Ann Microbiol (2016) 66:397–405
DOI 10.1007/s13213-015-1122-1

ORIGINAL ARTICLE

Microbial community distribution and genetic analysis in a sludge active treatment for a complex industrial wastewater: a study using microbiological and molecular analysis and principal component analysis

Giulio Moretti[1] · **Federica Matteucci**[1] · **Claudia Ercole**[1] · **Francesco Vegliò**[2] · **Maddalena Del Gallo**[1]

Received: 21 October 2014 / Accepted: 16 June 2015 / Published online: 9 July 2015

Abstract Liquid industrial chemical wastes are often treated with complex and integrated chemical and biological processes to attain the required standard for water discharge into the environment. Such wastewaters are potentially dangerous and of unknown composition. Biological treatment represents the cheapest means of pollution abatement. In the present work, a microbiological and genetic analysis was performed for the biological pool of an industrial wastewater treatment plant located in Civita Castellana (Viterbo, Italy). This study focused on active sludge treatment and on the subsequent secondary sedimentation system. The parameters of the biological section (the sludge active plant) are normally constantly monitored, but the biological efficiency of the microbial community should be better evaluated to understand the dynamics of the microbial community and its relationship to the overall standard quality parameters that are usually monitored. For this study, Biolog community level physiological profiling (CLPP) on EcoPlates and PCR-amplified 16S rRNA denaturing gradient gel electrophoresis (DGGE) were used in comparison and combined as ecological techniques to characterize an anthropic closed ecosystem. Biolog CLPP provides the potential metabolic pattern and DGGE analyses helps to explain the structure and complexity of the microbial community. The results suggest that these techniques could be predictive and more useful when used together than alone. In addition, a principal component analysis (PCA) performed on the Biolog assay over time can relate principal components predictive wells to the biological and standard chemical analysis used to control and monitor the activity of this type of industrial wastewater treatment plant, providing a selection method for further DGGE community analysis.

Keywords Industrial wastewater treatments · Community level physiological profiling · Denaturing gradient gel electrophoresis · Principal component analysis · Microbial community analysis

Introduction

Biological wastewater treatment has been a constantly growing research field since the introduction of non-biodegradable or recalcitrant pollutants into the environment. Biological wastewater systems are the easiest and cheapest way to lower the biological and chemical oxygen demand (BOD, COD) and toxicity from industrial liquid wastes prior to discharge into receiving waters. Although the functionality of a biological reactor can be monitored by physical and chemical parameters, there is a lack of information about the functionality and structure of the microbial community as it operates in biological reactors and in lagoon sedimentation systems. One of the major problems of this type of analysis is the unknown composition of both the introduced wastes and the composition of the microbial community inside the plant. The aim of this work was to characterize the microbial community of an industrial waste treatment plant located in Civita Castellana (Viterbo, Italy). The plant receives chemical wastes, landfill leachate and potentially dangerous wastes of unknown

✉ Maddalena Del Gallo
maddalena.delgallo@univaq.it; delgallo@aquila.infn.it

[1] Department of Life, Health and Environmental Sciences, University of L'Aquila, via Vetoio, Coppito 1, 67010 L'Aquila, Italy

[2] Dipartimento di Ingegneria Industriale e dell'Informazione e di Economia, University of L'Aquila, via G. Gronchi 18, 67100 L'Aquila, Italy

composition as input; they are treated by upstream processes such as the Fenton reaction, salt precipitation, and solvent distillation at the inlet, followed by entry into a 4000 m^3 oxidizer, which is the core of the process. The plant also has an anoxic reactor for nitrates abatement and a lagoon sedimentation system.

Isolation-based methods of study are nearly impossible to perform because nearly 90 % of microorganisms in nature are refractory to selective enrichment culture (Wayne et al. 1987; Ward et al. 1992). In this study, non-isolation-based methods were used to analyze the community's functional potential and structural complexity. Biolog community level physiological profiling (CLPP) was performed as described by Garland and Mills (1991), by directly inoculating onto Biolog EcoPlates after cell washing and saline suspension. This method is a redox system based on sole carbon source utilization. The Biolog EcoPlate has 96 wells that contain 31 substrates and controls in triplicate (Hitzl et al. 1996). The oxidation of the carbon sources used by bacteria to grow will yield reduced coenzymes, such as NADH, which react with the tetrazolium dye in each well. The violet color that forms can be measured by taking the assorbance at 590 nm and optical density. A mathematical analysis and interpretation of optical density distributions provides useful information about the metabolic potential of dominant community members (Frąc et al. 2012). CLPP was performed with classical biodiversity indexes, and principal component analysis (PCA) was conducted to reduce the number of variables and relate them to reactor parameters. To investigate the structure and distribution of the microbial community from the oxidation reactor and the sedimentation lagoon system, a PCR-amplified 16S bacterial rRNA was evaluated by denaturing gradient polyacrylamide gel electrophoresis (DGGE). DGGE is a separation technique for PCR-amplified DNA fragments based on the sequence properties rather than fragment length (Fischer and Lerman 1980). Within a denaturing gradient, a double-stranded DNA fragment will migrate until the less stable region denatures, a transition from a helical to a partially melted structure occurs, and the partially melted conformation will halt in the gel (Myers et al. 1985). Because of the different melting properties of nucleotide couples, the sequence variations of particular fragments will stop at different positions in the gradient and can then be visualized in the gel (Muyzer et al. 1993).

Biolog CLPP and DGGE have been used together, in parallel, to assess the carbon substrate utilization and genetic diversity of the rhizosphere (Travis et al. 2007) and activated sludge (Song et al. 2011), the effects of herbicides on soils (El Fantroussi et al. 1999), or in combination (Smalla et al. 1998; İnceoğlu et al. 2012). In these latter articles, the metabolic patterns and genetic diversity of the different microbial communities was examined carefully, while the correlation between metabolic patterns and biodiversity has not.

In the present work, a combination of both methods was used to evaluate the structure of a microbial community present in an industrial wastewater system with the aim of finding a mathematical correlation with plant functionality to use in routine analysis. A PCA of CLPP is a widespread method used to assess changes in soil metabolic patterns from pollutants (Schmitt et al. 2004; Shi et al. 2005), but it does not provide information about the microbial community structure. A reduction factor analysis of the principal components (PCs) was performed to find representative wells of a functionality pattern from the microbial community into the plant. Contextually, we investigated the loss of biodiversity using Biolog EcoPlates' wells, which represent hidden variables (PCs) with high correlation values.

Material and methods

Collecting samples

Liquid samples were taken from: (1) the input stream to the oxidation pool from upstream treatments, (2) the oxidation pool, and (3) the lagoon system. All the samples were taken in triplicate at four different times during years 2011/2012 as follows: A: 30 May 2011, B: 27 June 2011, C: 8 September 2011, D: 21 June 2011; samples were taken in late spring and early autumn to minimize seasonal changes. The samples were kept refrigerated and analyzed within a short time (3–4 h) to prevent changes in the chemical, physical and biotic conditions (Shishido and Chanway 1998; Preston-Mafham et al. 2002). Samples of the two commercial inoculants that were employed in the oxidation pool were also taken. For every sample, environmental parameters such as COD abatement, pH, TSS (total suspended solids), NH_4^+ abatement were provided by the company (data not shown). To preserve the native conditions of the samples, only the necessary steps needed to inoculate the Biolog® EcoPlates (Biolog, Hayward CA) were performed. The samples from the oxidation pool were too dense to be inoculated directly, thus they were centrifuged at 500*g* for 2 min to eliminate the crude fraction, and the supernatant was then used. All the samples were washed twice at 10,000*g* and the bacterial pellets were resuspended into 40 mL NaCl 0.9 % physiological solution.

Biolog®

Sample washing was necessary to remove all the organic substances that could interfere with sole carbon source utilization, and samples were then inoculated onto Biolog® EcoPlates (150 μL per well) and incubated at 28 °C. The absorbance at 590 nm and optical density of each well (OD_I) were measured on a Biolog® Microstation every 24 h until reaching a plateau. The plates were then frozen at −20 °C until DNA extraction. A

serial dilution and CFU count of all samples were conducted on Nutrient Agar (Difco) in triplicate.

OD_I data were transferred to worksheets, normalized by subtracting the blank well values (control wells were provided with dye but with no carbon source), and the average well color development (AWCD) was calculated as follows:

$$AWCD = \Sigma OD_I/31$$

which gives the index of the total metabolic potential of the community (Garland and Mills 1991; Preston-Mafham et al. 2002; Frąc et al. 2012). A growth curve as a function of time was drawn for each plate with a median value of the triplicate. For each plate, the richness of response was calculated as the ratio of positive wells to all substrates, for a 0 to 1 index. The evenness of the response was expressed as a Shannon-Weaver Index, calculated as follows:

$$H = -\Sigma p_I \ln (p_I)$$

where p_I is the ratio of activity (OD_I) of each well to the sum of all the OD_I values in the plate (Frąc et al. 2012). This index is utilized to describe the richness of an ecosystem by the range of species diversity in the community, with a value that ranges from 0 to ln (S), where S is the number of species. For the CLPP performed on EcoPlates, H has a maximum value of ln (31)=3.4, and describes the probability that a random member of the microbial community can grow on the plate.

An alternative method that enables a comparison of functional diversity in microbial communities—by quantifying the inequality of carbon source utilization—is the Gini coefficient (commonly utilized to measure the distribution of resources within human populations) as derived from the Lorenz curve obtained from the CLPP (Harch et al. 1997). The Gini coefficient based on CLPP measures the functional distribution of the community. The Gini coefficient is twice the area between the plotted Lorenz curve and the line of perfect evenness, which is a 45° positive line from the origin. A lower Gini coefficient represents higher functional distribution, which is caused by similar responses across all carbon sources (Preston-Mafham et al. 2002).

Principal component analysis

The PC is an ordination technique based on the reduction of variables by projecting the initial set of variables into new axes (PCs); each new axis is determined as an eigenvalue of the correlation (or covariance) matrix that is obtained from the multivariate matrix of the data set, so a limited number of axes extracts the highest possible variance, and eigenvectors associated with eigenvalues determine each PC direction. The combination of new values that define each original sample is called the Score; from the Score graph, it is possible to compare the relative similarity among samples. The correlation of the original variables with the PC is called Loading, and from the Loadings graphs, it is possible to evaluate which of the original variables are responsible for the PCs (Garland and Mills 1991; Victorio et al. 1996; Garland 1997). The PCA was performed with IBM SPSS 20 with the correlation method and Varimax with Kaiser Normalization as the rotation method. PCA was used to monitor the substrate-utilization pattern of the oxidizing reactor's microbial community over time. Data obtained from the reactor, which recorded the loss in COD and NH_4^+, were added to the multivariate matrix prior to data normalization.

Denaturing gradient gel electrophoresis

DNA from plant samples and from the selected Biolog wells was extracted with the Norgen Soil DNA Isolation Kit. Biolog wells were selected according to the PCA correlations. This soil isolation kit was chosen because it removes humic acids and other PCR contaminants. The densities of purified DNA were checked using Thermo Scientific Nanodrop 2000 (Thermo Scientific, Waltham, MA). Extracted DNAs were used as a template for the PCR amplification of V3–V5 regions from bacterial 16S rRNA. Polymerase chain reactions (PCR) were performed on Thermoelectron PCR Sprint. Selected regions were amplified using 20 μL reaction mixtures containing 10 μL Amplitaq Gold Fast PCR Mix (2x), 0.5 μM primer 341 F=(5′-CCTACGGGAGGCAGCAG-3′), 0.5 μM primer 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) and 40 ng of DNA template. A GC clamp (5′CGCCCGGGGC GCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3′) was added to the 5' end of 341 F (Muyzer et al. 1993). Nearly all base substitution in the DNA fragments that are joined to a GC-Clamp can be detected by DGGE (Myers et al. 1985).

PCR amplification was performed with an initial denaturation step (95 °C×10 min) followed by 35 cycles consisting of 3 s of denaturation at 96 °C, 3 s of primer annealing at 54 °C and 6 s of primer extension at 68 °C. A final extension (72 °C×10 s) was added to reduce the proportion of single-stranded DNA fragments.

The PCR products were analyzed on a 1 % agarose gel to confirm their sizes and yields, in comparison with SmartLadder™.

DGGE analysis was performed using previously described protocols on a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) (Green et al. 2009; Valášková and Baldrian 2009). A 40–60 % denaturing gradient [7 M urea plus 40 % (w/v) deionized formamide] was employed, 20 μL of each sample (10 μL amplified DNA plus 10 μL 2x loading buffer) was loaded into the wells and then the gel was run at 60 V, 60 °C overnight.

After the runs, each 8 % acrylamide gel was stained with ethidium bromide (35 μL in 500 mL TAE buffer) for 30 min and then washed with deionized water.

Gel images were acquired with a GelDoc XR+ System (Bio-Rad) and ImageLab Software, and the band intensities, relative front and volumes were measured for each lane.

The data were inserted into a worksheet to calculate biodiversity indexes as follows:

Simpson's diversity index (D), as proposed by E.H. Simpson in 1949, expresses the probability that two random individuals selected from a community will be from the same species. Used in reciprocal form, 1−D is a 0 to 1 index of biodiversity. In analyzing the DGGE bands by using band intensities as the number of individuals for each population (n_I) and the sum of band intensities as the total population (N), the 1−D is calculated as follows:

$$1-D = 1-\Sigma_I(n_I/N)^2$$

Simpson's evenness index (Ed) is the relative abundance of the species. It has a maximum value when there are more species of similar dimensions. This index is linked to D through the equation

$$\frac{1-D}{1-(1/M)}$$

where M is the number of species.

The range-weighted richness (Rr) is a biodiversity index that is based on the relative front (distance run by bands in the gel) and is expressed as follows:

$$M^2 \cdot \Delta g$$

where Δg is the amount of denaturing gradient needed to describe all the bands in the sample. An Rr<10 is associated with an adverse environment, 10<Rr<30 is an intermediate value and Rr>30 indicates high microbial diversity (Marzorati et al. 2008).

The functional organization (Fo) describes the structural organization of a community based on the relative quantities of bands by Lorenz curve.

Results

Community level physiological profiling

The average well color development (AWCD), richness of response (R) and Shannon-Weaver index (H) were used to compare two different pure inoculants that were inserted into the oxidation pool at different times (Table 1). The first has minimal metabolic potential and functional diversity, as characterized by a preference for i-erythritol, Tween 80 and N-acetyl-D-glucosamine as carbon sources. However, the second inoculant has ten times the metabolic potential, as characterized by a very high response in each well of the plate but 2-hydroxy-benzoic acid, and showed Evenness near the asymptotic maximum value.

The same indexes were utilized to monitor the oxidizing pool trend, prior to the more accurate PC pattern analysis (Table 2).

None of the samples exhibited growth in the wells containing 2-hydroxy-benzoic acid (C3) and β-hydroxy-butyric acid (E3). Data from these plates were inserted into a vector matrix of 31 × 1, in which the daily COD and NH_4^+ abatement values were added, prior to normalizing the data by subtraction of the matrix median value. By inserting the plant efficiency values into the sole carbon utilization matrixes, it is possible to relate the meaning of the PCs to the plant function and to assess which substrates are predictive of the functionality of the microbial community. Eight PCs satisfy the selection condition, and the system is reduced to one-third of the original variables. The cumulative variance associated with the first eight components is 94.69 % (Table 3).

The rotated component matrix-defined values of variables on the new axes (PCs) are called "loadings" as shown graphically (Fig. 1). The loading plot shows variables that behave the same way and others that are outliers (G2, glucose-6-phosphate and C3, 2-hydroxy-benzoic acid).

Numerical confirmation is obtained from the reproduced correlations matrix that shows the distances between the values in the new vectorial space as defined by PCs (Table 4).

The score plot shows sample measurements (three times for each sample) through sample values into the space of components. The scores appear to be clustered into four groups defined by positive/negative values in space, according to plant functionality over time (Fig. 2).

The pattern obtained by comparing absorbance values between samples allows for the characterization of plant functionality according to PC scores, as determined each time new data are added. The PC's precision and predictive meaning increase with the number of samples (matrix vectors) added to the matrix. Thus, this method seems to be valuable for evaluating the functional patterns of the same microbial community over time. The loading values indicate which variables are most predictive of the pattern and of the functional potential of the community.

Denaturing gradient gel electrophoresis

DGGE gels were performed to compare the structure of microbial communities that were sampled from two environments inside the plant, namely the oxidizer (Ox) and the sedimentation (Sed pool). Furthermore, DNA extracted from representative PC wells were compared, in the same gel, with

Table 1 Difference in community level physiological profiling (CLPP) indexes between the two microbial inoculants used in the plant. *AWCD* Average well color development, *R* richness of response, *H* Shannon-Weaver index

Sample	AWCD	R	H
Inoculant 30 May 2011	0.121 (±0.005)	3/31 (C2, D1, E2)	1.677 (±0.093)
Inoculant 8 September 2011	1.625 (±0.024)	30/31 (all but C3)	3.338 (±0.015)

DNA extracted from the native solution. The aim was to observe the loss of biodiversity in the Biolog wells as reported by Smalla et al. (1998), and to relate this finding to the PC expressed variance.

Samples were processed at the same time and with the same extraction, amplification, and run parameters. DGGE biodiversity indexes, in comparison with their respective Biolog functional potential indexes, are summarized in Table 5.

Both samples showed good well color development, Ox exhibited metabolic potential and functional organization values that were slightly higher than the Sed values. Sed has greater response and response evenness on the plate, as confirmed by the Rr and Simpson index of the species structure. In addition, the Simpson evenness (Ed) index, which demonstrates the distribution of individuals into species, seems to be related to the Gini coefficient, which shows the response distribution across the wells.

Lorentz curves obtained from DGGE band intensities, showing the Fo, and color development across wells—from where Gini coefficient was calculated—are reported in Fig. 3 as follows:

The functional organization obtained from DGGE bands showed that, although the sedimentation pool is a stable environment with an equal distribution of members into species, the oxidizer is a dynamic environment with large populations of some predominant species and many small resilient populations present with a lower number of individuals. This finding reflects the functional potential of the plates (AWCD), because higher Fo values indicate a higher capacity to adapt to environmental changes. The Lorenz distribution obtained from CLPP analysis on Biolog Plates exhibited high response efficiency values for both samples, with good evenness of wells' OD_i values. These data confirm that higher biodiversity will give the community higher probability to be able to metabolize a given random carbon source.

A DGGE gel was run to assess the loss of biodiversity in Biolog wells (Fig. 4).

Lanes represent 16S rDNA extracted from Biolog wells that were selected according to their correlation with PCs.

There are two bands common to all lanes, with variable proportions; however, these bands were not the dominant ones in the original inoculum. Some other bands exhibited no growth on Biolog EcoPlates, and others are typical of some substrates, or are not present in some lanes, expressing the selectiveness of the substrates provided by the EcoPlate. This gel was used to calculate biodiversity indexes, the values of which are summarized in Table 6.

Range-weighted richness (Rr) values, in comparison with the native environment confirm that the Biolog wells strongly reduce the species number by applying selective force on the community, and they should be considered in many cases to be adverse environments (Rr <10).

These results confirm the hypothesis that the CLPP profiles that were obtained from Biolog Plates derived from the activity of only a part of the original community, and we should be very careful when drawing conclusions about the structure or function of a whole community (Stefanowicz 2006). It is possible to state that each well of the plate selects its own fraction of the community.

Table 2 Difference in CLPP indexes among pool samples taken at different times

Sample	AWCD	R	H
OX1 (30/05/2011)	1.268 (±0.061)	28/31	3.318 (±0.011)
OX2 (27/06/2011)	1.326 (±0.071)	23/31	3.173 (±0.014)
OX 3 (08/09/2011)	1.370 (±0.067)	28/31	3.216 (±0.001)
OX 4 (21/06/2012)	1.540 (±0.061)	27/31	3.294 (±0.010)

Table 3 PCs with expressed data variance

Component	Initial Eigenvalues		
	Total	% of variance	Cumulative %
1	11.516	37.148	37.148
2	5.590	18.031	55.179
3	3.904	12.593	67.772
4	2.331	7.200	75.292
5	1.938	6.250	81.543
6	1.580	5.098	86.640
7	1.432	4.620	91.261
8	1.063	3.430	94.690

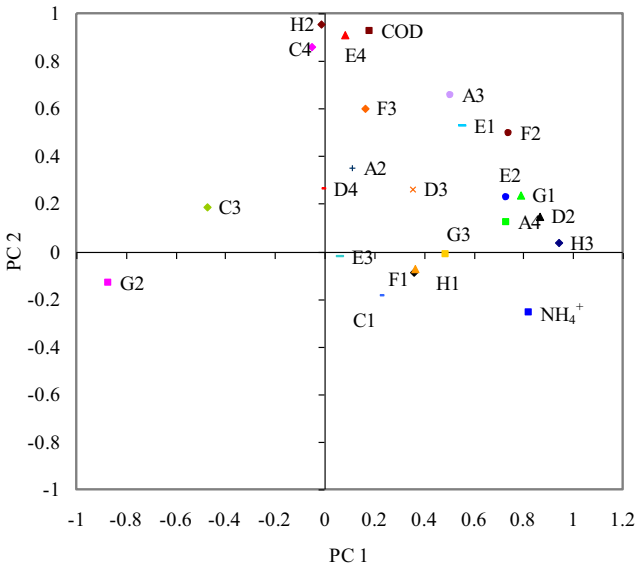

Fig. 1 Loadings plotted on principal component (PC) PC1 vs PC2, as obtained by creating a principal component analysis (PCA) correlation matrix to show the relation between parameters and PCs. Redundant or non-significant wells are not shown. *COD* Organic carbon abatement; *NH_4^+* Ammonium ion abatement; *A2* β-methyl-D-glucoside; *A3* D-galactonic acid γ-lactone; *A4* L-arginine; *C1* Tween 40; *C3* 2-hydroxybenzoic acid; *C4* L-phenylalanine *D2* D-mannitol; *D3* 4-hydroxybenzoic acid; *D4* L-serine; *E1* α-cyclodextrin; *E2* N-acetyl-D-glucosamine; *E3* γ-hydroxybutyric acid; *E4* L-threonine; *F1* glycogen; *F2* D-glucosaminic acid; *F3* itaconic acid; *G1* D-cellobiose; *G2* glucose-1-phosphate; *G3* α-ketobutyric acid; *H1* α-D-lactose; *H2* D,L-α-glycerol phosphate; *H3* D-malic acid

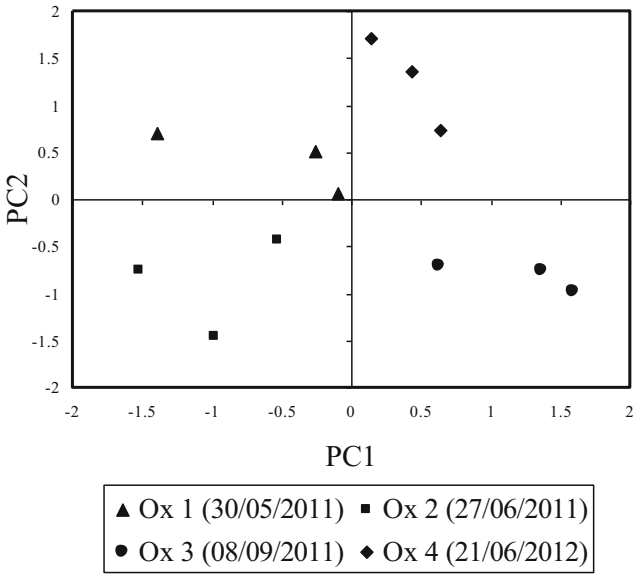

Fig. 2 Score Plot PC1 vs PC2 demonstrate trial score distributions within the PC's vectorial space

The Rr values obtained from the DGGE gel of PCs representative wells were inserted into a graph in decreasing order (Fig. 5) as follows:

Biodiversity richness seems to be related to the variance expressed by the first PCs, and to the correlation values of wells with PCs. Wells H2 and H3 had high correlation values with PC2 and PC1, respectively, and they have good Rr values. Wells F1 and A2 correlated with PC3 and PC5, with median values on the PC1/PC2 loading plot. Other wells representing minor PCs show almost null values for biodiversity, which was maybe influenced more by their relation with the first PCs than the last ones, thus expressing a minimal matrix variance. Two wells (G2 and D3) selected very few bacterial species. G2 (glucose 1-P) showed an inverse correlation with PC1; and D3 (4-hydroxybenzoic Acid), represented PC4 with an inverse correlation to biodiversity.

Table 4 Correlation among EcoPlate wells and plant parameters (COD and NH_4^+ abatements)

Well	Carbon source	Reproduced correlation
COD abatement		
E4	L-Threonine	0.944
H2	D,L-α-Glycerol phosphate	0.902
C4	L-Phenylalanine	0.854
NH_4^+ abatement		
H3	D-Malic acid	0.812
G1	D-Cellobiose	0.740
D2	D-Mannitol	0.732

Discussion

These results presented here show that the Civita Castellana plant is characterized by high sludge biodiversity and functionality, which demonstrate good operating conditions. The biodiversity indexes extrapolated from the oxidizing and sedimentation pool samples analysis show high species richness (Rr) Ox=26 and Sed >30, which translates into Ox=27/31 and Sed=30/31 well response in Biolog EcoPlates. This result shows that the greater the number of species (or metabolic types) in a specific environment, the higher the probability that when inserting any random compound, there will be at least one species, or set of species, that are able to metabolize that compound. The Shannon Weaver Index (H), showed very high response evenness on plates, reaching mathematical asymptotic values for both samples. The relative abundance of

Table 5 A comparison of CLPP and denaturing gradient gel electrophoresis (DGGE) indexes for Ox and Sed samples

Biolog index	AWCD	R	H'	Gini
Ox	1.540 (±0.061)	27/31	3.294 (±0.010)	0.206
Sed	1.383 (±0.039)	30/31	3.293 (±0.041)	0.185
DGGE index	Fo	Rr	1-D	Ed
Ox	60 %	26.3	0.824	0.888
Sed	40 %	36	0.908	0.969

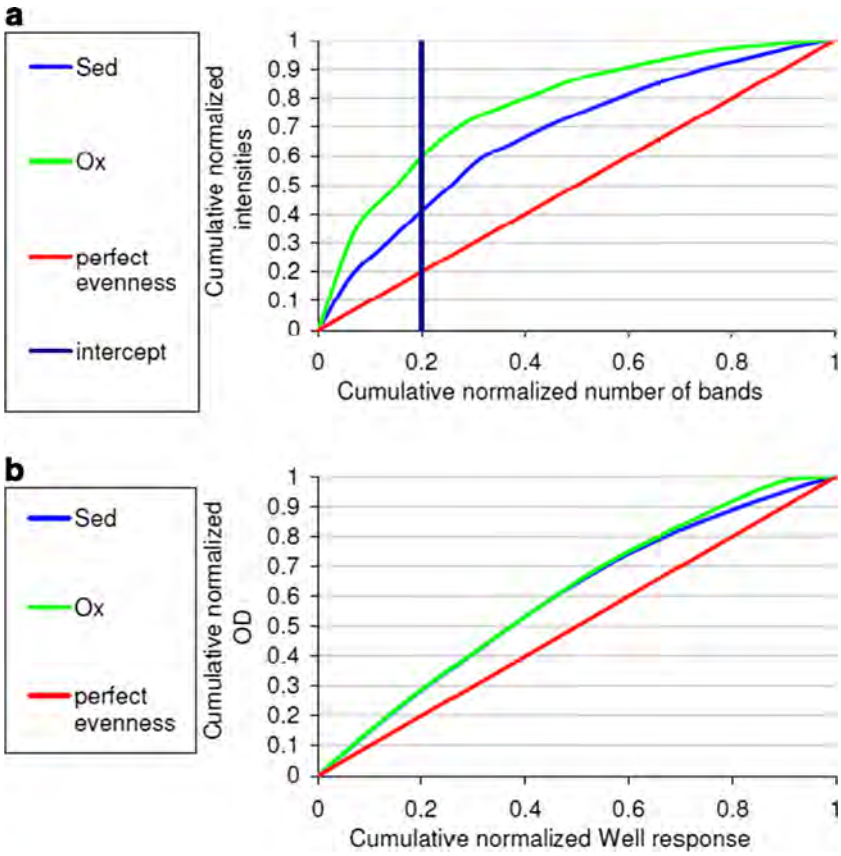

Fig. 3 **a, b** Plotted Lorenz curves obtained from DGGE (**a**) and CLPP (**b**)

species, measured by Simpson Inverse Index (1-D), shows values of 82 % in the oxidation pool and 90 % in the sedimenter. DGGE profiles confirm that the higher the number of species in an environment, the higher the probability that they will exploit all the available resources.

The Gini Index obtained from Lorenz curves that were applied to OD_i plate values is an inverse evenness index: lower values indicate a higher even color response. The Gini values Ox=20 % and Sed=18 % show well-equilibrated environments, with a good number of "metabolic types" that are able to share resources and cooperate to metabolize them, instead of competing for them. The Simpson Evenness index of the ribotype confirm high evenness values of species, with Ox=89 % and Sed =97 %. This finding indicated that the sedimentation pool is an equilibrated environment with balanced species, while the continuous nutrient input into the oxidizer selects faster growing species (the r strategists).

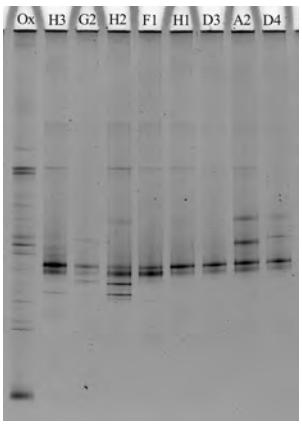

Fig. 4 A 40–60 % denaturing gradient, 8 % gel of the original inoculum (OX) and selected wells from the Biolog EcoPlate at the growth plateau. Lanes: *Ox* Original inoculum; *H3* D-malic acid; *G2* glucose-1-phosphate; *H2* D,L-α-glycerol phosphate; *F1* glycogen; *H1* α-D-lactose; *D3* 4-hydroxybenzoic acid; *A2* β-methyl-D-glucoside

Table 6 Biodiversity indexes obtained from a DGGE gel of the original sample and EcoPlate wells related (PC 1, 2, 3, 4, 5, 6) or inversely related (PC −1, PC −4) to principal components (PCs)

Lane		#	1-D	Ed	Rr	
	OX	14	0.824	0.888	26.31	
D-Malic acid	H3	7	0.659	0.769	4.473	Pc 1
Glucose-1-phosphate	G2	4	0.615	0.820	0.355	Pc −1
D,L-α-Glycerol phosphate	H2	9	0.784	0.882	7.648	Pc 2
Glycogen	F1	5	0.569	0.712	2.105	Pc 3
α-D-Lactose	H1	4	0.480	0.641	1.274	Pc −4
4-Hydroxybenzoic acid	D3	2	0.386	0.773	0.012	Pc 4
β-Methyl-D-glucoside	A2	6	0.726	0.872	1.966	Pc 5
L-Serine	D4	4	0.532	0.709	0.437	Pc 6

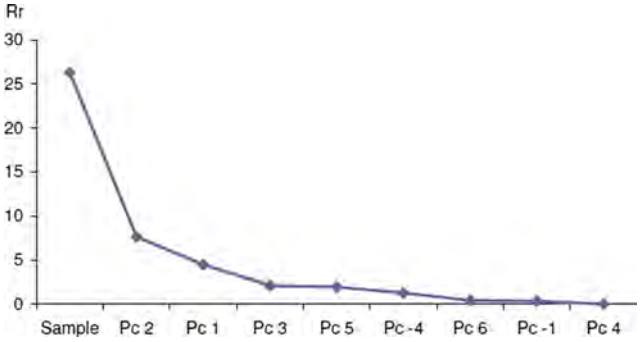

Fig. 5 The biodiversity richness (Rr) of the Ox sample and different EcoPlate wells that were inoculated with the same sample

The functional organization index (Fo) obtained by taking the Lorentz curve of relative band quantities showed how the oxidizer community is organized so that it could respond actively and more quickly to environmental changes. Higher nutrients and air input contribute to community dynamics.

The AWCD obtained from Biolog EcoPlates show maximum (plateau) values of Ox=1.5 and Sed=1.3. This value measures the functional potential of a community to respond positively to environmental changes, in which this change is the reduction in a sole carbon source, that is, a minimal and selective environment: a higher population dynamic reflects a higher metabolic potential.

PCA was performed with a correlation matrix on samples that were collected from the oxidation pool at different times. Plant functionality parameters were added to the multivariate data matrix. Regression analysis sorted eight hidden variables (PCs), and the first three of them explain most of the data variance. PCs were used to select information about discriminant, redundant and outlier wells. The first component (PC1) was linked to ammonium abatement, and the second (PC2) to organic carbon chemical abatement (COD), with wells distributed onto an inverse proportionality line between the PCs. This distribution gives a score to each trial, defined positive/negative space, according to the plant function under different conditions. Wells with significant—positive or negative—correlations to PCs were selected for DGGE analysis. The results showed that wells that were positively related to PCs 1 and 2 exhibited higher species richness than unrelated ones, and the inversely related wells (PC-1, PC-4; Table 6, Fig. 5) selected only one fast-growing ribotype. This finding confirms that the variance displayed by PCs reflects the real functionality of the treatment plant, and the substrate-driven selection of the microbial community.

Conclusions

Biolog CLPP and DGGE techniques for analyzing complex microbial communities are more likely to be used together than alone, whether for scientific or for monitoring purposes; CLPP provides information about the potential function of the microbiota, and DGGE biodiversity indexes give an image of the structure of the microbial community. The Biolog CLPP method was conceived to minimize preliminary work, focusing attention on data interpretation. However, the time required for plate growth (48 h–96 h) is usually longer than the entire DGGE setup, and both methods can be performed at the same time.

In future investigations, DGGE bands of interest could be excised and sequenced, for identification purposes, and custom DGGE ladders can be constructed as controls, and can be applied to routine wastewater treatment analysis.

Our results confirm that phylogenetic diversity appeared to be associated with the metabolic diversity in the activated sludge microbial community as also reported by Song et al. (2011), but these latter authors did not utilize mathematical methods to correlate microbial populations with process performance. We suggest performing aPCA of carbon source utilization (Biolog®) with insertion of environmental or performance data as variables in the multivariate matrix.

Smalla et al. (1998) suggest that "Whether the bacterial populations that are numerically dominant in the inoculum remain dominant in the wells of BIOLOG GN plates may in part depend on the history of the microbial community. For example, there was no significant decrease in the number of populations in wells inoculated with bacteria recovered from an activated sludge reactor (glucose fed)".

In our case the reactor was not fed with glucose, but rather with a large number of pollutant organic compounds. Our DGGE analysis instead shows that the choice of wells based on the variance of the components greatly affects the loss of biodiversity. In fact, the glucose-6-P well shows very low growth and minimal biodiversity. This method can be helpful in monitoring this kind of plant without going through more expensive DNA sequencing, which, however, remains important for research purposes.

Here, we suggest a possible strategy to monitor the microbial community in correlation with some environmental parameters that can be measured easily:

(1) Make a cell count from the original sample, assuring that the cell densities are of the same order of magnitude. After receiving the sample, quickly inoculate the Biolog Plate and freeze the sample for subsequent DNA extraction.
(2) Make Biolog readings, and perform 16S rDNA PCR-DGGE on the sample, comparing it with previous samples and/or with specifically built DGGE ladders.
(3) Insert data obtained from Biolog into a multivariate data Matrix with the reactor parameters and perform PCA (PCA becomes more accurate each time new data are added), giving a score to the sample.

(4) Compare CLPP and DGGE biodiversity indexes to discover if the structure or function of the community is changing.
(5) A Biolog and DGGE profile of the inocula inserted into the reactor could be useful for easy monitoring.
(6) Additionally, it is possible to perform PCR-amplified 16S rRNA DGGE of the communities selected in the different wells, to compare with the original sludge sample community, as shown in this work. PCA will help to reduce the number of significant wells prior to DGGE analysis.

This method could be useful when a better understanding of the microbial community in association with specific metabolic patterns is needed, especially when these patterns are responsible for the majority of pollutant abatement.

References

El Fantroussi S, Verschuere L, Verstraete W, Top EM (1999) Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. Appl Environ Microbiol 65(3):982–988

Fischer SG, Lerman LS (1980) Separation of random fragments of DNA according to properties of their sequences. Proc Natl Acad Sci USA 77(8):4420–4424

Frąc M, Oszust K, Lipiec J (2012) Community Level Physiological Profiles (CLPP), characterization and microbial activity of soil amended with dairy sewage sludge. Sensors 12:3253–3268. doi:10.3390/s120303253

Garland J (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. FEMS Microbiol Ecol 24:289–300

Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole-carbon-source utilization. Appl Environ Microbiol 57:2351–2359. doi:10.1007/978[--]1-60761-439-5_16

Green SJ, Leigh MB, Neufeld JD (2009) Denaturing gradient gel electrophoresis (DGGE) for microbial community analysis. In: Timmis KN (ed) Microbiology of hydrocarbons, oils, lipids, and derived compounds. Springer, Heidelberg, pp 4137–4158

Harch BD, Correll RL, Meech W, Kirkby CA, Pankhurst CE (1997) Using the Gini coefficient with BIOLOG substrate utilization data to provide an alternative quantitative measure for comparing bacterial soil communities. J Microbiol Methods 30:91–101

Hitzl W, Rangger A, Sharma S, Insam H (1996) Separation power of the 95 substrates of the BIOLOG system determined in various soils. FEMS Microbiol Ecol 22:167–174. doi:10.1111/j.1574-6941.1997.tb00368.x

İnceoğlu Ö, Falcão Salles J, Van Elsas JD (2012) Soil and cultivar type shape the bacterial community in the potato rhizosphere. Microb Ecol 63(2):460–470. doi:10.1007/s00248-011-9930-8

Marzorati M, Wittebolle L, Boon N, Daffonchio D, Verstraete W (2008) How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ Microbiol 10:1571–1581

Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59(3):695–700

Myers RM, Fisher SG, Lerman LS, Maniatis T (1985) Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. Nucleic Acids Res 13(9):3131–3145

Preston-Mafham J, Boddy L, Randerson PF (2002) Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles—a critique. FEMS Microbiol Ecol 42:1–14

Schmitt H, Van Beelen P, Tolls J, Van Leeuwen CL (2004) Pollution-induced community tolerance of soil microbial communities caused by the antibiotic sulfachloropyridazine. Environ Sci Technol 38(4):1148–1153. doi:10.1021/es034685p

Shi W, Bischoff M, Turco R, Konopka A (2005) Microbial catabolic diversity in soils contaminated with hydrocarbons and heavy metals. Environ Sci Technol 39(7):1974–1979

Shishido M, Chanway CP (1998) Forest soil community responses to plant growth-promoting rhizobacteria and spruce seedlings. Biol Fertil Soils 26(3):179–186

Smalla K, Wachtendorf U, Heuer H, Liu WT, Forney L (1998) Analysis of BIOLOG GN substrate utilization patterns by microbial communities. Appl Environ Microbiol 64(4):1220–1225

Song C, Yang C, Liu R, Li Q, Li B, Wang S, Mulchandani A (2011) Phylogenetic diversity and metabolic potential of activated sludge microbial communities in full-scale wastewater treatment plants. Environ Sci Technol 45(17):7408–7415. doi:10.1021/es2010545

Stefanowicz A (2006) The Biolog plates technique as a tool in ecological studies of microbial communities. Pol J Environ Stud 15(5):669–676, **and references therein**

Travis ER, Hannink NK, Van der Gast CJ, Thompson IP, Rosser SJ, Bruce NC (2007) Impact of transgenic tobacco on trinitrotoluene (TNT) contaminated soil community. Environ Sci Technol 41(16):5854–5861. doi:10.1021/es070507a

Valášková V, Baldrian P (2009) Denaturing gradient gel electrophoresis as a fingerprinting method for the analysis of soil microbial communities. Plant Soil Environ 55(10):413–423

Victorio L, Gilbride KA, Grant Allen D, Liss SN (1996) Phenotypic fingerprinting of microbial communities in wastewater treatment systems. Water Res 30(5):1077–1086

Ward DM, Bateson MM, Weller R, Ruff-Roberts AL (1992) Ribosomal RNA analysis of microorganism as they occur in nature. Adv Microbial Ecol 12:219–286. doi:10.1145/267521.267546

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Truper HG (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematic. Int J Syst Bacteriol 37:463–464. doi:10.1099/00207713[--]37-4-463