**Sewage sludge addition modifies soil microbial communities and plant
performance depending on the sludge stabilization process**

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Abstract

Despite the widespread use of sewage sludge as an organic amendment to improve soil
stability and plant productivity, relatively little is known about how the different sludge
stabilization processes affect the microbial composition and diversity of the sludge and
the soil microbial populations as well as plant performance. In this study, the effects

caused by addition of thermophilic aerobic (ATAD) and mesophilic anaerobic (MAD) sludge and inorganic fertilization on soil microbial community structure and diversity was assessed by pyrosequencing of 16S and 18S rRNA genes. Melon (*Cucumis melo* L., cv. Giotto) was used as model crop and its performance (growth and physiological state) was monitored together with changes in soil chemical parameters. Our results showed that the stabilization process of sewage sludge determined the feasibility of the final by-product as an organic amendment by altering in different manner the soil environment and modifying the soil microbial community structure and functioning. Changes in soil microbial community were related more to changes in the soil chemical environment rather than to the introduction of sludge-borne microorganisms. We also have shown that changes in a single physicochemical parameter (electrical conductivity) due to sludge application are associated with a pronounced shift in microbial community structure and activity as well as in plant performance. Along these lines, we showed that the application of ATAD sludge into soil resulted in less pronounced changes in its chemistry and microbial community structure, while enhancing soil microbial activity and plant performance. This study shows, therefore, that ATAD sludge could be applied as an excellent alternative to MAD sludge or inorganic fertilization.

Keywords: *organic amendment, pyrosequencing, bacterial community, fungal community, substrate utilization, plant performance*

1. Introduction

More than ten million tons of sewage sludge (dry matter) are produced in Europe annually, of which approximately 37% are used in agriculture (Milieu et al., 2010). In

Spain, over one million tons per year (83% of the sludge produced) are applied to land (MARM, 2011; Milieu et al., 2010). Land application of sewage sludge, which is encouraged by the Council Directive 91/271/EEC (European Commission, 1991), provides nutrients and organic matter that confer significant positive agricultural benefits (Insam et al., 2015; Singh and Agrawal, 2008), and is of special interest in the Mediterranean region due to a widespread lack of soil organic matter (García et al., 2000). The quality of sewage sludge is strongly dependent on three main factors: (1) the original inputs into the sewers; (2) the wastewater treatment process; and (3) the subsequent sludge stabilization process (Milieu et al., 2010). Autothermal thermophilic aerobic digestion (ATAD) is listed as an “advanced” treatment in the Proposal for a Directive of the European Parliament and of the Council on spreading of sludge on land (European Commission, 2003), as well as a technology capable of producing Class A biosolids (USEPA, 2003). Mesophilic anaerobic digestion (MAD), on the other hand, is one of the most widely used processes for the stabilization of sludge in wastewater treatment plants (Ahn and Forster, 2000) and is considered a “conventional” treatment and a technology capable of producing Class B biosolids (European Commission, 2003; USEPA, 2003).

The presence of oxygen in ATAD produces a more efficient mineralization that reduces the concentration of labile carbon (C) and bioavailable nutrients (Bernal et al., 1998; Odlare et al., 2011). Likewise, higher process temperatures result in larger stabilization rates and a higher efficiency of solids’ degradation (Juteau, 2006). Aerobiosis, temperature and physical-chemical parameters are the main factors controlling microbial communities in organic residues (Han et al., 2011; Hayes et al., 2011; Insam et al., 2010; Kim et al., 2002; Liu et al., 2010; Piterina et al., 2006; van

Lier et al., 1993), and thus it is to be expected that the sludge microbiota are ultimately determined by the stabilization process.

The addition of sewage sludge alters the physical and chemical properties of soils due to organic matter input. This addition may significantly modify soil structure, increase soil moisture and porosity, and enhance humus content and cation exchange capacity (Barzegar et al., 2002; Korentajer, 1991). Furthermore, the application of sludge to soil leads to a reduction in pH and an increase in electrical conductivity (EC) (Antolín et al., 2005; Pascual et al., 2009). Overall, the use of organic amendments has been associated with an acceleration of microbial development and activity (Bailey and Lazarovits, 2003), together with changes in microbial community composition (Marschner et al., 2003). These shifts are likely associated with changes in the functional capabilities of soil microbial communities (Fierer et al., 2012), and can in turn alter soil fertility and crop production (Kelley et al., 1984; Min-Jian, 1997; Singh and Agrawal, 2008). Finally, elevating nitrogen (N) inputs, as with sludge amendments, might increase plant performance (LeBauer and Treseder, 2008).

Here, we integrated the effects of sewage sludge addition on soil chemical properties, microbial genetic and functional profiles, as well as plant performance, in a comparative study of two different sludge stabilization processes (ATAD vs MAD). Specifically, we aimed to evaluate: 1) how the distinct stabilization processes impacted the sludge microbial community; and 2) the effects of the addition of ATAD and MAD sludge separately on the soil physical-chemical properties, soil microbial community structure and activity, and plant growth and physiology, in relation to the addition of mineral fertilization. The stabilized sludges were produced from the same original incoming raw sludge and their application into an agricultural soil was performed at a microcosm scale. We hypothesized that: 1) the sludge stabilization process would shape

its chemistry and its microbial community composition; and 2) different nutrient sources (ATAD and MAD sludge versus inorganic fertilizer) would influence the soil microbial community as well as plant performance. We postulated that due to the nature of the different stabilization processes, amending soils with ATAD sludge would affect to a lesser extent the soil chemical properties, having therefore a reduced impact on the soil microbiota (in terms of changing community structure, activity and metabolic profiles) but leading to a decreased crop production relative to the application of MAD-treated sludge.

2. Material and methods

2.1. Soils, organic amendments, experimental layout and growth conditions

Soils were collected from an agricultural land in Murcia (Southeast Spain). The topsoil layer (0-20 cm) was sampled and sieved (< 3 mm). Both types of sewage sludge were collected from the Molina de Segura wastewater treatment plant (WWTP) (Murcia, Spain). The sludges were produced after: 1) ATAD digestion with thermal treatment as described by Lloret et al. (2012); and 2) MAD digestion (Table 1). Both sludges and soils were collected and stored at 4 °C until the set-up of the microcosm experiment. Four experimental treatments were performed in 2.8 L pots: 1) none (control soil); 2) mineral fertilizer; 3) ATAD sludge; and 4) MAD sludge. The mineral fertilizer (NH_4NO_3 and KH_2PO_4) and sewage sludges were applied at a rate of 1.8 g N kg^{-1} and 0.13 g P kg^{-1} soil (dry weight). After an equilibration period of 24 h, one melon seedling (*Cucumis melo* L., cv. Giotto) was transplanted into each pot. All plants were grown in a controlled environment greenhouse (28 °C) and harvested after 66 days when fructification became the predominant process. Pots were irrigated as needed to adjust soil water content to 60% water holding capacity (WHC) throughout the

experiment. Soil samples were taken at the beginning and at the end of the experiment (before planting and after harvesting, respectively). One composite subsample from each pot was air-dried and used for chemical analysis. Another subsample was stored at 4 °C and used for soil microbiological parameters and another stored at -80 °C until molecular analysis. A full description of the soils, the organic amendments, the experimental layout and the growth conditions are shown in the Supplementary Material.

2.2. Soil chemical and microbiological analysis and community-level physiological profiles (CLPPs)

Soil chemical analyses were performed at the start and at the end of the experiment. The chemical parameters that were analyzed included EC, pH, C and N content and other macro- and micronutrients as well as heavy metals. The analysis of microbiological parameters included basal respiration and microbial biomass C (C_{mic}). Soil basal respiration was measured as CO₂ evolution from moist soil samples using continuous flow infrared gas analysis (IRGA) (Heinemeyer et al., 1989). From basal respiration, organic C and C_{mic} , the microbial quotient (C_{mic}/C_{org}) and the metabolic quotient (qCO_2 , $\mu g\ CO_2-C\ g^{-1}\ C_{mic}\ h^{-1}$) were calculated. The CLPPs were assessed by using a micro-respiration technique (MicroRespTM) which is a convenient and sensitive method to perform catabolic assays to characterize the functional potentials of the communities (Campbell et al., 2003). The different C sources consisted of six carbohydrates, five amino acids, and four carboxylic acids. Further details of the chemical and microbiological analysis and community-level physiological profiles (CLPPs) can be found in the Supplementary Material.

2.3. DNA extraction and pyrosequencing of SSU rRNA gene

Total nucleic acids were extracted from 0.1 g of ATAD and MAD sludges and from 0.5 g of soil samples taken at the end of the experiment (n = 5). Sludges were freeze-dried and soils ground prior to DNA extraction. DNA extraction was performed according to the protocol described by Ivanov et al. (2009) including some modifications. Soil DNA samples were PCR amplified using a universal primer pair targeting the SSU rRNA V9 region, and PCR reactions were performed using Takara Ex Taq Hot Start polymerase (Takara, Madison, WI, USA). The barcoded PCR reactions were analyzed for quality and quantity on a Bioanalyzer 2100 using a DNA 7500 chip (Agilent Technologies, Santa Clara, CA, USA). Sequencing libraries were created using the SV emu-PCR kit (Lib-A, Roche, Indianapolis, IN, USA) and sequenced on a 454 GS-FLX sequencer (Roche, Indianapolis, IN, USA) at the Veterans Medical Research Foundation (La Jolla, CA, USA). Raw 454 sequences were uploaded, denoised and processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). The sequence data have been submitted to the European Molecular Biology Laboratory (EMBL) under accession number PRJEB7592. Further details of DNA extraction, SSU rRNA PCR and pyrosequencing data processing can be found in the Supplementary Material.

2.4. Plant growth, photochemical efficiency and relative chlorophyll content

Plant growth was determined as the plant fresh weight recorded immediately after the plants were harvested. To assess the physiological status of the plant in the short term as a proxy of plant stress, indicators of the efficiency of the photosystem II (PSII) (F_v/F_m), protective mechanisms against photooxidative damage (NPQ), and the metabolic efficiency of the plant regarding the use of absorbed nutrients (RCC) were

measured in all the plants shortly before harvesting. Modulated chlorophyll fluorescence (F_v/F_m and NPQ) was measured in dark-adapted (30 min) leaves using a chlorophyll fluorometer OS-30 (OptiSciences, Herts, UK). The relative chlorophyll content (RCC) was estimated using a SPAD-502 chlorophyll meter (Minolta Camera Co., Ltd, Osaka, Japan). Full details of the photochemical efficiency and the RCC measures are described in the Supplementary Material.

2.5. Statistical analysis

To test the effect of the treatment and incubation time on soil chemical parameters, data were subjected to a two-way ANOVA. The effect of the treatments on soil microbiological parameters, CLPPs and plant performance were tested by a one-way ANOVA. The overall variations in catabolic activity of the CLPPs were analyzed with a principal component analysis (PCA) and PCA scores were analyzed by a one-way ANOVA. When significant F values were obtained, a further analysis with Tukey's HSD (honestly significant difference) comparison procedure was performed as a *post hoc* test ($p < 0.05$) to control for multiple testing. Data were tested for normality and transformed when necessary. When the data did not meet the normality condition, they were subjected to non-parametric tests for several independent samples (Kruskal–Wallis test) and pair-wise comparisons between different types of sludge were performed using the Mann–Whitney U test ($p < 0.05$). The effect of treatments on the community structure of bacteria, archaea and fungi and on their relative abundance were assessed through permutational multivariate analysis of variance (Oksanen et al., 2013) and by generalized linear models. The effect of the sludge stabilization process on sludge diversity, as well as the effect of the treatments on soil microbial community structure, was tested by a Student's *t*-Test and one-way ANOVA, respectively. To test for

similarities between the microbial community structure in ATAD and MAD sludges as well as on the corresponding amended soils, we computed matrix correlations between OTU abundance distance matrices (Mantel, 1967; Oksanen et al., 2013). Finally, to test whether the similarity of the prokaryotic and the fungal community structure in soils submitted to different treatments was related to their chemical and microbiological properties as well as their plant production, Mantel correlations were performed. Weighted unifrac distance matrices were used to visualize the soil community composition through ordination in a correspondence analysis (Okansen et al., 2013). All statistical analyses were performed in R (R Core Team, 2012). Additional details can be found in the Supplementary Material.

3. Results

3.1. Soil chemical and microbiological properties

Both ATAD and MAD sludge significantly increased soil EC, total organic C (TOC), total N, P, and Na compared to those of the control soils (Table 2 and Table S1). This increase took place immediately after the addition of sludge and was still evident after a 2-month incubation period (start and end of the experiment, respectively). EC, TOC, and total N were significantly higher in MAD-amended soils compared to ATAD-amended soils. Conversely, a small but significant reduction of soil pH was observed in the sludge-amended pots (Table 2). Mineral fertilized soils followed a similar trend to that of the sludge-amended soils (Table 2). Concentrations of several micronutrients and heavy metals such as Cu, Mo, Pb, S, and Zn, were also increased by the addition of sludge compared to the control soils (Table 2 and Table S1). Macronutrients such as N, P, and K significantly decreased at the end of the study in most of the treatments (Table 2).

Microbial biomass C (C_{mic}) and the ratio C_{mic}/C_{org} were strongly stimulated by the addition of mineral fertilizer and ATAD sludge (Table 3). Basal respiration tended to increase in all of the treatments compared to the control soils, but differences were only statistically significant in the ATAD-amended soils (Table 3).

3.2. Community-level physiological profiles (CLPPs)

The CLPPs of the soil-borne microorganisms of the different treatments were studied in MicroRespTM plates. Fig. 1A depicts the first two principal components (PCs) of the overall analysis, which explained 58.7% and 12.1% of the variance, respectively. The effect of sludge was evident, resulting in a unique cluster for both types of sludge-amended soils, thereby indicating different substrate utilization patterns from those of the control and mineral fertilized soils. Treatments were significantly separated along PC1 ($p = 0.003$). Most of the C sources (11 out of 15) were strongly positively correlated with PC1, their loadings ranging from 0.71 to 0.92, whereas only one C source (malic acid) was weakly negatively correlated with PC1 with a loading of -0.12 (Table S2). Substrate utilization of each C source across the different treatments is shown in Fig. 1B. The strongest effect was observed after the addition of ATAD sludge. Substrate utilization of the sludge-amended soils differed from that of the control soils in their ability to increase the catabolism of 8 and 5 C substrates for ATAD and MAD sludge, respectively.

3.3. Sewage sludge microbial community structure

The prokaryotic community structure was significantly different depending on the sludge stabilization process ($r^2_{1,8} = 0.518$; $p = 0.006$). This result remained the same when bacterial ($r^2_{1,8} = 0.518$; $p = 0.006$) and archaeal communities ($r^2_{1,8} = 0.398$; $p =$

0.01) were analyzed independently. The fungal population was not analyzed as fungal sludge sequences were only detected in 6 out of 10 sludge samples, constituting less than 1.7% of the overall sludge microbial community. Differences in the microbial community structure between ATAD and MAD sludges are depicted in a principal coordinates analysis (PCoA) of the bacterial and archaeal operational taxonomic units (OTU) abundance matrices, with PC1 explaining 80.9% of the variance (Fig. 2).

Microbial communities in the MAD sludge were significantly more diverse than in the ATAD sludge, both in terms of phylogenetic diversity (Faith's PD) and species richness (Chao1) (Table S3). The Firmicutes dominated the ATAD sludge community composition, while the Actinobacteria and Thermotogae were also significant, albeit smaller, components of the sludge (Fig. 3A). The MAD sludge showed greater diversity with large contributions attributable to members of the Firmicutes, Actinobacteria, Thermotogae, Synergistetes, Proteobacteria, Chloroflexi and Bacterioidetes (Fig. 3A). The archaeal community of the ATAD sludge consisted of one major dominant taxon (Thermoplasmatales), whereas the MAD sludge had a community formed almost equally by members of the Thermoplasmata and Halobacteriales (Fig. 4A).

3.4. Soil microbial community structure

The mineral and sludge amendments had a strong influence on the composition of soil microbial communities, which significantly differed across the treatments after two months of incubation ($r^2_{1,18} = 0.347$; $p = 0.001$). This pattern was confirmed separately for bacterial ($r^2_{1,18} = 0.349$; $p = 0.001$), archaeal ($r^2_{1,18} = 0.289$; $p = 0.001$) and fungal communities ($r^2_{1,18} = 0.264$; $p = 0.001$). In a PCoA representing the overall soil microbial community structure, four different small clusters can be observed

corresponding to each treatment (Fig. 5), with PC1 explaining 59.0% of the variance. The addition of MAD sludge provoked the strongest shift in the microbial community of the soils. The ATAD-amended and mineral fertilized soils showed strong similarity. Soil microbial diversity significantly decreased in the mineral fertilized and sludge-amended soils compared to the control soils, as indicated by the metrics of phylogenetic diversity (Faith's PD), and species richness (Chao1) (Table S3). The indigenous bacterial community of the control soil was largely dominated by Actinobacteria (44% of the total bacteria) and Proteobacteria (20%), followed by Chloroflexi, Gemmatimonadetes, Planctomycetes, and Acidobacteria (Fig. 3B). The addition of sludge resulted in a significant decrease in the relative abundance of Actinobacteria, Acidobacteria and Cyanobacteria, and an increase in the relative abundance of Proteobacteria, Bacteroidetes and Firmicutes. These changes were particularly apparent following the addition of MAD sludge (Fig. 3B). Archaeal communities in the control soils were dominated by Methanosarcinales, Thermoplasmatales, and Halobacteriales (Fig. 4B). The addition of either mineral fertilizer or sludge significantly increased the relative abundance of 16S rRNA of the Korarchaeota, which became the predominant archaeal taxon in the mineral fertilized and the ATAD-amended soils. There was a subsequent decline in the relative abundance of Thermoplasmatales and Halobacteriales. Individual additions of ATAD and MAD sludge resulted in a significant increase in the relative abundance of members of the Thermococcales and Methanosarcinales, respectively (Fig. 4B). Among fungi, Basidiomycota dominated the control soils followed by Ascomycota, while Glomeromycota increased significantly following the addition of mineral fertilizer and both sludges (Fig. 6).

3.5. Plant growth, photochemical efficiency and relative chlorophyll content

Plant performance was substantially altered by all treatments compared to the control pots (Table 3). Plant growth was strongly stimulated by the addition of mineral fertilizer and ATAD sludge and to a lesser extent by MAD sludge. The maximum photochemical efficiency of PSII (F_v/F_m) was enhanced by the addition of both sludges and to a lesser extent by mineral fertilization. The non-photochemical quenching (NPQ) response was significantly higher in the control soil and lower in the mineral fertilized and ATAD sludge-amended soils. Finally, RCC was significantly affected by all the treatments, the greatest increase being produced by the addition of ATAD sludge, followed by the MAD sludge and mineral fertilizer.

3.6. Relationships between the soil environment, microbial communities and plant performance

Similarities in the community structure of the sludges (ATAD and MAD) did not correlate to similarities in the sludge-amended soils (Table S4). Instead, the composition of the microbial communities were significantly related to the soil chemical properties (TOC, pH, EC, N, P and K) following the amendments ($r_{\text{mantel}} = 0.662$, $p = 0.001$). EC was the single chemical parameter explaining the majority of the community composition variance in the overall community structure ($r_{\text{mantel}} = 0.652$, $p < 0.001$), but also in bacterial ($r_{\text{mantel}} = 0.652$, $p < 0.001$) and archaeal communities ($r_{\text{mantel}} = 0.508$, $p = 0.015$). This was followed by pH ($r_{\text{mantel}} = 0.404$, $p < 0.001$), which was also the chemical variable that was most highly correlated to the fungal community ($r_{\text{mantel}} = 0.548$, $p = 0.003$), and TOC ($r_{\text{mantel}} = 0.374$, $p = 0.004$). Chemically similar soils showed similar plant performance (growth and physiological state) ($r_{\text{mantel}} = 0.18$, $p = 0.026$). Plant performance was significantly correlated with the microbial community structure ($r_{\text{mantel}} = 0.233$, $p = 0.01$) and performance (as assessed by microbial biomass,

metabolic and microbial quotients and CLLPs; $r_{\text{mantel}} = 0.72$, $p = 0.001$). A marginally significant relationship was found between plant weight and EC ($r_{\text{mantel}} = 0.126$, $p = 0.069$).

4. Discussion

The application of sewage sludge to the soil modified the chemical environment and the community structure and function of soil prokaryotes and microbial eukaryotes, as well as plant performance. The magnitude of these changes was associated with the stabilization process of sewage sludge. This showed that the biotreatment technology used for processing sewage sludge determines its suitability as an organic amendment.

4.1. Sludge stabilization process determined sludge microbial community structure

The microbial communities of sewage sludge were strongly influenced by the stabilization process, as shown by the significantly different community structure found in the ATAD and MAD sludges. Aerobic thermophilic digestion generated a sludge characterized by the low diversity primarily represented by spore-forming (Firmicutes and Actinobacteria) and thermophilic taxa (Thermotogae, Thermoplasmatales and Crenarcheota) (Goberna et al., 2009; Hayes et al., 2011; Liu et al., 2010; Piterina et al., 2006). Similarly, the archaeal population was dominated by Thermoplasmatales and Crenarcheota, both considered to be thermophilic (Blochl et al., 1997; Huber and Stetter, 2006). On the other hand, the MAD stabilization process generated a highly diverse sludge, with diversity evenly distributed across seven bacterial phyla. This indicates a favorable environment for the growth of a wider range of microbes that is probably triggered by the presence of more labile C in this kind of system (Bernal et al., 1998; Odlare et al., 2011). The presence of Halobacteriales among the archaeal

population of the MAD sludge is likely due to the presence of a saline environment (Garrity and Boone, 2001), represented by a higher EC compared to ATAD sludge.

4.2. Changes in the microbial community following sludge addition are dependent on the sludge stabilization process

The mineral and sludge amendments altered the soil microbial community structure, reduced the taxonomic and functional diversity, and influenced microbial activities. As we postulated, ATAD-amended soils resembled the control soils to a greater extent than the MAD-amended soils. Nevertheless, although previous studies show that sludge application modifies soil microbiota (Borrero et al., 2004; Pascual et al., 2008), here we report that these changes are determined by the stabilization process that the sludge has previously undergone. The changes imposed by the organic amendments can be induced either directly through the addition of exogenous microorganisms, or indirectly through changes in the soil environment (Innerebner et al., 2006). Our results indicated that sludge-borne microorganisms did not significantly displace the soil indigenous microbiota since we found no correlation between the community structure of sludge and sludge-amended soils.

Alternatively, we showed that shifts in soil chemical parameters (mainly EC, pH and TOC) were associated with the alteration of soil microbial communities. These abiotic variables, which can be strong determinants of the microbial phylogenetic composition and functioning (Fierer and Jackson, 2006; Goberna et al., 2012; Lozupone and Knight, 2007), were significantly modified by the addition of sludge (Antolín et al., 2005; Pascual et al., 2009), especially after the addition of MAD sludge. The content of soil nutrients (N, P, K), however, proved to have less prominent effect in shaping the soil microbiota. EC explained most of the variance in the soil microbial community

composition, most likely by selecting salinity-tolerant microbes and in turn not favoring those physiologically less adapted organisms (Šimek et al., 1999; Thirukkumaran and Parkinson, 2000). The MAD-amended soils, with the highest EC value, also had lower microbial activity (as measured by basal respiration), and their microbes proved less efficient in using organic substrates as indicated by the C_{mic}/C_{org} ratio (Anderson, 2003; Rietz and Hayes, 2003). Another main factor associated with soil microbial community was pH. As expected, similar microbial communities were found at a similar pH value, probably due to the narrow pH ranges for optimal growth of bacteria (Rousk et al., 2010). This parameter regulates thermodynamics and the availability of nutrients in addition to having direct impact on cell physiology. As previously reported by Marschner et al. (2003), pH was the factor most strongly correlated with the fungal community composition. Finally, the changes in the levels of TOC also correlated with shifts in the community structure. Soil microbial communities are frequently C- limited (Zak et al., 1994), and the organic substrates added with the sludge become a primary nutrient source. As a consequence, soil microorganisms undergo a selective enrichment in response to C additions, and this may account for the reduced taxonomic and functional diversity of the amended soils compared to the control soils (Goldfarb et al., 2011). In the present work, although ATAD and MAD sludge application altered in a significant different manner the soil microbial community structure, we did not obtain a remarkable difference in their substrate utilization profiles, maybe due to functional redundancy.

Specifically, the shifts in the composition of the soil bacterial communities were attributable to the decrease in the numbers of the dominant Actinobacteria and the increase in Proteobacteria levels. In particular, the control soils shifted to soils co-dominated by Actinobacteria and Proteobacteria when subjected to mineral fertilization

and ATAD amendments or to Proteobacteria-dominated soils following the addition of MAD sludge. Actinobacteria are among the most important litter decomposers in soil (Kopecky et al., 2011), and can reach high abundances in arid soils with low C contents (Okoro et al., 2009). Certain classes of Proteobacteria become superior competitors, able to displace other soil bacteria, under C-enriched conditions (Goldfarb et al., 2011). Thus, artificial C amendments typically increase the abundance of some Proteobacterial classes (Fierer et al., 2007; Goldfarb et al., 2011). The shift in the composition of the archaea was primarily due to the increased prevalence of Korarchaeota following the addition of both types of sludge. The ATAD-amended soils showed an increase of the Thermococcales, a thermophilic heterotrophic taxon, whereas the addition of MAD sludge increased the prevalence of the methanogens, such as Methanosarcinales. Generally, all of the amendments increased the relative abundance of members of the Glomeromycota. As this group is known to comprise mutualistic symbionts of land plants that form arbuscular mycorrhiza (Smith and Read, 2008), an improved supply of water and nutrients could be expected in the amended plants.

4.3. The sludge stabilization process influenced plant growth and physiology

The addition of sludge or mineral fertilizer resulted in bigger and healthier plants as demonstrated by increased plant weight, enhanced F_v/F_m , lower NPQ and higher RCC. The Mantel tests indicated that differences in plant performance across the treatments were related both to the direct changes in the soil properties and the environmentally-mediated shifts in soil microbial community structure, as well as in the metabolic abilities of decomposers. However, contrary to our predictions, improved plant growth and physiology were higher with use of ATAD-amended treatments. In fact, plant growth was significantly higher for ATAD sludge and mineral fertilizer

treatments, which resembled one another in their microbial community composition. Bearing in mind that N and P were applied at the same rate in all amended treatments, the fact that plant growth and N assimilation were lower in the MAD-amended soils points to increased EC in the MAD-amended soils as the most likely reason for poorer plant performance. It is known that a higher EC affects plant growth, nutrient cycling and biological activity negatively and that an elevated osmotic pressure around the roots increases water stress to the plant (Munns, 2002; Smith and Doran, 1996). Consequently, the functional capabilities as obtained by the CLPPs do not relate to plant biomass accumulation.

The significantly lower value of F_v/F_m in the control soils indicates a degraded photochemical activity in these treatments, suggesting photoinhibition and subsequent photodamage of the PSII (Maxwell and Johnson, 2000). This may be due to the effect of oxidative processes on chlorophyll as indicated by the lower RCC. It seems clear that, although heat dissipation was promoted in the control plants (higher NPQ) it was not sufficiently high enough to prevent damage to the leaves. On the contrary, F_v/F_m levels of melon crops in the ATAD and MAD-amended soils were within the values of a healthy plant, which are estimated to be optimal between 0.79 and 0.84 (Björkman and Demming, 1987). Our data are in agreement with Pascual et al. (2008), who found increased photosynthesis in pepper plants amended with the ATAD and MAD sludge compared to the control plants. RCC differed across all of the treatments, with the highest values in the ATAD-amended soils, probably due to the fact that synthesis of chlorophyll is known to be highly dependent on N availability (van der Meer and de Jong, 2006). We also postulate that this can be due to a more efficient N mineralizing microbial community.

5. Conclusion

Our results show that the stabilization process of sewage sludge stands out as a pivotal agent determining the feasibility of the final by-product as an organic amendment, shaping the sludge microbial community structure while altering in a different manner the soil microbial community. ATAD sludge produced smaller changes in the soil microbiota and in the soil chemical properties, whilst enhancing soil microbial activity and plant growth and performance. This conforms with the classification of ATAD digestion as an advanced sludge treatment (European Commission, 2003; Lloret et al., 2012), and indicates that this alternative stabilization process could replace the conventional MAD digestion and also be used as an excellent surrogate to inorganic fertilization. As reported by several authors, the positive response of crops to sludge application is not a general phenomenon, and it depends not only on the type of sludge applied and the technology used to process the raw material but also on the soil type (Kim et al., 2007; Richards et al., 2000; Singh and Agrawal, 2008). Hence, repeating this experiment with a battery of different soil types would be necessary to guarantee the agronomic quality of ATAD sludge. At the same time, further investigation is needed in a longer field experiment to assess the dynamics between the original and the newly established microbial community, as well as to evaluate the prevalence of the induced chemical changes in order to determine the sludge application frequency to assure enhancement of plant growth.

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Table 1. Chemical characteristics of ATAD and MAD sewage sludge (values on dry weight basis).

Parameters ^a	ATAD sludge	MAD sludge	UE limits ^b
Dry matter (%)	1.2	1.4	
pH	9.5	7.8	
EC (dS m ⁻¹)	8.9	14.0	
TOC (g kg ⁻¹)	346	315	
Total N (g kg ⁻¹)	183	129	
Total P (g kg ⁻¹)	16.7	14.3	
Total K (g kg ⁻¹)	8.0	8.0	
Total Cd (mg kg ⁻¹)	0.80	2.2	10
Total Cr (mg kg ⁻¹)	25.0	57.1	1000
Total Cu (mg kg ⁻¹)	208	314	1000
Total Fe (g kg ⁻¹)	14.8	29.1	
Total Ni (mg kg ⁻¹)	41.7	157	300
Total Pb (mg kg ⁻¹)	33.3	96.3	750
Total Zn (mg kg ⁻¹)	825	550	2500

^a EC: electrical conductivity; TOC: total organic carbon. Both sludges were applied to soil at the same nitrogen rate.

^b Heavy metal limits for soils (European Commission, 2003).

Table 2. Chemical characteristics of the control, mineral fertilized, ATAD and MAD sludge-amended soils at the beginning and at the end of the experiment (values on dry weight basis). Standard deviation is given in parentheses. Letters indicate significant differences between the treatments and asterisks indicate significant differences between the sampling times ($p < 0.05$).

Parameters ^a	Sampling time	Control soil	Mineral fertilized soil	ATAD-amended soil	MAD-amended soil
pH	Start ^b	8.0 (0.1) b	8.0 (0.1) b	7.8 (0.1) a	7.9 (0.1) ab
	End	8.2 (0.2) c	7.9 (0.0) b*	7.8 (0.1) ab	7.7 (0.1) a*
EC (dS m ⁻¹)	Start	0.48 (0.06) a	0.48 (0.06) a	2.0 (0.2) b	2.8 (0.1) c
	End	0.88 (0.11) a*	1.09 (0.08) ab*	1.4 (0.0) b*	2.5 (0.4) c
TOC (g kg ⁻¹)	Start	7.2 (0.9) a	7.2 (0.9) a	8.4 (0.4) b	9.2 (1.1) c
	End	6.6 (1.0) a	7.5 (0.7) a	8.8 (1.0) b	10.2 (0.3) c
N (g kg ⁻¹)	Start	0.70 (0.10) a	0.70 (0.1) a	1.5 (0.1) b	1.6 (0.0) b
	End	0.40 (0.12) a*	0.50 (0.2) a	0.70 (0.13) b*	1.1 (0.1) c*
P (g kg ⁻¹)	Start	0.30 (0.00) a	0.30 (0.01) a	0.50 (0.02) b	0.60 (0.11) b
	End	0.20 (0.00) a	0.30 (0.01) b	0.40 (0.10) c	0.50 (0.00) c*
K (g kg ⁻¹)	Start	8.5 (0.7) a	8.5 (0.7) a	8.2 (0.4) a	8.5 (0.6) a
	End	7.7 (0.4) a*	8.0 (0.3) a*	7.7 (1.0) a*	7.2 (0.2) a*
Cd (mg kg ⁻¹)	Start	BDL ^c	BDL	BDL	BDL
	End	BDL	BDL	BDL	BDL
Cr (mg kg ⁻¹)	Start	67.5 (4.4) b	67.5 (4.4) a	59.7 (7.3) a	57.8 (4.7) a
	End	81.7 (12.7) b	62.1 (11.2) a	61.2 (7.5) a	58.5 (6.5) a
Cu (mg kg ⁻¹)	Start	16.4 (0.9) a	16.4 (0.9) a	23.0 (0.8) b	26.0 (1.0) c
	End	16.5 (2.7) a	17.7 (1.1) a	21.6 (2.0) b	22.2 (0.6) b*
Fe (g kg ⁻¹)	Start	18.4 (1.9) a	18.4 (1.9) a	20.0 (1.0) a	19.6 (1.0) a
	End	18.6 (1.0) a	17.3 (1.8) a	19.2 (1.5) a	18.5 (1.3) a
Ni (mg kg ⁻¹)	Start	30.3 (2.5) b	30.3 (2.5) a	30.5 (3.7) ab	29.8 (2.4) ab
	End	37.0 (4.5) b	28.6 (4.8) a	29.9 (3.5) ab	29.1 (2.6) ab
Pb (mg kg ⁻¹)	Start	11.2 (0.9) a	11.2 (0.9) ab	11.5 (0.6) ab	13.1 (0.7) b
	End	10.6 (0.9) a	11.6 (0.6) ab	11.8 (1.3) ab	11.6 (0.3) b
Zn (mg kg ⁻¹)	Start	28.4 (3.9) a	28.4 (3.9) a	32.0 (2.2) b	36.5 (2.0) b
	End	28.2 (2.4) a	26.9 (1.7) a	31.8 (2.5) b	31.6 (1.2) b

^a EC: electrical conductivity; TOC: total organic carbon.

^b Marginally significant differences: differences were found after ANOVA ($p < 0.05$) but not after Tukey HSD test ($p \geq 0.05$).

^c BDL: below detection limits.

Table 3

Table 3. Soil microbiological parameters and plant fitness in control, mineral fertilized, ATAD and MAD sludge-amended soils at the end of the experiment. Standard deviation is given in parentheses. Letters indicate significant differences between the treatments ($p < 0.05$).

	Control soil	Mineral fertilized soil	ATAD-amended soil	MAD-amended soil
Soil biological parameters ^a				
C_{mic} ($\mu\text{g C g}^{-1}\text{soil}$)	109 (13) a	288 (30) b	336 (32) b	177 (23) a
C_{mic}/C_{org} (%)	1.65 (0.42) a	3.94 (1.06) b	3.80 (0.79) b	1.74 (0.50) a
Basal respiration ($\mu\text{g CO}_2\text{-C g}^{-1}\text{soil h}^{-1}$)	0.46 (0.21) a	1.32 (0.21) ab	2.04 (0.41) b	1.48 (0.40) ab
$q\text{CO}_2$ ($\text{mg CO}_2\text{-C g}^{-1} C_{mic} \text{ h}^{-1}$)	3.87 (1.67) a	4.58 (0.64) a	5.92 (0.77) a	8.05 (1.85) a
Plant growth and photochemical efficiency				
Plant weight (g)	22.0 (4.07) a	206 (12.70) c	206 (12.29) c	94.6 (7.99) b
F_v/F_m	0.34 (0.081) a	0.69 (0.077) b	0.78 (0.019) c	0.78 (0.013) c
NPQ	0.39 (0.111) c	0.13 (0.039) a	0.16 (0.059) a	0.21 (0.086) b
RCC	0.21 (0.086) a	16.4 (4.4) b	33.6 (7.5) d	26.7 (5.8) c

^a C_{mic} : microbial biomass; C_{mic}/C_{org} : microbial quotient; $q\text{CO}_2$: metabolic quotient; F_v/F_m : maximum photochemical efficiency of photosystem II; NPQ: non-photochemical quenching; RCC: relative chlorophyll content.

Figure Captions

Fig. 1. PCA plot showing community-level physiological profiles across treatments (MicroRespTM) (A) and substrate utilization for 15 carbon sources (MicroRespTM) in the control, mineral fertilized, ATAD and MAD sludge-amended soils at the end of the experiment. Bars represent standard errors ($n = 4$). Letters indicate significant differences between treatments ($p < 0.05$) (B)

Fig. 2. Weighted principal coordinates analysis of the overall microbial community of ATAD and MAD sludge

Fig. 3. Relative abundance of bacterial phyla in ATAD and MAD sludge (A) and in the control, mineral fertilized and sludge-amended soils (B). Bars represent standard errors ($n = 5$). Asterisks indicate significant differences between treatments relative to the control or within the type of sludge ($p < 0.05$)

Fig. 4. Relative abundances of archaeal orders in ATAD and MAD sludges (A) and in the control, mineral fertilized and sludge-amended soils (B). Bars represent standard errors ($n = 5$). Asterisks indicate significant differences between treatments relative to the control or within type of sludge ($p < 0.05$)

Fig. 5. Weighted principal coordinates analysis of the overall microbial community of control, mineral fertilized and sludge-amended soils

Fig. 6. Relative abundances of fungal phyla in the control, mineral fertilized and sludge-amended soils. Bars represent standard errors ($n = 5$). Asterisks indicate significant differences between treatments relative to the control or within type of sludge ($p < 0.05$)

Figure 1A

Figure 1B

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6