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Comparative analysis of metagenomes of Italian top soil improvers

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ABSTRACT

Biosolids originating from Municipal Waste Water Treatment Plants are proposed as top soil improvers (TSI) for their beneficial input of organic carbon on agriculture lands. Their use to amend soil is controversial, as it may lead to the presence of emerging hazards of anthropogenic or animal origin in the environment devoted to food production. In this study, we used a shotgun metagenomics sequencing as a tool to perform a characterization of the hazards related with the TSIs. The samples showed the presence of many virulence genes associated to different diarrheagenic *E. coli* pathotypes as well as of different antimicrobial resistance-associated genes. The genes conferring resistance to Fluoroquinolones was the most relevant class of antimicrobial resistance genes observed in all the samples tested. To a lesser extent traits associated with the resistance to Methicillin in *Staphylococci* and genes conferring resistance to Streptothricin, Fosfomycin and Vancomycin were also identified. The most represented metal resistance genes were cobalt-zinc-cadmium related, accounting for 15–50% of the sequence reads in the different metagenomes out of the total number of those mapping on the class of resistance to compounds determinants. Moreover the taxonomic analysis performed by comparing compost-based samples and biosolids derived from municipal sewage-sludges treatments divided the samples into separate populations, based on the microbiota composition. The results confirm that the metagenomics is efficient to detect genomic traits associated with pathogens and antimicrobial resistance in complex matrices and this approach can be efficiently used for the traceability of TSI samples using the microorganisms' profiles as indicators of their origin.

1. Introduction

The recycling of bio-waste represents a resource for energy, water and nutrients in agriculture (ISWA, 2013). The bio-waste category includes sewage sludges derived from biological or chemical treatment of industrial or municipal wastewater and from the agri-food sector; manure from livestock and compost from both green wastes and organic fraction of the household wastes (Saveyn and Eder, 2014).

The solid organic matter from sewage treatment plants is often defined as Biosolid (BSO). BSO can be stabilized through an aerobic fermentation process by which mesophilic and thermophilic micro-organisms decompose organic matter into simpler nutrients. The process consumes oxygen and produces heat, with temperature rising up to 60 °C (Liang et al., 2003; Trautmann and Olynciw, 2010).

In the last years an increase in the use of BSO from Municipal Waste Water Treatment Plants (WWTP) in agriculture as top soil improvers (TSI) (1,000,000 t/year in Italy, mean 5 t/hectare/year) was observed (ISPRA, 2012). Such a trend relates to the presence of useful compounds of potential environmental value, such as organic carbon, nitrogen, phosphorous and potassium and to lesser extent, calcium, sulphur and magnesium (Usman et al., 2012). This approach allows facing intensive cropping while reducing the need to make use of the more expensive mineral fertilizers, making the use of BSO sustainable and economical due to nutrient cycling and disposal of sewage sludge (Usman et al., 2012).

In contrast to the proposed benefits, the use of BSO as TSIs has drawbacks. It has been shown that BSO and the derived TSIs can be 10–100 fold more contaminated with persistent organic pollutants (POPs) than the animal manure (Brambilla et al., 2016). The use of such products to improve the fields fertility may cause the soils to be persistently contaminated in the range of the hundreds or thousands of ng/g dry matter for certain POPs (Gottschall et al., 2012; Zennegg

Abbreviations: BSO, Biosolid; WWTP, Municipal Waste Water Treatment Plants; TSI, Top Soil Improver; POP, Persistent Organic Pollutants; STEC, Shiga Toxin producing *E. coli*; CO, Compost; MCO, Mixed Compost; AMR, Antimicrobial Resistance; COG, Cluster of Orthologous Groups; Kegg, Kyoto Encyclopaedia of Genes and Genomes; PCoA, Principal Coordinate Analysis; EAEC, Enteroaggregative *E. coli*; EPEC, Enteropathogenic *E. coli*

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et al., 2013; Suominen et al., 2014). The top soil intake by grazing animals represents the main determinant for the chemical carry-over to food and exposure through food consumption in humans (Brambilla et al., 2011). Beside the chemical contamination, the use of TSIs of anthropogenic and farm origin may cause the spreading of zoonotic and human enteric pathogens in pastures (Yergeau et al., 2016). Recently, virulence genes associated to important human pathogens, such as Shiga Toxin producing *E. coli* (STEC) and enteric viruses, were detected by Real Time PCR in TSIs (Tozzoli et al., 2016). A recent study shown that biosolids contain numerous virulence-associated genes related with many pathogens as assessed by metagenomics shotgun sequencing (Yergeau et al., 2016). It is noteworthy that a long term survival (more than 200 days) has been observed for STEC O157 in manure-amended soil (van Hoek et al., 2013). Noro- and Rota-viruses are resistant to wastewater treatment and can persist in the raw sewage and run-off waters from WWTPs as well as in surface waters used in agriculture for several months (López-Gálvez et al., 2016; Zhou et al., 2016). Similarly, parasites as *Cryptosporidium parvum* and *Giardia lamblia* have been shown to persist in soils for long times (Helmi et al., 2008). Besides the possibility of transmission of microbial pathogens, transfer of antimicrobial resistance genes from manure to soil bacteria has been described (Heuer and Smalla, 2007; Binh et al., 2008). Sengeløv et al. (2003) reported that resistance to Tetracycline, Macrolides and Streptomycin was measured for a period of 8 months in soil bacteria obtained from farmland treated with pig manure slurry (Sengeløv et al., 2003). A recent study on the antibiotic resistance in sewage treatment plants has shown that antimicrobial resistance genes can be enriched during sludge treatment process (Bengtsson-Palme et al., 2016).

We used a shotgun metagenomics sequencing approach, to perform the characterization of biological hazards related to the use of TSIs in agriculture in Italy. We selected eight samples including BSO, compost (CO) and mixed compost (MCO) and determined the microbiological profile diversity and to assess the presence of genomics traits related with pathogenic *E. coli* and antimicrobial resistance (AMR) determinants.

2. Materials and methods

2.1. Sampling and samples origin

The specimens analysed in this study were selected among a collection of 24 Top soil improvers samples collected in 2013 from different Italian regions and used in a previous study (Tozzoli et al., 2016). In particular, eight of these have been used for the metagenomics analysis: four municipal sewage sludges (BSO1, BSO2, BSO3, BSO4), obtained from a tertiary wastewater treatment plants with merged inputs from urban and intensive farmed pigs settlements; three samples of compost, (CO1, CO2 and CO3) derived from green wastes only; and one mixed compost sample (MCO) containing contributions from household wastes, green wastes and urban sewage.

Sampling was performed in accordance with UNI 10802/2004 guideline and the provision of law for the analysis of heavy metals. Biosolids from Waste Water Treatment Plants were stored in glass jars, in the dark, at − 30 °C, until the analyses were performed.

2.2. Nucleic acid extraction and DNA sequencing

DNA was extracted from 0.25 g of each untreated sample using the Power Soil DNA isolation kit (MO BIO Laboratories inc., Carlsbad, CA, USA) following the manufacturer's instructions.

Sequencing libraries were prepared from 100 ng of the DNA extracted from each sample, using the NEBNext Fast DNA Fragmentation & Lirbary Prep kit (New England BioLabs, New England, USA). In detail, the DNA was enzymatically fragmented to obtain fragments of about 400 bp, through an incubation at 25 °C for 15 min, followed by 10 min at 70 °C. The fragmented DNA was subjected to link with adaptors and size selection of 450 bp fragments by electrophoresis on E-Gel SizeSelect 2% (Invitrogen, Carlsbad, USA) followed by PCR amplification as indicated in the NEBNext Fast DNA Fragmentation & Library Prep kit manual (New England BioLabs, USA). The libraries were amplified individually through emulsion PCR with an Ion OneTouch 2 and sequenced with an Ion Torrent Personal Genome Machine (Life Technologies, 118 Carlsbad, USA), using the 400 bp sequencing protocol. The eight samples were sequenced individually in eight different runs using a 316 V2 chip per run.

2.3. Bioinformatics analysis

The sequences were analysed using the open source webserver for processing metagenomics sequences data MG-RAST to characterize the relative microbiota composition (http://metagenomics.anl.org) (Meyer et al., 2008). In detail, the sequence data were filtered for the sequences of human origin and analysed for the microbial content and then the resulting reads were compared against M5NR (the M5 non-redundant) protein database, using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15 aa for protein and 15 bp for RNA databases (Meyer et al., 2008).

The bioinformatics analyses of the metagenomes were also performed using the tools available on the ARIES public webserver (https://w3.iss.it/site/aries/).

In detail, the raw reads were subjected to a quality check and consequent trimming to remove the adaptors and to accept 20 as the lowest Phred value. The identification of the presence of *E. coli* virulence genes was performed through the pipeline Virulotyper, which employs Bowtie2 algorithm (http://bowtiebio.sourceforge.net/bowtie2/) (Langmead and Salzberg, 2012) to map the sequencing reads against the *E. coli* Virulence genes database (Joensen et al., 2014). Virulence genes showing coverage above 1X were considered present in the sample.

Comparative analysis of all samples was performed by using COMMET (COmpare Multiple METagenomes) (Maillet et al., 2014). This tool allows to observe the homology between the different samples on the basis of all-against-all comparisons of the non-assembled reads (http://colibread.inria.fr/commet/) (Maillet et al., 2014).

Functional metagenomics analysis was performed using the DIAMOND tool (Buchfink et al., 2015). Sequence data in a FASTA format were used to search the COG (Cluster of Orthologous Groups) reference database (Tatusov et al., 1997).

The results of the DIAMOND alignments were analysed and visualized using the MEGAN (MEta Genome ANalyzer) software version 5 (http://ab.inf.uni-tuebingen.de/software/megan5/) (Huson et al., 2007). The SAM files produced with the DIAMOND tool were imported into MEGAN 5 together with the FASTA files containing the sequence data. The KEGG (Kyoto Encyclopaedia of Genes and Genomes) (Kanehisa and Goto, 2000), COG and SEED subsystems (Overbeek et al., 2005) content was determined using the MEGAN internal Reference sequence maps. MEGAN 5 software was also used to perform the rarefaction analysis (Gotelli and Colwell, 2001) and to calculate the distances between the different samples through the principal coordinate analysis (PCoA) (Smith et al., 2007). For the latter analysis a stress of 0.41 and the Bray Curtis ecological index were used.

3. Results

3.1. Metagenomes quality check

On average, 2,861,113 reads per sample were retained after the quality check and have been used for the subsequent analyses.

The metagenomics datasets are available at the MG-RAST website, under the identification numbers: 4639314.3 (CO1); 4639313.3 (CO2); 4639304.3 (CO3); 4631825.3 (MCO); 4633025.3 (BSO1); 4632987.3