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GENEPEASE

Genomic tools for assessment of pesticide effects on the agricultural soil ecosystem

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Title:

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Authors:

Carsten Suhr Jacobsen; Louise Feld; Mathis Hjort Hjelmsø; Morten Schostag Nielsen, Regin Rønn, Flemming Ekelund, Anders Johnsen, Jesper Givskov Sørensen, Paul Henning Krogh

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Foreword

This project has been funded by the Danish Ministry of Environment, through the Environmental Protection Agency (EPA) program for pesticide research. We are gratefully acknowledging that the funding authorities have been supporting this research into a new field.
Specifically we would like to thank people that have supported the development of the conceptual model that the project has been following. This includes the steering group of the project that has been led by Jørn Kirkegaard, Danish EPA.
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Summary

In this study, we evaluated different methods based on DNA and RNA analysis to describe effects of pesticides on the soil ecosystem. In brief short term effects of pesticides was investigated on soil microorganisms by measuring the level of ammonia monoxygenase gene (amoA) transcription in soil, and on soil collemboela. The long term effects on community structure was evaluated by measuring diversity changes in the bacteria and soil protozoan communities by analysing 16SrRNA and 18SrRNA as well as their corresponding genes (DNA).

In the project we developed a qPCR assay targeting mRNA transcripts of the ammonia monoxygenase gene (amoA) from bacteria and Archaea. The method was compared to the OECD nitrification testing protocol using an alternative colorimetric based assay for measurement of nitrate. When no ammonium sulphate was added to the soil the OECD test was not sensitive enough to show any changes due to pesticides addition – even with the fumigant Basamid. In this case the developed amoA expression method was more sensitive in the evaluation of pesticide side effects than the OECD method, since a clear decrease of amoA transcript could be detected from both Archae and bacteria.

mRNA transcripts were also used to evaluate the effect of pesticides on the whole transcriptome in the soil collembolan Folsomia candida. The iSQ microarray chip was able to detect induced stress at a relevant soil concentration level of one third of the EC_{10}, which is a level where conventional toxicity tests could not detect effects due to relatively high variability. Centralising and accumulation transcriptomic information will improve the usefulness of this soil collembolan as an indicator of decreased soil quality, e.g. due to pesticide residues.

Pesticide effects on community structure of soil bacteria and soil protozoa was evaluated using – respectively - 16S rRNA and 18S rRNA based techniques on both DNA and RNA level. In both cases pyrosequencing revealed that the fumigant Basamid indeed had a profound effect on the bacterial as well as the protozoan community. The basamid impacted soil showed a strong decrease in numbers of bacterial cells and a fast recovery of bacterial numbers after the initial decline. The fastest colonizing species was the protoebacteria Burkholderia sp and the firmicute Bacillus sp both known as bacterial species representing R-strategist. At day 12 the active part of the bacterial community measured as fraction of pyrosequences based on ribosomal RNA was showing an increased diversity compared to the DNA based analysis indicating that after the initial depression of the bacterial community a high number of bacteria species is active in the soil.

The bacterial community structure was evaluated using pyrosequencing of 16S DNA and rRNA a clear distinction between the soils sprayed with basamid and the non sprayed soil, while no differences was found between the bacterial community in the fungicide treated soil and the non treated soil.

In a larger scale experiment it was shown that the microbial activity after an initial decline – followed by a stimulation - was back at normal activity after 28 days.

Sammenfatning

I projektet har vi evalueret brugbarheden af forskellige DNA og RNA metoder til at beskrive effekter af pesticider på jordens økosystem. Korttidseffekter af pesticider blev undersøgt ved at bestemme niveauet af amoA ekspression i jord som et mål for nitrifikation og tilsvarende ved at måle regulering af gener i springhaler. Langtidseffekter af pesticider blev målt ved at undersøge ændringer i populationsstrukturen i jordens bakterielle populationer såvel som i protozoepopulationerne. Dette blev gjort ved at analysere ændringer i 16SrRNA og 18SrRNA såvel som i det tilsvarende DNA.

I projektet blev en metode udviklet til at måle transskription af gener der koder for ammonium monoxygenase genet amoA fra både bakterier og archaea. Kvantificering af amoA ekspression blev sammenlignet med OECD testen for nitrifikation, idet dog en alternativ kolorimetrisk metode til måling for nitrat blev anvendt. Sammenligningen viste, at når der ikke blev tilsat ammonium sulfat til jorden kunne man ikke se nogen effekt med OECD metoden – selv af Basamid tilsætning - mens kvantificering af amoA ekspression tydeligt faldt når Basamid blev tilsat.

For at undersøge pesticideffekten på springhaler blev mRNA transcripter kvantificeret på en microarray.

Pesticideffekter på populationsstrukturen af jordbakterier og jordprotozoer blev målt ved pyrosekventering af 16SrRNA og 18SrRNA gener – både på DNA og RNA niveau. For både bakterier og protozoer viste pyrosekventeringen at Basamid havde en meget kraftigt effekt. Den basamidbehandlede jord viste en stærk nedgang i totalantallet af bakterieceller, men efter at Basamid var fordampet skete der en hurtigt genvækst af bakterier – dog kun nogle få arter. De hurtigst koloniserende arter af bakterier var protoebakterien Burkholderia sp og firmicuten Bacillus sp der i begge i andre sammenhænge er vist at være hurtige kolonisatorer (R-strateger). På dag 12 viste RNA baseret måling en højere diversitet end DNA – formodentlig indikerende at der allerede her er ved at etablere sig et mere forskelligartet bakterielt samfund efter den initiale ødelæggelse.

I et mesokosmosforsøg på større skala blev det vist, at den mikrobielle aktivitet efter den initielle reduktion bliver stimuleret igen og efter 28 dage er tilbage på det normale aktivitetsniveau.

1. Background and aim

Soil processes are performed by soil organisms; hence soil functional diversity, and thus health, largely depends on composition and activity of the community of soil organisms. Soil microbes are responsible for many ecosystem services, such as litter degradation - reviewed in Schneider et al (2010), promotion of plant growth as reviewed in Hayat et al. (2010), and nutrient cycling (van der Heijden et al. 2008). Soil invertebrates intimately interacts with microorganisms through detrivory and microbivory and as modifiers of the soil matrix. These functions are of great importance to both the farmer and society and therefore it is of great importance to establish if application of molecular techniques can be used for sensitive, specific and cost-efficient assays to investigate soil health status. Yet, despite OECD focus on adopting genomic approaches in risk assessment of chemicals, no molecular-based standard protocols have so far been suggested to assess malicious side-effects on soil organisms.

In contrast to DNA, mRNA transcripts are highly labile messenger molecules that are only present during periods of gene expression. Therefore, abundance of specific mRNA transcripts is potentially better correlated to enzyme activity and hence process rates, as was previously shown for mineralization of the pesticide MCPA, which correlated well with the number of *tfdA* transcripts but not with gene copy number in soil (Bælum et al 2008).

Currently, the analyses requested by the European Commission to cover risk assessment of pesticides on soil microorganisms include a carbon and a nitrogen transformation test (European Commission 2002). The carbon test measures general microbial activity by glucose-induced respiration and the nitrogen test measures nitrification activity by production of nitrate. For these tests, standard OECD guidelines exist (OECD, 2000a and OECD 2000b).

Likewise, analyses are requested on the toxicity of pesticides towards a series of different soil animals including the microbial feeding collembolans (OECD 2009). The current OECD toxicity test guideline programme does not yet include any 2nd generation ecotoxicogenomics tools, obviously because these test are far from being available for routine work. Such tests also depend on bioinformatics and accumulation of transcriptomic information databases, tools that are not completely mature for routine applications. Currently, OECD is stimulating adoption of *omics* techniques in the “Adverse Outcome Pathways, Molecular Screening and Toxicogenomics”.

The influence of pesticides on overall microbial diversity is not currently a part of the European legislation process for pesticides. This is likely mainly due to the lack of methods that in a standardised manner can describe if any changes have been recorded due to the spraying with pesticides. In a recent review by Jacobsen and Hjelmsø (2014), the ISI indexed literature was searched and no examples were found on the use of next generation sequencing to describe effects of pesticides on microbial populations in soil. Thus, it seems that these otherwise widespread methods have not been put into work in scientifically studies of side effect of pesticides on microbial ecosystems in agricultural soil. Diversity of soil invertebrates is at the research stage and NGS approaches have been employed for e.g. earthworms (Biernert et al. 2012) and insects including collembolans (Yu et al. 2012). Transcriptomic approaches is now applicable to some main soil invertebrate groups including nematodes, earthworms, enchytraeids and collembolans supported by species specific microarrays (e.g. Liu et al. 2010; Novais et al. 2012). All in all very few international examples of testing pesticide effects on soil ecosystems using a genomic or

transcriptomic approach. Thus the GENEPEASE project are a methodological driven project, it asks if we possible can perform risk assessment of pesticides using RNA based approaches. In the project we have worked with two pesticides, Basamid and Tridex. Basamid is a soil fumigant that is designed to kill all potentially harmful soil organisms – thus it's expected to be very harmful to any organisms in the soil. It can be seen as a worst case effects scenario of a pesticide application. Tridex is a fungicide and is not expected to have a direct impact on soil bacteria and bacterial driven process – however it's expected to influence eukaryotic organisms.

1.1 Hypotheses of the project

1. Quantification of gene expression in soil can measure the impact of pesticides directly on important soil functions
2. Microbial diversity changes following pesticide application can be described using rRNA based pyro-sequencing
3. Gene expression of field populations will reflect possible stress induced by pesticides
4. Basamid and Tridex will have different effects on the soil ecosystem. Basamid will, as a soil fumigant, affect all species, while the fungicide Tridex mainly will affect eukaryotic organisms
5. Basamid will immediately hamper expression of *amo*A genes in soil, while Tridex will not influence *amo*A expression.

In this report, we evaluate new bio-indicator tools to assess pesticide side-effects on agricultural soil ecosystems. One of these tools is based on reverse transcription qPCR that target the ammonia monooxygenase gene (*amoA*) from bacteria and archaea. Here, we quantify the abundance of *amoA* mRNA transcripts in response to treatment with the pesticides Tridex DG or Basamid GR after N-amendment. We further evaluate the changes in bacterial and protozoan communities in soil applying a metagenomic and metatranscriptomic technique based on pyro taq sequencing of the 16SrRNA gene and transcripts as well as the 18SrRNA genes and transcripts using primers covering major parts of the bacterial soil community as well as major parts of the protozoan soil community. We finally in a two phase experiment test 1) which genes are strong and semipermanently down regulated in collembolans after pesticide exposure and then 2) if these genes can be quantified using a reverse transcription qPCR method as applied for the amoA genes above.

2. Materials and Methods

2.1 Soil

The soil was an agricultural sandy loam. We sieved the soil (4 mm mesh) and stored it at 5 °C in the dark for 8 months prior to the experiment. The laboratory tests were done with soil collected on Dec. 14, 2010, from the ploughing layer at an agricultural field at the experimental station at Askov, Jutland (Lat/Long 55°28'20N, 9°6'36E) The soil had the following composition (common batch used for all the GENEPEASE WP2 experiments): Coarse sand: 37.3%; Fine sand: 41.7%; Silt: 9.8%; Clay: 9.1%; Organic matter: 2.1%; pH: 6.4; C: 1.24%; N: 0.27%; C:N: 4.7; P: 3.33 mg kg^{-1}; K: 11.1 mg kg^{-1}; Mg: 6.7 mg kg^{-1}; NO3- 19.9 mg kg^{-1}; and NH4+: 1.53 mg kg^{-1};
A simple determination of the waterholding capacity WHC of 30% was based on OECD 232 (2009). Soil analyses was determined by OK Laboratorium for jordbrug, Viborg Denmark, according to Danish procedures (Plantedirektoratet, ´1994). Soil treatment is described in details in the different sections but in brief: Soil used in microcosms was air dried at room temperature and sieved through a 2 mm sieve. Soil used in single species collembolan experiments was sieved through a 2 mm sieve and dried at 80 °C and stored in dry condition until use. Soil used in mesocosm experiment was fresh and not sieved.

2.2 Pesticides

We tested two formulated pesticides, Basamid GR and Tridex DG. Basamid is a soil disinfectant with the active compound dazomet (tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione). Basamid was obtained as granulate from SiLa Plantebeskyttelse (Hillerød, Denmark). Tridex is a fungicide with the active compound mancozeb [[1,2-ethanediylbis[carbamodithioato]](2-)]manganese mixture with [[1,2-ethanediylbis[carbamodithioato]](2-)]zinc. Tridex was obtained from DLG Garta (Odense, Denmark) as granulate from United Phosphorus Ltd. The concentrations used in the experiments were 266 mg kg^{-1} for Basamid and 13.3 mg kg^{-1} for Tridex. The concentration of Basamid corresponded to field dose and for Tridex it was five times the field dose. These doses were calculated assuming distribution of the pesticides within the top 5 cm of the soil and a soil density of 1.5 g cm^{-1}.

2.3 Microcosm setup

Soil was air dried at room temperature in the dark for 3 days and then homogenised by sieving (2 mm mesh). We then prepared microcosms by adding 10 g of sieved soil to 50 ml polypropylene tubes. Pesticide and/or ammonium sulphate (100 mg N kg^{-1} soil) in sterile distilled H_2O was added to each microcosm to a final soil moisture content of 60% of water holding capacity. Thus, we set up a full factorial design with six treatments; Basamid, Tridex no pesticide, with or without ammonium sulphate. All microcosm-tubes were covered with polyvinylchloride film and stored in the dark at 20 °C. We harvested triplicate tubes five times; after 1 hour and after 3, 12, 21 and 28 days. At each harvest, a subsample of 500 mg of soil was flash-freezed in liquid nitrogen for later extraction of DNA and RNA, and the remaining soil sample was stored at -20 °C until nitrate determination.

2.3.1 Nitrate analysis

In the microcosms experiment the nitrate was extracted by mixing soil (9.5 g moist) with KCl (0.1 M) to a total volume of 47.5 ml. We deviated from the OECD 216 guideline (2) in that the 50- ml extraction tubes were more than half full. To compensate for this, we shook the tubes manually

until all silt and clay particles were in suspension, and then placed the containers on an end-to-end shaker for 60 min according to the guideline. To test for effects of co-extracted compounds, we spiked the non-amended extracts from days 7, 12 and 28 with NO_3^--N at 1.00 mg l^{-1}.
The nitrate concentrations of soil extracts were determined by a chromogenic microplate method (Hood-Nowotny et al 2010). In this assay, nitrate is reduced to nitrite by VCl_3 in acid solution. Nitrite then forms a colour complex with N-naphthyleethylenediamine and sulfanilamide in a Griess reaction hence, the result is the total sum of nitrate and nitrite present in the sample. Degradation of ammonia to nitrite is usually the rate limiting step of nitrification and the nitrite content is consequently insignificant in most soil samples. Nitrate standards (5.0-0.078 mg NO_3^--N l^{-1}) were added in duplicate to each microplate. The soil extracts (diluted to 0.2-2.0 mg NO_3^--N l^{-1}) were added in quadruplicate of which duplicates were added Griess reagents and VCl_3, and duplicates served as humus controls. Wells at the edges of the microplates were only added water due to edge effects. After incubation (90-100 min, 37 °C), the absorbance was read at 590 nm, and converted to nitrate concentration.

2.4 Extraction of nucleic acids, cDNA synthesis and qPCR

DNA and RNA were extracted simultaneously from soil using a phenol-chlooform extraction procedure (Griffiths et al. 2000) with the following modifications: In order to minimize DNA loss by sorption to clay particles, 0.5 ml G2 (GEUS, Copenhagen, DK) was added to 1.4 mm ceramic bead tubes (Mo Bio Laboratories, Inc, Carlsbad CA, US), and the tubes were freeze-dried prior to use. Hexadecyltrimethylammonium bromide (CTAB) and phenol chloroform was added to the bead tubes along with the frozen soil samples and bead beating was performed at speed 5 for 20 s in a FastPrep FP120 (BIO 101, Farmingdale, NY). Two runs of bead beating were performed with an intermediate cooling step for 1 min at ice. The aqueous phase was separated by centrifugation for 10 min (16.000 × *g*) at 4 °C. After removal of phenol with chloroform-isoamyl alcohol, 1 µl of glycogen (Roché, Basel, Switzerland) was added instead of polyethylene glycol, and the samples were placed on ice for 2 h to facilitate nucleic acid precipitation. Samples were kept on ice during all steps of the extraction.
RNA/DNA extracts were purified using the RNA purification kit (NucleoSpin RNA Clean-up XS kit, Macherey-Nagel, GmbH & Co. KG) and eluted in 20 µl of RNase-free H_2O; the samples were subsequently split in two. One part was diluted 10 times with RNase-free H_2O and stored at -80 °C until DNA analysis. An undiluted aliquot of the extract was immediately subjected to DNase-treatment using the RTS DNase Kit (Mo Bio) according to the manufacturers' instructions. A subsample of the DNase-treated sample was used as template in cDNA production using random hexamer primers (Fermentas) and the RevertAid Premium RT kit (Fermentas) in a RT-PCR procedure according to the manufactures' protocol. Extraction, DNase treatment and reverse transcriptase were done in one working-day. The quantity and quality of extracted RNA was checked on an Agilent 2100 Bioanalyzer with the prokaryote total RNA pico chip, which uses the relative signal intensity of 16S and 23S rRNA to calculate the RNA integrity number (RIN).

2.4.1 amoA real-time PCR assays

The numbers of *amo*A gene copies and mRNA transcripts were quantified by real-time PCR using primers amoA-1F and amoA-2R (Rotthauwe et al 1997) for bacterial ammonia oxidizers and amoA19F (Leininger et al 2006) and CrenamoA616r48x (Schauss et al 2009) for archaeal ammonia oxidizers. Reactions were carried out in a final volume of 20 µl containing dH_2O, SYBR Premix Ex Taq™ (Tli RNaseH Plus) (Takara RR420A), 5 pmol of each primer and 1 µl DNA or cDNA template. The real-time PCR reactions were performed in an iCycler Thermocycler (Bio-Rad) with the following cycling conditions; 95 °C for 1 min continued by 40 cycles of 95 °C for 30 s, 58 °C/55 °C (bacterial *amo*A/archaeal *amo*A) for 30 s and 72 °C for 45 s.
As a standard for bacterial *amo*A quantification we used the *amo*A gene of a *Nitrosomonas europaea* ATCC19718 derived lux-marker strain (pHLUX20). The gene was PCR amplified using primers amoA-1F and amoA-2R and cloned in the *E. coli* pCR 2.1-TOPO vector (Invitrogen)

according to the manufacturers' protocol. As standard for archaeal *amo*A, fosmid clone 54d9 (Treusch et al. 2005) was used. Standard curves were made from the extracted plasmids using 10-fold dilutions in the range 10 to 10^7 bacterial *amo*A copies per µl and 3×10 to 3×10^6 archaeal *amo*A copies per µl.
Negative controls of DNase-treated RNA samples were included in the qPCR to ensure the absence of contaminating DNA. The specificity of the PCR amplification was tested by inspection of the melting curves prepared at the end of each PCR run. A subset of the PCR products was also run on a gel to verify the presence of a single band of the correct size.

2.4.2 16SrRNA gene and 16SrRNA real-time PCR assays

The numbers of 16SRNA gene copies and 16SRNA transcripts were quantified with the use of real time PCR (CFX96™ Real-Time System, Biorad). *E. coli* was used as standard. Each triplicate soil sample was run in technical duplicates. Mastermix consisted of 2 µl BSA, 10 ul SsoFast EvaGreen Supermix (Biorad), 0.8 µl of forward primer 341F: 5´-CCTAYGGGRBGCASCAG-3' (10 µM) , 0.8 µl of reverse primer 806R: 5´-GGACTACNNGGGTATCTAAT-3' (10 µM), 1 µl diluted template and water to a total of 20 µl. PCR conditions were: 98°C for 15 min, followed by 35 cycles of 98°C for 30s, 56°C for 30s and 72°C for 30s (with RFU measurements) and ended with 72°C for 7min.

2.4.3 Preparation of 16SrRNA gene and 16SrRNA libraries for pyrosequencing on a Roche Titanium 454 platform

Samples from day 12 - previous shown to have the largest variation compared to day 0 controls (Hjelmsø et al 2014) - were selected for further analysis. Each sample was amplified with a mastermix containing : 4µl of 5x Phusion HF buffer (Thermo Scientific), 0.4 µl of 10 mM dNTP mixture, 0.2 µl Phusion Hot Start II DNA Polymerase (2units/ µl, Thermo Scientific), 1µl of forward primer 341f, 1 µl of reverse primer 806r ,1 µl of 10x diluted template and H_2O to a total of 20µl. PCR conditions were 98°C for 30s, followed by 30 cycles of 98°C for 5s, 56°C for 20s and 72°Cfor 20s, and final extension at 72°C for 5 min. The samples were then run on a 1.25% agarose gel and specific bands were cut out and purified using the Montage DNA Gel Extraction Kit (Millipore).

Tags were then added with a 2.round PCR using same primers as 1. round PCR with 10bp long individual tags added. Purified PCR products were used as template, and only 15 cycles were used in the PCR program. Conditions and mastermix were otherwise the same as the first round PCR. Second round PCR products were then purified as before and DNA concentrations were measured on a Qubit (Invitrogen). Samples were then mixed together creating a equimolar solution to a total of 1 µg. Adapter-ligation, Emulsion PCR and 454 sequencing were done by Beckman Coulter Genomics on a 454 GS FLX Titantium (Roche) (1/2 titanium plate) producing, on average, 5229 sequences per sample.

2.4.4 Preparation of 18SrRNA gene and 18SrRNA libraries for pyrosequencing on a Roche Titanium 454 platform

Chosen samples were amplified with a mastermix containing : 4µl of 5x Phusion HF buffer (Thermo Scientific), 0.4 µl of 10 mM dNTP mixture, 0.2 µl Phusion Hot Start II DNA Polymerase (2units/ µl, Thermo Scientific), 1µl of forward primer 18S-cercozo_F (5'-TGT TGC AGT TAA AAA GCT CGT-3'), 1 µl of reverse primer 18S-cercozo_R (5'- TGA ATA CTA GCA CCC CCA AC -3') ,1 µl of 10x diluted template and H20 to a total of 20µl. PCR conditions were 98°C for 30s, followed by 30 cycles of 98°C for 5s, 55°C for 20s and 72°Cfor 20s, and final extension at 72°C for 5 min. The samples were then run on a 1.25% agarose gel and specific bands were cut out and purified using the Montage DNA Gel Extraction Kit (Millipore).

Tags were then added with a 2. round PCR using same primers as 1. round PCR with 10bp long individual tags added. Purified PCR products were used as template, and only 15 cycles were used in the PCR program. Conditions and mastermix were otherwise the same as the first round PCR. Second round PCR products were then purified as before and DNA concentrations were measured

on a Qubit (Invitrogen). Samples were then mixed together creating an equimolar solution to a total of 1 µg. Adapter-ligation, Emulsion PCR and 454 sequencing were done by Beckman Coulter Genomics on a 454 GS FLX Titantium (Roche) (1/2 titanium plate) producing ~10000 sequences per sample.

2.5 Fate studies

Mineralisation and sorption of the fungicide Tridex in the soil was analysed by ^{14}C-labelling of the active component mancozeb and radioactivity measurements. The labelled mancozeb was purchased from Institute of Isotopes Co. (Budapest, Hungary) and had a specific activity at 64.16 mCi g^{-1}. We did not use the formulated compound as it is not available as ^{14}C-labelled. Mancozeb was labelled in a position so that ^{14}C was not released until the compound was completely mineralized and the last daughter compound ethylenurea degraded to CO_2. Triplicates were set up in 100 ml airtight flasks with 20 g soil with or without ammonium sulphate (100 mg N g^{-1} soil) and water regimes corresponding to 60% of WHC. Tridex was added to the soil at 2.7 mg kg^{-1} and ^{14}C-labeled mancozeb at 10 000 DPM pr. flask. A base trap containing 2 ml of NaOH was placed in each flask to collect $^{14}CO_2$. During two months incubation at 20 °C, the base traps were replaced regularly and the radioactivity was measured by liquid scintillation counting on a Perkin Elmer Tri-Carb 2810 TR scintillation counter using 10 ml of Wallac OptiPhase HiSafe 3 scintillation cocktail (Perkin Elmer, Turku, Finland) per sample.

Sorption of Tridex in the soil was measured as a function of the pesticide concentration by a procedure modified from the OECD guideline (OECD 2000). Sorption isotherms were determined by setting up triplicate samples of 1 g of soil in glass vials with Teflon caps. Initially, the soil was equilibrated with 1 ml of a 0.01 M $CaCl_2$ solution for 12 h. Solutions of Tridex and ^{14}C-labeled mancozeb were subsequently added to the vials to reach a 1:10 soil to solution ratio and final concentrations of 0, 0.2, 0.4, 1.0, 2.0 and 4.0 mg Tridex l^{-1} and 2500 DPM ml^{-1} soil solution. The vials were mixed on an end-to-end rotator for 2 h and 24 h at room temperature. After a two-step centrifugation, the radiation of the supernatant was measured by liquid scintillation counting as in the mineralisation experiment.
The fumigant Basamid is degraded rapidly in moist soil via a chemical conversion of the active component dazomet to methyl isothiocyanate (MITC) (Ruzo 2006). The volatilization of MITC was analyzed by application of ^{14}C-labeled MITC (American Radiolabeled Chemicals, Missouri, USA) to air-dried or moist (60% WHC) soil in airtight serum flasks with Teflon stoppers. The flasks were placed either on ice (0 °C), or at 20 °C, and volatilization was measured during a 72 h-period. Subsamples were redrawn from the headspace using a hypodermic needle and injected into new airtight flasks with scintillation liquid. The radioactivity of MITC in the gas-phase was counted in a gas-liquid equilibrium.

2.6 Conventional toxicity assessment of *Folsomia candida*

Conventional lethal- and sublethal toxicity tests were performed with the model pesticides and the collembolan *F. candida* similar to the present international standards (ISO, 1999; OECD, 2009). It was obtained from permanent cultures at Department of Bioscience, Aarhus University, of the FCDK/Berlin strain (Simonsen and Christensen, 2001; Tully et al., 2006) where it had been cultured for about 25 years since its arrival in Denmark from BBA in Germany in the late 80-ies.

We conducted studies of acute and chronic toxicity of the two model pesticides on the springtail *F. candida*. The setup of the experiments included range-finding studies and definitive tests. The outcome of these tests was used for the selection of test concentrations of animals for transcriptomic analyses with microarrays. Basamid was exposed to *F. candida* only for 48 hours, as no further exposure would happen beyond this period due to the fast degradation of Basamid, DT_{50} (20^{o} C) 2 hours – 5 days (EFSA, 2010).

2.6.1 Range-finding tests with Basamid and Tridex for effects on mortality and reproduction of *F. candida*

F. candida at age 9-12 days was exposed in the Askov soil substrate, 25 g dry weight and 30 g fresh weight per replicate, for 48 hours in an acute toxicity range-finding test with Basamid and a 3 week reproductive test with Tridex at the concentrations:

Basamid: 0, 0.25, 0.5, 1.0, 2.0 and 3.0 mg kg^{-1}
Tridex: 0, 0.5, 1.0, 5.0, 10, 100 and 500 mg kg^{-1}

There were two replicates per treatment concentration. The purpose of the range-finding test was to identify concentrations where a 10% effect occurred. Ten adult 22-25 days old *F. candida* per replicate were exposed to Basamid for 2 days in Askov soil in the increasing soil concentrations. The numbers of adult *F. candida* that were either dead, not recovered by flotation, or affected otherwise by Basamid were considered affected and subject to toxicity assessments

Range-finding of Tridex was performed similar to standard toxicity testing, e.g. (OECD, 2009), where the range-finding test concentration series was partly geometrical with 0, 0.5, 1, 5, 10, 100, 500 mg kg^{-1}.

2.6.2 Definitive test with Basamid and Tridex for effects on mortality and reproduction of *F. candida*

The procedure was identical to the range-finding except the age of *F. candida* was 22-25 days, 4 replicates per treatment instead of 2, using 30 individuals per replicate container and 30 g moist Askov soil for the acute Basamid test and 60 g moist soil for the Tridex chronic test. To have enough RNA 30 animals was added to the soil. The 60 gram soil was chosen to avoid density dependent reproduction as the triple times initial adults, i.e. 30 adults, compared to the normally 10 juveniles for 30 gram soil could lead to such an inhibition of the reproduction. The mortality was assessed as the number of adults not recovered alive and well from the flotation process.
The following concentrations series was employed for the definitive tests:
Tridex: 0, 50, 100 mg kg^{-1}
Basamid: 0, 2.0, 3.0, 5.0 and 10 mg kg^{-1}

2.6.3 Preparation for transcriptomics of *F. candida*

Based on the estimates of LC's and EC's (see Table 1) with *F. candida* we selected two concentrations for transcriptomic responses: the highest concentration was about the LC_{10}/EC_{10} and the lowest was one third of the LC_{10}/EC_{10}. This was a "guestimate" based on the expectation that if we ran even lower concentrations we would not be able to detect gene expression effects. Thus, for the *F. candida* test specimen for the microarray transcriptomic analyses we have run the following test concentrations for each of the 4 replicates per treatment concentration:

Tridex: 0, 10, 30 mg kg^{-1}
Basamid: 0, 1.0, 3.0 mg kg^{-1}

Again, we used 30 g moist Askov soil in microcosm containers for the Basamid test but 60 g moist soil for the Tridex reproduction tests, and we added 45 *F. candida* adults into each replicate. Thirty individuals were used for transcriptomics and 15 were stored at – 80 ^{o}C for possible qPCR analysis in Silkeborg, acting as control and cross-references between the microarray animals and the qPCR animals for the last occasion of the temporal series if needed. Thus, the extra 15 individuals is be available for qPCR analyses of selected gene expressions, e.g. to be done for selected gene transcripts responding to the pesticide.

The animals were dispatched in deep frozen condition with dry-ice for microarray analysis.

2.7 The microarray transcriptomic analyses of *F. candida*

Animals from the experiment was collected by water flotation of the test soil and transferred to liquid nitrogen in 1.5 ml Eppendorf tubes for quick freezing to prevent responses by the collembolans due to the handling. They were shipped in frozen condition to VU in late January 2012. At VU animal RNA was extracted and checked for RNA quality.

2.7.1 RNA integrity

RNA integrity was confirmed on a 1% agarose gel and RNA quantities were assessed with a nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) on all the 24 samples used subsequently for microarray analysis. It ranged between 130 and 420 ng μL^{-1} of total RNA except in one sample where it was 17 ng μL^{-1}, which was still acceptable. However, in all cases the threshold ratio of ~2.0 for the 260 nm/280 nm absorbance ratio was exceeded in all samples, therefore the RNA is considered pure, and there was no interference of protein or other contaminants that absorb strongly around 280 nm. The secondary measure of nucleic acid purity, the 260/230 ratio, was mostly above 2.2 except in 4 cases, where it was between 1.7-2.0. So the 260/280 and 260/230 is considered in this study to be free from protein contamination and (organic) salts.

2.7.2 Microarray procedure

Gene expression analyses was performed for the 24 RNA samples of the F. candida exposed to Basamid for 48 hours and to Tridex for 3 weeks (see section 2.6.3). RNA preparation, labelling and hybridisation were performed as described by van Ommen Kloeke et al. (2012) and Nota et al. (2009). The microarray platform "GPLxxxx iSQ Chip *F. candida* Collembola VU 75K version 3.0" (platform records forthcoming on the Gene Expression Omnibus (GEO) repository www.ncbi.nlm.nih.gov) was employed at the Department of Ecological Science, VU university Amsterdam. It is a custom made 60-mer, 8 × xxK oligo microarray and contains 75K unique probes representing 75K different gene clusters from Collembase www.collembase.org). All probes were spotted randomly in triplicate (Nota et al., 2009). For hybridisation a loop design was used. Single-colour methods were employed so there was no need to normalize between colours as in previous approaches. Therefore, only the 'normexp' background correction and 'quantile' normalization were used. The array data analysis does not make use of reference genes or house-keeping genes because the full transcriptome was available with iSQ ver 3.0.

After feature extraction (Agilent FE software version 9.5.1.1) microarray data was further analysed using the package 'Limma' (version 2.18.3 (Smyth, 2004) in the statistical environment R (version 2.9.0). In short, normexp background correction (Ritchie et al., 2007), LOESS normalization and aquantile normalization (Hahne et al., 2008) were performed for the whole dataset. Using Blast2GO (Conesa et al., 2005), part of the differentially expressed genes were annotated to known genes, assigned to a gene ontology (GO) term or referenced to an InterPro number, setting the hit threshold at e-value <1.0e-6. The raw and processed microarray data will be available from the NCBI Gene Expression Omnibus (GEO).

GO term enrichment analysis was made for both pesticides. The GO term Enrichment Analysis was performed for the significant log fold changes (logFC) of expressed genes as detected by statistical testing (de Boer et al 2011) to assess which biological processes (BP) and molecular functions (MF) were mostly affected.

Significant transcripts compared to the control were selected by ANOVA by running the Limma package for R. The widely used Fisher's exact test was employed for each GO term to ascertain the significance compared to a random gene selection. The procedure is as follows: the probability that a gene will be flagged under the GO term is p_1 and the probability that it will not is p_2. The null hypothesis H_0: $p_1 = p_2$ will be true if genes are flagged under the GO term purely by chance, and there is no significant difference in the two categories. The Fisher's exact test is a conditional test given the sufficient statistics $n_f/n,(N_f-n_f)/(N-n)$ where: n_f is the number of flagged genes under the GO term; n is total number of genes under the GO term; N_f is the number of flagged genes on the

microarray; and N is the total number of genes on the microarray. The SAS FREQ procedure was used to calculate the Fisher's exact test statistics (SAS Institute Inc. 2013).

The significant common gene transcripts among the treatment groups were calculated and visualized by a Venn diagram produced by running a SAS macro (SAS Institute, 2013) on our microarray data.

2.8 qPCR of *F. candida*

2.8.1 Experimental design

Single species

The experiment dedicated to generating samples for qPCR investigation consisted of exposing 15 22-25 days old adult F. candida to Basamid and Tridex at soil concentrations of 0, 0.1, 0.3, 1.0 and 3 mg kg^{-1} and Tridex at 1, 3, 10, 30 mg kg^{-1} for increasing number of days: 2, 7, 14, 21 and 28 days. All treatments were replicated four times. The high test concentration of 3 mg kg^{-1} for Basamid and 30 for Tridex was made to enable a comparison with the same maximum concentrations of the microarray study and for only one period, i.e. 2 days and 21 days, respectively.

Mesocosms

After the mesocosm experimentation was terminated, the animals were extracted from soil samples by water flotation and immediately frozen in liquid nitrogen, and then stored at -80°C until analysis.

2.8.2 RNA extraction and cDNA synthesis of *F. candida*

Extraction of RNA from each sample (10-15 adult animals) was done by using the RNeasy Mini kit with on-column DNAse treatment (Qiagen, Copenhagen, Denmark) according to the instructions provided by the manufacturer. Due to the number of samples, the extraction was divided in several independent runs, with the sequence of samples randomized to avoid systematic effects. For the samples of the mesocosm experiment, fewer animals were available, and these samples consisted of 6-10 animals extracted in a single block. The concentration of RNA was determined by using an Implen NanoPhotometer (spectrophotometer, AH Diagnostics, Aarhus, Denmark). Following the manufacturer's instructions, cDNA was synthesized from 500 ng total RNA using the Omniscript Reverse Transcriptase kit (Qiagen) and Anchored Oligo $(dT)_{20}$ primers (Invitrogen A/S, Taastrup, Denmark). cDNA was synthesized in two blocks, with all replicate 1 and 2 samples in one run and all replicate 3 and 4 samples in the other run. cDNA for the mesocosm samples were synthesized in a single block. Finally, cDNA was diluted in nuclease free water to a concentration equivalent to 4 ng total RNA μL^{-1}, and stored at -20 °C until further use.

2.8.3 Gene expression (qPCR) of *F. candida*

The sequences of the analysed genes were obtained from a transcriptome of *F. candida* (D. Roelofs et al., unpublished, forthcoming on NCBI/GEO) used for generating the microarray. Preliminary annotated isotigs of interest were identified from the array experiment and primers for these candidate genes were designed by using Primer3 and were synthesised by Sigma-Aldrich. Stratagene Brilliant® II SYBR® Green qPCR Mastermix (AH Diagnostics, Aarhus, Denmark) was used for Real-time quantitative polymerase chain reaction (qPCR) conducted on a Stratagene MX3005P (AH Diagnostics, Aarhus, Denmark). Each reaction contained 5 µL of cDNA template (equivalent to 20 ng total RNA) along with 900 nM primers in a final volume of 15 µL and these reactions was run in duplicate. The amplification was performed under the following conditions: 95 °C for 10 min to activate the DNA polymerase, then 40 cycles of 95 °C for 10 s and 60 °C for 60 s. Melting curves of the raw qPCR data were inspected to confirm the presence of a single amplification product with no primer-dimers.

2.9 Mesocosm experiment - experimental setup

Soil from Askov (see above) was air-dried to a water content of 4% (w/dw). Portions of 520 g of this soil, corresponding to 500 g dry soil, were weighed into glass jars (2 l volume). In order to stimulate microbial activity and as a source of nitrogen for the nitrifiers we mixed the soil with 2.5 g of dried, ground maize leaves. In total we prepared 54 mesocosms with maize addition. In addition, we also prepared 21 mesocosms treated in the same way but without maize addition. These 21 mesocosms without were used for respiration measurements and allowed us to evaluate the maize-induced decomposition activity (see below). All microcosms were then amended with 60 ml of tap water and the soil was gently stirred to ensure homogenous distribution of water. The water addition raised the water content in the soil to 16 % (w/dw). We then incubated the jars at 20 °C for two days before we added the pesticides. Two days after the initial construction of the mesocosms we started the pesticide treatment. The two day preincubation period ensured that the microorganisms were active when they were exposed to the pesticides. Basamid and Tridex were added with 20 ml of water resulting in the same soil concentrations as used in the microcosm experiment (266 mg kg^{-1} for Basamid and 13.3 mg kg^{-1} for Tridex). Control samples received 20 ml of distilled water and the resulting water content in the mesocosms (20%, w/dw) corresponded to 60% of water holding capacity. After pesticide addition we closed the jars and incubated them at 20°C. During the whole incubation period, we regularly weighed the jars and added water to constant weight in order to maintain soil moisture at approximately 60% of water holding capacity. Three days after pesticide addition, we added collembolans (*F. candida*), enchytraeids (*Enchytraeus crypticus*) and predatory mites (*Hypoaspis aculeifer*).

2.9.1 CO_2 measurements in Mesocosms

The mesocosms were incubated for three months. At intervals during this time, we closed the jars for a period of up to a few days to allow accumulation of CO2 in the headspace. At the end of each of these incubation periods, we measured the concentration of CO2 in the headspace (six replicates of each treatment) by injecting 0.5 ml gas samples into a gas chromatograph with a thermal conductivity detector and 1.8m×3mm Poropak Q column operated at 35°C. For these measurements, we also included the mesocosms without maize addition. Hence, in total we measured gas from 36 mesocosms at each occasion (six replicates from each of the three pesticide treatments with maize addition and six replicates from each of the replicates without maize addition). Furthermore, at the five occasions, on day 1, 3, 10, 31, 53, we transferred 8.0 ml headspace to 5.9 ml Exetainer vials for 13C/12C ratio analysis on Gas Chromatography Isotope Ratio Mass Spectrometer (performed at Risø National Laboratory)

2.9.2 Destructive sampling in Mesocosms

Immediately after pesticide addition and at three occasions during the incubation (day 3, 31 and 87) we destructively sampled three mesocosms from each of the three pesticide treatments (water, Tridex and Basamid). Only mesocosms amended with maize were used for destructive sampling. The sampling was carried out by carefully removing soil from the jars and gently homogenizing the soil in a tray. At each sampling, we took samples for analysis of ammonium and nitrate (15 g soil) and enumeration of protozoa (5 g soil). At the samplings after 31 and 87 days, we carefully transferred the soil back into the jars and transported them to DMU, Silkeborg, for extraction of soil animals.

2.9.3 Enumeration of protozoa in Mesocosms

Protozoa were enumerated by homogenizing 5 g soil samples with 100 ml amoebae saline by mixing in a kitchen blender for one minute. Dilution series were prepared in microtitre plates (Costar, No. 3598; Biotech Line ag., Slangerup, Denmark) by a modified version of the “most probable number method” using dilute Tryptic Soy Broth (0.1 g•l-1, Difco, BD, Brøndby, Denmark) as medium (Rønn et al. 1995). Microtitre plates were incubated at 15°C in darkness, and individual wells were inspected for the presence or absence of protozoa after 1 and 3 weeks by an Olympus CK 2 inverted microscope (×200 magnification, phase contrast).

2.10 Data analyses and statistics

2.10.1 Nitrate measurement and *amo*A qPCR quantification

Reported values are the means from triplicates. Comparison of treatments with pesticides and N-addition was done using the Wilcoxon test on triplicate samples from all time points. Comparison of development between two time points was done using the Student's t-test on triplicate samples.

2.10.2 Bioinformatic analyses of cercozoan sequences

The samples from the microcosm and mesocosm experiment (11 and 23 samples respectively) were analysed together on one half plate (Beckman-Coulter Genomics). The 454 sequencing run produced 521153 reads. We analysed these data using Mothur (Schloss et al. 2009) discarding all singletons, sequences that had a quality score below 35, more than 10 homopolymers, any mismatches in the primer or MID tag sequences and a length outside 200-360 bps. The remaining sequences were checked for chimeras and all suspected chimeras were removed from the data set. The remaining sequences were pre-clustered to 5% and the data set was divided into two separate sets from the microcosm and mesocosm experiment respectively. Two of the samples from the mesocosm experiment contained few sequences and were discarded. We then subsampled the samples from the two experiments to obtain the same number of sequences from each sample. This resulted in 1020 sequences from each sample in the microcosm experiment and 1188 sequences in the mesocosm experiment. The 30 dominant OTU's (5% level) found in each of the two experiment were manually blasted against GenBank. We chose the 5% OTU level since previous work showed that this value is reasonable for analyses of cercozoan data (Harder et al. 2014). To get an overview of the overall structure of the cercozoan communities we classified the OTU's into larger taxonomic units (mainly family level). Furthermore, we analysed the composition of the communities from each treatment by principal coordinate analyses using the facility provided in Mothur.

2.10.3 Dose-response data for *F. candida*

The mortality of *F. candida*, in the Basamid test was subject to a probit analysis according to the sigmoid model:

$$m = c + (1 - c)\Phi(a + b \cdot conc)$$

and point estimations of LC_{10} and LC_{50}:

$$LC_{10} = \frac{\Phi^{-1}\left(\frac{0.1 - c}{1 - c}\right) - a}{b}$$

$$LC_{10} = \frac{\Phi^{-1}\left(\frac{0.5 - c}{1 - c}\right) - a}{b}$$

where Φ is the cumulative normal distribution function and Φ^{-1} is the inverse, i.e. the probit function; c is control mortality; a and b are shape parameters. The mortality was always modelled according to a binomial distribution.

The reproduction of the range-finding test with Tridex was modelled according to an exponential decay model:

$$\text{Juveniles} = c \cdot e^{-a \cdot conc}$$

The mortality of both the Basamid range-finding and the Tridex and Basamid definitive test was modelled according to a second degree polynomial model:

$$\text{Mortality} = c + b \cdot conc + a \cdot conc^2.$$

All EC and LC modelling was performed with the SAS procedure PROC NLMIXED (SAS-Institute-Inc., 2011).

2.10.4 Microarray with *F. candida*

Each gene was represented by four data points (biological replicates/microarrays). Differential expression was then assessed by means of linear models and empirical Bayes methods. Finally the Benjamini–Hochberg's false discovery rate method (Benjamini and Hochberg, 1995) was used for multiple testing corrections (adjusted p\0.05 was considered significant). MA-plots and boxplots were used for quality control of the data for each array. The expected and observed log ratios of the Agilent spike-in control probes showed a R^2 >0.95 for all arrays. Differential expression analysis was performed for several contrasts between the treatments resulting in a mean $\log_2$ expression ratio (treated/untreated) and a p-value for each probe on the array.

2.10.5 qPCR of *F. candida*

Data Analysis for Real-Time PCR (DART-PCR) (Peirson et al., 2003) was used for analysing the raw qPCR data. For every sample DART-PCR enables calculation of threshold cycles and amplification efficiencies. Calculated efficiencies indicated around 2-fold amplification per PCR cycle for all genes. In the few cases where outliers were identified by DART-PCR, they were removed from the dataset prior to further analysis. The resulting data set was normalised using the data-driven NORMA-Gene normalization method (Heckmann et al., 2011).

3. Results

3.1 qPCR quantification of amoA genes and mRNA transcripts

Practically equal counts of archaeal and bacterial *amoA* genes and transcripts were detected in the soil prior to addition of pesticides and N-amendment. The development in population size and activity of the two communities was highly influenced by addition of ammonium sulphate. In amended soil, bacterial *amoA* transcripts became fifty times more abundant than archaeal *amoA* transcripts. Oppositely, in non-amended soil, archaeal *amoA* transcripts became thirty times more abundant than bacterial transcripts (Fig. 1).

The numbers of archaeal and bacterial *amoA* genes were present in similar quantities (app. 3×10^6 copies g^{-1} soil) (Fig. 2) prior to pesticide treatment and N-amendment. Likewise, the initial *amoA* transcript numbers for the two populations were similar (app. 2×10^5 transcripts g^{-1} soil). Ammonium sulphate significantly stimulated bacterial ammonia oxidizers, resulting in a doubling of the population size and a five-fold increase in *amoA* transcripts, which subsequently decreased to initial levels (Fig. 1 + Fig. 2). In contrast, no bacterial growth occurred in non-amended soil and bacterial *amoA* transcripts decreased tenfold. Oppositely, amendment with ammonium sulphate led to a significant decrease in *amoA* transcripts of the archaeal ammonia oxidizers, whereas an increase was observed in non-amended soil (Fig. 1).

Treatment with Tridex seemed to inhibit bacterial ammonia oxidizers by causing a delay (though non-significant, P = 0.06) in the induction of *amoA* transcription and a retarded peak in transcript numbers at day 21 in N-amended soil (Fig. 1). A similar trend was observed for Tridex on bacterial *amoA* genes, which reached the highest abundance at day 21 as opposed to day 12 in N-amended soil without pesticide (Fig. 2). In Basamid-treated samples, a large reduction in both bacterial and archaeal *amoA* gene copies (from app. 2×10^6 to 2×10^4) indicated killing of ammonia oxidizers (Fig. 2). Immediately after exposure (T = 1 h), Basamid also caused a substantial decrease in both bacterial (×400-1000) and archaeal (×100) *amoA* mRNA copies. As for nitrification, there was a trend to recovery of both bacterial and archaeal ammonia oxidizers upon Basamid treatment, however a significant increase in *amoA* levels was not recognized even after 28 days (Fig. 1 + Fig. 2).

TABLE 1

COEFFICIENTS OF DETERMINATION (R^2) FOR NITRIFICATION RATES AND AMOA. THE COEFFICIENTS HAVE BEEN DETERMINED ACCORDING TO THE SOURCE OF AMOA (BACTERIAL, ARCHAEAL OR TOTAL SUMMED AMOA) AND BY GROUPING OF ALL SAMPLES (CONTROL)

amoA	+ NH_4SO_4	No N-amendment
Bacterial transcripts	0.55***	0.16*
Archaeal transcripts	0.11^{NS}	0.03^{NS}
Total transcripts	0.47***	0.11^{NS}
Bacterial genes	0.45***	0.18*
Archaeal genes	0.10^{NS}	0.13*
Total genes	0.31***	0.16*

3.2 Nitrification

Nitrate production in the soil was significantly stimulated by ammonium sulphate and followed a sigmoid trend with an initially increasing production rate pursued by a decreasing rate towards the end of the incubation (Fig. 1). A calculation of N-pools implies that this development was due to an exhaustion of the added ammonium substrate during the experiment. Hence, the produced amount of NO_3^--N in the amended controls (200±4 mg NO_3^--N kg^{-1}) approximated the amount of nitrogen added to the system as ammonium sulphate plus the nitrate developed in the non-amended control (190±2 mg NO_3^--N kg^{-1}). In non-amended soil, a significant increase in nitrate concentration was also observed during the experiment however, it was approximately seven times lower than in N-amended soil (Fig. 1).

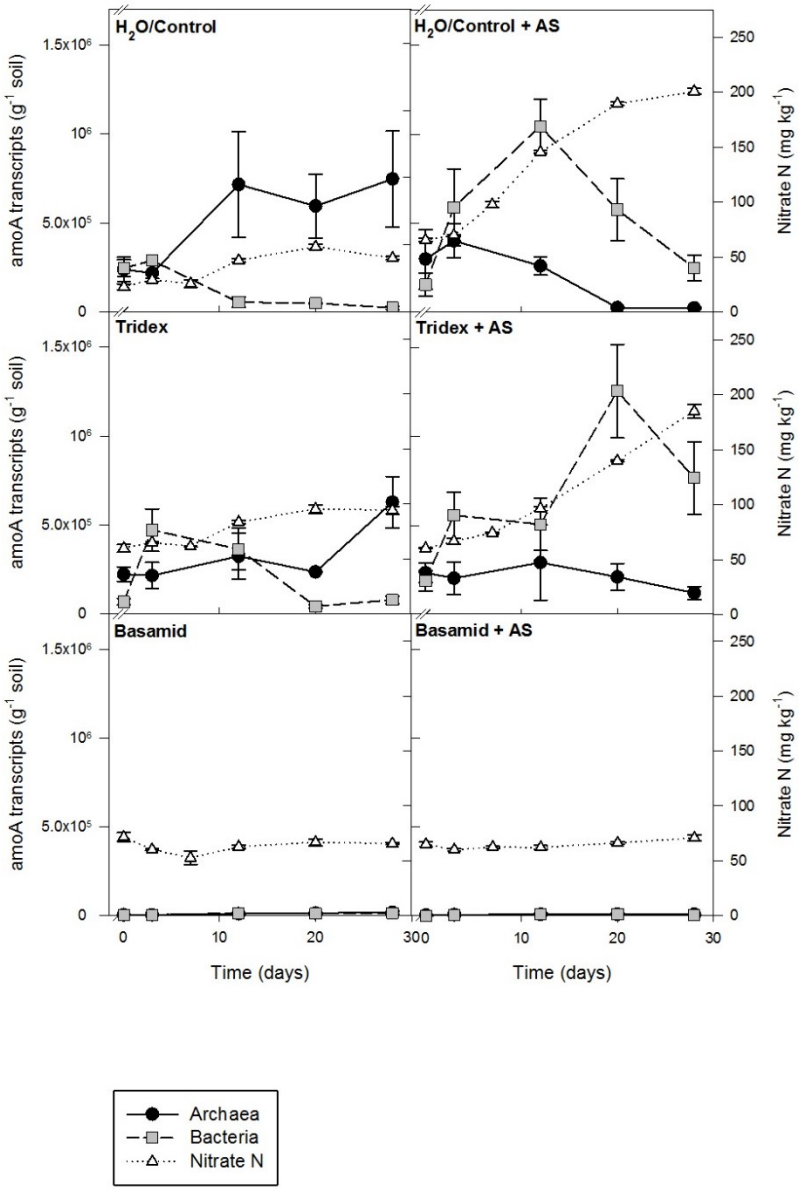


FIGURE 1
ABUNDANCE OF AMOA TRANSCRIPTS AND NITRIFICATION IN SOIL WITH AND WITHOUT TREATMENT WITH PESTICIDES AND AMENDMENT WITH AMMONIUM SULPHATE (AS). LEGENDS ARE; BACTERIAL AMOA TRANSCRIPTS (GREY SQUARES), ARCHAEAL AMOA TRANSCRIPTS (BLACK CIRCLES) AND NITRATE N (OPEN TRIANGLES). DEPICTED VALUES ARE MEANS OF TRIPLICATE SAMPLES AND ERROR BARS INDICATE THE STANDARD ERROR.

Tridex significantly inhibited nitrification as reflected in a delayed increase in nitrate concentration in the N-amended soil (Fig. 1). After a week, the effect of Tridex was gradually relieved and nitrate concentration increased. Basamid in contrast, caused an almost complete inhibition throughout the study. Towards the end of the study an increasing nitrification trend was observed in the amended soil, yet this was non-significant (Fig. 1).

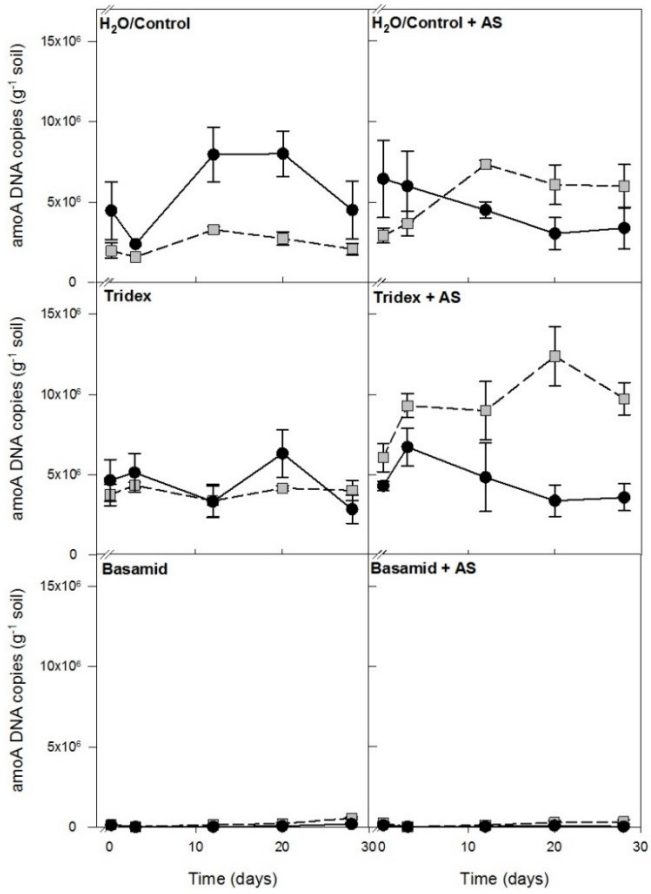


FIGURE 2
ABUNDANCE OF AMOA GENE COPIES IN SOIL WITH AND WITHOUT TREATMENT WITH PESTICIDES AND AMENDMENT WITH AMMONIUM SULPHATE (AS). LEGENDS ARE; BACTERIAL AMOA TRANSCRIPTS (GREY SQUARES) AND ARCHAEAL AMOA TRANSCRIPTS (BLACK CIRCLES). DEPICTED VALUES ARE MEANS OF TRIPLICATE SAMPLES AND ERROR BARS INDICATE THE STANDARD ERROR.

3.3 amoA quantification and nitrification rate

The number of bacterial *amoA* transcripts was significant but weakly correlated with the nitrification rate both in N-amended soil ($R^2 = 0.55$) and in non-amended soil ($R^2 = 0.16$) (Table 1 + Fig. 3). Archaeal *amoA* transcripts showed no correlation with nitrification rates in amended or in non-amended soil ($R^2 \leq 0.11$) (Table 1 + Fig. 3). This was further accentuated by the R^2 for the total (archaeal + bacterial) number of *amoA* transcripts ($R^2 = 0.47$), which was lower than the correlation obtained for bacterial transcripts alone. The abundance of gene copies of either bacterial or archaeal *amoA* was also evaluated as biomarkers for nitrification, and again the best fit was obtained with bacterial *amoA* in amended soil ($R^2 = 0.45$). Yet, the best biomarker for nitrification overall, was bacterial transcripts rather than gene copies (Table 1).

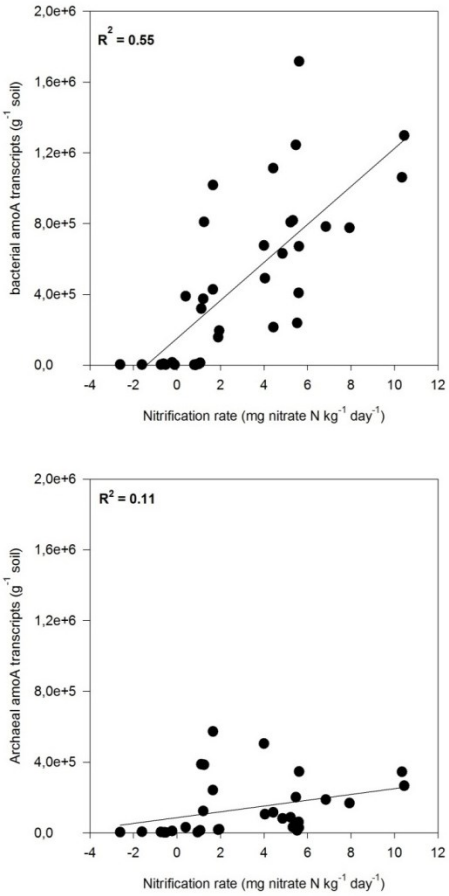


FIGURE 3
REGRESSION PLOTS FOR BACTERIAL (TOP) AND ARCHAEAL (BOTTOM) AMOA TRANSCRIPTS VERSUS NITRIFICATION RATES IN SOIL AMENDED WITH AMMONIUM SULPHATE. THE NITRIFICATION RATES ARE CALCULATED AS NET DEVELOPMENT IN NITRATE CONCENTRATION BETWEEN EACH SAMPLING DAY IN THE EXPERIMENTAL PERIOD. THESE VALUES ARE PLOTTED AGAINST THE AMOA ABUNDANCE AT THE LAST OF THE RESPECTIVE SAMPLING DAYS. INDIVIDUAL SAMPLE VALUES FOR EACH TREATMENT (WITHOUT PESTICIDE, WITH TRIDEX, WITH BASAMID) ARE DEPICTED.

3.4 Fate of pesticides in the soil

In order to estimate the availability of Tridex and Basamid in the soil system, and thus the length of time that the microbial community was influenced, we investigated the fate of the pesticides in the soil. The mineralization of Tridex followed 1st order kinetics ($R^2 = 0.99$), with the mineralization rate decreasing with time and with concentration of Tridex (data not shown). The total amount of Tridex mineralized after 62 days was 23% in the soil without N-amendment. Ammonium sulphate significantly increased the mineralization ($P<0.05$) and in the same period a total of 31% of Tridex was mineralized.

Sorption of Tridex in the soil seemed to be determined primarily by the concentration in the aqueous phase (data not shown). The sorption could be fitted by a Freundlich isotherm with K_F values of 1.91 l kg^{-1} after 2 h sorption and 2.00 l kg^{-1} after 24 h sorption ($R^2 = 0.99$). The regression constant calculated for this isotherm was 1.04 and 1.01 l kg^{-1} for 2 and 24 h sorption, respectively.

Volatilization of MITC (the active component of Basamid) occurred rapidly in the soil at water and temperature settings corresponding to those of the nitrification/*amoA* study (data not shown). The volatilization measurements showed that approximately 60% had evaporated after 1 h and that practically everything was gone after 72 h. As expected, the volatilization was considerably faster at 20 °C and in moist soil than at 0 °C or in dry soil.

3.5 Effects of Tridex and Basamid on total number of bacteria and total number of bacterial ribosomes in soil

The effects of Tridex and basamid was measured on both DNA and rRNA (cDNA) level. The numbers of bacteria per gram of soil drops from more than $3x10^9$ 16S genes per gram of soil, to 1x108 genes per gram of soil at day 3 in the case of basamid addition (figure 4). Thus the basamid treatment did result in a significant decrease of the total numbers of bacteria. We have at a previous occasion counted the average number of 16SrRNA genes in a soil bacteria to about 4 using cell extracts from soil as well as bioinformatics search in full genomes of soil bacteria (Glæsner et al 2013). Looking at the total number of bacteria it thus declined from about $1x10^9$ cells per gram to about $5x10^7$ cells per gram. After this initial decline the bacterial population in the basamid treated soil at day 12 grew to a number comparable to the original number of bacteria.
In figure 4 both the nonamended soil as well as for the soil microcosms originally amended with alfalfa meal (OECD standard) or with ammonium sulphate (ISO standard). The overall picture shows in all cases the same – soils treated with basamid show a strong decrease in gene numbers at day 0 and day 3, and at day 12 the total number of genes is high again – with one exception – the soils treated with ammonium sulphate and basamid does not recover fully.

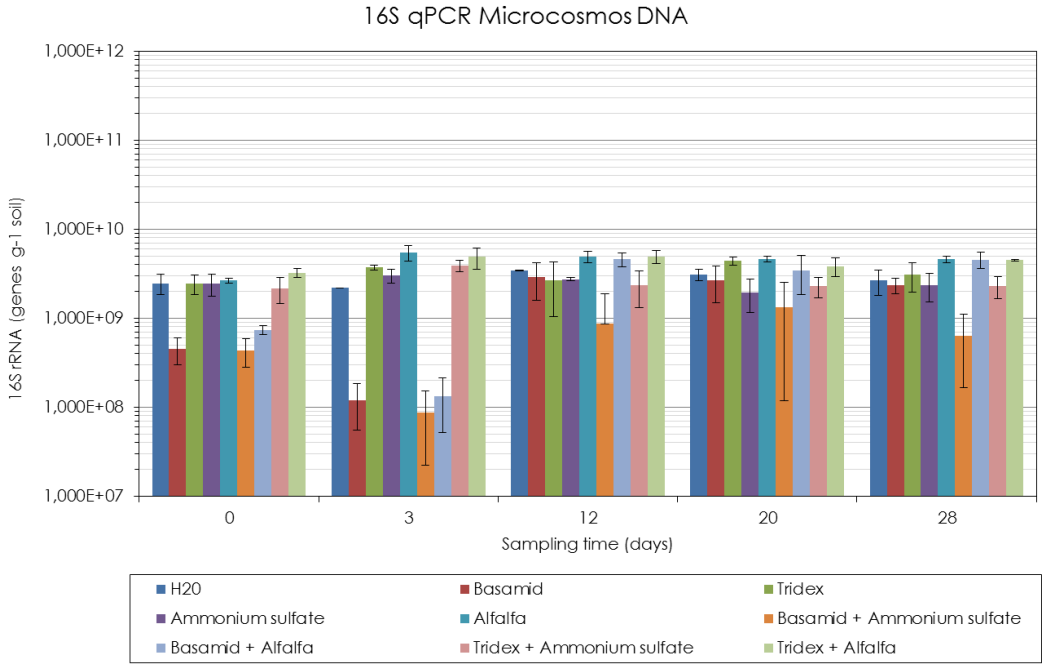


FIGURE 4
TOTAL NUMBERS OF 16SRRNA GENES PER GRAM OF SOIL TREATED WITH WATER OR WITH BASAMID OR WITH TRIDEX SHOWN FOR THE SAMPLING DAYS 0, 3, 12, 20 AND 28. THE DARK BLUE BARS ARE THE SOILS THAT ONLY RECEIVE WATER, THE DARK RED BARS ARE THE SOILS THAT WAS SPRAYED WITH BASAMID AND FINALLY THE DARK GREEN COLUMNS ARE SOIL THAT ARE SPRAYED WITH TRIDEX. THE PURPLE AND THE MEDIUM BLUE BARS ARE SOIL RECEIVING N SOURCE AS AMMONIUM SULPHATE OR AS ALFALFA MEAL – RESPECTIVELY. THE LIGHT BROWN BARS ARE BASAMID + AMMONIUM SULPHATE AND THE LIGHT BLUE BARS ARE BASAMID + ALFALFA MEAL. FINALLY THE LIGHT PURPLE AND LIGHT GREEN BARS ARE TRIDEX WITH RESPECTIVELY AMMONIUM SULPHATE AND ALFALFA MEAL.

The total numbers of 16SrRNA copies in the soil samples are shown in figure 5. In contrast to the total number of genes (DNA) the number of 16S rRNA (cDNA) are more or less the same irrespective of the treatment. The numbers of ribosomes are about 100 times higher than the number of genes (DNA).

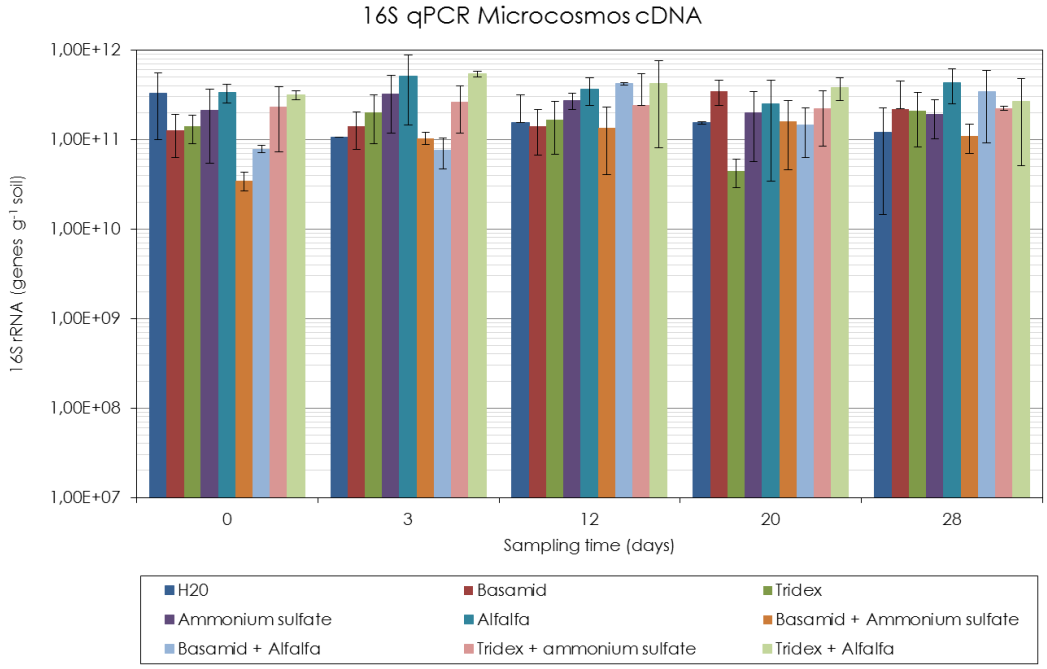


FIGURE 5
TOTAL NUMBERS OF 16SRRNA PER GRAM OF SOIL TREATED WITH WATER OR WITH BASAMID OR WITH TRIDEX, SHOWN FOR THE SAMPLING DAYS 0, 3, 12, 20 AND 28. THE DARK BLUE BARS ARE THE SOILS THAT ONLY RECEIVE WATER, THE DARK RED BARS ARE THE SOILS THAT WAS SPRAYED WITH BASAMID AND FINALLY THE DARK GREEN COLUMNS ARE SOIL THAT ARE SPRAYED WITH TRIDEX. THE PURPLE AND THE MEDIUM BLUE BARS ARE SOIL RECEIVING N SOURCE AS AMMONIUM SULPHATE OR AS ALFALFA MEAL – RESPECTIVELY. THE LIGHT BROWN BARS ARE BASAMID + AMMONIUM SULPHATE AND THE LIGHT BLUE BARS ARE BASAMID + ALFALFA MEAL. FINALLY THE LIGHT PURPLE AND LIGHT GREEN BARS ARE TRIDEX WITH RESPECTIVELY AMMONIUM SULPHATE AND ALFALFA MEAL.

3.6 Changes of the bacterial community in soil

In order to assess the impact of the two pesticides on the bacterial community in soil PCR targeting 16SrRNA genes and transcripts were performed. The DNA and cDNA samples were sequenced with primers directed to the 341-806 region of the gene. It can be seen from figure 6 and 7 that while the samples from soil treated with water or Tridex shows only small changes compared to the control, the soil treated with basamid had a profound different composition compared to the control. The proteobacteria has increased markedly from approximately 25% to 50% and the firmicutes has increased from approximately 5% to between 30% and 40%. The dominant species among the proteobacteria was members of the order Burkholderiales (mainly *Oxalobacteraceae* sp.) while the dominant order in the increased group of firmicutes was Bacillales (mainly two species: *Paenibacillus* sp. *Bacillus* sp.) Comparing DNA and RNA based analysis of the microbial community in the basamid treated soil at day 12 (figure 6 and 7) it can be seen that with the RNA level we can see more abundant species than at the DNA level. This is likely due to that the species that where the first initial colonizers (and thus the most abundant at the DNA level) is less active when the first available food sources have been depleted.

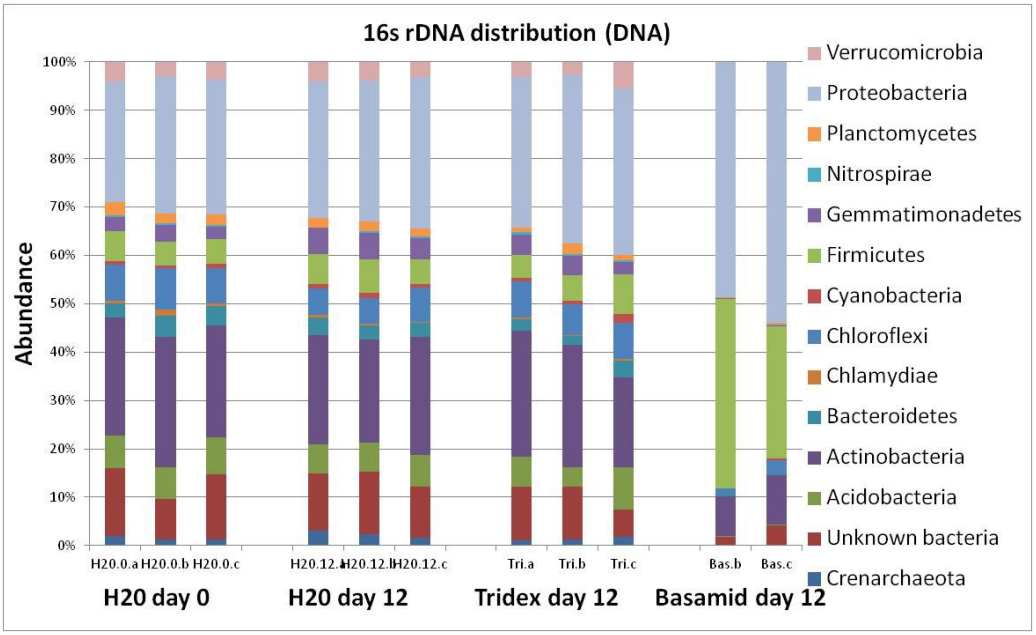


FIGURE 6

RESULTS FROM 16S RRNA GENE SEQUENCING OF SOILS FROM DAY 0 (WITH ONLY ADDITION OF WATER) AND DAY 12 (ADDITIOON OF EITHER WATER, TRIDEX OR BASAMID) EACH COULUM REPRESENTS APPROXIMATELY 2500 INDIVIDUAL SEQUENCES. ONLY SMALL CHANGES IS VISIBLE BETWEEN THE CONTROL AND TREATMENTS WITH WATER AND TRIDEX, WHILE TREATMENT WITH BASAMID RESULTED IN A LARGE INCREASE OF THE FAST GROWING BURKHOLDERIA CEPACIA (PROTEOBACTERIA) AND THE FAST GROWING BACILLUS SP. (FIRMICUTES).

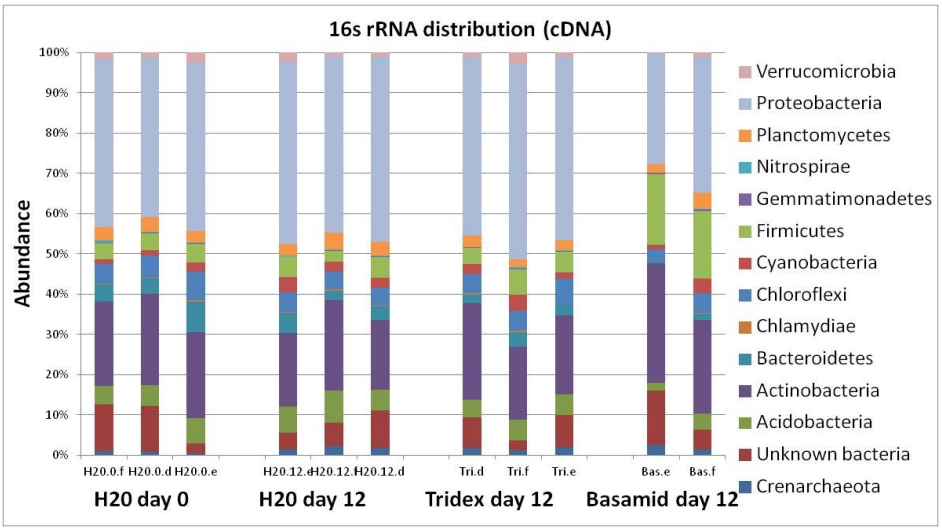


FIGURE 7

RESULTS FROM SEQUENCING OF 16S RIBOSOMAL RNA OF SOILS FROM DAY 0 (WITH ONLY ADDITION OF WATER) AND DAY 12 (ADDITION OF EITHER WATER, TRIDEX OR BASAMID). EACH COLUMN REPRESENTS APPROXIMATELY 2500 INDIVIDUAL SEQUENCES. ONLY SMALL CHANGES IS VISIBLE BETWEEN THE CONTROL AND TREATMENTS WITH WATER AND TRIDEX, WHILE TREATMENT WITH BASAMID RESULTED IN A SMALL DECREASE OF THE PROTEOBACTERIA AND THE FAST GROWING BACILLUS SP. (FIRMICUTES).

The samples analysed by 454 pyro sequencing were clustered using the “weighed-unifrac metric” in Qiime. The basamid samples were clustered away from the Tridex and H_20 samples. The clustering methods were able to discriminate between the Tridex and H_20 amended samples.

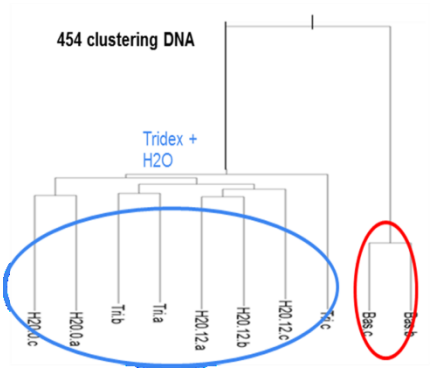


FIGURE 8
CLUSTERANALYSIS SHOWING THE RESULTS 454 PYROSEQUENING OF THE SAME GENE ANALYSED USING THE "WEIGHED-UNIFRAC METRIC" IN QIIME.

3.7 Range finding mortality tests and reproduction tests for collembolans

The response by subadult 9-12 days old *F. candida* to Basamid followed a typical sigmoid curve which was modelled as described in section 2.10.3. After the exposure of 2 days the highest dose of 10 mg kg^{-1} killed all the adults (Fig. 9). The 3 week test with Tridex also resulted in a complete inhibition at the highest test concentration of 500 mg kg^{-1}. and both mortality and reproducution declined according to sigmoid curve (Fig. 10).
The estimates obtained from the Basamid™ range-finding mortality tests (Fig. 9) were:

LC_{10} = 4.6 [4.0 - 5.2][1] mg kg^{-1} LC_{50} = 6.3 [5.3 - 7.2] mg kg^{-1}

and for Tridex™ it resulted in:

EC_{10}: 11 [7.4 - 15] EC_{50}: 72 [49 - 95]

for the reproduction and effects on mortality gave:

LC_{10}: 191 [22 - 361] LC_{50}: 440 [353 - 526]

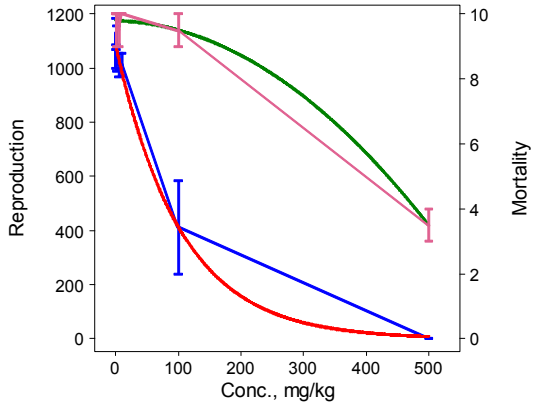


FIGURE 9
ACUTE EFFECTS OF BASAMID™ ON MORTALITY OF F. CANDIDA IN A RANGE-FINDING TEST. BLUE LINE: OBSERVATIONS; RED CURVED LINE MODELLED RESPONSE ACCORDING TO A PROBIT MODEL.

[1] 95% confidence limits in square brackets.

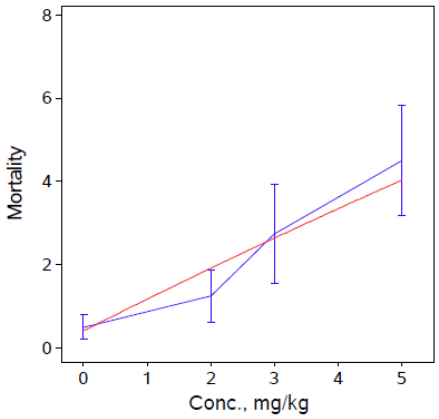
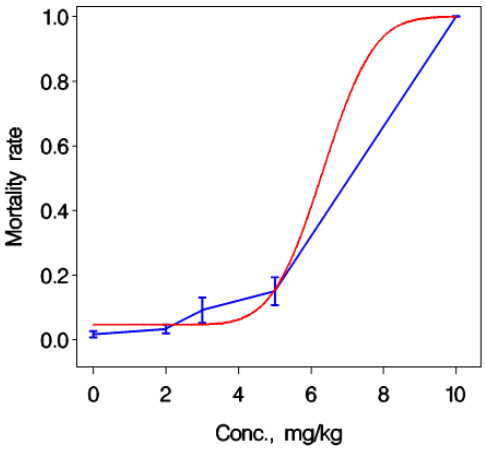


FIGURE 10
EFFECTS OF TRIDEX™ ON MORTALITY AND REPRODUCTION OF F. CANDIDA IN A RANGE-FINDING TEST WITH 10 ADULTS IN EACH REPLICATE CONTAINER. ADULT SURVIVAL: PINK LINE: OBSERVATIONS; GREEN LINE 2ND ORDER POLYNOMIAL MODEL FIT. BLUE LINE: OBSERVED NUMBER OF JUVENILES; RED CURVED LINE IS THE MODELLED RESPONSE ACCORDING TO AN EXPONENTIAL DECAY MODEL.



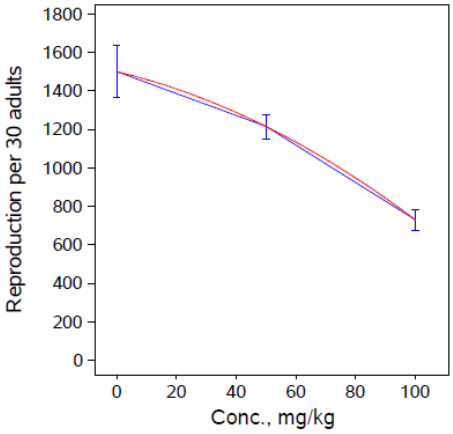


FIGURE 11 (LEFT)
EFFECTS OF BASAMID™ ON MORTALITY OF F. CANDIDA IN A DEFINITIVE TEST WITH 30 ADULTS IN EACH REPLICATE CONTAINER HOLDING 30 G OF MOIST ASKOV SOIL . BLUE LINE: OBSERVATIONS; RED CURVED LINE IS THE MODELLED RESPONSE ACCORDING TO A SECOND DEGREE POLYNOMIAL MODEL.

FIGURE 12 (RIGHT)
EFFECTS OF TRIDEX™ ON REPRODUCTION OF F. CANDIDA IN A DEFINITIVE TEST WITH 30 ADULTS IN EACH REPLICATE CONTAINER HOLDING 60 G OF MOIST ASKOV SOIL. BLUE LINE: OBSERVED JUVENILE NUMBERS; RED CURVED LINE IS THE MODELLED RESPONSE ACCORDING TO A SECOND ORDER POLYNOMIAL.

To obtain sublethal effect levels, definitive tests were run to establish appropriate concentrations about the EC10 for the transcriptomic testing. Effect levels obtained from the range-finding tests were largely reproduced. Fig. 11 and 12 present the observations and the model-fitting used for the LC and EC estimation.

The definitive test with Basamid™ gave estimates of: LC_{10} = 4.0 [2.1 - 6.0] mg kg^{-1}, when employing a second degree polynomial model (Fig. 11 red line):
$mortality = c + b \cdot conc + a \cdot conc^2$

For Tridex we obtained the estimates for reproductive output during 3 weeks (Fig. 12):

EC_{10}: 30.5 [0.5 - 60] EC_{50}: 98.3 [82 -114]

Overall mortality of adults was only 2% .

3.8 Expression analysis of Collembola

A total of 74,528 unique geneprobes was monitored on the iSQ v3.0 microarray. The increase in concentration elicited a ten-fold differential geneexpression between the treatment and the control (Table 2). The most striking response of the overall transcriptomic changes was due to the difference between 24-27 days old adults and the 50-53 days old adults incubated in the soil for 2 days and 21 days, respectively. They may have been in different instars where the 52 days old may be in an ovipository stage (instar no. 16) and the 25 days old may be in a non-fertile stage (instar no. 7). But due to the variability of the duration of stages depending on environmental conditions and properties of the particular strain they could easily be in the opposite stages.

GO terms were more abundant for Basamid than for Tridex. At the highest exposure 169 GO terms were significantly enriched compared to the control for Basamid (Appendix 3:), while for Tridex 76 GO terms were enriched (Appendix 2:). Among common GO terms of the two pesticide treatments are “Proteolysis” (GO:0006508) and “Oxidation reduction” (GO:0055114).

TABLE 2
DIFFERENTIAL TRANSCRIPTOME RESPONSE TO PESTICIDES IN TERMS OF THE NUMBER OF SIGNIFICANT (P<1%) UP “UP” OR DOWN “DOWN” REGULATION OF THE 74528 GENES, OR NO SIGNIFICANT REGULATION, “O”. B0 AND T0: CONTROL; B1: 1 MG KG^{-1}; B3: 3 MG KG^{-1}; T10: 10 MG KG^{-1}; T30: 30 MG KG^{-1}.

	Basamid			Tridex			Basamid/Tridex		
	B1-B0	B3-B0	B3-B1	T10-T0	T30-T0	T30-T10	B0-T0	B1-T10	B3-T30
Up	1149	12954	13035	505	2791	735	15848	19265	16771
o	72926	52681	52053	73945	68915	72816	42743	38532	44439
Down	453	8893	9440	78	2822	977	15937	16731	13318

As the primary aim of the qPCR was to detect general responses to soil relevant concentrations of pesticides a set of genes were selected while employing this criterion. Appendix 1 displays the first selection of candidates all having a highly significant response to the pesticides compared with the control. In Table 3 up-regulated genes of choice are listed.

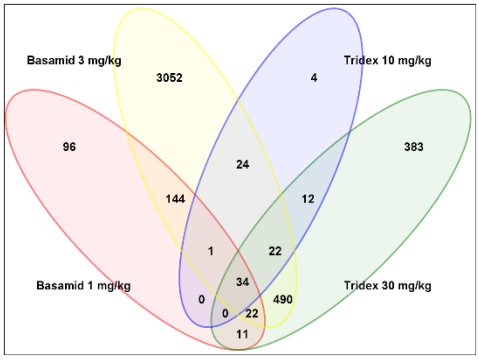


FIGURE 13
VENN DIAGRAM OF ALL SIGNIFICANTLY EXPRESSED GENES (COMPARED WITH THE CONTROL TREATMENT) SHOWING HOW MANY GENE RESPONSES ARE SHARED AMONG THE TREATED ANIMALS. THE TOTAL NUMBER OF SIGNIFICANTLY EXPRESSED GENES ACROSSE TREATMENTS WERE 4323.

TABLE 3
SUGGESTED TRANSCRIPTS FROM THE MICROARRAY ANALYSIS FOR QPCR AND THEIR MICROARRAY FOLD CHANGE IN RELATION TO THE CONTROL. PRIMER SEQUENCES (F: FORWARD; R: REVERSE) AND AMPLICON LENGTHS (BASE PAIRS) FOR GENES INVESTIGATED BY QPCR. RESULTS FROM MICROARRAY ARE GIVEN IN LOG_2 FOLD CHANGE RELATIVE TO CONTROLS (B0 AND T0, RESPECTIVELY; N.S.: NON SIGNIFICANT).

EST Id.	NCBI Ref. ID	Annotation	Primer sequences	Amplicon (bp)	Basamid Log_2FC		Tridex Log_2FC	
					B1	B3	T10	T30
a2675_404	gi|170041657|ref|XP 001848571.1|	Peritrophic membrane chitin binding protein	F:TTGTACCGCAAAGGACATGA R:TGGATGATTTCTGCATTCCA	107	0.9	2	1.3	2.2
a9093_103	gi|321469723|gb|EFX80702.1|	E3 ubiquitin-protein ligase	F:TGCCGATTCAAGGGTTTAAG R:AATTACTTGTGGGCGCTTCA	96	2.7	2.9	0.8	1.7
a16899_81	gi|321476270|gb|EFX87231.1|	Cytochrome family subfamily polypeptide 4	F:AAGGCCTTCCAGCAGTGATA R:CATCTTGGCACGTCTCATGT	101	N.S.	N.S.	1	1.2
a69968_58	gi|260800093|ref|XP 002594971.1|	Cytochrome P450	F:GTTGGAATTTGGCATGCTTT R:TTGCTGTCTATCTGCCATCG	118	1.2	1.6	0.6	1.1
a88355_150	gi|291224582|ref|XP 002732282.1|	Inhibitor of growth ing4	F:TATTGGATATGCCCGTCGAT R:TCCAATGTGGAACCATTCAA	119	1.7	1.5	0.9	1.1
a263859_29	IPR001128; PTHR24290 (PANTHER), PTHR24290:SF146 (PANTHER), SignalPNohitNN(euk) (SIGNALP)	Cytochrome P450	F:GAGGTGTGGATGGCTTAGGA R:CGCCATCTTGATCTCGTACA	118	2	4.1	N.S.	N.S.
a9056_164	gi|26996830|gb|AAH41016.1|	Succinate dehydrogenase	F:AGGGTACGGAAGGGCATACT R:CCAAATCTTGGTTGGGAAGA	101	2.9	3.6	1.7	2.9
a177253_9	no IPS match	Conserved plasmodium protein			N.S.	N.S.	-2	-2

TABLE 4
SIGNIFICANT EFFECTS ON THE EXPRESSION LEVEL OF THE QPCR TRANSCRIPTS (EST ID) CAUSED BY THE CONCENTRATION LEVEL OF THE PESTICIDES AND THE TIME ACCORDING TO AN ANOVA. ***: P<0.1%.

	Tridex			Basamid		
EST Id	Day	Conc.	Day*Conc.	Day	Conc.	Day*Conc.
a2675_404	***	***		***		***
a9093_103	***	***		***		
a16899_81						
a69968_58			***	***		***
a88355_150	***			***		
a263859_29	***				***	***
a9056_164	***			***		***

There were no clear trends in the response of the transcripts in the sense of either monotonically increasing or decreasing with the concentration but a time trend was more often significant (Table 4). There was a significant positive correlation with the concentration at day 21 but one week later this had turned to an opposite trend.

Differentially expressed genes (DEG) were abundant even at the low levels of exposure. However, when employing GO terms that gives more biological sense to the groups of transcripts there was a loss of signals, and only 3 significant GO terms for Tridex and 6 for Basamid. Even this could nevertheless support the use of transcriptomics, as the signals were still significant and could be interpreted as response to chemicals.

The number of significant (and annotated – required for a GO-term assignment) genes used for the GO-term analysis were: Tridex; T30-T0: 1002, T10-T0: 97 and Basamid; B3-B0: 3817, B1-B0: 336). GO-term enrichment analyses revealed an overall pattern comparable to the pattern of significant differential expressed genes, where at the highest exposure 169 GO terms were significantly enriched compared to the control for Basamid (Appendix 3:), while for Tridex 76 GO terms were enriched (Appendix 2:). The gene lists for the low concentration exposures contained rather few genes which makes enriched pathways harder to detect significantly, and only few significantly enriched GO-terms were identified for these lists.

When comparing enriched GO-terms among chemicals we found 36 shared GO-terms. This overlap corresponds to approximately half of the significantly enriched GO-terms found for the high concentration of Tridex exposure (36 out of 76). When comparing this to the corresponding overlap of genes among treatments we find a similar relation. Thus, half of the genes for the high concentration of Tridex exposure (490 out of 1002) were shared with the high concentration exposure to Basamid. Thus, lokking at functional groups rather than individual genes do not yield a stronger overlap, but will reveal the functional properties of the identified candidate genes.

The most significant common GO terms of the two pesticide treatments are “Proteolysis” (GO:0006508). Although difficult to interpret this suggests an effect on protein breakdown and possibly on protein turnover. A number of processes and enzymatic pathways of the fatty acid

metabolism (especially biosynthetic pathways) are shared among the chemical treatments. It is difficult to interpret broad general metabolic pathways, however, the shared pathways suggest a specific effect on fatty acid metabolism. The GO-term “structural constituent of cuticle” was also shared possibly related to an effect on molting cycle.

Specifically significant GO-terms for Tridex included lipid and amino acid transport and stress response pathways related to: response to toxin, chemical stimulus and heat, respectively.

3.9 Mesocosm experiment

Addition of basamid to the soil had a dramatic effect on the microbial activity in the soil amended with maize leaves. The respiration rate was severely reduced after addition of basamid and the rate remained low during the first 5-7 days of the incubation (Fig. 14). From day 7 and onwards the respiration rate in the Basamid treatment was higher than the treatments with water and Tridex. This reversal of the effect of basamid probably reflects that Basamid in the beginning inhibited the decomposition of the easily available carbon substrates and after a few days when the effect of the volatile Basamid had decreased there were more available carbon present in the Basamid treatment. In contrast Tridex had a much smaller impact on soil respiration although there was a tendency that respiration was slightly lower in the Tridex treatment than in the control treatment during the first few days (Fig. 14).

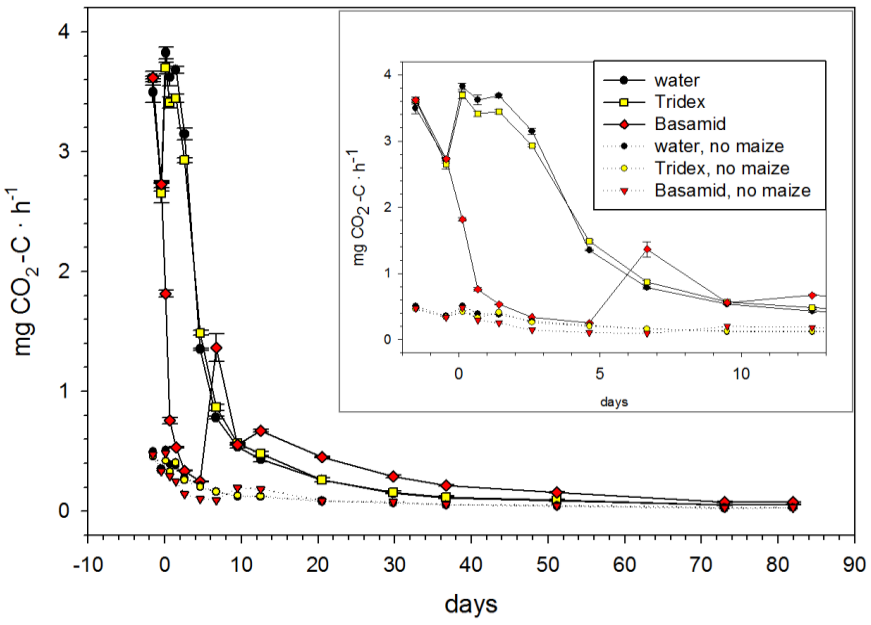


FIGURE 14
RESPIRATION RATE FROM SOIL MESOCOSMS WITH OR WITHOUT MAIZE ADDITION AND EITHER WITHOUT PESTICIDE ADDITION OR WITH ADDITION OF EITHER TRIDEX OR BASAMID. EACH DATA POINT REPRESENTS THE AVERAGE OF SIX REPLICATE MESOCOSMS. ERROR BARS REPRESENT +/- ONE STANDARD ERROR.

The amount of nitrate remained low in the Basamid treated soil and at the same time ammonium accumulated (Fig. 15) strongly suggesting that Basamid severely inhibited nitrification. The amount of ammonium and nitrate in the Tridex treatment did not appear to be different from the control treatment.

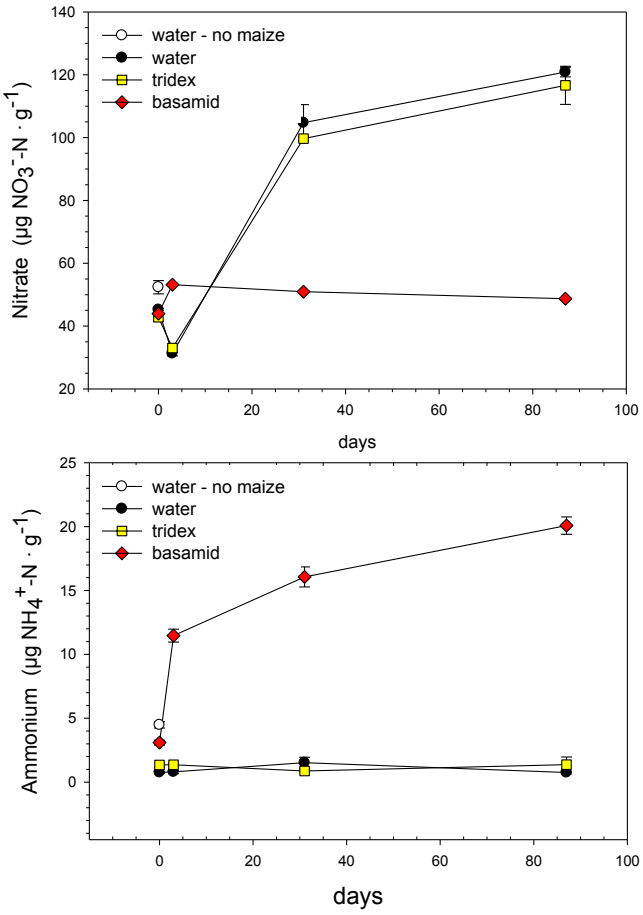


FIGURE 15
EXTRACTABLE NITRATE AND AMMONIUM IN SOIL MESOCOSMS AMENDED EITHER ONLY WITH WATER OR WITH EITHER TRIDEX OR BASAMID. DURING THE INCUBATION WE ONLY SAMPLED MESOCOSMS AMENDED WITH MAIZE BUT AT THE INITIAL SAMPLING AT DAY 0 WE ALSO SAMPLED THREE MESOCOSMS WITHOUT MAIZE ADDITION. VALUES REPRESENT MEANS OF THREE REPLICATE MESOCOSMS. ERROR BARS REPRESENT STANDARD ERRORS.

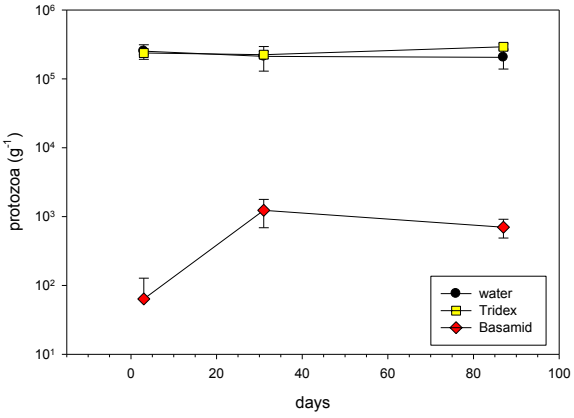


FIGURE 16
NUMBER OF PROTOZOA DURING THE EXPERIMENT IN SOIL MESOCOSMS ADDED WATER, TRIDEX AND BASAMID. VALUES REPRESENT MEANS OF THREE REPLICATE MESOCOSMS. ERROR BARS REPRESENT STANDARD ERRORS.

The strong effect of Basamid on microbial activity was also reflected in the protozoan community. The number of protozoa was severely reduced by Basamid addition and the effect lasted throughout the experiment (Fig. 16). In accordance with this we found that the species richness of cercozoan sequences that was found in cDNA from the Basamid treatment was much lower than the species richness in the control and Tridex treatment. Hence the average number of OTU's found in cDNA in the Basamid treatment was 14 compared to 46 and 51 for the control and Tridex treatment respectively (Fig. 17a).

Ordination analyses of the cercozoan sequence distribution also indicated more severe effect on cercozoan communities of Basamid than of Tridex. Hence the principal coordinate analysis shown in fig. 17b indicates that the community structure of the water and Tridex treatment at the 28 day sampling is rather similar whereas the Basamid treatment differs. The ordination analysis also suggests that there are stronger effects on the community structure evaluated by cDNA than by DNA.

More than 99% of the sequences found in cDNA in the Basamid treatment was clustered into either of two OTU's which showed a close similarity to sequences from *Polymyxa* spp. (belonging to the group Plasmdiophoridae). In fact these two OTU's dominated all samples as indicated by the large dominance of Plasmodiophoridae in all treatments in fig. 17a.

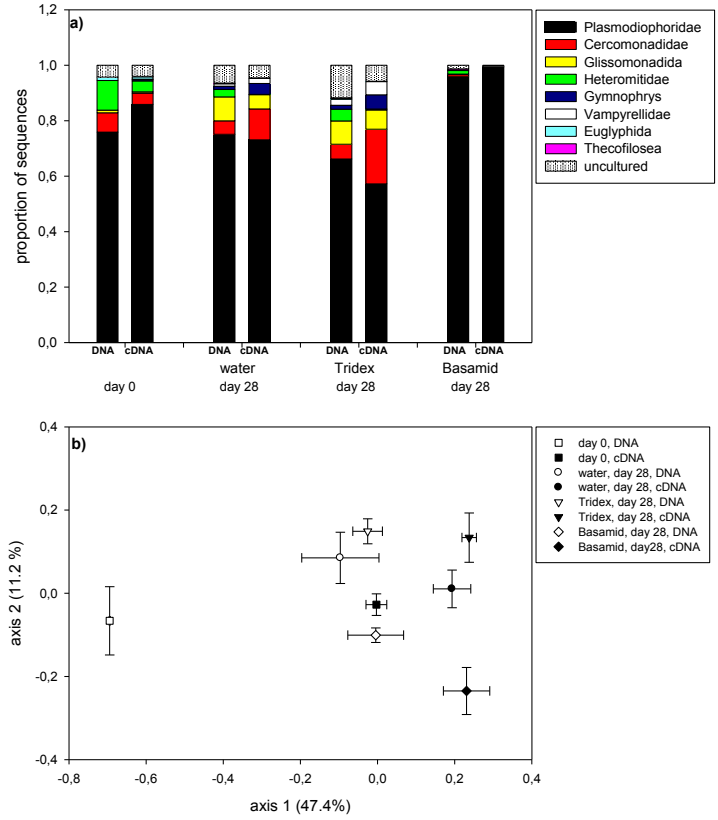


FIGURE 17

EFFECTS OF PESTICIDE ADDITION ON CERCOZOAN COMMUNITIES EVALUATED BY 454 SEQUENCING OF THE V4 REGION OF 18S. THE ANALYSIS WAS PERFORMED BOTH ON DNA AMPLIFIED DIRECTLY FROM DNA EXTRACTED FROM SOIL AS WELL AS ON EXTRACTED RNA AMPLIFIED BY RTPCR. THE TOP PANEL (A) SHOWS THE OVERALL DISTRIBUTION OF SEQUENCES AFFILIATED WITH MAJOR TAXONOMIC GROUPS OF CERCOZOA. THE DATA FOR EACH TREATMENT REPRESENT THE AVERAGES OF THREE (OR IN SOME CASES TWO) REPLICATE MESOCOSMS. THE LOWER PANEL (B) SHOWS A PRINCIPAL COORDINATE ANALYSIS (BASED ON A DISTANCE MATRIX CALCULATED BY THE THETAYC ALGORITM) OF THE STRUCTURE OF THE CERCOZOAN COMMUNITIES IN THE DIFFERENT TREATMENTS. SEQUENCES WERE CLUSTERED INTO OTU'S AT THE 5% LEVEL. POSITIONS OF THE COMMUNITIES ACCORDING TO THE TWO FIRST ORDINATION AXES ARE SHOWN. EACH POINT REPRESENTS THE AVERAGE OF TWO-THREE MESOCOSMS AND THE ERROR BARS REPRESENT THE STANDARD ERROR OF THE POSITION ON EACH OF THE TWO AXES.

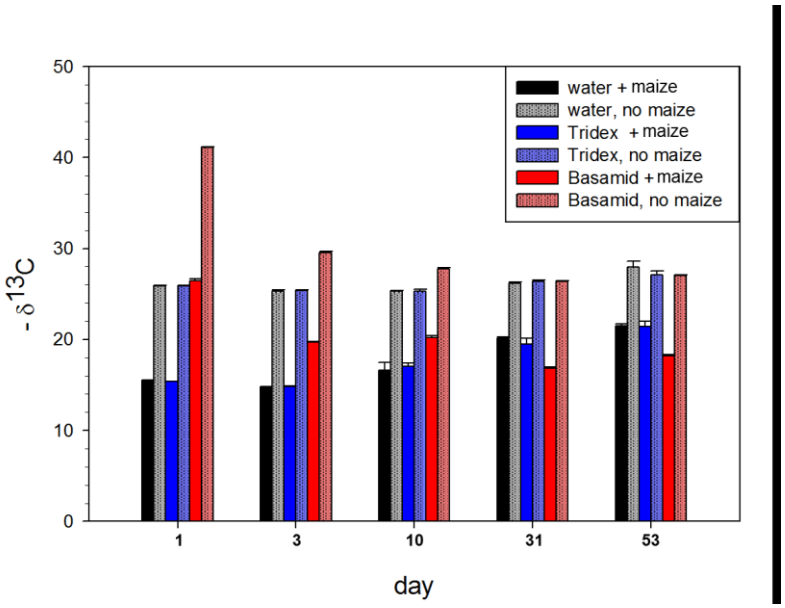


FIGURE 18
MEASUREMENTS OF ^{13}C IN CO_2 PRODUCED FROM THE SOIL IN MESOCOSMS WITH OR WITHOUT MAIZE ADDITION AND WITH OR WITHOUT ADDITION OF EITHER OF THE TWO PESTICIDES TRIDEX AND BASAMID. THE BARS SHOW THE NEGATIVE VALUE OF THE $\Delta^{13}C$ VALUE. THIS MEANS THAT THE HIGHER THE BAR THE LESS ^{13}C IS PRESENT IN THE RESPIRED CO_2. THE SOIL HAS A $\Delta^{13}C$ VALUE AROUND -28 ‰, WHEREAS MAIZE LEAVES HAVE A VALUE AROUND -11 ‰.

The analysis of $^{13}C/^{12}C$ ratios in the respired CO_2 allows us to some extent to evaluate whether the pesticides affected the decomposition of newly added plant material differently from the way they affect the degradation of older soil organic matter. The soil used in the experiment was an agricultural soil which has always been cultivated with C3 plants (e.g. wheat, barley etc). Hence the ^{13}C signature of this soil is close to the signature of C3 plants ($\delta^{13}C \approx -28‰$). Maize, on the other hand is a C4 plant and it has a $\delta^{13}C$ -value around -11‰. The results in Fig. 25 clearly show that addition of maize leaves shifts the signature in CO_2 towards the signature of maize. This reflects that a major part of the respired CO_2 stems from added material. It is also evident that Tridex does not appear to affect the ratio between newly added and old organic material in the respired CO_2 (compared to the control treatment), whereas there is a dramatic effect of Basamid during the first few days of the experiment.

4. Discussion

4.1 Pesticide effects on microbial activity in soil with specific focus on the nitrification process

Pesticides are applied in extensive doses to agricultural fields all over the world, yet using analyses presently at hand, it is difficult to assess the damage imposed on the microbiological ecosystem. Here, we evaluated a molecular assay with a potentially wide application range. By relatively simple modification, this assay can be set up to monitor a wide array of microbial processes. After RNA/DNA extraction and cDNA production, which is the most time-consuming part of the protocol, the sample can be used for qPCR using primers that target any characterized gene of interest.

In the present study, we focused on the *amoA* gene coding for ammonia monooxygenase. To our knowledge, this is the first study that investigates the impact of pesticides on transcriptional activity of both ammonia oxidizing bacteria and Archaea. Recently, the impact of the insecticide Profenofos on abundance of ammonia oxidizing bacteria and Archaea was examined (Liu et al 2012). Exposure to Profenofos caused the potential nitrification rate to decrease significantly, whereas in contrast the number of *amoA* genes remained unchanged. , qPCR quantification of *amoA* genes was considered inappropriate to measure the effect of pollutants on nitrification (Liu et al 2012). In our experiment, a weak but significant correlation (R^2=0.45) between bacterial *amoA* gene copies and nitrification was obtained in N-amended soil. This correlation was further improved (R^2=0.55) using bacterial transcripts of *amoA*.

We measured transcription of *amoA* under a diverse set of conditions that represented both lethal (Basamid) and nonlethal (Tridex) inhibition. In the case of Basamid, an immediate decrease (up to a 1000-fold) in the number of *amoA* transcripts was observed. An immediate decrease in the number of *amoA* gene copies was also observed, but whereas the transcripts dropped to a minimum after 1 hour exposure, the number of gene copies decreased further until day 3. Data for volatilization of the active component MITC in Basamid illustrate that the majority of the compound evaporated within 1 h and that the rest disappeared from the soil within 3 days. Thus, the fate of the pesticide correspond well with the qPCR results that demonstrate an instant inhibition of bacterial transcription and an effective killing of cells, which either remained as intact dying or dead non-degraded DNA until day 3. Despite a presumed fast removal of inhibiting compound from the soil, Basamid was shown to be a very potent fumigant with long-term effects. Hence, no significant recovery of ammonia oxidizers was recorded even 28 days after Basamid exposure, although a small increase was suggested both by the nitrification and the qPCR assay.

With Tridex treatment, we observed a similar development in the number of bacterial *amoA* transcripts and genes, in accordance with an inhibition of nitrification from day 3 to 12 in N-amended soil. The mineralization and sorption experiments for Tridex suggest that the bioavailability of Tridex correlated negatively with nitrification, with a decrease in bioavailability and an increase in nitrification around day 12. Curiously, Tridex did not inhibit bacterial transcription until day 3, probably because the less harmful parent compound mancozeb was degraded relatively fast to the more harmful metabolites ethylenthiourea (ETU) and ethylenurea (EU) before the compound was completely mineralized.

Several studies have stated that bacteria rather than Archaea are the major ammonia oxidizers in nitrogen-rich soil ecosystems. In agricultural soil microcosms amended with ammonium sulphate, nitrification was previously shown to parallel the abundance in bacterial but not archaeal *amoA* gene copy numbers (Jia and Conrad 2009). Likewise, nitrification was correlated ($R^2 = 0.38$) with bacterial but not archaeal *amoA* gene copies in a grassland soil amended with urine-N substrate (Di et al 2010). In fact, the abundance of archaeal *amoA* genes showed a negative relationship with N-fertilization in both of these studies. Similarly, we observed that amendment with ammonium sulphate resulted in a 10-fold decrease in archaeal *amoA* mRNA transcripts and a small but insignificant decrease of archaeal *amoA* gene copies.

Although functional dominance of bacterial ammonia oxidizers in N-fertilized soils has been accepted as a general phenomenon, the reason for this niche differentiation between communities is still much debated (Hatzenpichler, 2012; Prosser and Nicol, 2012; Taylor et al., 2012). In the present study, archaeal *amoA* abundance dominated in the non-amended soil, but was yet invalid to describe nitrification rates. This discrepancy may be explained by mixotrophic growth (Jia and Conrad, 2009). The archaeal ammonia oxidizer *Nitrososphaera viennensis* was recently shown to display higher cell yield and *amoA* gene abundance per event of ammonia oxidation under mixotrophic conditions compared to autotrophic growth (Tourna et al, 2011). However, we found that both *amoA* genes and *amoA* transcripts showed a weaker correlation with nitrate formation in non-amended soil than in ammonium-ammended soil. This suggests a change in the relationship between *amoA* transcription and ammonia monooxygenase activity in response to shifting ammonia availability. The bacterial nitrificants, *Nitrosospira briensis* and *Nitrosomonas europaea*, display two levels of ammonia oxidation activity; a basal level as well as a substrate-induced level (Bollmann et al. 2005; Stein et al., 1997). These two levels are partly regulated by a constitutive as well as an inducible mRNA transcript production. Furthermore, an increase in the half-live and stability of *amoA* transcripts has also been suggested upon substrate deprivation (Bollmann et al., 2005). Finally, translational selection or posttranslational modification has been argued to play a role in regulation of ammonia monooxygenase activity (Stein et al., 1997). Altogether these studies indicate a complex regulation of enzyme activity in the ammonia oxidizing communities and we need a deeper understanding of both archaeal and bacterial regulation of ammonia oxidation to explain these results.

4.2 Influence of Basamid on bacterial community composition in soil

A recent study using the same fumigant and application of DGGE and meltcurve analysis showed a marked influence on the bacterial community study (Hjelmsoe, et al. 2014). Common to all of the studies were that the observed shifts in community structure were quite prolonged and lasted between 1 and 3 months depending on the experimental setup (Zobiole, et al. 2011).
The sequencing of 16S genes implied that recovery of bacterial population numbers after Basamid treatment was caused by a confined fraction of potentially recalcitrant and fast-growing bacteria. The bacterial species richness after restoration of the population density was significantly decreased and the community was largely dominated by members of the order *Bacillales* and *Burkholdereales*. A large fraction of the *Burkholdereales* classified within the family *Oxalobacteraceae*, which have previously been shown to express the characterisitics of r-strategists i.e. they are early colonists with high growth rates (Ofek et al., 2012). The dominance of *Bacillales* may be due to the capability to produce stress-resistant endospores (Nicholson et al., 2000), and a high prevalence of *Bacillus* spp. has also previously been observed after fumigant exposure (Ibekwe et al., 2001).

4.3 Influence of Basamid and Tridex on protozoan community composition in soil

Protozoa are very important predators on bacteria and play a key role in soil food webs (Ekelund and Rønn 1994). They are involved in intimate interactions with the bacteria and any disturbance of the soil system that affects bacteria and bacterial processes is likely to cause changes in the activity or structure of the protozoan communities. On the other hand, in cases where the protozoa are more sensitive to a given pesticide than the bacteria themselves, then application of this pesticide could affect bacterial processes indirectly by changing the grazing pressure on the bacteria by reducing protozoan activity or altering the protozoan communities. This possible effect is particular relevant for pesticides, such as Tridex, that target eukaryotes.

It is tedious and difficult to investigate protozoan communities in great detail by traditional methods (Ekelund et al. 2001) but next generation sequencing techniques offer a more efficient possibility for obtaining detailed information of protozoan community structure. In this project we wanted to investigate whether pyrosequencing could be used to detect relatively subtle changes in protozoan community structure caused by pesticide use. We hypothesized that basamid would have a very strong effect whereas Tridex would lead to minor effects. Further, we expected that the most active population would be stronger affected than the less active populations and therefore we expected larger effects for the pyrosequencing analysis performed on cDNA than on DNA.

As expected Basamid had a more severe effect than Tridex on protozoan community structure (Fig. 17). However, the strong effect on community structure was coupled with a strong effect on population size (Fig. 16). We had expected the protozoan populations to have recovered 28 days after Basamid exposure but it appears that the Basamid treatment had such a strong effect on the protozoa that they were not able to recover in four weeks. Hence, we do not know whether Basamid would have a more lasting effect on community also after the populations would have had time to recover from the initial reduction. Our results also largely confirmed the hypothesis that the pesticides would have larger effects on the community structure evaluated by cDNA than by DNA. This supports our initial idea that a method for detecting subtle effects of pesticides should be based on cDNA.

Full interpretation of these results is not possible since the knowledge on the diversity and function of cercozoa in soils is still limited (Harder et al. 2014). For example, it was a bit surprising in the Basamid treatment that more than 99% of the sequences found in cDNA was clustered into either of two OTU's which showed a close similarity to sequences from *Polymyxa* spp. (belonging to the group Plasmodiophoridae) since Plasmodiophoridae is a group of plant parasitic cercozoa. The experimental conditions applied in this study is unlikely to have favoured plant parasites and presently we do not have a good explanation for the high dominance of these sequences in our samples. It is possible that the sequences represents a hitherto unknown free-living organism related to the plasmodiophorids or that some cercozoan sequences in GenBank has been wrongly annotated as *Polymyxa*. However, we cannot rule out the possibility that there is also some kind of PCR bias involved in this highly intriguing result.

4.4 Influence of Basamid and Tridex on collembolans

Normal field rates of Basamid to agricultural soil result in 250 mg a.i. kg^{-1} soil and for Tridex is 1.3 mg a.i. kg^{-1} soil. However, collembolans do respond to much lower concentration of MITC, the active ingredient of Basamid, in the order of 5 mg kg^{-1} for Tridex doses need to be at least 30 mg kg^{-1} for a 10% effect on the reproduction. When testing ecotoxicogenomic tools the only requirement is that, an effect is detectable on the biological non-target organisms and of secondary importance whether this effect is of practical relevance.

In general it was found that the responses to the applied compounds were quite modest, with changes usually below three fold. Still the microarray analyses identified a large number of genes being potential biomarkers for the exposure. In our attempt to push the detection level of transcripts to their limit we have realised that the microarray although considered semi-quantitative was very responsive to the pesticides spiked to soil.

In our experiments on time series and concentration ranges designed to validate the detection limits of the identified genes, the results were much less clear. There are a number of reasons as to why we found such a low correspondence between array and qPCR results. Both techniques are quite sensitive and can be influenced by a range of factors originating from both biological and technical variation. As the animals used for the two different molecular investigations are from different experiments, some degree of differences could be expected. As the responses were modest in terms of fold changes the influence of variation of various sources are inflated. However, we were disappointed by the lack of correspondence, and the relative high variation found in the results of the qPCR validation. Even though gene expression is a part of a very complex molecular network, the quality of this dataset seems not to be of highest standard.

On a more encouraging side, the results of the array data are quite promising with respect to developing biomarkers for detecting exposure to the compounds used here. However, more data are required to validate these markers, with respect to repeatability between samples with different exposure history, age of animals and repeatability across biological replicates. A future attempt to compare ITC's across studies such as by van Ommen Kloeke et al. (2012).

Furthermore, the mechanisms of action of the compounds should be investigated with a targeted approach to elucidate the effects of exposure and discriminate between biomarkers suggesting pathological responses in general from gene expression responses specifically involved in the metabolism of the compounds in question.

Mancozeb, the active ingredient of Tridex, belongs to the group of fungicides commonly known as ethylene bis dithiocarbamates (EBDCs), along with the active ingredients maneb, metiram and nabam. It has a range of mode of actions (MoA) such as reactive oxygen species (ROS) and apoptosis (Calviello et al. 2006). Basamid can cause DNA damage and oxidative damage, which is supported by the GO term. The rather long list of significant GO terms may be part of these processes, and still reveal that the organism is in an abnormal environment leading to general metabolic responses.

For Basamid, a different signal from stress related genes were found as response to other organism (immune reponse), DNA damage stimulus and oxidative stress were significant. Thus, a subtle but distinct different type of stress response was detected among the two substances. Further, we found processes related to sugar metabolism and transport differently expressed, which also suggest a different type of metabolism was affected by Basamid compared to Tridex. Finally, Basamid treated animals showed enrichment in processes related to transcription and translational. This could, in combination with the signal from oxidative stress and following DNA damage, indicate a need for a continued strong molecular up-regulation (greater turn-over) to keep the cellular homeostasis.

4.5 Bioavailability of Basamid and Tridex in soil

In the current study the effect of a fumigant was immediate, and after less than 4 days the fumigant was evaporated. Thus after this period the compound was non-existing and the microbial communities could start regaining. If in this case no knowledge had been available on the disappearance of the compound from the soil systems it would have been difficult to explain the dynamic of the system found.

5. Conclusion

The project focussed on validating RNA based methods as potential genomic tools in assessment of agricultural soil ecosystems. It was shown that our mRNA based technique was very sensitive and the effects were seen in the same situations as when the OECD nitrification assay showed an effect. After 12 days the 16SrRNA based pyrosequencing of bacterial communities differed from the same analysis based on DNA. This is indicating that the bacterial communities were recovering after initial disturbance caused by pesticide addition – in particular basamide that had strong influence on the microbial community. Finally, a microarray analysis was compared to the traditional test for toxicity testing of *Folsomia candida* and showed a more sensitive response to the pesticide exposure.

Both bacterial and archaeal populations of *amoA* genes and transcripts decreased significantly in response to Basamid treatment, which also completely blocked detection of nitrification by the nitrate assay. Basamid showed a strong negative effect on overall agricultural ecosystem. The microbial activity during the initial phase of decomposition of newly added organic material was strongly hampered, but the negative effect of Basamid on general decomposition disappears after a few days. The bacterial and protozoan abundance and diversity was strongly affected by Basamid and Basamid had a strong influence on *Folsomia candida* and showed that 30 mg kg^{-1} was the effective concentration where 10% of the populations were affected (EC10).

Contrastingly, Tridex treatment did not result in any negative effect on overall microbial activity, but a small but significant inhibition of nitrification was found in the microcosms experiment while in the mesocosms no effect of Tridex was found on nitrate formation. In the microcosms experiment we found that in contrast to the nitrate assay that bacterial *amoA* genes and transcripts abundance was not significant different from the soil treated with water. Further Tridex treatment of soil had no effects on bacterial and protozoan abundance and diversity. In contrast to the protozoan and bacterial populations Tridex did influence the population of *F. candida* and a toxic effect was found at the same concentration as with Basamid (EC10 = 30 mg kg^{-1}

Hypothesis	Conclusion
Quantification of gene expression in soil can measure the impact of pesticides directly on important soil functions	Yes: mRNA quantification of *amo*A genes was able to describe impact on nitrification processes.
Microbial diversity changes following pesticide application can be described using rRNA based pyro-sequencing	Yes: Next Generation Sequencing approaches does describe accurately changes in the communities
Gene expression of field populations will reflect possible stress induced by pesticides	partly: Microarray of *Folsomia candida* was more sensitive than traditional test, however the result could not be validated by qPCR
Basamid and Tridex will have different effects on the soil ecosystem. Basamid will, as a soil fumigant, affect all species, while the fungicide Tridex mainly will affect eukaryotic organisms	Yes: Basamid showed effects on all organisms while Tridex did not influence the bacterial communities
Basamid will immediately hamper expression of *amo*A genes in soil, while Tridex will not influence *amo*A expression.	Yes: Basamid had – as with other processes large impact on amoA expression – this was not the case when the soil was exposed to Tridex

This study has for the first time used mRNA quantification in soil as a tool in assessing unwanted side effects of pesticides directly on the soil microbial community. The effect of the fumigant basamid was very strong on the nitrifying bacterial community in soil – leading to a total shut down of the transcriptional activity. This was shown with an increased sensitivity compared to the chemical measuring of nitrate transformation. This result indicates that use of mRNA of *amoA* genes can be used to assess also the effect of other treatments on the microbial nitrification in soil.

From a perspective of improving future risk assessment of pesticides or other man-made chemicals on the agricultural soil ecosystem the positive results from this project calls for other genes to be explored as potential indicators of unwanted side effects of pesticides.

6. Perspectives

6.1 Scientific perspectives

For the time being only few genes in soil microorganisms are as well studied as the amoA genes coding for nitrification. Other important microbial driven processes can be thought of - especially many fungal processes including the formation of compounds involved in the stabilization of soil physical structure – like glomalin produced by mychorrhiza fungi. Thus from a scientific perspective it could be of interest to increase the knowledge base of genes involved in such processes.

Further we found using the investigated combination of soil and pesticides that the applied amplicon sequencing method using 16SrRNA gene as a target showed interesting effects of Basamid on the bacterial community.

The qPCR for *F. candida* was not convincing in detecting effects even at the 10% level, thus presently it cannot be recommended to employ it for detection of sub-sublethal concentrations collembolans exposed to field collected soil. Contrary with the microarray transcriptomic iSQ chip, which could detect significant effects at the low concentration of one third of EC_{10}. It rejuvenates the old dream of using biomarkers, but now with much higher reliability, due to the coverage of an extensive number and diversity of individual biological processes.

6.2 Administrative perspectives

We believe that mRNA transcript quantification has a potential to be used in a broader sense for evaluation of pesticide side-effects on the soil ecosystem. The strength of the direct in-situ transcript assessment is that it may be used on complex processes that are problematic to measure. Thus, if the gene responsible for any particular process is identified, then quantification of mRNA transcripts may facilitate real-time measurement of pesticide effects on that particular process.

On the same node it can be proposed to further investigate the use of general housekeeping genes in soil animals in future assessments of unwanted side effects of pesticides.

The long term effects of pesticides on soil microbial populations could be measured with a highly increased level of accurateness using Next Generation Sequencing of selected microbial communities, i.e. the bacterial community and the cercomonas community. The precision of these new sequencing technologies allows also an assessment of the potential loss of important species.

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Appendix 1: Selection of significantly expressed genes (LOG2FC P<0.1 % for most of the fold changes). These genes were suggested for QPCR and in particular the red, yellow and green highlighted were considered promising candidates .
EST: Expressed sequence tags

EST Id.	Fold change Log_2FC				NCBI Reference ID	Gene annotation
	B1-Bo	B3-Bo	T10-To	T30-To		
a12084_435	3.1	4.7	1.5	3.1	gi|322787091|gb|EFZ13312.1|	Elongation of very long chain fatty acids protein 6
a9056_164	2.9	3.6	1.7	2.9	gi|26996830|gb|AAH41016.1|	Succinate dehydrogenase
a7929_384	3.4	4.4	1.7	2.9	gi|24266981|gb|AAN52387.1|	Ribosomal protein s13
a34610_283	1.8	1.7	1.6	2.8	gi|321477204|gb|EFX88163.1|	Villin 1
a15590_255	2.4	2.9	1.9	2.8	gi|332028268|gb|EGI68315.1|	Neurogenic locus notch protein
a912_462	2.0	3.4	1.7	2.7	gi|91088343|ref|XP_971105.1|	L-xylulose reductase
a745_782	2.7	4.0	1.3	2.7	gi|187115160|ref|NP_001119681.1|	Ribosomal protein s17
a58544_102	1.5	1.9	1.3	2.4	gi|307207918|gb|EFN85479.1|	Esterase fe4
a5432_423	2.6	3.2	1.1	2.4	gi|328899359|gb|AEB54635.1|	Eukaryotic translation initiation factor 3 subunit k
a6383_114	2.6	2.5	1.1	2.4	gi|332023177|gb|EGI63433.1|	G protein-coupled receptor 98 partial
a2230_317	1.8	2.9	1.5	2.3	gi|345482355|ref|XP_001608062.2|	Carboxypeptidase a-like
a2675_404	0.9	2.0	1.3	2.2	gi|170041657|ref|XP_001848571.1|	Peritrophic membrane chitin binding protein
a55032_198	2.6	3.2	1.1	2.2	gi|2665654|gb|AAB88449.1|	Polyadenylate-binding protein 1
a44991_216	1.6	2.0	0.9	2.2	gi|157132518|ref|XP_001656050.1|	Alcohol dehydrogenase
a483975_5	1.8	2.5	1.4	2.1	gi|366991919|ref|XP_003675725.1|	Actin
a6477_557	2.7	2.9	1.4	2.1	gi|307185019|gb|EFN71248.1|	Ras-related protein rab-11b
a9391_104	2.8	3.1	1.3	2.1	gi|189238960|ref|XP_001814187.1|	Vacuolar h
a19312_253	1.6	2.0	0.8	2.1	gi|260793432|ref|XP_002591716.1|	Tetratricopeptide repeat protein 36-like
a3570_611	2.2	3.0	1.3	2.1	no_hit	Secreted salivary protein
a5642_150	2.8	2.2	1.0	2.0	gi|242013333|ref|XP_002427365.1|	Glucosyl glucuronosyl transferases
a11850_235	2.5	2.8	1.1	2.0	gi|321466881|gb|EFX77874.1|	Isoform a
a960_323	1.6	2.5	0.7	1.9	gi|326320025|ref|NP_001191880.1|	Nadh-cytochrome b5 reductase-like
a39185_122	2.3	2.4	0.9	1.9	gi|91083817|ref|XP_973463.1|	Large neutral amino acids
a2214_248	2.7	3.7	1.4	1.9	gi|227955305|gb|ACP43442.1|	Arginine kinase
a11660_79	1.0	2.2	1.3	1.9	gi|221130006|ref|XP_002168244.1|	Long-chain fatty acid transport protein 4
a56392_71	1.0	2.9	1.1	1.8	gi|291230900|ref|XP_002735404.1|	Dimethylaniline monooxygenase
a741_607	2.0	2.4	0.8	1.8	gi|170045840|ref|XP_001850501.1|	60 kda heat shock mitochondrial
a9582_192	1.2	1.6	1.0	1.8	gi|171473635|ref|YP_001798467.1|	Nadh dehydrogenase subunit 2
a2729_304	1.7	2.4	0.8	1.8	gi|321460466|gb|EFX71508.1|	Alcohol dehydrogenase
a14074_186	2.3	2.7	1.5	1.8	gi|328784765|ref|XP_624408.2|	Protein
a9161_119	2.4	2.6	1.2	1.7	gi|195441048|ref|XP_002068343.1|	Translocation protein sec63-like protein
a57144_135	1.0	1.5	1.1	1.7	gi|241998158|ref|XP_002433722.1|	60 kda heat shock mitochondrial
a37635_274	1.6	2.9	1.4	1.7	gi|156537053|ref|XP_001601575.1|	Short-chain dehydrogenase
a9093_103	2.7	2.9	0.8	1.7	gi|321469723|gb|EFX80702.1|	E3 ubiquitin-protein ligase
a145156_42	2.4	2.5	0.9	1.7	gi|91092412|ref|XP_967539.1|	Dihydrouridine synthase 1-like (cerevisiae)
a23698_75	2.2	1.9	1.1	1.6	gi|348543582|ref|XP_003459262.1|	Gastrula zinc finger
a337268_6	1.4	2.0	1.0	1.6	gi|323307184|gb|EGA60467.1|	Eft2p

EST Id.	Fold change Log_2FC				NCBI Reference ID	Gene annotation
	B1-Bo	B3-Bo	T10-To	T30-To		
a16296_51	2.0	2.6	0.7	1.6	gi|291241127|ref|XP_002740468.1|	Zinc finger rna binding protein
a17556_714	2.2	-6.5	2.1	1.6	gi|312382729|gb|EFR28084.1|	Endocuticle structural glycoprotein bd-4
a91466_137	1.1	1.4	1.1	1.6	no_hit	Conserved hypothetical protein [Culex quinquefasciatus]
a30961_115	2.3	2.1	1.0	1.6	gi|66499443|ref|XP_394248.2|	Ring finger and spry domain-containing protein 1
a136740_51	1.8	1.7	1.0	1.5	gi|332026284|gb|EGI66421.1|	Ubiquitin carboxyl-terminal hydrolase 33
a96358_71	1.8	1.8	0.8	1.5	gi|322798091|gb|EFZ19930.1|	Beta-transducin repeat containing
a1836_82	1.9	2.6	1.0	1.5	gi|321476616|gb|EFX87576.1|	Isoform c
a33276_174	1.4	2.1	1.1	1.5	gi|321468810|gb|EFX79793.1|	Translocon-associated protein subunit beta
a104381_43	1.8	2.9	0.9	1.5	gi|242011684|ref|XP_002426577.1|	Endoplasmic reticulum protein isoform cra a
a2984_179	1.7	1.8	0.8	1.5	gi|291234145|ref|XP_002737010.1|	Ebna2 binding protein
a43820_164	1.8	1.6	0.8	1.5	gi|307175350|gb|EFN65369.1|	Max-like protein x
a40489_76	2.3	2.3	0.8	1.5	gi|242017130|ref|XP_002429045.1|	Tyrosine-protein phosphatase non-receptor
a27576_176	1.7	2.1	1.1	1.5	gi|194760181|ref|XP_001962320.1|	Nadh dehydrogenase
a121203_74	0.9	1.2	1.1	1.5	gi|40548515|gb|AAR87378.1|	Tropomyosin
a4916_157	1.0	2.5	1.1	1.5	gi|221116104|ref|XP_002160160.1|	Chito-oligosaccharidolytic beta-n-acetylglucosaminidase
a3094_71	2.1	1.7	0.9	1.4	gi|345481105|ref|XP_003424289.1|	Nad-dependent deacetylase sirtuin-4
a955_116	2.1	1.9	0.9	1.4	gi|91078766|ref|XP_968956.1|	Transmembrane protein 170
a36908_55	2.2	1.9	1.1	1.4	gi|242018343|ref|XP_002429637.1|	Ensangp00000014982 isoform 2
a48139_49	2.2	2.1	1.2	1.4	gi|321468712|gb|EFX79696.1|	Pyrroline 5-carboyxlate reductase
a40854_100	1.9	1.9	1.0	1.4	gi|115749044|ref|XP_001196745.1|	Hydroxymethylglutaryl-mitochondrial-like
a74368_61	2.3	2.2	0.8	1.4	gi|345484029|ref|XP_003424930.1|	Natural resistance-associated macrophage protein
a27801_201	1.3	1.9	0.9	1.4	gi|156551692|ref|XP_001601820.1|	Dipeptidyl peptidase iii
a181115_53	1.6	1.2	0.8	1.3	gi|156392140|ref|XP_001635907.1|	General control of amino acid synthesis protein 5-like 2
a41369_323	1.6	4.2	1.1	1.3	gi|115928361|ref|XP_780550.2|	Regucalcin
a24052_98	1.2	0.6	0.7	1.2	gi|189235118|ref|XP_971727.2|	Adenylyl cyclase 76e
a16410_637	0.6	1.3	0.9	1.2	no_hit	Secreted salivary protein
a16899_81	1.1	2.8	1.0	1.2	gi|321476270|gb|EFX87231.1|	Cytochrome family subfamily polypeptide 4
a81514_96	0.8	0.7	0.7	1.2	gi|321469925|gb|EFX80903.1|	Acetyl-coenzyme a transporter 1
a15720_150	2.0	1.3	0.7	1.2	gi|321461416|gb|EFX72448.1|	Cytoplasmic polyadenylation element binding protein 1
a19097_45	0.9	1.5	0.6	1.1	gi|307212370|gb|EFN88165.1|	Pantothenate kinase 4
a69968_58	1.2	1.6	0.6	1.1	gi|260800093|ref|XP_002594971.1|	Cytochrome p450
a88355_150	1.7	1.6	0.9	1.1	gi|291224582|ref|XP_002732282.1|	Inhibitor of growth ing4
a15806_331	0.8	1.1	0.9	1.1	gi|242023873|ref|XP_002432355.1|	Long form-like
a63826_103	1.3	1.7	0.6	1.1	gi|325189572|emb|CCA24058.1|	Peptidyl-trna hydrolase mitochondrial
a274239_19	0.6	0.4	0.7	1.0	gi|357610212|gb|EHJ66872.1|	Calcium-binding atopy-related autoantigen 1
a29285_75	1.4	1.6	0.8	1.0	gi|189239344|ref|XP_973859.2|	28s ribosomal protein mitochondrial
a218661_29	1.2	2.0	0.9	1.0	gi|307167444|gb|EFN61020.1|	Neuroendocrine convertase 2
a142109_13	0.7	1.2	0.9	1.0	gi|242023873|ref|XP_002432355.1|	Long form
a648247_9	1.2	0.7	0.8	0.9	no_hit	Peptidyl-glycine alpha-amidating monooxygenase a
a334091_12	1.1	1.7	0.8	0.9	gi|123488984|ref|XP_001325288.1|	Xpg n-terminal domain containing protein
a35868_145	1.1	1.1	0.7	0.9	gi|321461942|gb|EFX72969.1|	Serine threonine-protein kinase

EST Id.	Fold change Log_2FC				NCBI Reference ID	Gene annotation
	B1-Bo	B3-Bo	T10-To	T30-To		
						38 (ndr2 protein kinase)
a12341_301	0.9	1.2	1.0	0.9	gi|171473643|ref|YP_001798475.1|	Nadh dehydrogenase subunit 4
a133811_75	0.5	1.0	0.8	0.9	gi|293341280|ref|XP_002724888.1|	Squamous cell carcinoma antigen 2
a3293_738	0.8	1.5	0.4	0.9	gi|156544938|ref|XP_001607263.1|	Cg12324 protein
a65010_42	1.2	1.8	0.6	0.9	gi|291227350|ref|XP_002733649.1|	Chromosome x open reading frame 15
a14753_178	0.8	1.7	1.0	0.8	gi|270016014|gb|EFA12462.1|	Isoform d
a47023_78	1.1	1.1	0.5	0.8	gi|321455352|gb|EFX66487.1|	Zinc finger protein
a21127_38	0.7	1.2	0.7	0.8	gi|54298435|ref|YP_124804.1|	Alkaline phosphatase
a18878_155	1.6	1.5	1.0	0.8	gi|322794371|gb|EFZ17475.1|	Isoform b
a80193_14	0.5	2.1	0.6	0.8	gi|86515340|ref|NP_001034497.1|	Ultrabithorax
a56699_78	0.9	1.0	0.5	0.7	no_hit	Tudor and kh domain containing
a20122_269	0.8	1.0	0.5	0.7	gi|242023708|ref|XP_002432273.1|	128up
a682837_10	0.5	1.5	0.6	0.6	gi|326921040|ref|XP_003206772.1|	Mitochondrial carnitine acylcarnitine carrier
a68501_84	1.2	1.6	0.6	0.5	gi|296483105|gb|DAA25220.1|	ktn1_protein

Appendix 2: Top most significantly enriched GO terms for Tridex with minimum 5 genes involved. 40 of the 76 significantly enriched GO terms is shown for the high level of Tridex, 30 mg kg^{-1}. P%: Fischer's exact test.

T30-T0		T10-T0	
GO term	**P %**	**GO term**	**P %**
Proteolysis	<0.0	Structural constituent of cuticle	<0.0
Serine-type endopeptidase activity	<0.0	**Oxidation reduction**	1.5
3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase activity	<0.0	Regulation of transcription	2.9
3-oxoacyl-[acyl-carrier-protein] reductase activity	<0.0		
[acyl-carrier-protein] S-acetyltransferase activity	<0.0		
Myristoyl-[acyl-carrier-protein] hydrolase activity	<0.0		
Oleoyl-[acyl-carrier-protein] hydrolase activity	<0.0		
Palmitoyl-[acyl-carrier-protein] hydrolase activity	<0.0		
Fatty acid biosynthetic process	<0.0		
Fatty acid synthase complex	<0.0		
3-oxoacyl-[acyl-carrier-protein] synthase activity	<0.0		
[acyl-carrier-protein] S-malonyltransferase activity	<0.0		
Biotin carboxylase complex	<0.0		
Biotin carboxylase activity	<0.0		
Serine-type endopeptidase inhibitor activity	<0.0		
Nucleic acid binding	<0.0		
Acyl carrier activity	<0.0		
Arginase activity	<0.0		
Oxidation reduction	<0.0		
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.1		
Monooxygenase activity	0.1		
Response to chemical stimulus	0.1		
Peptidase activity	0.1		
Cofactor binding	0.1		
Electron carrier activity	0.1		
Electron transport	0.2		
Hemocyte proliferation	0.2		
Transferase activity, transferring acyl groups other than amino-acyl groups	0.2		
Sulfotransferase activity	0.2		
Catalytic activity	0.2		
Lipid biosynthetic process	0.3		
Triglyceride biosynthetic process	0.3		
Zinc ion binding	0.3		
Biosynthetic process	0.3		
Lipid binding	0.3		
Polyamine transport	0.4		
D-amino acid transmembrane transporter activity	0.4		
D-amino acid transport	0.4		
L-amino acid transmembrane transporter activity	0.4		
L-amino acid transport	0.4		

Appendix 3: Top most significantly enriched GO terms for Basamid with minimum 5 genes involved. 47 of 168 significantly enriched GO terms is shown for the high level of Basamid, 3 mg kg^{-1}. The common GO terms with Tridex in bold. P%: Fischer's exact test.

B3_B0		B1_B0	
GO term	**P %**	**GO term**	**P %**
ATP binding	<0.0	Peptidase activity	0.1
Proteolysis	<0.0	**Proteolysis**	0.9
Extracellular region	<0.0	**Oxidoreductase activity**	2.7
Structural constituent of cuticle	<0.0	Membrane	2.7
Chitin binding	<0.0	Sodium ion transport	4.8
Serine family amino acid metabolic process	<0.0	Chitin metabolic process	5.0
Chitin metabolic process	<0.0		
Protein binding	<0.0		
Oxidation reduction	<0.0		
DNA binding	<0.0		
Hydrolase activity	<0.0		
Serine-type endopeptidase activity	<0.0		
Transporter activity	<0.0		
Nucleus	<0.0		
Heme binding	<0.0		
Carbohydrate metabolic process	<0.0		
Metabolic process	<0.0		
Peroxidase reaction	<0.0		
Oxidoreductase activity	<0.0		
Odorant binding	<0.0		
Cation binding	<0.0		
Fatty acid biosynthetic process	<0.0		
2-alkenal reductase activity	<0.0		
Catalytic activity	<0.0		
Protein serine/threonine kinase activity	<0.0		
Nuclear mrna splicing, via spliceosome	<0.0		
Perinuclear region of cytoplasm	<0.0		
Nucleic acid binding	<0.0		
Ubiquitin-protein ligase activity	<0.0		
Hydrolase activity, hydrolyzing O-glycosyl compounds	<0.0		
Transport	<0.0		
Cytosolic small ribosomal subunit	<0.0		
Peroxidase activity	<0.0		
Cytoplasm	<0.0		
Structural constituent of chitin-based cuticle	<0.0		
Protein kinase activity	<0.0		
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	<0.0		
Ribosome biogenesis	<0.0		
Oxygen transport	0.1		
Helicase activity	0.1		
Phosphorylation	0.1		
Protein amino acid phosphorylation	0.1		
Chitin catabolic process	0.1		
Acyl carrier activity	0.1		
Electron carrier activity	0.1		
Zinc ion binding	0.1		
Chitinase activity	0.1		

GENEPEASE

The project focussed on validating RNA based methods as potential genomic tools in assessment of agricultural soil ecosystems. It was shown that the mRNA based technique was very sensitive and the effects was seen in the same situations as when the OECD nitrification assay showed an effect. 16SrRNA based pyrosequencing of bacterial communities in soil was shown to report different than just DNA based analysis and indicated unlike the DNA measurement that the community was developing. Finally microarray analysis was compared to traditional test for toxicity testing of *Folsomia candida* and showed a more sensitive response to the pesticide exposure.

Ministry of Environment and Food of Denmark
Environmental Protection Agency
Strandgade 29
1401 Copenhagen K, Denmark
Tel.: (+45) 72 54 40 00

www.mst.dk