Supplementary information

# Impact of long-term manure and sewage sludge application to soil on the incidence of pathogenic microorganisms and antibiotic resistance genes

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## MATERIALS AND METHODS

### *Preparation of samples for 16S rRNA gene standard calibration curve*

As a reference for the total abundance of 16S rRNA gene copies, the DNA from *Pseudomonas stutzeri* JM300 was used for the calibration curves. *P. stutzeri* JM300 is a gram-negative soil bacterium with 919,568 copy numbers of 16S rRNA gene per 1 ng of DNA (Ginard et al., 1997). Due to its high conservative character, it was used as a reference for 16S rRNA gene quantification. First, the bacterium was cultivated on LB medium agar plates (37 °C, 24 h) and formed colonies were subjected to DNA isolation using QIAmp® DNA Mini Kit (Qiagen, USA). The DNA concentration was determined fluorimetrically using Qubit (Thermo Fischer, USA) and based on the know number of gene copies in 1 ng of DNA, the number of 16S rRNA gene copies in the isolated sample was derived.

### *Preparation of samples for ARGs standard calibration curve*

*Generation of ARG-amplicons*

Samples for ARG standard calibration curves contained fragments of pDRIVE plasmid with inserted amplicon of target ARG. First, the amplicons of target ARG (Supl. Table 1) were generated from the metagenomic DNA of environmental samples, in which the presence of ARGs was the most expected (samples of manure and sewage sludge). The metagenomic DNA of such samples was isolated using a FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) and purified with a Genomic DNA Clean and Concentrator kit (ZYMO Research, Irvine, CA, USA) according to the manufacturer's instructions. Then, the DNA concentration was diluted to 10 ng/µl and used for the following polymerase chain reaction (PCR). The PCR was carried out with the following cycle conditions (35

cycles): 3 min at 98 °C, 10 s at 95 °C, 10 s under annealing temperature (Supl. Table 1), 15 s at 72 °C and a final extension of 5 min at 72 °C. The master mix (25 µl) for one reaction was prepared as following: 12.5 µl of KAPA HiFi HotStart ReadyMix (Kapa Biosystems, USA), 10.5 µl of water for molecular biology (Sigma-Aldrich, USA), 0.5 µl of each primer (100 mM, Sigma-Aldrich, USA), and 1 µl of DNA (10 ng/µl). The ARG-amplicons were separated using agarose gel (1.5 %) electrophoresis, and the bands of the interest were excised and purified using a Zymoclean Gel DNA Recovery Kit (ZYMO Research, Irvine, CA, USA).

*Insertion of the target ARG-amplicon into a cloning vector (TA cloning)*

Insertion of the target ARG-amplicon into a cloning vector was performed with QIAGEN PCR Cloning Kit (Qiagen, Hilden, Germany), containing pDRIVE plasmid as a bacterial vector for cloning and expression of PCR product. First, the ARG-amplicon previously isolated from electrophoretic gel was adenylated. The adenylation master mix (15 µl) contained 12.65 µl of extracted DNA, 0.3 µl of dNTP (dA, TermoFischer, Massachusetts, USA), 0.3 µl of bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO, USA), 0.25 µl of DyNAzyme II DNA polymerase 2 U/µL (TermoFischer, Massachusetts, USA), and 1.5 µl of 10X Optimized DyNAzyme Buffer (TermoFischer, Massachusetts, USA). The samples subjected for adenylation were incubated at 72 °C for 15 min. After the adenylation, the ARG-amplicons were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany).

Subsequently, the adenylated ARG-amplicon was inserted into a cloning vector via ligation process. QIAGEN PCR Cloning Kit and its pDRIVE Cloning Vector were used for ligation of ARG-amplicons into a cloning vectors (Qiagen, Hilden, Germany). Ligation reaction was performed using 5 µl of 2x Ligation Master Mix, 2 µl of distilled water, 2 µl of the adenylated ARG-amplicon, and 1 µl of pDrive Cloning Vector. Reaction mixture was incubated for 1 h at 16 °C.

*Transformation of competent cells*

The recombinant plasmid vector was subsequently transformed into the competent cells (Escherichia coli DH11S) using heat-shock transformation procedure. The heat-shock transformation was performed by adding 5 µl of the recombinant plasmid vector (containing gene of the interest) to the 100 µl of E. coli DH11S competent cells and slightly mixed. After 30 min incubation on ice, the sample was incubated for 1 min at 42 °C with following incubation for 3 min on ice. Then 500 µl of sterile SOC medium (TermoFischer, Massachusetts, USA) was added, and another incubation was perform (1 h, 37 °C, on a shaker). The culture was centrifuged at 300 × g for 3 min in a room temperature, and 500 µl of supernatant was discarded. The pellet was resuspended in 100 µl of the residual media and spread on LB-agar plates. The plates were prepared 1 day earlier by using LB medium and agarose (1.5 %). After autoclave sterilization, 150 µl of isopropyl β-d-1-thiogalactopyranoside (IPTG, TermoFischer, Massachusetts, USA), 150 µl X-gal (TermoFischer, Massachusetts, USA), and 75 µl kanamycin (TermoFischer, Massachusetts, USA) were added. The plates with culture were incubated at 37 °C for 24 h.

*Colony PCR and the final preparation of ARG-calibration curve samples*

After obtaining blue and white colonies, a half of several white colonies was resuspended in 15 µl of water for molecular biology, incubated for 15 min at 99 °C and centrifuged for 1 min at 20,000 × g in a room temperature. The supernatant was transferred into a clean microcentrifuge tube and used for further colony PCR. The colony PCR was performed to determine the presence or absence of inserted target ARG-amplicon in plasmid vector. During this reaction, M13 forward (5′-GTCGTGACTGGGAAAAC-3′) and M13 reverse (5′-AACAGCTATGACCATG-3′) primers targeting on sequences on both ends of the cloning site were used. Master mix (10 µl) for one reaction was prepared as following: 5 µl of 0.02 U/µl KAPA HiFi HotStart ReadyMix, 3.4 µl of water for molecular biology, 0.3 µl of each M13 primers (10 mM), and 1 µl of the supernatant. The colony PCR was carried out with the following cycle conditions: 98 °C for 2 min, 98 °C for 20 sec, 50 °C for 10 sec (30 cycles), 72 °C for 30 sec 72 °C for 2 min. Same procedure was performed with a few blue colonies (suspected for not being transformed) in order to obtain a negative control (pDRIVE without inserted ARG-amplicon). Presence or absence of the ARG-amplicon inserted in the vector were determined on an agarose gel based on the size of the product length (ARG-amplicon + 258 bp).

The other half of white colonies was transferred into a sterile LB medium (with kanamycin) and incubated at 37 °C overnight. Based on the result of gel electrophoresis performed after colony PCR, plasmid DNA was isolated from the grown-cultures that were suspected of being successfully transformed using QIAmp® DNA Mini Kit (Qiagen, USA). The isolated plasmid-DNA together with M13 forward and M13 reverse primers (each primer separately) were sent to European Genome and Diagnostics Centre (Germany) for Sanger sequencing. Sanger sequencing was used to verify the successful ligation of the target ARG-amplicon. The sequence of the ARG-amplicon was searched using the Basic Local Alignment Search Tool (BLAST) algorithm in the Genbank database (National Center for Biotechnology Information, NCBI).

Based on the BLAST search, the isolated plasmids with successfully inserted ARG-amplicons were again amplified in PCR reaction with M13 primers. The reaction master mix and the temperature program used were the same as for the colony PCR. Agarose gel electrophoresis was performed, and bands of correct length (ARG-amplicon + 258 bp) were excised and purified using a Zymoclean Gel DNA Recovery Kit (ZYMO Research, Irvine, CA, USA). The generated purified plasmid-fragments with inserted ARG-amplicon were subsequently used for further qPCR assays as samples for standard calibration curves. The DNA concentration in such samples was measured on Qubit (Thermo Fischer, USA) fluorimetrically. The number of the ARG copies (CN = copy number) was calculated using the following formula (Lee et al., 2006):

$$\mathrm{CN} = \frac{\text{DNA concentration} \cdot (6{,}022 \cdot 10^{23})}{(\text{length of the DNA fragment}) \cdot (660 \cdot 10^{9})} \cdot \text{Volume of the sample}$$

**Supl. Table 1:** List of primer sets (Sigma Aldrich, Sigma-Aldrich, USA) targeted to 16S rRNA and antibiotic resistance genes (ARGs) used in qPCR assays.

| Gene Target | Label | Primer 5′ to 3′sequence | Amplicon length | Annealing temperature | Reference |
|---|---|---|---|---|---|
| 16S rRNA gene | 786-F | GATTAGATACCCTGGTAG | 140 bp | 55 °C | (Baker et al., 2003; Shyu et al., 2007) |
| | 926-R | CCGTCAATTCCTTTRAGTTT | | | |
| Resistance to tetracycline | tetA - F | CAGGCAGGTGGATGAGGAA | 210 bp | 67 °C | (Zhang and Zhang, 2011) |
| | tetA - R | GGCAGGCAGAGCAAGTAGAG | | | |
| | tetW - F | GAGAGCCTGCTATATGCCAGC | 168 bp | 61 °C | (Aminov et al., 2002) |
| | tetW - R | GGGCGTATCCACAATGTTAAC | | | |
| Resistance to sulfonamide | Sul1 - F | CCGTTGGCCTTCCTGTAAAG | 60 bp | 67 °C | (Bergeron et al., 2015) |
| | Sul1 - R | TTGCCGATCGCGTGAAGT | | | |
| | Sul2 - F | TCCGGTGGAGGCCGGTATCTGG | 189 bp | 67 °C | (Xu et al., 2015) |
| | Sul2 - R | CGGGAATGCCATCTGCCTTCAG | | | |
| Resistance to erythromycin | ermB - F | TAAAGGGCATTTAACGACGAAACT | 172 bp | 61 °C | (Stedtfeld et al., 2018) |
| | ermB - R | TTTATACCTCTGTTTGTTAGGGAATTGAA | | | |
| Integrase | Intl1 - F | CGAACGAGTGGCGGAGGGTG | 312 bp | 70 °C | (Stedtfeld et al., 2018) |
| | Intl1 - R | TACCCGAGAGCTTGGCACCCA | | | |
| Resistance to vancomycin | vanA - F | AAAAGGCTCTGAAAACGCAGTTAT | 150 bp | 61 °C | (Stedtfeld et al., 2018) |
| | vanA - R | CGGCCGTTATCTTGTAAAAACAT | | | |

**Supl. Table 2:** Comparison of meteorological data at different sampling points.

| Last fertilization | Sampling | Air temperature [°C]* | Deviation from normal [°C] | Precipitation [mm]* | Deviation from normal [mm] |
|---|---|---|---|---|---|
| 0 | September 2015 | 13.7 | 0.2 | 20 | -27 |
| 0.5 year | March 2016 | 4.0 | 0.3 | 25 | -15 |

*the monthly means of air temperature and precipitations
Deviation from long-term normal 1981-2010, (https://www.chmi.cz/historicka-data/pocasi/uzemni-teploty?l=en)

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