# Slutrapport

*New DNA method for analyzing root-knot nematode in soil*

## Projektnummer: R-18-25-0022

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## Del 1: Utförlig sammanfattning


Rotgallnematoder är allvarliga skadegörare i frilandsodlade grönsaker. I Sverige är *Meloidogyne hapla* den vanligast förekommande arten, och orsakar ekonomiska skördeförluster särskilt i morötter. Jordanalys av nematodförekomst är en viktig åtgärd för att bedöma risken för skördeförluster i fält och för att planera hållbara växtföljder. Tillgång till en robust analysmetod av jord kan bidra till att flera prover analyseras inom grönsaksproduktionen. Nuvarande analysmetoder för nematodförekomst i jord är antigen konventionella metoder baserade på utdrivning av nematoder från jord följd av artbestämning under mikroskop, eller DNA-baserade metoder som realtids-PCR (rt-PCR). En sådan metod är kommersiellt tillgänglig för analys av rotgallnematoden, men används endast för artbestämning. Det övergripande målet med projektet var att ge odlare, rådgivningsbranschen och hela näringen tillgång till en snabb och säker DNA-baserad analysmetod för att kvantifiera rotgallnematoder i jord. Syftet var att validera praktisk tillämpning av en tidigare utvecklad DNA-metod baserad på ”Loop-Mediated Isothermal Amplification” (LAMP) teknologi. Nu har vi tagit arbetet vidare för att testa och tillämpa metoden i praktiken.

Med syfte att minska analystid och kostnad för odlarna, utvecklades en DNA extraktion procedur ”SKMM” som jämfördes med ett kommersiellt DNA- extraktionskit följt av två extra kit för DNA rengöring. DNA extraherades från tre grupper av jordprover: 1) rotgallnematodfria sand- och lerjordar med tillsatta *M. hapla*, juveniler i andra stadiet (J2), i nivåer från 4 till 64 J2 per 250 gram jord, 2) fältprover erhållna från HS Nematodlaboratorium, insamlade 2019 från 20 olika fält i södra Sverige och Danmark, samt 3) fältprover insamlades i april 2020 från sex fält i Skåne, där förekomst av nematoder konstaterats i tidigare provtagningar. Förfrukten i dessa fält var lök (fält 1), vårkorn+rödsvingel (fält 2), vårkorn (fält 3–4) och potatis (fält 5–6). I den sista gruppen från 2020, togs ett samlingsprov i ”W” över varje fält samt 4 enskilda prover, dvs totalt samlades 30 prover in. Resultatet visade att DNA som extraherades med SKMM proceduren


Projekt har fått finansiering genom:

hade högre DNA koncentration och var renare från jordsubstanser, som humussyra, jämfört med DNA som extraherades och rengjordes med kommersiella kit.

Realtids-LAMP (rt-LAMP) analyserna genomfördes med en speciell LAMP utrustning ”Genie® II” och för att kunna kvantifiera antalet juveniler eller genkopior användes DNA från *M. hapla* samt en syntetisk DNA standard i olika koncentrationer (spädningsserier). Linjärt samband mellan $T_t$-värde (tid som tas för att få positiv signal i minuter med Genie® II) och DNA koncentrationer eller genkopior av den syntetiska DNA standarden, användes för att beräkna motsvarande antal juveniler eller genkopior per jordprov. Av de artificiellt infekterade sand- och lerjordproverna var sandproverna med 8 och 64 tillsatta juveniler positiva (SKMM), respektive sandproverna med 32 och 64 tillsatta juveniler (kit). De flesta lerproverna var däremot negativa för DNA som utvunnits med både SKMM och kitet. Detta kan bero på otillräcklig malning av jordproverna innan DNA extraktionen, eftersom DNA mängden från dessa jordprover inte ökade proportionellt med antal tillsatta juveniler.

Förekomsten av *M. hapla* i fältproverna från 2019 var under detektionsgränsen i rt-LAMP-analyserna. Tre jordprover var positiva när DNA extraherades med SKMM och det uppskattade antalet juveniler var 184, 37 och 6 J2s per 250 gram jord. Motsvarande tätheter som beräknades i rt-LAMP-analyser då DNA extraherades med kitet var 15, 0 och 6 J2s. Fältproverna som samlades in 2020 analyserades med två molekylära metoder, rt-LAMP och rt-PCR. Båda metoderna visade positiv förekomst av *M. hapla* DNA i prover från två av sex fält. Dessa sex fält valdes ut baserat på tidigare provtagning och morfologisk analys under hösten/vinter innan provtagningen i april 2020 och den morfologiska analysen visade hög förekomst av *M. hapla* i samtliga sex fält. Trots detta minskade populationerna kraftigt i fyra av fälten under vintern 2019/2020. Den uppskattade nematodtätheten enligt rt-PCR i ”W”-samlingsprovet i dessa två fält, var 50 och 4 J2s per 250 gram jord. Motsvarande tätheter enligt rt-LAMP var 183 och 12 (SKMM) samt 110 och 15 J2s per 250 gram jord (kit). Våra resultat visar också en inomfälts-variation i förekomsten, särskilt i det fält som hade den högsta nematodtätheten. Värdena på de fyra enskilda proverna enligt rt-PCR varierade mellan 12 till 51 J2s per 250 gram jord i detta fält och 7 till 17 J2s per 250 gram jord i fältet med lägre förekomst. När det gäller rt-LAMP varierade förekomsten i fältet med högst förekomst mellan 0 till 86 (kit) och 0 till J2s per 250 gram jord (SKMM). Motsvarande tätheter i de fyra proverna som var tagna från det andra fältet varierade mellan 0 till 22 (kit) och 0 till 23 J2s per 250 gram jord (SKMM).

Vårt resultat tyder på att jordprovtagning och analys av ett samlingsprov som tas i ett ”W” över ett fält kan ge vägledande information, men på grund av den fläckvisa förekomsten av *M. hapla*. behövs flera delprover för att lokalisera de områden där nematoderna finns. Resultatet visade också att nematodpopulationer kan minska under vintern i vissa fält och därmed kan det vara mer lämpligt att provtagning sker tidigt på våren. Fortsatta undersökningar med provtagning vid flera tidpunkter behövs för att identifiera den optimala tidpunkten för jordprovtagning.

# Del 2: Rapporten (max 10 sidor)

## Background

The root-knot nematode (RKN), *Meloidogyne hapla*, is the most common root-knot nematode species in Sweden, especially in carrots. When roots are deformed by RKNs, the carrots that are produced will be unsellable at the consumer level. Therefore, growers must cultivate carrots in root-knot nematode free soil. Carrot production is an important part of the Swedish horticulture industry and considered as the second largest vegetable crop after strawberry in open field cultivation [1]. Both production and consumption have increased steadily, while the number of producing farms has decreased. Carrots are grown intensively in a limited area in Sweden, especially in Skåne county, where the problem with *M. hapla* is noticeably increasing [2]. Other known hosts, such as sugar beets, potatoes, onions, and lettuce are usually grown in the same crop rotation as carrots, hence *M. hapla* can continuously reproduce in soil. Control strategies against RKNs in Swedish vegetables production rely mostly on crop rotation and weed control. Most weed species occurring in Swedish fields that has been tested for resistance against RKNs have been found to be susceptible [3]. To achieve a successful control strategy, soil analysis stands out as an extremely important measure to be able to decide when there is a need for management actions to keep nematode populations below the tolerance level. In practice, fields to be cultivated with carrots without jeopardizing yield and quality, can only be selected based on soil analysis in autumn before carrot cultivation in spring the following year. The patchy occurrence of *M. hapla* in fields suggests that it is better to take multiple soil samples instead of a pooled sample, to evaluate the nematode occurrence more accurately in a specific field. Nematode extraction from soil followed by morphological identification, though useful but time consuming, has also low capacity and requires nematode expertise. On the other hand, molecular identification using DNA-based techniques is more reliable. Generally, those techniques require highly purified DNA, especially when DNA is extracted from soil. We have previously developed a molecular identification assay for *M. hapla* based on Loop-mediated Isothermal Amplification (LAMP) technology [4]. LAMP is a robust and specific DNA technique for detecting and quantifying microbial DNA used for diagnosing of different plant pathogens [5] and nematodes using simple lab equipment and quick colorimetric detection. LAMP can also be used for quantification of pathogens and nematodes, using real-time platforms. In general, DNA-based analysis consists of two parts, first DNA extraction followed by DNA amplification and finally detection and quantification. Extraction of DNA from soil samples is the most important step as inhibition is likely to occur [6]. The most significant benefit of LAMP is being less sensitive to DNA inhibitors from samples, therefore simple DNA extraction procedures can be used instead of expensive commercial kits. This makes LAMP cheaper than other DNA-based techniques such as PCR, thus provide a low-cost analysis option to growers [7; 8]. **The overall goal** of this project was to provide vegetables growers with a cheap and reliable DNA-based soil analysis method for the root-knot nematode, *M. hapla*, based on LAMP technology. **The aim** was to validate the practical application of LAMP for soil analysis.

*The objectives were to:*

1. Develop a simple extraction method for root-knot nematode DNA from soil.
2. Validate the previously developed LAMP method for quantification of *M. hapla* in naturally infested field soils using the simple DNA extraction method.
3. Compare real-time LAMP with standard extraction of nematodes from soil followed by morphological identification, for identifying and quantifying *M. hapla* juveniles in soil.

# Materials and methods

The project comprised of three working packages (WP) performed mostly during 2018 – 2021. In 2022 the project group focused on communicating the results to the Swedish growers as well as to the international scientific community.

*Further developments of the LAMP assay*

Specificity of the developed LAMP assay [4] was tested against other RKN species using both colorimetric and real-time LAMP (rt-LAMP). DNA of two populations of *M. chitwoodi* in addition to one population of *M. fallax*, *M. arenaria*, *M. incognita* and *M. javanica* were kindly provided by Prof. Nicole Viaene, Instituut voor Landbouw-, Visserij- en Voedingsonderzoek (ILVO), Belgium, Dr. Åsa Olsson, HS Nematode Laboratory (HS NL), Alnarp, Sweden and Intertek ScanBi Diagnostics (ISD), Alnarp, Sweden. Details on the colorimetric LAMP were presented in our published article in "*Horticulturae*" [9].

**WP 1: Development of a simple method to extract DNA from sand and clay soils artificially inoculated with juveniles**

*Soil sampling and soil preparation*

Root-knot nematode free sandy and clayey soils were collected from two fields in Uppsala [9]. These soils were sieved and air-dried at room temperature for 14 days, and later artificially inoculated with *M. hapla* second-stage juveniles (J2s). The juveniles (4, 8, 16, 32 and 64) were hand-picked under a stereomicroscope and placed in 2 ml tubes to which, 500 mg soil and glass beads were added. The homogenized inocula in 500 mg of soil were then added to the final amount of 250 gram dry soil followed by a second homogenization, twice at 2000 and 4000 rpm, with Mortar Grinder RM 200 (ISD, Alnarp, Sweden). This generated artificially infested clayey and sandy soil samples at a range of 4 – 64 J2s 250 $g^{-1}$ soil ($n$ = 3).

*Development of manual soil DNA extraction procedure*

I. Soil washing

A pre-washing step of soil, prior to DNA extraction, was tested in the sandy and clayey soils to minimize DNA contamination with humic acids originating from clay particles. In short, four buffers were tested: (1) TNP (100 mM Tris, 100 mM $Na_4P_2O_7$, 1% PVP,100 mM NaCl, 0.05% Triton X-100, and 4% skim milk, pH 10.0) [10]; (2) TENP (50 mM Tris, 20 mM EDTA, 100 mM NaCl, 1% polyvinylpolypyrrolidone) [11]; (3) EDTA (Ethylenediamine tetracetic acid); (4) sodium phosphate buffer (MP Biomedicals). A volume of 500 µl from each buffer was added to 250 mg soil, and the soil was then mixed, centrifuged and the supernatant was removed. The color of the supernatant was visually inspected, and aliquots were analyzed by gel electrophoresis. To assess the effect of soil washing on the following DNA quantity and quality, DNA was extracted from the washed samples with FastDNA kit for soil (MP Biomedicals).

II. SKMM manual procedure

An extraction method originally developed to extract DNA from plants [12] was used for DNA extraction from soil, but with modifications. The extraction buffer in the original protocol was replaced by a buffer based on skim milk solution and Sodium Dodecyl Sulfate (SDS). Soil sample NI6-19 was selected for developing the manual procedure [9], here referred to as SKMM (Skim milk method). DNA was extracted from 10 g of soil which was mixed with extraction buffer (8 ml 10 $g^{-1}$ soil). After centrifugation 1 ml was used in the rest of the process [9].

*DNA extraction with commercial FastDNA kit*

Parallel DNA extractions were performed with FastDNA kit for soil, here referred to as FD kit (kit 1), with some modifications. First, the soil washing prior to DNA extraction was excluded from the protocol. Second, after addition of extraction buffer (11 ml 10 $g^{-1}$ soil), homogenization of the soil and centrifugation, 1 ml of the supernatant was withdrawn and used in the rest of the extraction process, according to the mini version of the kit. DNA was purified with Wizard DNA kit, Promega (kit 2) followed by MicroSpin S-300 HR Columns, Cytiva (kit 3) [13]. DNA from all samples was quantified by NanoDrop spectrophotometer.

**WP 2: Analyses of field samples**

*Field samples collected in 2019*

Soil samples were collected from commercial fields in southern Sweden and in Denmark, and these were morphologically identified and subsamples of 250 g from the original samples were kindly provided by HS NL, Alnarp, Sweden [9]. The samples were processed as mentioned in WP1. DNA was extracted from 10 g subsamples ($n$ = 3), using SKMM manual procedure developed in WP1 as well as the commercial kits.

**WP3: Investigation of in-field variation of *M. hapla* occurrence in commercial fields**

*Field samples collected in 2020*

Thirty new soil samples were collected from 6 commercial vegetable fields in southern Sweden, April 2020 [9]. These fields were pre-selected based on nematode analysis in autumn/winter 2019/2020. One pooled sample collected in a W-pattern and 4 individual samples were collected per field. The samples were mixed thoroughly by hand and divided into two sets of subsamples (250 g each). Samples in the first set were processed as in WP1 and WP2 and DNA was extracted from soil. Samples in the second set were used for recovery of nematodes from soil by the Baermann funnel method [14] (Vildrosen Jord & Hund AB), followed by DNA extraction and real-time PCR analysis (ISD, Alnarp, Sweden). Soil chemical properties of all soil samples were analyzed at Eurofins Food & Agro Testing Sweden [9].

*Real-time LAMP*

Fluorescence based rt-LAMP was performed with Genie® II (OptiGene Ltd, West Sussex, UK) (quantification) or qPCR machine (detection). The master mix contained per reaction: 15 µl ISO-001 isothermal master mix (OptiGene Ltd); 5 µl primers mix and 5 µl DNA template. The reaction conditions were amplification at 65 ºC for 30 min, annealing at 98 ºC – 80 ºC and ramping at 0.05 ºC $s^{-1}$ to generate melting curves.

*Quantification of gene copies and juveniles*

To quantify the number of gene copies in a soil sample, standard curves were generated from a synthetized DNA (gBlock$^{HSP}$) at 193 000 to 193 gene copies per reaction (IDT, Iowa, USA) ($n$ = 3). To estimate the number of juveniles in a soil sample, standard curves were generated from *M. hapla* genomic DNA extracted from 16 juveniles and used at 1/10$^{th}$ dilutions to give 1.6 to 0.0016 juveniles per reaction ($n$ = 3). The gene copies or number of nematodes were plotted against the respective time threshold values ($T_t$) generated by the LAMP equipment, Genie® II, to calculate the slope and intercept.

*Statistics*

The results were analyzed using JMP statistical software (ver.9.0) (USA).

## Results and discussion

Detailed results are accessible through the open access article published in "*Horticulturae*" special issue "Recent Advance in the Identification and Diagnostics of Plant-Parasitic Nematodes" [9].

**Can LAMP analysis differentiate between *Meloidogyne hapla* and other RKN species?**
The LAMP assay used in this study was originally developed to identify and analyze *M. hapla* [4] as it was the only RKN species prevalent in vegetable fields in Sweden. However, in 2017 and 2018 two additional root-knot nematode species, *M. chitwoodi* and *M. fallax*, were reported for the first time in potato crops in Sweden [15]. Both species are classified as quarantine pests by the European Plant Protection Organization (EPPO). Therefore, it was crucial to assess the LAMP specificity against these species and other RKN species that probably may find their way to Sweden through infested plant or soil materials. The results confirmed the specificity of the LAMP assay towards *M. hapla*, where genomic DNA from *M. hapla* was exclusively amplified in both colorimetric and rt-LAMP and none of the other RKN species were detected by LAMP [9]. The specificity of the assay was previously tested against other nematodes prevailing in vegetables, sugar beet and potato crops in Sweden, including sugar beet and potato cyst nematodes, stubby-root nematodes, needle nematodes and root-lesion nematodes [4; 9].

**Dose soil washing prior to DNA extraction improves DNA quality?**
Soil is a complex matrix containing different substances including phenols, polysaccharides, salts and humic acid [16]. Therefore, DNA extraction from soil is a real challenge as some of these substances may co-purify with DNA [17]. Humic acid, which originates from clay particles is an essential contaminant of DNA, usually indicated by discoloration of DNA. To overcome this problem, soil washing has been used to remove as much color as possible prior to DNA extraction [10; 18]. The four tested buffers resulted in different color intensities from both sandy and clayey soils (Fig. 1a). The strongest color was produced by the TNP buffer, which was accompanied by clear DNA loss as seen by gel electrophorese (Lane 1) (Fig. 1b). Very high DNA concentrations were obtained after washing of the clayey soil with EDTA, sodium phosphate and TNP buffers indicating high DNA contamination (Fig. 1c). It seems that the concept of soil washing is more suitable when the chemicals phenol and chloroform are used as part of the DNA extraction process where the extract is partitioned into two phases, an aqueous layer containing the DNA a layer of phenol-chloroform that contains salts, proteins and other contaminants [10]. We decided from the start not to use phenol-chloroform since these are very toxic solutions. Both FD kit and SKMM procedure involves DNA binding to silica particles to separate DNA from the buffers during the extraction (instead of phenol-chloroform), but other substances may also by absorbed by the silica particles. When DNA is eluted with water or a buffer, the absorbed color will be released again and contaminate the DNA. Therefore, this step was no longer considered in the development process of the SKMM procedure and it was also excluded from the FD kit.

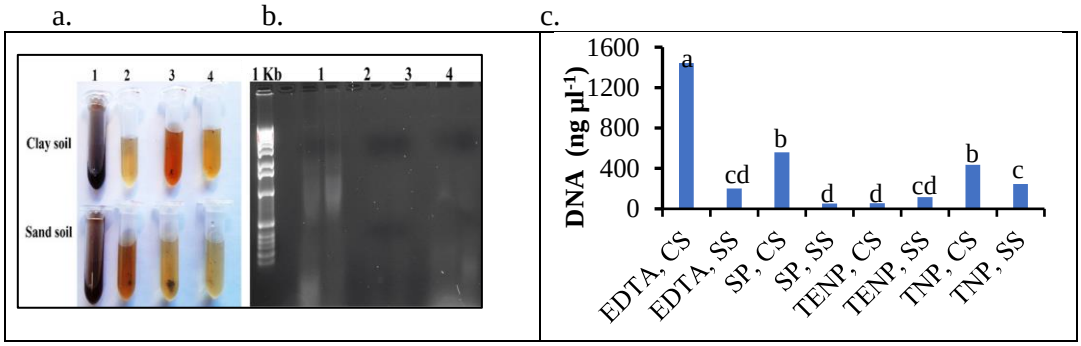


Figure 1. Soil washing with four buffers prior to DNA extraction, a: aliquot of washes from clayey (CS) and sandy soil (SS) with (1) TNP; (2) TENP; (3) EDTA; (4) sodium phosphate buffer (SP), b: corresponding gel electrophoresis showing DNA loss during washing, c: DNA concentrations extracted from the washed soils with FD kit.

**Skim milk-based manual DNA extraction "SKMM"**

Specific criteria were considered when developing the SKMM manual procedure: it should 1) be efficient, 2) work across different soil types, 3) not be time consuming and 4) a lower cost than the commercial kits. Given the spatial heterogeneity of nematodes in soil, large amounts of soil (approx. 200–250 g) are usually used for morphological identification [19]. Molecular identification usually involves DNA extraction from as little as 0.25 – 1 g soil, however, using larger soil amounts was found to be more reliable, but at the same time more time consuming [20]. In this study, we chose to start with a larger amount of soil, 10 g to have a homogenous starting material, but only 1 ml of the extract was used in the rest of the extraction procedure in both the SKMM and the kit (Fig. 2). A similar strategy has previously been adopted for DNA extraction from soil [21]. This shortened the time for the whole process and reduced the overall cost for one sample with two replicates, which was approx. half the cost of commercial kits, 276 *vs* 649 SEK. Concerning the effectiveness in terms of high DNA concentration and quality, the two important components in the extraction buffer, SDS and skim milk were first optimized, and the detailed results are presented in [9]. Briefly, the purest DNA from proteins ($A_{260}/A_{280}$) and humic acid ($A_{260}/A_{230}$) was produced by the highest volume of SDS (100 µl). On the other hand, different concentrations of skim milk neither resulted in significantly different DNA concentrations nor absorbance ratio ($A_{260}/A_{230}$). The final optimized SKMM procedure consisted of (10 $g^{-1}$ soil): 5 ml skim milk (6 %), 1 ml SDS (10%) and 2 ml MQ water. The silica product, Celite 545 AW, was used for DNA binding. The SKMM procedure resulted in purer DNA from sample NI6-19 compared with the FD kit (Fig. 2).

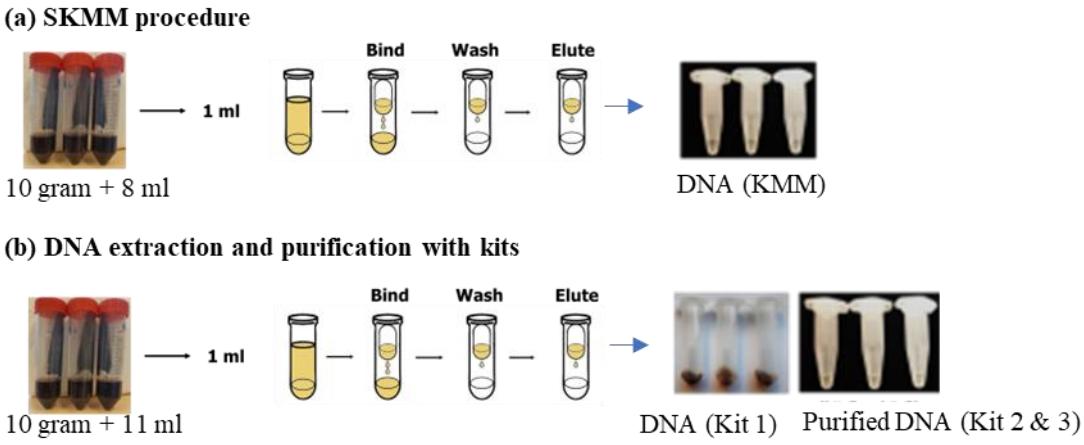


Figure 2. Illustration of DNA extraction from soil sample NI6-19 with (a): SKMM where 8 ml of extraction buffer with Skim milk and SDS was added to10 gram of soil; (b): FastDNA kit (kit 1) where 11 ml buffer from the kit was added to 10 gram soil, the DNA was then purified with two additional kits, Wizard DNA kit (kit 2) and MicroSpin S-300 HR Columns (kit 3).

**SKMM *vs* commercial kits**

The highest average amount of DNA extracted from artificially infested soil samples was obtained by the FD kit (kit 1), however, the DNA concentration decreased after purification with kit 2 and kit 3 (Table 2). This was accompanied by a significant increase in DNA purity, especially from humic acid. The same tendency was observed in the field samples collected in 2019 and 2020 (Table 1). The FD kit is a well-established commercial kit, however, low DNA quality is sometimes reported [22]. The values of the obtained absorbance ratios ($A_{260}/A_{280}$ and $A_{260}/A_{230}$) before DNA purification agrees well with other studies [23]. Apparently, multiple purification steps after DNA extraction with the FD kit are necessary to avoid inhibition of

DNA amplification in both rt-LAMP and rt-PCR [13]. The corresponding average soil DNA concentrations extracted with SKMM from both clayey and sandy soils was as in kit 3, however, significantly higher concentrations and purer DNA was obtained from field samples collected in 2019 and 2020 (Table 1). The non-fat skim milk probably made the SKMM procedure superior over the kits in terms of DNA purity from humic acid measured as absorbance ratio ($A_{260}/A_{230}$). It is generally difficult to develop a universal DNA extraction method that can apply across different soil types. A few studies have developed extraction procedures with modifications to make it suitable for DNA extraction from different soil types [24]. Our results showed that the developed SKMM procedure worked well in all tested soil samples including soils with high organic matter and total carbon as soil NI6-19.

**Detection limit in artificially infested soil**

*Meloidogyne hapla* DNA was poorly detected in the artificially infested samples. Positive DNA amplification in rt-LAMP was obtained from one out of the three technical replicates of the clayey and sandy soils inoculated with 64 J2s (250 $g^{-1}$) and extracted and purified with kits. The lowest detected level in sandy soil was 32 J2s (250 $g^{-1}$). DNA extracted with SKMM showed positive amplification from the sandy soil inoculated with 8 and 64 J2s (250 $g^{-1}$), but none of the clayey soil samples. This inconsistency might be due to insufficient soil homogenization, as the amount of extracted DNA from all samples regardless of the extraction method, did not proportionally increase with the number of added juveniles [9]. The range of 4 – 64 juveniles 250 $g^{-1}$ was chosen in consultation with HS NL as it represents the most common densities usually found in the commercial vegetable fields in Sweden. The aim here was to determine the detection limit of rt-LAMP and compare it with the tolerance level used in practice, 1 – 4 J2s (250 $g^{-1}$ soil). In another LAMP study, artificially inoculated samples were generated by adding as much as 10,000 J2s to 100 g of soil, to generate artificially infested samples with the RKN *M. chitwoodi* [25]. Probably, adopting similar inoculation strategy, then diluting soil with nematode-free soil to generate different densities, could have been more successful than adding separate numbers of juveniles per sample.

***Meloidogyne hapla* populations decline over time**

In general, *M. hapla* DNA was under the detection limit of rt-LAMP in most of the 20 samples collected in 2019. The samples were processed immediately and were kept in cold storage until DNA extraction and rt-LAMP analysis in 2021. In general, it is recommended to analyze soil immediately or shortly after sampling [24]. On the other hand, when soil is air-dried and homogenized, it can be kept at room temperature and used within 6 months without a risk of DNA degradation [24]. DNA was amplified from two samples (kits) and three samples (SKMM) out of the 20 soil samples. The difference between the two DNA extractions methods was clearly shown by the estimated number of juveniles, especially in sample NI6-19. Using DNA extracted with SKMM, positive DNA amplification in rt-LAMP was reached after 12 min and the average number of juveniles was 184 J2s (250 $g^{-1}$ soil), which is closer to the density estimated by morphological analysis in 2019 (Table 2). The amplification time for DNA extracted with the kits from the same soil sample was 16 min and the corresponding number of juveniles was 15 J2s (250 $g^{-1}$ soil). These results indicate a strong inhibition in rt-LAMP performed with DNA extracted and purified with kits, in form of a signal delay or failure of DNA amplification [9]. LAMP is known to be more resilient than PCR when it comes to inhibition of DNA amplification by humic acid and it can tolerate up to 100 ng $\mu L^{-1}$ per reaction [26]. Therefore, it can be speculated that DNA extracted from sample NI6-19 with the commercial kits contained an amount of humic acid exceeding the reported tolerance level [26].

Table 1. DNA extraction from naturally and artificially *Meloidogyne hapla* infested soil samples using the manual procedure SKMM and the commercial kit FastDNA kit (kit 1)

followed by DNA purification with kit 2 and 3. Total DNA concentration and absorbance ratios indicating purity from protein ($A_{260}/A_{280}$) and humic acid ($A_{260}/A_{230}$) were measured by a spectrophotometer. Correlation between DNA concentration and soil organic matter is indicated by r-values of correlation coefficient (Corr. Coeff)

| | **DNA** (µg $g^{-1}$ soil) | | **Absorbance ratios** | | | | **Corr. Coeff[8]** |
|---|---|---|---|---|---|---|---|
| | | | ($A_{260}/A_{280}$) | | ($A_{260}/A_{230}$) | | **(r)** |
| NF-17[1] | | | | | | | |
| Kit 1[4] | 15 | a | 1.80 | b | 0.22 | c | |
| Kit 2[5] | 11 | b | 1.83 | a | 0.36 | b | |
| Kit 3[6] | 8 | c | 1.81 | b | 0.90 | a | 0.98 |
| SKMM[7] | 8 | c | 1.80 | b | 0.99 | a | 0.83 |
| *p* | *<0.0001* | | *0.0005* | | *<0.0001* | | |
| *CV* | *37,1* | | *1.9* | | *66.1* | | |
| NI-19[2] | | | | | | | |
| Kit 1 | 28 | a | 1.65 | c | 0.20 | c | |
| Kit 2 | 10 | b | 1.78 | a | 0.17 | c | |
| Kit 3 | 6 | d | 1.74 | b | 0.57 | b | 0.93 |
| SKMM | 7 | c | 1.76 | a | 0,81 | a | 0.83 |
| *p* | *<0.0001* | | *<0.0001* | | *<0.0001* | | |
| *CV* | 167,9 | | 6,2 | | 69,3 | | |
| NI-20[3] | | | | | | | |
| Kit 1 | 13 | a | 1.75 | b | 0.10 | c | |
| Kit 2 | 5 | b | 1.79 | a | 0.13 | c | |
| Kit 3 | 3 | c | 1.80 | a | 0.49 | b | 0.60 |
| SKMM | 6 | b | 1.72 | b | 0.62 | a | 0.86 |
| *P* | *<0.0001* | | *<0.0017* | | *<0.0001* | | |
| *CV* | *106* | | *7* | | *82* | | |

[1]NF-17: root-knot (RKN) nematode free soil collected in 2017; [2]NI-19: naturally RKN infested soil collected in 2019; [3]NI-20: naturally RKN infested soil collected in 2020; [4]Kit 1: FastDNA kit for soil (FD kit 1) (extraction); [5]Wizard DNA kit (kit 2) (DNA purification step 1); [6]MicroSpin S-300 HR Columns (kit 3) (DNA purification step 2); [7]SKMM (Skim milk procedure); [8]correlation between DNA concentration and soil organic matter content.

Table 2. Comparison of nematode densities estimated by morphological analysis and real-time LAMP analysis of *Meloidogyne hapla* DNA in three naturally infested soil samples collected in 2019. DNA used in real-time LAMP was extracted from soil and purified using commercials or the manual procedure SKMM

| **Soil sample** | **Morphological analysis** | **LAMP-Kit 3** | **LAMP-SKMM** |
|---|---|---|---|
| NI6-19 | 261 | 184 | 15 |
| NI11-19 | 9 | 37 | 0 |
| NI13-19 | 8 | 6 | 6 |

**The patchiness of *Meloidogyne hapla* occurrence in fields**

According to the original plan in WP3 and objective three, the LAMP assay was supposed to be compared with the classical soil analysis with Seinhorsts elutriation and microscopic

identification. Due to the introduction of *M. chitwoodi* and *M. fallax* in Sweden, it was necessary to use a soil analysis method that can reliably identify and differentiate between these species and *M. hapla*. It was also obvious to compare LAMP with another molecular technique such as rt-PCR. The rt-PCR analysis that was used in this study was performed by a commercial company (ISD). Both rt-LAMP and rt-PCR confirmed the occurrence of *M. hapla* DNA in samples collected from only two out of six fields in 2020. The estimated densities with rt-LAMP were significantly higher than those estimated by qPCR (Fig. 3). According to rt-PCR analysis, nematode densities in the "W" pooled soil samples from the two fields, NI5–2020 and NI6–2020, were 50 and 4 J2s (250 $g^{-1}$ soil), respectively. The corresponding densities estimated by rt-LAMP were 110 and 15 (Kit 3) (Fig. 3a) compared to 183 and 12 J2s (250 $g^{-1}$ soil) (SKMM) (Fig. 3b), but the difference was not significant. Within-field distribution of *M. hapla* ranged from 12 to 51 in field NI5-20 and from 7 to 17 in field NI6–20 (Fig. 3a). The distribution range according to rt-LAMP was 0 to 86 (field NI5–20) and 0 to 22 (field NI6–20) (Kit 3). The corresponding range with DNA extracted with SKMM was 0 to 173 and 0 to 23 J2 250 $g^{-1}$ soil (Fig. 3b). The accuracy of the rt-PCR was influenced by the indirect approach of DNA extraction adopted by the commercial lab, ISD. In this case the motile juveniles were collected from soil prior to DNA extraction. Total DNA extraction from soil, which involves all life stages of *M. hapla*, probably explains the difference between the molecular methods in their estimated *M. hapla* populations.

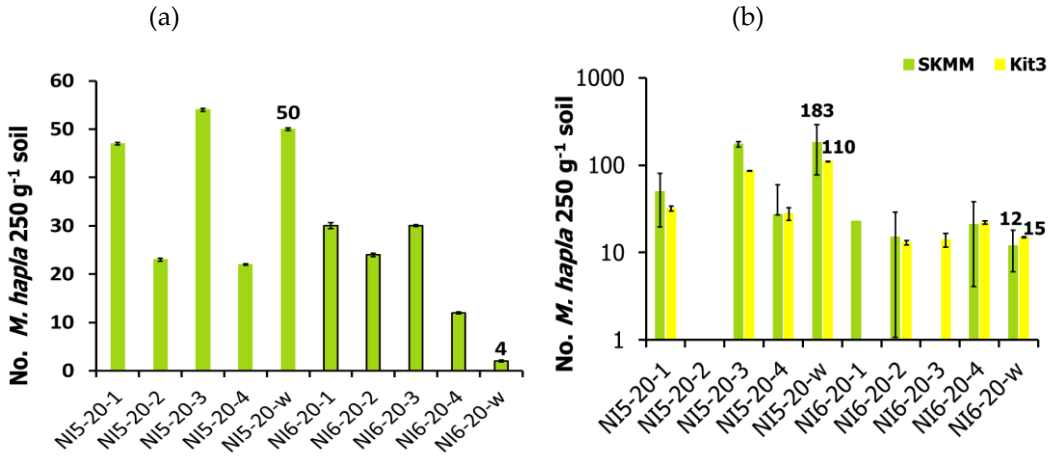


Figure 3. Analysis of *Meloidogyne hapla* in soil samples collected from field NI5-20 and NI6-20 (n=5): (a) with rt-PCR; (b) analysis with rt-LAMP using DNA extracted with SKMM and FastDNA kit followed by DNA purification with Wizard DNA and MicroSpin S-300 HR Columns commercial kits. Note: Log on y-axis (b).

**Is the "W" pooled sample representative?**

The results showed that both molecular methods, rt-LAMP and rt-PCR, confirmed the spatial distribution of *M. hapla* especially when the densities are high (>100). In this case, the "W" pooled sample gave an estimation of nematode density over the whole field without identifying "hot spots" that probably need more focus when planning control measures as in field NI5-20 (Fig. 4). A similar tendency has been shown for other soil-borne plant pathogens [13] as *Plasmodiophora brassicae* casing clubroot in Brassica crops. Such patches or hot spots can be as large as one hectare, in the case of carrots may leads to an economical loss of 50 000 SEK/ha. Worth mentioning is that, although the population densities in some fields were quite high according to the morphological determination in the preceding autumn/winter [9], both rt-LAMP and rt-PCR failed to detect *M. hapla* DNA in these fields the following spring. The

northern root-knot nematode, *M. hapla*, is known for its cold tolerance, hence it is more adapted to temperate regions [27]. The sharp decline of the populations in these fields was probably related to biotic and/or other abiotic factors than the cold temperature during Swedish winter.

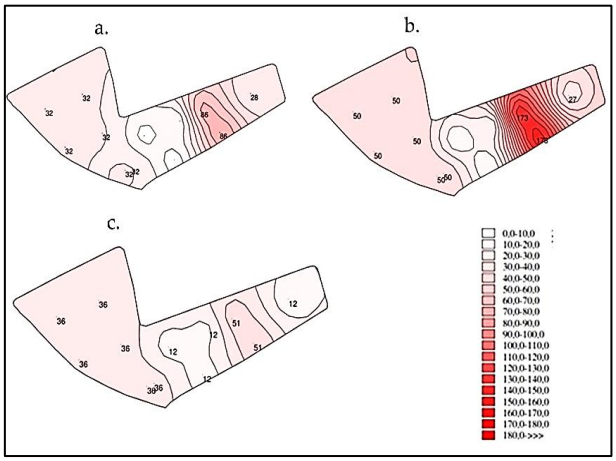


Figure 5. In-field variation of *Meloidogyne hapla* occurrence in commercial field NI5-20 according to: rt-LAMP analysis conducted with a) DNA extracted and purified with commercial kits and b) DNA extracted with SKMM; c) rt-PCR.

**Conclusions**

- An efficient and cost-effective DNA extraction procedure (SKMM) was developed in this project. Together with rt-LAMP it gives the growers an excellent low-cost soil analysis for *M. hapla*.
- SKMM worked across different soil types and the obtained DNA concentration was higher and purer compared to the commercial kits.
- To avoid decline of nematode populations, soil samples should be analyzed immediately or shortly after sampling.
- Real-time LAMP was unsuccessful in detecting *M. hapla* DNA at the tolerance level in the artificially inoculated clayey and sandy soils, but in some naturally infested soils the estimated populations were close to the one used in practice (1-4 J2s 250 $g^{-1}$ soil.
- Multiple samples are important to accurately assess the occurrence of *M. hapla* within a specific field.
- Apart from the fact that high nematode densities occurred in the six fields in autumn/winter 2019, *M. hapla* was only detected in two fields in spring 2020. Further research is needed to better understand the population dynamics and decide the optimal time point for soil sampling.

**Benefits for the agricultural industry**

Molecular identification of *M. hapla* by rt-PCR is commercially available to Swedish growers, but the method is used for detection rather than for quantification of *M. hapla* in soil. Quantification is important to be able to plan sustainable management strategies because yield loss by *M. hapla* is density dependent. In this project, a DNA analysis based on rt-LAMP was proven to be specific and was able to quantify *M. hapla* in different naturally infested soil samples. As DNA extraction is the most expensive part of molecular soil analysis, it would be desirable to use an efficient but at the same time cost-effective DNA extraction method prior to DNA analysis by either rt-PCR or rt-LAMP. The developed DNA extraction procedure

"SKMM" was found to be as efficient as the three commercial kits and even superior when extracting DNA from certain soil samples. The estimated material cost is approximately half the one of the commercial kits. This reduction in DNA extraction costs reduces the overall cost of rt-LAMP analysis, which is in line with the overall goal of the project. We also reached out to the industry and discussed the potential of commercializing the rt-LAMP.

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# Del 3: Resultatförmedling

*Ange resultatförmedling av projektet, inklusive titel, referens, datum, författare/talare, och länk till presentation eller publikation om tillämpligt. Planerade publiceringar (med preliminära titlar) ska ingå i tabellen. Ytterligare rader kan läggas till i tabellen.*

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|---|---|
| **Vetenskapliga publiceringar** | **Omer, Z.S; Wallenhammar, A-C; Viketoft, M**. Development of Loop-Mediated Isothermal Amplification Assay for Rapid Detection and Analysis of the Root-Knot Nematode *Meloidogyne hapla* in Soil. *Horticulturae* **2022**, *8*, 87-104. https://doi.org/10.3390/horticulturae8020087<br><br>(Special Issue Recent Advance in the Identification and Diagnostics of Plant-Parasitic Nematodes) |
| **Övriga publiceringar** | **Omer, Z.S; Andersson, S; Wallenhammar, A-C; Viketoft, M**. 2022. Rotgallnematod bestäms med ny DNA-baserad analysmetod. *Viola* (Grönsaker), nr. 4, pp: 20–21.<br>**Omer, Z.S; Andersson, S; Wallenhammar, A-C; Viketoft, M**. 2022. Rotgallnematod bestäms med ny DNA-baserad analysmetod. *Viola* (Potatis), nr. 2, pp: 23–24.<br>**Omer, Z.S; Andersson, S; Wallenhammar, A-C; Viketoft, M**. 2022. 'Ny teknik kan artsbestemme rodgallenematoder' *Gartner Tidende*, In press |
| **Muntlig kommunikation** | **Omer, Z.S.** 2019. Analys av rotgallnematod i jord med ny DNA-metod. SLF trädgårdsdag, 2 april, Alnarp<br><br>**Omer, Z.S**. 2022. Analysis of *Meloidogyne hapla* in soil using Loop-mediated Isothermal Amplification technique. 7th International Congress of Nematology, May 6, Antibes Juan-les-Pins, France.<br><br>Participation in the congress was financed by KSLA (travel grant number: GFS2019-0080) |
| **Studentarbete** | Alexander Pettersson: Agronomprogrammet – mark/växt Kurs: Kvalificerad agronompraktik 1.<br>Two weeks practical work with DNA extraction from soil |
| **Övrigt** | **Omer, Z.S; Wallenhammar, A-C; Viketoft, M; Andersson, S.** 2018. Analys av rotgallnematod i jord med ny DNA-metod. Brunnby lantbrukardagar. Poster presentation |
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| | **Omer, Z.S; Viketoft, M; Wallenhammar, A-C; Andersson, S.** 2018. Analys av rotgallnematoder i jord med ny DNA-metod. Nationell Växtskyddskonferens, 14-15 November, Uppsala. Poster presentation |
| | **Omer, Z.S; Viketoft, M; Wallenhammar, A-C; Andersson, S.** 2018. Loop-mediated Isothermal Amplification assay for rapid detection of Meloidogyne hapla in carrots. European Society of Nematologists Conference, 9-13 September, Ghent, Belgium. Poster presentation<br><br>Participation in the conference was financed by HS Konsult AB |