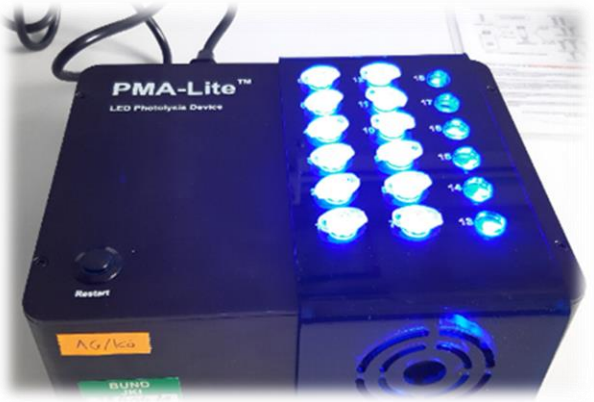


Julius Kühn-Institut
Bundesforschungsinstitut für Kulturpflanzen
Federal Research Centre for Cultivated Plants

Methods for viability testing in Nematology – A proof for treatment efficacy to control potato cyst nematodes (PCN) in residual soils

König, S., Berger, B., Aukamp-Timmreck, C., Reimann, K., Daub M.

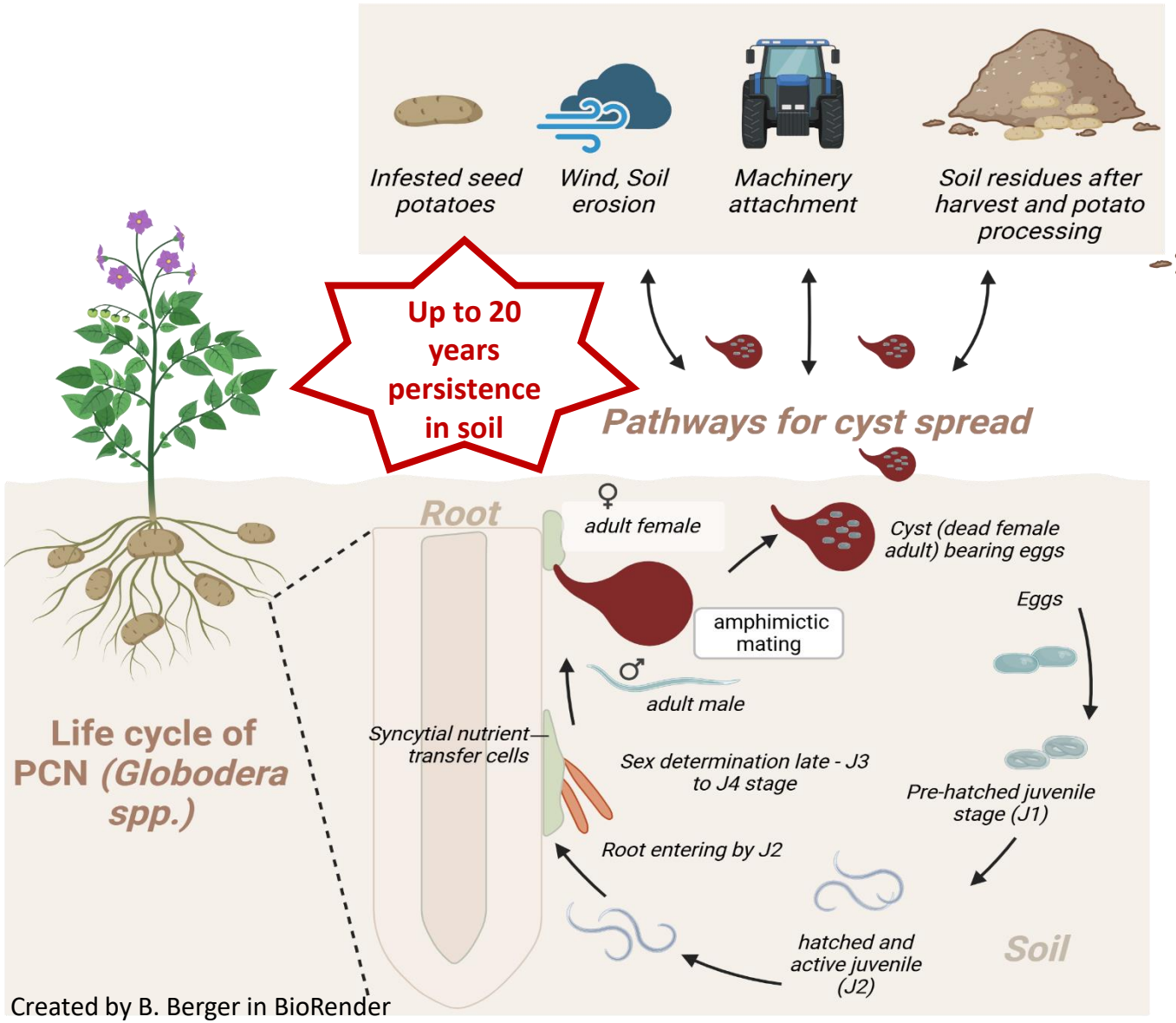
6th EPPO Workshop for Heads of Plant Pest Diagnostic Laboratories; Saku (EE), 2025-03-06/07

Julius-Kuehn Institute – Federal Research Centre for Cultivated Plants stephan.koenig@julius-kuehn.de

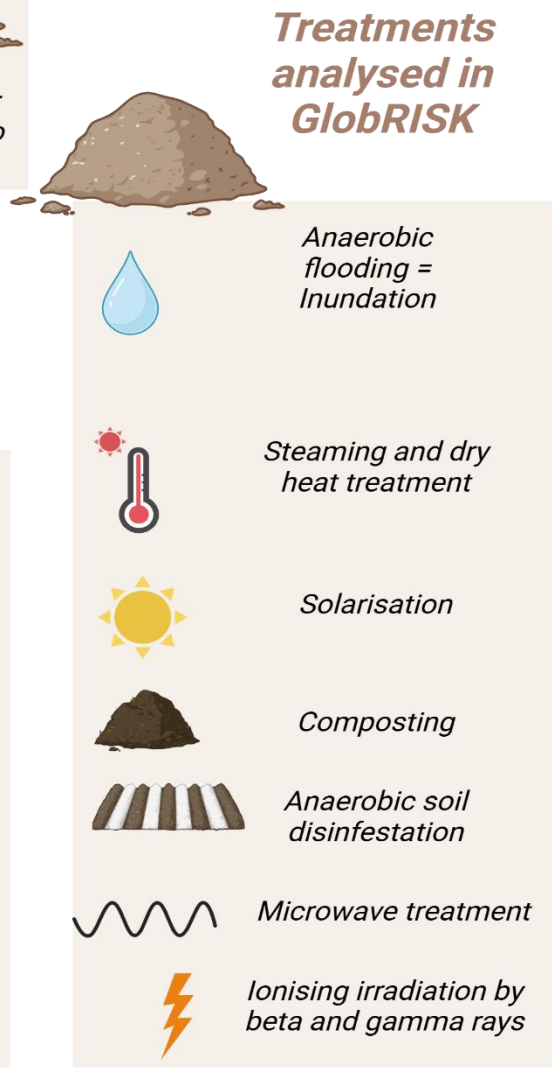
www.julius-kuehn.de

PCN* biology, spread & treatments

* *Globodera pallida* (STONE) SKARBILOVIC & *G. rostochiensis* (WOLLENWEBER) SKARBILOVIC



Created by B. Berger in BioRender

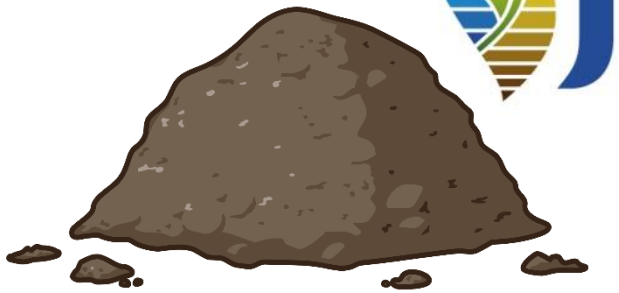


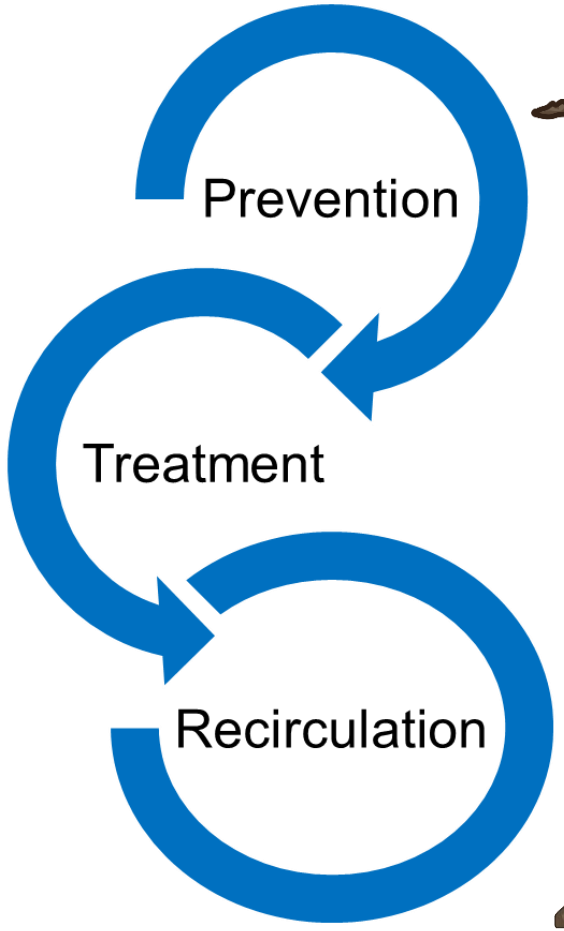
www.julius-kuehn.de

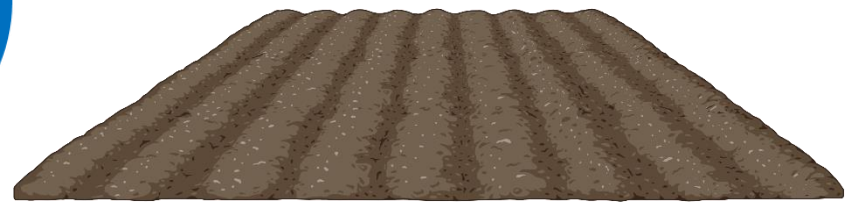
Project GlobRISK

Project objectives of GlobRISK

- >**1 million** tons of soil and plant residues from beet and potato processing every year = **2.2 to 3.2 t** per ha
- Minimising the spread of cyst nematodes
- Sustainable production of “healthy” potatoes
- Inexpensive recycling of huge amounts of nematode infested soils
- Protection of soil resource



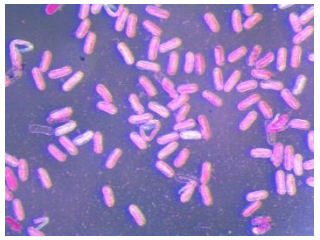


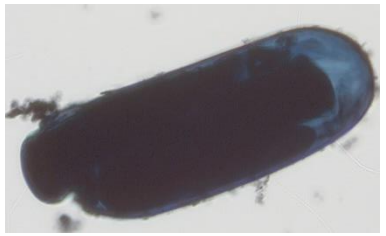


Definitions

Viability

direct assessment of the vital potential of eggs and juveniles in a sample by means of hatching test, morphological characteristics, after lethal staining, biochemical or molecular biological examination





Reproducibility

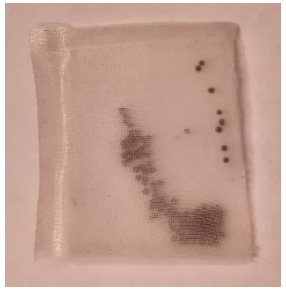
analysis of a sample of juveniles or cysts in their ability to go through the entire development cycle until the emergence of new offspring in a (semi-)natural system on host plants suitable for reproduction

➡ Quantitative detection of the viability and, if possible, developmental capacity of the nematodes is required to assess the effectiveness of the treatments for nematode control

Bioassays - PCN

Methods & Materials*:

PCN-fully susceptible potato variety ‘Desiree’, 9x9.5cm TEKU plastic containers, loess soil, 3-4M Osmocote® fertilizer, 20-25 nematode cysts (=10-15 eggs/g soil) placed in gauze bags (initial population P_i) close to the host plant, termination after reaching a daily temperature sum of 1850 K; washing through a 250 µm sieve and counting of the newly formed cysts outside the gauze bag (final population P_f)



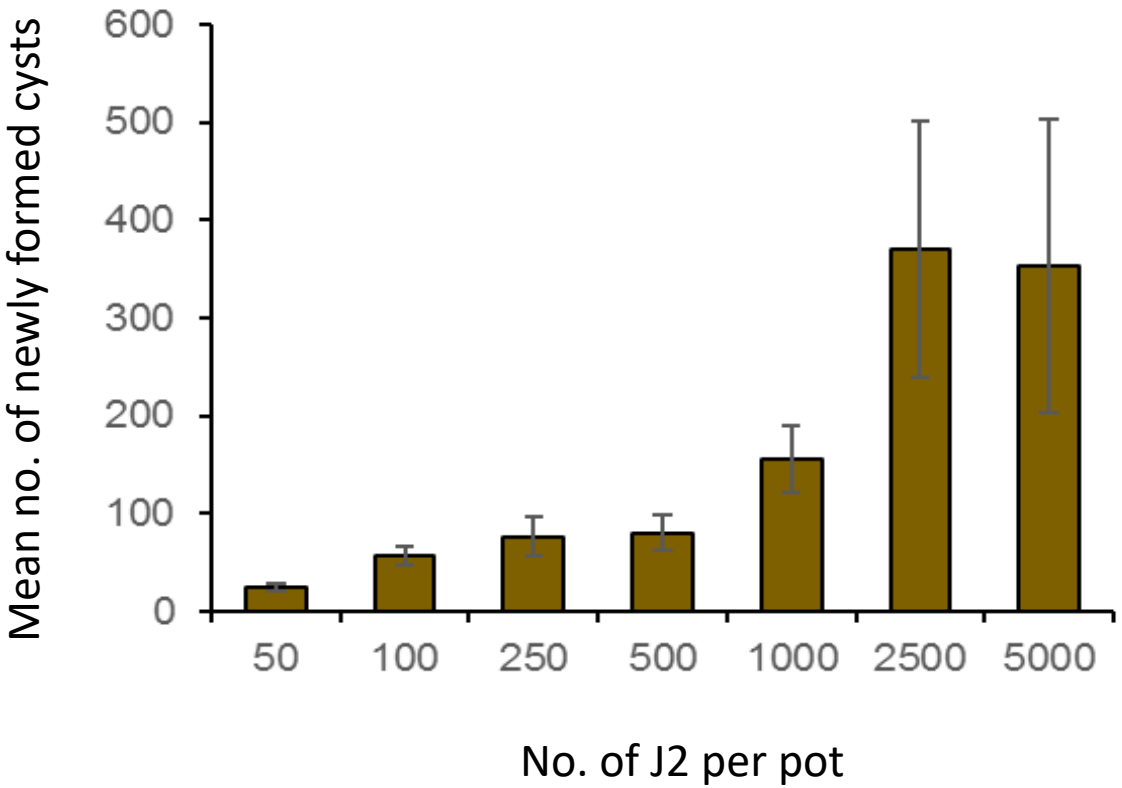
Pros & Cons:

- Only test method that also simulates natural environment of nematode development in the greenhouse
- Time-consuming (~100 d)
- Breaking the nematodes dormancy at 4°C not always successful
- Limitation of implementation between (February) March and June (July) allows a maximum of 2 test approaches per year

*PM7/40(6) and KORT, J. et *al*: International scheme for identifying and classifying pathotypes of potato cyst-nematodes *Globodera rostochiensis* and *G. pallida*. 1977 Nematologia 23:333-339

Bioassays

Preliminary testing of juveniles instead of cysts



- The preparative effort to release the juveniles is time-consuming and destroys up to 20% of the eggs/juveniles
- Optimal linear correlation between 50 to 250 J2 per pot, density dependent negative effect starting at 2.500 J2
- ~ Optimum in abundance for 15 eggs per g soil (identical to the results of Kort et *al*. 1977)
- Using 15 to 20 cysts per replicate fits this ratio for quantification within a linear range
- The formation of approx. 50% females indicated a balanced nutrient supply in the individual pots (n=3)

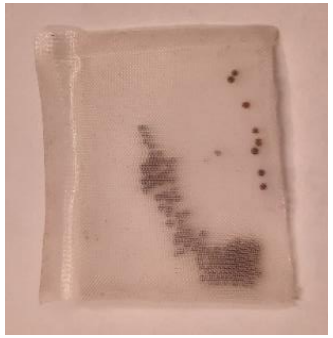
Kort, J., Ross, H., Stone, A.R. & Rumpenhorst, H.J. (1977). An international scheme for identifying and classifying pathotypes of potato cyst-nematodes *Globodera rostochiensis* and *G. pallida*. *Nematologica*, 23, 333–339.

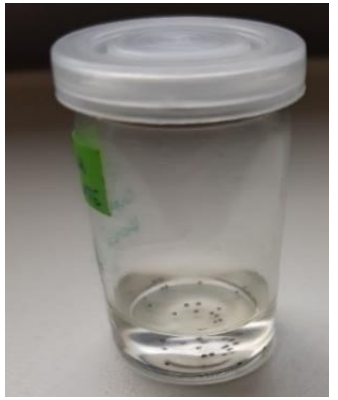
Hatching test

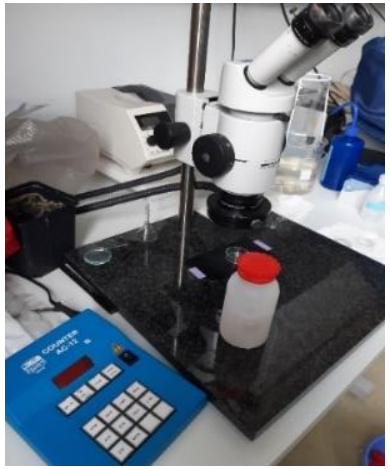
Stimulation of juvenile hatching with potato root diffusates (PRD) or with picrolonic acid (PA)

Methods & Materials

- PRD liquid obtained by leaching growing potatoes (cv. ‘Desiree’, 3 weeks, sand matrix) with tap water
- Cysts of *G. pallida* ‘Kalle’ with diameter >500 µm pre-soaked in water for 2 days at RT
- Treatment of 3-5 sample replicates with 20 cysts each over a period of at least 49 days with PRD or picrolonic acid (PA) of various concentrations at 20°C
- Counting of hatched juveniles at weekly intervals under the binocular





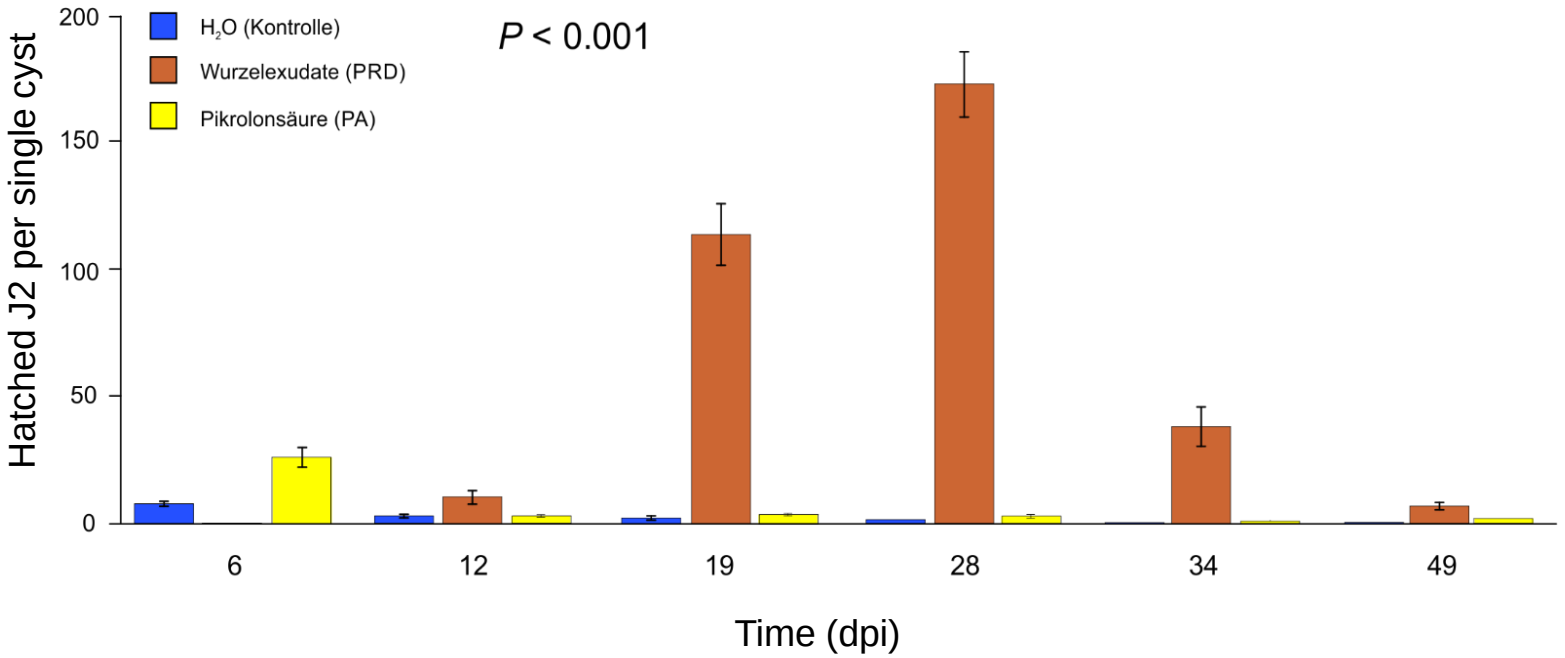


Pros & Cons:

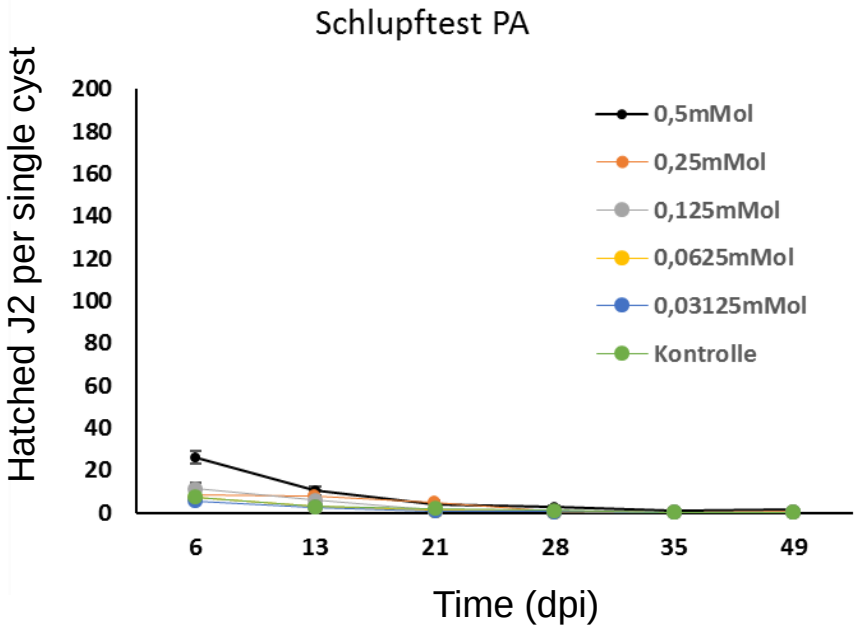
- Less time-consuming than bioassays (~49 d)
- Apparently higher susceptibility to endogenous dormancy of nematodes
- No seasonal restrictions

Results hatching test

Hatching activity using PRD, PA & tap water



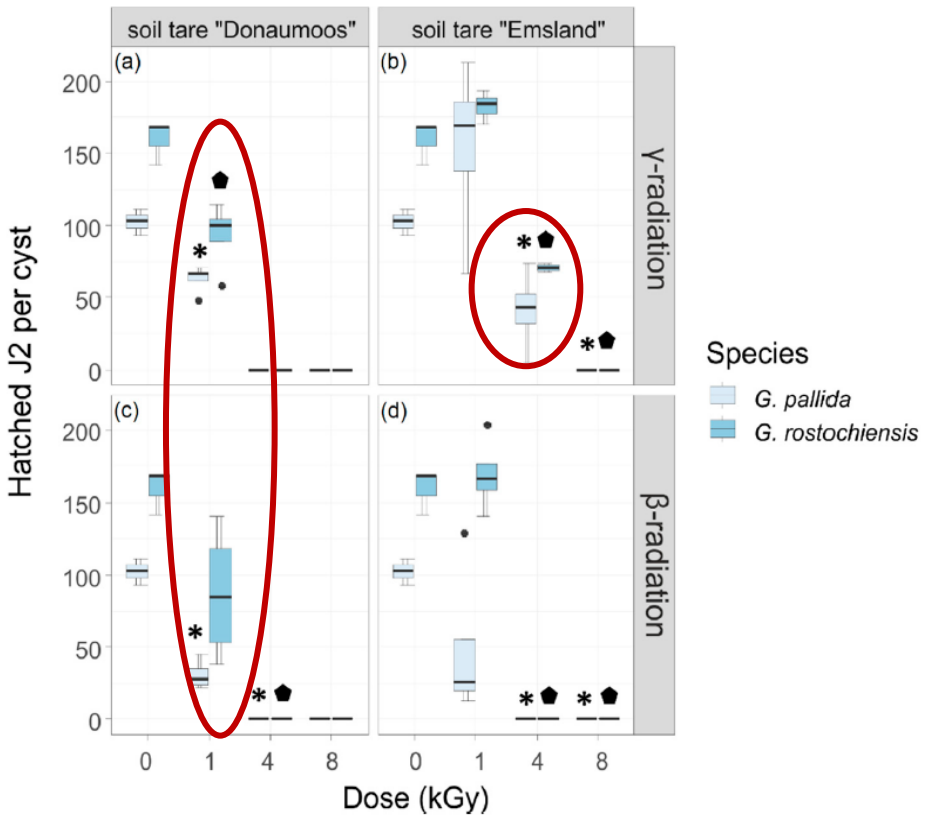
Concentration PA



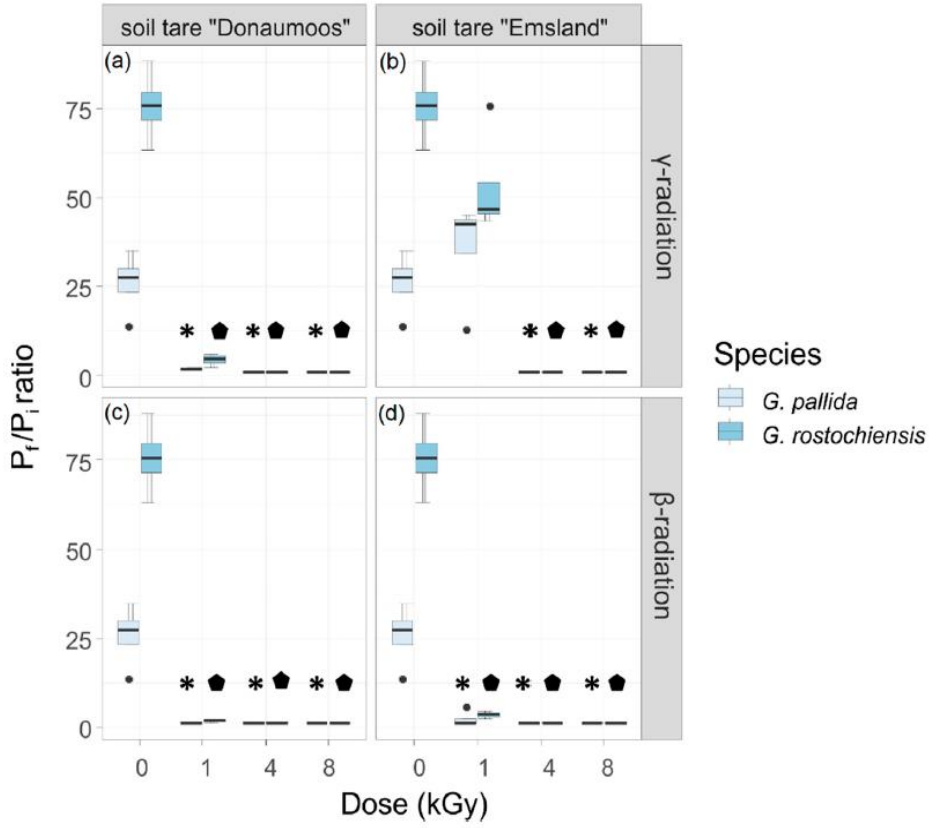
Stimulation of PCN juvenile hatching by complex root excretions (PRD) better than by artificial single component as PA – Nevertheless, for some cyst nematodes (e.g. *Heterodera schachtii*) artificial hatch stimulants e.g. 5mM $ZnCl_2$ work excellent

Comparison between bioassay & hatch test in “practise”

Hatching test



Bioassay



Bioassays mostly confirmed results of nematode viability from hatching tests - with the exception of studies on the β- and γ-irradiation of residual soils

Difference in irradiation treatment - hatching test showed high rate of viable nematodes at dose 0 and 1 KGy while bioassay after irradiation showed no or lower reproduction, the last in denser parabrown ‘Emsland’ soil – **this indicates that juveniles can hatch BUT NOT reproduce after irradiation at a dose of 1 (4) KGy**

Berger, Beatrice; Schumann, Lisa; Daub, Matthias; König, Stephan (2022): An Evaluation of Irradiation Treatment to Disinfect Soil Tare from *Globodera* spp.. Agronomy. 12 (2), 464. **Doi:** 10.3390/agronomy12020464 Link OpenAgrar

Morphological viability assessment

- Frequently used method
- Evaluation according to characteristics such as strongly angled posture of the juveniles, black coloration of the tissues and vacuole formation in the abdominal cavity
- Subjective factor - dependent on the person carrying out the examination
- Mostly underestimation of the potential of viable nematodes
- Labor-intensive and time-consuming for exact quantification
- Qualitatively meaningful; illustration of treatment successes

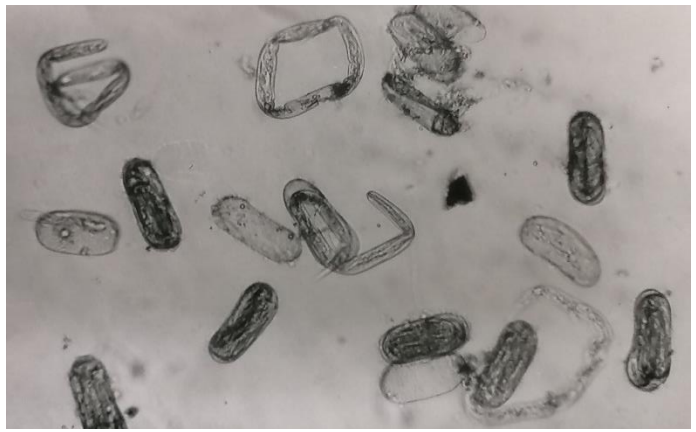
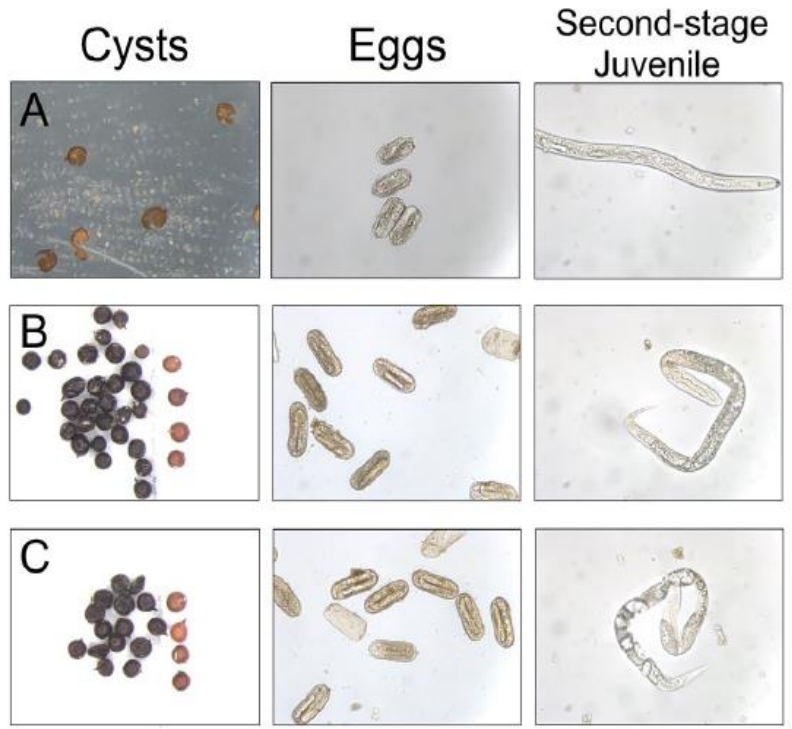


Photo from a viability study of PCN in waste water (E. Lücke 1964)

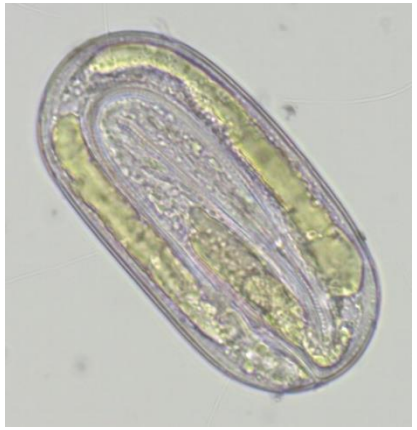


Cysts, eggs and juveniles in an untreated control (**A**); after 10 d of fermantation (**B**) after fermentation and 14 d of composting (**C**)

Schumann, L.; Berger, B.; Daub, M.; Böhlke, T.; König, S. (2023): Industrial-scale composting process as a successful method for inactivation of potato cyst nematodes (*Globodera* spp. Skrabilovich) and sugar beet cyst nematode (*Heterodera schachtii* Schmidt). JPDP. 130 (6); 1317-1330.

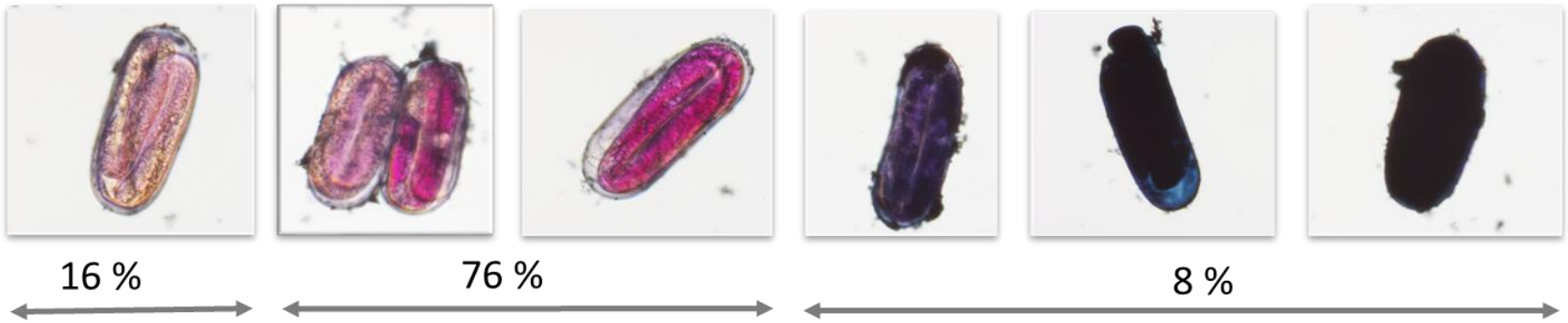
Lethal staining methods

- Various dyes (MELDOLA BLUE as the strongest dye according to Shepherd 1962; NILE-BLUE A; Malachite green)
- Principle of lethal coloration means that only eggs and juveniles with an defect in egg membrane or cuticle/hypodermis absorb the dye in the body tissue (pseudocoelom)
- Evaluation under a light microscope (250x/400x magnification)
- Subjective evaluation - mostly overestimation of viability
- Qualitative statement possible – correct quantification?
- Not in EPPO Standard PM7/40 - dyes often difficult to obtain



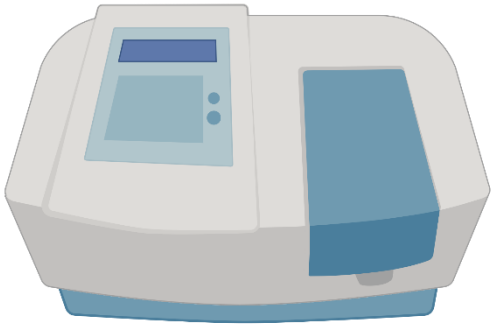
Dark blue or purple -> **non-viable nematode**
Uncoloured or only stained in intestinal granules -> **viable nematode**

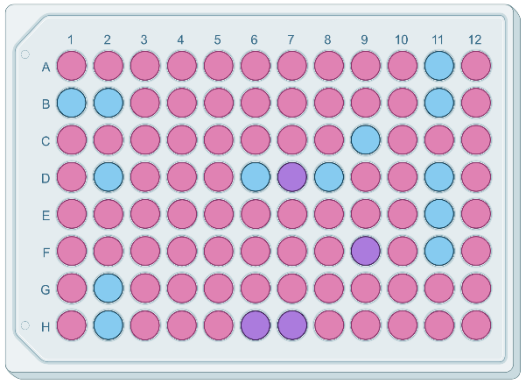
Practice: Quantitative differentiation between dead and alive by sequential colour gradients not reliable (depending on incubation time and subjective decision of the processor)



Biochemical quantification by trehalose concentration

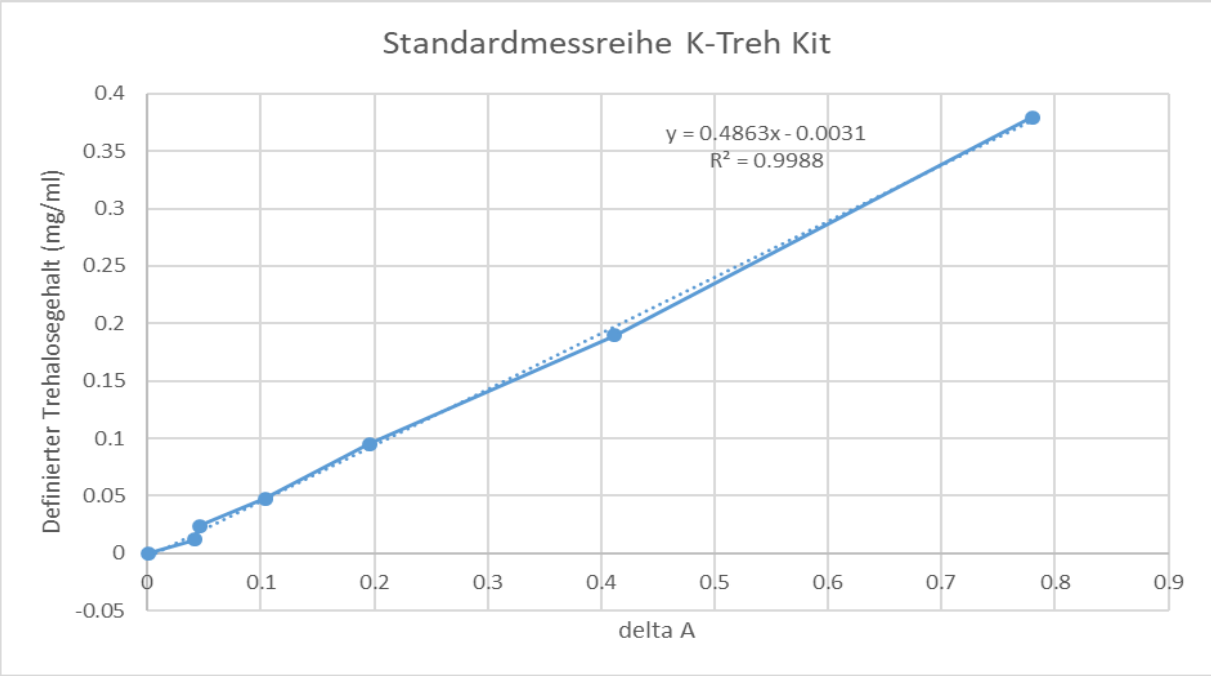
- Species-independent; Beniers et al 2014 and EPPO PM 7/40(5) Appendix 12
- Marker trehalose as a disaccharide 1,1-glycosidically linked glucose molecules in perivillin fluid of living eggs
- 2 d incubation of the cysts in approx. 200 µl MilliQ-Water -> 30 min heating to 99°C -> release of the egg contents with trehalose
- **K-Treh Kit** (Megzyme Ltd., Wicklow, IE) Kit reagents: Trehalase cleavage into 2x glucose -> phosphorylated by ATP -> glucose-6-P -> electron transfer to NADP+ -> $NADPH/H^+$ -> measurement in spectrophotometer at wavelength λ = 340 nm
- **Absolute quantification** along standard curve using kit reagents
- **Size of a single test approach limited to max. 1-2 cysts** - larger amounts of cysts require dilution = increase in measurement uncertainty



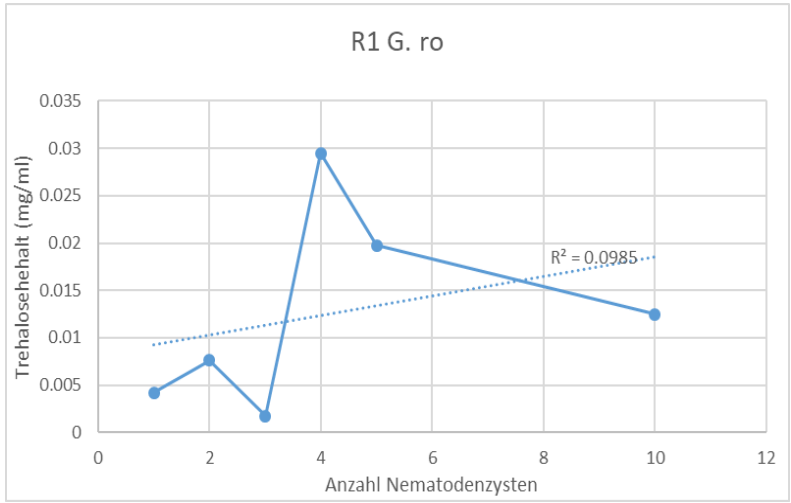


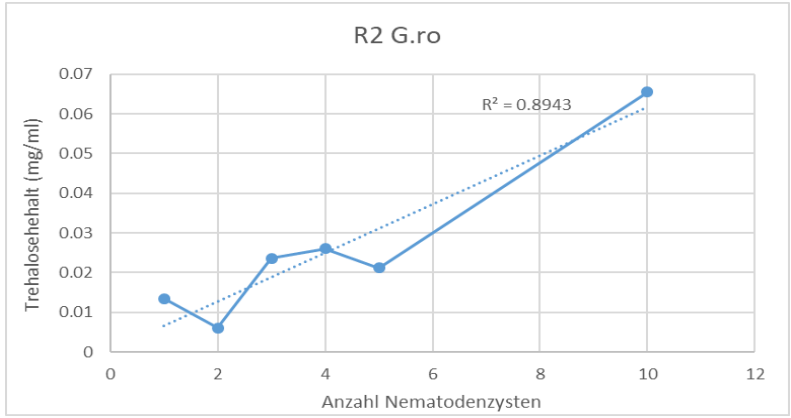
Beniers, J.E., Mendes, O., Van Gent-Pelzer, M.P., Van Der Lee, T.A. & Been, T.H. (2014). Quantification of viable eggs of the potato cyst nematodes (*Globodera* spp.) using either trehalose or RNA-specific Real-Time PCR. *Nematology*, 16, 1219–1232.

Biochemical quantification by trehalose concentration



R^2 = 0.9988; linear photometrically determined trehalose yield



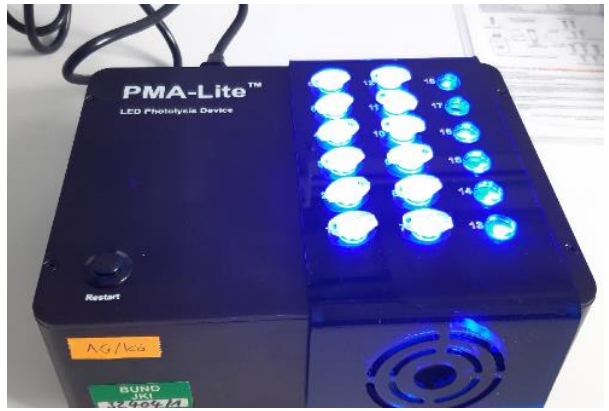


- Measurement series of 1-10 cysts (from 3 cysts dilutions 1:2 to 1:5)
- Technically not reliably replicable
- Quantitative measurement not linear
- Qualitative statement usable

Molecular Methods – DNA blocking

Propidium-Monoazid (PMA) DNA Blocking

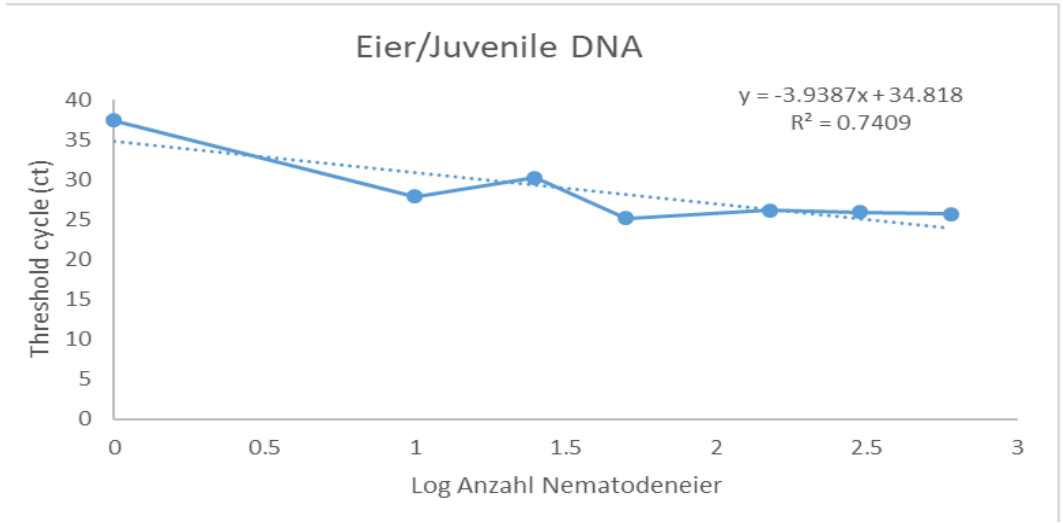
- Method not in the EPPO protocol; published by Christoforou et al. 2014
- DNA can persist in the soil for weeks to years; detection by PCR/qPCR thus gives no indication of the viability of the analysed nematodes
- DNA blocking can also be used regardless of species; subsequent identification and quantification with species-specific molecular tools
- Principle: PMA intercalates photoactively in DNA double strand, cannot penetrate cytoplasmic membrane due to molecule size, but binds to free DNA
- Sample preparation: Cysts incubated for 2 d in tap water, carefully opened 1-600 eggs or juveniles separated
- Working solution: dissolve 10 mg PMA in 19.6 ml dimethethyl sulphoxide = **1 mM PMA**
- Procedure: 10 µl PMA working solution per batch - incubate for 30 min in the dark, then for 30 min at light intensity > 650 W halogen lamp or in the PMA Lite LED photolysis device

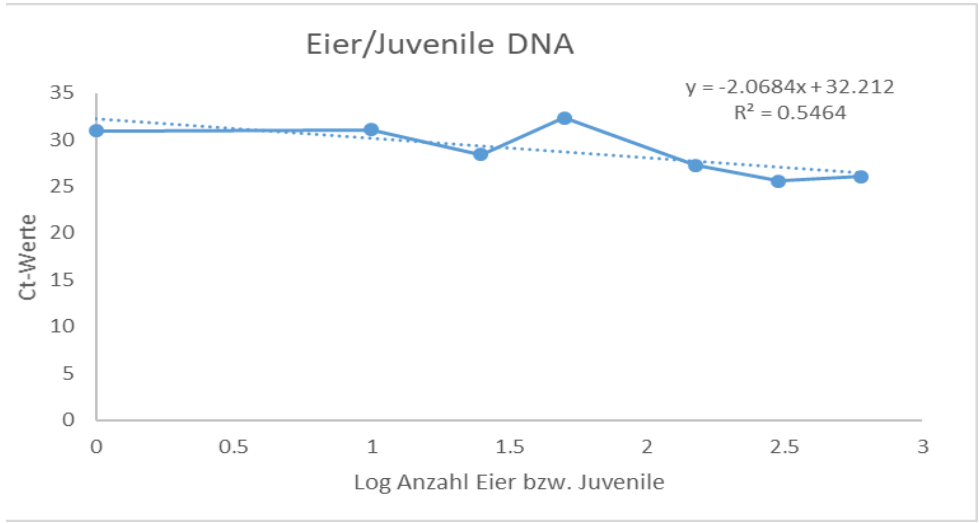


Christoforou, M., Pantelides, I.S., Kanetis, L., Ioannou, N. & Tsaltas, D. (2014). Rapid detection and quantification of viable potato cyst nematodes using qPCR in combination with propidium monoazide. *Plant Pathology*, 63, 1185–1192.

Molecular Methods – DNA blocking

Log J2 count in the real-time TaqMan assay according to Gamel et al. 2017





Globodera pallida; R^2 = 0.74; PCR-efficiency = 204 %

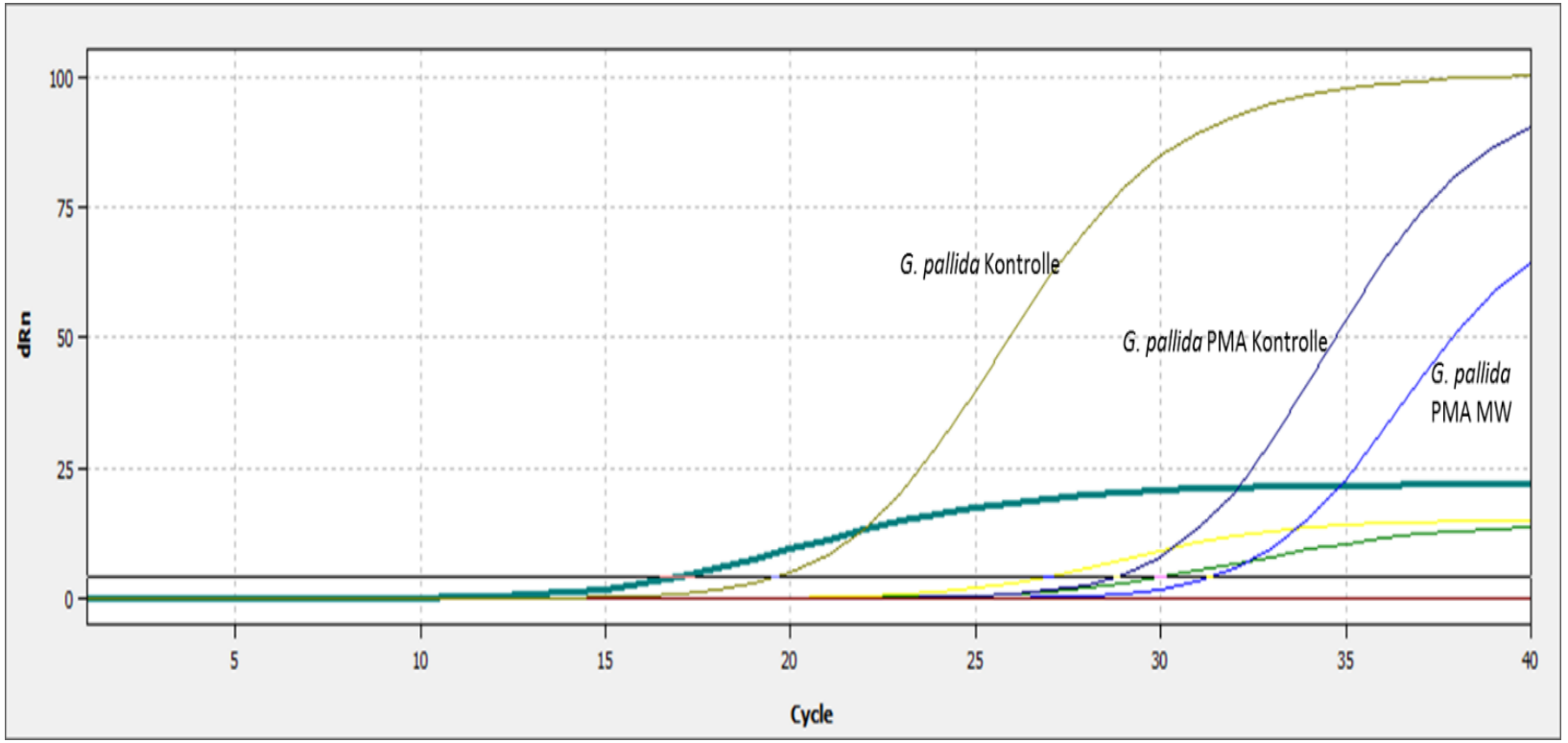
G. rostochiensis; R^2= 0.55; PCR-efficiency = 79 %

Calculation of PCR-efficiency: $E = 10 \exp\left(-\frac{1}{slope}\right)$

Optimal PCR efficiency (doubling every 3.32 cycles) 95-105%

No linear correlation = absolute quantification of nematode specimen impossible

Molecular Methods – DNA blocking



Examined cysts of *G. pallida* (untreated control; control incubated with PMA; cysts incubated in PMA heated to >80°C for 5 min in microwave) - after heating < 1 live egg remains in absolute quantification

- Detection limit with the preparation mostly >1 egg
- Result is consistent with hatching tests
- **Reliable in a qualitative way – exact quantification?**

Conclusions

- <u>Clear disadvantages of hatching test and bioassays are:</u>
  - dependence on the endogenous state of the cysts (dormancy - diapause or quiescence)
  - availability of host plants in the appropriate developmental stage for infection for biotesting
  - time effort
  - quarantine requirements
  - ➢ Relative rates depending on controls with the same development cycle (population and propagation stage with subsequent storage at ±4°C) allow quantitative evaluation in controlled experiments
  - Staining and morphological evaluation are subjective, labor-intensive with often overestimation in staining (especially Meldola's blue according to Shepherd 1986) and incorrect evaluation in morphological comparison
  - **Quantification** in trehalose detection and PMA blocking approach not/only partially reliable

> **For qualitative testing of viability, all approaches can be used in practice**

Gefördert durch

Bundesministerium für Ernährung und Landwirtschaft

BÖL BUNDESPROGRAMM ÖKOLOGISCHER LANDBAU

JKI

aufgrund eines Beschlusses des Deutschen Bundestages

Funding no.: 2815NA120
Project term: 11/2018 – 8/2023
Researchers: Dr. B. Berger, L. Schumann, Dr. A. Elhadi
Assistants: K. Schuldt, K. Reimann, C.-M. Gottwald….

…and thanks to you for your attention!!

www.julius-kuehn.de