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Inorganic and organic fertilizers impact the abundance and proportion of antibiotic resistance and integron-integrase genes in agricultural grassland soil

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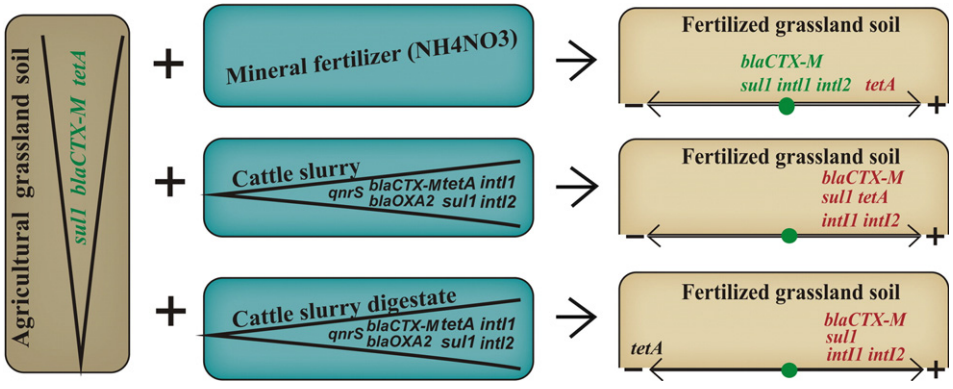
HIGHLIGHTS

- Cattle slurry and its digestate were considerable ARG sources.
- Fertilization of agricultural grassland soil significantly affected its ARGs content.
- Organic fertilizers enhanced *sul1*, *intI1* and *intI2* abundance in grassland soil.
- Cattle slurry digestate amendment significantly enhanced *blaCTX-M* level in soil.
- Mineral fertilizer usage significantly enhanced *tetA* abundance in soil.

GRAPHICAL ABSTRACT



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ABSTRACT

Soil fertilization with animal manure or its digestate may facilitate an important antibiotic resistance dissemination route from anthropogenic sources to the environment. This study examines the effect of mineral fertilizer (NH_4NO_3), cattle slurry and cattle slurry digestate amendment on the abundance and proportion dynamics of five antibiotic resistance genes (ARGs) and two classes of integron-integrase genes (*intI1* and *intI2*) in agricultural grassland soil. Fertilization was performed thrice throughout one vegetation period. The targeted ARGs (*sul1*, *tetA*, *blaCTX-M*, *blaOXA2* and *qnrS*) encode resistance to several major antibiotic classes used in veterinary medicine such as sulfonamides, tetracycline, cephalosporins, penicillin and fluoroquinolones, respectively. The non-fertilized grassland soil contained a stable background of *tetA*, *blaCTX-M* and *sul1* genes. The type of applied fertilizer significantly affected ARGs and integron-integrase genes abundances and proportions in the bacterial community ($p < 0.001$ in both cases), explaining 67.04% of the abundance and 42.95% of the proportion variations in the grassland soil. Both cattle slurry and cattle slurry digestate proved to be considerable sources of ARGs, especially *sul1*, as well as integron-integrases. *Sul1*, *intI1* and *intI2* levels in grassland soil were elevated in response to each organic fertilizer's application event, but this increase was followed by a stage of decrease, suggesting that microbes possessing these genes were predominantly entrained into soil via cattle slurry or its digestate

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application and had somewhat limited survival potential in a soil environment. However, the abundance of these three target genes did not decrease to a background level by the end of the study period. *TetA* was most abundant in mineral fertilizer treated soil and *blaCTX-M* in cattle slurry digestate amended soil. Despite significantly different abundances, the abundance dynamics of bacteria possessing these genes were similar ($p < 0.05$ in all cases) in different treatments and resembled the dynamics of the whole bacterial community abundance in each soil treatment.

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1. Introduction

Antibiotic resistance is a threat to human and animal health worldwide (Berendonk et al., 2015). Although antibiotic resistance is of natural origin, numerous anthropogenic sources contribute to the spread of resistance, including antibiotic use in clinical and agricultural settings (Allen et al., 2010). Studies have shown that the quantity of antimicrobials used in animal husbandry can be several-fold greater than for human use (Maron et al., 2013), making farms an important source of antibiotic residues and antibiotic resistant bacteria possessing antibiotic resistance genes (ARGs) (Forsberg et al., 2012; Zhu et al., 2013). The common practice of soil fertilization with animal manure facilitates the dissemination of antibiotic residues, ARGs and associated mobile genetic elements (i.e. plasmids, integrons) from the farm to the environment (Binh et al., 2008; Heuer et al., 2011). Especially, integrons (genetic elements that allow efficient capture and expression of exogenous genes) are widely regarded for their role in the dissemination of antibiotic resistance (Gillings, 2014). In manure-amended soil ARGs tend to persist (Ghosh and LaPara, 2007; Jechalke et al., 2013) and have increased transfer frequency to indigenous soil bacteria via horizontal gene transfer (Riber et al., 2014). It has been shown that manure fertilization can cause ARG blooms in soil, even if the producing animals have never been treated with antibiotics, suggesting the enrichment of soil resistome (Heuer and Smalla, 2007; Kyselková et al., 2013; Udikovic-Kolic et al., 2014). From agricultural fields, antibiotic resistant bacteria can enter the human food chain (Rahube et al., 2014) or ARGs can be transferred to clinically relevant pathogens (Riber et al., 2014).

In addition to the direct field application, animal manure is also increasingly utilised in biogas plants for energy production. The waste product of this technology is a digestate that can be used for soil fertilization, similarly to manure (Insam et al., 2015). The available data on the effect of the more commonly used mesophilic anaerobic digestion on the initial manure resistome is, thus far, inconclusive. It has been shown that mesophilic digestion of cattle manure reduced cultivable multidrug resistant bacteria and putative pathogenic bacteria by >90% (Beneragama et al., 2013, Resende et al., 2014a). Mesophilic digestion was also found to reduce several ARGs concentrations in cattle manure digestates (Resende et al., 2014b). On the other hand, mesophilic digestion could not reduce ARGs encoding for erythromycin and tetracycline resistance in swine manure (Chen et al., 2010). Moreover, digestates have been shown as a potential source of transferable broad host-range antibiotic resistance plasmids, making them, much like manure, a potential reservoir of antibiotic resistant bacteria (Wolters et al., 2015).

Both manure and digestate amendments introduce substantial amounts of nutrients into the soil, increasing its carbon and nitrogen content, as well as influencing its pH, which are likely to affect indigenous soil microbial communities, including the portion carrying antibiotic resistance determinants (Ding et al., 2014, Poulsen et al., 2013). Studies have shown that inorganic fertilizer amendments (especially mineral N) significantly affect soil bacterial community biomass (Truu et al., 2008) and also structure (Fierer et al., 2012), but the effect of this treatment on antibiotic resistome of soil indigenous bacteria is almost unknown. Only results from a study by Udikovic-Kolic et al. (2014) show no effect of inorganic fertilization on soil bacteria harbouring genes for resistance to β-lactam antibiotics. A need to connect soil resistome data with key soil properties such as its type, organic matter content, moisture, and pH has been recognized, but seldom addressed (Cytryn, 2013). Therefore, addressing these aspects is essential to understanding the contribution of agricultural practices to the environmental ARGs pool in more detail.

The aim of this study was to assess the effect of fertilization with cattle slurry, cattle slurry digestate and mineral fertilizer on a) the abundance dynamics of the bacterial community, five ARGs (*blaCTX-M*, *blaOXA2*, *tetA*, *sul1*, and *qnrS*) conferring resistance to the main antibiotic classes, and two classes of integron-integrases; b) the relationships between targeted genes (16S rRNA gene, ARGs and integron-integrases) and soil parameters in experimental plots of an agricultural grassland.

2. Materials and methods

2.1. Experimental setup and soil sampling

The experiment was conducted at the Eerika experimental station of the Estonian University of Life Sciences, Estonia (58°23′32″ N, 26°41′31″ E, elevation 60 m above sea level). The soil at the study site was categorised as *Stagnic luvisol* (World Reference Base for Soil Resources (WRB) classification) with a sandy loam soil texture (FAO, 2006). The meadow-type grassland was established on a former extensive cereal crop production field in 2008. Prior to 2008, the experimental field had received only mineral fertilizers on a few random years with application rates of 50–70 kg (N) ha^{-1} per year. No fertilizers were applied to the established grassland during the period of 2008–2011. In 2012, 12 treatment plots (8.8 m^2 each) were created and three types of fertilizers (mineral N-fertilizer (NH_4NO_3), cattle slurry, and cattle slurry digestate) were applied to three replicate plots each, situated in a randomised block system. The remaining three plots served as non-fertilized controls. The application rates of organic fertilizers were calculated based on their NH_4-N content. Fertilizers were manually surface-applied (60 kg (N) ha^{-1}) three times during the vegetation period. The application pattern and rates of fertilizers were calculated according to the yearly norm of the grassland (180 kg (N) ha^{-1}) and Estonian Water Law requirements. The start of the experiment in 2012 marked the first applications of organic fertilizers to the experimental field. The dominant plant species at the study site were *Poa pratensis* L. and *Festuca rubra* L. On non-fertilized control plots, small-leaved *Trifolium repens* L. was dominating also. During the experiment the grass was mown thrice per year; no grazing occurred. The climatic conditions of the experimental period of 2012–2013 are described in Suppl. Fig. A.1.

This study was conducted in the experimental plots during the period of April to September 2013 (second year of fertilizations). The fertilizers were applied on plots on Days 1, 47, and 96 of the experimental period. Untreated cattle slurry and cattle slurry digestate were collected, before application, from a dairy farm (Tartu County) and Oisu biogas plant (Järva County), respectively. In the biogas plant the cattle slurry was fermented together with smaller quantities of solid manure, hay and silage at mesophilic conditions (37–38 °C). An overview of antibiotic usage on the dairy farm prior to the experiment is presented in Suppl. Table A.1.

Composite bulk soil samples (200 g) were collected with a soil auger from the topsoil layer (0–10 cm) from each experimental plot (each composite soil sample constituted of 20 piercings from a specific experimental plot). Sampling was conducted throughout the vegetation period, on the day prior (Days 0, 46, and 95) as well as subsequent (Day 2,

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