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# Long-term application of Swedish sewage sludge on farmland does not cause clear changes in the soil bacterial resistome

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ABSTRACT

The widespread practice of applying sewage sludge to arable land makes use of nutrients indispensable for crops and reduces the need for inorganic fertilizer, however this application also provides a potential route for human exposure to chemical contaminants and microbial pathogens in the sludge. A recent concern is that such practice could promote environmental selection and dissemination of antibiotic resistant bacteria or resistance genes. Understanding the risks of sludge amendment in relation to antibiotic resistance development is important for sustainable agriculture, waste treatment and infectious disease management. To assess such risks, we took advantage of an agricultural field trial in southern Sweden, where land used for growing different crops has been amended with sludge every four years since 1981. We sampled raw, semi-digested and digested and stored sludge together with soils from the experimental plots before and two weeks after the most recent amendment in 2017. Levels of selected antimicrobials and bioavailable metals were determined and microbial effects were evaluated using both culture-independent metagenome sequencing and conventional culturing. Antimicrobials or bioavailable metals (Cu and Zn) did not accumulate to levels of concern for environmental selection of antibiotic resistance, and no coherent signs, neither on short or long time scales, of enrichment of antibiotic-resistant bacteria or resistance genes were found in soils amended with digested and stored sewage sludge in doses up to 12 metric tons per hectare. Likewise, only very few and slight differences in microbial community composition were observed after sludge amendment. Taken together, the current study does not indicate risks of sludge amendment related to antibiotic resistance development under the given conditions. Extrapolations should however be done with care as sludge quality and application practices vary between regions. Hence, the antibiotic concentrations and resistance load of the sludge are likely to be higher in regions with larger antibiotic consumption and resistance burden than Sweden.

## 1. Introduction

Sewage sludge contains substantial amounts of nutrients, providing a rationale for its use as fertilizer for arable land in accordance with circular economy principles. A reduced need for import of conventional mineral fertilizer has economic value for individual farmers and promotes sustainable usage of finite resources such as phosphorus. There is also an ecological dimension as efficient plant uptake of nutrients alleviates negative effects coupled to eutrophication in recipients caused by e.g. runoffs or landfill leakage. Around 200,000 tons (dry weight) of sludge are produced annually in Swedish municipal sewage treatment plants (STPs) but only approximately a third is used for agrarian purposes (SCB, 2018). Historically, the top argument against the application of sludge to agricultural fields has been the risk for accumulation of heavy metals in sludge-amended soil or crops (McBride, 2003). This concern has largely been solved in Scandinavian

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countries thanks to an improved handling and recycling of metal wastes in society as a whole, causing lower metal loadings of municipal waste water (Börjesson et al., 2014, Lekfeldt et al., 2014). More recently concerns have also been raised for antibiotics and resistant bacteria (Bondarczuk et al., 2016). As common STPs are not designed to degrade pharmaceuticals, sewage contains a mixture of residual drugs in addition to a heavy load of bacteria of various origins, including human gut microbiota known to carry a plethora of resistance determinants (van Schaik, 2015). STPs are hence considered key discharge points for antibiotics and other potentially co-selective agents as well as for antibiotic resistant bacteria (ARBs) and the causative antibiotic resistance genes (ARGs) (Rizzo et al., 2013) into the environment. To what degree selection occurs in STP recipients (waterways or fields receiving treated effluents or digested sludge) is yet debatable and it has been proposed that elevated levels of resistance identified in those milieus could be due mainly to fecal contamination from the continuous STP discharges (Karkman et al., 2019). In addition, bacterial composition can change in the absence of an antibiotic selection pressure, e.g. due to changes in temperature or oxygen access. Thereby, some bacterial taxa carrying resistance genes can be favored or disfavored coincidentally. Changes in ARG abundances should therefore be combined with data on shifts in bacterial composition (Bengtsson-Palme et al., 2016). Ideally, each ARG should be linked to its host to enable detection of within-species selection. Although emulsion, paired isolation and concatenation PCR (epicPCR) is a recent technology that to some extent allow such linkages, it is not yet quantitative or provide sufficient taxonomic resolution (Hultman et al., 2018).

Understanding if and how the environment contributes to the development of resistance is essential since it can direct measures ultimately aiming to diminish risks. The debate of the value and risks of using sludge from sewage treatment plants as soil enrichment and fertilizer on arable land has been going on for decades in Scandinavia, and initiatives to provide knowledge and risk analyses are equally long-standing. For example, in a large-scale field study in Skåne, Sweden, digested sludge from a municipal STP has been spread at crop fields of a local farm every fourth year since 1981. Concentrations of macronutrients in sludge and soil as well as the effects of long-term sludge application on soil characteristics and crop yields have been published previously (Andersson, 2015). The present study took advantage of this long-term field trial and aimed to add knowledge on concentrations of selected antimicrobials and bioavailable metals in soil after sewage sludge amendment and effects on the bacterial resistome and community composition. Total metal concentrations have been continuously monitored in both sludge and soil (Andersson, 2015), but are often poor predictors of metal toxicity in soils (Smolders et al., 2009). Hence the bioavailable fraction exerting the selective pressure was specifically measured for Cu and Zn, two of the most significant metals for co-selection of antibiotic resistance (Seiler and Berendonk, 2012, Song et al., 2017). Metagenome sequencing enabled a broad approach to study effects on resistance genes, both for antibiotics and, with regard to co-selection and cross-resistance, metals and biocides (MRGs and BRGs). Additionally, effects on the abundance of mobile and mobilizing genetic elements (MGEs) (Lupo et al., 2012) influencing the risk for dissemination of resistance genes across strains and species, as well as shifts in bacterial communities in response to the various treatments were estimated based on metagenomic sequencing data. Metagenome reads matching crAssphage (Dutilh et al., 2014), a bacteriophage highly abundant in human feces, were analyzed in all samples to facilitate discrimination between on-site selection and ARB/ARG build-up in amended soils caused by remnants of human fecal matter in applied sludge. Differences in bacterial resistance levels to ampicillin, tetracycline, and ciprofloxacin, representing three major classes of antibiotics, were investigated using conventional culturing.

## 2. Materials and methods

### 2.1. Soil and sludge sampling

Soil was sampled from an agricultural field trial designed to investigate long-term effects of sewage sludge application on soil quality and crop yields (Andersson, 2015). The soil was a sandy loam, typical for the area, with approximately 2% organic matter and 14% clay content (Börjesson et al., 2014). The field is divided into 36 plots ($6 \times 20$ $m^2$ each), each applied with either 0, 4, or 12 metric tons (dry weight) of sludge per hectare every four years (Table 1) (Andersson, 2015). Cultured crops, winter wheat, sugar beets, spring barley and oilseed rape, have been rotated in a four year cycle since the initiation of the experiment in 1981. Yearly, soil plots have been supplied with 0, 50 or 100% of the amount of nitrogen fertilizer required by each crop, in the last four year cycle corresponding to 140 kg/ha for wheat, 120 kg/ha for beets and barley and 170 kg/ha for rape. The plots given nitrogen have also been fertilized with phosphorus and potassium in individual doses depending on crop, 18 and 63 kg/ha for wheat, 42 and 150 kg/ha for beets and 33 and 63 kg/ha for rape, respectively. No additional PK fertilizer was needed for barley. In 1998, the whole field was limed (4 tons per hectare) due to decreasing pH levels and in 2017, the pH ranged from 6.7 to 7.1. Supplementary File 1, Table 1 lists herbicides and insecticides used for the agricultural field trial during the latest completed crop rotation cycle 2014–2017.

In total the soil plot sample matrix contains nine different combinations of sludge amendment and nitrogen fertilization, each with four replicates. Due to practical and economic limitations, we decided to exclude the plots given ½ the normal amount of nitrogen leading to 24 of the 36 plots being sampled (plots with bold text in Table 1). In 2017, two rounds of soil sampling were performed, on August 22 and September 26, i.e. almost four years after the sludge application in 2013 and 15 days after the most recent amendment in 2017, respectively (referred to as sampling time-point s1 and s2 below). Twelve soil cores were taken on the diagonal of each plot using a sampler reaching to an approximate depth of 20 cm, mixed thoroughly in a bucket and about 35 ml of the mixture was transferred into sterile 50 ml tubes. The

**Table 1**
Schematic view of the long-term field trial outside Malmö, Sweden. Every 4 years, either 0, 4 or 12 metric tons of sludge per hectare (dry weight) have been spread on each plot in combination with 0, 50 or 100% of the normal amount of nitrogen fertilizer (N) for the particular crop to be grown (rotated in a 4 year cycle). Each combination is present in four replicates. In the present study, soil from 24 of the 36 plots, (indicated in bold in table), were sampled twice in 2017. The first sampling took place almost four years after the most recent application of digested and stored sludge and the second sampling took place a month later, 15 days after the 2017 amendment.

| | | | | | |
|---|---|---|---|---|---|
| **0t/ha, no N** | **4t/ha, no N** | **12t/ha, no N** | **4t/ha, no N** | **12t/ha, no N** | **0t/ha, no N** |
| 0t/ha, half N | 4t/ha, half N | 12t/ha, half N | 4t/ha, half N | 12t/ha, half N | 0t/ha, half N |
| **0t/ha, full N** | **4t/ha, full N** | **12t/ha, full N** | **4t/ha, full N** | **12t/ha, full N** | **0t/ha, full N** |
| 0t/ha, half N | 4t/ha, half N | 12t/ha, half N | 4t/ha, half N | 12t/ha, half N | 0t/ha, half N |
| **0t/ha, no N** | **4t/ha, no N** | **12t/ha, no N** | **4t/ha, no N** | **12t/ha, no N** | **0t/ha, no N** |
| **0t/ha, full N** | **4t/ha, full N** | **12t/ha, full N** | **4t/ha, full N** | **12t/ha, full N** | **0t/ha, full N** |

sampler and bucket were rinsed with 70% ethanol between soil plots. Anaerobically digested (21 days at 35 °C) and centrifuged sludge stored in open air for eleven months was applied to the experimental field plots on September 11, 2017. A composite sample of the sludge (referred to as digested and stored) was transported to our laboratory at the University of Gothenburg. After the amendment, all field plots, including plots that did not receive any sludge, were plowed to a depth of approximately 25 cm. Between sludge amendment and the second round of soil sampling, the total precipitation was 40 mm and the average temperature was 13.3 °C (data from the Swedish meteorological and hydrological institute's closest observational point, located approximately 8 km north east from the study site). The same database provided average annual meta-data for total precipitation (696 mm) and temperature (9.4 °C) for the five previous years (2012–2016). Basic properties of the digested and stored sludge were as follows: 30% dry mass, pH 8.5, Tot-N 3% of dry mass, Tot-P 2.8% of dry mass. On two occasions, grab samples were also taken from undigested sludge (referred to as raw) and sludge directly from the chamber during the digestion process (referred to as semi-digested). The STP where sampling of sludge took place is located approximately 12 km northwest of the agricultural field trial and is one of Sweden's largest STPs with approximately 300,000 connected residents and an average flow of 1,350 l/s (VASYD, 2018). All samples were kept cool during transport. At the laboratory, sludge and soil samples to be used for chemical analyses and DNA extraction and subsequent sequencing were frozen at -20 °C and samples used for culturing were kept at 4 °C and spread on agar plates within 24 h. In total, 24 + 24 soil samples and 1 + 2 + 2 sludge samples were analyzed (n = 53).

### 2.2. Chemical analysis

The concentrations of 15 antibiotics (amoxicillin, azithromycin, cefadroxil, ciprofloxacin, clarithromycin, clindamycin, doxycycline, erythromycin, metronidazole, nitrofurantoin, norfloxacin, ofloxacin, oxytetracycline, sulfamethoxazole, tetracycline) and one antibacterial biocide (cetyltrimethylammonium chloride, CTAC) were determined in soil and sludge samples using liquid chromatography-tandem mass spectrometry. Soil and sludge samples (0.1 g) were extracted sequentially after addition of 50 ng of each internal and surrogate standard. Three sequential extractions were done: 1.5 ml methanol and water (7:3) with 0.1% formic acid; 1.5 ml acetonitrile and 1.5 ml 5% triethylamine in methanol/water (1/3). Samples were homogenized for four minutes at 42,000 oscillations per minute, using a Mini Beadbeater (Biospec. Bartlesville, USA) with zirconium beads and then centrifuged at 14,000 revolutions per minute for 10 min. This protocol was followed for all three eluent mixtures individually and the supernatants were combined, evaporated to 20 µl and reconstituted in 20 µl water and methanol (1:1 mixture) with 0.1% formic acid.

Samples were analyzed using a system with a triple-stage quadrupole mass spectrometer (Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, USA) coupled with a liquid chromatographic pump (Accela, Thermo Fisher Scientific) and an autosampler (PAL HTC, CTC Analytics AG, Zwingen, Switzerland). Heated electrospray ionization (HESI), krypton 10.6 eV, in positive ion mode were used for ionization of the analytes. This method is based on a previous method and all specific details related to the chromatography and general settings of the mass spectrometer are identical and described elsewhere (Grabic et al., 2012). HESI ionizations, precursor/product ions, collision energies, tube lens values, are given in Supplementary File 1, Table 2. To ensure proper quality assurance and quality control, two MS/MS transitions were used for positive identifications of analytes with the criterion that the ratio between the transitions was not allowed to deviate more than ± 30% from the ratio in the corresponding calibration standard. Retention times for all analytes also had to be within ± 2.5% of the retention time in the corresponding calibration standard. Limit of quantification (LOQ) was determined from standard curves based on repeated measurements of low level spiked soil samples, and the lowest point in the standard curve that had a signal/noise ratio of 10 was considered to be equal to the LOQ. A seven-point matrix adjusted calibration curve over the range of 0.05–100 ng/l was used for linearity evaluation and quantification. Carry-over effects were evaluated by injecting standards at 100 ng/l followed by two mobile phase blanks. Several instrumental and field blanks were included in the analytical runs.

### 2.3. Bioreporter analyses of Cu and Zn bioavailability

Bioavailable copper (Cu) and zinc (Zn) were determined in soil extracts using bioluminescent whole-cell bacterial bioreporters essentially as described previously (Brandt et al., 2008, Song et al., 2017). Hence, bioavailable Cu and Zn were operationally defined as dissolved Cu or Zn species that were able to induce expression of metal-regulated *luxAB* genes in Cu- or Zn-specific bioreporter strains. Soil extracts were prepared by mixing 5 g of soil with 25 ml of Milli-Q water. Soil-water slurries were shaken at 250 rpm for 2 h at room temperature and subjected to centrifugation (10,000*g*, 10 min, 22 °C). Soil extracts (i.e. supernatants) were transferred to sterile polystyrene tubes and stored at −18 °C until bioreporter analyses. Bioavailable Cu was determined by

**Table 2**
Bioavailable Cu determined with dual strain *Pseudomonas fluorescens* bioreporter assay in soils sampled almost four years after the latest application of digested and stored sewage sludge (0, 4 or 12 metric tons of sludge per hectare (dry weight)) and with or without nitrogen fertilizer (full N/no N).

| Treatment | IF | $[Cu]_{bio}$ | $[Cu]_{total}$[a] | $[Cu]_{bio}/[Cu]_{total}$ | pH[a] |
|---|---|---|---|---|---|
| | | (µg/g) | (µg/g) | (%) | |
| **0t/ha, no N** | 1.6±0.07 | 0.003±0.000 | 10 | 0.03±0.001 | 7.1 |
| **0t/ha, full N** | 1.5±0.07 | 0.002±0.000 | 9 | 0.03±0.001 | 6.9 |
| **4t/ha, no N** | 3.1±0.32 | 0.005±0.000 | 14 | 0.03±0.003 | 6.9 |
| **4t/ha, full N** | 2.6±0.26 | 0.004±0.000 | 14 | 0.03±0.003 | 6.9 |
| **12t/ha, no N** | 7.5±0.8 | 0.008±0.000 | 19 | 0.04 ±0.001 | 6.8 |
| **12t/ha, full N** | 5.9±1.1 | 0.007±0.000 | 20 | 0.03 ±0.002 | 6.8 |

IF-bioreporter induction factor (bioluminescence), $[Cu]_{bio}$- bioavailable soil Cu, $[Cu]_{total}$-total soil Cu concentrations. Means ± standard deviations ($n$ = 4) are shown.
[a] Data from Andersson (2015).

bioreporter analysis of soil extracts with *Pseudomonas fluorescence* strain DF57-Cu15 (Tom-Petersen et al., 2001) as described previously (Brandt et al., 2008) except that a MOPS buffered medium was used as recently recommended by Hansen and co-workers (Hansen et al., 2019). Bioavailable Zn was determined using *Pseudomonas putida* strain KT2440.2431 (Hynninen et al., 2010) using a bioreporter protocol described previously for another closely related Zn bioreporter strain (Song et al., 2017). Standards used for bioreporter calibration were as follows: 0, 5, 10, 13, 20, 26, 39, 78, 156, 313, 625, 1250 nM for $CuSO_4$ and 0, 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 32 μM for $ZnSO_4$, respectively. For each sampling time (i.e. before or after sludge amendment), all samples and standards were analyzed ($n = 2$) in the same microtiter plate for each bioreporter strain to eliminate risks of bioreporter cell batch-to-batch variability (Hansen et al., 2019). Bioluminescence was quantified in a BMG FluoStar Optima plate reader (BMG Labtech, Offenburg, Germany). Bioavailable Cu ($[Cu]_{bio}$) and Zn ($[Zn]_{bio}$) were quantified with reference to the freely dissolved $Cu^{2+}$ or $Zn^{2+}$ concentration in external metal sulfate standard solutions assuming zero bioavailability of particle-associated Cu and Zn (Brandt et al., 2006). The bioreporter strain *P. fluorescens* DF57-40E7 constitutively expressing the *luxAB* genes (Tom-Petersen et al., 2001) was used in parallel to metal specific bioreporters to correct for sample matrix interference (i.e. masking of light) as described in detail previously (Brandt et al., 2008).

### 2.4. Shotgun metagenome sequencing

DNA was isolated from soil and sludge samples (48 + 5 samples) in a randomized order using E.Z.N.A. soil DNA kit (Omega bio-tek, Norcross, GA, USA), following the manufacturer's instructions. DNA concentrations were determined with Qubit (Invitrogen) and sent to National Genomics Infrastructure, SciLife Lab in Stockholm where libraries were constructed using Illumina TruSeq PCR-free preparation kit followed by metagenomic sequencing by NovaSeq 6000 on a S2 flow cell and a paired-end read length of 150 bp. TrimGalore v0.4.4 was used to trim sequencing adapters and remove low quality bases (-q 25) from resulting reads. Using DIAMOND v.0.8.36.98 blastx with an identity threshold of 90% and a minimum open reading frame length of 20 amino acids, reads were annotated as ARGs, from sequences contained in the manually curated database ResFinder (downloaded September 2019), as BRGs or MRGs from predicted and experimentally verified sequences in Bacmet (v2) (Pal et al., 2014), as MGEs from manual extractions from Resqu (v1.1) (https://1928diagnostics.com/resdb/). As many genes have multiple functions, some reads were represented in more than one of the four annotated categories. Gene variants were grouped together by clustering each database using a 90% sequence similarity threshold (USEARCH v8.0.1445). A read that mapped to a sequence that was part of a cluster was assigned the ID of the cluster's centroid sequence. The second read in each pair was only considered if the first read failed to reach threshold values when mapped against reference sequences. Counts of reads mapping to database entries with the same gene name were analyzed cumulatively. Only ARGs, MRGs, BRGs and MGEs reaching prevalence and abundance thresholds, corresponding to detection in at least 33% of all samples and with average counts of five per sample, were included in the subsequent statistical analyses of individual genes. 16S rRNA gene sequences were identified and classified using metaxa2 (v2.1) (Bengtsson-Palme et al., 2018). Phylogenetic analyses were performed on phylum, class, order, family and genus level.

The crAssphage genome (NC_024711.1) was downloaded from GenBank and metagenome reads were mapped against it using bowtie2 (Langmead and Salzberg, 2012). Only properly paired reads were counted. Phage abundance is shown as the average genome coverage, calculated using SAMtools (Li et al., 2009), normalized by the number of reads matching the bacterial 16S rRNA gene.

Raw reads are accessible under the NCBI Bioproject number PRJNA562849.

### 2.5. Enumeration of colony-forming units

Soil and raw sludge (0.5 g/ml), semi-digested and digested and stored sludge (0.25 g/ml) were homogenized by intense vortexing (2 min) in sterile tubes with saline (0.85%) and glass beads (4 mm ø Marienfeld, Lauda-Königshofen, Germany). Suspensions were left to settle for 5 min before 4 ml were poured to new tubes. From this, decimal serial dilutions were made and aliquots of 100 μl were spread on agar plates in triplicates. Two different types of plates were used, ECC (CHROMagar, Paris, France) for enumeration and distinguishability of presumptive *Escherichia coli* and other presumptive coliforms and R2 agar (R2A) (Oxoid, Basingstoke, UK) to determine the number of aerobic heterotrophs. Both types of agar plates were prepared both without antibiotics and with the addition of ampicillin 8 μg/ml, tetracycline 8 μg/ml, or ciprofloxacin 0.5 μg/ml, resulting in eight types of plates in total. The antibiotic concentrations were derived from EUCAST's non-species related clinical breakpoints (http://www.eucast.org/clinical breakpoints/) or, in case of tetracycline, the ECOFF value (an upper limit of the measured wildtype MIC distribution) for *E. coli* (EUCAST, 2018). We acknowledge the difficulties in distinguishing intrinsic resistance from acquired resistance when colonies are not determined to species, as on the R2A plates. Still, we chose to complement the more selective ECC plates with R2A plates as this media covers a broad set of species, and, as an increase in intrinsically resistant bacteria may also reflect an antibiotic selection pressure, although much less conclusive. Colonies growing on plates after incubation, 18–20 h in 37 °C for ECC plates and 2 days in room temperature for R2A plates, were enumerated and results are presented as colony-forming units (CFUs) per gram dry matter (CFUs/g wet weight divided with dry-to-wet mass ratio after drying soil and sludge at 99 °C overnight). Detection limits were defined as the presence of one colony on one of the plates in the triplicate of the lowest dilution and half this value was used as a proxy for plates without any colonies found. On a single occasion, (one sample in the group receiving no sludge and full nitrogen fertilizer on ECC plates with ampicillin), plates were uncountable due to high numbers of colonies. To reduce the bias introduced if omitting the data point, instead an estimated value of 450 CFUs/plate for the highest dilution included in the analysis were used.

### 2.6. Statistical models

All statistical analyses were performed in R v3.5.1. Relative gene abundances in metagenome data were analyzed using an overdispersed generalized linear model assuming a quasi-Poisson distribution (Jonsson et al., 2016). All abundances were normalized against the sample-specific number of bacterial 16S rRNA gene sequences by using them as offset in the model. For ARGs, MRGs, BRGs and MGEs, both the cumulative read counts for each gene category and the abundance of individual genes were evaluated (Supplementary File 2). For culturing data, CFUs/g were log transformed and the relative resistance levels for ampicillin, tetracycline and ciprofloxacin were determined by normalization with the sample-specific read count on the corresponding agar plate without added antibiotics. Differential abundance of both total colony counts and relative resistance levels were analyzed with a linear model.

Soil effects of the maximum sludge dose from the most recent sludge application were first evaluated by comparing only the plots from the second round of sampling that received either 0 or 12 tons of sludge/ha but without additional nitrogen fertilization. Irrespective of which group of genes that were studied, a single soil sample in the group receiving 12 tons of sludge/ha always stood out as a clear outlier with gene counts resembling those found in sludge samples rather than soil.

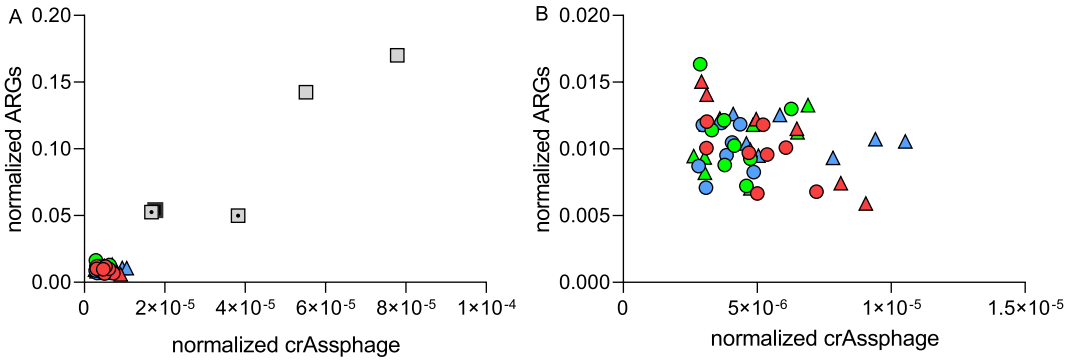


**Fig. 1.** Correlation of ARG and crAssphage abundance in soil and sludge samples (A) ($R^2$ = 0.93) and soil only (B) ($R^2$ = 0.06). Phage abundance is shown as the average genome coverage, calculated using SAMtools (Li et al., 2009) related to 16S rRNA gene abundance. ARG read counts are normalized to 16S rRNA gene counts. Soil were sampled twice, four years after (circles) and 15 days after (triangles) application of treated sewage sludge (red; no sludge, green; 4 tons/ha (dw), blue; 12 tons/ha (dw)). Grab samples of raw sludge (gray squares), semi-digested sludge (grey squares with black dot), completely digested and long-term stored sludge (black square, partially overlapped).

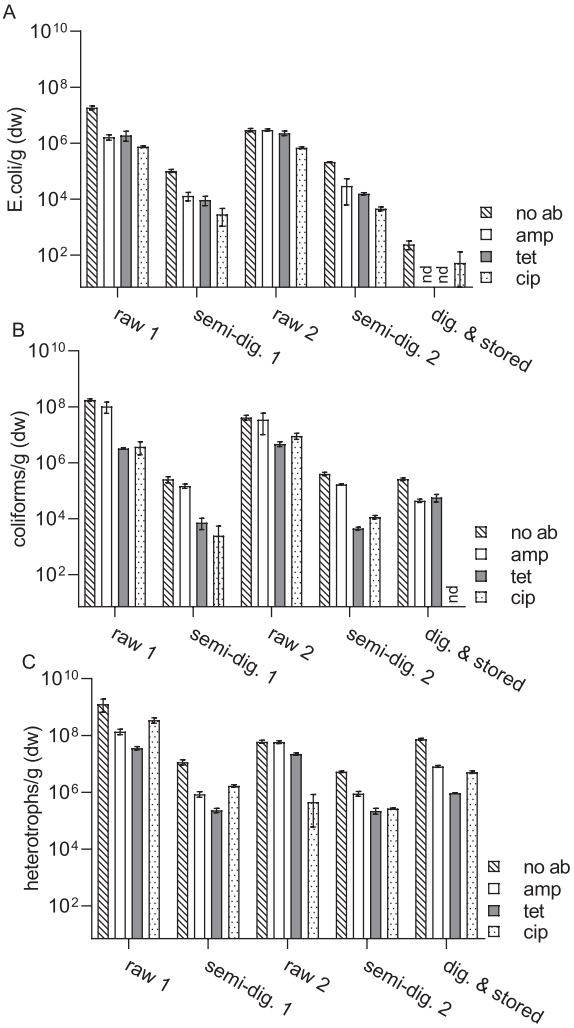


**Fig. 2.** Average CFUs/g sludge (dw) of presumptive *E. coli* (A), other coliforms (B) and heterotrophs (C) on plates without antibiotics or supplemented with ampicillin 8 μg/ml (amp), tetracycline 8 μg/ml (tet) or ciprofloxacin 0.5 μg/ml (cip). Raw and semi-digested sludge were sampled on two occasions (grab samples) and the digested and long-term stored sludge was sampled on the same day it was spread on fields by mixing several sub-samples. Error bars represent the standard deviation of three technical replicates. nd- no colonies detected.

It is possible that a higher than expected proportion of sludge to soil, e.g. due to a mistake during plowing or a contamination from a shoe sole was accidently sampled on this soil plot. The outlier was therefore excluded from all further analyses (Supplementary File 1, Figs. 1 and 2) meaning that the first analysis included 4 + 3 soil samples. Later, all data points (n = 24 + 23) were included in the analysis and effects of sludge amendment, nitrogen fertilization or sampling time-point were assessed, including interactions between variables, by determining the difference in residual sum of squares between a full model and a null model containing all the covariates of the full model except for the effect parameter. Interactions between the three independent variables were also assessed. Differences between groups were evaluated using F-tests and shown as fold changes where 1 means no change and a value above or below 1 denotes an increase or decrease, respectively. Obtained p-values were corrected for multiple testing when applicable using the Benjamini-Hochberg false discovery rate (fdr) (Benjamini and Hochberg, 1995). An fdr less than 0.05 was considered significant.

## 3. Results

### 3.1. Antimicrobial concentrations in sludge and soil

For raw and semi-digested sludge grab samples, azithromycin, ciprofloxacin, clarithromycin, clindamycin, CTAC, doxycycline, norfloxacin, ofloxacin and tetracycline were found in three of the four samples, the fourth containing all but tetracycline in quantifiable levels. The same eight compounds were also detected in the digested and stored sludge sample that was spread on the fields. The concentration range of the selected compounds in sludge spanned over four orders of magnitude, from 3 to 75,339 μg/kg (dry weight), CTAC being found in the highest concentration in all samples (Supplementary File 1, Table 3). Most compounds were found in lower concentrations even after the partial digestion except doxycycline and tetracycline which were present in higher levels in semi-digested sludge than in raw sludge at both time-points. The digested and stored sludge used for the application on field plots though had lower concentrations of all detected compounds compared to semi-digested sludge.

None of the 16 analyzed compounds could be detected in soil samples taken before the sludge application. Two weeks after sludge amendment, ciprofloxacin, clarithromycin, clindamycin, cetyltrimethylammonium chloride (CTAC) and tetracycline could be quantified in at least one soil sample but no sample had more than two detected compounds (Supplementary File 1, Table 4). No antibiotics could be detected in samples without sludge amendment but CTAC was

**Table 3**
Summary of statistically significant differences (fdr < 0.05) on the five investigated taxonomic ranks in soil samples according to the overdispersed generalized linear model with three independent variables (sludge application, sampling time-point and nitrogen fertilization). Plus and minus signs respectively represent an increase or decrease in read counts for specific taxa upon application of sludge, at the second round of sampling and with the addition of nitrogen fertilizer. For details on the variation of specific taxa, please see Supplementary File 1, Table 6. Taxa are given in alphabetical order for each variable. For less decipherable taxon names, additional information on taxonomical rankings up to phylum level is given in parentheses.

| | | | |
|---|---|---|---|
| **phylum** | sludge | + | Elusimicrobia |
| | | - | |
| | time-point | + | Nitrospirae |
| | | - | |
| | nitrogen fert. | + | |
| | | - | Cyanobacteria |
| **class** | sludge | + | TK10 (Chloroflexi) |
| | | - | |
| | time-point | + | Anaerolineae, Clostridia, Deltaproteobacteria, Nitrospira, OPB35-soil group (Verrucomicrobia) |
| | | - | Alphaproteobacteria |
| | nitrogen fert. | + | Acidimicrobiia |
| | | - | Cyanobacteria, Deltaproteobacteria, Nitrospira, KD4-96 (Chloroflexi), Ktedonobacteria |
| **order** | sludge | + | |
| | | - | |
| | time-point | + | Chromatiales, Clostridiales, Desulfuromonadales, Nitrospirales |
| | | - | Sphingomonadales, SubsectionIV (Cyanobacteria, Cyanobacteria) |
| | nitrogen fert. | + | Acidimicrobiales, Micrococcales, Streptomycetales, Xanthomonadales |
| | | - | Acidithiobacillales, Acidobacteriales, Bacterium.Ellin6529 (KD4-96, Chloroflexi), Candidatus Solibacter (Acidobacteria, Acidobacteria), DA052 (Acidobacteria, Acidobacteria), Desulfobacterales, Desulfovibrionales, Desulfuromonadales, Ktedonobacterales, Sphingomonadales, SubsectionIV (Cyanobacteria, Cyanobacteria), Syntrophobacterales |
| **family** | sludge | + | |
| | | - | |
| | time-point | + | *Desulfobacteraceae, Geobacteraceae, IGE-018* (Candidatus Chloracidobacterium, Acidobacteria, Acidobacteria), *Nitrospiraceae, Rhizobiaceae, Ruminococcaceae* |
| | | - | *Erytrobacteraceae, Microbacteriaceae, Sphingomonadaceae* |
| | nitrogen fert. | + | *Bradyrhizobiaceae, Cellulomonadaceae, Erythrobacteraceae, Iamiaceae, Microbacteriaceae, Nitrosomonadaceae, Nocardiaceae, Phyllobacteriaceae, Streptomycetaceae, Xantomonadaceae* |
| | | - | *Acidithiobacillaceae, Acidobacteriaceae, Cystobacteraceae, Desulfovibrionaceae, Desulfuromonadaceae, Geobacteraceae, Rhodospirillaceae, Sphingomonadaceae, Syntrophobacteraceae, Thermomonosporaceae, Family I* (Subsection I, Cyanobacteria, Cyanobacteria), *Usitatus Ellin6076* (Candidatus Solibacter, Acidobacteria, Acidobacteria) |
| **genus** | sludge | + | |
| | | - | |
| | time-point | + | *Desulfovibrio, Geobacter, Iamia, Nitrospira* |
| | | - | *Brevundimonas, Ferruginibacter, Luteolibacter, Microbacterium, Novosphingobium, Pedobacter, Rhizobium, Sphingomonas* |
| | nitrogen fert. | + | *Agromyces, Iamia, Ilumatobacter, Lysobacter, Mezorhizobium, Microbacterium, Nitrospira, Pedobacter, Rhodococcus, Streptomyces, Xanthomonas* |
| | | - | *Acidithiobcaillus, Azospirillum, Desulfovibrio, Geobacter, Pelobacter, Roseiflexus, Sporangium* |

detected once. The concentrations of the compounds detected in soil ranged from 1 to 68 μg/kg (dry weight).

### 3.2. Cu and Zn bioavailability in soil

Bioavailable Cu ($[Cu]_{bio}$) were slightly higher in sludge-amended plots compared to controls in soil from the first round of sampling, almost four years after the latest sludge application. The relative bioavailability (i.e. $[Cu]_{bio}/[Cu]_{total}$) was 0.03–0.04% irrespective of field treatment (Table 2). Bioavailable Cu fell below the quantification limit in soil samples taken two weeks after the most recent sludge amendment (data not shown). However, this may be explained by approximately 3-fold higher $[Cu]_{bio}$ quantification limits (~0.007 μg/g) for the bioreporter cell batches used for soils sampled after sludge amendment (repeated twice) as compared to the cell batch used for samples prior to sludge amendment (Table 2). Bioavailable Zn concentrations consistently fell below the detection limit for the used Zn bioreporter for all treatments both before and after sludge application (data not shown). Collectively, our bioreporter data indicate that levels of bioavailable Cu and Zn were low across all treatments with only minor effects of sludge application rate on $[Cu]_{bio}$.

### 3.3. Metagenome sequencing

Metagenome sequencing resulted in 32–73 million reads per sample. Four genes (the ARGs *oqxB*, *aadA2* and *ant(3'')-Ia* and the MGE integron integrase *intI6*) with much higher read counts than all others were excluded from the cumulative analysis of ARGs reaching prevalence and abundance thresholds, but not from the analyses of individual genes, in order to avoid masking of abundance changes by just these genes. Comparing only the plots from the second round of sampling that received either 0 or 12 tons of sludge/ha with no additional nitrogen fertilization showed no significant differences for ARGs, MRGs, BRGs, MGEs or bacterial community composition. Therefore, reported below are only results from the analysis including all soil samples and three independent variables (sludge application, sampling time-point and nitrogen fertilization). Results from the overdispersed generalized linear model on resistance genes and bacterial community composition in metagenome data are summarized in Supplementary File 1, Tables 5 and 6.

#### 3.3.1. ARGs, MRGs, BRGs, MGEs, and crAssphage

No significant differences in cumulative ARGs were found between soil treatments. In total, 335 different ARGs were detected in soil and sludge samples. However, a majority of these genes were represented by very few or even single reads and only six genes, *oqxB, otrA, tetV, aadA2, ant3''-Ia* and another *otrA* variant (annotated as 'tet' in ResFinder) exceeded the prevalence and abundance threshold values mentioned in section 2.4 and were thus included in the analysis of individual ARGs (see Supplementary File 3 for the accession numbers for all centroids of gene clusters). The only ARG with significantly different read counts was *oqxB* which was increased in nitrogen-fertilized soils (FC = 1.15, fdr = 0.008) but decreased in the second round of sampling compared to the first (FC = 0.91, fdr = 0.004). No significant alterations of ARG levels were observed when effects of sludge application were evaluated. No significant differences were identified for the cumulative number of MRGs. When individual MRGs were studied, nitrogen fertilization increased the abundance of *ctpC* (FC = 4.25, fdr = 0.013) and *nikR* (FC = 2.00, fdr = 0.022) while the level of *ziaA* decreased (FC = 0.40, fdr = 0.013). Neither sludge amendment nor sampling time point affected the levels of individual MRGs. The total number of BRGs did not differ significantly between treatments. However, on individual gene level, plots from the second round of sampling showed a decreased abundance of *actA* (FC = 0.65, fdr = 0.040) while

the same gene was increased in nitrogen fertilized soil plots (FC = 1.64, fdr = 0.020) together with *bepC* (FC = 1.91, fdr = 0.020). The total MGE abundance was significantly higher in soils from the second round of sampling (FC = 1.26, p = 0.030). Neither sludge application nor nitrogen fertilization had any significant effects on the total MGE read count but when the analysis was performed on individual genes, IS*CR2* was significantly more abundant in sludge-amended soils (fdr = 0.042, FC = 1.73 and FC = 1.24 for the group receiving 4 and 12 tons of sludge per hectare respectively).

The relative abundance of both ARGs and crAssphage decreased during sludge treatment and were positively correlated (Fig. 1A). However, if only soil samples were included, no correlation between these two could be found (Fig. 1B).

### 3.3.2. Microbial community composition

Only few and minor effects of long-term sludge application could be observed on soil bacterial community composition. The read counts for one phylum, *Elusimicrobia,* and one class (lineage TK10 from phylum *Chloroflexi*) were significantly enriched in sludge amended soils. Of the 47 soil samples included in the analysis, there were 31 samples where no reads mapped to the *Elusimicrobia* phylum. Thus, with such high proportion of samples where counts were below the detection limit, a reliable fold change of this phylum between the different conditions could not be calculated. TK10 was enriched 2.10 and 2.77 times for the soils receiving 4 tons or 12 tons of sludge per hectare respectively. The limited effects of sludge application on bacterial community composition stand in contrast to sampling time-point or nitrogen fertilization which affected 34 and 63 taxa respectively (Table 3). However, the effect sizes were rather modest and ranged from FC = 0.05–2.87 for the two covariates (Supplementary File 1, Table 6).

The resolution of the phylogenetic analyses used in this study does in general not allow determination of specific species with certainty, but eleven genera encompassing Gram negative pathogens (strict or opportunistic) that were found in raw sludge were selected and their abundances compared between samples. Digestion showed clear effects as no reads for seven of the genera could be detected in semi-digested sludge. The total relative read count from the remaining genera was on average seven-fold lower compared to raw sludge. The digested and stored sludge sample was similar to the semi-digested samples in that no reads were detected for the genera *Aeromonas, Citrobacter, Escherichia, Enterobacter, Klebsiella, Vibrio* or *Yersinia*, but the relative abundance of *Pseudomonas* was 100-fold higher, exceeding the levels found in raw sludge. A similar pattern was observed in soil samples where reads matching *Pseudomonas* were found in all samples in addition to scattered detections of *Bacteroides*, *Acinetobacter*, *Aeromonas* and *Campylobacter*. The total relative read count of these eleven genera in soil samples were about one order of magnitude lower than in applied sludge and were not affected by sludge amendment, sampling time point or nitrogen fertilization.

As the abundance of spore-forming Gram-positive clostridia have been reported to increase in sludge-amended soils in Canada (Scott et al., 2018), we investigated their presence here as well. A higher relative abundance of reads matching the genus *Clostridium* were found in digested and stored sludge compared to raw sludge, however no significant differences were seen among soil samples, irrespective of sludge application (fdr = 0.90), nitrogen fertilization (fdr = 0.94) nor sampling time point (fdr = 0.69, Supplementary File 1, Fig. 3).

## 3.4. Culturing data

### 3.4.1. Culturable bacteria in sludge

Semi-digested sludge gave lower CFUs/g (dry weight) than raw sludge on all types of plates used for culture analyses. The decrease was 10 to 250-fold for *E. coli* (Fig. 2A), 100 to 1,500-fold for other coliforms (Fig. 2B) and 2 to 200-fold for heterotrophic bacteria (Fig. 2C). The number of *E. coli* in digested and stored sludge was reduced additionally

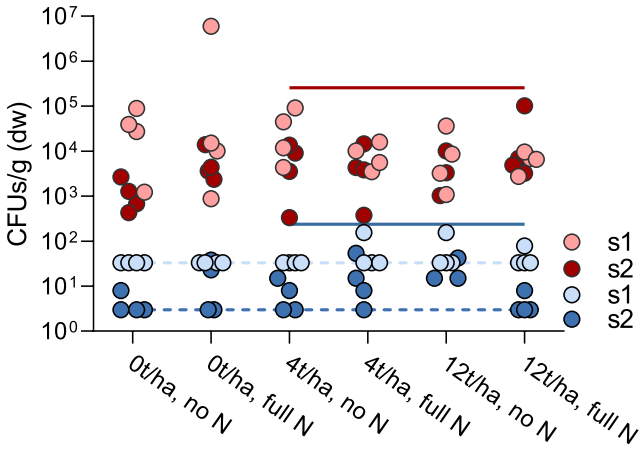


**Fig. 3.** Colony counts of *E. coli* (blue) and other coliforms (red) on ECC plates from soil sampled at two different time-points (s1 and s2), four years and 15 days after sewage sludge application (0, 4 or 12 metric tons per hectare (dw)) and with or without nitrogen fertilizer (full N/no N) added. Colony counts from the applied sludge are given as solid lines. Detection limits for *E. coli* at the two time-points are represented by dashed lines in the corresponding colors. No statistically significant differences in the abundance for presumptive *E. coli* were observed for any of the treatment conditions. The number of coliforms were significantly lower in the second round of sampling (FC = 0.29, fdr = 0.018) while the application of sludge or nitrogen fertilizer did not cause significantly different results.

430 to 880-fold on ECC without antibiotics and not detected at all on plates supplemented with ampicillin or tetracycline. Only a single *E. coli* colony was found on plates with ciprofloxacin (Fig. 2A). For coliforms, no ciprofloxacin-resistant colonies were detected in the digested and stored sludge but for the other antibiotics, the substantial reduction seen for *E. coli* was not observed, and tetracycline-resistant coliforms were even more abundant in fully treated sludge than in semi-digested sludge (Fig. 2B). A similar pattern was seen for R2A plates where the digested sludge stored for 11 months in open air had 3 to 19 times higher colony counts than semi-digested sludge (Fig. 2C). However, after normalization to the colony counts on unsupplemented plates, the relative resistance levels of the three investigated antibiotics were lower in digested and stored sludge than the average levels for the raw and semi-digested sludge grab samples on R2A plates.

### 3.4.2. Culturable bacteria in soil

Using only the subset of data from plots receiving no nitrogen fertilizer and either 0 or 12 tons of sludge/ha two weeks prior to sampling, no statistically significant differences in abundances of presumptive *E. coli*, other coliforms or heterotrophic bacteria were observed between treatment groups. Results reported below thus come from the more complex analysis including all soil samples and three independent variables (sludge application, sampling time-point and nitrogen fertilization). Statistically significant results from culturing are summarized in Supplementary File 1, Table 7.

*3.4.2.1. Enumeration of presumptive E. coli and other coliforms in soil.* Based on color, two types of colonies were enumerated on ECC plates, *E. coli* (blue) and other coliforms (red), including, but not limited to e.g. *Klebsiella, Citrobacter* and *Enterobacter*. In the first round of sampling, presumptive *E. coli* were found on ECC plates without antibiotics from three of the 24 soil samples. In the second round of sampling, 15 days after the most recent sludge application, *E. coli* could be detected in 11 of the 23 soil samples but, importantly, on this occasion ten times less diluted soil samples were spread on the ECC plates and hence the detection limit was lowered by one order of magnitude (Fig. 3). If the same detection limits were used as in the first round of sampling, *E. coli* could again be found in only three samples,

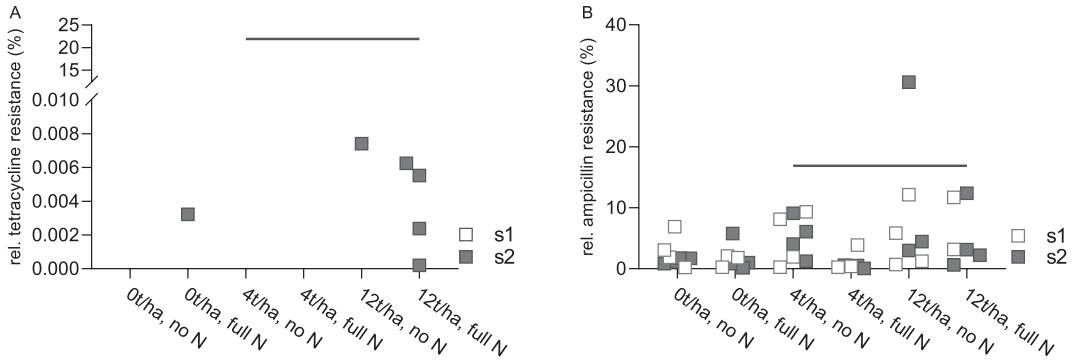


**Fig. 4.** The relative resistance levels of coliforms on ECC plates supplemented with tetracycline 8 μg/ml (A) or ampicillin 8 μg/ml (B) in soil sampled at two different time-points (s1 and s2) four years and 15 days after sewage sludge application (0, 4 or 12 tons per hectare (dw)) and with or without nitrogen fertilizer (full N/no N) added. Note that all but six samples (all from the second round of sampling) were below detection limit in (A). The solid line represents the relative resistant coliforms in the digested and stored sludge. For the relative abundance of tetracycline-resistant coliforms, the only statistically significant difference was an increase in the second round of sampling (FC = 5.16, fdr = 0.016). The relative abundance of ampicillin-resistant coliforms was not significantly different between treatments (B). No graph is shown for ciprofloxacin-resistant coliforms since all samples were below the detection limit.

one from each sludge treatment group (0, 4 or 12 tons of sludge/ha). The abundance of presumptive *E. coli* on unsupplemented ECC plates did not change significantly for any of the three variables (sludge fdr = 0.21, sampling time-point fdr = 0.63, nitrogen fertilizer fdr = 0.63). No *E. coli* resistant to ampicillin, tetracycline or ciprofloxacin was found from any of the soil samples.

The absolute abundance of red colonies, representing coliforms other than *E. coli*, growing on unsupplemented ECC plates was reduced in the second round of sampling (FC = 0.29, fdr = 0.018) (Fig. 3). The opposite were found for ECC plates with tetracycline where no coliforms were found in the first round of sampling but colonies could be detected in six samples from round 2 (FC 1.47p = 0.008). Five of these came from plots that had received the maximum sludge load leading to an fdr = 0.030 for sludge amendment (FC = 1.61 for 12 tons/ha but FC = 0.94 for 4 tons/ha). However, after normalization, the increase in relative abundance of tetracycline-resistant coliforms was significant only for sampling time-point (FC = 5.16, fdr = 0.016) (Fig. 4A). The relative abundance of ampicillin-resistant coliforms was not significantly different between treatments (Fig. 4B) and ciprofloxacin-resistant coliforms were not found in soil samples at either sampling time-point.

*3.4.2.2. Heterotrophic bacteria in soil.* The abundance of culturable heterotrophic bacteria was lower in the second round of sampling compared to the first for unsupplemented R2A plates (FC = 0.65 fdr = 0.006) (Fig. 5A). The relative resistance level for ampicillin was not significantly different between treatment groups while both tetracycline and ciprofloxacin-resistant heterotrophs were decreased in the second round of sampling compared to the first (FC = 0.38 fdr = 0.0005 and FC = 0.60 fdr = 0.0002 respectively) (Fig. 5B-D). No significant differences due to nitrogen fertilization or sludge application were found on the relative resistance levels of the three investigated antibiotics on R2A plates.

## 4. Discussion

None of the investigated antibiotics were detected in soils sampled before the most recent sludge application which indicates that 36 years of sludge amendment have not led to accumulation of these antibiotics in the soils. In the soil sampling performed 15 days after sludge application, all samples where antibiotics were detected came from amended plots though none of the compounds were consistently found in sludge-treated soils and detected concentrations were not obviously related to the amount of applied sludge. Laboratory experiments have shown that sub-inhibitory antibiotic concentrations can select for antibiotic resistance (Gullberg et al., 2011, Lundström et al., 2016, Kraupner et al., 2018). The antibiotics detected in digested and stored sludge and in soil after sludge application were all found in concentrations exceeding the predicted no effect concentrations (PNECs) for antibiotics in aquatic environment (Bengtsson-Palme and Larsson, 2016). However, these PNECs cannot easily be extrapolated to sludge or soils as many antibiotics adsorb strongly to particular matter, radically limiting their bioavailability (Subbiah et al., 2011, Song et al., 2017). It should also be noted that the metabolic state of the bacteria in sludge and soil may also influence their susceptibility to antibiotics and hence the likelihood for selection (Lin et al., 2017). We found ciprofloxacin in concentrations up to 11.6 mg/kg in raw sludge (Supplementary File 1, Table 4). This is a concentration exceeding minimal selective concentrations in culture media with two competing strains by more than 10,000-fold (Gullberg et al., 2011), more than 1,000 times higher than the lowest observed selective concentrations in complex aquatic biofilms (Kraupner et al., 2018) and it exceeds the concentration that completely inhibits growth of the great majority of investigated wild-type bacteria (EUCAST, 2018). Still, the proportion of ciprofloxacin resistant *E. coli* isolated from the raw sludge was modest (Fig. 5A). In the digested and stored sludge, the concentration of ciprofloxacin was 7–65 times lower, further reducing the probability for selective effects.

Given the current lack of defined selective antibiotic concentrations in sludge and soil (Brandt et al., 2015), risks for resistance selection is probably better assessed by studies of the resistant bacteria and resistance genes themselves. We did not find any significant changes in ARGs as a result of sludge amendment, in line with no selective effects of antibiotic residues in the amended soils. One should acknowledge that even if sludge amendment had led to increased levels of ARGs and ARBs, this would not necessarily infer on-site selection, as it could simply be a result of the input of bacteria from the sludge carrying more ARGs. For example, amendment of soil with manure can lead to increases in ARGs despite lack of detectable antibiotic residues (Kyselkova et al., 2015). As shown by Karkman et al (2019), increased ARG levels can often be explained by the presence of fecal residues. Indeed, the levels of crAssphage as an indicator of human feces and ARGs were well correlated in sludge samples as both decreased in a similar manner during the treatment process (Fig. 1A). Hence, despite higher levels of antibiotics in sludge than in soils, we found no support for resistance selection in the sludge. The lack of correlation between ARGs and crAssphage in soil samples (Fig. 1B) is expected as their background levels (i.e. read counts detected in non-amended control soil) vastly exceeded the estimated addition from the applied sludge.

The proportions of bacteria resistant to ampicillin, tetracycline and ciprofloxacin were not higher in soils amendment with sludge. This is in agreement with no onsite selection by residual antibiotics. Also, the addition of already resistant bacteria from the sludge was so small that

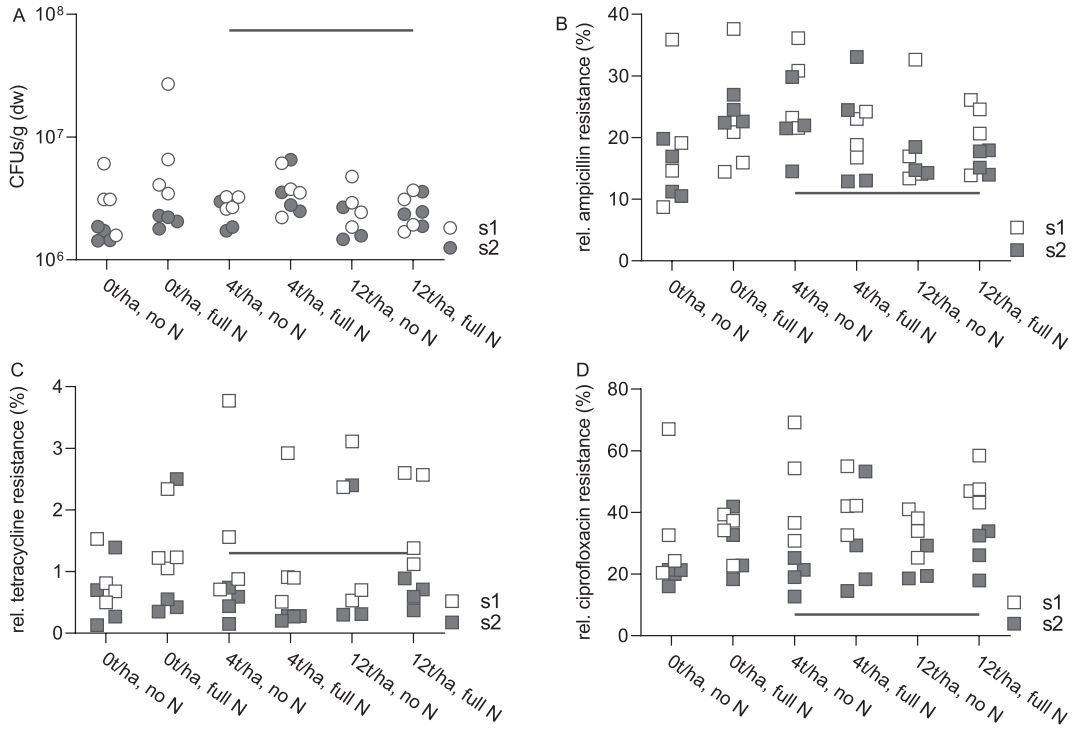


**Fig. 5.** Colony counts on R2A without antibiotics (A) and the relative resistance levels to ampicillin 8 μg/ml (B), tetracycline 8 μg/ml (C) or ciprofloxacin 0.5 μg/ml (D) in soil sampled at two different time-points (s1 and s2), four years and 15 days after sewage sludge application (0, 4 or 12 tons per hectare (dw)) and with or without nitrogen fertilizer (full N/no N) added. Relative resistance is calculated as CFUs on antibiotic-supplemented plates divided by CFUs on plates without antibiotics. Corresponding data for the digested and stored sludge are represented by solid lines. The only significant result from unsupplemented R2A plates (A) was that the number of bacteria decreased in soils from the second round of sampling compared to the first (FC = 0.65 fdr = 0.006). No significant differences were observed for the relative ampicillin resistance levels (B). Fewer tetracycline and ciprofloxacin-resistant heterotrophs were detected in the second round of sampling compared to the first (FC = 0.38 fdr = 0.0005 and FC = 0.60 fdr = 0.0002 (C and D respectively), but sludge application or nitrogen fertilization did not cause significant differences in the relative resistance levels of any of the three investigated antibiotics on R2A plates.

it would most likely pass undetected. Compared to raw sludge, the number of ARGs per 16S rRNA gene decreased from 0.16 to 0.05 in digested and stored sludge, and *E. coli* and other coliforms on unsupplemented plates decreased by approximately four and two orders of magnitude, respectively. Assuming a plowing depth of 25 cm and a soil density of 1,500 kg/m$^3$, the sludge bacteria were diluted approximately 900-fold and 300-fold in the soil for the two sludge doses, respectively. These dosages reflect the normal rates of sludge addition in Sweden reasonably well. Accordingly, *E. coli* resistant to ampicillin, tetracycline or ciprofloxacin could not be detected at all in the soils. Additionally, CFUs of (non-resistant) coliforms and heterotrophs in soil were often unchanged or even lower in the second round of sampling compared to the first, also in plots receiving no sludge. An explanation for the lower counts could be that fecal bacteria from wild animals, e.g. birds feeding on crop leftovers, present in topmost soil layers would have been less accessible for sampling after plowing that took place soon after sludge amendment. This is in line with a recent article, where remote Arctic soils showed elevated levels of ARGs, suggested to originate from fecal inputs from wildlife (McCann et al., 2019).

The most common resistance genes identified here are most likely intrinsic to different members of the soil microbiome. Different variants of *otrA* are intrinsic to the soil-dwelling oxytetracycline producer *Streptomyces* (Petkovic et al., 2006), and *tetV* is a chromosomal gene in Mycobacteria identified from agricultural soil (Kyselkova et al., 2012). These genes were detected in all soil samples, but only in two of five sludge samples and close to the detection limit. The *oqxB* gene, as found in clinical isolates, encodes part of the OqxAB efflux pump, which consists of the products of the genes *oqxA* and *oqxB*. Though the *oqxA* gene was identified in the undigested sludge, it was not detected in the digested sludge and only sporadically in a minority of field samples, close to detection limit. This indicates that the considerably more prevalent *oqxB* genes are not part of the OqxAB efflux pump present in clinical isolates. The comparably high counts of reads mapping to *oqxB* is therefore likely due to the gene encoding part of another efflux pump, with unknown substrate specificity, in soil bacteria. Reads mapping to the gene clusters represented by *aadA2* and *ant(3'')-Ia* are by far the most abundant ARGs detected in this study, in both sludge and field samples. However, the great majority of reads mapping to these genes map at exactly the same position on the reference gene. This suggests that this particular region is highly conserved and found in other proteins as well. Reads mapping to this region can thus not be reliably counted as mapping to a clinical resistance gene. This argument is supported by equally high counts in untreated and treated fields.

Cetyltrimethylammonium chloride, CTAC, was the substance detected in the highest concentration in all sludge samples. The level in the digested and stored sludge was in the lower range of what has been previously reported in digested sludge from Swedish treatment plants (Östman et al., 2017). CTAC was also the most frequently detected compound in soils, found in four samples albeit in 100–1,000 times lower concentrations than in the applied sludge. On a single occasion, CTAC was detected in soil that had not received any sludge. We speculate that this is an unsolicited carry-over from amended plots. However, effects from the potential contamination are considered limited as none of the 15 antibiotics were found neither in this particular sample nor in any of the other control soils. CTAC contains quaternary ammonium cations (QACs), a group of chemicals produced in large volumes for various purposes such as surfactants and softeners in household products such as shampoos and laundry detergents, but also as disinfectants due to their antimicrobial properties. Genes conferring resistance to QACs are associated with co-selection as they are often found on mobile genetic elements also harboring other resistance determinants (Chapman, 2003). While neither antibiotic, metal or biocide

resistance gene levels were altered when sludge application was used as the explanatory variable, a single MGE, I*SCR2* (insertion sequence common region 2), was significantly increased in sludge-amended soils compared to controls. However, the effects were modest and did not follow the logical dose-response pattern, i.e. soils that had received 4 tons of sludge per hectare showed a larger effect than plots given 12 tons/ha. Hence, we found no support for effects of residual antibacterial biocides in the soil.

Although metals such as Cu and Zn clearly have the potential to co-select for antibiotic resistance genes (Berg et al., 2010, Song et al., 2017, Zhao et al., 2019) our bioreporter data indicate that that levels of bioavailable Cu and Zn were consistently low across all treatments with only minor effects of sludge application rate on $[Cu]_{bio}$. The observed levels of $[Cu]_{bio}$ in the sludge-amended soils (Table 2) were almost certainly too low to exert a selection pressure for resistance development to Cu or antibiotics. Hence, the levels of $[Cu]_{bio}$ were far lower than the levels needed to cause selection in soil bacterial communities in previous studies (Hagerberg et al., 2011, Song et al., 2017). Furthermore, the levels of $[Cu]_{total}$ in the sludge amended soils (Table 2, Andersson, 2015) were far lower than PNECs calculated for European agricultural soils (Monteiro et al., 2010). Our results are also consistent with results from recent Danish studies simulating more than 100 years of farmland application with high-quality sewage sludge derived from modern sewage treatment plants according to current environmental regulations (Poulsen et al., 2013, Lekfeldt et al., 2014). Hence, long-term application of sewage sludge only resulted in minor changes of soil bacterial community composition and did not lead to changes in bacterial community tolerance to Cu. In line with this, our analyses of metal resistance genes did not indicate selection either.

Chemical analysis, metagenome sequencing and bacterial culturing did not reveal any signs of accumulation of antimicrobials or enrichment of resistance genes or resistant bacteria in soil amended with digested and stored sewage sludge the past decades. Most of the observed differences between plots instead linked to nitrogen fertilization, For example, two of the resistance genes found to be significantly *increased* in nitrogen-fertilized soils, *oqxB* and *bepC*, code for parts of efflux pumps responding to elevated resistance levels of toxic compounds e.g. QACs (Hansen et al., 2007, Posadas et al., 2007). However, the complementing genes of the functional pump complexes were not significantly changed, and in the second round of sampling *oqxB* levels were significantly *decreased*. The level of three additional resistance genes, *ctpC*, *actA* and *nikR*, coupled to Zn efflux (Padilla-Benavides et al., 2013), Cu sensitivity (Tiwari et al., 1996) and Ni regulation (De Pina et al., 1999) respectively, were higher in nitrogen-fertilized soils compared to controls, while *ziaA*, another gene associated with export of Zn (Thelwell et al., 1998) was instead decreased in identically treated soils. Again, in the second round of sampling one of the genes that was *higher* in amended soils (*actA*) was now significantly *lower* compared to controls. Such patterns are not consistent with a direct role of the metals, biocides or antibiotics in promoting bacteria harboring resistance genes, but rather point towards indirect changes in resistance gene abundances as a result of taxonomic changes. Inorganic fertilizer has previously been shown to have a strong effect on ARG composition in soil microbial communities, and in line with our interpretation, it was suggested to be a consequence of alterations in phylogeny rather than extensive horizontal gene transfer (Forsberg et al., 2014). Data on bacterial community composition in soil in the different treatment groups showed that read counts from more than 30 and 60 taxa respectively were significantly altered in the models using sampling time point and nitrogen fertilization as independent variables. In contrast only two taxa, *Chloroflexi* and *Elusimicrobia,* both previously found in activated and digested sludge (Björnsson et al., 2002, McIlroy et al., 2015, Lin and Li, 2018) were significantly different in abundance after sludge application compared to control plots. A similar result were recently described in a Spanish study where 24 years of annual amendment with 80 t/ha of anaerobically digested sewage sludge did not lead to any significant changes in soil prokaryotic composition (Urra et al., 2019). Competitive exclusion (Eldridge et al., 2017) of sludge derived bacteria is likely contributing to the lack of effects observed on the microbial community composition in our study and the study by (Urra et al., 2019).

Effects on ARGs after long-term sewage sludge application have been investigated previously. However, comparisons across studies are not always straightforward as the composition, sludge treatment, added volume, frequency and duration of applied sludge varies vastly. Furthermore, different studies focus on different types of effects (genes, living bacteria in various matrices such as sludge, soil, vegetables etc). Still, there are clearly situations where certain ARGs are increased in sludge-amended soils (Chen et al., 2016, Urra et al., 2019). In these two studies, the frequencies and/or amounts of applied sludge were higher than in the present study (18–80 tons per hectare, spread annually, biennially or quadrennially). In fact, when the amount of applied sludge was in the same range as in our study, no enrichment of ARGs compared to control plots was observed (Chen et al., 2016), suggesting that the dosage can be critical. The relative abundance of ARGs in the sludge could of course also be of importance. Sweden has both lower antibiotic usage and less widespread problems with antibiotic resistance than most other countries, which is also reflected in the ARG abundance in Swedish sewage (Hendriksen et al., 2019). Still, despite that both antibiotic residues and ARGs were likely higher in the Chinese study by Chen et al (2016), at least in the raw sludge, similar dosing as here did not lead to significant increase of ARGs in the Chinese soils. Many coliforms, including *E. coli* often do not survive well in soils, which can also contribute to limited effects of sludge amendment on the ARG content of soils. For example, a recent study showed that anaerobically digested sludge containing similar levels of coliforms and approximately 100-fold higher numbers of *E. coli* than in our study did not cause an enrichment of these bacteria in soil compared to control plots when analyzed 7 and 30 days after application (Murray et al., 2019). Similarly, Rahube et al. (2014) found no increase of viable coliforms in soil sampled 100 days after a single amendment neither when 10 tons digested sludge/ha (with similar levels of coliforms as our study) or 28 tons raw sludge (containing a much higher bacterial load) was applied. In additional concordance with our results they observed no ciprofloxacin resistance, a very low tetracycline resistance and a frequent ampicillin resistance with large variation between replicates among coliforms in soil without significant differences in comparison with control soils. However, there is a possibility that sludge-derived *E. coli* and coliforms may be present in the amended soil in a viable but non cultivable state (Fu et al., 2014). Taken together, while there are places and practices that are associated with increased ARGs in soils, and possibly also risks for the emergence and transmission of resistance, the practices investigated here did not indicate a significant risk.

In summary, we could not find evidence, neither on a short- nor on a long-term scale, for accumulation of antibiotics or an enrichment of ARGs or ARBs in soil amended with digested and stored sewage sludge in doses up to 12 tons per hectare every four years. No evidence of co-selection via metals or biocides measured as abundance of MRGs and BRGs could be found. Additionally, very few alterations in the microbial community structure were observed due to the sludge application. We acknowledge that while metagenome sequencing is a broad and explorative methodology, it is less sensitive than e.g. PCR why alterations in low abundance genes may remain undetected. At the same time, there are often other challenges with interpreting PCR analyses, including specificity. It should also be noted that Sweden has a favorable position compared to many other parts of the world when it comes to antibiotic consumption and clinical resistance levels. As municipal sewage reflects the resistance situation of the contributing population (Hutinel et al., 2019), it is possible that sludge from countries with a higher antibiotic use, higher resistance burden or less efficient sewage treatment than Sweden have more pronounced effects on the soil resistome if applied on to arable land.

## CRediT authorship contribution statement

**Carolin Rutgersson:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Stefan Ebmeyer:** Methodology, Software, Formal analysis, Data curation, Writing - review & editing. **Simon Bo Lassen:** Investigation, Writing - review & editing. **Antti Karkman:** Methodology, Software, Formal analysis, Writing - review & editing. **Jerker Fick:** Methodology, Investigation, Resources, Writing - review & editing. **Erik Kristiansson:** Methodology, Formal analysis, Resources, Writing - review & editing, Supervision. **Kristian K. Brandt:** Methodology, Investigation, Resources, Writing - review & editing, Supervision. **Carl-Fredrik Flach:** Conceptualization, Methodology, Writing - review & editing, Supervision. **D.G. Joakim Larsson:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The study was partially supported by the Swedish Water & Wastewater Association (Svenskt vatten utveckling), an organization with interest in the management of wastewater and sludge, where most members are municipalities (publicly owned). Also, the experimental plots are managed by “Swedish Rural Economy and Agricultural Society Malmöhus” (Hushållningssällskapet), a Swedish organization encompassing private farmers. The design of the experiment (given the available soil plots), all analyses, interpretation and writing was done without the involvement of any of the above organizations. The authors have no competing interests to declare.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105339.

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