 40 |
| ddPCR | 100 | 80 | 40 | 40 | 0 | 0 | 40 | | 42.9 |
| **Spinach** | Real-time PCR | 100 | 100 | 20 | 40 | 20 | 20 | 0 | | 42.9 |
| ddPCR | 100 | 80 | 60 | 60 | 20 | 0 | 20 | | 45.7 |
| **Soil** | Real-time PCR | 100 | 100 | 80 | 0 | 0 | 0 | 0 | | 40 |
| ddPCR | 100 | 100 | 80 | 80 | 80 | 20 | 40 | | 60 |