Laboratory measurements of *Aporrectodea caliginosa* earthworm counts using qPCR in agricultural soils

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Abstract

Traditionally, the abundance of earthworms in soil is perceived as a good biological indicator of soil health. While the direct relationship between earthworms and soil health is perhaps uncertain, it is well known that earthworms do provide benefits to the soil. The conventional method of determining the abundance of earthworms is a physical count within a spade square of sampled soil, which is a manual and time-consuming process, and where speciation is desired, requires technical expertise. Earthworm abundances vary spatially and hence the values derived will be dependent on the sites selected for measurement. Hill Labs in conjunction with AgResearch have developed a quantitative PCR (qPCR) test to determine the concentration of environmental DNA (eDNA) of the earthworm *Aporrectodea caliginosa. A. caliginosa*, is the most abundant earthworm in agricultural New Zealand. The eDNA contained within routine (7.5 cm transect cores, 38°C dried and < 2 mm ground) agronomic soil samples, was correlated to abundance. The field calibration was highly correlated with a log equation having an R^2 of 0.68, and the remaining variation largely explainable by the precision of the measurements. While eDNA appears to degrade in the field-moist samples post collection, the eDNA is stable once the soil samples are dried and ground within the lab. The analysis is run in triplicate to account for inherent variation and potential for 'spikes' of genomic DNA within the subsampling process. While there is evidence that a single result is as precise as the field-sampling technique, we believe testing in triplicate and using the average value (excluding outliers), provides a result that is more reflective of the earthworm population. We believe this method is overall fit-for-purpose for determining the state of earthworms in New Zealand.

Keywords: earthworms, eDNA, soil health

Introduction

It is well documented that earthworms provide many benefits including the maintenance of soil quality through their contribution to soil aggregate building, pore structure creation, nutrient cycling, and decomposition of plant material (Edwards and Bohlen 1996). Similarly other studies have found abundance correlated with increased soil macroporosity, water infiltration, increased plant nitrogen and plant growth (Schon et al. 2021). The strong presence of earthworms has also traditionally been perceived as representing a 'healthy soil', and their abundance has been directly linked to increased pasture production (van Groenigen et al. 2014). Schon et al. (2023) recommend total earthworm abundances should be >400 ind/m2, and that the three different earthworm ecological groups (epigeic, endogeic and anecic) all be represented for maximum benefit.

Aporrectodea caliginosa (an endogeic species), while not a native species is now widely spread across agricultural New Zealand, and in general represents around 80% of the total earthworm population typically found on New Zealand pastoral systems (Fraser and Boag 1998). The endogeic group of earthworms, primarily live and feed within soil, and live typically within the top 20 cm of the topsoil. Thus, measuring and quantifying *A. caliginosa* will be highly correlated to the total earthworm population and typically largely represent the abundance of earthworms.

The conventional method of assessing the state of earthworms is a physical observation within a square spade of sampled soil. This is a manual and time-consuming process, and where speciation is desired, requires technical expertise. Earthworm abundances vary spatially, even within a single paddock, and hence the values derived are dependent on the sites selected for measurement.

All organisms shed DNA into the environment (eDNA) as they move through it. This eDNA can be detected by molecular techniques such as PCR and can be used as an indicator of organism presence without needing to physically observe the organism in question. Molecular techniques are increasingly being applied to measure various organisms within soil ecosystems. This includes helping to understand microbial communities within soil (Labouryrie et al. 2023), soil fauna (Kirse et al. 2021) and indeed understanding earthworm populations (Llanos 2023; Jackson et al. 2017). The technique can also be used for quantitative assessments, using standard curves, especially with the correction

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with an internal standard to account for variations with extraction efficiencies (Yang et al 2018).

This work builds on the research published in the Our Land and Water report by Hsu et al. (2023) and Schon et al (2025). It was earlier shown that a method for qPCR measurements of *A. caliginosa* could be created, and a correlation with the abundance found in the field obtained. In converting the research method into a practical, efficient, high throughput laboratory method, the method needed to be further understood in terms of robustness, accuracy, and precision, and it was necessary to derive a calibration for an estimate of the DNA concentration itself, rather than instrument signals (cycle times) (which are method and instrument dependent). The earlier work showed improved DNA detection in dried soils than field-moist soils. Drying stabilised the DNA giving more accurate measurements over time, as the DNA in field-moist soils appeared to degrade, especially when stored at room temperature. For the test to be commercially practical, it was desired for it to utilise the standard agronomic soil transects (7.5 cm cores), with as minimal extra preparation as required of the lab.

Thus, this research further develops the test, improving the robustness and reliability, and to validate the field calibration. The aim of this research was to understand the variation and factors affecting the laboratory DNA measurement, and to derive a calibration for the abundance of earthworms found in the field.

Materials and Methods

Hill Labs in conjunction with AgResearch developed a quantitative PCR (qPCR) test to determine the concentration of environmental DNA (eDNA) of the earthworm *A. caliginosa* contained within routine (7.5 cm transect cores, 38°C dried and finely ground) agronomic soil samples, and have correlated this to the abundance of *A. caliginosa*, the most abundant earthworm in agricultural New Zealand.

Laboratory qPCR method

The DNA is extracted from a sub-sample of dried and finely ground soil using a magnetic bead extraction kit. Purified DNA is then tested for the presence of *A. caliginosa* eDNA using quantitative real time PCR (qPCR). *A. caliginosa* eDNA detected is quantified against a standard curve of known target gene concentration and is reported as pg DNA per gram of soil. The *A. caliginosa* qPCR test is specific for the detection of *A. caliginosa*.

To create the calibration curve, a known concentration of *A. caliginosa* DNA was 10-fold serially diluted and spiked into a soil sample that had previously been tested and had shown no detectable amplification of *A. caliginosa* DNA. The spiked soil samples were then extracted in triplicate and tested on the *A. caliginosa* qPCR to generate a standard curve. An internal standard, of known concentration (non-related) DNA is added pre-extraction, including the spiked soil used to create the standard curve, to correct for differing extraction efficiencies within different soil samples. This enables each sample to be quantified against the same standard curve, converting the Cq value (the cycle number at which the amplification of DNA is first detected) to concentration of *A. caliginosa* extracted (pg DNA/g soil). While the thermocycler does not tend to drift, the internal standard would also correct for drift within the method / between instruments. The calibration and internal standard can be re-derived at any point in time with a new soil with no detectable *A. caliginosa* DNA. The exact efficiency is not required to be determined, as all cases are simply relative efficiencies to the sample that the calibration curve has been created from.

Various experiments were conducted in the laboratory to develop and validate the methodology, including repeat measurements at various parts of the process to understand uncertainty of measurement, and spiking of known amounts of *A. caliginosa* DNA into various soils and reading back the difference between the original and the spiked amount.

Field calibration

For the field calibration, 73 different samples from around New Zealand including different sample types, across dairy, sheep and beef, were analysed via the traditional spade square method and the qPCR test to derive the calibration.

All samples for eDNA were taken as per a typical pasture based agronomic nutrient analysis using a 7.5 cm auger, with a transect of 8 to 10 cores across each sample site. Samples were analysed by qPCR as described above.

Counts of *A. caliginosa* were done via collecting a spade square (20 cm x 20 cm) and physically counting the earthworms present. All earthworms were identified, counted and weighed. Juveniles (unidentifiable to the species level) were not included in the count for each species. The average number of *A. caliginosa* (excluding juveniles) from 3 spade squares, expressed as ind/m^2, was used to compare to qPCR data.

Precision of each method

In a field precision experiment, 6 different paddocks were analysed in replicate to determine the relative precision of both the qPCR and the traditional spade square method. This experiment included 5 different transects in each paddock. Along each of the 5 transects 3 spade square measurements (15 total) for traditional earthworm assessment were obtained for each paddock.

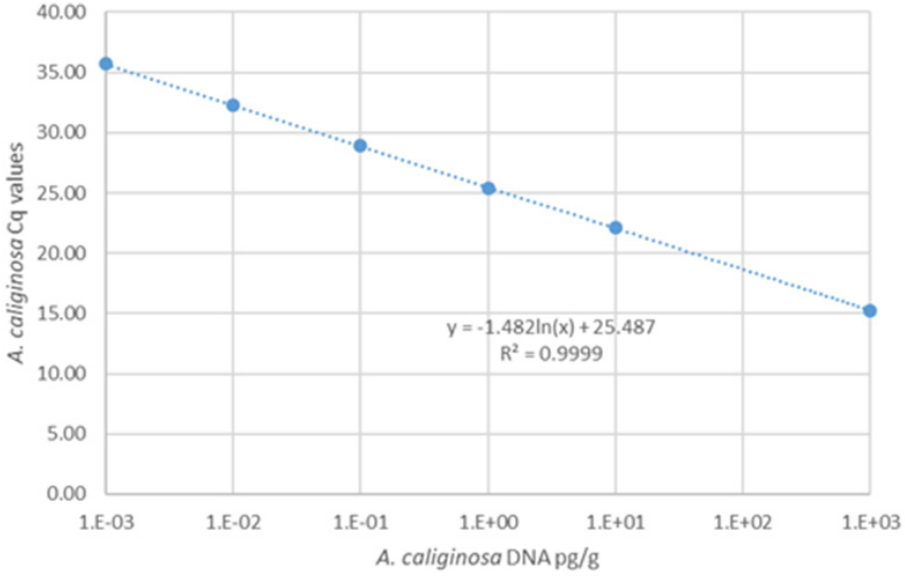

Figure 1 Standard curve for a soil applied to varying amounts of A. caliginosa DNA (pg/g)

Precision within the laboratory

In a separate experiment, 6 samples within the laboratory (lab-sample) were replicated at different stages of the analytical method. Two subsamples (~30g < 2 mm ground subsamples) for fine grinding from the processed 2 mm soil fraction, were obtained for each sample, duplicated across two different batch / extraction runs, with two extracts per sample on each run (4 fine-ground 0.3 g subsamples). The resulting 48 extracts were then repeated in duplicate across two different PCR instruments (total 96).

Statistical analysis

The field calibration was created by regressing the obtained DNA, transformed into the log10 domain as the dependent variable against the abundance of *A. caliginosa* as the independent variable. The best fit was found to be a log equation, and thus a linear equation in the log domain for the abundance physical count.

For the field precision experiment, a two-tailed T-test was conducted against the means for each paddock to compare the traditional method against the DNA method after conversion to a modelled abundance value based on the derived field calibration equation, to test statistical non-equivalence of the means. An F-test was also conducted to compare each of the variances to test statistical non-equivalence of the variances.

For the within-laboratory precision, each potential duplicate at the various repeatability levels were compared against its pair. The pairs were assuming 'first' and 'second' for each replicate stage, e.g. for the batch comparison, the first batch was compared against the second batch for samples where everything else was the same, that is the first extractions were paired together. This has the effect of not comparing the first extraction to the second extraction (and all the other similar extra comparisons at the other levels). A precision estimate was derived as the standard deviation of the difference between the duplicates dividing by the square-root of two (assuming homoscedasticity in the replicates). An F-test was conducted the various precision estimates to statistically test for non-equivalence of these variances (with respect to the above caveat). All statistical analysis were conducted in Excel (Microsoft Office 2016).

Results

The creation and serial dilution of the standard curve for the *A. caliginosa* gBlock DNA as applied to a single soil provided an extremely strong calibration (Figure 1). The curve then serves as the basis to convert any other sample Cq value to *A. caliginiosa* DNA pg/g values. In developing the method to determine the amount of eDNA, rather than simply measuring the Cq values it was observed that different soils have very different extraction efficiencies of DNA in soil (effectively the percent of DNA measured to what is present). Hence an internal standard was added to account for the different extraction efficiencies. While this causes a decrease in the precision of the overall method due to having to measure two DNA signals and correct one with the other, it improves ability to compare across different soils. The internal standard Cq values have been shown to vary between 23 and 37 cycles, suggesting potentially 4 orders of magnitude range of extraction efficiencies between different soils.

Table 1 Two soils that had no initial A. caliginosa DNA and were subsequently spiked with 100 pg/g DNA

	A. *Caliginosa* Cq values	Internal Standard Cq values	Corrected A. *Caliginosa* Cq values	DNA Concentration (pg)	
				Uncorrected	Corrected
Soil 1	23.2	31.0	18.9	5	87
Soil 2	19.3	27.4	18.5	65	111

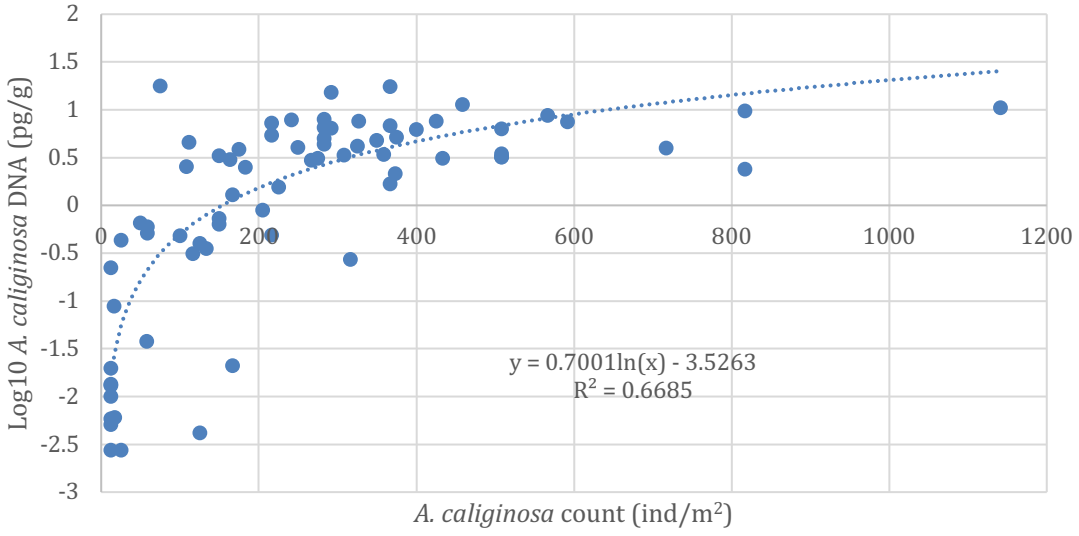

Figure 2 Field calibration of A. caliginosa DNA pg/g against the measured count per m2

Results from two soils with no *A. caliginosa* present (both in count and no DNA amplification) spiked with 100 pg/g of *A. caliginosa gBlock DNA*, returned 87 and 111 pg/g, respectively, after correcting for extraction efficiency using the internal standard (Table 1). If the internal standard was not used to correct for the difference in the extraction efficiency then the obtained values would have been 5 and 65 pg/g DNA, respectively (Table 1), and thus those cases had a far lower extraction efficiency than the soil that was used for the calibration curve. The same phenomenon can be observed through the large differences between the Cq values in the uncorrected spikes (23.2, compared to 19.3). These differences were reflected in the internal standard results, and thus the corrected results are more similar (18.9 compared to 18.5).

Each cycle (represented by Cq value) of the qPCR test represents a doubling of DNA, and thus every cycle represents exponential amplification of the extracted DNA. Potential error within the Cq units of ±1 thus represents 50% to 200% expansion of the potential DNA concentration, and therefore underlying abundance. As the underlying measurement uncertainty is proportional to these 'cycles', it therefore makes sense to plot and consider the DNA pg/g measurement within the log domain. The results from the field calibration, within a log domain, shows a strong correlation between the log DNA (pg/g) and the log count of *A. caliginosa* (Figure 2, $R^2 = 0.67$).

The overall repeatability of a single transect DNA measurement, showed near equivalent repeatability to the traditional 3x spade square average, on average across the 6 different paddocks when modelled through to a similar measurement (abundance). Four of the six paddocks after modelling to abundance (for comparison purposes, based on the equation from Figure 1), were statistically equivalent to the traditional method. Paddocks 4 and 5 were not and had elevated DNA levels compared to the observed physical count. The underlying instrument signals (cycles or Cq values) were precise and considered within typical precision for a PCR method (having an average SD of 0.5, Table 2).

Within the internal repeatability trial, variation within a single Lab-sample was largely explained by variation across different batches (Table 3). For Lab-sample 4 a pair of outliers were observed, appearing to contain an increase of DNA measured within both replicates (across PCR instruments) from a single 'extraction' (Figure 3). The increase was equivalent to

Table 2 Field precision estimates for the qPCR method compared to the traditional 3 x spade square average counting method

	DNA (Cq values)		DNA (pg/g)		Traditional (ind/m^2)		Modelled from DNA (ind/m^2)	
Paddock	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	26.1	0.45	2.2	0.75	373	114	251*	51
2	26.9	0.33	1.3	0.28	167	43	181**	24
3	27.4	0.77	1.0	0.51	205	112	150**	48
4	24.3	0.41	7.8	1.98	327	69	548	89
5	25.0	0.42	4.7	1.21	112	30	401	66
6	25.6	0.85	3.5	2.25	163	40	322*	125
Average	25.9	0.5	3.4	1.2	224	68	309**	67

* Statistically equivalent at the >1% level
** Statistically equivalent at >5 % level

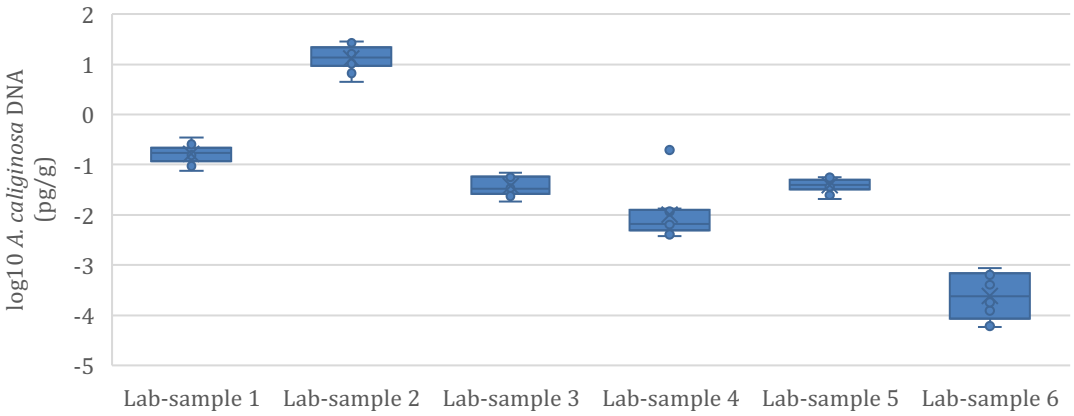

Figure 3 Internal lab precision experiment precision data containing all 16 replicates per lab-sample.

0.2 pg/g DNA or 50 extra numbers of earthworms per m^2. The Lab-sample 6 set was also more variable than the other cases, which is explainable by it being below the detection limit (DNA pg/g of 0.001, or log10(DNA pg/g) of -3). Samples near or below the detection level can often be more variable. While the fine-grind step appears to add some variation to the method, this was not statistically significant ($p = 0.20$) compared to the inter-batch variation with the 41 comparisons (excluding Lab-sample 6 and the outliers in Lab-sample 4). The instrument precision was overall very good and was shown to be only a very small contributor to the overall variation to the method (Table 3).

Table 3 Average precision estimates (repeatability: Sr) at different method level for both Cq values and log10 (DNA pg/g) (excluding Lab-sample 6 and the outliers in Lab-sample 4)

Precision Comparison	Cq values	Log10 (DNA pg/g)
Fine-Ground subsample	0.67	0.20
Batch replicate	0.55	0.16
Extraction subsample	0.38	0.11
PCR replicate (instrument precision)	0.22	0.06

Discussion

There was very good correlation between the observed DNA concentration and the abundance of *A. caliginosa* earthworms obtained during sampling, especially given that earthworms are highly mobile, and abundances vary across a single paddock. Furthermore, factors such as the impact of rain on both the earthworm behaviour, and the potential leaching of DNA (Pietramellara et al. 2009) would also have potential for increased variance between these two measures in any given sampling event.

The eDNA is stable once the soil samples are dried and ground within the lab, but the eDNA appears to degrade in the field-moist conditions post sampling

(Schon et al. 2024). Hence there will be a degree of degradation that occurs prior to a sample being dried, based on the abundance present, and the degradation rates. These degradation rates would be expected to be both environmental (moisture and temperature) and soil biology dependent, and thus variable for each site. Previous work showed degradation rates of up to 40% decrease in DNA per day of fresh soil stored at room temperature.

Variations in the extraction efficiency across different soils is thought to arise from interference via DNA absorption onto various soil particles during the extraction steps, for example from clay or humic acids and potentially other factors causing DNA to shear (break down more), during the bead-beating stage (Lloyd-Jones and Hunter 2001; Roose-Amsaleg et al. 2001; Wnuk et al. 2020). All these factors will play a role in soil specific extraction efficiency values, which are difficult to assess through traditional DNA abundance or quality metrics (UV-vis ratios). While an internal standard is used to overcome this, however, there is the potential for the type of DNA (synthetic gBlock) that was used within the standards (both the *A. caliginosa* and the internal standard) to behave differently than the 'real' target DNA in the soil. Indeed, evidence from some workers have shown biases can emerge from different extraction methodologies from this effect between types of DNA in soil, such as bacterial compared to fungal DNA (Feinsten et al. 2009). It is unknown how much bias this will contribute to the overall method for estimating the true amount of *A. caliginosa* DNA, although our research found a good correlation between the observed DNA concentration and the abundance of *A. caliginosa* earthworms across a range of soils.

As observed within the laboratory precision experiment, large concentrations of DNA (spikes) were observed rarely (2% of extractions within that experiment). Similar observations were also seen during early experiments (data not shown). The outlier spike of DNA was only observed within a single subsample (0.3 g) from the 30 g fine ground subsample and hence was not observed throughout the entire fine-ground fraction, with other subsamples being with the expected precision limits. The spike could be caused from the sampling of eggs, cocoons and small juveniles too small to be visible and removed from the soil sample, but acting as highly concentrated piece of DNA material that gets subsampled, rarely. One methodology to avoid this robustness problem would thus be analysing in replicate (e.g. triplicate) and discarding any elevated result, providing some protection against such erroneous cases, and improving the overall precision of the method considerably (in reporting the average result).

Given all the factors and measurement variations observed within each test, it is surprising that such a strong correlation can be created at all. The variation observed in each measurement from replicates, almost completely explains the observed variation within the correlation (variation inferred between studies), suggesting that the underlying relationship is very strong. This was highlighted again within the field-precision experiment, where individual spade measurements can misrepresent the overall 'true' abundance of the field, and even the standard 3 spade square average, was shown to be highly variable. While there is evidence that a single qPCR result is as precise as the field-sampling technique, we believe testing in triplicate and using the average value (excluding outliers), provides a result that is more reflective of the field conditions.

Conclusions

We believe this method, particularly when run in triplicate avoiding any 'spikes' in DNA and in general reporting the average, is overall fit-for-purpose for determining the state of earthworms in New Zealand.

Samples need to be sent to the lab as soon as possible or kept cool to ensure accurate results. The test better represents the spatial variability of any given sample site, by being able to better take representative samples, and easily fits into existing agronomic samples for nutrient testing. Future planned work includes field-calibrations of 0-15 cm soil samples.

The developed qPCR earthworm test at Hill Labs could replace the existing field-based measurement, enabling easier monitoring of this component of soil biology for farmers and agronomists.

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