Journal of Hazardous Materials, Volume 181, Issues 1-3, 15 September 2010, Pages 1163-1169

# Effect of pine bark and compost on the biological denitrification process of non-hazardous landfill leachate: focus on the microbiology

Cristina Trois[a*], Frédéric Coulon[b], Cécile Polge de Combret[a], , Jean M.F. Martins[c], Laurent Oxarango[c],

[a] Centre for Research in Environmental, Coastal and Hydrological Engineering, School of Civil Engineering, Surveying and Construction, University of KwaZulu-Natal, Howard College Campus, Durban, 4041, South Africa.

[b] Centre for Resource Management and Efficiency, School of Applied Sciences, Cranfield University, MK43 0AL, United Kingdom

[c] Laboratoire d'étude de Transferts en Hydrologie et Environnement, UMR 5564 CNRS/INPG/IRD/UJF), Université de Grenoble, BP 53, 38041 Grenoble Cedex 9, France

*Corresponding author: (Prof. C. Trois) troisc@ukzn.ac.za ; Tel: +27 (0)31 260 3065

**Abstract:** In an attempt to optimize the cost-efficiency of landfill leachate treatment by biological denitrification process, our study focused on finding low-cost alternatives to traditional expensive chemicals such as composted garden refuse and pine bark, which are both available in large amount in South African landfill sites. The overall objective was to assess the behaviour of the bacterial community in relation to each substrate while treating high strength landfill leachates. Denitrification processes in fixed bed reactors were simulated at laboratory scale using anaerobic batch tests with immature compost and pine bark. High strength leachate was simulated using a solution of water and nitrate at a concentration of 500 mg $l^{-1}$. Results suggest that pine bark released large amounts of phenolic compounds and hydroxylated benzene rings, which both can delay the acclimatization time and inhibit the biological denitrification (only 30% efficiency). Furthermore, presence of potential pathogens like *Enterobacter* and Pantoea agglomerans prevents the applicability of the pine bark in full-scale operations. On the other hand, lightly composted garden refuse (CGR) offered an adequate substrate for the formation of a biofilm necessary to complete the denitrification process (total nitrate removal observed within 7 days). CGR further contributed to a rapid establishment of an active consortium of denitrifiers including *Acinetobacter, Rhizobium, Thermomonas, Rheinheimera, Phaeospirillum* and *Flavobacterium*. Clearly the original composition, nature, carbon to nitrogen ratio (C/N) and degree of maturity and stability of the substrates play a key role in the denitrification process, impacting directly on the development of the bacterial population and, therefore, on the long term removal efficiency.

**Keywords**: denitrification; landfill leachate; compost; pine bark; microbial diversity

## Introduction

The majority of municipal solid waste landfills, including those that previously co-disposed hazardous materials continue to receive a significant proportion of bioreactive wastes which produce mainly greenhouse gases and wastewater known as leachate [1]. Landfill leachate contains organic and inorganic pollutants including humic acids, ammonia, heavy metals, persistent organic pollutants and inorganic salts at high concentrations (e.g. Chemical Oxygen Demand (COD) between 2000 - 6000 mg $l^{-1}$, ammonia between 1000-1600 mg $l^{-1}$ and chloride between 1500-2600 mg $l^{-1}$) [2]. If they are not collected carefully and not discharged safely, they may become a potential pollution source which threats soil, surface water and groundwater [3]. Therefore, landfill leachate is recognized as an important environmental problem by modern societies. In the treatment of landfill leachate, biological systems such as nitrification-denitrification processes are frequently used [4-6]. Even though, these systems ensure a high Biological Oxygen Demand (BOD) removal efficiency, they are usually insufficient in degrading high-molecular-weight fractions and decolouring, and their efficiency is often susceptible to the presence of toxic substances and presence of refractory organics such as humic acids and surfactants [7]. In old sanitary landfills, the amount of organic materials having high molecular weight in leachate is high [7]. In the treatment of these wastewaters, therefore, combined systems including many processes such as aerobic–anaerobic decomposition, chemical oxidation, coagulation–flocculation and adsorption are preferred to single-process solutions [1]. However these combined treatment processes are often costly in terms of capital investment, energy requirements and frequent use of additional chemicals [1,7]. Other methods such as reverse osmosis, active carbon adsorption and advanced oxidation processes have been recently

pointed out as more versatile methods, however they only transfer the pollutants without solving the environmental problem and their full-scale application is not often economically feasible [1,8]. Cleary there is a need to re-evaluate the methods to remove contaminants from landfill leachate in order to shift from "waste treatment" to exploitation of landfill leachate as a resource that can be processed for recovery of energy, nutrients and other constituents.

Biological denitrification is one of the most promising and versatile approaches in the treatment of landfill leachate [7, 8]. In this process, an external organic substrate (i.e. methanol, ethanol, acetic acid) or electron donor is needed [9, 10]. While these compounds are expensive and potentially dangerous, some complex substrates such as tree barks, wood chips, corncobs, sawdust, compost [11] and newspapers [12] have proved to be efficient carbon sources for denitrification and generally more suited to treat high strength effluents [7, 11]. These natural substrates are normally cheaper than the synthetic ones and can be derived from a typical waste stream [13, 14].

Biological denitrification of landfill leachate is often undertaken in sequencing batch reactors (SBR) [7] or in constructed wetlands (CW) [15]. Both treatments are known for their flexibility in terms of adaptation to leachate nature and collection strategies [16, 17]. However, the influence that specific substrates have on the development and nature of active microbial populations is not yet widely understood [15]. Indeed, performance and stability of a bio-denitrification process, as of any biological process, depend on the concentration of the active species and on their metabolic activity. Little is known about their diversity, distribution, metabolic potential and functional roles. The nitrate-based

microbial communities of which they are members remain uncertain as well as the identity of their major and minor players and the ecological parameters that influence denitrification. This information is crucial to better understand the bio-denitrification process particularly in high strength landfill leachate and for the development of knowledge-based technologies to accelerate and optimize this treatment.

The objective of this study was to investigate the influence of garden refuse compost and pine bark on the microbial diversity and denitrification activity in the treatment of high strength nitrified landfill leachates (nitrate concentrations ranging between 500 up to 2000 mg $l^{-1}$). The growth of the microbial community was followed using a spread plate enumeration technique; the colonization of the substrates was assessed through Environmental Scanning Electronic Microscopy (ESEM), and insight into the composition of the bacterial community was obtained by phylogenetic analysis.

## 1. Materials and methods

### 1.1. Leachate selection

To avoid analytical interferences, treated leachate from an SBR was simulated in the laboratory with a synthetic solution of potassium nitrate and distilled water with a concentration of 500 mg $l^{-1}$ of $NO_3^-$.

### 1.2. Carbon sources selection

Commercial (CGR) and domestic (DGR) garden refuse and pine bark (PB) were collected at the Mariannhill landfill site, Durban, South Africa. The garden refuse was composted for 10 weeks in pilot-scale forced aerated vessels at the University of KwaZulu-Natal, Durban,

South Africa. The properties of the solid substrates were characterized according to standard analytical methods as published by the American Public Health Association [18]: moisture content, total solids, volatile solids, C/N, Dynamic Respiration Index at 7 days ($DRI_7$), determined with an OxiTop® respirometric system. Eluate tests were conducted to assess amount and nature of the compounds leached-out from the substrates in distilled water during 24 hours, using a 10/1 Liquid to Solid ratio (L/S) [11]. The following parameters were measured: total solids, volatile solids, pH, conductivity (ρ), COD, $BOD_5$, $NH_3$, NOx and C/N ratio [18]. All analyses were carried out in triplicate.

### 1.3. Batch tests

Batch experiments were designed to study the denitrification patterns of the synthetic leachate using the three substrates as carbon sources. Duplicate tests were conducted in 1.5 L anaerobic bottles equipped with two airtight silicone septa that allow for continuous sampling avoiding air ingress. Each substrate (S) was mixed with the synthetic leachate (L) at L/S=10/1 to ensure full saturation in the batch reactors (Table 1). As the size distribution and consistency of the three substrates were different, and the pine bark chips were reduced to 2-3 cm, varying amounts of materials (masses) were used in setting up the reactors, as reported in Table 1. A control test with distilled water was also carried out for each substrate. Optimal environmental conditions and full liquid to solid transfer were obtained by performing the experiments at a controlled temperature of 25 ºC and by shaking at 150 rpm. The batches were flushed with $N_2$ to set anaerobic conditions.

For nitrate and pH testing, 2 mL samples were collected with a precision syringe connected

to a 0.45 μm filter after 5, 10, 15, 30 and 60 min and then every hour during the first day. Afterwards, samples were collected four times a day. Nitrate and nitrite concentrations were analyzed using Nitrate Test Sticks type Merckoquant (MERCK). This method was selected to avoid large variations of the L/S ratio with time maintaining a reasonable accuracy (error within 10-15%). 1.5 mL samples were taken three times a day with a sterile syringe for microbiological analyses from Batch 1 for each substrate (Fig. 1, 2, and 3). The experiment was stopped when total denitrification was achieved, except for the pine bark for which the final concentration never fell within the discharge limits during the experimental time. The output COD, ammonia and pH were then analyzed on the filtered eluates.

### 1.4. Batch inoculation

In order to investigate the effect of inoculation on the reaction rate and the acclimatization time, 5 ml of solution of the first CGR test, were used to inoculate a second CGR batch prepared in the same conditions.

### 1.5. Semi-quantitative analysis of the bacterial community

The effect of the substrates on the growth of the bacterial populations was assessed during the batch tests. The 9215-C spread plate method [18] was applied to enumerate the aerobic cultivable microflora. A laminar flow cabinet was used to work in a sterile atmosphere. Samples were diluted in sodium chloride solution at 9 g $l^{-1}$ and 100 μl of each dilution ($10^{-3}$ to $10^{-7}$) were spread on 90 mm agar plates using the Luria-Bertani Broth. The glass rods

were replaced by 4 mm glass beads spread on the plate. Plates were incubated at room temperature (25 ºC) in the dark and enumeration was carried out visually after 3 days [19].

### 1.6. Microscopic analysis of the bacterial community

Colonization of the different solid substrates was assessed using an Environmental Scanning Electronic Microscope (ESEM Philips, FEI XL 30). Samples were fixed in 3% (v/v) glutaraldehyde, washed twice in 0.05 M cacodylate buffer (pH 7.1) for 10 min and dehydrated in an alcohol series (10 min each in 30, 50, 70, 80, 90%, and 3×10 min in 100%) in a fume cupboard. The specimens were then transferred into critical point drier baskets under 100% alcohol and dried in a pre-cooled Hitachi HCP-2 critical point drier. After gold palladium sputter coating (Polaron Equipment Limited SEM, coating unit E5100), the samples were examined in the ESEM at 10 keV.

### 1.7. Genetic analysis of the bacterial community

DNA extraction from the PB (at 2.5 h, 96 h and 263 h) and the CGR (at 3 h, 74 h and 162 h) liquid samples was carried out using the Zymo Research Fungal/Bacterial DNA extraction kit as described by Lejon et al. [20]. Purified DNA was suspended in 50 µL of sterile water and examined by agarose gel electrophoresis. All extracted genomic DNA samples were stored at –20 °C until further processing. The 16S rRNA gene was amplified by PCR using universal bacterial 16S primers 27-F and 1492-R [21]. PCR amplification was performed using Lucigen EconoTaq plus Green master mix. PCR products (approximately 1400 bp) were analyzed on a 1% agarose gel and cleaned with PCR purification kit (Qiagen). PCR products were then cloned using the CloneJet kit

(Fermentas) according to the manufacture's specifications. The screening of inserts from the transformants was performed by direct PCR amplification from colonies using primers for the pJET1.2F and pJET1.2R sites on the plasmid. Amplified inserts were identified on gel electrophoresis and cleaned by using the ZR-DNA Sequencing Clean-up kit™ (Zymo Research Corp). DNA sequences were determined by using an ABI 3130XL genetic analyzer and the BigDye terminator v3.1 cycle sequencing kit (PE Applied Biosystems). Sequences were compared to the GenBank nucleotide database library by BLAST on-line searches [22].

## 2. Results and discussion

### 2.1. Substrates characterization

Characterization of the solid matter showed that different origins and composition of the domestic garden refuse in relation to the commercial sample are evident (Table 2). Primarily large palm leaves, grass and twigs constituted the former, while the latter contained largely woody waste, tree bark and branches that made it more similar to the pine bark. These differences in composition, associated with the substrates' origins and collection methods, reflect also the amount and nature of the available carbon for denitrification which was two times higher in CGR than in DGR (Table 2). The high C/N ratio for the pine bark fell within the expected range as in literature, while the low value for the compost suggested an IV and V degree of maturity for the CGR and DGR, respectively (DIN 4187), with levels slightly higher than the optimum range of 13-16 for stabilised garden refuse compost [11]. Overall, CGR and PB displayed similar characteristics with respect to their composition, origin and C/N before composting suggesting a similarity in

the way carbon is released during denitrification. It is also worthy to note that high amounts of nitrogen, COD and TS are released from the DGR after 24 hours contact time with water (eluate tests) as well as during the batch tests (Table 3) through mechanisms of bio-leaching as observed also by other authors [6, 13, 33]. During this initial period ammonia is promptly converted into nitrites by nitrifiers as oxygen is still trapped in the water, while denitrification is limited by the availability of electron donors with a consequent increase in nitrate concentrations [6].

### 2.2. Batch tests

Although no significant differences were observed for the DGR, CGR and PB Batch tests in term of nitrate removal, each substrate showed a distinct biotransformation rate. In the test with the CGR, complete removal of nitrates in solution was achieved within 8 days (Fig. 1). The DGR tests showed a large initial release of nitrate (500 mg $l^{-1}$) in solution by the substrate, independently of the input nitrate concentration (Fig. 1). However, the nitrate consumption rate remained close to that of the CGR tests and the complete denitrification was achieved within 8 days. The onset of denitrification was generally slower in the tests with PB and complete nitrate removal was not achieved, as the final concentration plateaued around 150 mg $l^{-1}$ after 11 days (data not shown). This finding suggests the occurrence of a strong inhibitory effect on the active denitrifier population. Further this could be explained by the low pH observed during the batch tests with PB (Table 4), as suggested by other studies [6, 13, 33]. Although a neutral pH in the batch tests with compost could suggest a more favourable condition for microbial activity, the high release of COD and nitrate in the DGR is of concern and would require further investigation (Table 4).

### 2.3. Initial inoculation effect

No direct effects were observed on nitrate removal in the inoculated batch test (data not shown). In a denitrification study using newspapers as a carbon source, Volokita et al. [12] found that an initial inoculation with a solid matrix was far more efficient than with a liquid inoculum. On the contrary, Ovez et al. [9] reported an inhibitory effect when inoculating their batches with bacteria from previous experiments. These contrasting effects might be explained by the extreme complexity of the microbial community established during the denitrification process, which is strongly dependent on the nature of the substrate and the experimental conditions. In general, inoculation using a solid substrate containing a well-established microflora should be preferred to an inoculum derived from the liquid phase.

### 2.4. Effect of the solid substrates on the size of the aerobic bacterial community

The number of colony forming units (CFU) for both the CGR and DGR (Fig. 2) was estimated to be $5.10^7$ CFU $ml^{-1}$ at the beginning of the experiment and decreased by five orders of magnitude during the first two days. The viable bacterial community present in the PB test at the beginning of the experiment was accounted to $3.10^8$ CFU $ml^{-1}$, which is ten times higher than in the compost tests (Fig. 2). A logarithmic decrease ($R^2$=0.94) was observed during the first 7 days, leading to a constant bacteria concentration of $1.10^7$ CFU $ml^{-1}$ until the test was stopped. Assuming CFU were mainly using carbon and nitrate for their development, it should be possible to establish a relationship between CFU numbers and denitrification rate. The correlation between these two parameters for the liquid phase of the PB batch tests was good ($r^2 > 0.80$) and confirmed that carbon and nitrate depletions

were mainly related to the microbial activity. Whilst this finding is in agreement with previous studies [6, 7, 8], it should be interpreted with much care. Indeed, the enumeration of the bacteria in the liquid phase does not account for those proliferating on the surface of the substrates (biofilms), and as such, it may not constitute a reliable indicator [7].

### 2.5. Effect of the solid substrates on the bacterial community

Phylogenetic analysis was performed on 16S ribosomal DNA for each treatment in order to obtain further insight into the bacterial community structure and dynamics (Table 2). Even if the same tendencies were observed for the three treatments (dominance of *Gamma-proteobacteria*, *Firmicutes* and *Bacteroidetes* in all libraries) differences were observed between composts and PB applications. During the acclimatization period, the bacterial community observed in the CGR and DGR tests was essentially composed of *Gammaproteobacteria* commonly found in natural environments, *e.g. Pseudomonas putida*, *Pseudoxanthomonas*, *Rheinheimera sp.* [23]. In contrast, the PB test was dominated by Enterobacteria including *Rahnella*, *Panteoa*, *Kluyvera* and *Enterobacter* which are typical of pine bark [24]. The population of *Rahnella sp.* largely dominated during the experiment while Pantoea agglomerans disappeared halfway through the experiment as being outcompeted by *Lactobacillus* and *Erwinia sp*. which both are known to be unable to reduce nitrate [25]. *Enterobacter sp.* and Pantoea agglomerans are potential human pathogens [24] and as such could prevent the applicability of the pine bark in full-scale operations. .

Bacteria capable of reducing nitrate into ammonia such as *Acinetobacter sp.* for *Gammaproteobacteria* [26] and *Clostridium sp.* for *Firmicutes* [27] as well as bacteria

capable of dissimilatory nitrate reduction such as *Rhizobium sp.* and *Thermomonas sp*. [28]) were identified after 74 hours treatment in the CGR eluates. In contrast, dominance of *Thermoactinomyces* in the DGR eluate after 74 hours suggests that these bacteria can first produce nitrous acid from nitrate followed by the generation of ammonium as they have both nitrate-reducing and ammonium-forming ability [29]. Over time, the bacterial community in the CGR eluate evolved towards a consortium of denitrifiers mainly composed of *Rheinheimera sp., Phaeospirillum sp*. and *Flavobacterium sp.* [23, 30, 31]. *Phaespirillum sp*. has been described as being able to use ammonia as a nitrogen source [31]. This suggests that it could counterbalance the presence of the ammonia-producing bacteria present in the second step of the experiment. This hypothesis is further supported by the low concentration of ammonia found at the end of the experiment ($NH_3$-N = 3 mg $L^{-1}$)

### 2.6. Bacterial colonization of the solid substrates

The interpretation of ESEM micrographs could be challenging as the preparation of the samples may significantly change the matrix structure through shrinking and deformation [32]. To overcome this limitation, solid substrates before and after treatment were compared. Before treatment, cocci and fungal spores were the two most abundant organisms colonizing the surfaces of CGR while numerous cocci and rod-shaped bacteria were observed on the surfaces of DGR (Fig. 3 and 4). After treatment, rod-shaped bacteria dominantly colonized CGR surfaces (Fig. 5) whilst no changes were observed in DGR tests (data not shown). This finding suggests that the composted domestic garden refuse (DGR) offers a favourable surface for the rapid development of a biofilm of denitrifiers and that

$NO_3^-$ exerts a selective pressure on promoting the growth of rod-shaped bacteria leading to the formation of a superficial biofilm.

Numerous cocci were visible on the surface of the PB before incubation (Fig. 6). After treatment, very few bacterial cells were observed in the control and nitrate-rich tests (data not shown) due to possible inhibitory effects or desorption of most of the bacteria from the surface of the pine bark into the liquid phase. Previous studies demonstrated that pine barks could release large amounts of phenolic compounds and hydroxylated benzene rings, which both can inhibit the activity of various bacterial enzymes [32, 33]. Added to this, a constantly low pH during the process did not contribute in creating favourable conditions to bacterial growth.

## 3. Conclusion

The composts (CGR and DGR) proved to be efficient substrates for denitrification, promoting the sustained development of a complex biofilm as a niche for the denitrifying communities. The phylogenetic analysis carried out on CGR and DGR samples showed that the bacterial community evolved from a diverse community towards a limited consortium of active denitrifiers. Pine bark was found to be far less efficient in promoting favourable conditions for microbial growth because of the combined effect of a low pH and the release of potentially inhibitory compounds leading to the irreversible release of biofilm forming cells into the leachate. Furthermore, potential pathogens have been detected in association with the pine bark, rendering unsuitable its use as a carbon source for the treatment of nitrate-rich leachates at a large scale. Overall, this study contributes in pointing out the different behaviour displayed by the microorganisms from different substrates in the solid

and liquid phases and highlights the important role of biofilms in the denitrification process and their sensitivity to prevailing environmental conditions.

**Acknowledgements:** This study was conducted under the aegis of the NRF (SA) and CNRS (France) Scientific Collaboration Agreement. The authors would like to thank Durban Solid Waste for financial support; the Microbiology and Plant Pathology Departments of the University of KwaZulu-Natal (Pietermaritzburg) for sharing their expertise; Sheila Arnold, Bjorn Plug (UKZN) and Inquaba Bio-Tech Laboratories for assisting with the analytical work. We also thank Ewan Vince of the University Joseph Fourier (Grenoble) for his technical assistance and the platform MOME from the pole Envirhonalp.

**References**

[1] A.A. Abbas, G. Jingsong, L.Z. Ping, P.Y. Ya, W.S. Al-Rekabi. Review on landfill leachate treatments. Am J Appl. Sci. 6 (2009) 672-684.

[2] D. Kulikowska, E. Klimiuk. The effect of landfill age on municipal leachate composition. Bioresour. Technol. 99 (2008) 5981-5985.

[3] D. Fatta, A. Papadopoulos, M. Loizidou. A study on the landfill leachate and its impact on the groundwater quality of the greater area. Environ Geochem Health 21 (1999) 175-190.

[4] V. Mateju, S. Cizinska, J. Krejci, T. Janoch, Biological water denitrification - A review, Enzyme Microb. Technol. 14 (1992) 170-183.

[5] K. Knox, Leachate treatment with nitrification of ammonia. Water Res. 13 (1985) 895-904.

[6] X.Z. Li, Q.L. Zhao, Efficiency of biological treatment affected by high strength of ammonium-nitrogen in leachate and chemical precipitation of ammonium-nitrogen as pretreatment. Chemopshere 44 (2001) 37-43.

[7] L. Tengrui, A.F. Al-Harbawi, L.M. Bo, Z. Jun, X.Y, Long, Characteristics of nitrogen removal from old landfill leachate by sequencing batch biofilm reactor. Am. J. Appl. Sci. 4 (2007) 211-214

[8] J.M. Lema, R. Mendez, R. Blazquez, Characteristics of landfill leachates and alternatives for their treatment: A review. Water air soil Pollut. 40 (1988) 223-250.

[8] L. J. Strachan, An Investigation into Biological Treatment Systems for Landfill Leachate, Master of Science in Engineering, University of Natal, Durban, 1999.

[9] B. Ovez, S. Ozgen, M. Yuksel, Biological denitrification in drinking water using Glycyrrhiza glabra and Arunda donax as the carbon source, Process Biochem. 41 (2006) 1539-1544.

[10] C. Costa, C. Dijkema, M. Friedrich, P. Garcia-Encina, F. Fernandez-Polanco, A. J. M. Stams, Denitrification with methane as electron donor in oxygen-limited bioreactors, Appl. Biochem. Microbiol. 53 (2000) 754-761.

[11] L. Tsui, I. G. Krapac, W. R. Roy, The feasibility of applying immature yard-waste compost to remove nitrate from agricultural drainage effluents: A preliminary assessment, J. Hazard. Mater. 144 (2007) 585-589.

[12] M. Volokita, S. Belkin, A. Abeliovich, M. I. M. Soares, Biological denitrification of drinking water using newspaper, Wat. Res. 30 (1996) 965-971.

[13] C. Trois, G. Pisano, L. Oxarango, Alternative solutions for the bio-denitrification of landfill leachates using pine bark and compost. J Hazard. Mater. 178 (2010) 1100-1105.

[14] S. Rajakumar, P.M. Ayyasamy, K. Shanthi, P. Thavamani, P. Velmurugan, Y. C. Song, P. Lakshmanaperumalsamy, Nitrate removal efficiency of bacterial consortium (Pseudomonas sp. KW1 and Bacillus sp. YW4) in synthetic nitrate-rich water, J. Hazard. Mater. 157 (2008) 553-563.

[15] C. Sundberg, K. Tonderski, P. E. Lindgren, Potential nitrification and denitrification and the corresponding composition of the bacterial communities in a compact constructed wetland treating landfill leachates, Water Sci. Technol. 56 (2007) 159-166.

[16] M. R. Boni, A. Chiavola, A. Corti, V. Luzi, Treatment of leachate from MSW sanitary landfill by sequencing batch reactor: results from a lab-scale experience, Sardinia 2001,

Eighth International Waste Management and Landfill Symposium: Leachate and Landfill Gas, S. Margherita di Pula, Cagliari, Sardinia (2001) 179.

[17] A. Cao, A. Carucci, P. Crasto, A. Muntoni, MSW landfill leachate treatment using constructed wetlands: a laboratory-scale experience, Sardinia 2001, Eighth International Waste Management and Landfill Symposium: Leachate and Landfill Gas, S. Margherita di Pula, Cagliari, Sardinia (2001) 249.

[18] A. D. Eaton, L. S. Clesceri, E. W. Rice and A. E. Greenberg, *Standard Methods for the Examination of Water and Wastewater*, 21st ed.,Washington: American Public Health Association, American Water Works Association, Water Environment Federation, 2005, pp. 9-169.

[19] V. Guiné, J. M. F. Martins, B. Causse, A. Durand, L. Spadini, Effect of cultivation and experimental conditions on the surface reactivity of the metal-resistant bacteria *Cupriavidus metallidurans* CH34 to protons, Cadmium and Zinc, Chemical Geology. 236 (2007) 266-280

[20] D. P. H. Lejon, V. Nowak, S. Bouko, N. Pascault, C. Mougel, J. M. F. Martins, L. Ranjard, Genetic structure and diversity of copper resistant bacterial communities according to soil types, organic status and copper contamination. FEMS Microbiol. Ecol. 61 (2007) 424-437.

[21] D.J. Lane, 16S/23S sequencing. In: *Nucleic acid techniques in bacterial systematics* Stackebrandt E. and Goodfellow M. (eds.). John Wiley & Sons, Chichester, United Kingdom. (1991) pp 115-175.

[22] S.F. Altschul, T.L. Madden, A.A Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25 (1997) 3389-3402.

[23] I. Brettar, R. Christen, M. G. Höfle, Rheinheimera perlucida sp. nov., a marine bacterium of the Gammaproteobacteria isolated from surface water of the central Baltic Sea, Int. J. Syst. Evol. Microbiol. 56 (2006) 2177-2183.

[24] J. M. Janda and S. Abbott, The Enterobacteria, 2nd. ed., ASM Press, 2006. pp. 411.

[25] J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley, S. T. Williams, Bergey's Manual of Determinative Bacteriology, 9th ed., Lippincott Williams & Wilkins, Philadelphia, USA, 2000.

[26] V. Barbe, D. Vallenet, N. Fonknechten, A. Kreimeyer, S. Oztas, L. Labarre, S. Cruveiller, C. Robert, S. Duprat, P. Wincker, L. N. Ornston, J. Weissenbach, P. Marlière, G. N. Cohen, C. Médigue, Unique features revealed by the genome sequence of Acinetobacter sp. ADP1, a versatile and naturally transformation competent bacterium, Nucleic Acids Res. 32 (2004) 5766-5779.

[27] Y. Seki, S. Seki, M. Satoh, A. Ikeda, M. Ishimoto, *Rubredoxin* from Clostridium perfringens: complete amino acid sequence and participation in nitrate reduction, J. Biochem. 106 (1989) 336-341.

[28] J. Mergaert, M. C. Cnockaert, J. Swings, Thermomonas fusca sp. nov. and Thermomonas brevis sp. nov., two mesophilic species isolated from a denitrification reactor with poly(e-caprolactone) plastic granules as fixed bed, and emended description of the genus *Thermomonas,* Int. J. Syst. Evol. Microbiol. 53 (2003) 1961-1966.

[29] K. Okamoto, N. Satou, Isolation, identification and characterization of effective bacteria on bioremediation from the waste parts of Stevia rebaudiana Berutoni, Enzyme Microb Technol 39 (2006) 407-413.

[30] M. A. Horn, J. Ihssen, C. Matthies, A. Schramm, G. Acker, H. L. Drake, Dechloromonas denitrificans sp. nov., Flavobacterium denitrificans sp. nov., Paenibacillus anaericanus sp. nov. and *Paenibacillus terrae* strain MH72, $N_2O$-producing bacteria isolated from the gut of the earthworm Aporrectodea caliginosa, Int. J. Syst. Evol. Microbiol. 55 (2005) 1255-1265.

[31] M.M. Williams, J.W.S. Domingo, M.C. Meckes, C.A. Kelty, H.S. Rochon, Phylogenetic diversity of drinking water bacteria in a distribution system simulator. J. Appl. Microbiol. 96 (2004) 954-964.

[32] D. G. Teclu, Bioremediation of arsenic contaminated groundwater, Doctor of Philosophy, University of KwaZulu-Natal, Pietermaritsburg, 2008.

[33] Q. Zhong, D. Li, Y. Tao, X. Wang, X. He, J. Zhang, J. Zhang, W. Guo, L. Wang, Nitrogen removal from landfill leachate via *ex situ* nitrification and sequential *in situ* denitrification. Waste Management 29 (2009) 1347-1353.

Table 1 Batch experiment set-up

| | **Mass of substrate** (g) | **Volume of the solution** (ml) | **Concentration of the solution** ($g.l^{-1}$ of $KNO_3^-$.) |
|---|---|---|---|
| CGR | 313 | 787 | 1.4 |
| DGR | 295 | 805 | 1.0 |
| PB | 196 | 905. | 0.9 |

Commercial (CGR) and (DGR) domestic garden refuse; Pine bark (PB)

Table 2 Solid matter characterisation for each substrate

| | **Moisture Content** (%) | **Total Solids** (%) | **Volatile Solids** (%) | **$RI_7$** ($mgO_2/g$ DM) | **C/N** (before composting) | **C/N** |
|---|---|---|---|---|---|---|
| CGR | 67 ± 1 | 33 ± 1 | 47 ± 2 | 8.5 ± 0.6 | 40 | 24 |
| DGR | 66 ± 6 | 34 ± 6 | 62 ± 12 | 14.1 ± 0.4 | 22 | 13 |
| PB | 49 ± 3 | 51 ± 3 | 97.1 ± 0.1 | 17.8 ± 0.4 | n.a. | 62 |

Table 3 Physicochemical characteristics of the eluates after 24 hrs

| | **Total Solids** ($g\ l^{-1}$) | **Volatile Solids** ($g\ l^{-1}$) | **pH** at 20°C | **ρ** (mS/cm) at 20°C | **COD** ($mg\ l^{-1}$) | **$BOD_5$** ($mg\ l^{-1}$) | **$NH_3$-N** ($mg\ l^{-1}$) | **NOx-N** ($mg\ l^{-1}$) | **C/N** |
|---|---|---|---|---|---|---|---|---|---|
| CGR | 2.4 ± 0.1 | 1.6 ± 0.1 | 6.9 | 0.81 | 2800 ±400 | 155 | 9.8 ± 1.2 | 0.19 ± 0.05 | 1.8 |
| DGR | 17 ±3 | 12 ±0.2 | 7.5 | 5.1 | 17600 ±1300 | 350 | 82.0 ± 0.4 | 8 ± 2 | 8.3 |
| PB | 3.6 ± 0.01 | 3.3 ±0.3 | 4.2 | 0.85 | 4500 ±450 | 297 | 8.5 ± 0.1 | 0.03 ± 0.01 | 3.6 |

Table 4 Evolution of COD, pH and $NH_3$ in the liquid phase during the batch tests

| | COD ($mg\ l^{-1}$) | pH at 20°C | NH3-N ($mg\ l^{-1}$) |
|---|---|---|---|
| CGR 24h | 2800 ± 400 | 6.9 | 9.8 ± 1.2 |
| CGR final | 3200 ± 100 | 7.5 | 3.0 ± 0.1 |
| DGR 24h | 17600 ± 1300 | 7.5 | 82.0 ± 0.4 |
| DGR final | 17800 ± 1100 | 7.6 | 87.2 ± 0.4 |
| PB 24h | 4410 ± 20 | 4.2 | 8.5 ± 0.1 |
| PB final | 14200 ± 1200 | 4.3 | 22.5 ± 10 |

Table 5: Summary of bacterial sequence identification (expressed as %) according to the closest matches to sequences in the Genbank database found by BLAST

| | PB eluate | | | CGR eluate | | | DGR eluate | | |
|---|---|---|---|---|---|---|---|---|---|
| Phylogenetic group / genus level | **2.5 h** | **96 h** | **263 h** | **3 hr** | **74 h** | **162 h** | **3 hr** | **74 hr** | **162 hr** |
| **α-proteobacteria** | | | | | | | | | |
| *Phaeospirillum* | | | | | | 26 | - | - | - |
| *Rhizobium* | | | | - | 20 | 8 | - | - | - |
| Alpha proteobacterium INAWF007 | | | | | | | - | 7 | 3 |
| Aquicella siphonis | | | | - | - | - | 21 | 12 | 8 |
| **γ - protebacteria** | | | | | | | | | |
| *Pseudoxanthomonas* | - | - | - | 30 | 4 | - | 4 | - | - |
| *Rheinheimera* | - | - | - | 25 | 9 | 14 | 11 | - | - |
| *Acinetobacter* | - | - | - | - | 12 | 2 | 4 | - | - |
| *Pseudomonas* | - | - | - | - | - | 17 | 12 | 8 | 5 |
| *Thermomonas* | - | - | - | - | 20 | 5 | - | - | - |
| *Rahnella** | 24 | 22 | 34 | - | - | - | - | - | - |
| *Pantoea** | 12 | - | - | - | - | - | - | - | - |
| *Kluyvera** | 6 | 6 | 4 | - | - | - | - | - | - |
| *Enterobacter** | 14 | 11 | 8 | - | - | - | - | - | - |
| Uncultured gamma proteobacterium clone 16S5 | 11 | - | - | - | - | - | - | - | - |
| *Erwinia** | - | 22 | 10 | - | - | - | - | - | - |
| **Firmicutes** | | | | | | | | | |
| Uncultivated clostridium sp clone 3.28 | - | - | - | 8 | 7 | - | - | - | - |
| *Geobacillus* | | | | 23 | 3 | - | - | - | - |
| *Bacillus* | - | - | - | - | - | - | 13 | 29 | 40 |
| *Thermoactinomyces* | - | - | - | - | - | - | 2 | 24 | 27 |
| *Lactobacillus* | - | 33 | 29 | - | - | - | - | - | - |
| **Bacteroidetes** | | | | | | | | | |
| *Flavobacterium* | - | - | - | - | - | 17 | - | - | - |
| *Pedobacter* | - | - | - | - | 18 | 3 | - | - | - |
| Unknown | 33 | 6 | 15 | 14 | 7 | 8 | 33 | 20 | 17 |

Phylogenetic grouping based on the highest identity score obtained after submitting the sequence to BLAST (sequence identity with > 97% homology). Data are expressed as % of 16S rRNA clones.

"-" : not detected; * Enterobacteria

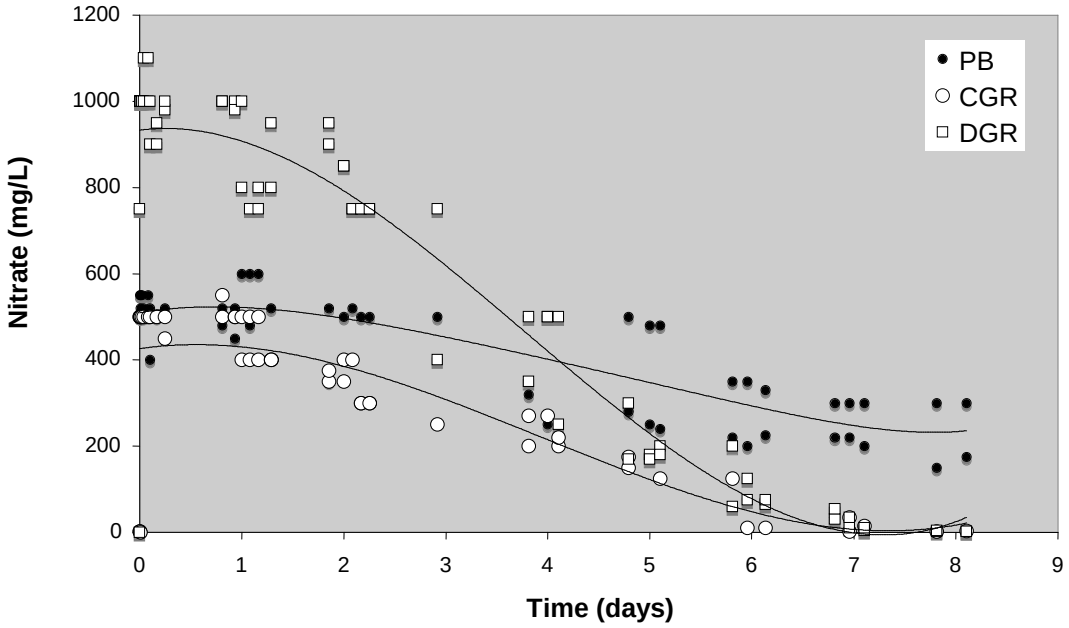


Fig. 1: Influence of Pine Bark (PB), Commercial Garden Refuse (CGR) and Domestic Garden Refuse (DGR) amendment on the nitrate removal in batch tests.

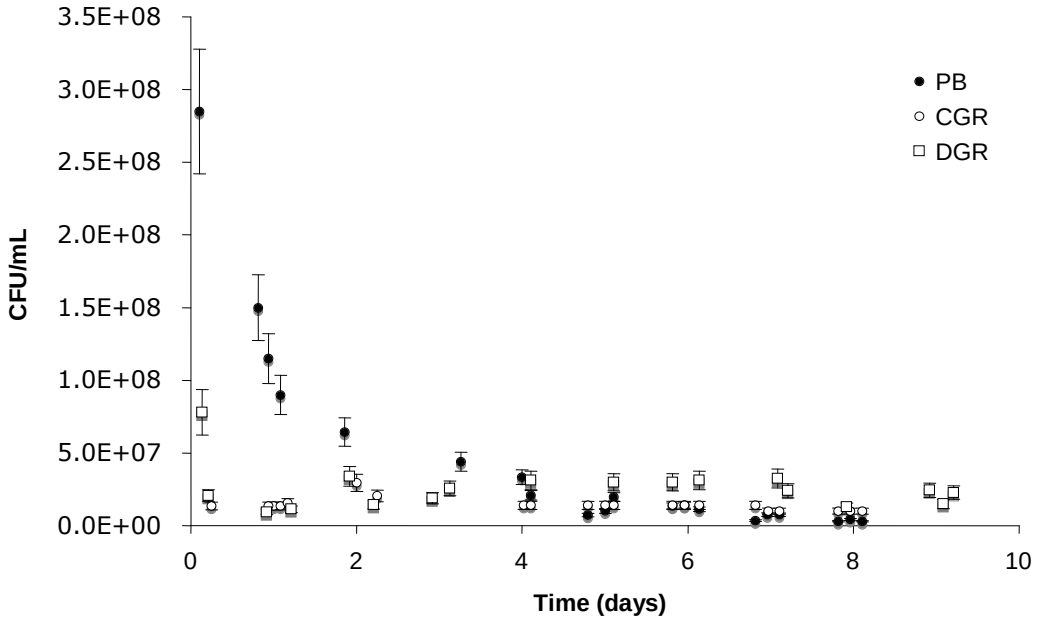


Fig. 2: Change in the abundance of the microbial population according to the carbon sources used in batch tests.

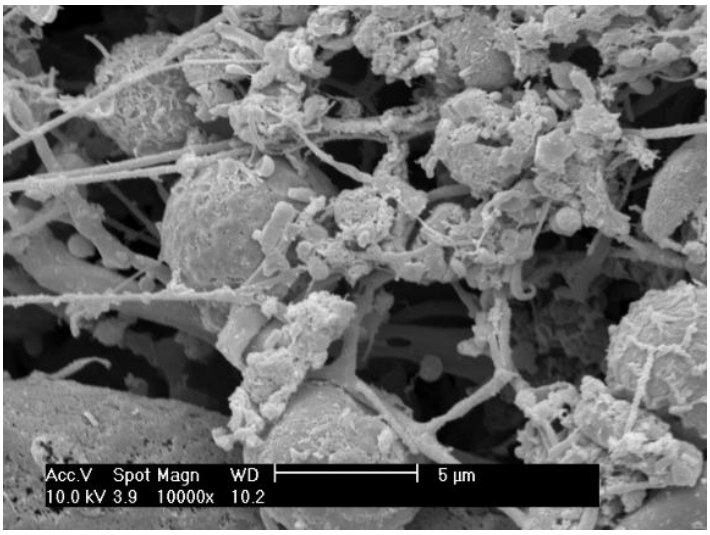


Fig. 3: ESEM micrograph of the surface of CGR before incubation

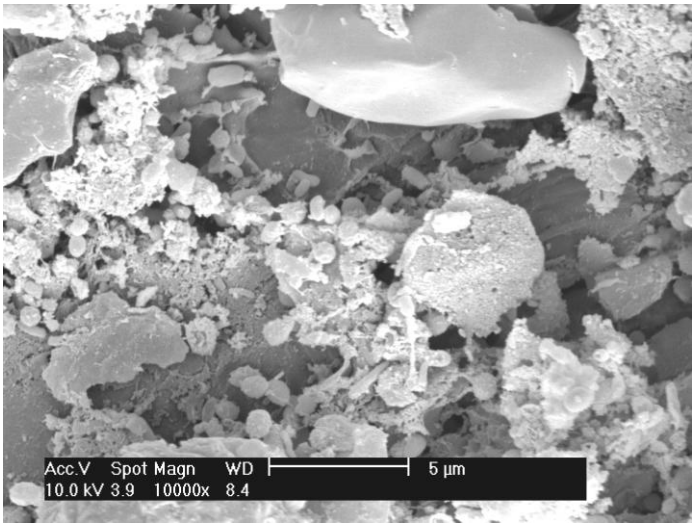


Fig. 4: ESEM micrograph of the surface of DGR before incubation

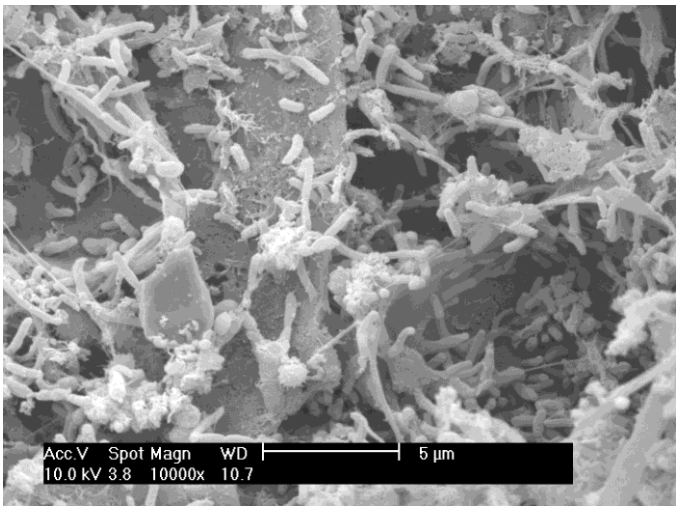


Fig. 5: ESEM micrograph of the surface of CGR 500 after 8 days

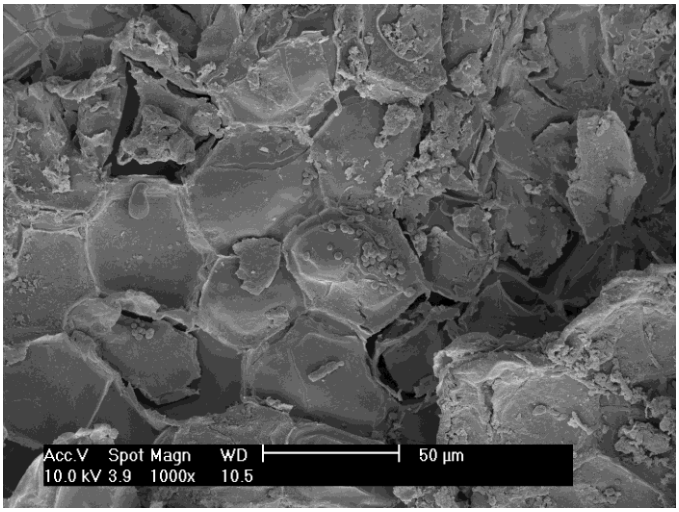


Fig. 6: ESEM micrograph (magn. x 10000) of the surface of pine bark (PB) before incubation