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Long-term fertilization with urban and animal wastes enhances soil quality but introduces pharmaceuticals and personal care products

Jeanne Vuaille¹ · Thorsten Klaus Otto Gravert² · Jakob Magid¹ · Martin Hansen² · Nina Cedergreen¹

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Abstract

Applying organic wastes such as manure or sewage sludge on agricultural fields improves soil physical structure and fertility, while giving purpose to otherwise disposed and burned waste products. Yet, xenobiotics, and especially pharmaceuticals and personal care products, may be present in these wastes and thereby enter the soil ecosystem. To investigate their occurrence and their potential effect on soil fauna, the CRUCIAL experimental fields, Denmark, were used. Here we combine, for the first time, a chemical wide-scope suspect screening analysis with a study of soil nematode faunal response on soils, which were fertilized with cattle manure, sewage sludge, human urine, and mineral fertilizers over 14 years. The suspect screening analysis was performed with a nanoflow ultra-high performance liquid chromatography coupled with high-resolution tandem mass spectrometry. The abundance of soil indigenous nematodes was determined in each treatment at two time-points, followed by laboratory tests with the nematode species *Caenorhabditis elegans* to assess its reproduction capacity in the soils. A total of 12 pharmaceuticals and personal care products were identified as being up- or down-concentrated in the different treatments compared to controls (mineral fertilizers): 8 and 2 were found to be up-concentrated in sludge- and urine-treated soils, respectively. Our results, however, showed a significantly greater abundance of nematodes in soils amended with sludge and manure, while *C. elegans* had the lowest reproduction capacity in the controls. The reproduction capacity tests did not reveal any chronic toxicity. A principal component analysis showed a clear correlation between nematode abundance and soil organic matter, water content, nutrient contents, and porosity. Overall, urban and animal wastes enhanced soil quality. Pharmaceuticals were detected, but adverse effects on the measured endpoints could not be demonstrated. Although further investigations are needed to examine other associated risks, recycling nutrients from urban areas into fertilizers can contribute to a more sustainable fertilization strategy.

Keywords Fertilization · Urban wastes · Ecotoxicology · Nematodes · High-resolution mass spectrometry · Suspect screening

1 Introduction

Agricultural production needs to be more sustainable. Using urban and animal wastes as organic fertilizers is an effective way of limiting the use of mineral fertilizers while recycling nutrients, and therefore an important step towards more sustainable food production systems. In particular, sewage sludge and cattle manure are well-known

for their benefits on soil fertility and structure due to their phosphorus (P), nitrogen (N), and organic matter contents, whereas human urine is known for its N richness (Heinonen-Tanski and van Wijk-Sijbesma 2005). These organic amendments have also been shown to enhance soil microbial biomass and soil microfauna abundance (Weiss and Larink 1991; Liang et al. 2009; Coors et al. 2016). Nevertheless, applying urban and animal wastes on agricultural soil can be a direct entry route for xenobiotics and heavy metals to the soil ecosystem (Ghirardini et al. 2020), which is the cause of several countries not allowing the recycling of sewage sludge. While heavy metals, polycyclic aromatic hydrocarbons and other organic compounds have been traditionally monitored and regulated in sewage sludge under the European Directive 86/278/EEC

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(European Commission 1986), non-monitored organic compounds are getting increased attention as potential emerging contaminants. Emerging contaminants include a wide panel of chemicals, e.g., flame retardants, microplastics, pharmaceuticals and personal care products (PPCPs) (Gottschall et al. 2012), including veterinary pharmaceuticals. PPCPs may occur in the soil after sewage sludge application at concentrations ranging from ng to $\mu\text{g/g}$ of soil (Walters et al. 2010). These diverse and unregulated substances present in organic wastes form a chemical cocktail of potentially bioactive compounds, which could be toxic to soil fauna and flora.

Adverse effects of sewage sludge and manure application on soil fauna have been investigated over short (< 5 years) and long-term periods (> 5 years), and especially using nematode abundance and community structure as recognized soil health bioindicators (Weiss and Larink 1991; Liang et al. 2009; Coors et al. 2016), without leading to strong conclusions. In their environmental risk assessment of sewage sludge amendment, Verlicchi and Zambello (2015) showed that some PPCPs, namely ciprofloxacin, ofloxacin, tetracycline (antibiotics), estradiol (hormone), ibuprofen (analgesic), caffeine (psychoactive), triclosan and triclocarban (bacteriocides), posed a potential risk for soil organisms. In a similar risk assessment, Magid et al. (2020) concluded that phthalates and triclocarban were the organic chemicals posing the highest potential risk to soil-living organisms in sludge-amended soils. Both studies were based on risk quotients, corresponding to the ratio between pollutant concentration and predicted no-effect concentration (PNEC). However, because only little data regarding chronic and acute toxicity of PPCPs for soil organisms is available, the PNEC in soil was estimated with the known PNEC in water of other organisms (e.g., daphnid) using the partition coefficient K_d of the compound of interest to calculate pore water concentrations. While pointing out the compounds of highest concern, these results also highlighted the significant uncertainty of the estimated risk using wide species extrapolations and associated high safety factors (Magid et al. 2020).

To combine occurrence of PPCPs with biological indicators of effect in a more realistic long-term scenario, we used soils from an experimental farm near Copenhagen in Denmark, where urban and animal wastes have been applied since 2003, in the framework of the CRUCIAL project (Magid et al. 2006). In order to represent worst-case scenarios for waste recycling, sewage sludge and cattle manure have been applied at very high rates, on average four times higher than the maximum application rate of 30 kg/ha y of P authorized by the Danish legislation (López-Rayó et al. 2016, average rates between 2003 and 2016 recalculated based on fertilizer analyses). The study of López-Rayó et al. (2016) showed that these extreme application rates presented no substantial risks related to the content of heavy metals in the soils.

In addition, the application of wastes to a subsection of plots was terminated 9 years before sampling to study persisting effects.

The aim of this study was first to investigate the presence of PPCPs in the soil plots fertilized with different organic wastes, i.e., cattle manure, sewage sludge and human urine, as compared to a control treated with mineral fertilizers (NPK), using a novel chemical wide-scope suspect screening analysis concept. Second, the effect of PPCPs on soil nematodes was studied over the abundance of indigenous free-living soil nematodes and the reproduction capacity of the *Caenorhabditis elegans* species (Fig. 1). We hypothesized that, after the application of three types of urban and animal wastes over 14 years, (i) PPCPs were more abundant in soils amended with manure, sludge and urine compared to NPK fertilization, (ii) PPCPs were less abundant in soils where sludge application terminated after 5 years when compared to a non-stop sludge application, and (iii) the soil physical properties as influenced by the fertilizers were driving factors for the nematode abundance.

2 Materials and methods

2.1 Experimental layout

The CRUCIAL experimental field is located in Tåstrup, Denmark, and belongs to the University of Copenhagen. The project was established in 2003 with 39 plots of 891 m² each, separated by 3-m wide strips of grass, and organized in a random block design with three blocks containing 12 treatments each (Magid et al. 2006). The sandy loam texture (Table 1) was generally homogeneous across the blocks but showed a slight difference in the mean clay content, ranging from 16.7% in block 1 to 14.7% in block 3. A 4-year crop rotation including oat (*Avena sativa*), spring barley (*Hordeum vulgare*), rape (*Brassica napus*) and spring wheat (*Triticum aestivum*) was implemented, except for the years 2010 and 2011 where ryegrass (*Lolium* sp.) and winter wheat (*Triticum* spp.) were cultivated. Spring barley was grown at the time of sampling for this study (2017). Ten types of urban and animal wastes have been applied since 2003, while three plots were left unfertilized and three others were fertilized with a conventional NPK mix. Since 2012, some of the plots have been subdivided into two parts. In order to study the persistence of the amendment effects, the application of the wastes was terminated on one-third of the plots. Instead of the waste application, a reduced quantity of nitrogen fertilizer was applied, at half the rate of N of the mineral fertilizer treatment plots. In this study, we focused on five of the treatments: cattle manure, sewage sludge, terminated sewage sludge, human urine

Fig. 1 Upper left: photograph of the CRUCIAL plots, Tåstrup, Denmark; upper right: soil sampling in a CRUCIAL plot; lower left: pressurized liquid extraction with a Dionex ASE 300 accelerated solvent extractor on soil samples collected after waste application; lower right: view of the laboratory nematode species *C. elegans* under microscope.



Table 1 Soil properties in the CRUCIAL treatments studied. Soil texture is expressed in the USDA system. Standard deviations are presented in brackets when available. SOMC, soil organic matter content; U, unknown. Estimated values are marked with e. $n=2$ for SOMC and $n=4$ for porosity.

Treatment	Block	Sand %	Silt %	Clay %	SOMC %	Porosity %
Cattle manure	1	65.1	17.1	17.8	4.70 (0.42)	49.8 (2.9)
	2	66.2	19.2	14.6	5.15 (0.35)	46.8 (2.8)
	3	66.1	19.4	14.5	4.20 (0.85)	49.5 (4.2)
Sewage sludge	1	68.6	15.7	15.7	4.35 (0.35)	42.0 (1.3)
	2	66.3	18.7	15.1	4.30 (0.28)	46.6 (1.9)
	3	67.6	17.5	14.8	4.65 (0.92)	46.0 (5.1)
Human urine	1	68.8	16.1	15.1	4.25 (0.07)	45.0 ^e
	2	67.3	17.4	15.3	3.10 (0.57)	41.7 ^e
	3	65.5	18.7	15.8	3.40 (0.14)	42.6 ^e
Control (NPK)	1	65.2	16.6	18.2	3.05 (0.78)	44.0 (1.1)
	2	66.8	18.0	15.2	3.65 (0.07)	42.1 (5.4)
	3	70.0	16.4	13.5	3.45 (0.07)	40.8 (2.5)
Sewage sludge terminated	1	68.6	15.7	15.7	3.40 (0.21)	U
	2	66.3	18.7	15.1	3.54 (1.05)	U
	3	67.6	17.5	14.8	3.61 (0.21)	U

and mineral fertilizers NPK, as presented in Fig. 2. We hypothesized that these specific urban and animal wastes were the most likely to carry PPCPs to the agricultural soils since they were excreta. Because mineral fertilizers are widely used on croplands, we assumed that they corresponded to the most conventional fertilization strategy and therefore defined it as our control.

2.2 Fertilizers

All fertilizers were incorporated after yearly application by ploughing down to approximately 20 cm. Table 2 gives the estimated average fertilizer inputs. The application rates varied from one year to another according to the type of crops and were based on the mineral fertilizer

Fig. 2 Experimental design of the CRUCIAL project fields. CM, cattle manure; S, sewage sludge; HU, human urine; NPK, mineral fertilizers; U, unfertilized. Hatched areas are not cultivated. Grey plots are other treatments not considered in this study. RN stands for terminated treatments with reduced nitrogen fertilization.

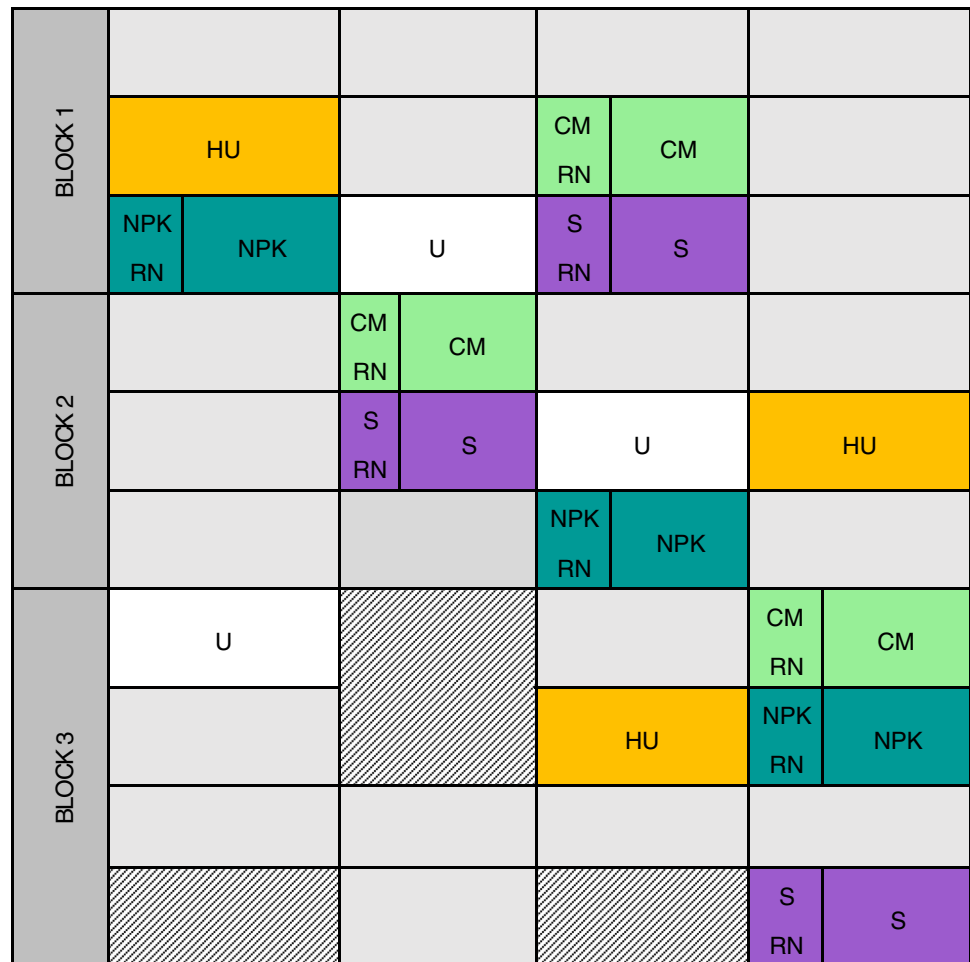


Table 2 Average ($n=14$) application rates, dry matter, total carbon, and total nitrogen contents of the urban and animal wastes applied between 2003 and 2016 on the CRUCIAL field plots. Standard deviations are presented in brackets. MFE stands for mineral fertilizer equivalent.

Treatment	Applied rate t/ha y	Dry matter (DM) content % fresh matter	Total C content % DM	Applied C t/ha y	Total N content % DM	MFE %	Applied MFE N t/ha y
Cattle manure	89.00 (19.52)	17.96 (3.36)	45.77 (1.91)	7.01 (1.70)	2.16 (0.12)	65	0.216 (0.054)
Sewage sludge	17.17 (4.84)	21.93 (1.42)	30.24 (2.11)	1.14 (0.29)	4.28 (0.51)	45	0.072 (0.018)
Human urine	74.96 (9.97)	0.46 (0.11)	-	-	40.63 (10.22)	65	0.091 (0.024)
Control (NPK)	0.52 (0.09)	100	-	-	20.6	100	0.108 (0.019)
Sewage sludge terminated	0.20 (0.02)	100	-	-	20.6	100	0.042 (0.005)

equivalent (MFE) of nitrogen input, as regulated by the Danish authorities. Cattle manure originated from a conventional farm located in Hedehusene (about 8 km from Tåstrup) and was applied at an average yearly rate of 89.0 t/ha (fresh matter, FM), which was equivalent to 216 kg MFE N/ha y. Degassed and dewatered sewage sludge was collected from a public treatment plant in Avedøre (about 15 km from Tåstrup), receiving mixed wastewaters from non-hazardous industries and private households (0.3

million people). The sludge was spread at about 17 t FM/ha on average, equivalent to 72 kg MFE N/ha y.

The application rates of manure and sludge were aberrations with respect to Danish legal practice. The legal limit for P fertilization is 30 kg P/ha y in Denmark, while cattle manure and sewage sludge typically have an N-to-P ratio of 3 and 1.5, respectively. Thus, the manure treatment had an average P fertilization of 115 kg P/ha y and the sludge treatment of 107 kg P/ha y. The purpose of such extreme application

rates was to investigate potential accumulation of heavy metals and other pollutants over time in a worst-case scenario. Human urine was supplied by an eco-village near Roskilde and a housing department in Albertslund (about 15 and 6 km, respectively, from Tåstrup) and stored in airtight tanks for at least six months before spreading at about 75 t/ha on average, equivalent to 91 kg MFE N/ha y. The average application rates of mineral fertilizers were 108 kg MFE N/ha on the control plots and 42 kg MFE N/ha on the terminated sludge plots.

2.3 Soil sampling

Soil samples were first collected in March 2017 about 2 weeks before spring application of urban and animal wastes (sampling campaign 1), with a 2-cm diameter auger and down to 25 cm. A number of 15 cores were collected in a re-sealable plastic bag for each treatment and block, resulting in 3 bags of 15 cores each per treatment and a total of 15 bags. The samples were kept at 4 °C for indigenous nematodes extraction, reproduction capacity tests and soil property measurements, and subsequently stored at –18 °C until further analysis for PPCPs. With the same material and method, the second sampling campaign was performed in June, about 9 weeks after wastes application (sampling campaign 2).

2.4 Soil property measurements

Gravimetric soil water content was determined for each treatment after drying around 10 g of soil at 105 °C for 24 h. Soil organic matter content was obtained afterward, following the loss on ignition method. For pH measurements, about 10 g of soil were mixed with 25 mL 0.01 M CaCl₂. The mixture was shaken 30 times before being left to settle for 1 h. The pH was measured after being shaken again and rested for 10 min.

Elemental compositions were measured by López-Rayó et al. (2016) in 2013 for all soils, except the terminated sludge treatment, and included macro and micronutrient analysis. Soil porosity of the control and the treatments manure and sludge was estimated based on dry bulk density measurements, using a particle density value of 2.65 g/cm³. In the study of Peltre et al. (2015), the soil bulk density and organic carbon content in the CRUCIAL plots were linearly correlated with a R² of 0.75. The bulk density of the urine treatment was therefore estimated based on this model using the organic carbon content.

2.5 Chemical analysis

2.5.1 Extraction of pharmaceuticals and personal care products

Sample extraction in triplicates was performed by pressurized liquid extraction with a Dionex ASE 300 accelerated solvent extractor on soil samples collected after waste application and a procedural blank sample. A 10-mL stainless steel extraction cell was packed from the bottom up with a glass fiber filter (Thermo-Fischer Scientific). Lyophilized soil (6 g) or burned (400 °C, 8 h) Ottawa Sand (6 g, Restek Inc., USA), for procedural blanks, was mixed with burned (400 °C, 12 h) diatomaceous earth (0.5 g, Restek Inc., USA). Each sample was transferred to an extraction cell. The remaining cell volume was filled with burned Ottawa Sand and topped with a glass fiber filter. The extraction program was as follows: cells were preheated 5 min at 80 °C, a solvent mixture of aqueous ammonium solution (1 mM, pH 9) and methanol (1:1 v/v, HPLC-grade, Merck KGaA, Germany) was applied (Pérez-Carrera et al. 2010) under 1500 psi with 5 min static time, before the sample was flushed with nitrogen (60% flush volume) into amber glass vials. The extraction cycle was performed five times on each cell. The combined extracts were concentrated to 4 mL under a gentle stream of nitrogen (40 °C). Solid-phase extraction was applied to remove remaining particles and salts. The concentrated extracts (4 mL) were diluted with water (20 mL) and loaded on a hydrophilic-lipophilic balance solid-phase extraction material (200 mg, OASIS Prime HLB, Waters Inc., USA), conditioned with methanol (5 mL) and water (5 mL). Sample extracts were purified on the HLB cartridges placed on a vacuum manifold. The HLB material was washed with water (3 × 2 mL) and dried for 20 min by applying vacuum. Analytes were eluted with methanol (2 × 3 mL), evaporated to 0.1 mL under nitrogen, and water (1 mL) was added. Finally, extracts were centrifuged (1000 g, 6 min) and the supernatant transferred to glass vials for liquid chromatography. All sample handling was performed with glass intraMark micropipettes (BLAUBRAND®, Wertheim, Germany). Extracts were stored at –20 °C until analysis. A quality control sample was prepared by pooling 50 µL of each sample into a new vial.

2.5.2 Nanoflow liquid chromatography–high resolution tandem mass spectrometry

The chromatographic separation was performed on a Dionex Ultimate 3000 NCS-3500RS Nano Proflow system (ThermoFisher Scientific), containing a two-column trap and elute system. The Dionex WPS-3000 TPL RS autosampler preserved samples at 8 °C. Samples of 5 µL were injected using partial loop injection onto an Acclaim PepMap column

(C18, 100 Å, 5 µm, 0.3 mm × 5 mm, ThermoFisher Scientific, Germany) for 3 min, focusing the sample. The loading pump was run at 30 µL/min with water. A valve switch eluted trapped analytes online to a capillary-LC column (PepMap RSLC, C18, 2 µm, 100 Å, 75 µm × 75 cm, ThermoFisher Scientific) under a flow rate of 200 nL/min for chromatographic separation. The mobile phase was composed of [A] water/acetonitrile/formic acid (97.9/2/0.1, v/v/v, Fisher Scientific, Finland) and [B] water/acetonitrile/formic acid (2/97.9/0.1, v/v/v, Fisher Scientific, Finland). Sample separation was attained by a gradient starting from 10% B at 0 min to 95% B at 57 min, which was held for 20 min. The initial conditions were restored over 1.5 min, and the column was re-equilibrated for 21.5 min, leading to a total runtime of 100 min. The column temperature was controlled at 40 °C. Quality control samples and blank solvent injections on every 5th injection were used to monitor chromatographic performance and analyte carryover.

The mass spectrometric analysis was performed on a high-resolution mass spectrometer (Q Exactive HF, ThermoFisher Scientific, Germany) in full scan and iterative data-dependent acquisition mode (Koelmel et al. 2017; Gravert et al. 2021). Analytes were ionized on an EasySpray (ThermoFisher Scientific) nanoESI source operated in positive mode (1.9 kV). The capillary temperature was held at 260 °C. Full scan spectra were recorded in profile mode between a mass-to-charge ratio (m/z) of 75 to m/z 1000, with 240,000 resolution at m/z 200, an automated gain control target of 3×10^6 , and a maximum ion injection time of 100 ms. Mass accuracy was corrected via lock mass 371.10120 (Schlosser and Volkmer-Engert 2003). Fragment mass spectra were recorded at a resolution of 15,000 (at m/z 200) and stepped normalized collision energy of 15 and 50. Calibration of the mass spectrometer was performed weekly with Pierce™ LTQ Velos ESI Positive Ion Calibration Solution (ThermoFisher Scientific).

2.5.3 Wide-scope suspect screening

The chemical analysis data for PPCP suspect screening was processed with Compound Discoverer (3.1.1.12, ThermoFisher Scientific, USA). Mass spectral peaks were picked, retention time aligned and grouped by isotopologues and adduct peaks into theoretical compounds. The chemical composition of these compounds was predicted based on exact mass and isotope pattern matching within preset limits. To remove background, compounds were filtered by a minimal peak intensity threshold (10^6) and needed to be six times higher in soil samples compared to solvent blanks. A gap filling method was applied for the statistical analysis to remove null values from the results list. If a theoretical compound was found in one sample, all other samples were reprocessed at the same retention time and m/z , ignoring

intensity thresholds and noise level. If still no corresponding compound peak was detected, a normal distribution was fitted to the baseline noise creating a small simulated positive peak area. The exact mass and fragmentation spectra of the theoretical compounds were then searched against the mzCloud database (HighChem LLC, Slovakia) and an in-house mass spectral database. Exact masses were further compared to environmental contaminant lists. The resulting annotated compounds were filtered and prioritized. Focus was placed on compounds originating from the organic waste application over shared contaminants, such as pesticides or natural compounds. Additionally, this approach increased the confidence in compound annotations. Compounds were removed if they did not fulfill following criteria; 1) presence of a fragmentation spectrum (MS2), 2) a peak area above 10^7 , 3) a heteroatom (i.e., O, N, S, P, I, Cl, Br, and F) in their elemental composition, which improves ionization via electrospray and excludes exotic molecules, and 4) a spectral database reference (MS1 and MS2) or 5) a reference standard measured in the same batch. Filtered results were assessed manually for peak shape (Gaussian, Signal-to-noise) and spectral database matching (Sfit, mzCloud score, mzLogic score). To compare relative concentrations between soils, compound peak areas were used and reported as fold-changes.

2.6 Indigenous soil nematodes abundance

Indigenous soil nematodes were extracted from each soil replicate of about 10 g twice before and twice after waste application based on the Baermann funnel technique described by Hooper (1986). Because keeping the soil at 4 °C after sampling drastically slowed down the biological activity, the samples were kept at room temperature (about 20–24 °C) for 12 days prior to extraction. After 12 days, nematodes from the five studied treatment plots were extracted simultaneously in triplicates (one random sample per block, 3 blocks). The setup corresponded to a 10-cm long rubber tubing attached to the bottom of a plastic funnel and closed with a clamp. The funnel had a diameter of 7 cm at the top and 1 cm at the bottom, and was mounted on a holder. The soil sample was placed on a plastic-mesh basket (mesh size of 0.5 mm) in the funnel. A piece of paper towel was added between the plastic-mesh basket and the soil, thereby preventing pieces of soil from mixing with water. The funnels were filled with tap water, so that the water level was about 5 mm above the soil surface. Submerging the soil allowed the nematodes to leave the soil matrix and settle down in the rubber tube. The extraction was carried out at room temperature, which is optimal for nematode movement. This method only allowed the recovery of active stages. After 48 h, the nematodes settled above the clamp were recovered into 15-mL centrifuge tubes, in which 50.0 µL of Rose Bengal at 1% (Sigma-Aldrich®—CAS: 632–69-9) were

added. The tubes were then left for 40 min before being heated at 55 °C for 1 h. This process led to straight and pink dead nematodes, easily visible with a microscope (Nikon SMZ800, magnification 1 to 6.5). The tubes were subsequently centrifuged (74 g, 1 min) to reduce the volume of liquid with nematodes to about 1 mL, allowing faster examination and counting under the microscope. The abundance of nematodes from the 10 g of fresh soil was converted into a number per 100 g of dry soil.

2.7 Reproduction capacity of *Caenorhabditis elegans*

The *C. elegans* strain N2 (wild type) was used to perform reproduction capacity tests in the sampled soils. The culture originated from the University of Minnesota (USA) and was maintained according to the ISO 10872 guidelines (ISO 2010). The culture was maintained on sterile growth medium agar plates at 20 °C in the dark with the *Escherichia coli* bacteria type OP50 as food source. The reproduction test was conducted for 72 h for each treatment in triplicates (one sample per block, 3 blocks). An amount of 0.3 g of gently crushed soil was added to 12-well culture plates, followed by 0.14 mL of a growth medium M9 (ISO 2010) and 0.5 mL of OP50 (absorbance of about 0.35 at 600 nm). Finally, one gravid nematode was introduced in each well. Three blank wells were prepared in the same manner without adding any test organism, to correct the reproduction results for the presence of indigenous nematodes. After 72 h, 50 µL of Rose Bengal at 1% were added to each well before heating the plates in an oven for 1 h at 55 °C. The worms were separated from the soil with a Ludox (2 mL, from Sigma-Aldrich®—CAS: 7631–86-9) centrifugation (800 g, 5 min) repeated 3 times, before examination and counting under the microscope. The results were expressed as number of offspring per adult nematode.

2.8 Statistical analyses

Statistical analyses were conducted with the R software, version 4.0.2 (R Core Team 2020). Nematode abundance, reproduction capacity and the soil water content measurements were individually fitted to a linear mixed-effects model using the lme4 package (Bates et al. 2015), with block as random effect. Interaction effects between the treatment and the sampling time was considered for both the abundance and the soil water content. The significance of main and interaction effects was determined with an analysis of deviance using the ANOVA function from the base R stats package. Residual- and quantile–quantile-plots were visually assessed to check the model assumptions of normality and homogeneity, and the data were log transformed to meet the assumptions. The estimated means with 95% confidence

intervals were reported. The single-step approach (Hothorn et al. 2008) was used to adjust the pairwise comparisons and the significance level was set to 0.05. A principle component analysis (PCA) was carried out with the factoextra package (Kassambara and Mundt 2020) to investigate the soil factors affecting nematode abundance. Nematode abundance, soil water content, soil organic matter content and pH determined at the two sampling points were combined with texture, porosity and nutrient content data from previous studies (Peltre et al. 2015; López-Rayó et al. 2016) for the treatments manure, sludge, urine and the control. The dataset was standardized before analysis, in order to reach a mean of 0 and a standard deviation of 1 for each variable. This prevented from introducing any bias in the PCA due to unit differences.

Regarding suspect screening, peak areas of filtered compounds were exported from Compound Discoverer (3.1.1.12) and log transformed, before a PCA using the base R stats package was made. A differential analysis of peak area with Benjamini–Hochberg correction was used to identify significant fold-changes between the treatments i.e., ratios of peak areas of the manure, sludge, urine or terminated sludge treatment to the control significantly higher or lower than 1. A fold-change greater than 1 with a *p*-value < 0.05 and an adjusted *p*-value < 0.1 indicated that a chemical was up-concentrated in manure, sludge, terminated sludge or urine compared to the control.

3 Results and discussion

3.1 PPCP occurrence in soil

A total of 114 compounds were annotated through the suspect screening approach. The PCA on Fig. 3 shows a spread of treatment groups over the second, and to a lesser extent, the first principal component (PC1). The widest distance laid between sludge and manure treated soils, indicating a very different chemical profile. This was supported by the six compounds in the positive loadings of PC2, which appeared to be pharmaceuticals common to sewage sludge (Gottschall et al. 2012), namely metoprolol, propranolol (beta-blockers), venlafaxine, citalopram (antidepressants), desmethylcitalopram (metabolite of citalopram), and tapentadol (analgesic), from top to bottom. The treatments urine and terminated sludge clustered in the middle of the PC2, together with the quality control samples. This might have been due to a lower abundance of treatment-specific compounds in these samples. Urine and terminated sludge in the PCA were connected to the detection of benzophenone (UV filter), cetirizine (antihistaminic), and methadone (analgesic), although a full separation was not possible on these principal components. Pooled quality control samples were

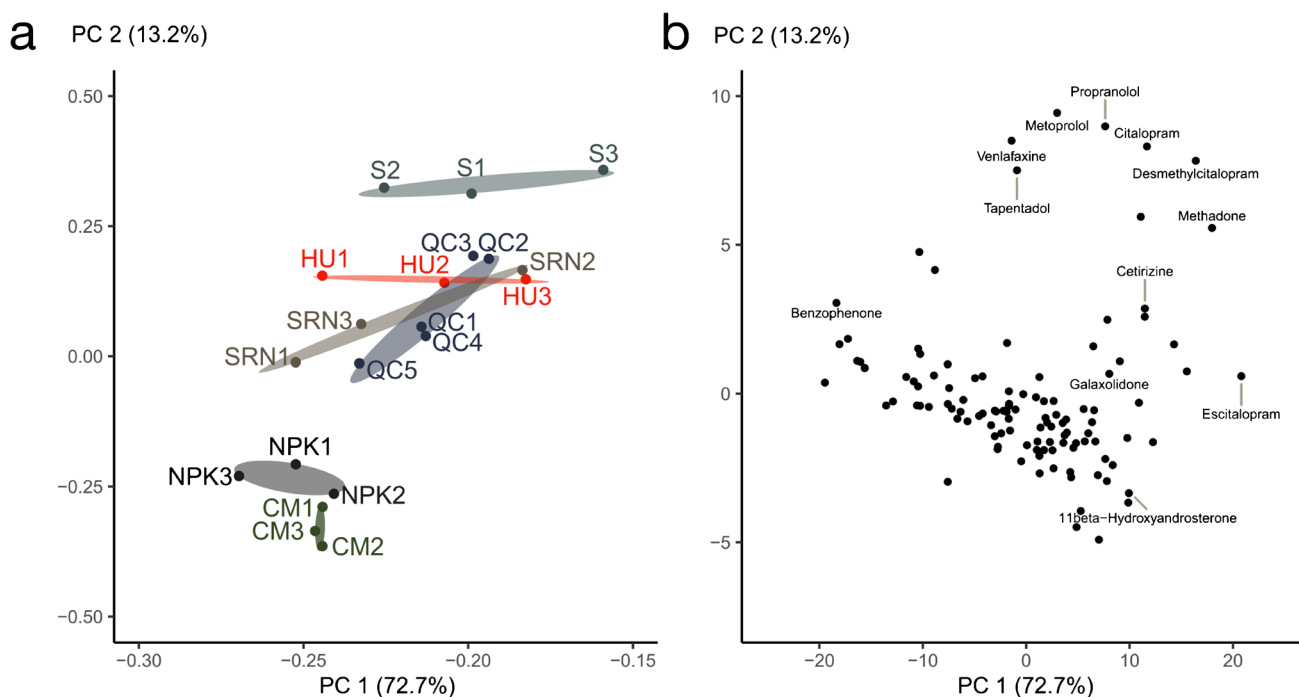


Fig. 3 Principal component (PC) analysis of 114 compounds annotated through suspect screening of the treated soils. **(a)** Score plot of soil samples for treatments with Steiner ellipse. CM, cattle manure; S, sewage sludge; SRN, sewage sludge terminated; HU, human

urine; NPK, control (mineral fertilizers); QC, pooled quality control. **(b)** Loading plot, with labels for a selection of pharmaceuticals and personal care products (PPCPs). Note that the PCs are unitless.

expected to have a lower concentration of compounds due to the dilution effect of mixing different soil extracts during preparation. The shared scores for quality control, urine, and terminated sludge, were a sign of a lower abundance of measured chemicals in these soils. Control and manure samples clustered closely together due to many shared compounds in similar concentrations. Fertilization with NPK in the control treatments was not expected to add PPCPs to the soil. Therefore, its proximity to manure was likely rather caused by the absence of specific contaminants and a higher abundance of shared soil compounds, such as fatty acids. Finally, manure might have been the origin of steroids, such as found 11beta-hydroxyandrosterone, expected in cattle manure (Horak and Möstl 2013). Noteworthy is the shared base structure and abundance of isomers for steroids, making structure identification based on accurate mass and fragmentation spectra alone difficult.

The differential analysis performed on each treatment group against the control treatment highlighted 12 compounds, which had at least twice the peak area in one of the treated soils compared to the controls, as shown in Table 3. Especially, the sludge treatment had the highest number of significantly up-concentrated compounds (8), followed by terminated sludge (3) and urine (2). The high presence of pharmaceuticals in the sludge-amended soil was in line

with previous studies examining contaminants in soil after sludge application (Walters et al. 2010; Gottschall et al. 2012; Verlicchi and Zambello 2015). The occurrence of two compounds only in the urine treatment might be related to the fact that urine was collected from only two small neighborhoods. The chemical profile of the treatment plot might therefore not be representative of the general population.

The antidepressant and analgesic venlafaxine and the beta-blocker metoprolol were found common to the three treatments sludge, terminated sludge and urine. However, the fold-changes of venlafaxine and metoprolol in terminated sludge were about 70 to 90% lower than in sludge, respectively (not significant), and about 86 to 97% lower than in urine (p -value = 0.012 for metoprolol and adjusted p -value = 0.003 for venlafaxine). Urine is the primary route of excretion of venlafaxine from the human body. But after ingestion, only 5% of the venlafaxine dose is recovered in urine as unchanged compound within 48 h (Kim et al. 2019), while sewage also contains urine. The larger abundance of venlafaxine in the urine samples compared to terminated sludge and sludge (adjusted p -value = 0.015) could rather be explained by an estimated half-life time (DT_{50}) of 2.1 days in water under field conditions, and 137 days when excluding photodegradation (Rúa-Gómez and Püttmann 2013). Storing urine in tanks for months before field application

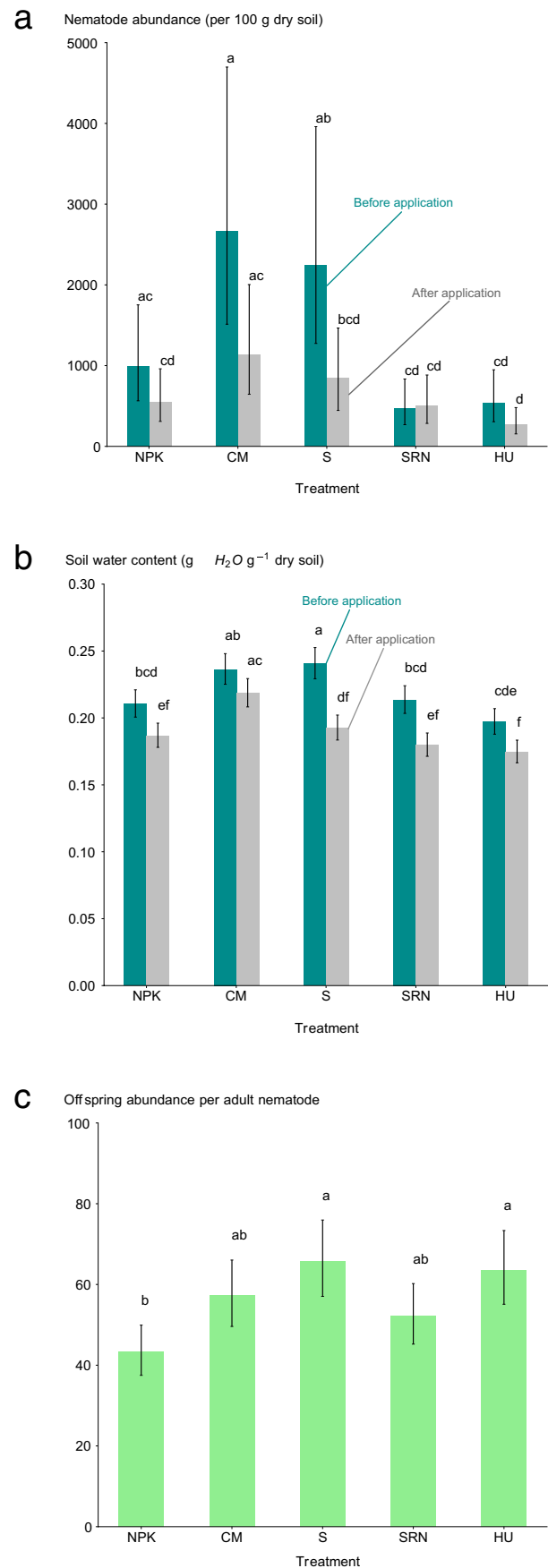
Table 3 List of annotated compounds identified as up- or down-concentrated in the treated soils compared to the control (NPK) treatment. The reported fold-changes are based on the ratios of chromatogram areas and are marked with ** for a p -value < 0.05 and *** for an adjusted p -value < 0.1. The usage categories were retrieved from PubChem (Kim et al. 2019).

Usage	Compound	Treatment			
		Cattle manure	Sewage sludge	Sewage sludge terminated	Human urine
Analgesic	Methadone	0.66	446***	6	1
	Propyphenazone	0.20**	0.40	0.63	1
	Tapentadol	2	687***	223***	3
Antidepressant					
Metabolite	Desmethylcitalopram	0.41	702.2***	5	48**
	Venlafaxine	0.68	102.6***	33.8***	1000***
Antihistaminic					
	Cetirizine	0.73	6	1	250**
Beta-blocker	Metoprolol	0.67	703***	72***	500***
	Propranolol	0.46	432***	4	100**
Calcium channel blocker					
Metabolite	2-(3,4-Dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile	0.46	312***	288**	21
Industrial ingredient					
Antistatic, disinfectant	Didecyldimethylammonium chloride	0.04**	2	1	0.88
Metabolite of synthetic musk	Galaxolidone	0.73	11***	4**	0.41
	1,3-Dihydro-1,3,3-trimethyl-2H-indol-2-ylidene acetaldehyde	0.39**	0.47	0.78	0.64

Fig. 4 (a) Abundance of free-living indigenous nematodes in the treated soils per 100 g dry soil ($n=6$ per treatment) before and after urban and animal waste application (1st and 2nd sampling campaigns, respectively); (b) gravimetric water content in the treated soils ($n=3$ per treatment) during the two sampling campaigns; (c) reproduction capacity of the nematode culture *C. elegans* in number of offspring per adult nematode in the treated soils ($n=3$ per treatment). NPK, control (mineral fertilizers); CM, cattle manure; S, sewage sludge; SRN, sewage sludge terminated; HU, human urine. Treatments with different lettering are significantly different. The error bars correspond to 95% confidence intervals.

may have prevented venlafaxine from degrading, resulting in a higher abundance in soil after application. This could also confirm our findings where the compound abundance in terminated sludge after 5 years of mineral fertilization exclusively was 70% lower than in sludge, but without statistical significance, potentially due to the variability in abundance between treatment replicates. Alternatively, the origin of the human urine could be an explaining factor of the high abundance of venlafaxine compared to sludge.

Nevertheless, the detection of venlafaxine in terminated sludge after 5 years of mineral fertilization also showed its persistence over years in the soil. With a log P of 3.20 (Kim et al. 2019), venlafaxine might not be very bioavailable for soil microbes and therefore hardly degradable. Furthermore, the persistence of such pharmaceuticals in soil is potentially much greater under field conditions than in a laboratory. The estimated field DT_{50} from Walters et al. (2010) for a series of PPCPs showed considerable discrepancies between predicted field half-lives and laboratory measurements. The DT_{50} of the antibiotic ciprofloxacin was for instance one order of magnitude greater in the field (2310 days on average) than the predicted half-life from the probability fate model BIOWIN (4.00, Microsoft Windows, US EPA) of 120 days. This large difference is likely to be explained by the fact that BIOWIN assumes a constant temperature of 25 °C whereas the average air temperature was 14 °C during the experiment in the field. In Denmark, mean soil temperatures are about 9 °C (Patil et al. 2010). Likewise, the analgesic tapentadol was found significantly up-concentrated in terminated sludge compared to control with a fold-change of 223, that is 68% lower than in sludge. This difference in fold-changes between the ongoing and terminated treatments was not statistically significant. For metoprolol, the average DT_{50} in different soils under dark laboratory conditions was determined to be 82.8 days by Kodešová et al. (2016), but our analysis showed that this compound was still present 5 years after sludge application, albeit a log P of 1.88 (Kim et al. 2019). On the other hand, the compounds desmethyl-citalopram, propranolol, galaxolidone (metabolite of synthetic musk), and methadone were not found significantly up-concentrated in terminated sludge contrarily to sludge, thereby showing a greater degradability in soil.



The soils treated with manure showed no known PPCP up-concentrated compared to the control, although the application rates had been extreme for 14 years. Three compounds were, however, identified with a higher abundance in the control compared to the manure treatment, though with adjusted p -values greater than 0.1: didecyldimethylammonium (adj. p -value = 1), 1,3-dihydro-1,3,3-trimethyl-2 h-indol-2-ylidene acetaldehyde (adj. p -value = 1) and propyphenazone (adj. p -value = 0.34). Didecyldimethylammonium chloride is an aliphatic alkyl quaternary used as a surface disinfectant (Kim et al. 2019), propyphenazone is a compound used as analgesic in veterinary products, and the compound 1,3-dihydro-1,3,3-trimethyl-2 h-indol-2-ylidene acetaldehyde is a precursor and possible hydrolysis product of widely used colorants, such as Basic Yellow 51. This colorant could have potentially been introduced through agrochemicals application, and its occurrence in the control treatment might demonstrate a difference in terms of biodegradability between the treatments. This compound might be present in sludge, terminated sludge and urine plots as well, but the high amount of organic matter added to the soil through manure application might have increased the microbial activity in this soil and allowed them to degrade faster than in the other treatments.

The occurrence of the analgesic and disinfectant compounds in the control treatment solely fertilized with mineral fertilizers was unexpected and their origin unclear. Carryovers from other treatments might have occurred even though it seemed unlikely. Given the size of the plots and the grass strips in between treatment plots, cross-contamination from natural processes is unexpected, but tillage operations, field sampling or laboratory preparation might have contaminated the samples with detected compounds. Alternatively, with adjusted p -values greater than 0.1, these compounds might have been falsely detected in the control samples. Compounds detected in suspect screening approaches require reference standards to be confirmed and rule out false positives. The detected analgesic and disinfectant compounds were not confirmed by reference standards.

3.2 Nematode abundance, reproduction capacity and soil quality

The abundance of indigenous soil nematodes in the investigated treatments before and after organic waste application (first and second sampling, respectively) is shown on Fig. 4A. The main effects in the mixed-effects model were significant (p -values < 0.01) but the interaction between treatment and sampling time was not (p -value = 0.19). With averages of 2666 (CI: 1513, 4698) and 2247 (1275, 3960) nematodes per 100 g dry soil, the nematode abundance in the manure and sludge treatments before application was

significantly higher than in urine and terminated sludge (p -values < 0.01), where the abundance was 538 (305, 949) and 473 (269, 834) nematodes per 100 g dry soil, respectively. In the control plots, the abundance tended to be higher than in urine and terminated sludge (995 (564, 1753) per 100 g dry soil), but the difference was not significant (p -values of 0.76 and 0.50, respectively). After application, only the manure and urine treatments were statistically different (p -value < 0.01).

The relative differences before and after application within each treatment in Fig. 4A were not significant, with p -values of 0.29, 0.14, 0.63, 0.78 and 1.0 for manure, sludge, urine, control and terminated sludge, respectively. Despite not being statistically significant, the abundance of nematodes decreased by half after fertilization across the different treatments. Since the first sampling before application was conducted in March and the second in June, the weather was a major difference between the two sampling campaigns. Figure 4B shows the gravimetric water content in the treated soils at the two time-points. The main effects of treatment and sampling time were significant (p -values < 0.01) as well as the interaction effect (p -value = 0.03). Figure 4B indicates that the soil tended to be drier after application, with statistical significance (p -values < 0.05) for all treatments except manure (p -value = 0.54).

Soil nematodes are mostly active and thrive in water films. Hence, the reduced amount of water in the soil after application could explain the decline in abundance between the sampling campaigns. In addition, the soils amended with manure and sludge generally had higher soil water contents, especially compared to urine, with which the difference was significant before application (p -values < 0.01), but they also had higher porosities and soil organic matter contents (Table 1). Therefore, Fig. 4A and Fig. 4B show that the addition of organic matter to the soil from manure and sludge applications tended to promote the development of a soil nematode population. We could hypothesize that the larger nematode population was due to the enhanced soil structure and water holding capacity, most likely promoting microorganism growth, a food source for fungal and bacterial-feeders nematodes.

The differences observed before application between treatments could thus be interpreted as long-term effects of the type of fertilizer used on the growth conditions of the native nematode community.

The enhancing effect of sludge or manure application on soil nematode abundance has already been reported (Weiss and Larink 1991; Liang et al. 2009). In the study by Coors et al. (2016), anaerobically digested and dewatered sludge application induced a significant rise in the free-living nematode abundance about 5 times greater than the control plots fertilized with mineral N (95.3 kg N/ha), with a lag time of 6 months.

Figure 4C presents the reproduction capacity of the nematode culture *C. elegans* in the treated soils from the first sampling campaign. The number of offspring was equivalent in all treatments, about 60 per adult nematode, yet with a tendency of a lower reproduction capacity in the control of

about 43 offspring, which was significantly lower than in sludge and urine. Because water and food were not limiting factors in the test, the soil structure and the chemical profile of the soil matrix, be it nutrients or xenobiotics such as PPCPs and metals, were the two parameters potentially

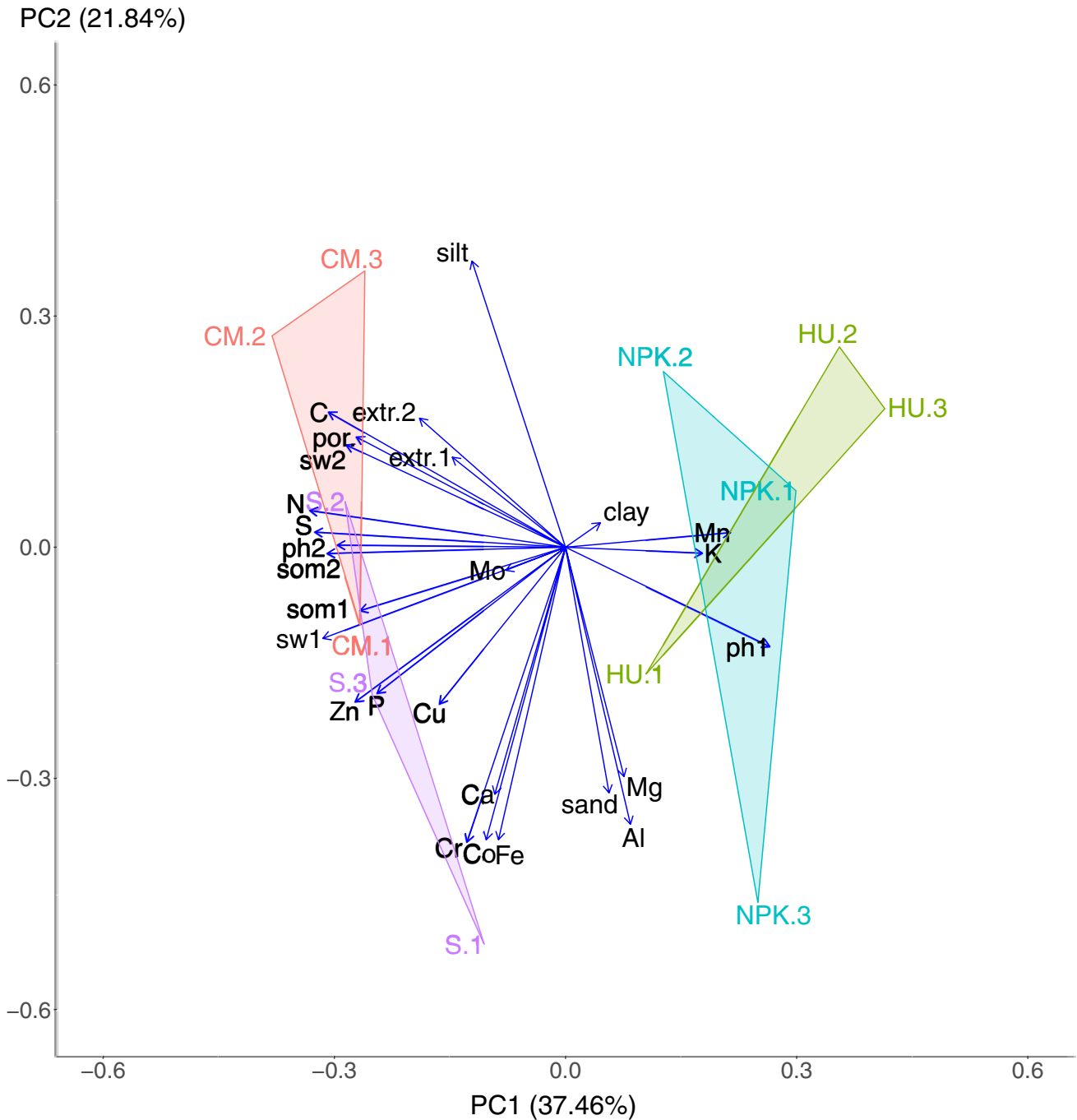


Fig. 5 Principal component (PC) analysis of physical, chemical and biological properties of soils treated with organic waste. Treatment coordinates on PC1 and PC2 plotted with the loading eigenvectors of the investigated variables. Extr. 1 and extr. 2, indigenous nematode abundance before and after waste application, respectively; sw1 and

sw2, soil water contents; som1 and som2, soil organic matter contents; por, porosity; CM, cattle manure; NPK, control (mineral fertilizers); S, sewage sludge; HU, human urine. Note that the PCs are unitless.

affecting the reproduction capacity. Hence, Fig. 4C shows that the urban and animal wastes did not negatively affect the offspring production when compared to the control, despite the detection of PPCPs in the treated soils. The poorer soil porosity (Table 1) and the mineral fertilizers themselves might explain the reduced number of offspring in the control. Due to the absence of tests carried out with soil from unfertilized plots, no conclusion could be drawn on the possible adverse effect of mineral fertilizers. Nevertheless, with respect to the conventional mineral fertilization strategy, the urban and animal wastes tested in this study did not appear to induce chronic toxicity towards soil nematodes.

The absence of toxic effects from PPCPs on soil organisms has been reported in several studies. Pino et al. (2015) investigated the acute toxicity of 18 pharmaceuticals in an artificial soil, based on bioassays with the earthworm *Eisenia fetida*. Their results showed that the most toxic compound was the analgesic ibuprofen, with a lethal concentration for 50% effect (LC_{50}) of 64.8 $\mu\text{g/g}$ soil (CI: 58.5, 71.8), which is at least two orders of magnitude higher than the range of reported concentrations in soils amended with sludge. Čadková et al. (2019) showed that the synthetic musk galaxolide inhibited the feeding activity of *C. elegans* after 24 h of exposure in a simulated soil solution, with an EC_{50} for this endpoint of 12.2 ± 2.2 mg/L. The predicted no-effect concentration (PNEC) was derived from this EC_{50} ($EC_{50}/1000$) to calculate a risk quotient (measured concentration in sludge/PNEC) of 0.14. This indicated that the compound posed a negligible risk to soil nematode in terms of feeding activity. For the antibiotic tetracycline, the EC_{50} in terms of nematode reproduction was determined to be 7.4 and 32.4 mg/L, for gel and liquid medium forms, respectively (Vangheel et al. 2014). More generally, the study of Domene et al. (2010) demonstrated that sludge toxicity towards collembolans in terms of reproduction and survival rates was directly linked to the amount of water extractable ammonium in soil treated with sludge. Higher survival and reproduction rates were found associated to higher soil organic carbon contents, most likely because of a higher sorption of ammonium (Domene et al. 2010). A higher organic carbon content in soil may thereby play a role in the bioavailability of PPCPs present in the applied sludge and their ecotoxicity.

Figure 5 shows the two first principal components from the PCA investigating the effect of soil physical–chemical properties on nematode abundance. The loadings of each investigated variable and the coordinates of the control and the treatments on PC1 and PC2 are visible. Together, PC1 and PC2 explained 59.3% of the total variance in the dataset. On PC1, the most contributing variables were N (8.7%), sulfur S (8.4%), and C (7.5%) contents, as well as soil water content during the first sampling campaign (sw1) (7.8%) and soil organic matter content during the second sampling campaign (som2) (7.5%), all directed towards negative values on

the axis. Thus, PC1 overall represented the organic matter content of the soils, with a higher content for lower coordinates. Soil porosity was also an important contributor to PC1 (5.8%). The silt content in the soil was the driving factor of PC2 in the positive part of the axis (loading of 10.9%), while the elements Fe, Cr, Co and Al were equivalently contributing to the negative part of PC2 (about 11% for each). Consequently, the PCA showed that the nematode abundance, with contributions of 2.8% and 2.2% on PC1 (negative part) and PC2 (positive part), respectively, was positively correlated to both organic matter and silt contents. It also tended to be negatively correlated to heavy metals. This allowed demonstrating that the clay gradient within the three blocks had some effect on nematode abundance, visible through a variation in the silt content. Yet, the treatments were clearly distinguishable on PC1 only. This was in line with a homogeneous soil texture across the different treatments. On PC1, manure and sludge clustered on the negative side, whereas urine and control were both on the positive side. Interestingly, these clusters were different from the ones observed in Fig. 3, implying that the chemical profile of the treatment plots did not coincide with the textural and structural properties.

The PCA results in terms of nematode abundance and soil organic matter content confirmed those from Gutiérrez et al. (2016) and Coors et al. (2016). In addition to nematode abundance, both studies examined the nematode community structure, which appeared to be affected by industrial pollution and sludge application, respectively. Gutiérrez et al. (2016) found that both heavy metals and pharmaceuticals were positively correlated to the presence of colonizer-type nematodes in the soil (calculated based on the ecological colonizer-persister gradient), known to be more opportunist and stress-tolerant than the persister nematode types. In Coors et al. (2016), soil texture had a greater influence on the structural index of the nematode community than sludge application. However, in one of the two investigated sites, a lower structural index in the treated field compared to the control could not be fully explained by soil texture. Thus, although adverse effects might not be visible when assessing the total nematode abundance, PPCPs might still affect the functioning of their food web. Indeed, several studies have shown that the nematode population was also increased after sludge application on contaminated soils (Weiss and Larink 1991), because of the high resistance to environmental stress of opportunist nematodes (Hlava et al. 2017). Therefore, the total abundance does not appear to be a sufficient endpoint for assessing soil health and may even be misleading.

Our nematode reproduction capacity test demonstrated that the laboratory culture used for risk assessment, *C. elegans*, was not sensitive to the presence of PPCPs added to the soil through 14 years of extreme sludge and manure application. Liang et al. (2009) reported an increase in the

total nematode abundance and in the number of colonizer bacterivores in soils treated with manure, compared to soils fertilized with mineral N only, whereas the number of persister bacterivores and fungivores were higher in the unfertilized controls. Thus, a shift in the trophic groups of soil nematodes after sludge and manure application might be due to the added organic matter only and might not constitute a clear signal of pollution.

Frequent exposure to antimicrobial agents and antibiotic-resistant bacteria, potentially present in manure and sludge, could also develop antibiotic resistance in the community of soil microorganisms (Riber et al. 2014). In their study, Riber et al. (2014) investigated the CRUCIAL plots treated with manure, sludge and compost, and reported general seasonal changes in the bacteria richness. The prevalence of antibiotic-resistant pseudomonads following amendment was observed to be transient and quickly declining towards basic levels. Thus, 9 weeks after fertilizer application, there was no difference between the treatments and the unfertilized control plot.

Overall, in the light of previous studies, our results suggest that the positive effects of organic matter enrichment on the soil structure, water holding capacity, and nutrient stocks outweigh the potential adverse effects from the occurrence of PPCPs. Such positive effects are of agronomic interest as crops may benefit from a higher soil available water content and porosity, although the magnitude of the effect on crop yield remains unclear (Singh and Agrawal 2008; López-Rayó et al. 2016).

Higher organic carbon contents in soil also promote aggregate stability, which may reduce soil erosion and improve water infiltration. However, sewage sludge cannot be applied at a rate as high as 100 kg N/ha without breaching the maximum legal P application rate of 30 kg/ha in Denmark due to an N-to-P ratio of about 1.5. In order to follow good agricultural practice mineral fertilizers would therefore have to be supplied according to the crop needs in N.

Due to the high diversity of PPCPs and their presence as a cocktail in the soil, more ecotoxicological tests are needed to elucidate their toxicity towards soil organisms fully. Our study focused on nematodes only, but extending tests to other groups of the soil fauna, such as collembolans and enchytraeids, would be necessary to consider different trophic levels and provide a more comprehensive assessment. Using effect-directed analysis could also further highlight harmful products by screening soils or organic wastes that present a toxicity.

4 Conclusion

Our chemical wide-scope suspect screening analysis allowed the detection of multiple PPCP suspects without predefinition of targets. In total, 12 PPCPs were identified and found

significantly up-concentrated in one or several of the treated soils compared to the control (mineral fertilizers). Most PPCPs were present in the sludge-treated soil, even after the termination of sludge application. However, the sludge treatment along with manure showed the highest indigenous nematode abundance and did not induce any chronic toxicity towards the culture *C. elegans*. Field-based research on the toxicity of PPCPs towards soil organisms is yet scarce and appears necessary to fully investigate the risks for the soil ecosystem. Furthermore, potential uptake of PPCPs or their metabolites by crops cultivated with urban waste amendments needs to be assessed, to avoid the transfer of potentially bioactive compounds to food products. Nevertheless, in our study combining for the first time a wide-scope suspect screening analysis and a study of soil nematode faunal response, no toxic effects of sludge, manure and urine application could be demonstrated, albeit a continuous application for 14 years and at extreme rates for the sludge and manure. In the light of previous studies conducted on the CRUCIAL plots, the treated soils appear strongly resilient. In addition, using sludge and manure as organic fertilizers enhanced the soil quality through a better structure, water holding capacity, and nutrient enrichment, while providing a valuable purpose for urban and organic wastes.

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Availability of data and material The datasets generated and analyzed during the current study are available in the CRUCIAL-NEMATODES-2020 repository at <https://doi.org/10.17894/ucph.cda2671e-34a9-4461-9b63-9710556919b1>.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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