

Unravelling the Mechanism of Pathogen Inactivation during Anaerobic Soil Disinfestation

W.T. Runia^a, T.C. Thoden,
L.P.G. Molendijk and W. van den Berg
Wageningen UR, Applied Plant Research
PO Box 430, 8200 AK Lelystad
The Netherlands

A.J. Termorshuizen^b
BLGG Research
Binnenhaven 5
6709 PD Wageningen
The Netherlands

M.A. Streminska and A.W.G. van der Wurff
Wageningen UR Greenhouse Horticulture
PO Box 20, 2665 ZG Bleiswijk
The Netherlands

H. Feil and H. Meints
Thatchtec BV
Agro Business Park 10
6708 PW Wageningen
The Netherlands

Keywords: anaerobic soil disinfestation, *Globodera pallida*, potato cyst nematode, soilborne plant pathogens

Abstract

Anaerobic soil disinfestation (ASD) for the control of multiple soilborne pathogens is a viable alternative to the application of biocides and soil steaming. ASD implies soil wetting, incorporation of fresh organic matter, and covering with airtight plastic foil for several weeks. To speed up the whole process, Thatchtec has developed a procedure based on defined agricultural products (referred to as Herbie). To further optimize ASD, the mechanism of pathogen inactivation should be unraveled. Therefore, we performed an incubation experiment in 11-L polypropylene containers with 6 soil types (glacial sand, dune sand, river clay, marine loam, peat soil, and an artificially composed soil lacking any organic matter) in triplicate. After wetting the soil to field capacity, Herbie was added to it (equivalent to 4 g crude protein L⁻¹ soil) and containers were filled with soil (8 L, head space 3 L). A similar treatment without Herbie served as control. Nylon mesh bags containing cysts of potato cyst nematode (PCN; *Globodera pallida*) were added and the containers were sealed off. Destructive sampling was performed 3, 7, 14, and 28 d after start of the experiment and biotic (cyst content survival, free-living non-plant parasitic nematode community, total and functional groups of bacteria, fungi and protozoa) and abiotic (organic matter, pH, nutrients, EC, fatty acids) parameters measured. Additionally, concentrations of several gases (O₂, H₂S, CH₄, CO₂, NH₃, N₂O) were measured in the head space before each destructive sampling. Survival of eggs within the cysts declined in all treatments to levels <0.5% at the end of the experiment. The rate of egg inactivation depended significantly on soil type, although texture (sand vs. clay) appeared unimportant. Egg survival in the treatments correlated negatively with concentrations of acetic, propionic and butyric acid. As a potential quick-to-measure proxy for incubation time and efficacy, level of O₂ combined with the density of free-living non-plant parasitic nematodes might be interesting.

INTRODUCTION

There is a multitude of soilborne plant pathogens that affect yields of many crops. Most soilborne pathogens are able to survive for multiple years in the absence of a host. Many pathogens have a wide host range (e.g., fungi: *Verticillium dahliae*; oomycetes: *Pythium* spp.; nematodes: *Pratylenchus penetrans*, *Meloidogyne chitwoodi*), thus hampering the choice of proper rotation crops. Also narrow host range pathogens (e.g.,

^a willemien.runia@wur.nl

^b aad.termorshuizen@blgg-research.nl

fungi: *Synchytrium endobioticum*, nematodes: *Globodera pallida* and *G. rostochiensis*) are sufficiently persistent to survive the generally narrow rotations. Methods to manage infestations of soilborne pathogens are limited. The great majority of fungicides available to suppress airborne pathogens are ineffective in soil. The use of nematicides is becoming more and more limited by regulations. Other practices like soil steaming are costly and energy-demanding. Solarization is not available in regions with a temperate climate. Wide-spectrum biocides, such as methyl bromide, have been banned by international treaties. Disease and pest resistant cultivars are often not available, produce lower yields or have some other suboptimal traits. Biocontrol agents are scarcely available or are insufficiently effective. So-called catch crops have a quite specific range of action (e.g., *Tagetes* specially targeting *Pratylenchus* spp. (Evenhuis et al., 2004) or *Solanum sisymbriifolium* hatching only *Globodera* spp. (Timmermans et al., 2009)). Organic amendments, such as compost, have only a limited effect on the disease suppressive capacity of soils. For a given field, farmers may have little choice to change rotation. The latter is especially true for greenhouse crops, which are typically cultivated continuously. To avoid soilborne pathogens in greenhouse crops, cultivation in renewable substrate (rockwool) has been introduced, but certain crops still rely on production in soil (e.g., chrysanthemum) while organic horticulture completely relies on production in soil.

Since about the mid-1990s, effects of specific amendments on reduction of infestations by soilborne pathogens are being investigated. Here, the composition of the amendments, soil pH and conditions of incubation determine the effects. In wet, alkaline soils, high-N amendments lead to production of ammonia, which is toxic against a wide range of pathogens (Conn et al., 2005; López-Robles et al., 2013). On the other hand, in neutral to acid soils readily decomposable amendments lead under anaerobic conditions (obtained by plastic covering or inundation) to production of toxic organic acids (Conn et al., 2005; Oka, 2010). In all these methods, the positive effects of pathogen control have to be weighed against potential negative side-effects like N-leaching (in the case of high-N amendments), environmental effects of plastic use, and other biotic effects affecting soil quality (e.g., reductions in non-pathogenic soil organisms). Currently also combinations of methods are being introduced, such as anaerobic incubation of organic amendments combined with induction of sublethal temperatures by solarization (Melero-Vara et al., 2012).

Until now, not a generally applied soil disinfection method is in use. Costs of a specific method and variability of the effects are crucial factors. In The Netherlands, anaerobic soil disinfection (ASD) was developed, originally to control *Fusarium oxysporum* f. sp. *asparagi* in asparagus (Blok et al., 2000) and later also *Verticillium dahliae* and *Pratylenchus penetrans* in strawberry (Lamers et al., 2010). ASD implies incorporation of fresh organic matter, soil wetting and covering with airtight plastic foil for several weeks. All kinds of organic matter may be used (Blok et al., 2000), but the enterprise Thatchtec (www.thatchtec.com) has developed an organic matter mixture based on defined agricultural products of plant origin (referred to as 'Herbie') that speeds up the process (from the original 6 weeks to 2-3 weeks) with similar but more reproducible results (Runia et al., 2012). First experiments were done in the lab and on pilot scale. These first experiments were followed by farmer's experiments and although generally the effects were quite successful, treatments with insufficient effects occurred as well. This raised questions about the requirements for a successful process and hence more insight into the mechanism involved was desired, so that performance of ASD can be monitored and predicted, so that failures could be avoided.

The goal of this research was to apply ASD in mesocosms of 6 different soils and to study the biotic and abiotic changes over time. Survival of eggs within cysts of *Globodera pallida* (potato cyst nematode; PCN) was determined and used as an indicator of plant pathogen survival. This pathogen was selected because it proved to be sensitive to ASD in previous experiments (Runia et al., 2012). Moreover, results of previous experiments are more consistent with PCN than with *V. dahliae* or *Meloidogyne hapla* and for PCN the complete ASD process is needed for efficacy and not only the lack of

oxygen which was sufficient to kill larvae of *P. penetrans*.

We hypothesized that the cause of reduction in living eggs within cysts of *G. pallida* might vary for the different soil types and that any of the biotic and abiotic factors measured during the experiment, varying in a similar way, would give us a clue on the mechanism involved. At the same time we expected that some parameters could predict better success rate of ASD than oxygen content.

MATERIALS AND METHODS

Overview

An incubation experiment was done in 11.2-L polypropene containers with 6 soil types (glacial sand, dune sand, river clay, marine loam, peat, and an artificially composed soil lacking any organic matter) in triplicate. The soil was mixed with organic material (Herbie, see below) or not (control). Nylon mesh bags containing cysts of the pathogen potato cyst nematode (PCN) *Globodera pallida* were incorporated into the soil and the containers were sealed off. Destructive sampling was performed 3, 7, 14, and 28 d after start of the experiment and biotic (cyst content survival, free-living non-plant parasitic nematode community, total and functional groups of bacteria, fungi and protozoa) and abiotic (organic matter, pH, nutrients, EC, fatty acids) parameters measured. Additionally, fatty acids concentrations in the soil and several gases (O_2 , H_2S , CH_4 , CO_2 , NH_3 , N_2O) in the head space were measured before each destructive sampling.

Soils, Organic Matter and Pathogen

Five different soils were collected from arable fields in The Netherlands (Table 1). Of each soil, about 0.5 m^3 was collected from the top 0-25 cm and stored in large bulk containers loosely covered with plastic to prevent drying up. Soils were collected in the two weeks before starting the experiment. In addition a sixth soil was composed artificially. This was done in order to have one completely reproducible object with respect to its 'soil' composition. This artificial soil consisted of a mixture of silver sand, clay and hydrogranules in a ratio of 4/0.7/1 w/w/w. From the bulk containers about 60 subsamples were taken to make up 1 kg of soil used for soil chemical analysis (Table 1). One day before starting the experiment, soils were wetted if necessary to approximately field capacity by spraying water and homogenizing the soil.

As source of organic matter the commercial product Herbie 22 (www.thatchtec.com; analysis of pure product in water extract: pH 5.1, 13.9 NH_4 , 6.5 NO_3 , 341 K, 10.9 Na, 4.4 Ca, 36.9 Mg, 34.1 Cl, 40.7 SO_4 , 36.1 P (all in mmol/L), and 740 Fe, 66 Mn, 240 Zn, 217 B, 18 Cu (all in $\mu\text{mol/L}$)) was used. An amount of 20 g equivalent to $4.08\text{ g crude protein L}^{-1}$ soil was added.

Cysts of *Globodera pallida* (strain E-400) were produced at Applied Plant Research in Lelystad by growing the susceptible cultivar 'Bintje' for 16 weeks on artificially infested soil.

Experiment

11.2-L polypropene containers (PP) were each filled with 8 L of soil and 160 g of Herbie and thoroughly shaken. The O_2 transmission rate (TR) of PP types in general is on average $2300\text{ ml/m}^2/\text{day}$ at 23°C and 0% RH (www.polyprint.com). This is approx. 10-fold higher than the O_2 TR of virtually impermeable films, used in the field for ASD. For CO_2 and N_2 , TR is approx. 3-5 \times higher and 2.5-5 \times lower than the O_2 TR, respectively. Bulk density of the soils, comparable with the field situation was imitated by pounding the containers with soil three times. The control treatment without addition of Herbie was shaken equally well. One nylon Monodur mesh bag (1.5 \times 5 cm; width/length) per container with 200 cysts of PCN were incorporated into the soil at 7-10 cm soil depth. The containers were sealed off with an airtight lid, and incubated in a climate room in the dark at on average $19.8(\pm 0.1)^\circ\text{C}$ and a relative humidity of 65%. Destructive sampling was performed 3, 7, 14, and 28 d after start of the experiment. Just before each destructive

sampling, fatty acids concentrations in the soil and several gases were measured in the head space as described below.

Measurements

Just before each destructive sampling, several gases (O₂, H₂S, CH₄, CO₂, NH₃, N₂O) in the head space were measured. The gases except O₂ were measured with an infrared photo acoustic spectrophotometer for (greenhouse) gases (Innova 1412, LumaSense Technologies). O₂ was measured with a portable gas monitor based on electro-chemical sensor technology (type Impact Pro, Zellweger Analytics Ltd.). For the quantification of fatty acids, immediately after opening a container 10 subsamples were taken with a diam. 1.8 cm auger to make up a single sample. After mixing in a plastic bag, a subsample of 50 g was taken, 100 ml demineralized water was added, shaken for 30 min, and centrifuged at 5000 rpm for 25 min. Then 0.5 ml of supernatant was transferred to a 1.5 ml eppendorf tube. To each tube, 0.5 ml of 0.1 M ortho-phosphoric acid containing 0.5 ml 19.7 mmol/L isocaproic acid was added as internal standard. Samples were stored at -20°C until measurement. The volatile fatty acids were separated by GC using Grace EC-1000 length 30 m, ID 0.53 mm, 0.2 µm as column and He as carrier gas and detected by FID. Quantification was based on comparison with a chemical standard after internal standard correction.

pH, organic matter, and major and minor nutrients were measured by BLGG AgroXpertus (Wageningen, The Netherlands) according to certified standard techniques shortly indicated in the footnotes of Table 1.

For *G. pallida* a hatch test was performed to establish vitality of the eggs (Been and Schomaker, 1998). In short, after soaking and crushing the cysts in water, its contents was placed on a small sieve and submersed in potato root extract (i.e., the hatching agent). Vital larvae pass this sieve and are weekly counted in the hatching agent, which is then replaced by fresh agent. Hatching was performed in duplicate per replicate with 3500 eggs per sieve and was finished after six weeks.

Protozoa were quantified according to a MPN-method (Darbyshire et al., 1975); amoebae, ciliates and flagellates were microscopically counted. Total number of free-living non-plant parasitic nematodes was determined microscopically after elutriation of 200 ml of soil (Oostenbrink, 1966). Hyphal length of fungi was determined according to Lodge and Ingham (1991). Bacterial densities were determined microscopically using a method described by Seiter et al. (1999). Active and inactive fungal and bacterial biomass was differentiated with fluorescein diacetat (FDA) as vital stain (Seiter et al., 1999).

DNA was isolated from the soil using PowerSoil DNA isolation kit (MoBio) according to the protocol recommended by the manufacturer. qPCR assays were conducted in polypropylene 96-well plates on an Mx3000P sequence detection system (Stratagene). Each 25-µl reaction contained the following: 12.5 µl of PCR Brilliant II SYBR Green Mastermix (Stratagene), 1 µl of each primer (10 µM), 9.5 µl H₂O and 1 µl template DNA. PCR conditions were 10 min. at 95°C, followed by 40 cycles of 95°C for 1 min, 30 s at the annealing temperature suggested for a specific primer pair, and 72°C for 1 min. For total bacteria, *Firmicutes* we used primer sets described by Fierer et al. (2005). Each qPCR plate included triplicate reactions per DNA sample and the appropriate set of standards. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal originated from specific PCR products and not from primer-dimers or other artifacts.

Phytotoxicity was tested using Phytotoxkits of MicroBioTests Inc. (Mariakerke, Belgium) with garden cress (*Lepidium sativum* L.) as test plant. In short, each test soil or a reference soil provided by the manufacturer is put into three small containers and 10 seeds are put on top of it. After three days, seed germination and root length is measured with a ruler. As the results showed no differences, only seed germination data are presented.

Data Analysis

To estimate the decline of the number of hatching eggs per day a generalized linear model (MacCullagh and Nelder, 1989) with the variance proportional to the mean and a logarithm link (quasi likelihood) was used to fit the model *number of hatching eggs* $= \alpha \times e^{\beta \times \text{day}} \times \varepsilon$, where α is the expected number of hatching eggs on day 0, e^{β} the expected fraction of surviving hatching eggs after 1 day, and ε a multiplicative error term. In the results we present the fraction of killed hatching eggs after 1 day, $1 - e^{\beta}$. After n days the number of killed eggs equals $1 - e^{\beta n}$.

With the R (R Core Team, 2013)-package Tree (Ripley, 2013), using default parameter inputs, a classification tree was constructed to see if (combinations of) quickly measurable parameters could correctly predict high levels of inactivation of eggs of PCN.

RESULTS

Hatching of eggs of PCN declined with time in all soils. In the control treatment, decline was significant ($P < 0.05$) in the glacial sand and river clay soil (decline by 70 and 65%, respectively), and for the other soils there was a clear tendency of a decline (18-62%) (Fig. 1). These data suggest that only sealing soil already affects PCN survival negatively. Decline in hatching of eggs of PCN in the Herbie-amended treatment was however much larger than in the control treatment, resulting in survival values $< 0.5\%$ after 28 d (Fig. 1). Three days after starting the experiments, viability of PCN was not significantly affected by the Herbie treatment. Seven days after sampling, a strong decline was observed for the glacial sand, marine loam and peat soil (91, 83 and 99% respectively), while this was significantly ($P < 0.05$) less for the dune sand, marine clay and artificial soil (25, 27, and 43% respectively) (Fig. 1). Average daily decline in the Herbie-treated soils significantly ($P < 0.05$) separated these two groups of soil (0.35-0.57 for the first and 0.14-0.20 for the second group) (Table 2). On average this is a $2.4\times$ difference between these two groups of soils in average daily decline of eggs of PCN.

In the controls, oxygen concentrations declined with time (Fig. 2), indicating breakdown of organic matter through microbial activity. Thus, the artificial soil lacking any organic matter showed no decline, and the peat soil with 11% organic matter declined to 1.1% O_2 28 d after incubation. In the Herbie-amended treatment, O_2 decline occurred promptly, with concentrations $< 1.3\%$ for all soils from day 3 onwards (Fig. 2). CO_2 behaved largely inverse to the observed O_2 patterns (Fig. 2). Except for some single peaks in the peat and artificial soil, levels of N_2O , CH_4 and NH_3 were low in the control treatments. In the Herbie-amended treatments, N_2O remained low except for a peak in the artificial soil (Fig. 2); CH_4 remained insignificant compared to the control except for the marine loam and river clay soils, where final concentrations $> 10,000$ ppm were measured (Fig. 2); NH_3 showed a slight increase to max. 5-25 ppm (Fig. 2); and H_2S often was in the order of magnitude of 5,000 to 10,000 ppm, with a few peaks as high as 10,000-55,000 ppm (Fig. 2).

Organic fatty acids in soil extracts occurred close or below the detection limit in the control soils. With time steadily increasing concentrations of acetic, propionic, and butyric acid were measured in the Herbie-amended soils (Fig. 3). Highest concentrations were found for acetic acid, all highest at the latest harvest (28 d) with levels > 60 mmol/L. Propionic and butyric acid occurred at lower concentrations of up to 35 and 50 mmol/L, respectively (Fig. 3). Production of these fatty acids in the soil were strongly correlated after lumping all the Herbie-amended data together (Pearson's correlations, acetic with propionic and butyric acid: $R^2 = 0.87$ and 0.95 respectively and propionic with butyric acid: $R^2 = 0.89$). Nearly complete or complete inactivation ($> 99.5\%$) of PCN occurred at concentration > 100 , > 10 , and > 30 mmol/L for acetic, propionic and butyric acid, respectively (Fig. 4).

pH did not change in time in the controls. In the Herbie-amended treatment, pH was about 0.5 unit lower than in the controls, with a weak tendency to decline in time except for the artificial soil (Fig. 5). Ammonium was significantly increased in the Herbie-amended treatment (Fig. 5), and increased also with time, indicating inhibition of

nitrification due to lack of oxygen. The strongest increase occurred in the two sandy soils, up to levels of 7-8 mmol/L. Nitrate was measured at about 1 mmol/L in the control treatment and was not detected in the Herbie-amended treatments (data not shown).

Among the protozoa, the flagellates, amoebae and ciliates were quantified separately. They occurred below the detection limit in the artificial soil. In the other soils, their numbers did not show clear patterns in time, neither in the control nor in the Herbie-amended treatments (data not shown). It is however interesting that initial amounts of flagellates and amoebae (at $t=0$, so before addition of Herbie) were clearly higher in the three soils showing a significantly stronger decline in viable eggs of PCN (Fig. 1, Table 2), i.e., the glacial sand, marine loam and peat soil, with flagellates $>9.8 \times 10^5$ and amoebae $>3.6 \times 10^4 \text{ g}^{-1}$ soil (dw) compared to the other soils (dune sand and river clay) with respective levels $<4.8 \times 10^3$ and $<0.62 \times 10^4 \text{ g}^{-1}$ soil (dw). Such differences between soils differing in PCN survival were not observed for the total number of nematodes (=total counts, pathogens+non-pathogens), but except for the artificial soil, in which only very low numbers of nematodes were recorded, there was a decline in time in the Herbie-amended treatments, while there was no change or even a slight increase in the control treatments (Fig. 6). From day 14 onwards, the total number of nematodes was more than halved compared to the amount counted in the day 3 control soils (Table 3).

Numbers of *Firmicutes* were consistently higher in the Herbie-treated soils than in the control treatment. The relative contribution of *Firmicutes* bacteria relative to the total bacterial population increased during the two first weeks of anaerobic incubation (Fig. 7).

It would be interesting to identify indicators that predict PCN decline quickly and at low cost. Therefore, a split in the Herbie-amended dataset was made at 50 viable eggs of PCN (equaling survival $<0.5\%$). Various variables, like acetic acids, gave a good separation between the class of data >50 and <50 viable eggs of PCN, but here we focused on indicators that can be done both quickly and at a low price. Other variables, including hyphal length and active and non-active fungal and bacterial biomass, showed no interesting results (data not shown). The most prospective result of this analysis is that objects with <50 viable eggs were uniquely characterized by low O_2 levels ($<1.1\%$) in combination with total number of nematodes $<120 \text{ g}^{-1}$ f.w. soil. In selected samples we identified the surviving nematodes, but no taxonomic class of nematodes predicted inactivation of PCN better than the total number. Differences between the soils with a relatively fast inactivation (glacial sand, marine loam and peat soil; Table 2) compared to soil with a relatively slow inactivation (dune sand, river clay and artificial soil) appeared in the organic matter content, N-total, N- NH_4 and P-total concentrations, and in the initial number of flagellates and amoebae (Table 4). Other data we measured, including total number of bacteria and fungi (either with DNA-based methods or microscopically) and sulphate-reducing bacteria did not yield interesting results and are not shown here.

Soils sampled at the end of the experiment appeared phytotoxic. Germination after 3 d of the test plant *Lepidium sativa* (garden cress) was $<50\%$ for the glacial sand, dune sand, peat soil and artificial soil compared to germination in a non-treated reference soil. The marine loam was somewhat less phytotoxic and the river clay showed no inhibition of germination. The sampled soil was stored at room temperature and again tested 1, 3, 7 and 28 d later. Phytotoxicity faded away in 3 d for the marine loam and peat soil, but it took between 7 and 28 d to fade away for the other soils. It is likely that phytotoxicity was caused by high EC levels, which were highest for the soils being most phytotoxic, i.e., 3.4 for the artificial soil and 2.7 for both the glacial and dune sand soil (data not shown).

DISCUSSION

A very strong reduction in survival of PCN was observed in all soils. Only after 3 d of incubation, survival was high and not different from the control, but already after 7 d decrease was significant for all Herbie-amended soils. Though inactivation of PCN was $>99.5\%$ in all soils after 4 weeks of incubation, it did not reach 100% in all cases. However, it is much better than effects of applying nematicides in general (e.g., Melero-Vara et al., 2012). More specifically, the decline of PCN after application of metam

sodium (the single approved nematicide in The Netherlands) is only up to 80% on sandy soils (Been and Schomaker, 1999), while on clay this fumigant is largely ineffective. Decline in survival of PCN was observed also in the control, indicating sensitivity of PCN to anaerobic conditions, but effects of Herbie were 8-29× larger (Table 2). Survival of PCN under various anaerobic treatments has been reported to depend on more than just absence of O₂. Thus, soil flooding results in significantly higher inactivation of PCN than anaerobic, non-flooded soil (Spaull et al., 1992). For PCN, this has been attributed to formation of fatty acids (e.g., Hollis and Rodriguez-Kabana, 1966; Lopez-Robles, 2013) and H₂S (Spaull et al., 1992).

Inactivation of PCN roughly paralleled with O₂ depletion (partial depletion in the control, quick and complete depletion in the Herbie treatment), CO₂ accumulation and accumulation of organic acids and to some extent also of NH₃, but not with accumulation of CH₄ and H₂S (Fig. 2). PCN survival was strongest correlated with accumulation of organic acids. Levels of 110, 11, and 30 mmol/L soil solution of acetic, propionic and butyric acid, respectively, without exception were associated with complete or nearly complete inactivation of PCN (Fig. 4). However, toxicity of organic acids is strongly pH-dependent, as only the undissociated forms are active (Oka, 2010; Banage and Visser, 1995; McElderry et al., 2005). Because of the low pK_a values of organic acids (<4.9), they are supposed to be a factor of importance only at pH levels roughly below 6, while in our experiment the bulk pH was usually >6 (Fig. 5). Perhaps there was variation of pH at microsite level (e.g., Strong et al., 1997), which we did not attempt to determine. Li et al. (2010) reported inactivation of *Streptomyces scabies* >99.8% after addition of 200 mmol/L soil suspension to a sandy loam with pH of 7.1, possibly also indicating variation in pH at microsites. Analogous to the organic acids, the levels of NH₃ measured in the Herbie-amended treatments were low compared to published dosages lethal to soilborne pathogens; in addition, formation of NH₃ is significant only in soils with pH>8 as below this level it takes the form of NH₄⁺ (pK_a=9.2) (Oka, 2010). The H₂S concentrations we measured in the head space in the containers with Herbie addition were high and potentially toxic (Fortuner and Jacq, 1976) and the relative proportion of *Firmicutes* (Fig. 7) is in line with this observation. However, variation in H₂S concentration did not correspond with variation in inactivation of PCN. As stressed by Oka (2010), many compounds are known to be formed from decomposing organic amendments, and it is likely that a multitude of them are contributing simultaneously to decline in PCN.

The fact that PCN declined more rapidly in three soils (the glacial sand, marine loam and peat soil) than in the three other soils (the dune sand, river clay and artificial soil) (Fig. 1, Table 2) is of interest. In the soils where decline took place relatively fast, initial values (so before starting the experiment, without Herbie amendment) of organic matter content, N-total, P-total, and the density of flagellates and ciliates were all consistently higher (Table 4). In addition, initial N-NH₄ was higher except for the artificial soil and butyric acid concentrations were higher except for the peat soil. No differences were observed in e.g., O₂ depletion or accumulation of other gases or of acetic and propionic acid. Thus, the ‘quick inactivation’ soils are richer than the ‘slow inactivation’ soils, indicating that biological activity is essential (Oka et al., 2010). Organic matter and N content may play a dominant role, since these are major components to stimulate biological activity needed for decomposition of Herbie. Because of lack of organic matter in the artificial soil, the inactivation mechanism was probably different from the natural soils we studied. We included this soil to have access to an object that is completely reproducible.

Predictability of pathogen inactivation is important as detection assays are costly, time-consuming and imprecise if their spatial distribution is heterogeneous. Moreover, with DNA-based assays, false-positives could appear if samples are studied where pathogens have died very recently (Scheda et al., 2013). Yet, growers aim at starting up crop production as soon as possible. From previous work it appeared that O₂ depletion is not the single prerequisite for pathogen inactivation (Blok et al., 2000). CH₄ production

indicates a strongly reducing environment, but since it was measured here in only low concentrations, it apparently does not predict pathogen inactivation. Protozoa appeared relatively resistant against ASD. The single parameter we measured that predicted well >99% inactivation of PCN was a density of <150 total number of nematodes/100 ml of soil, given presence of O₂ depletion (O₂<1.1%).

Phytotoxicity of the ASD-treated soils was observed. As toxic gases like NH₃ and organic acids are quickly nitrified and decomposed, respectively, in aerated soils, it is likely that EC levels incited phytotoxicity. Growers can manage this by irrigating their soils prior to planting, but this is costly. Therefore, the provider of Herbie has lowered the EC content, and phytotoxicity should be not problematic anymore.

The results of our experiment show that ASD can inactivate PCN by more than 99.5% in a wide range of soils, including an artificial soil lacking any organic matter. Inactivation occurs faster for soils relatively high in organic matter content. Most likely a combination of factors causes the decline in PCN. More attention needs to be paid to processes occurring at soil microsites. Efficacy of ASD may be well-predictable using easy-to-determine parameters such as total number of nematodes in combination with O₂ concentration.

ACKNOWLEDGEMENTS

We thank Leon de Jonge (WU Animal Sciences) for his kind advise on fatty acids extraction and measurement. This study was financed by the Dutch province of Gelderland with European Regional Development Fund.

Literature Cited

- Banage, W.B. and Visser, S.A. 1965. The effect of some fatty acids and pH on a soil nematode. *Nematologica* 11:255-262.
- Been, T.H. and Schomaker, C.H. 1998. Quantitative studies on the management of potato cyst (*Globodera* spp.) in The Netherlands. Chapter 4: Ways to improve the accuracy of hatching tests for nematodes *Globodera* spp. With special emphasis on nematicide trials. Ph.D. Thesis Wageningen University, p.71-103.
- Been, T.H. and Schomaker, C.H. 1999. Fumigation of marine clay soils infested with *Globodera pallida* and *G. rostochiensis* using 1,3-dichloropropene and additional top soil treatments. *Nematology* 1:3-14.
- Blok, W.J., Lamers, J.G., Termorshuizen, A.J. and Bollen, G.J. 2000. Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. *Phytopathology* 90:253-259.
- Conn, K.L., Tenuta, M. and Lazarovits, G. 2005. Liquid swine manure can kill *Verticillium dahliae* microsclerotia in soil by volatile fatty acid, nitrous acid, and ammonia toxicity. *Phytopathology* 95:28-35.
- Darbyshire, J.F., Wheatley, R.E., Greaves, M.P. and Inkson, R.H.E. 1974. A rapid micromethod for estimating bacterial and protozoan populations in soil. *Rev. Écol. Biol. Soil* 11:465-475.
- Evenhuis, B., Korhals, G.W. and Molendijk, L.P.G. 2004. *Tagetes patula* as an effective catch crop for long-term control of *Pratylenchus penetrans*. *Nematology* 6:877-881.
- Fierer, N., Jackson, J.A., Vilgalys, R. and Jackson, R.B. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* 71:4117-4120.
- Fortuner, R. and Jacq, V.A. 1976. In vitro study of soluble sulfides to three nematodes parasitic on rice in Senegal. *Nematologica* 22:343-353.
- Hollis, J.P. and Rodriguez-Kabana, R. 1966. Rapid kill of nematodes in flooded soil. *Phytopathology* 56:1015-1019.
- Lamers, J.G., Runia, W.T., Molendijk, L.P.G. and Bleeker, P.O. 2010. Perspectives of anaerobic soil disinfestation. *Acta Hort.* 883:277-283.
- Lazarovits, G., Conn, K.L., Abbasi, P.A. and Tenuta, M. 2005. Understanding the mode of action of organic soil amendments provides the way for improved management of

- soilborne plant pathogens. *Acta Hort.* 698:215-224.
- Lazarovits, G. 2010. Managing soilborne disease of potatoes using ecologically based approaches. *Am. J. Pot. Res.* 87:401-411.
- Lin, Y., He, Z., Roskopf, E.N., Conn, K.L., Powell, C.A. and Lazarovits, G. 2010. A nylon membrane bag assay for determination of the effect of chemicals on soilborne plant pathogens in soil. *Plant Dis.* 94:201-206.
- Lodge, D.J. and Ingham, E.R. 1991. A comparison of agar film techniques for estimating fungal biovolumes in litter and soil. *Agric. Ecosyst. Environ.* 34:131-144.
- López-Robles, J., Olalla, C., Rad, C., Díez-Rojo, M.A., López-Pérez, J.A., Bello, A. and Rodríguez-Kábana, R. 2013. The use of liquid swine manure for the control of potato cyst nematode through soil disinfection in laboratory conditions. *Crop Prot.* 49:1-7.
- Lynch, J.M. 1978. Production and phytotoxicity of acetic acid in anaerobic soils containing plant residues. *Soil Biol. Biochem.* 10:131-135.
- McElderry, C.F., Browning, M. and Amador, J.A. 2005. Effect of short-chain fatty acids and soil atmosphere on *Tylenchorhynchus* spp. *J. Nematol.* 37:71-77.
- Melero-Vara, J.M., López-Herrera, C.J., Basallote-Ureba, M.J., Prados, A.M., Vela, M.D., Macías, F.J., Flor-Peregrín, E. and Talavera, M. 2012. Use of poultry manure combined with soil solarization as a control method for *Meloidogyne incognita* in carnation. *Plant Dis.* 96:990-996.
- Oka, Y. 2010. Mechanisms of nematode suppression by organic soil amendments – a review. *Appl. Soil Ecol.* 44:101-115.
- Oostenbrink, M. 1960. Estimating nematode populations by some selected methods. *Nematology* 6:85-102.
- R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL www.R-project.org.
- Ripley, B. 2013. Package ‘tree’, version 1.0-34. <http://cran.r-project.org/web/packages/tree/index.html>.
- Runia, W.T., Molendijk, L.P.G., Ludeking, D.J.W. and Schomaker, C.H. 2012. Improvement of anaerobic soil disinfection. *Comm. Appl. Biol. Sci.* Ghent University 77(4):753-762.
- Schena, L., Nicosia, M.G., Li, D., Sanzani, S.M., Faedda, R., Ippolito, A. and Cacciola, S.O. 2013. Development of quantitative PCR detection methods for phytopathogenic fungi and oomycetes. *J. Plant Pathol.* 95:7-24.
- Seiter, S., Ingham, E.R. and William, R.D. 1999. Dynamics of soil fungal and bacterial biomass in a temperate climate alley cropping system. *Appl. Soil Ecol.* 12:139-147.
- Spaull, A.M., Trudgill, D.L. and Batey, T. 1992. Effects of anaerobiosis on the survival of *Globodera pallida* and possibilities for control. *Nematologica* 38:88-97.
- Strong, D.T., Sale, P.W.G. and Helyar, K.R. 1997. Initial soil pH affects the pH at which nitrification ceases due to self-induced acidification of microbial microsites. *Aust. J. Soil Res.* 35:565-570.
- Timmermans, B.G.H., Vos, J. and Stomph, T.J. 2009. The development, validation and application of a crop growth model to assess the potential of *Solanum sisymbriifolium* as a trap crop for potato cyst nematodes in Europe. *Field Crops Res.* 111:22-31.

Tables

Table 1. Chemical properties of the soils studies.

Soil code	Soil type	Origin	pH ²	C-org ³ (%)	Lutum ⁴ (%)	EC ⁵ (mS cm ⁻¹ , 25°C)	N-total (mg kg ⁻¹)	N-total soluble (mg kg ⁻¹)	N-NH ₄ ² (mg kg ⁻¹)	N-NO ₃ ² (mg kg ⁻¹)
a	Glacial sand	Vredepeel	5.8	2.2	<1.0 ⁷	0.1	1405	22.1	2.5	13
b	Dune sand	Lisse	7.2	0.8	1.2	0.05	350	3.7	<1.2	2.8
c	River clay	Randwijk	7.6	0.7	7	0.45	585	19.6	<1.2	9.5
d	Marine loam	Lelystad	7.5	1.2	17	0.1	1140	9.65	3.8	1.4
e	Peat soil	Valthermond	4.9	11	<1.0	0.1	4255	37.5	8.4	22
f	Artificial soil ¹	-	6.1	<0.1	6	0.38	<200	34.3	31	20

Soil code	C/N	P-total (mg P ₂ O ₅ 100 g ⁻¹)	K (mg K ₂ O 100 g ⁻¹)	S-total (mg kg ⁻¹)	CEC ⁶ (mmol+ kg ⁻¹)	Ca-CEC (%)	Mg-CEC (%)	K-CEC (%)	Na-CEC (%)
a	15.7	124	7.5	250	63	74	15	1.8	3.6
b	22.9	92	3	120	42	96	3.1	1.2	<0.1
c	12.0	73	15	580	84	90	6.1	3.4	1.3
d	10.5	127	23	610	152	93	3.4	3.5	0.3
e	25.9	117	13	785	242	72	7.5	1.1	0.5
f	-	16	16	225	0	0	0	0	0

¹ Artificial soil: preparation see text.

² In 0.01 M CaCl₂.

³ Determined with loss-on-ignition.

⁴ Particles <2 µm.

⁵ Electrical conductivity.

⁶ Cation exchange capacity at pH=7.

⁷ Below the detection limit indicated.

Table 2. Estimated fraction of hatching eggs of *Globodera pallida* inactivated per day. Cysts were incubated in 6 different soils at 20°C in closed (airtight) 11.2-L containers filled with 8 L of soil with (Treatment) or without (Control) Herbie (a type of readily decomposable organic matter) added.

Soil	Control ¹	Treatment	Treatment corrected ²
Glacial sand	0.0480 c	0.4166 b	0.3966
Dune sand	0.0205 ab	0.2006 a	0.1965
River clay	0.0409 bc	0.2049 a	0.1965
Marine loam	0.0118 ab	0.3521 b	0.3479
Peat soil	0.0208 ab	0.5652 b	0.5535
Artificial soil	0.0078 a	0.1435 a	0.1424

¹ Within the control and treatment columns, numbers without common characters differ significantly (P<0.05).

² “Treatment corrected” is the calculated effect of Herbie addition under airtight conditions (Treatment) minus the effect of airtight conditions without Herbie (Control). Since this calculation is based on averages, no statistics could be applied. Note that these values are very close to the Treatment values, indicating the large additional effect of Herbie addition.

Table 3. Proportion of free-living non-plant parasitic nematodes relative to the amount counted at day 3 in the control treatment as function of time incubation in 6 different soils at 20°C in closed (airtight) 11.2-L containers filled with 8 L of soil with Herbie (a type of readily decomposable organic matter) added.

Day	Soil type				
	Glacial sand	Dune sand	River clay	Marine loam	Peat soil
3	0.04	0.34	1.16	0.67	0.58
7	0.03	0.07	0.69	0.39	0.09
14	0.04	0.01	0.41	0.25	0.01
28	0.01	0.01	0.12	0.12	0.02

Table 4. Contrasts between soils with a rapid decline in PCN (glacial sand, marine loam and peat soil) compared to soils with a slower decline in PCN (dune sand, river clay and artificial soil) (viz. Table 2).

	Rate of decline of PCN ¹ day ⁻¹	C-org ² (%)	N-total ² (mg kg ⁻¹)	N-NH ₄ ² (mg kg ⁻¹)	P-total ² (mg P ₂ O ₅ 100 g ⁻¹)	Initial # flagellates (#)	Initial # amoebae (#)
Quick inactivation soils a, d, e ²	>0.34	>1.2	>1140	>2.5	>117	>9.8×10 ³	>3.6×10 ⁴
Slow inactivation soils b, c ²	<0.20	<0.8	<585	<1.2	<92	<4.8×10 ³	<0.62×10 ⁴

¹ cf. Table 2.

² cf. Table 1.

Figures

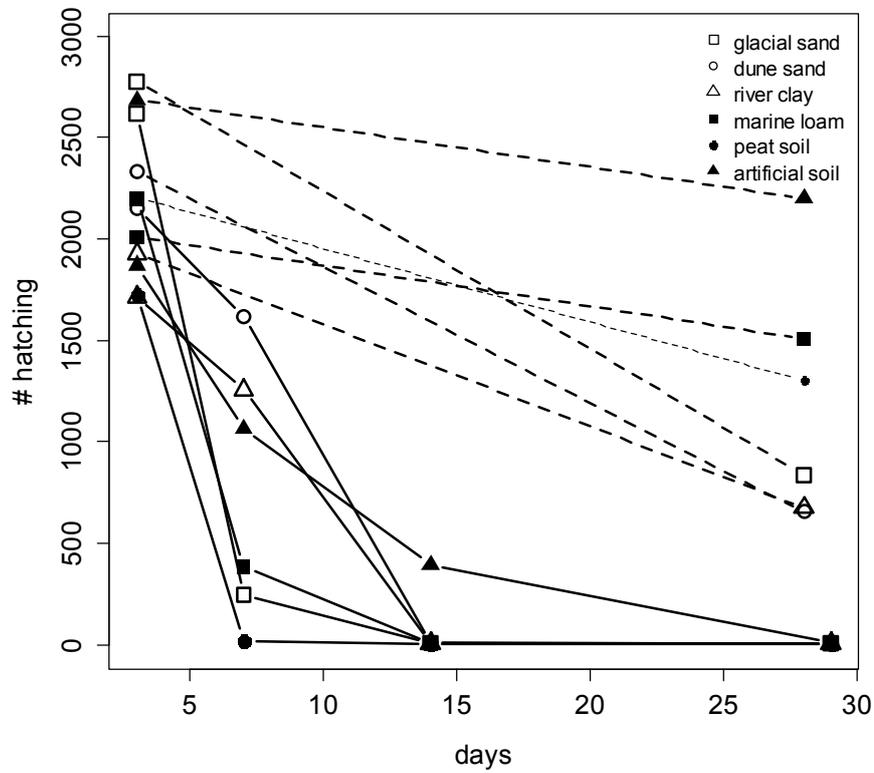


Fig. 1. Hatching of eggs of *Globodera pallida* as function of time incubated in 6 different soils at 20°C in closed (airtight) 11.2-L containers filled with 8 L of soil with (solid lines) or without (dashed lines) Herbie (a type of readily decomposable organic matter) added.

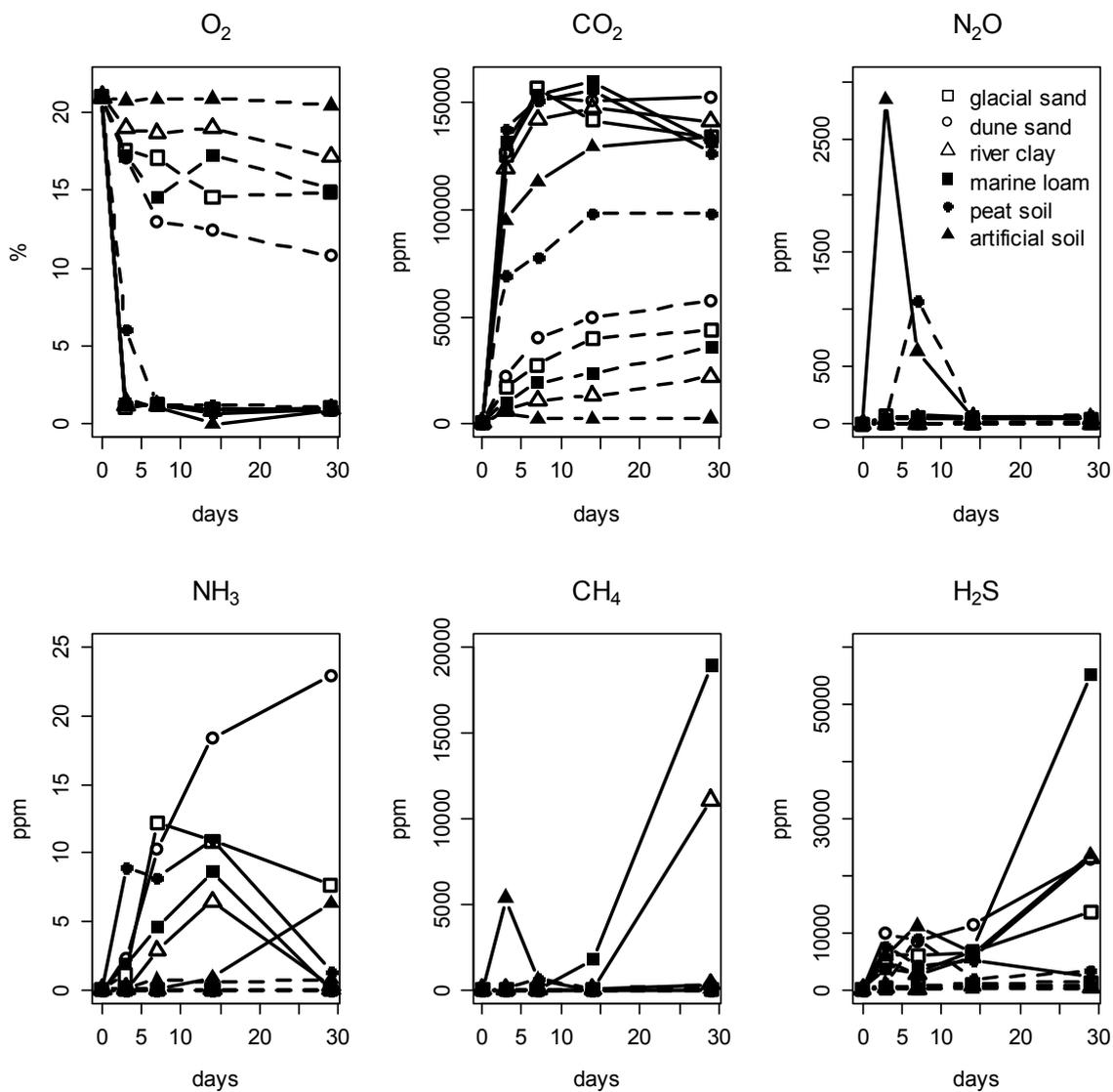


Fig. 2. Concentrations of O₂, CO₂, N₂O, NH₃, CH₄, and H₂S as function of time incubated in 6 different soils at 20°C in closed (airtight) 11.2-L containers filled with 8 L of soil with (solid lines) or without (dashed lines) Herbie (a type of readily decomposable organic matter) added.

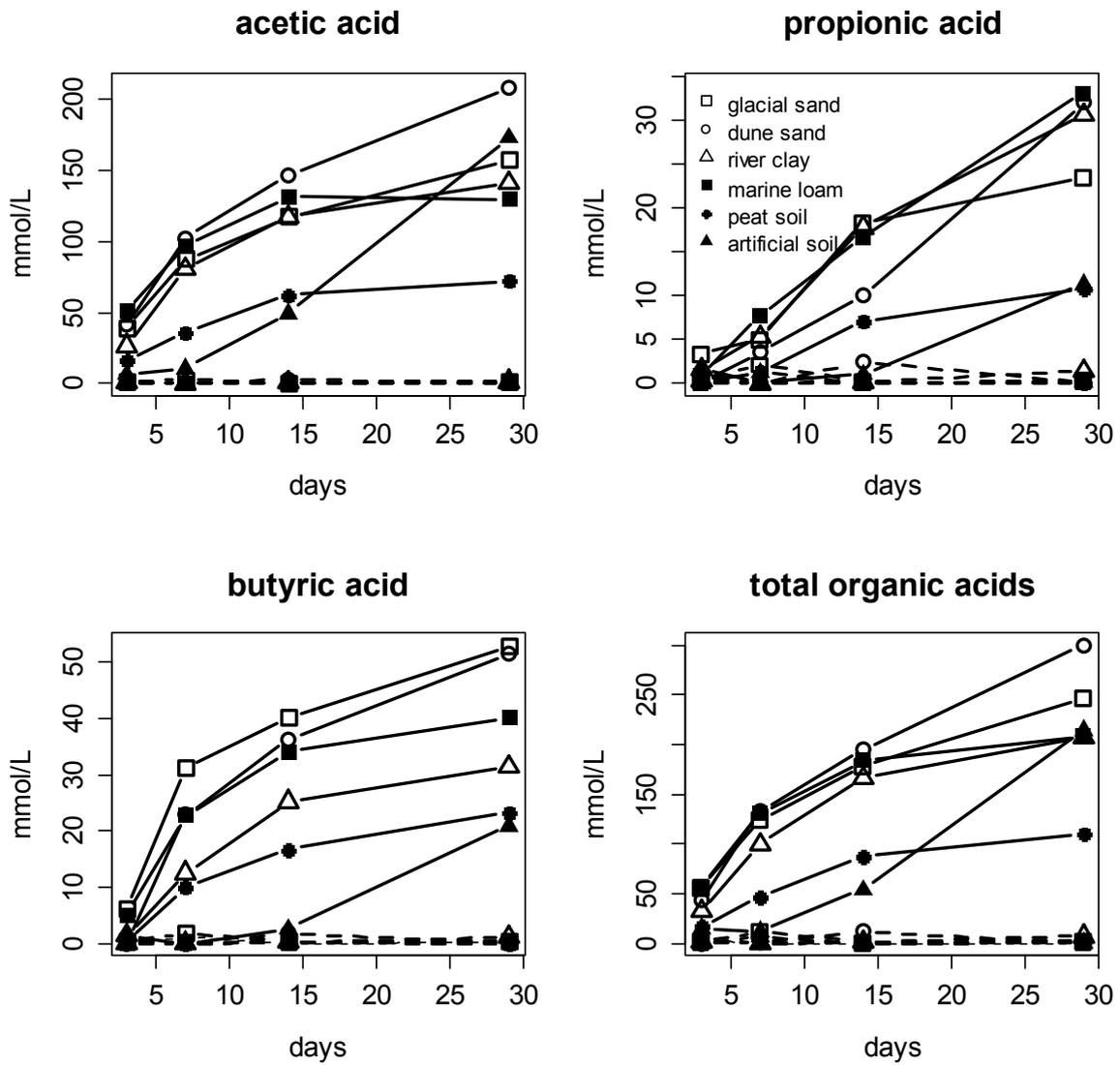


Fig. 3. Three organic acids and total organic acids (= acetic + propionic + butyric + iso-butyric + valeric + iso-valeric acid) concentrations in the soil solutions in 6 Herbie-amended (solid lines) and control soils (dashed lines).

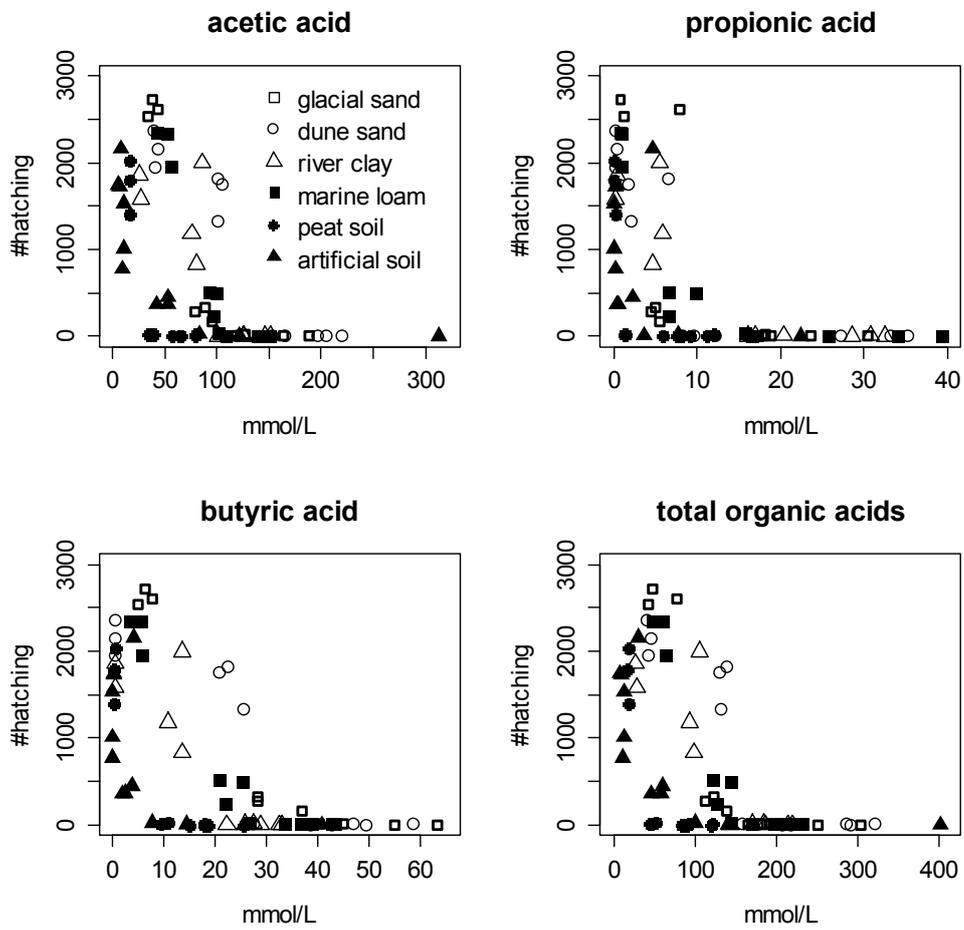


Fig. 4. Scatterplots for three organic acids and total organic acids (= acetic + propionic + butyric + iso-butyric + valeric + iso-valeric acid) concentrations in the soil solutions with hatching of eggs of *Globodera pallida*.

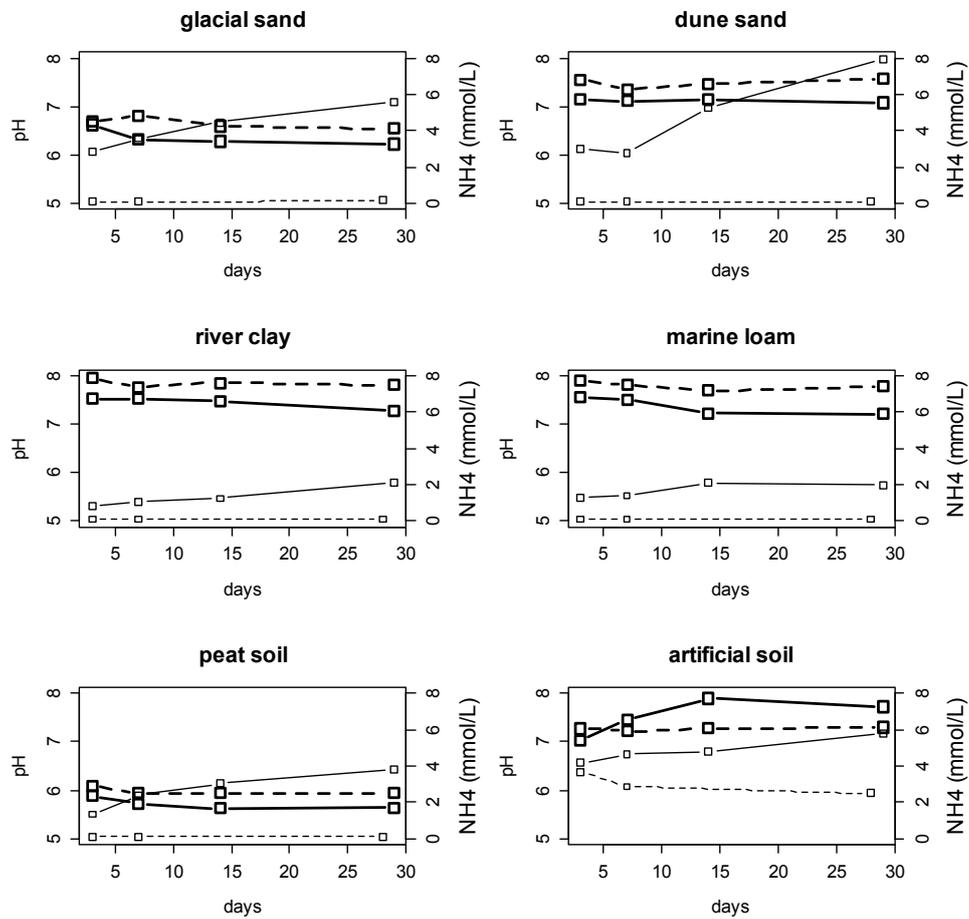


Fig. 5. pH (in H₂O; thick lines and left Y-axis) and NH₄ (in 0.01 M CaCl₂ extract; thin lines and right Y-axis) as function of time incubated in 6 different soils at 20°C in closed (airtight) 11.2-L containers filled with 8 L of soil with (solid lines) or without (dashed lines) Herbie (a type of readily decomposable organic matter) added.

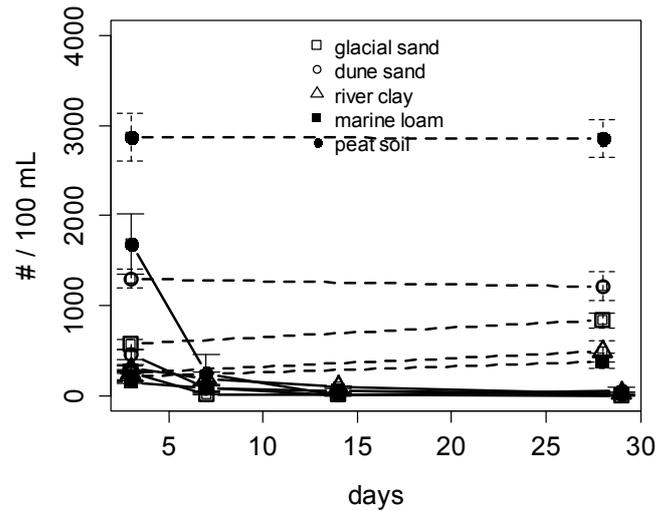


Fig. 6. Total number of free-living non-plant parasitic nematodes as function of time incubated in 5 different soils at 20°C in closed (airtight) 11.2-L containers filled with 8 L of soil with (solid lines) or without (dashed lines) Herbie (a type of readily decomposable organic matter) added. The 6th soil (artificial soil) contained only negligible numbers of free-living nematodes in all treatments.

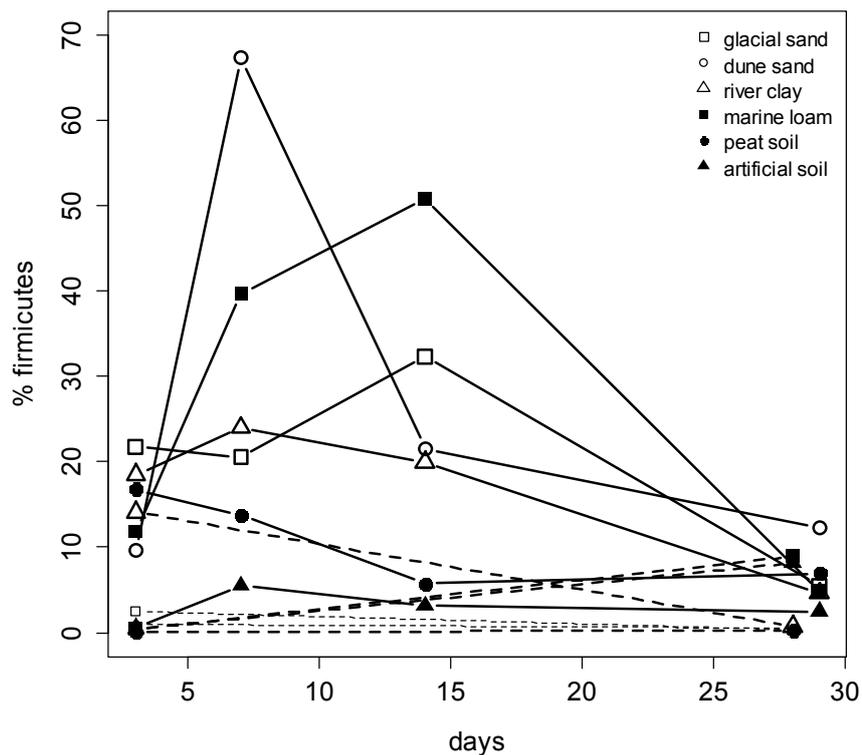


Fig. 7. Relative abundance of *Firmicutes* (as % total bacteria) as function of time incubated in 6 different soils at 20°C in closed (airtight) 11.2-L containers filled with 8 L of soil with (solid lines) or without (dashed lines) Herbie (a type of readily decomposable organic matter) added.

