Physicochemical, microbiological and ecotoxicological characterization of urban sewage sludge destined for agricultural reuse

R.I. ZOGHLAMI[1,3*], S. MOKNI-TLILI[1], H. HAMDI[*], M. NACEUR KHELIL[2], N. BEN AISSA[3], N. JEDIDI[1]

[1] Water Research and Technology Center, University of Carthage, P.O.Box 273, Soliman 8020, Tunisia,, inesrahma.zoghlami@gmail.com, helmi.hamdi@certe.rnrt.tn, mt_sonia@yahoo.fr, Naceur.Jedidi@certe.rnrt.tn
[2] National Institute for Research in Rural Engineering, Water and Forestry, P.O.Box 10, Ariana 2080, Tunisia, khelil_mn@yahoo.fr
[3] National Institute of Agronomy, 43 Av. Charles Nicolle, 1002 Tunis, Tunisia

*Corresponding author: inesrahma.zoghlami@gmail.com

Abstract - Sewage sludge characterization is important for an efficient use as an agricultural soil amendment. The present work reports on the outcomes of a preliminary investigation of an urban sewage sludge that has been reused by local farmers as an organic amendment. Polymorphism analysis of isolated bacteria showed the existence of two major phenotypic groups: a group P (non-mycelia bacteria) and a group G (mycelial bacteria), also showing that the 50 isolated strains were more sensitive to copper than to zinc. This sludge was very phytotoxic to lettuce seeds; the effective concentration (EC_{50}) was very low around 0.36%. This may be caused partly by heavy metal contents which were, however, in accordance with French and Tunisian standards or by the high salinity of the sludge which could be an obstacle to its agricultural reuse. On the other hand, C/N ratio was equal to 15 indicating that the nitrogen supplied by this sludge provides good decomposition of the organic matter, which is an important indicator to improve the fertility of agricultural soils.

Keywords: sewage sludge, polymorphism, heavy metals, Phytotoxicity, C/N ratio

1. Introduction

The activated urban sewage sludge system consists of the separation of the flocculated biosolids from the treated urban wastewater in a settling tank after undergoing the secondary biological treatment (Liwarska-Bizukojca et al. 2015). The successful reuse of activated sludge in agricultural soils as an organic amendment is largely based on the effectiveness of the treatment and stabilization procedures provided at treatment plants (Nansubuga et al. 2015; Alvarenga et al. 2015). Consequently, sludge quality depends on its physico-chemical, biological and toxicological properties (Sciubba et al. 2015).

As a biowaste, the addition of sewage sludge to croplands contributes to the recycling of nutrients in the soil due its relatively high content of organic matter. Sludge mineralization in the soil is a highly dynamic process in which microorganisms play a major role when edaphic factors are favorable (Hamdi et al. 2006). In this regard, microbial metabolic processes degrade complex carbon and nitrogen-containing polymers such as cellulose, hemicelluloses and proteins (Partanen et al. 2010), resulting in a more simplified matter (minerals and humus) that can be used as a plant growing medium or soil conditioner. The techniques used to identify the microbial populations involved in sludge biodegradation include qualitative techniques such as respiration indices where evolved CO_2 or absorbed oxygen are measured (Ní Chualáin and Prasad 2009), and semi-quantitative and quantitative techniques, e.g. plate counts using selective media for bacteria and fungi culturing (Chroni et al. 2009). For instance, proteobacteria, actinobacteria, nitrosomonas have been shown to be abundant in different activated sludges (Sinthusith et al. 2015).

Nevertheless, sewage sludge as a complex biosolid originally contained in raw wastewater contains also various pollutants such as toxic heavy metals, persistent organic pollutants, emerging contaminants, detergents, and pathogens (Hamdi et al. 2006; Meulepas et al. 2015). This can limit the

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agricultural reuse and pose potential risks to human health and the environment. Consequently, data should be gathered on the presence of contaminants in urban sewage sludge in order to determine environmental risks amid land application.

In this context, the current study focuses on the characterization of an urban sewage sludge produced at the wastewater treatment plant of Korba, Tunisia, and partly destined for agricultural reuse. Sludge potential as a fertilizer, toxic chemical and biological parameters, and the resistance of some bacterial isolates to heavy metals are hereby presented and discussed.

2. Materials and Methods

The urban sewage sludge used in this study was collected from the drying beds at Korba Wastewater Treatment Plant in Nabeul, Tunisia. This sludge is continuously generated by an activated sludge treatment process and partly used by the local farmers for fertilization purposes. At the laboratory, the fresh sludge sample was divided into two parts. The first was immediately stored at -20°C for microbial characterization. The second part was air dried at room temperature, crushed then sieved at 2 mm and stored at 4°C for physico-chemical and phytotoxicity analyses.

2.1 Physico-chemical parameters

Moisture content was estimated by drying 100 g of air-dried sludge at 105°C for 24 h. Sludge pH was measured in distilled water (1:2) according to NF T90-008. Electrical conductivity (EC) was determined in sludge-water slurry (1:5) according to NF T90-110. Total heavy metals were extracted with aqua regia by acid digestion (3:1, v/v, $HCl:HNO_3$) (ISO standard 11466). Total Kjeldahl nitrogen (TN) was determined according to Bremner (1996). Total organic carbon was determined by the Walkley and Black method (1934). Total available phosphorus (P) was analyzed by the Olsen method (1954). Exchangeable bases (K^+, Na^+ and Ca^{2+}) were determined by flame photometry after sample mineralization.

2.2 Sludge phytotoxicity

The absolute phytotoxicity of sewage sludge was evaluated using the 120-h seed germination/root elongation inhibition test method (US.EPA 1996) using lettuce seeds (*Lactuca sativa* cv. Merveille des quatre saisons). Seed germination rate and root elongation were combined into an index of germination Ig according to the following equation (Barbero et al. 2001):

$$Ig = (Gs \times Ls) / (Gc \times Lc)$$

where Gs is the number of germinated seeds of the sample and Ls is the corresponding root length mean mm; Gc and Lc are the corresponding values for the washed sand control.

Consequently, percent root elongation inhibition could be calculated as follows:

$$\% REI = (1 - Ig) \times 100$$

The different values of REI were treated graphically using Origin 6.0 program to determine the effective concentration (EC_{50}) of sewage sludge in the mixture that causes 50% of REI as compared to sand control.

2.3 Microbiological parameters

The microbiological assessment of the fresh sludge sample was carried out to determine the microbial populations that would be eventually added to croplands after amendments, and to assess the pathogenic potential of this biowaste as well.

2.3.1 Total germs and fecal pollution indicators

A dilution series was first prepared from a sludge-sterilized distilled water suspension (1:10) under strong mechanical stirring to ensure a better desorption and release of the major microbial groups. Total numbers of microorganisms were expressed as Colony-Forming Units (CFU) according to ISO 7218 (ISO 1996); each colony represents a microbial unit. Subsequently, colonies that showed the most distinctive morphologies were isolated and stored. The conservation of purified bacterial isolates was carried out in glycerol (20%) at -20°C.

On the other hand, fecal pathogens in sewage sludge were determined using the most probable number method (MPN) (Rompre et al. 2002). The method is based on the enumeration and the possible

identification of thermo-tolerant fecal coliforms (including *Escherichia coli*) as well as fecal streptococci.

2.3.2 Morphological and biochemical identification of bacterial isolates

Using the purified bacterial isolates preserved at -20°C, an initial morphological classification was conducted based on macroscopic and microscopic observation. In particular, the following characters were considered: color and aspect of the colony, presence or absence of aerial and vegetative mycelium, presence or absence of spores, release of diffusible pigments, melanin release, and finally microscopic shape of cells.

Besides, the biochemical identification aims to classify isolates according to their enzymatic activities. In this study, we addressed the microbial activities of catalase, cellulase, amylase and protease.

The combined polymorphism analysis of morphological and biochemical data was carried out using numerical taxonomy provided by the software "MVSP 3.13n" (MultiVariate Statistical Package), which uses the UPGMA algorithm method (Unweighted Pair-Group Method algorithm) and the Pearson coefficient. Consequently, a dendrogram was drawn to illustrate the classification of different isolates.

2.3.3 Physiological identification: heavy metal resistance

This test was performed to classify isolates according to their sensitivity or resistance to heavy metals. In this regard, Zn and Cu were chosen as model heavy metals for this study since they were the most prevalent in sewage sludge (Table 1). Solid media containing increasing concentrations of dissolved Zn or Cu (0, 200, 400, 800 and 1000 mg L^{-1}) was then prepared and tested on all isolates. After incubation at 30°C during 3 days, we were able to estimate the maximum tolerated concentrations (MTC) for each metal and all isolates.

3. Results and discussion
3.1 Sludge physico-chemical properties

The urban sewage sludge used in this study was characterized by a very low moisture content (2%) as compared to higher values (33-42%) often reported for the same sludge upon collection from the drying beds (Lassoued et al. 2013; 2014). This was a result of the supplementary air-drying performed at the laboratory, which may have positive impacts on the physico-chemical and phytotoxicity characterization.

Compared to analytical results reported for the same urban sludge, it was found that the C/N ratio in our study was relatively higher (15.7, Table 1). In fact, most of previous reports indicate that the C/N ratio of Korba's freshly collected sewage sludge varied between 6 and 10 (Lassoued et al. 2014; Kchaou et al. 2015).The current high C/N ratio can be explained by the long period that separated collection from characterization, which caused a greater biodegradation of nitrogen content (1.2%). In general, outcomes published previously on the same sludge pointed out consistently higher values for water content up to 53% (Rejeb et al. 2011) and nitrogen concentration: 3.87% (Jedidi et al. 2000), 2.25% (Gabteni and Gallali 1988), 2.16% (Bahri 1992), and 1.5% (Kchaou et al. 2015). It is likely that these sludge samples were analyzed directly after sampling without preliminary drying, which explains the lower C/N values. For instance, Bahri and Annabi (2011) proceeded to sludge drying prior to analysis and found a C/N ratio greater than 19. In any case, it is admitted that a biowaste is suitable for soil amendment when C/N ratio is lower than 20 (Lemos et al. 2013). As illustrated in Table 1, heavy metal content in the studied sludge is in accordance with the Tunisian standards for land application (NT 106.20, 2002). Actually, the contaminating potential of urban sludges in croplands depends on several factors: heavy metal content, applied dose, frequency of application, climatic and edaphic conditions, as well as cropping system (dry farmed or irrigated). In addition, the contamination of agricultural soils is reflected by heavy metal content and availability from one side, and by the level of metals that exceed thresholds in the edible tissues of crops from the other side (Kassaoui et al. 2009).

In our study , copper and zinc (respectively 174 and 342 mg kg^{-1}) were the most prevalent heavy metals though their concentrations were still below the Tunisian regulation (respectively 1000 and 2000 mg kg^{-1}) (ISO 1996).

Table 1: Physicochemical properties of the urban sewage sludge collected from wastewater treatment plant of Korba

Physico-chemical parameters	
Moisture content (%)	2.6
pH-water (1:2)	7.7
EC (µS/cm) (1:5)	1702
Total organic carbon (%)	18.5
Total nitrogen (%)	1.2
C/N	15.4
Available phosphorus (mg/kg)	220
Potassium (mg/kg)	9.54
Calcium (mg/kg)	113.5
Sodium (mg/kg)	12.3
Limestone (%)	11.8
Iron (g/kg)	2.08
Cadmium (mg/kg)	4.04
Zinc (mg/kg)	342
Copper (mg/kg)	174
Nickel (mg/kg)	22.2
Lead (mg/kg)	35

3.2 Sludge Phytotoxicity

The relationship between lettuce root elongation inhibition (%REI) and sewage sludge amount is represented in Figure 1. It was observed that when sludge amount in the medium exceeded 5%, there was a complete inhibition of lettuce seed germination. This points out a high phytotoxic potential reflected by a very low effective concentration ($EC_{50} = 0.36\%$) (Fig. 1). Numerically, this corresponds to 0.36 g sludge per 100 g of washed sand or to the addition of approximately 11 t ha^{-1} of the same sludge to a light-textured soil at 0-20 cm depth. This phytotoxicity may have various sources. On one hand, the high salinity of this sludge (1702 µS cm^{-1}, Table 1) could severely hinder germination and root elongation of lettuce seeds during the 5-d bioassay. Lettuce is known to be a very sensitive model plant to high levels of soluble salts in the exposure medium (Hamdi et al. 2007). These soluble salts in the soil solution can completely inhibit seed germination or reduce root growth by limiting water availability due to its high osmotic pressure (Hassanpouraghdam et al. 2009). If bioavailable, phytotoxic heavy metals at high levels may also affect the germination of lettuce seeds (Di Salvatore et al. 2008). In our case, the low concentration of heavy metals in the 100% inhibiting mixture sludge-sand (<5%) in addition to the neutral pH of the medium suggests that the analyzed heavy metals are not directly involved in sludge phytotoxicity. As sewage sludge is a complex matrix, the possible existence of other non-analyzed phytotoxic compounds should be also considered (Hamdi et al. 2007).
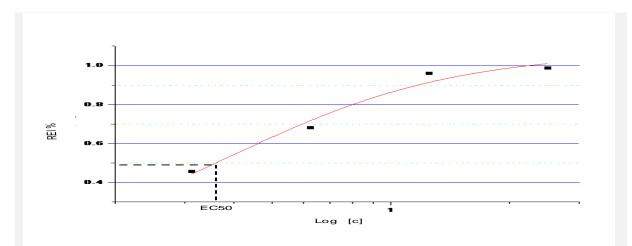

Figure 1: Graphic determination of the effective concentration of sewage sludge that inhibits the root elongation (REI) lettuce seed by 50% (EC_{50}) with respect to inert sand. [C] represents the % concentration of sludge in the mixture sludge-sand.

3.3 Microbiological characterization

3.3.1 Fecal pollution indicators and total microorganisms

In this study, streptococci and fecal coliforms were consistently at the limit of detection by the most probable number method (MPN) in the fresh sludge sample. Figure 2A shows that fecal coliforms reached 900 germs g^{-1}. In addition, the presence of fecal streptococci at a concentration of 20.10^5 germs g^{-1} was also observed. In comparison to Tunisian Standards NT 16.21 and NT 16.22, which report that the number of fecal streptococci and fecal coliforms must not exceed 2.10^3 and 2.10^6 g^{-1} of dry sludge respectively, we can conclude that the current sewage sludge is heavily contaminated by fecal streptococci. However, this contamination is low for fecal coliforms and *E. coli*.

We also counted all microbial colonies (total cultivable communities) and colonies morphologically similar to those of actinomycetes (presence of vegetative and aerial mycelia, presence of spores). The bacterial fraction was the highest and reached more than 125.10^7 CFU g^{-1} of dry sludge (Fig. 2B). Actinomycetes and heterotrophic fungi were less present in the sludge sample (33.10^7 and 3.10^7 CFU g^{-1}, respectively). Thus, actinomycetes accounted for approximately 25% of total cultivable bacteria, which indicates that these organisms occupy a large fraction of the total cultivable bacteria. In fact, actinomycetes provide a metabolic richness to the sludge that involves the synthesis of significant amounts of secondary metabolites required for the efficient degradation of organic matter (Pelmont, 2005).
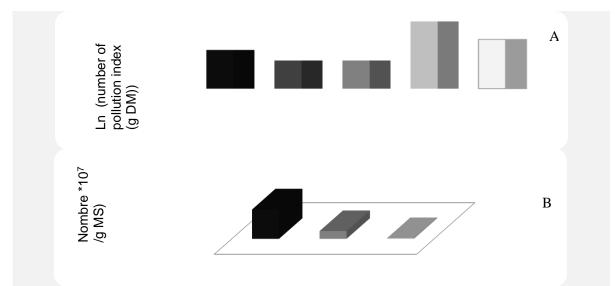

Figure 2: Number of pollution indicators (A) and total cultivable microorganisms (B) per gram of sewage sludge dry matter (average of 3 repetitions).

3.3.2 Phenotypic Isolation and identification of isolates

During the first investigation on bacteria enumeration, we were able to isolate 50 strains with diverse and distinctive morphologies.

3.3.2.1 Morphological identification of isolated bacteria

The outcomes of this classification showed the presence of two major groups: group G and group P. The tested media (GN, PCA or R2A) favored the formation of an abundant vegetative mycelium for isolates of group G (Figures 3a, b and c). The aerial mycelium may be attached to the agar (G1 and G4 groups) or detachable (G2-G3 and G5-G6). The orange-brown pigment observed on the surface of the colonies diffused in most cases on the medium where diffusible pigments could be observed (Figures 3b and c). Besides, the production of a melanoid pigment was noticed for groups G1 and G6 (Figure 3b). The microscopic form of these bacteria was essentially filamentous. These filaments are long or short (Figures 3 g and h) and are entangled to form clusters of mycelium. In contrast, isolates of group P (Figures 3d, e and f) did not have mycelia or spores. Their microscopic shape is spherical in most cases (Groups P1 and P3; Figure 3i). All these observations testified to the presence of a mosaic of bacterial genera and species reflecting a high phylogenetic diversity in the urban sludge described in this study. The comparison between the characteristics of the isolates and the criteria for determining

bacteria described in "Bergey's Manual of Determinative Bacteriology" (Lechevalier 1989), allows us to approximately identify the affiliation of these bacteria and conclude that: group G is for mycelial bacteria corresponding to the actinomycetes group and the P group belongs to non-mycelial bacteria.
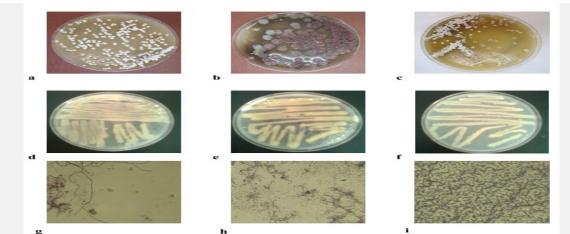

Figure 3: Photos of macro and microscopic observations of some strains isolated from the sewage sludge of Korba.

3.3.2.2 Biochemical identification of isolates

The enzymatic activities of the isolated strains were tested and visualized by observable responses around the colonies. The percentage of strains producing enzymes with respect to the total number of strains (50 isolates),is relatively high. It varies between 30% and 100% (Table 2). These bacteria are mainly producing catalase (95%) and amylase (65%). The outcomes of this biochemical test showed that the majority of active strains belong to the actinomycetes group (G), which has high percentages of producing strains of cellulase and protease (80%). These percentages are higher than those of the P group. In the latter, we noted 50% and 34% of producing strains of cellulase and protease, respectively. All these calculated percentages indicate that the metabolic activity in sludge is high and this allows this biowaste to have a dual role. First, it can act as a natural medium for the production of secondary metabolites (Mehul 2004), and second it is a potential source for the isolation of bacteria producing new bioactive molecules. Bacteria isolation from this sludge led to the constitution of a valuable heterotrophic bacteria collection. Morphological characteristics as well as hydrolytic activities differed considerably from one strain to another.

Table 2: Percentage of bacteria producing enzymes: catalase, amylase, cellulase, and protease (average of 3 repetitions).

Enzyme activities	Percentage of bacteria producing enzymes
Catalase	96%
Amylase	64%
Cellulase	56%
Protease	52%

Accordingly, the analysis by UPGMA algorithm associated to the Pearson coefficient led to the construction of a dendrogram constituted by different groups (Fig.4). Considering the lowest similarity coefficient (0.2), the presence of two major phenotypic groups, P and G was finally confirmed. The G group represents 26% of bacteria and corresponds to mycelial bacteria. It is composed of actinomycetes and consists of 4 phenons. Group P represents 74% of bacteria and corresponds to non-mycelial bacteria consisting of 19 phenons.
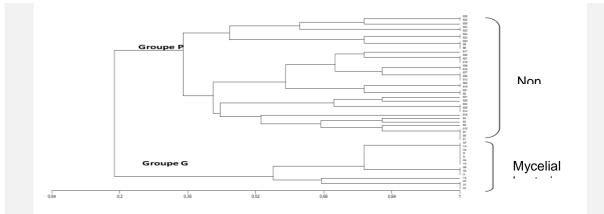
Figure 4: Dendrogram showing 23 bacteria isolated phenotypes constructed following their morphological and biochemical characteristics through the program MVSP V 3.13n (Pearson coefficient / UPGMA algorithm)

3.3.3   Heavy metal resistance

The physicochemical characterization of the sludge sample showed that it was highly contaminated with heavy metals, mainly Zn (342 mg/kg) and Cu (174 mg/kg) (Table 1). Isolates were more resistant to high levels of Zn. In fact we noted that more than 40% of isolates are tolerant to the highest exposure concentration of 1000 mg L^{-1} (Fig. 5). A significant behavior difference was then noticed between the two metals. At low concentrations of Cu from 200 to 400 mg L^{-1}, the number of tolerant isolates varied from 90% to 80%. Once the concentration was increased in the medium, bacterial tolerance decreased significantly to reach 14% to 4% for 800 mg L^{-1} to 1000 mg L^{-1} of Cu respectively. Microbial growth is inhibited in the presence of high copper content ($\geq$ 1,000 mg L^{-1}). Thus, the balance can be changed inducing reverse dominance (Diaz-Baath and Rovina 1996). It has been shown that changes in communities due to the presence of Cu could cause an increase in the relative proportion of gram-negative bacteria (Proteobacteria) or, on the contrary, gram- positive bacteria (Firmicutes) (Ekelund et al. 2003).
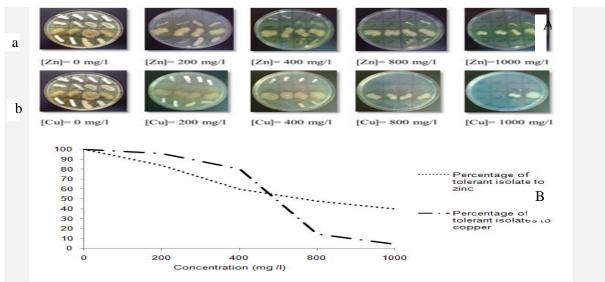

Figure 5: (A) Photos showing colony growth on different metal concentrations: Zinc (a) and copper (b), (B) Percent variation of tolerant isolates to zinc and copper.

4.   Conclusion

In general, the sludge investigated in this study respects the Tunisian standards for agricultural reuse and has been already valorized by farmers of the region, especially for citriculture. Nevertheless the relative high salinity of this sludge could affect its agricultural use on the long term. It is therefore advised to avoid sowing crops that are sensitive to salinity just after amendments. The polymorphism analysis of morphological characteristics obtained by macro and microscopic observation, as well as by enzyme assays, showed the existence of two major phenotypic groups, P (not mycelial bacteria)

and G (mycelial bacteria). Metal-resistance testing of bacterial isolates to major heavy metals in this sludge revealed that the 50 bacterial isolates are more sensitive to copper than zinc. However, interactions with the soil system after addition have to be deeply investigated.

Acknowledgements

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