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Relationship between Microbial C, Microbial N and Microbial DNA Extracts During Municipal Solid Waste Composting Process

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1. Introduction

The municipal solid waste composting process has been defined as a controlled aerobic microbial process widely used to decompose organic matter to obtain a stable product consisting of a humus-like substance (Michel *et al*., 1995). The end product or compost is available for agricultural use. However, the main requirement for the safe use or application of compost to agricultural lands is its degree of stability, which implies stable organic matter content (Castaldi *et al*., 2004, 2008; Mondini *et al*., 2004). This practice is becoming one of the most promising ways for the reclamation of degraded soils in semiarid areas of the Mediterranean countries like Tunisia (Bouzaiane *et al*., 2007 a). Optimization of the composting process depends on optimization of environmental conditions that promote the development and activity of microbial communities. In fact the microbial biomass (MB) amount plays an important role on the biochemical transformations, on the optimization and on the quality of the end product (Mondini *et al*., 2002; Jedidi *et al*., 2004).
The chloroform- fumigation–extraction (CFE) is currently the most common method used to quantify the microbial biomass in soil samples (Vance *et al*., 1987). Some authors have applied the CFE technique on compost substrates (De Nobili *et al*., 1996, Hellmann *et al*., 1997, Mondini *et al*., 1997; Ben Ayed *et al*., 2007).
On the other hand, the application of molecular methods to study the composting process and the microbial communities governing the transformation of the organic matter presents some unique challenges. One such challenge is the dynamic nature of the process, characterized by rapid changes in microbial population, temperature and oxygen gradients, and the availability of nutrients for microorganisms. The analysis of nucleic acids extracted from environmental samples allows researchers to study natural microbial communities without the need for cultivation (Peters *et al*., 2000; Dees and Ghiorse, 2001). Although there have been many published studies on methods for the extraction DNA from environmental samples, very few have focused upon the extraction of DNA from compost. Compost samples may also contain 10–100 times greater humic acid concentrations than mineral soils (Pfaller *et al*., 1994). Humic acids co-purify with DNA during many purification steps (Ogram *et al*., 1987). These factors combine to make DNA quantification in compost

exceptionally difficult. Methods designed to extract DNA from soils and sediments have been adapted to obtain DNA from composts (Blanc *et al*., 1999; Kowalchuk *et al*., 1999). However, the relative effectiveness of extraction and purification methods for isolating compost DNA of sufficient purity has not been examined. Also, potential bias introduced by different extraction protocols has not been investigated yet. In this paper, we adopted the Fast DNA Kit for Soil DNA extraction and purification procedures to extract and purify DNA from compost.

In the present study, we attempted to evaluate (i) the evolution of microbiological parameters such as microbial biomass C, N and DNA content during municipal solid waste composting process and (ii) the relationship between microbial biomass C, N and DNA concentration during municipal solid waste composting process and possibly use these parameters to find out the compost stability.

2. Materials and methods

2.1 Composting process

The compost was prepared at the pilot composting station of Beja City, situated about 100 km to the west of Tunis. At the entry of the composting station, the wastes were stocked on big pile with a pyramidal shape (3.0 m length x 2.5 m width x 1.5 m x high) during 2 months without any previous treatment. The non-biodegradable coarse wastes (mostly plastics and glasses) were manually removed; therefore the remaining wastes were subsequently crushed and sieved to 40 mm in order to decrease the waste heterogeneity. Sawdust and green wastes were added to the wastes and these wastes were stocked on new pile during 3 months for stabilization.

Temperature and humidity were controlled daily, and pile was turned and watered (humidity regularly adjusted to 50%) as soon as the inner temperature of the pile reached or exceed 65°C. These operations of turning and watering were performed almost twice per month on an average.

2.2 Sampling of organic wastes during the composting process

Ten samples (approximately 5 kg each) were collected every 15 days from day 5 to day 139 from ten randomly selected locations in the pile by digging a small pit to 1 m depth with a shovel. At each sampling time, samples were mixed thoroughly and three portions of 1 kg each were separated. The first portion was stored at -20°C to constitute a collection of samples, the second was for pH determination, and the third was for microbiological analyses.

2.3 Temperature and pH determination during the composting process

Temperature inside the windrows was measured, every day during the composting period, with a special sensing device stuck introduced to 60 cm depth in randomly selected points. For pH, 400 g of compost were placed in an Erlenmeyer flask containing 2 l of distilled water and stirred for 3-5 min. The mixture was allowed to settle for 5 min and the pH was measured using a pH meter. For dry weight, 400 g of fresh compost was dried at 105 °C until the weight remained constant.

2.4 Determination of microbial biomass C and N

Microbial biomass C and N were determined by the CFE method, according to Vance *et al*. (1987) and Brookes (1995), respectively. Twenty grams were fumigated with ethanol-free

$CHCl_3$ for 24 h at 25°C in a dessicator. After removing the fumigant the samples were extracted for 60 min with 80 ml 0.5 mol l^{-1} K_2SO_4 solutions (1/4, w/v) and then filtered through a Whatman filter paper. Non-fumigated samples were extracted as above at the time the fumigation started. The amounts of soluble C in the fumigated and non-fumigated compost extract are used to determine biomass C. Organic C was quantified by the potassium dichromate oxidation method (Jenkinson and Powlson, 1976) and subsequent back-titration of the unreduced dichromate. The sample microbial biomass C (MBC) was estimated using the following equation (Jenkinson and Powlson, 1976):

$$MBC = CE/0.35$$

Where CE was the difference between organic C extracted from fumigated and non-fumigated treated samples.
Total N in the extracts was determined according to the Kjeldahl methods as described by Brookes *et al*., 1985.
The microbial biomass N was estimated using the following equation:

$$MBN = NE/0.68$$

Where NE was the difference between total N extracted from fumigated and non fumigated samples. Amounts of microbial biomass C or N were expressed (mg C or N kg-1 dry weight) on air-dry soil basis and represent the average of three determinations (repeated three times on a single sample).

2.5 DNA extraction

About 0.5g of compost was weighed into DNA extraction matrix tubes using the Bio 101 Fast DNA Kit for Soil (Biogene, France). All extraction steps were carried out according to the manufacturer's instructions. DNA was eluted in 100µl of elution buffer. Purified DNA was quantified by spectrophotometer (Bio-RAD Smart Spec TM Plus, France) (Leckie *et al*., 2004). Reserve aliquots were stored at - 20°C and working stocks at 8°C.
The spectrophotometric *A260 /A280* and *A260 /A230* ratios were determined to evaluate levels of protein and humic acid impurities, respectively, in the extracted DNA (Ogram *et al*., 1987; Steffan *et al*., 1988).

2.6 Statistical analysis

The ANOVA analysis was carried out using the SPSS statistical program for Windows (SPSS Inc., Chicago, IL). The means were compared according to the Newman and Keuls multiple range-test at $P < 0.05$.

3. Results

3.1 Physico-chemical parameters of composting process

The physicochemical characteristics evolution obtained during the municipal solid waste composting process was presented in Table 1.
In this study the temperature progress vary according the two phases of composting process, digestion and maturation (Fig. 1). The phase of digestion starts with a mesophilic phase in which the temperature reached 42°C. During this mesophilic step, the humidity rate was up to 45%. After 20 days of composting, the temperature reached 65°C and the

thermophilic step started. In this step the humidity decreased significantly. Then, the temperature decreased gradually to reach 40°C. At the 62 days, and after the addition of sawdust and green wastes in order to enhance the microbial activity, the maturation phase took place. In this phase, like in the digestion phase, the temperature increased gradually to reach 50°C, stabilised for a short period then decreased. In this phase there was also mesophilic, thermophilic and cooling steps.

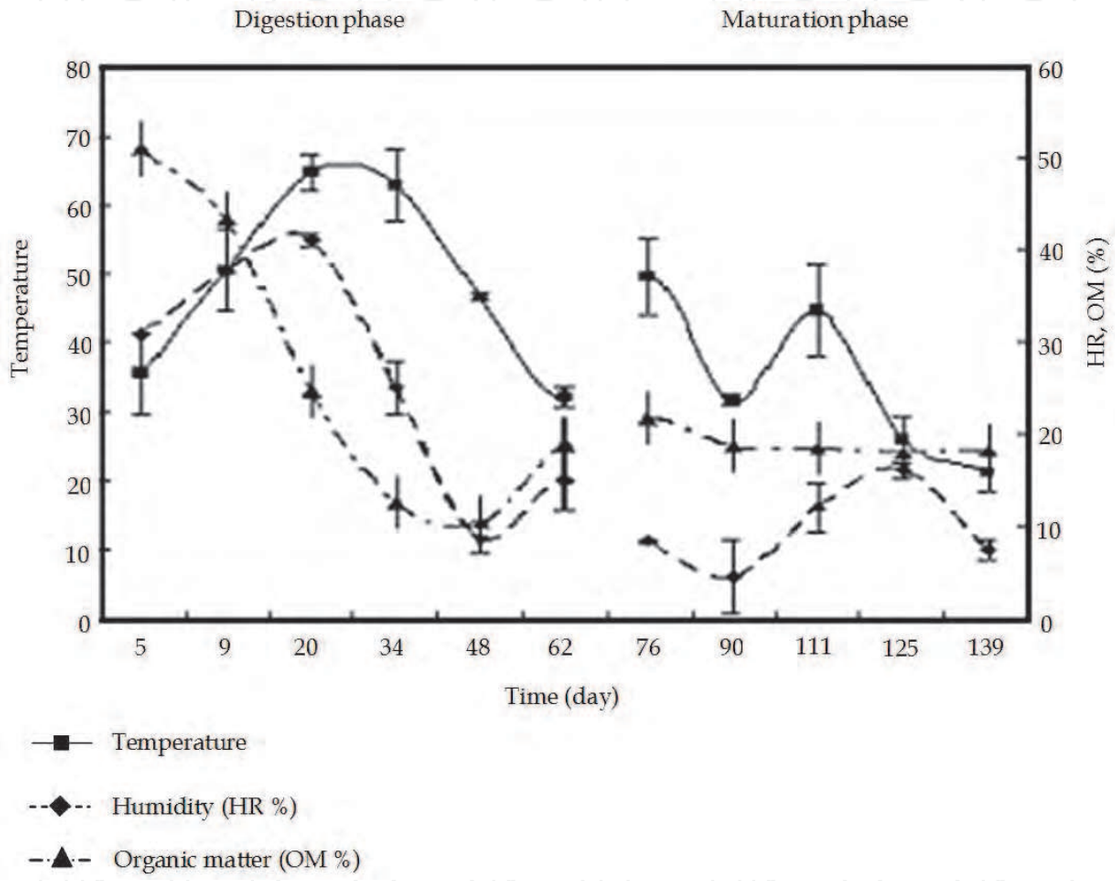

Fig. 1. Progress of temperature, humidity and organic matter during composting process

3.2 Evolution of microbial biomass C, microbial N and microbial DNA extracts during composting process

The progress of microbial biomasses (BC and BN) over time marked a real variation, particularly with a decrease of BC, BN and DNA concentration registered during the digestion and maturation phases (Figure 2). During the digestion phase of composting process microbial biomass C (*B*C) and microbial biomass N (*B*N) ranged from 4.86 to 1 µg kg^{-1} and from 1.472 µg kg^{-1} to 0.65, respectively. During the maturation phase these values decreased to reach 0.44 mg kg^{-1} for BC and 0.26 mg kg^{-1} BN. DNA content evolution ranged from 51.9 to 39 µg g^{-1} of dry matter in digestion phase and this content decrease to reach 18.5 µg g^{-1} of dry matter in the end product.

The BC/BN values registered in digestion phase indicate the dominance of three types of microbial communities. Homogeneous microbial community was found during mesophilic and thermophilic steps of municipal solid waste process was found particularly with BC/BN values of 3.3. Heterogeneous microbial communities were found particularly with BC/BN values of 7.92 and 1.54 (Table 1).

The BC/BN values registered in maturation phase indicate the dominance of two types of microbial communities. Heterogeneous microbial communities were found particularly with BC/BN values of 2.3 and 1.6.

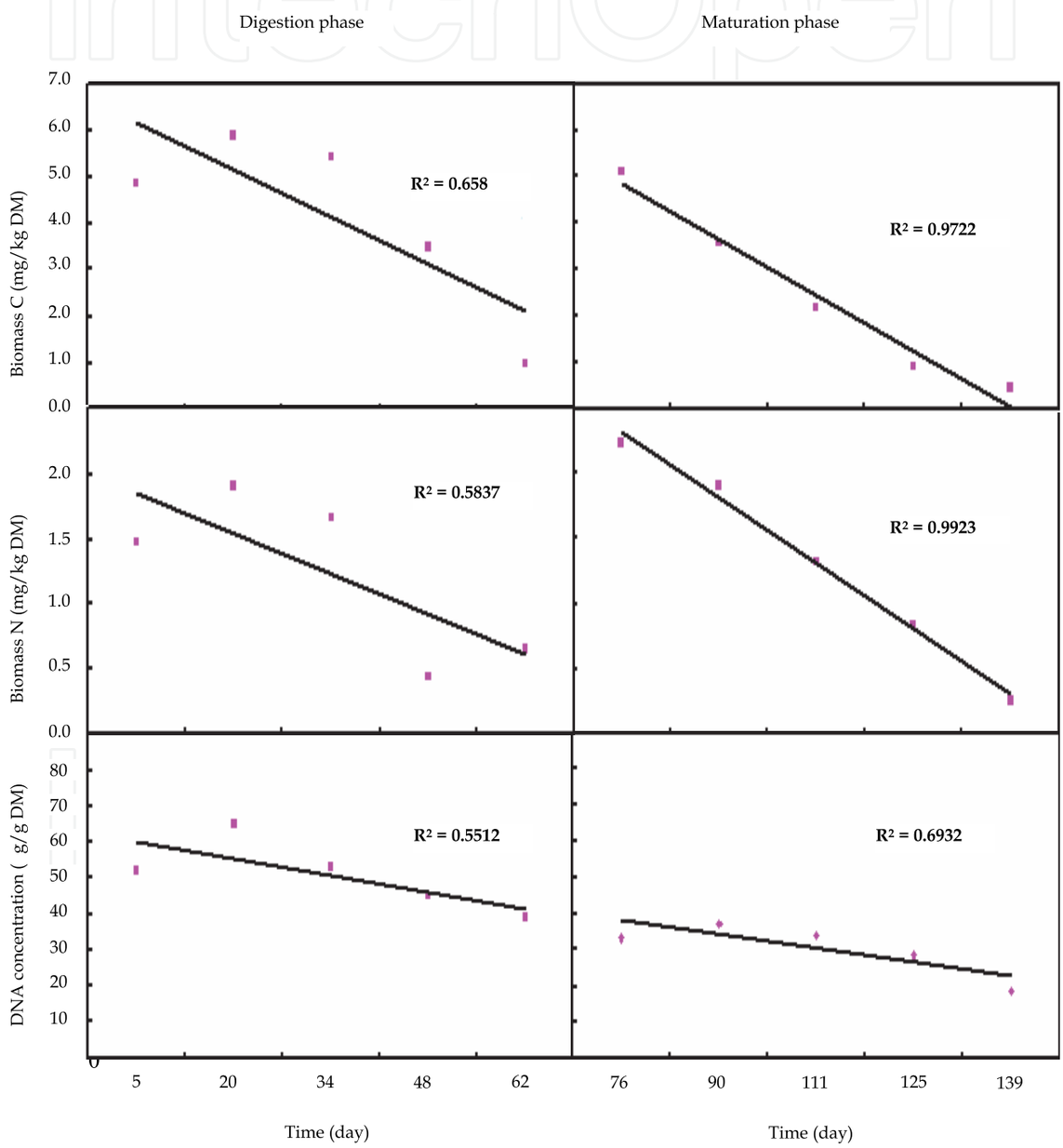

Fig. 2. Progress of microbial biomass C, microbial biomass N and microbial DNA extracts during composting process

The addition of sawdust and green wastes is considered to be a source of organic matter that stimulates microbial biomass. In fact, the addition of sawdust and green wastes affect the structure and composition of the microbial communities that colonize the municipal solid waste.

Days of composting	pH	TOC (mg kg^{-1}DM)	TN (mg kg^{-1}DM)	C/N	BC/BN
5	6.50	31.67	1.14	27.78	3.299
20	6.97	22.10	1.22	18.11	3.077
34	7.81	18.05	1.33	13.57	3.274
48	7.04	14.00	1.78	7.86	7.923
62	7.19	30.32	2..30	13.18	1.540
Addition of sawdust and green wastes					
76	7.41	14.18	1.14	12.44	2.301
90	7.60	15.79	1.21	13.05	1.889
111	7.32	16.54	1.41	11.73	1.662
125	7.52	15.51	1.54	10.07	1.080
139	7.65	10.36	1.14	9.09	1.698

TOC, Total organic carbon; TN, total nitrogen; C/N, carbon: nitrogen ratio; DM: dry matter

Table 1. Physicochemical properties obtained during municipal solid waste composting process

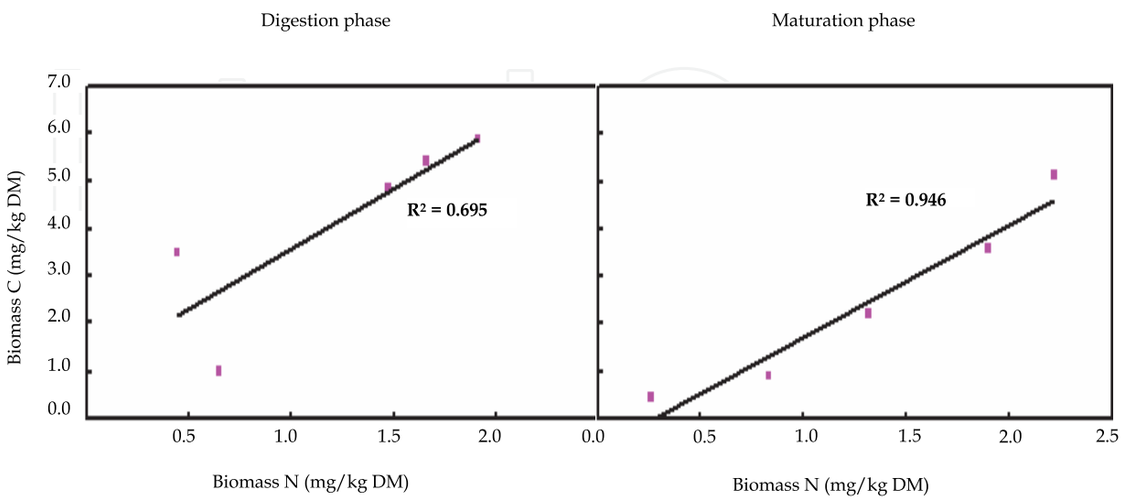

Fig. 3. Relationship between biomass N and biomass C during digestion and maturation phases of composting process.

3.3 Relationship between microbial biomasses BC and BN and DNA content

A good linear relation between microbial BC and BN was found during the digestion and maturation phases, with *r* coefficients of 0.69 and 0.94, respectively (Figure 3). The result showed clearly (*r* coefficients) that the microbial biomasses BC and BN obtained in the digestion phase were higher in comparison with those obtained during the maturation phase.

A linear relationship between biomass C and DNA concentration was found (Fig. 4A and B). DNA concentrations and BC were highly correlated during the digestion phase of municipal solid waste composting process with *r* coefficients of 0.80 (Fig. 4A).

On the other hand there is a linear relationship between biomass N and DNA concentration (Fig. 4C and D). DNA concentrations and BN were highly correlated during the digestion and maturation phases of municipal solid waste composting process with *r* coefficients of 0.78 and 0.76, respectively (Fig. 4B). Nevertheless, the DNA concentration was generally proportional to the BC or to BN and both methods seemed to give reliable values of compost microbial biomass. Our results indicate that BC and BN and DNA contents of the compost can be related with biological and chemical parameters in a combined way.

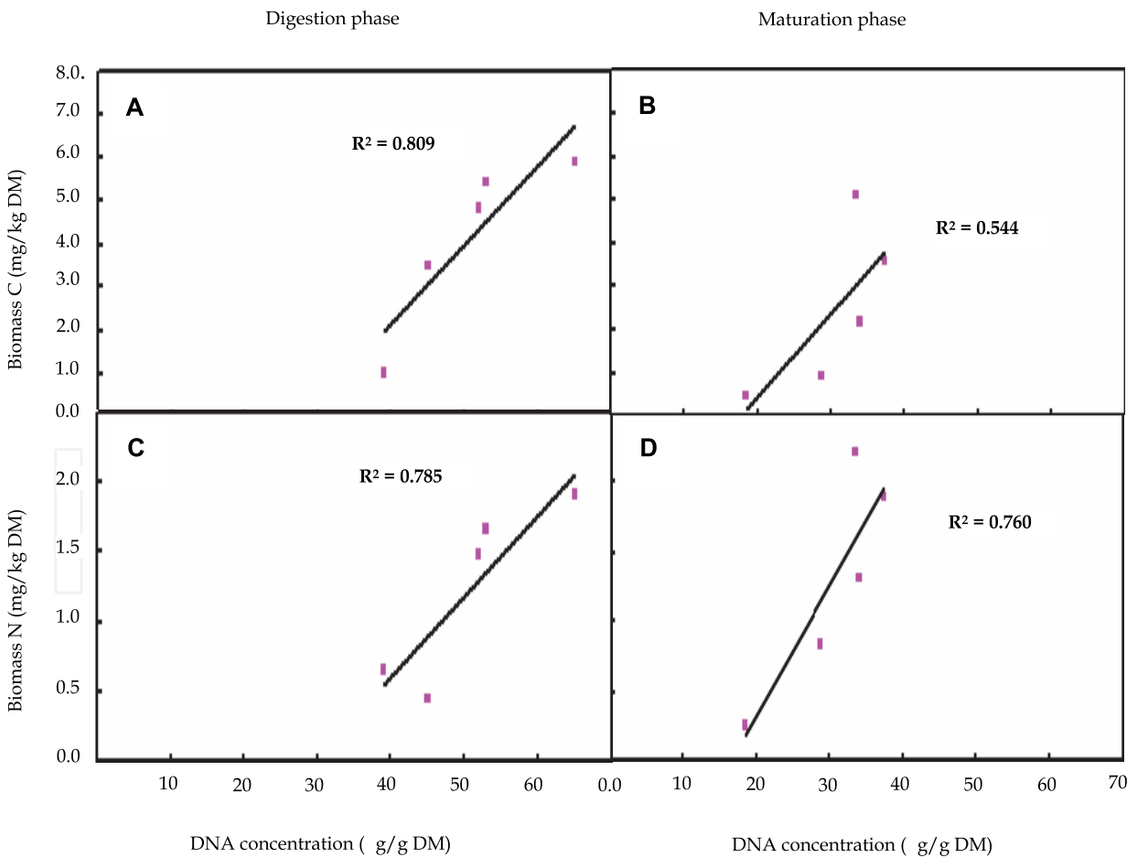

Fig. 4. Relationship between DNA concentration and biomass C (A and B) and biomass N (C and D) during digestion and maturation phases of composting process

3.4 Humic acid and protein impurities during composting

The *A260 /A230* and *A260 /A280* ratios for compost DNA were significantly lower than the ratios for DNA solutions from pure cultures showing that compost DNA was coextracted with humic compounds (Table 2).

DNA extracts from the cooling stage of maturation phase showed the lowest ratio *A260/A280* and *A260/A230* ratios than those obtained with the other stage of composting which may due to the high proportion of humic acids with the composting progress. Accordingly, the decrease in the microbial biomass DNA concentration in the cooling stage of composting could be explained by the DNA binding to compost humic acids and the formation of humic-DNA complexes.

The extracted DNA with low *A260 /A230* or unsuitable *A260 /A280* ratio decreases the efficiency of PCR amplification.

The extraction method will be suitable for the DNA purity. The purity will determine the extent to which the microbial DNA template can be amplified by PCR during the composting analysis. However, in this study the humic acid content could not interfere with PCR. Then the PCR products were successfully used for DGGE analysis (data not shown).

The DNA extract was thus suitable to be used for molecular studies on the microbial communities in municipal solid waste composting process.

Days of composting	DNA yield (µg DNA g^{-1} DM)	A260/A280 ratio	A260/A230 ratio
5	51.90	1.33 (0.04) d	1.02 (0.01) d
20	65.00	1.31(0.05) d	1.03 (0.04) d
34	53.00	1.32 (0.02) d	1.02 (0.02) d
48	45,00	1.22 (0.02) c	0.94 (0.04) c
62	39.00	1.22 (0.03) c	0.95 (0.03) c
Addition of sawdust and green wastes			
76	33.50	0.98 (0.03) b	0.71 (0.01) b
90	37.40	0.98 (0.01) b	0.73 (0.03) b
111	34.00	0.89 (0.03) a	0.72 (0.04) b
125	28.70	0.87 (0.04) a	0.66 (0.04) a
139	18,50	0.88 (0.02) a	0.65 (0.02) a
Pure culture		1.89	1.57

Pure culture: DNA from Gram positive bacteria. n = 3 determined by spectrophotometry at 260 nm (A260), 280 nm (A280) and 230 nm (A230); (In brackets): standard deviation; within a column different letter after bracket means that the value is significantly difference according to Student-Newmann-Keuls test at $P < 0.05$; DM: dry matter

Table 2. Comparison of compost DNA yields and purity

5. Discussion

5.1 Physico-chemical parameters of composting process

The composting process at the microbial level involves several interrelated factors, namely temperature, ventilation (O2 imputed), moisture content and available nutrients. Based on temperature, the process of aerobic composting can be divided into three major steps, a mesophilic-heating step, a thermophilic step and a cooling step (Mustin, 1987). During the mesophilic step, the temperature and the water content increased as a consequence of biodegradation of organic compounds. The temperature increment is the consequence of the organic matter oxidation (Hassen *et al*., 2001). The mesophilic step is followed by the thermophilic step. The latter step occurred between days 20 and days 34 of the composting process. As mentioned by Hachicha *et al*., 1992 and Marrug *et al*., 1993, a temperature above 60 °C seriously affect the decomposition rate of the organic waste as a result of a reduction in microbiological activity. The temperature started to decrease after 48 days, and then increased again after the addition of fresh organic matter. A second decrease of the temperature then occurred after 111 days of the process, this decrease led to the depletion of organic matters and the carbon/azotes (C/N) ratio tended to stabilize. By the end of the composting process, the average temperature inside the windrow showed a decrement and reached approximately 30 °C at the end of the process (Ben Ayed *et al*., 2007).

Composting is a self-heating, aerobic, solid phase, useful way of transforming organic wastes into valuable organic matter for use as an organic amendment for soils. The composting process can provide stable and valuable substrates through the bio-oxidation of the organic fraction deriving from different waste matrices (Castaldi *et al*., 2004, 2008). Many tests have been considered as maturity indices for compost, and most of them focus on the chemical and physical properties of compost. The most common parameters include compost temperature, pH, cation exchange capacity, dissolved organic C, C/N ratio, humification index, plant growth bioassay, spectroscopic methods, etc. (Garcia *et al*., 1992; Castaldi *et al*., 2004).

5.2 Evolution of microbial biomass C, microbial N and microbial DNA extracts during composting process

The evolution of microbial biomass C, microbial N and microbial DNA extracts during composting process is probably related to the availability of readily decomposable substrates; in fact when organisms are presented with a substrate they normally multiply rapidly until the substrate is nearly exhausted, when numbers reach a maximum (Joergensen *et al*., 1990; Ben Ayed *et al*., 2007). Thereafter, with the exhaustion of these substances caused by the intense microbial activity and by ongoing humification, the microbial biomass decreased. The BC and BN decreased possibly due to the degradation of the depletion of organic substrates available for micro-organisms growth (Manuael *et al*., 2009).

With the progress of the process the DNA content decrease and the extraction and purification method yielded 18.5 µg DNA/g of dry compost in the end of the process. Howeler *et al*., 2003 found 18.2 µg DNA/g of wet compost yielded by extraction and purification method from compost.

This result could be explained by (i) the microbial DNases degradation or by (ii) the protection of the DNA by binding to compost humic acids. The formation of humic-DNA complexes should be considered as a process related to the changes in compost matrix, i.e. formation of humic like substances, which is one of the main purposes for the composting process.

Biological parameters such as microbial biomass are useful indicators of biological activity in ecosystems (Benitez *et al*., 1999). Since, during the composting process microbial biomass C, microbial biomass N and DNA contents could indicate compost stability, defined as the degree of decomposition of the readily biodegradable organic matter.

5.3 Relationship between microbial BC and BN and DNA

A good linear relationship between microbial BC and BN, during the different stages of composting process. The same result was found during three consecutive years of compost amendment at the level of the upper and deep horizon of non cultivated soil (Bouzaiane *et al*., 2007 a). Jedidi *et al*., 2004 found the same linear relationship between BC and BN in amended soil and in laboratory conditions. Franzluebbers *et al*., 1995 found the same linear relationship between BC and BN with $r = 0.86$.

In the composting process the humification and mineralization of organic substances occurs simultaneously. The DNA content, BC and BN could be related to the humification index and degree of polymerisation evolutions.

In the digestion phase we think that the micro-organisms diversity is due to the incorporation of extra-cellular DNA from degrade microbial in to bacterial genome as possible source of genetic instructions (transformation, conjugation and transduction).

Similar results were obtained by Bouzaiane *et al*., 2007b who found a strong relationship between BC, BN estimated by CFE, and extracted DNA in cultivated-compost-amended soil. Marstorp *et al*., 2000 found also a strong relationship between BC, estimated by CFE, and extracted DNA in a mineral soil. They suggested that DNA could be used as a measure of microbial biomass in agricultural soils with low organic matter content. Tejada *et al*., 2009 were found a strong correlation between biological and chemical parameters during municipal solid waste composting process.

Tejada *et al*., 2009 suggested that humification index (HI) and degree of polymerisation (DP) of the compost can be related with biological and chemical parameters in a combined way.

5.4 Humic acid and protein impurities during composting

The humic acid increased during municipal solid waste composting process. Also Tejada *et al*., 2009 showed that the humic index and degree of polymerisation parameters, both increased during composting process (66% and 41%, respectively at the end of the composting process when compared to values at 0 days).

Composting DNA was often contaminated with humic acid or proteins that interfered with accurate quantification of DNA by UV absorbance at 260 nm (Tebbe and Vahjen, 1993; Kuske *et al*., 1998). In this work, we used Fast DNA Kit for Soil DNA extraction and UV absorbance at 260 nm to detect very low DNA concentrations in diluted samples (typically 100 to 1000 fold), so that the effect of humic acid contamination could be ignored. UV absorbance at 260 nm was an excellent method for DNA quantification of samples extracted from environmental sources containing high levels of humic acids. A simple and accurate method of humic acid quantification (e.g. absorbance) should also be used to determine the correct dilution required for DNA quantification and to measure the progress of humic acid.

6. Conclusion

It can be concluded that the microbial biomass C and N and DNA content during the municipal solid waste composting process can be of great use in understanding the

compost stability state. This fact does not mean that the study of these biological properties diminishes the study of the chemical properties, but rather, both types of properties can be combined to indicate the compost stability. In fact the linear regression analysis developed in this work indicates a strong relationship between the biological properties. On the other hand the commercial method for extraction DNA was suitable for PCR-DNA amplification of microbial analysis during the composting of municipal solid waste and of the end product such as the compost that could be used for the detection of microbial pathogens.

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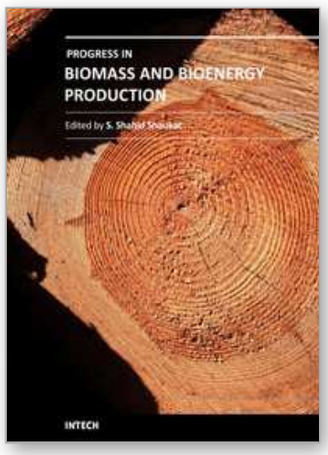

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Alternative energy sources have become a hot topic in recent years. The supply of fossil fuel, which provides about 95 percent of total energy demand today, will eventually run out in a few decades. By contrast, biomass and biofuel have the potential to become one of the major global primary energy source along with other alternate energy sources in the years to come. A wide variety of biomass conversion options with different performance characteristics exists. The goal of this book is to provide the readers with current state of art about biomass and bioenergy production and some other environmental technologies such as Wastewater treatment, Biosorption and Bio-economics. Organized around providing recent methodology, current state of modelling and techniques of parameter estimation in gasification process are presented at length. As such, this volume can be used by undergraduate and graduate students as a reference book and by the researchers and environmental engineers for reviewing the current state of knowledge on biomass and bioenergy production, biosorption and wastewater treatment.

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