Degradation of the sulfamethoxazole antibiotic in an agricultural soil

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Introduction

Livestock raising in most countries involves the use of antibiotics in veterinary practices and to increase animal productivity ("production-purpose uses") [1]. These compounds are designed to be quickly excreted from the threated body, through animal feces, either unaltered or as metabolites. The use of livestock manure or of the digestate (anaerobic digestion of the manure) as amending and fertilizer in agricultural practices, implicates the introduction of antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) in terrestrial ecosystem [2].

Due to their continual input into the environment and antimicrobial nature, antibiotics are classified as emerging pseudo-persistent organic pollutants (pseudo-POPs) [3]. Unlike the other conventional POPs, antibiotics not only can kill or inhibit natural bacterial population involved in ecosystem functioning, but also cause the appearance and rapid spread of resistant bacteria in the environment [4]. Indeed antibiotics, ARB and ARGs are recently included in the contaminant list of emerging concern [5].

The synthetic compound sulfamethoxazole (SMX) is one of the most popularly prescribed and consumed antibiotics in human and veterinary medicines leading to its presence in aquatic and terrestrial ecosystems. SMX is bacteriostatic, inhibiting the synthesis of folic acid metabolism and causing bone marrow suppression. This compound is among the top ten drugs with high priority identified in the European assessment of pharmaceuticals and personal care compounds [6].

The current knowledge about the persistence and the potential effects of SMX addition on the natural microbial community of an agricultural soil are still scarce. The aim of the present study was to evaluate the possible degradation of SMX and the response of the microbial community of a cattle manure amended and SMX-treated soil. Microcosm biodegradation experiments were performed by using microbiologically active (containing the natural microbial community) or sterilized cattle manure amended soil. The role of the natural microbial community on the degradation of the antibiotic and the effects of the latter on the microbial structure and functioning were investigated. Furthermore, a molecular marker for antibiotic resistance [7], the *intI* 1 gene was also analyzed.

Materials and methods

Soil and cattle manure sampling

Unamended soil sample was collected from a semiarid agricultural field close to Rome (Italy). Sampling was done manually from the surface layer (0–20 cm). The soil was air-dried and then passed through a 2-mm sieve to remove stones, gravel and plants.

The cattle manure was collected from an anaerobic digestion plant that produces biogas by utilizing principally as fed-in biomass a mix of cattle manure.

The soil sample was then amended with the cattle manure to achieve an organic fertilizer application level of 3% (mg soil with mg digestate, w/w).

Microcosms set-up

The experimental sets consisted of microcosms each of 1000 mL capacity filled with 600 g of cattle manure amended soil.

The experimental conditions (2 replicates for each condition) were the following:

- ✓ Cattle manure amended soils treated with SMX standard solution to reach a final concentration of 20 mg/Kg (Soil+ Cattle manure +SMX).

- ✓ Cattle manure amended soils previously sterilized and then treated with the antibiotic (Sterile soil+cattle manure+SMX).
- ✓ No-treated amended soils in presence of the natural microbial community (Soil+ Cattle manure).
- ✓ Pre-sterilized amended soils (Sterile soil+ Cattle manure).

The water content was 60% of the soil maximum water holding capacity. All microcosms were incubated in the dark under aerobic conditions and at 20±1 ◦C for 61 days.
At selected times, two sub-samples from each single microcosm were collected for chemical or microbiological analysis. All analytical results were referred to dry weight.

Analytical and microbiological methods

SMX extraction from unamended soil, cattle manure and Soil+Cattle manure was performed by using a Pressurized Liquid Extraction system (PLE), followed by an analytical determination with RP-HPLC interfaced with an UV detector and LC-MS/MS [8].
The overall effects of SMX occurrence on the microbial community were evaluated in terms of cells abundance (DAPI count), viability (LIVE/DEAD method) [9] and activity. The dehydrogenase activity was analyzed via colorimetric determination [10].
The changes in microbial community structure were investigated by analysing the ester-linked fatty acids (ELFAs). ELFAs were extracted from soil samples and then analyzed with Gas Chromatography coupled to a flame ionization detector (GC-FID)[11].
The *intI 1* gene prevalence was analysed by DNA extraction from different soil samples (unamended soil, cattle manure, Soil+ Cattle manure and Soil+cattle manure+SMX conditions) and at different sampling times (0, 7 and 61 days) and subsequent qPCR assays.

Results and discussion:

SMX content and *intI*1 gene prevalence in unamended soil, cattle manure and cattle manure amended soil are listed in Table 1. The absence of SMX and antibiotic resistance gene prevalence in unamended soil suggests that the microbial community was not adapted to its presence. Consequently, the occurrence of the *intI*1 gene in the cattle manure amended soils can be ascribable to resistant bacterial populations introduced through this agricultural practice.

Table 1 Main chemical analysis

	Unamended Soil	Cattle manure	Soil+ Cattle manure
SMX (µg/Kg)	< LOD	10	< LOD
***IntI1* gene (prevalence)**	< LOD	$9.59*10^{-02}$	$7.51*10^{-03}$

The degradation rates of SMX in amended soil (Soil+cattle manure+SMX) and in pre-sterilized soil (Sterile Soil+Cattle manure+SMX) during the experimental time (61 days) are shown in Fig. 1.

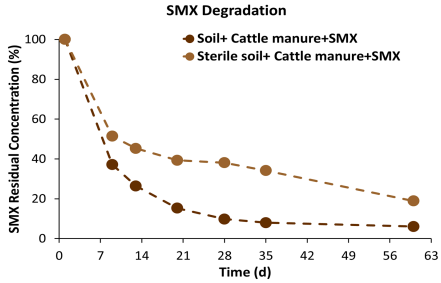

Figure 1 Concentrations (%) of sulfamethoxazole (SMX) *vs* time (days) in amended soil and pre-sterilized amended soil. The vertical bars represent the standard errors.

No lag phase was observed in both treatments and a fast SMX degradation until day 7 ($p< 0.05$) followed by a slow degradation was observed. At the end of the experiment, the SMX residual concentrations were lower than

3 mg Kg^{-1} in both treatments. However, the pre-sterilized soils showed a slower removal ratio (0.022 mg Kg^{-1} *day^{-1}) than the non- sterile conditions (0.045 mg Kg^{-1} *day^{-1}) and, consequently, a higher DT_{50} value (31 days for Sterile Soil+Cattle manure+SMX *vs* 15 days Soil+Cattle manure+SMX).

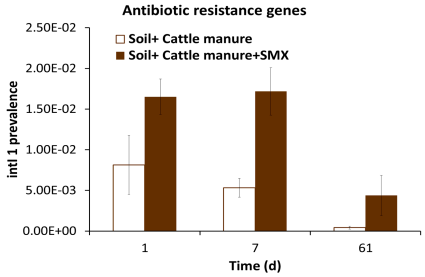

Figure 2 Variation of the *IntI*1 gene prevalence in amended soil and amended soil treated with antibiotic *vs* time (days). The vertical bars represent the standard errors.

The change in antibiotic resistance gene prevalence during the 61 days incubation period is shown in Figure 2. SMX addition promptly promoted the *intI*1 gene spread among the soil bacterial populations. At days 1 and 7, the antibiotic resistance gene prevalence was significantly (ANOVA, $p < 0.01$) higher in the antibiotic treated soils (Soil+Cattle manure + SMX) than in the untreated ones (Soil+Cattle manure). This phenomenon can occur by horizontal gene transfer; in fact, the class 1 integrons are often embedded in DNA mobile elements such as plasmids and transposons. The change in microbial abundance (N. live cells g^{-1} soil) during the experimental time is reported in Figure 3.

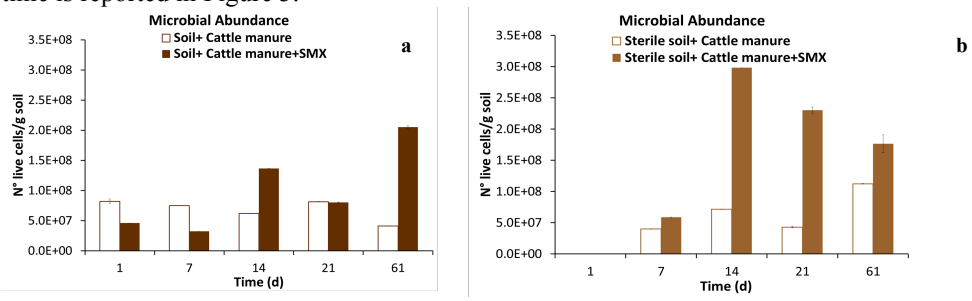

Figure 3 Microbial abundance (N. live cells g^{-1} soil) *vs* time (days) in all experimental conditions. The vertical bars represent the standard errors.

SMX addition had an initial detrimental effect on the microbial abundance (Fig. 3a). However, this toxic effect was transient and the number of live cells increased from day 14. At day 7, a colonization of bacterial cells (survived sterilization) was observed in the pre-sterilized soils (Fig. 3b) and the number of live cells increased during the experimental time and in line with SMX degradation (Fig. 1).

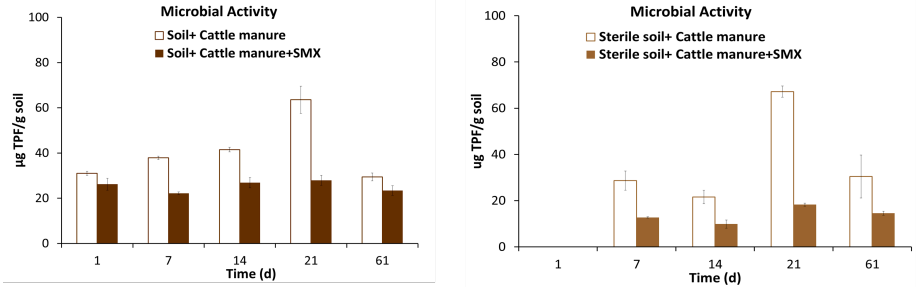

Figure 4 Microbial activity *vs* time (days) in all experimental conditions. The vertical bars represent the standard errors.

Antibiotic addition affected the microbial activity (Fig. 4). This result might be due to the negative effect on the populations susceptible to SMX. However, despite the decrease in the antibiotic concentration in soil, the dehydrogenase activity was constant over the experimental time.

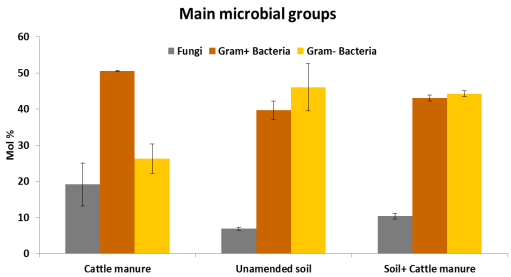

Figure 5 Soil microbial community structure evaluated by ELFA analysis. The vertical bars represent the standard errors.

The microbial group identified by ELFAs were Fungi, gram-positive and gram-negative bacteria (Fig. 5). Bacteria were more than Fungi in all conditions. In the cattle manure gram+ bacteria predominated over gram- bacteria.

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