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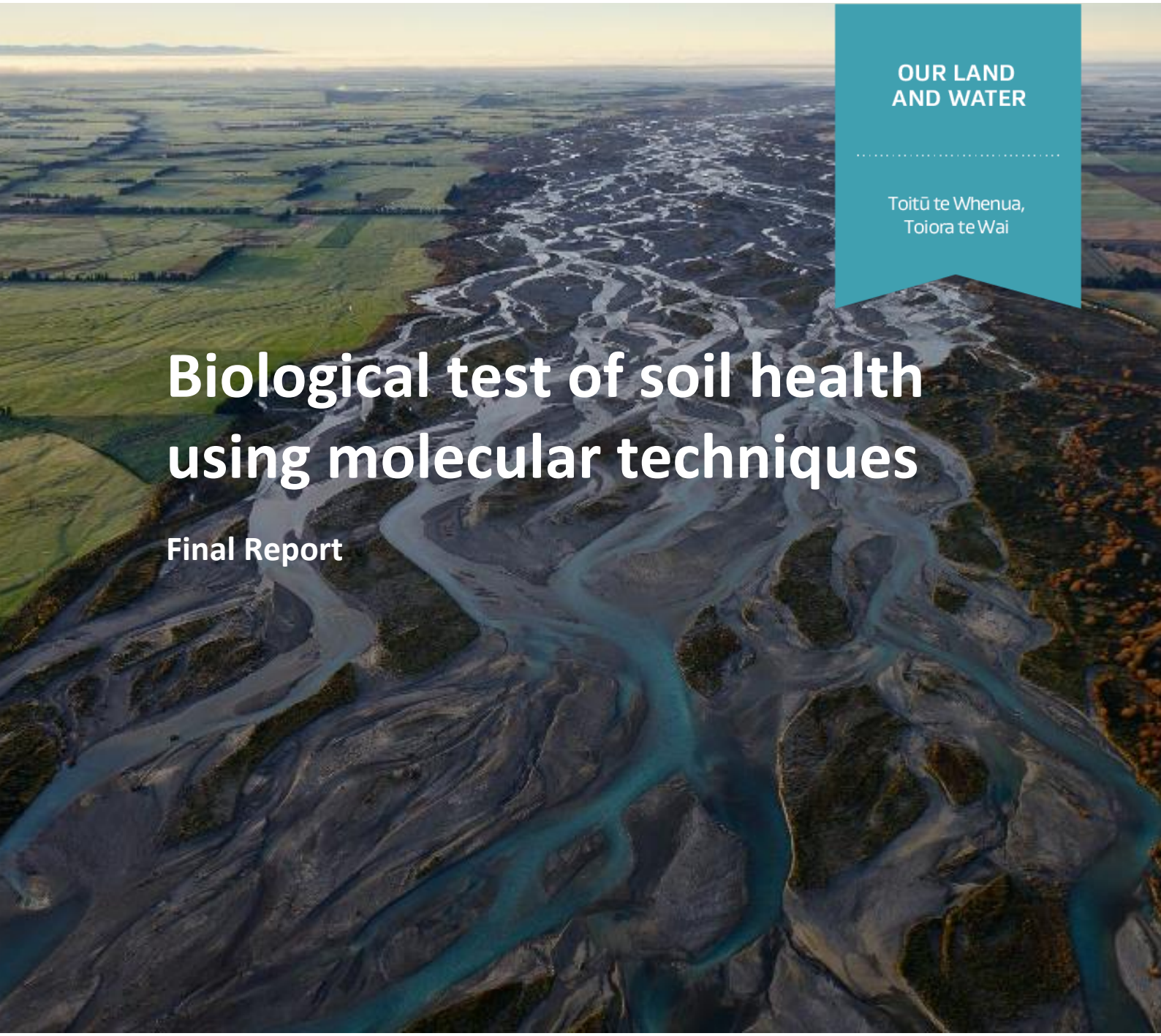

OUR LAND AND WATER

Toitū te Whenua, Toiora te Wai

Biological test of soil health using molecular techniques

Final Report

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1 Executive Summary

Project aim:

This project aimed to develop a molecular test to assess earthworm abundance and diversity, to provide a commercial test of soil biological health.

Team:

RJ Hill Laboratories and AgResearch, also Ngāi tahu and Pastoral Nutrient Management.

Key findings:

During the feasibility stage of the project, we were able to identify suitable primers for the most common earthworms found within each earthworm ecological group in New Zealand (e.g. *Lumbricus rubellus* as epigeic, *Aporrectodea caliginosa* as endogeic and *Aporrectodea longa* as anecic). The primers used could specifically identify target earthworm species using Real-Time PCR (RT-PCR). Soil samples collected from five sites during the development stage of the project showed good correlation between molecular and morphological assessment (R^2 0.907). The test also showed good extraction efficiency and precision, especially from dried and fine grind samples. Further samples analysed during the validation stage of the project reduced the strength of correlation between molecular and morphological assessment of earthworms (R^2 0.193). Investigation into factors causing this are ongoing. However, the test was sensitive to changes in earthworm abundance at individual sites. Further data needs to be collected prior to the test becoming commercially available to ensure confidence in the test developed, and the ability to predict earthworm abundance and diversity.

Recommendations:

To use earthworms as indicators of soil biological health an assessment of both abundance and ecological diversity is required. The collection of further data to help assess whether the test is appropriate for commercial use will be ongoing in aligned research. Understanding factors that can cause variation in results is important for the development of a reliable test.

2 Introduction

Soil is one of our most important natural resources, being important for a range of soil ecosystem functions and services such as sustainable plant production, nutrient cycling, water purification and regulation, carbon sequestration and greenhouse gas regulation, and maintenance of soil biodiversity (Doran and Parkin 1994). Soil health includes soil fertility and organic matter, soil physical condition as well as biological activity. Maintaining and improving the health or quality of the soil benefits the functions that a soil can provide. To improve soil health, we need to be able to measure and assess it and identify which aspects are not at optimum and may require attention.

There is no universally accepted methodology available for assessing soil health (Bünemann et al. 2018). Assessment of soil health should include all aspects of soil health (soil fertility, organic matter properties, soil physical condition as well as biological activity). In New Zealand a set of soil quality indicators are used for State of Environment Reporting (New Zealand's Environmental Reporting Series: Our land 2021 MfE & Stats NZ. 2021). However, biological indicators are often neglected because they require specific knowledge, are difficult to assess, and are not readily available through routine sampling and laboratory testing. Instead, the mineralisable nitrogen test is used as a proxy for biological activity. So there is an increasing recognition of the importance of biology within the soil system and a greater desire to understand biological indicators.

Earthworms are a key component of the soil biology and abundant populations are recognised as a sign of a healthy soil (Booth et al. 2019, Pauli et al. 2016). Current methods used to identify earthworm populations are labour intensive, requiring soil to be physically broken up and earthworms collected. Furthermore, the inclusion of earthworm ecological groups: epigeic or dung earthworm; endogeic or topsoil earthworm; and anecic or deep-burrowing earthworm (for more information on the ecological groups refer to www.earthworms.nz) can provide greater insight into earthworm activity and function (Schon et al. 2022), however this requires specialist knowledge which is challenging for farmers and the public.

This project aims to develop a biological soil health test that measures earthworm abundance and diversity within a soil profile using molecular techniques. This is a novel approach aiming to assess earthworm abundance from a soil sample. Importantly, the test would also identify the different ecological groups of earthworms present in the soil to give a better indication of soil function. The vision is that knowledge from this test can inform the farmer of the biological health of the soil and ultimately be used to improve on-farm management of soil health.

Although molecular extractions are usually conducted on fresh soil, ideally the test would be performed on the soil samples dried and sieved as part of routine testing. For soil fertility advice typically 20 soil cores (25 mm diameter and 75 mm depth) are collected along a paddock transect before being dried, sieved <2 mm, and mixed thoroughly, ensuring the sample is representative of the area. Ultimately, the RT-PCR test developed needs to be able to provide insights into earthworm abundance and diversity. Sufficient data needs to support the test prior to being made available commercially to ensure confidence in the test.

In this report we provide results on the test:

1) Feasibility, with the aim to identify relevant primers for identification of earthworm species and to have tested their specificity.

2) Development, with the aim to establish a Real-Time Polymerase Chain Reaction (RT-PCR) test that appears to perform adequately for the intended purpose and to understand the correlation between the RT-PCR test and initial earthworm assessments (morphological counts and identification)
3) Validation and interpretation, with the aim to perform the RT-PCR test on an expanded range of farms to test correlations across a wider range of sites.

3 Methods

3.1 Feasibility stage

3.1.1 DNA extraction

Three species of common New Zealand pasture earthworms, representing the different ecological groups were collected from across New Zealand (Waikato, Bay of Plenty, Manawatu, Canterbury and Southland) during spring 2021 to autumn 2022. Soil samples (20x20x20 cm) were hand-sorted, and species morphologically identified. Ten earthworms of the three species, *Lumbricus rubellus* (epigeic), *Aporrectodea caliginosa* (endogeic), and *Aporrectodea longa* (anecic) were stored at 4°C in a 400 ml container with soil (Figure. 3.1.1A & B). Individual earthworms were transferred into a 50 mL falcon tube with 35 mL deionised water at 4°C for 48 hours prior DNA extraction (Figure 3.1.1C). Each earthworm was briefly washed in 70% ethanol before dissecting into 2 mm pieces. Earthworm genomic DNA (gDNA) was extracted from 200 mg of earthworm tissue using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A sample of gDNA was also collected from the earthworm species *Allobophora chloritica* to test primer pair specificity.

Figure 3.1.1. Earthworms were collected and sorted manually into three separate species, *Lumbricus rubellus* (epigeic), *Aporrectodea caliginosa* (endogeic), and *Aporrectodea longa* (anecic) (A & B). Earthworms were placed in 50 mL falcon tubes for 48h prior to DNA extraction (B). Soil around earthworms (red circle) are collected and environmental DNA from soil were extracted before PCR and sequencing (C).

Soil collected from, on and around the earthworm body (approximately 400 mg fresh weight) was analysed for environmental DNA (eDNA using NucleoSpin® Soil Genomic DNA from soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The quality and concentration of extracted eDNA were measured by NanoDrop ND2000c spectrophotometer (NanoDrop Technologies, Thermo Scientific, MA, USA) and Quanti-iT™ dsDNA BR assay kit using a Qubit fluorometer (Life Technologies, Auckland, New Zealand).

To test the consistency of Real-Time Polymerase Chain Reaction (RT-PCR) results, a single 20x20x20 cm soil sample was collected from site 8 and 10 (Figure 3.1.2). Earthworms were hand-sorted, counted and identified. Soil samples were sieved through an 8mm coarse sieve thoroughly and 300 mg of fresh soil was extracted twice and analysed by RT-PCR in duplicate for each earthworm species (DNA extraction is usually conducted on fresh soil).

Figure 3.1.2. Map showing sampling locations and soil orders. During the feasibility stage sites 8 and 10 were sampled. During the development stage sites 1-5 were sampled. During the validation stage sites 2, 6-10 were sampled.

3.1.2 Polymerase chain reaction and gel electrophoresis

Polymerase chain reactions were carried out by amplification of 16S ribosomal RNA genes for *A. caliginosa* (116 bp) (Palumbi 1991), mitochondrial cytochrome oxidase subunit I (COI) for *A. longa* (213 bp) (King 2010) and mitochondrial cytochrome oxidase subunit II (COII) for *L. rubellus* (164 bp) (Heethoff 2004) (Supplemental 1). The PCR amplification was carried out by using Platinum SuperFi II DNA Polymerase High-Fidelity PCR Enzyme (ThermoFisher Scientific, Waltham, Massachusetts, United States) according to the manufacturer's instructions. A PCR temperature gradient ranging from 45℃ to 60℃ was performed for each primer pair to determine the optimal annealing temperature. The PCR reaction were carried out as follows: one cycle of 98℃ for 2 mins; 40 cycles of 98℃ for 10s, 45℃–60℃ for 30s, and 72℃ for 10s; one cycle of 72℃ for 5 mins; one cycle of 4℃ for infinity. Ten microliters of the PCR products were separated and visualised using agarose gel electrophoresis (1% agarose gel) under 110V for 30 mins.

The resultant prominent PCR bands with correct size were purified and concentrated using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) followed by Sanger sequencing using ABI Prism 3130xl Genetic Analyser (Applied Biosystems™, Waltham, Massachusetts, USA). Sequence results were analysed by MUSCLE alignment from Geneious Prime® (Biomatters Ltd., Auckland, New Zealand), and were searched against the NCBI database using the BlastN algorithm.

3.2 Development stage

3.2.1 Singleplex vs. Multiplex RT-PCR

Single RT-PCR assays were conducted using three different earthworm primers and probes. The earthworm species, *A. caliginosa*, *A. longa*, and *L. rubellus* were labelled with HEX (555 nm), Cy®5 (668 nm), and FAM (520 nm), respectively. The initial RT-PCR reagent was set up as follows; 10 uL of PerfeCTa® qPCR ToughMix™ (Quantabio, United States), 1 µL of 10 µM forward primer (500nM), 1 µL of 10 µM reverse primer (500 nM), 0.4 µL of 10 µM RT-PCR probes (200nM), 1.5 µL of earthworm gDNA or soil eDNA, and 6.1 µL of RNase free molecular water to make up a total volume of 20 µL. Details of the primer sequences used in this study are outlined in Supplemental 1. The RT-PCR reaction was carried out using QuantStudio™ 5 Dx RealTime PCR (Thermo Fisher, United States) with the thermocycling profile below; one cycle of 95℃ for 1 min followed by 45 cycles of 95℃ for 5s and 50℃ for 30s. A serial dilution of using earthworm gDNA as a template was performed to determine the RT-PCR efficiency.

The multiplex RT-PCR reagent was set up to assess whether the steps in the process could be minimised. Set up was as follows; 10 uL of PerfeCTa® qPCR ToughMix™ (Quantabio, United States), forward primer (final concentration of 125 nM), reverse primer (125 nM), RT-PCR probes (100 nM), 1.5 µL of earthworm gDNA or soil eDNA, and RNase free molecular water to make up a total volume of 20 µL. The multiplex RT-PCR reaction was carried out using QuantStudio™ 5 Dx RealTime PCR with the thermocycling profile same as the singleplex RT-PCR. A serial dilution of using *A. caliginosa*, *A. longa*, and *L. rubellus* earthworm gDNA as a template was performed.

3.2.2 Sample processing

For this test to be suitable for commercial laboratories, the test would ideally be performed on the soil samples already being collected for assessment of soil fertility, and not require a separate sample collection. Typically, 20 soil cores (25 mm diameter, 75 mm depth) are dried, sieved <2 mm, and mixed thoroughly, ensuring the sample is representative of the area. As the proposed DNA test involves testing only 0.4 g of soil, further homogenisation may be necessary to achieve satisfactory precision, and so further fine grinding and mixing was undertaken and compared against the <2mm ground fraction.

Soil samples (20x20x20 cm) from sites across New Zealand (sites 1-5, Figure 3.1.2) were hand-sorted for earthworms and species identified. Three subsamples of soil were used for RT-PCR testing. Fresh soil was sieved through an 8 mm sieve and homogenised prior to analysis (Fresh). A second sample was subsequently dried in the oven at 38°C for 16 hours and sieved through 2 mm cone grinder (Sieve). A third sub-fraction was prepared by fine grinding approximately 10g of the Sieve sample with a mortar and pestle (Grind). Samples were analysed in quadruplicate using singleplex RT-PCR.

Statistical analysis was used to assess the correlation between *A. caliginosa* Cq values and *A. caliginosa* abundance (m^{-2}) using log-linear regression analysis for sites sampled during the development state (sites 1-5). The regression analysis was performed on data from different sample methods. Each regression analysis was fitted for either 'fresh', 'sieve' or 'grind'. All regression analyses were carried out with statistical software R.

Statistical analysis was conducted on the Cq values of each earthworm species to assess how fresh, and sieve and grind samples differed. The Cq values were compared between treatments in an analysis of variance (ANOVA). The ANOVA was applied to data from each species separately from the other species – i.e., three independent ANOVAs were performed, one for each earthworm species. Sieve and grind data collected from all 10 sites (Figure 3.1.2 and detailed in section 3.3. validation and interpretation) were used in this analysis. All analyses were carried out with statistical software SAS version 9.4.

3.2.3 Changes in DNA remaining in soil through time

To investigate how the storage of dried samples may affect the RT-PCR test results, samples were analysed over time. Dried soil samples from site 6 and 7 (Figure 3.1.2) were analysed for DNA using singleplex RT-PCR (for *A. caliginosa* and *L. rubellus*) on both the sieved and grind fractions. Sieve samples were stored at room temperature and analysed weekly for three weeks.

To determine whether the test was responsive to management, and potential changes in earthworm abundance, a test to assess changes in DNA following organism removal was conducted. Earthworms were hand-sorted and identified (from site 8 and 10, Figure 3.1.2), and the remaining soil (with earthworms removed) was stored in differing conditions. Closed bags were left either in the chiller (4°C), or at room temperature (18°C), and an open bag was left outside exposed to the elements in Lincoln. Samples were analysed initially and retested after approximately 3 months to determine amplification changes through time. Samples were analysed using singleplex RT-PCR on fresh soil.

3.2.4 Earthworm spiking experiment

To establish earthworm concentration curves different abundances of earthworm species were added to soil known to lack earthworms (site 9 recently converted from forestry to pasture Figure 3.1.2). A RT-PCR test confirmed an absence of earthworms. Treatments established included low, medium, high and very high abundances of *A. caliginosa,* as well as low and high abundances of *L. rubellus* and *A. longa*. Abundances corresponded to 2, 5, 10 and 20 earthworms in 2 L of soil for the low, medium, high and very high treatments, respectively. The soil was tested after approximately 2 months. At this point in time, it was noted that the soil was <20% moisture with low earthworm activity and additional water (50 mL) was added to the containers before being analysed again at nearly 6 months. Samples were analysed using singleplex RT-PCR on fresh soil.

3.3 Validation and interpretation stage

3.3.1 Correlating RT-PCR test to earthworm abundance

Soil was collected from six farms (sites 2, 6-10, Figure 3.1.2). On each farm a transect was sampled following procedures for standard soil fertility testing, bulking 20 soil cores (25 mm diameter, 75 mm depth). Along this transect four spade squares (20x20x20 cm) were collected for the traditional assessment of earthworm populations using hand-sorting and morphological identification. Alongside each spade square a minimum of four soil cores (25 mm diameter, 75 mm soil depth) were also collected (Figure 3.3.1). Soil from the 'transect', 'spade-square' and 'cores' adjacent to the spade-square were analysed using singleplex RT-PCR and compared to the results of the traditional earthworm assessment. RT-PCR was carried out on dried soil (38°C for 16 hours) which was either 2 mm sieved or finely ground.

Figure 3.3.1. Example showing cores (25 mm diameter, 75 mm soil depth) collected from alongside a spade square (20x20x20 cm) to assess RT-PCR results in comparison to traditional earthworm assessment.

Statistical analysis was used to assess the correlation between *A. caliginosa* Cq values and *A. caliginosa* and total earthworm abundance (m^{-2}) using log-linear regression analysis for sites 2, 6-10. The regression analysis was performed on grind data from different sample. All regression analyses were carried out with statistical software R.

4 Results and Discussion

4.1 Feasibility phase

During the feasibility phase of the project the aim was to identify relevant primers for identification of earthworm species and to test their specificity. Primers established by Palumbi *et al.,* (1991) for detecting *A. caliginosa* (endogeic), Heethoff et al., (2004) for detecting *L. rubellus* (epigeic), and King *et al.,* (2010) for detecting *A. longa* (anecic) were used to determine species specificity of the PCR reactions. Earthworm gDNA was extracted and a temperature gradient PCR was performed for each primer pair to determine the optimal primer annealing temperature for the PCR reaction. Results show annealing temperature at 50°C is optimal for all primer pairs used for detecting three common earthworm species, *A. caliginosa* and *L. rubellus* (Figure 4.1a), and *A. longa* (result not shown here). This indicates when designing probes for RT-PCR, the annealing temperature for probes should also be approximately at 50°C. The PCR products from using earthworm gDNA as a template (annealing temperature at 50°C) were then subjected to the purification process followed by Sanger DNA sequencing.

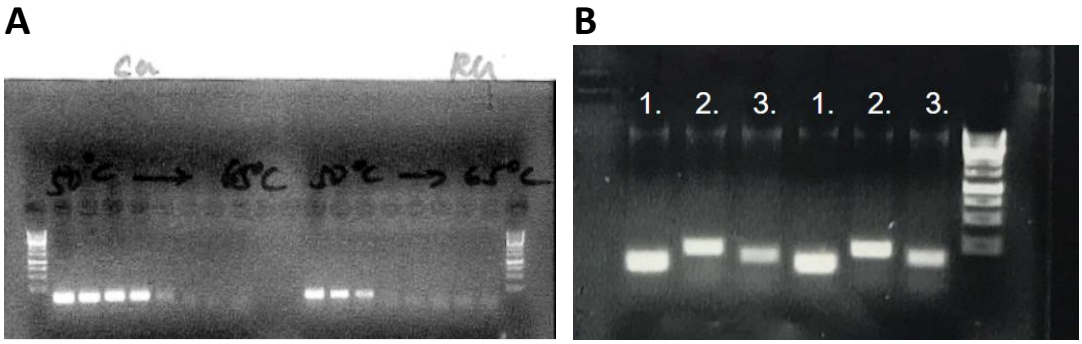


Figure 4.1. A) PCR temperature gradient (50°C–65°C) of *Aporrectodea caliginosa* (left (Ca), 116 bp) and *Lumbricus rubellus* (right (Ru), 164 bp) using earthworm genomic DNA as template. B) PCR of lane (1) *Aporrectodea caliginosa*, (2) *Aporrectodea longa* and (3) *Lumbricus rubellus* using extracted soil environmental DNA as template. Annealing temperature at 50°C and each PCR reaction was repeated twice.

Soil samples around the earthworms was extracted for eDNA. The PCR reaction for each primer pair was carried out at annealing temperature at 50°C, and the result shows that the ability to amplify earthworm DNA from soil samples (Figure 4.1B). The resultant PCR products were observed on a 1% agarose gel electrophoresis and demonstrated each primer pairs were amplifying at the corresponding size; *A. caliginosa* at 116 bp, *A. longa* at 213 bp and *L. rubellus* at 164 bp. The PCR products were then subjected to the purification process followed by Sanger DNA sequencing.

The resultant PCR products were subjected to purification and concentration prior to DNA Sanger sequencing. The sequence results were BLAST searched against the NCBI sequence database and showed high DNA conservation for each species (Supplemental 2). *A. caliginosa* gDNA sample was also subjected to PCR using primer pairs specific for *A. longa* and *L. rubellus,* and *vice versa*. Results show there was no amplification (data not shown), which suggests each primer pair is specific to the designated species. Furthermore, gDNA extracted from *Aporrectodea chloritica* were subjected to PCR using primer pairs for *A. caliginosa, A. longa* and *L. rubellus*. Results showed that no amplification was observed, indicating primer pair specificity.

The DNA sequencing results also revealed a high degree of DNA conservation between earthworm gDNA and soil eDNA. Sequencing results demonstrate that PCR product amplifying from soil eDNA is indeed earthworm DNA and is specific to species level. DNA sequence analysis showed that the resultant PCR product from lane 1 shared 96.3% pairwise sequence identity to *A. caliginosa* voucher 2010-NCCT-01 (KT818833). PCR product from lane 2 shared 98.3% sequence identity to *A. longa* voucher L63 (MG820217), and the band from lane 3 is 100% identical to *L. rubellus* isolate HAP20 (KT308192) (Figure 4.1B, Supplemental 2).

However, it is also important to note that the primer pair (16s-Ac-F1 and WORM-16s-R1) used in this study to amplify *A. caliginosa*, also amplified *Aporrectodea rosea* (data not shown). The sequencing data was able to locate the forward primer, but the reverse primer was found to be less specific. Since *A. rosea* is an endogeic earthworm like *A. caliginosa* and has the same ecological role within the soil, it is acceptable to also include this species within the amplification. *A. caliginosa* was chosen as the most abundant endogeic earthworm in New Zealand but is not the only endogeic earthworm and this will need to be clear in test reporting. It is also noted that the primer pair used for amplifying *A. longa* (COI-Al-F2 and COI-Al-R2) was only able to amplify five out of six samples of *A. longa* earthworm gDNA using PCR. This indicates that not all *A. longa* earthworm variants can be detected using this primer pairs. *In silico* sequence analysis shows that gene encodes for *A. longa* Cytochrome Oxidase subunit I is highly conserved, apart from one *A. longa* variant that may not be able to be amplified using the primer pair selected. The primer pair for amplifying *L. rubellus* (COII-Lr-F3 and COII-Lr-R2) was only able to amplify two out of six *L. rubellus* earthworm gDNA successfully. Three *L. rubellus* gDNA samples had weak amplification and one did not amplify. This indicates that not all *L. rubellus* earthworm variants can be detected using this primer pair. *In silico* sequence analysis shows that gene encodes for Cytochrome Oxidase subunit II is highly diverse, which accounts for lack of amplifications observed. Successful PCR products were confirmed by Sangar sequencing (Supplemental 2).

Duplicate extractions conducted on soil samples show RT-PCR Cq differences of the three species tended to be within 0.5 Cq of each other, with the maximum difference of 2.23 (Table 4.1). This indicates that each soil extraction is consistent and the reproducibility for detecting each earthworm species using RT-PCR is high. Lower Cq values indicate higher numbers of target DNA present, and higher Cq values indicate lower numbers of target DNA present. As the Cq values are slightly lower at site 10, it would suggest higher abundances of earthworms. This does not match the results of the traditional earthworm assessment, although sampling occurred during summer and the results are likely to be atypical. The PCR test also detected all three earthworm species, although all three species were not recovered from within the same spade square assessed using traditional assessment. However, both species have been found at these locations previously and suggest the test to be sensitive for the detection of earthworm species.

Table 4.1. Results showing RT-PCR Cq value from spade squares alongside abundance of earthworms (m^{-2}) using traditional assessment (e.g. hand-sorting spade square samples).

	A. caliginosa		***L. rubellus***		***A. longa***	
	RT-PCR (Cq)	Abundance	RT-PCR (Cq)	Abundance	RT-PCR (Cq)	Abundance
Site 8 Rep1	33.21	425	30.15	0	29.68	75
Site 8 Rep2	32.92		30.40		30.78	
Site 10 Rep 1	32.30	275	26.78	50	31.19	0
Site 10 Rep 2	30.07		26.61		31.99	

Molecular techniques to identify both earthworm abundance and species in soil have not been used in New Zealand. Kim et al. (2017) examined molecular differences between some native and exotic earthworm species in New Zealand. Overseas, the use of molecular techniques to identify earthworm community composition showed positive results (Bienert et al. 2012). The feasibility stage of this project shows the ability to identify and differentiate the three earthworm species representative of the earthworm ecological groups. The primers were specific to the species ecological groups. There is potential for the test to be a useful method to assess earthworm populations and further development of the test is required.

4.2 Development stage

During the development stage of the project the aim was to establish a RT-PCR test to perform adequately for potential use within a routine laboratory setting. The test was optimised and correlation between the RT-PCR test and observed earthworm abundance explored.

4.2.1 Singleplex vs. Multiplex RT-PCR

To optimise the efficiency of soil testing and laboratory processing time, multiplexing probes were designed based on the DNA sequences obtained from earthworm gDNA and soil eDNA (Supplementary 2). The RT-PCR probe was designed to detect a signal at the HEX channel (555 nm) for detecting *A. caliginosa*, Cy®5 channel (668 nm) for *A. longa*, and FAM channel (520 nm) for *L. rubellus*. The ideal RT-PCR efficiency is that the amplicon concentration doubles every PCR cycle (100%). The Cq value of each ten-fold dilution will have a 3.3 cycles difference if the efficiency is at 100% and the efficiency should be between 90–110%. The RT-PCR efficiency of the probes designed in this study is determined by a ten-fold serial dilution using gDNA from each earthworm species as template (Figure 4.2.1).

The results for the gDNA show RT-PCR efficiency for amplifying *A. caliginosa* is 106.43% (slope = -3.177, R^2 = 0.999), *A. longa* is 98.29% (slope = -3.363, R^2 = 0.999) and *L. rubellus* is 100.08% (slope = -3.320, R^2 = 0.998). Multiplex was successfully implemented with combined earthworm gDNA, however, multiplexing RT-PCR did not work with the soil eDNA. A reason for failed multiplexing on soil eDNA could be due to interferences of *A. longa* and *L. rubellus* primers and probe with *A. caliginosa* primers and probe, especially when *A. longa* and *L. rubellus* DNA are found in low abundance/concentrations in actual farm soil samples. Test development continued using singleplex RT-PCR.

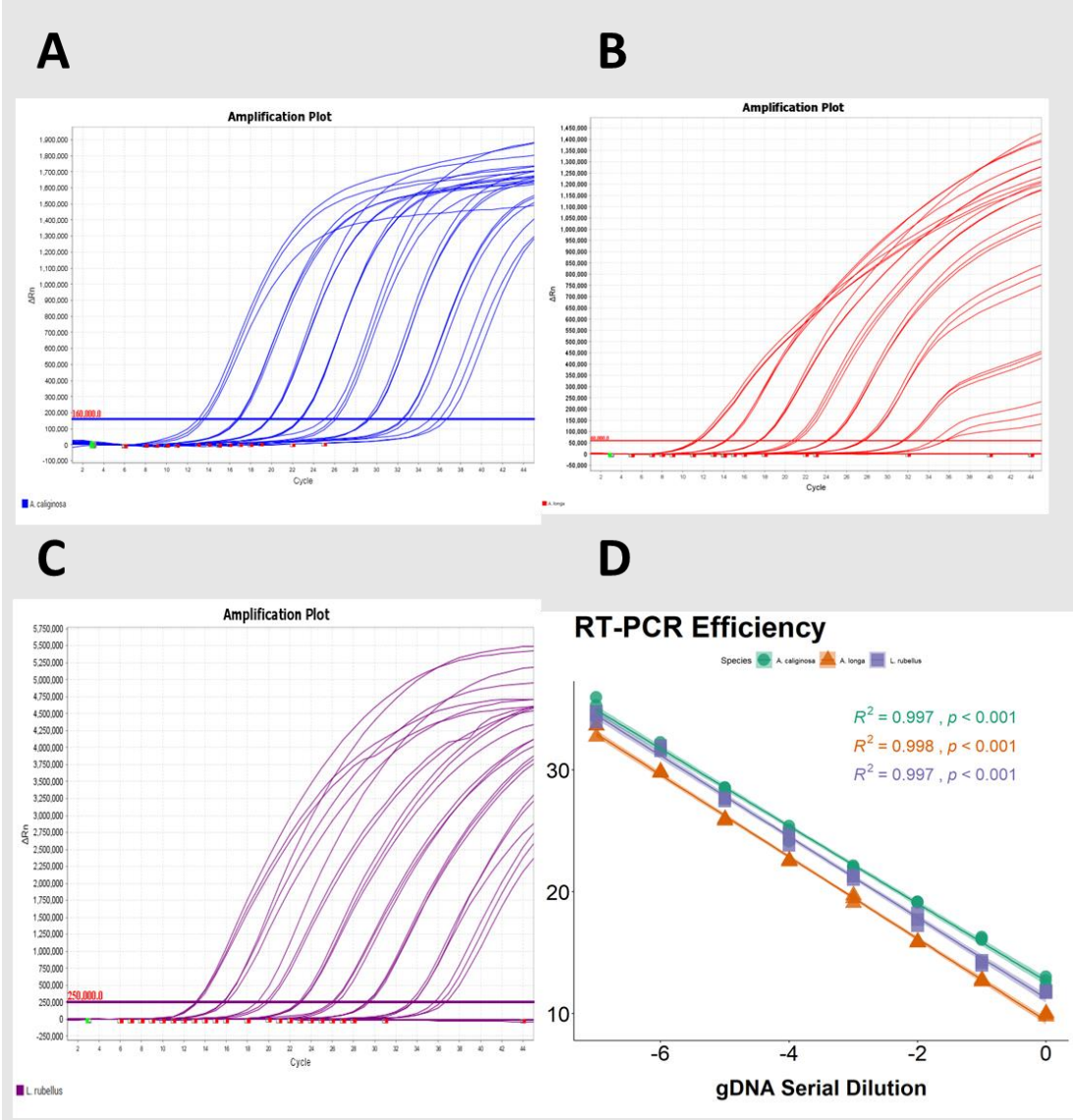


Figure 4.2.1. RT-PCR amplification plot of *A. caliginosa* gDNA serial dilution (0 to 10^{-7}) (A), *A. longa* (B) and *L. rubellus* (C). Linear regression of the gDNA serial dilution of *caliginosa* (green circle) and *A. longa* (orange triangle) and *L. rubellus* (purple square) for calculating the RT-PCR efficiency (B).

4.2.2 Sample processing

Results from the sieve and grind fractions showed improved detection efficiency in comparison to the fresh sample (Table 4.2.2). The DNA concentration extracted was lower in the fresh samples and increased in the dried sieve and grind samples, this may in part reflect the lower moisture in the dried soil, and an effective concentrating of the DNA. Statistical analysis showed the mean Cq value was significantly larger in fresh samples in comparison to sieve and grind samples for all three earthworm species (*A. caliginosa*, *A. longa* and *L. rubellus*). Between sieve and grind samples there was no evidence of a statistically significant difference in average Cq value for all three earthworm species, although the grind samples had a lower coefficient of variation, with improved test precision from grind fractions.

Table 4.2.2. RT-PCR Cq values from spade squares for *A. caliginosa*, *A. longa* and *L. rubellus* as an average across sites 1-5. The average coefficient of variation (CV%) is given. Fresh, sieve and grind samples were analysed in quadruplicate.

Method (sites)		*A. caliginosa* (SEM)	*A. longa* (SEM)	*L .rubellus* (SEM)
Fresh (1-5)	Average	32.03 (0.72)[a]	33.91 (1.16)[a]	40.37 (0.81)[a]
	Average CV%	4.57	8.28	3.81
Sieve (1-10)	Average	28.49 (0.26)[b]	30.60 (0.56)[b]	33.34 (0.33)[b]
	Average CV%	3.53	7.90	4.26
Grind (1-10)	Average	28.40 (0.32)[b]	29.73 (0.46)[b]	32.87 (0.34)[b]
	Average CV%	1.61	2.07	2.63

[a, b, c] indicates significant difference in methods used.

Samples collected during the development stage from a range of sites showed good correlation between *A. caliginosa* Cq values and observed abundances of *A. caliginosa* (Figure 4.2.2). The relationship between Cq value and the species *L. rubellus* and *A. longa* was not significant. The species *A. caliginosa* was observed across five sites through both RT-PCR and traditional methods (hand-sorting from spade square). Log-linear regression across these five locations shows RT-PCR Cq value from fresh samples were not significantly correlated with observed earthworm abundance ($R^2 = 0.090$, $p = 0.198$), whereas results from sieve and grind samples are significant ($p < 0.001$) and with R^2 value of 0.527 and 0.906 respectively (Figure 4.2.2). *L. rubellus* was detected at two sites using PCR but was detected at six sites through traditional assessment. *A. longa* was detected through PCR at three of four sites where they were detected using traditional assessment and was detected at a further two sites where it was not observed within the spade square. This is consistent with previous gDNA results that not all *A. longa* and *L. rubellus* variants were able to be detected using RT-PCR with selected primer pairs.

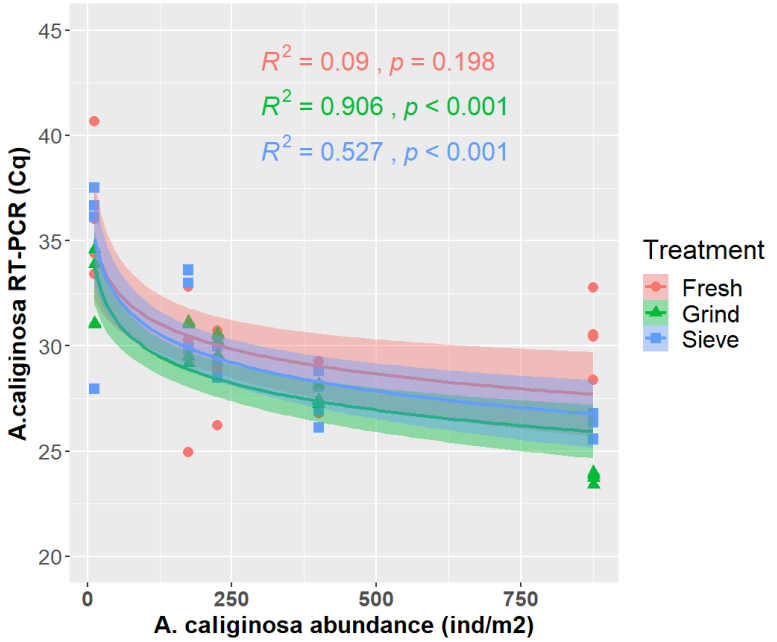


Figure 4.2.2. Correlation between *A. caliginosa* Cq values and observed abundance from spade squares collected at sites 1-5. Fresh (red), sieve (blue) and grind (green) samples. Shaded area indicates 95% confidence interval.

These results suggest that dried and fine grind soil samples are the best fraction for eDNA extraction, with greater precision of Cq values and a high correlation to observed earthworm abundance.

4.2.3 Changes in DNA remaining in soil through time

To determine the stability of earthworm DNA after the removal of organisms from soil, dried samples (38°C for 16 hours) were stored at room temperature and tested every week for three weeks (Figure 4.2.3). The difference in *A. caliginosa* Cq across the weeks sampled (grind) is less than 0.50 Cq at site 6 and 0.46 Cq at site 7. The difference in *L. rubellus* Cq across the weeks sampled (grind) is less than 1.05 Cq at site 6 and 1.50 at site 7. This indicates the test method is consistent three weeks after different extraction, although variability increases over time as seen on *A. caliginosa (*Figure 4.2.3B*)*. To ensure consistent results, samples should be analysed within a week of drying.

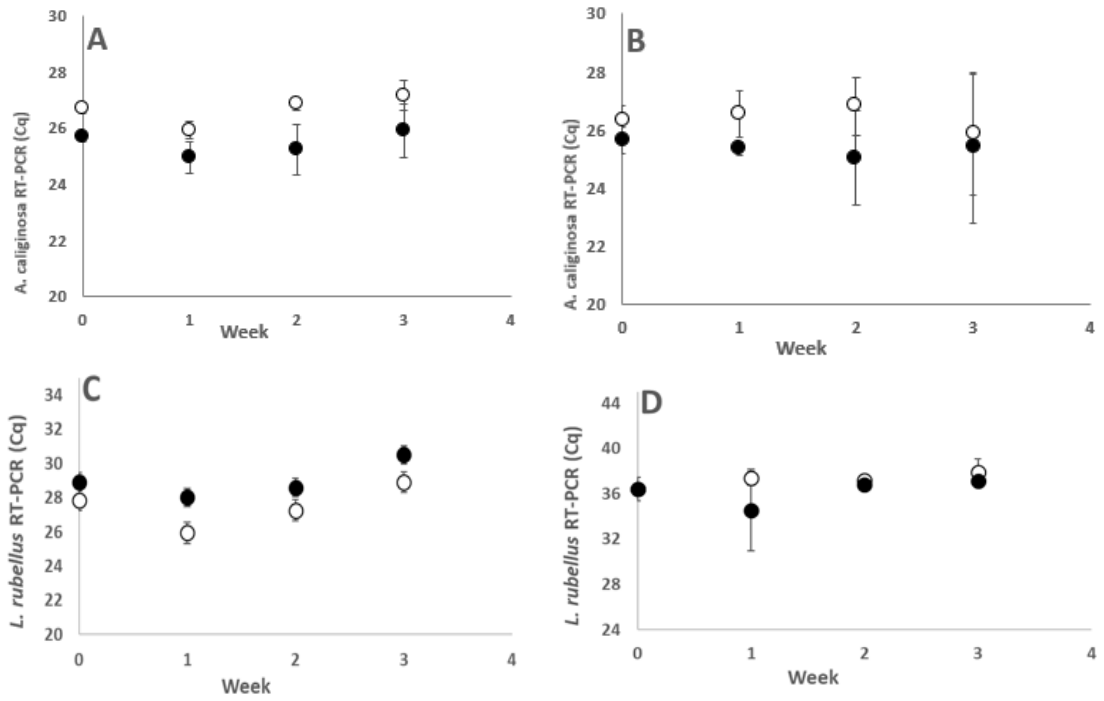


Figure 4.2.3. Change in Cq over time of *A. caliginosa* from site 6 (A) and site 7 (B), and *L. rubellus* from site 6 (C) and site 7 (D). Results for dried sieve (●) and grind (○) samples are given, error bars are standard deviation.

To test whether earthworm DNA remains detectable after a period, earthworms were removed from soil and left in different conditions for a 3-month period. At the time of soil sampling results showed good Cq values (Site 10, Table 4.1). Results from subsequent RT-PCR showed all earthworm species were not detected (data not shown). This indicates earthworm DNA appears to degrade from within fresh soil relatively quickly, suggesting the test will be suitable to detect real time changes in management.

4.2.4 Earthworm spiking experiment

Sampling of soil with different earthworm abundances resulted in little detection of earthworms after two months. At the second sampling date (after approximately 6 months) the detection of earthworms through RT-PCR was high, and there was no difference in concentration with increasing earthworm abundance. It is likely that activity was low initially, and through the addition of water earthworm activity was increased. Earthworms across all abundance treatments increased activity and throughout the whole soil profile, making it difficult to detect any differences in abundance.

4.3 Validation and interpretation stage

During the validation and interpretation stage the aim was to perform the RT-PCR test on an expanded range of farms and test correlations with the visual counting of earthworms from spade squares across a wider range of sites. The correlation between *A. caliginosa* Cq value and *A. caliginosa* abundance from soil collected from spade squares was good during the development stage. However, when more data was collected during the validation stage of the project the correlation between *A. caliginosa* abundance and the Cq value was no longer as strong (Figure 4.3.1). The correlation between *A. caliginosa* abundance and Cq value from spade square samples was significant (p = 0.017) but had a low R^2 (0.193) (Figure 4.3.1A). The correlation between *A. caliginosa* abundance (from spade square) and Cq value from the adjoining core samples was not significant (R^2 = 0.018, p = 0.541, Figure 4.3.1B). There may be several factors involved with sample collection and processing that may have contributed to the increased variability.

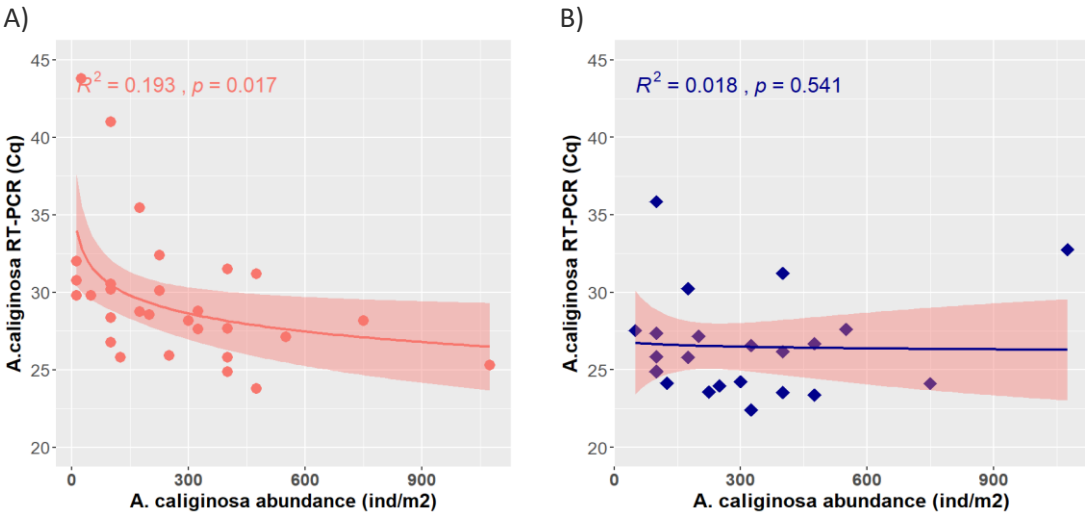
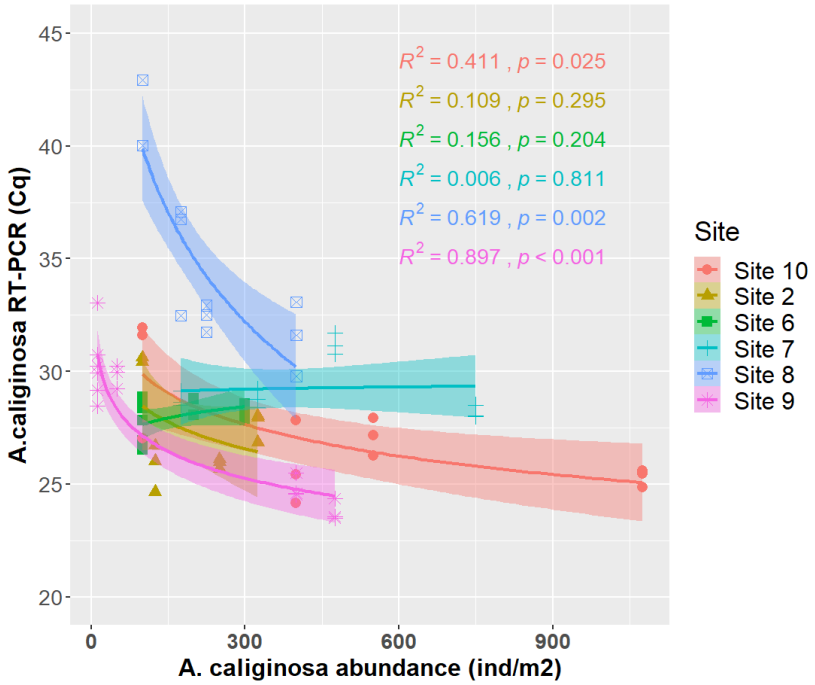


Figure 4.3.1. Relationship between *A. caliginosa* Cq value and *A. caliginosa* abundance using traditional assessment. Results show grind samples collected from A) spade square (from all sites or B) core samples (only for sites 2, 6-10). Shaded area indicates 95% confidence interval. Each data represents an average Cq value of biological samples in triplicates.

At individual sites there was a tendency for the *A. caliginosa* Cq value to decline as *A. caliginosa* abundance increased. At some individual sites this correlation was significant with either the spade square or the core samples (i.e. site 9, R^2 = 0.897, p < 0.001 and R^2 = 0.679, p = 0.006, respectively, Figure 4.3.2). This suggests that the test remains sensitive to differences in earthworm abundances. Earthworm populations vary through space, and this is reflected in both the Cq values as well as the traditional earthworm assessment. Although the impact of spatial variability could be reduced by increasing the number of samples collected, a farmer field-sampling method with a small number of replicates was found to give a reasonable estimate of the earthworm population size (Fraser et al. 1999). Earthworm populations also vary through time, being seasonally active, with greater activity during the wetter months.

A)



B)

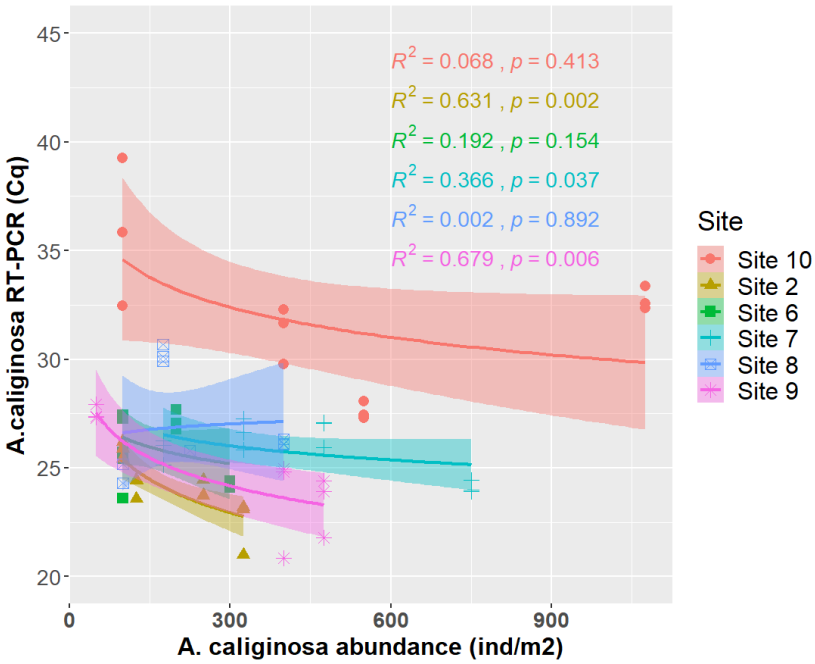


Figure 4.3.2. Relationship between *A. caliginosa* Cq value and *A. caliginosa* abundance using traditional assessment. Results show grind samples collected from A) spade square or B) core samples. Each site shown as separate colours. Shaded area indicates 95% confidence interval.

The Cq value tended to be lower from the core samples in comparison to the spade square samples. The lower Cq value represents a higher DNA concentration, with more earthworm eDNA present. This may reflect a dilution of soil from the spade square which is collected down to 20 cm depths as well as the soil collected from within the hand-sorted spade square having

less root material. Hence, the use of core samples may be appropriate for the collection of soil for analysis, and the transect sample may provide a good overview of earthworm communities. It would be convenient as core sampling is currently standard practice for collecting soils for fertility assessment. However, the correlation between *A. caliginosa* abundance and core samples was not significant (Figure 4.3B), and further investigations are required to understand the variable nature of these results. The transect samples had the lowest *A. caliginosa* Cq, although from the small number of samples there was no correlation with observed samples (Table 4.3). It is recommended that further samples are collected, with the need to analyse 'transect' data and compare to average earthworm communities.

Table 4.3. Results showing RT-PCR average Cq value of fine grind transect sample alongside average abundance (m^{-2}) of earthworms through traditional assessment.

Location (#)	***A. caliginosa***		***L. rubellus***		***A. longa***	
	Cq value (CV%)	Abundance (CV%)	Cq value (CV%)	Abundance (CV%)	Cq value (CV%)	Abundance (CV%)
Waikato (6)	26.65 (0.49)	175 (0.47)	27.85 (2.51)	44 (1.10)	n.d.	75 (0.41)
Bay of Plenty (2)	25.79 (0.67)	200 (0.46)	32.83 (0.86)	44 (0.85)	n.d.	n.d.
Manawatu (7)	26.30 (1.64)	431 (0.49)	36.40 (1.21)	19 (0.58)	n.d.	25 (1.22)
Canterbury (8)	27.03 (0.80)	225 (0.49)	27.62 (3.53)	81 (0.70)	28.85 (3.71)	19 (1.11)
Canterbury (9)	23.87 (1.98)	231 (0.90)	32.367 (3.89)	19 (0.58)	n.d.	n.d.
Otago (10)	29.19 (4.06)	531 (0.66)	34.70 (1.77)	19 (1.73)	23.59 (3.90)	119 (0.69)

n.d. not detected

There may be several factors involved with sample collection and processing that may have contributed to variability between *A. caliginosa* Cq and observed abundance. Soil type may influence the extraction efficiency. For example, allophanic soils have been reported to have lower extraction efficiencies of DNA from the soil (Huang et al. 2016). Results from sites sampled show the amount of DNA extracted and *A. caliginosa* Cq values to be similar between allophanic and sedimentary soils (Figure 4.3.3A). The relationship between *A. caliginosa* Cq and observed abundance was also similar for both soils (data not shown). Time between sample collection to sample processing for DNA extracted (Figure 4.3.3B), as well as timing of sampling following rainfall events (Figure 4.3.3C) show no obvious differences in either the amount of DNA extracted or *A. caliginosa* Cq values, however these factors continue to be investigated. By collecting further data and being able to understand some of the factors causing these variabilities we hope to improve the relationship between *A. caliginosa* Cq and observed abundance. Since *A. caliginosa* is the most common species present in agricultural soils, and their abundance drives total earthworm abundance (Figure 4.3.3D).

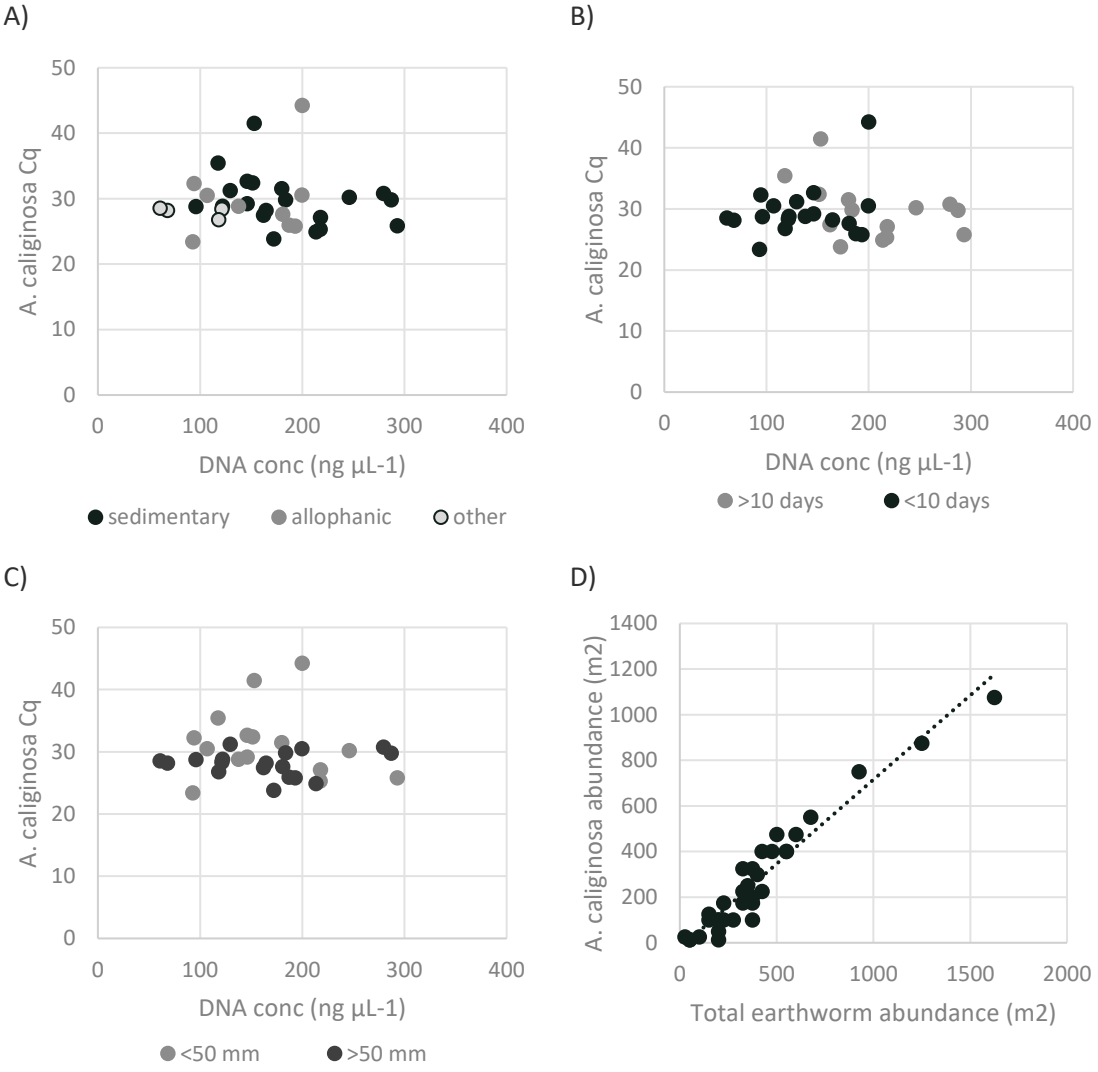


Figure 4.3.3. Relationship between *A. caliginosa* Cq value and DNA concentration for A) sedimentary and allophanic soils, B) days between sample collection to sample processing and C) estimated rainfall 2 weeks prior to sampling. Results show grind samples collected from spade square. D) Relationship between *A. caliginosa* abundance and total earthworm abundance from spade squares.

5 Recommendations and Conclusions

This study has shown that earthworm eDNA in the soil can be measured successfully and, in many cases, correlate well with the earthworm counts by traditional visual assessment. The development of a RT-PCR test for earthworms as an indicator of soil biological health is based on current knowledge of earthworms, and with the aim to provide an indication of earthworm abundance (through *A. caliginosa* Cq values) as well as earthworm ecological diversity (through the presence of *L. rubellus* and *A. longa*). The drying of soil as used for routine laboratory analysis of soil fertility has been shown to be effective at improving Cq values reported. Fine grinding also improves the analytical precision as was expected when such small quantities are used in the test.

A key factor in commercialising the test is to be able to convert the Cq value to an estimated number of earthworms, as this would provide comparability with the current earthworm assessment procedure. Initially, this appeared to be a straightforward exercise with the correlation between *A. caliginosa* Cq value and observed abundance high (R^2 0.906). Subsequent additional data shows good correlation at individual sites. However, across the diversity of sites, the correlation between *A. caliginosa* Cq value and observed abundance from spade square samples remains significant, but the variability increased (R^2 0.193). A smaller number of samples collected from core and transect samples did not show correlation with observed abundances of *A. caliginosa,* although show improved Cq values. There appears to be unidentified factors either affecting DNA residues in the soil or the presence of earthworms observed in the visual counts. Before the test can be offered commercially, it is important that possible causes for variability are further investigated and understood (e.g. spatial variability, fresh sample storage, soil type, rainfall and seasonal impacts). This will ensure guidance for sample collection and that interpretation of results are reliable.

The test was developed to detect the most common species represented within each ecological group (*A. caliginosa*, *L. rubellus* and *A. longa*). There may be some species that are inadvertently detected but are not necessary to distinguish as they are within the same ecological group (e.g. *A. rosea* and *A. caliginosa*). The detection of the epigeic *L. rubellus* and anecic *A. longa* was possible but did not always correlate to observed populations. These species are typically less abundant, and timing of sampling may have influenced results collected in this project through traditional assessment. Further investigation of whether this is an artefact of sample timing and their low abundance or whether methods can be modified to improve identification of potential subspecies is required.

The collection of further data to help assess the commercial viability of the test will be ongoing in aligned research projects (e.g. SFFF: Advancing soil health on-farm). These samples will be collected at a time typically suitable for earthworm sampling and processed to remove sampling bias. The RT-PCR test will be used to determine correlation with earthworm abundance, and earthworm species present. The additional data collected is vital to ensure confidence in the test developed. Once the data has been collected and analysed results will be incorporated into a publication for submission to a scientific publication and enable a decision on proceeding with the biological test of soil health as a commercial test and the development of an interpretation guide (to be made available to OLW).

The development of a biological test of soil health is aimed to benefit farmers and growers. Soils with 'healthy' earthworm populations, are those with abundances over 400 m^{-2} with all three ecological groups present. Increased earthworm abundance and ecological diversity has been shown to have greater benefits to soil functions as well as pasture production. The test will provide greater knowledge on the condition of the soil, providing opportunity to alter management on farm to improve soil condition for improved farm performance and environmental outcomes. Since earthworm eDNA in fresh soil appears to degrade over a short period, the test is useful in terms of considering the impact of management. Such knowledge may improve decision making on farm. Managing soils for improved soil health will have the benefit of requiring fewer inputs to maintain plant growth and is expected to help improve decision making.

6 Acknowledgements

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8 Appendix

Supplemental 1. Details of primer and probe sequences (5'–3') used or developed in this study.

Species	Gene	Size	Primer	Primer Name	Sequence	Reference
Aporrectodea caliginosa	16S ribosomal RNA	116 bp	Forward	16S-Ac-F1	CTAAATTCTGACCCTTATTC	Palumbi *et al.*, (1991)
			Reverse	WORM-16S-R1	CCTAAGCCAACATCGAGGTG	Palumbi *et al.*, (1991)
			Probe	Ca_22P	TGCCAACCCCCACTATTGAT	This study
Aporrectodea longa	Cytochrome Oxidase subunit I	213 bp	Forward	COI-Al-F2	TGGCTTCTACCTCTAATACT	King *et al.*, (2010)
			Reverse	COI-Al-R2	ATGAAGGGAGAAGATGGCCA	King *et al.*, (2010)
			Probe	Lo_26P	GGGCCCCTGACATAGCATT	This study
Lumbricus rubellus	Cytochrome Oxidase subunit II	164 bp	Forward	COII-Lr-F3	AGACGGTAATCTCCTGGAAGT	Heethoff *et al.*, (2004)
			Reverse	COII-Lr-R2	CTTCGTATTCTCTATATCACA	Heethoff *et al.*, (2004)
			Probe	Ru_28P	CAGARGTTGGAAGTATGTATGA	This study

Supplemental 2. DNA sequence alignments of earthworm genomic DNA and soil environmental DNA reveals high-degree conservation to *Aporrectodea caliginosa* (KT818833), *Aporrectodea longa* (MG820217) and *Lumbricus rubellus* (KT308192) sequences from NCBI database. Consensus sequences are highlighted in grey. Sequences used to design RT-PCR probes for each earthworm species are in bold and highlighted in yellow.

Aporrectodea caliginosa

Consensus	TCYCCTAAGCCAACATCGAGGTGCCAACCCCCACTATTGATAAGGACTCTTTAGTGAGATTAGCCTGTATCCCTAAGGTA	80
A. caliginosa (KT818833)	——CCTAAGCCAACATCGAGGTGCCAACCCCCACTATTGATAAGGACTCTTTAGTGAGATTAGCCTGTTATCCCTAAGGTA	77
A. caliginosa worm gDNA	——TCCTAAGCCAACATCGAGGTGCCAACCCCCACTATTGATAAGGACTCTTTAGTGAGATTAGCCTGTATCCCTAAGGTA	78
A. caliginosa soil eDNA	TCCTAAGCCTAACGATCGAGGTGCCAACCCCCACTATTGATAAGGACTCTTTAGTGAGATTAGCCTGTATCCCTAAGGTA	80

Consensus	GGYSTGTTA	89
A. caliginosa (KT818833)	G————	79
A. caliginosa worm gDNA	GCTGT———	83
A. caliginosa soil eDNA	GGCCTGTTA	89

Aporrectodea longa

Consensus	TTTTGGCTTCTACCTCTAATACTAGGGGCCCCTGACATAGCATTTCCCCGACTAAATAACATAAGATTTTGGCTATTACC		80
A. longa (MG820217)	———————————————————— CATAGCATTTCCCCGACTAAATAACATAAGATTTTGGCTATTACC	45	
A. longa worm gDNA	TTTTGGCTTCTACCTCTAATACTAGGGGCCCCTGACATAGCATTTCCCCGACTAAATAACATAAGATTTTGGCTATTACC		80
A. longa soil eDNA	—TTGGCTTCTACCTCTAATACTAGGGGCCCCTGACATAGCATTTCCCCGACTAAATAACATAAGATTTTGGCTATTACC		78

Consensus	CCCATCGCTGATTCTTCTAGTTTCCTCAGCCGCTGTAGAAAAGGGCGCAGGGACCGGCTGAACAGTATATCCCCCCTTAG	160
A. longa (MG820217)	CCCATCGCTGATTCTTCTAGTTTCCTCAGCCGCTGTAGAAAAGGGCGCAGGGACCGGCTGAACAGTATATCCCCCCTTAG	125
A. longa worm gDNA	CCCATCGCTGATTCTTCTAGTTTCCTCAGCCGCTGTAGAAAAGGGCGCAGGGACCGGCTGAACAGTATATCCCCCCTTAG	160
A. longa soil eDNA	CCCATCGCTGATTCTTCTAGTTTCCTCAGCCGCTGTAGAAAAGGGCGCAGGGACCGGCTGAACAGTATATCCCCCCTAG	157

Consensus	CTAGAAACTTVVCBCVCCGBVCCCCCY	187
A. longa (MG820217)	CTAGAAACTTAGCTCACGCGGGCCCCT	152
A. longa worm gDNA	CTAGAAACTAGCTCACGCGCCCCCCCC	187
A. longa soil eDNA	CTAGAACTAGTCACGCGCCGTACCC———	182

Lumbricus rubellus

Consensus	AGACGGTAATCTCCTGGAAGTAGGTCAGAKRTTGGDA-GADDTDTDDAATCTATTTCACGTTTATAAAGTCAGTATATTC	79
L. rubellus (KT308192)	AGACGGTAATCTCCTGGAAGTAGGTCAGAGGTTGGAAGTATGTATGAATCTATTTCAACGTTTATAAAGTCAGTATATTC	80
L. rubellus worm gDNA	————————————————————————————CGCTG-GAGATGTATGATCTATTTCACGTTTATAAAGTCAGTATATTC	47
L. rubellus soil eDNA	———————————————————————————— ATTGGGA-GTATGTATGAATCTATTTCACGTTTATAAAGTCAGTATATTC	50

Consensus	ATATCTTCAGTATCATTGGTGGCCGATAGTTTTTACAGTGATTGATGGTTGTCTTACTTCGTCTGTGATATAGAGAATAC	159
L. rubellus (KT308192)	ATATCTTCAGTATCATTGGTGGCCGATAGTTTTTACAGTGATTGATG————————————————————————	160
L. rubellus worm gDNA	ATATCTTCAGTATCATTGGTGGCCGATAGTTTTTACTGTGATTGATGGTTGTCTTACTTCGTCTGTGATATAGAGAATAC	127
L. rubellus soil eDNA	ATATCTTCAGTATCATTGGTGGCCGATAGTTTTTACAGTGATTGATGGTTGTCTTACTTCGTCTGTGATATAGAGAATAC	130

Consensus	GAAGA	164
L. rubellus (KT308192)	————	127
L. rubellus worm gDNA	GAAGA	132
L. rubellus soil eDNA	GAAGA	135