# A methodology for a quantitative interpretation of DGGE with the help of mathematical modelling: application in biohydrogen production

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## ABSTRACT

Molecular biology techniques provide valuable insights in the investigation of microbial dynamics and evolution. Denaturing gradient gel electrophoresis (DGGE) analysis is one of the most popular methods which have been used in bioprocess assessment. Most of the anaerobic digestion models consider several microbial populations as state variables. However, the difficulty of measuring individual species concentrations may cause inaccurate model predictions. The integration of microbial data and ecosystem modelling is currently a challenging issue for improved system control. A novel procedure that combines common experimental measurements, DGGE, and image analysis is presented in this study in order to provide a preliminary estimation of the actual concentration of the dominant bacterial ribotypes in a bioreactor, for further use as a variable in mathematical modelling of the bioprocess. This approach was applied during the start-up of a continuous anaerobic bioreactor for hydrogen production. The experimental concentration data were used for determining the kinetic parameters of each species, by using a multi-species chemostat-model. The model was able to reproduce the global trend of substrate and biomass concentrations during the reactor start-up, and predicted in an acceptable way the evolution of each ribotype concentration, depicting properly specific ribotype selection and extinction.

**Key words** | anaerobic digestion, DGGE, fingerprint, image analysis, model

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## INTRODUCTION

In engineered ecosystems, characterised by closely related biotic and abiotic components, there is still a striking need for a better understanding of the relationship between process function and ecosystem characteristics in terms of density, diversity, structure and activity (Briones & Raskin 2003; Dumont *et al.* 2009). During the last two decades, molecular biology techniques (MBT) have been increasingly used as a tool for opening the black box of biological reactors and getting insight into communities development, selection and adaptation (Cabrol & Malhautier 2011). Denaturing gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridisation (FISH) with DNA probes are among the most employed non-culture-based MBT (Sanz & Köchling 2007).

DGGE is applied for the analysis of whole bacterial community structure and allows the separation of small polymerase chain reaction (PCR) products, commonly up to 400 bp. The separation of the DNA fragments is achieved as a function of their different G + C content and distribution. Consequently, the fingerprinting pattern is built according to the melting behaviour of the sequences along a linear denaturing gradient (Fromin *et al.* 2002).

Anaerobic digestion (AD) is a widely accepted technology for producing biogas as well as for stabilising solid organic waste. Methane is the most common gas obtained in the AD process. However, increasing importance, and thereby research effort, has been given to the hydrogen production, which is an intermediate of the whole process. In the context of AD, the use of MBT is particularly attractive and relevant as it enables overpassing the microorganism's isolation and identification of difficulties inherent to the complexity of the

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AD process such as syntrophic interactions, low growth rates, unknown growth requirements and obligate anaerobic conditions (Ramirez & Steyer 2008). In the case of biohydrogen production, several MBT have been applied to gain insight in the process, among which the DGGE stands out (Li *et al.* 2011). For example, the DGGE enabled to identify the dominant bacterial populations involved in the biohydrogen production process under various operating conditions (Mariakakis *et al.* 2011) and to consistently link the variation of hydrogen production rate and the succession of dominant bacteria during the fermentation process (Huang *et al.* 2010).

Several mathematical models have been developed, implemented and validated in AD systems (Donoso-Bravo *et al.* 2011). Most of the models consider several microbial population as state variables, however the specific species concentration are unknown variables due to difficulties in performing measurements. This issue may trigger some identification problems since some parameters cannot be determined independently (Bernard *et al.* 2001; Noykova *et al.* 2002), and may cause some inaccurate model predictions (Batstone *et al.* 2004).

The integration of microbial data and ecosystem modelling is currently a challenging issue for improved system control (Harmand *et al.* 2008). Among the attempts to integrate both approaches, Dumont *et al.* (2009) proposed a generic method coupling fingerprinting and mathematical tools to achieve the functional assigning of bacteria detected in microbial consortia involved in a nitrification bioprocess. A dynamic model of the biomass concentration of each functional community was designed from the available measurements on nitrifying performance.

The aim of this study is to develop and validate a simple procedure to convert a typical DGGE fingerprinting in a quantitative measurement of the individual bacterial ribotypes concentration in a bioreactor, for further use as a variable in mathematical modelling. This approach was applied during the start-up of an anaerobic reactor aiming to produce hydrogen, where significant changes of the population characteristics are expected to take place.

## METHODS

### Experimental set-up

A glass-made reactor operating as a continuous stirred tank reactor (CSTR) of 2 L was used for the experiment. The reactor was connected to auxiliary equipment for pH control, influent feeding and effluent draw off and media agitation The system was maintained at 37 °C by using a heated jacket built into the reactor. A hydraulic retention time of 12 h was set for population selection of hydrogen producing bacteria glucose (5 g $L^{-1}$) was used as the sole carbon source (Tapia-Venegas *et al.* 2013). The biogas production was measured by liquid displacement, and the composition of biogas was measured by gas chromatography, using GC Perkin Elmer Clarus 500, with nitrogen as the carrier. Biomass concentration was measured by volatile suspended solids (VSS) through gravimetric method.

### 16S rDNA PCR-DGGE fingerprinting

Aliquots of well-homogenised biomass samples (120 mL) were taken from the CSTR every day during the first 7 days of operation. After centrifugation at 10.000 rpm for 10 min, total genomic DNA was extracted from biomass sample pellet using Powersoil DNA Isolation Kit, MO BIO Laboratories Inc. (Carlsbad, CA, USA), according to the supplier's instructions. DNA extraction was verified by 1.2%-agarose gel electrophoresis. The V6 to V8 region of bacterial 16S rRNA genes was amplified by PCR using the primers U968-f (5′-ACC GCG AAG AAC CTT AC-3′) and L1401-r (5′-CGG TGT GTA CAA GAC CC-3′) (Nübel *et al.* 1996). A 40-base GC clamp was attached to the primer U968-f at the 5′ end (Muyzer *et al.* 1993). PCR amplification was carried out in 50 μL reaction mixture with 1.25 U Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA), 3 mM MgCl2, 200 nM each primer, 200 μM dNTP and 1 μl template DNA, in a GeneAmp 9700 thermocycler (Life Technologies, Carlsbad, CA, USA) through 35 cycles of denaturation at 95 °C (30 s), hybridisation at 52 °C (40 s) and elongation at 72 °C (90 s). Correct size PCR amplification was verified by 1%-agarose gel electrophoresis. PCR products were separated by DGGE on a 8% polyacrylamide gel with a linear gradient ranging from 30 to 60%, according to the protocol of Muyzer *et al.* (1993). Denaturing solutions were prepared on the basis of a 100%-denaturing solution containing 7M urea and 40% formamide. The migration was carried out for 16 h at 85 V and 60 °C, in 0.5X TAE buffer, using the DCode System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Gels were stained with silver nitrate as described by Sanguinetti *et al.* (1994) and scanned at 400 dpi in an Epson Perfection V750 PRO (Epson, USA).

### Estimation of operational taxonomic units (OTUs) concentration

DGGE gel was analysed by the program *Image J* (National Institute of Health, USA). Each DGGE profile was

converted in a densitometric curve where each band was represented by a peak of given width and intensity. The area under each of the detected peaks (above the cut-off threshold of 2% of total area) was computed using an algorithm built in *Scilab*. According to the classical DGGE postulate, a single band was related to a single sequence, called ribotype or OTU, and the ratio between the area of the peak and the total area of the pattern (rather than the peak height) was assumed to be an estimator of the ribotype relative abundance in the community (Loisel *et al.* 2006). These were the main assumptions of this study and their limits will be discussed later on. Then, by knowing the total biomass density for each sample (measured as VSS), the concentration of each ribotype could be estimated.

## Mathematical model

### Mathematical model with several species groups: model description

A simple species-based chemostat model was developed, based on four main hypotheses:

1. Each species has the same substrate-biomass yield coefficient ($y$).
2. Interactions between species only result from the competition for the common substrate.
3. Each specific growth rate is a function of the substrate only (independently of the presence of the other species).
4. The Monod functions have been chosen to represent the microbial growth rate.

Under these assumptions, we consider the classical multi-species chemostat model (Equations (1) and (2)):

$$\frac{dX_i}{dt} = \mu_i(S)X_i - DX_i \tag{1}$$

$$\frac{dS}{dt} = \frac{1}{y}\sum_{i=1}^{n} \mu_i(S)X_i - D(S_{in} - S) \tag{2}$$

where $X_i$ represents the concentration of the species $i$, $S$ is the substrate concentration in the reactor, $D$ is the dilution rate, $S_{in}$ is the concentration of the substrate in the influent and $n$ represents the total number of species. Each function $\mu_i$ is represented by the form (Equation (3)):

$$\mu(S) = \frac{\mu^{\max} S}{S + K'} \tag{3}$$

where $\mu^{\max}$ is the maximum specific growth rate and $K$ is the affinity constant.

### Mathematical model with several species groups: model identification

Estimating separately the yield coefficient and the maximum growth rates is quite complicated since these parameters are highly correlated (Batstone 2006). Therefore the experimental data (concentration of the species, substrate concentration, dilution rate) were fitted to this model, to identify the parameter $y$ and the pairs ${\mu_i}^{\max}$ and $K_i$ for each species $i$, using a least squares method. First of all the coefficient $y$ has been estimated with the measurement of the total biomass $X$ and substrate $S$, as the following dynamical property is satisfied (Equation (4)):

$$\frac{dZ}{dt} = D(yS_{in} - Z) \tag{4}$$

where the variable $Z$ is defined as (Equation (5)):

$$Z = \sum_i X_i + yS \tag{5}$$

The variable $Z$ represents a reaction invariant (total biomass) which is independent of the kinetics (Bastin & Dochain 1990).

## RESULTS AND DISCUSSION

### Evolution of reactor performance and community structure

Figure 1 shows the biogas composition evolution during the first 7 days of operation as well as the DGGE profiles of the bacterial community during the first 7 days. As expected, the gas composition was changing throughout the first week of operation. The $CH_4$ production faded away after the fourth day, indicating the washout of the methanogens (confirmed by the negative PCR of archaeal 16S RNA genes, data not shown), while the $H_2$ content rose up to reach a stable value of 60% at day 5, suggesting the enrichment/selection of hydrogen production biomass population. The $CO_2$ content stabilised around 40% after a continuous decrease from the initial 70%. The evolution of gas composition may be related to the evolution of the community structure in the reactor.

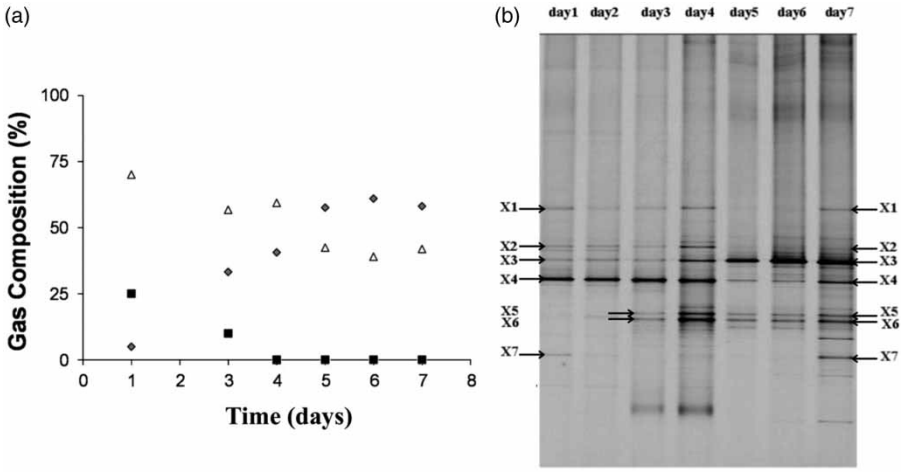


**Figure 1** | (a) Gas composition evolution during the first 7 days of operation. Grey diamond: hydrogen; black squares: methane; white triangles: carbon dioxide. (b) DGGE pattern of bacterial 16S rDNA during the first week of operation. The bands are identified from $X_1$ to $X_7$.

The evolution of the bacterial community structure is reflected in the DGGE pattern (Figure 1(b)). Based on densitometric peak areas, the seven most intense bands were selected and each was assumed to correspond to a single ribotype ($X_1$ to $X_7$) whose concentration was estimated on the basis of DGGE band relative intensity and the total biomass concentration (Figure 2). According to this quantitative interpretation of the DGGE fingerprint, there was a clear selection for $X_3$ ribotype, whose relative abundance exhibited the highest increase during the evaluation period (from day 4 onwards). The correlation between the increase of hydrogen production and the increase of $X_3$-abundance suggests that this specific ribotype might be related to hydrogen production. Despite the decrease of relative abundance of $X_4$ ribotype on days 4, 5 and 6, it can be inferred that it also participates in the hydrogen production, the latter being more abundant in day 7.

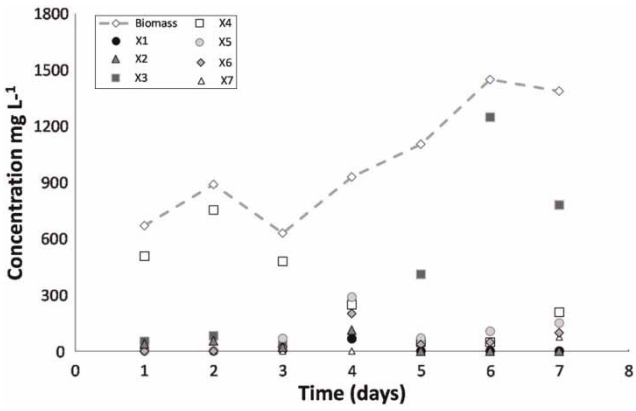


**Figure 2** | Concentration estimation of the seven dominant bacterial ribotypes from the total measured biomass and DGGE band intensities, during the first 7 days of reactor operation.

VSS has been used as a rough approximation of the total biomass concentration inside a reactor (Bernard *et al.* 2001; Lopez & Borzacconi 2009). However, and apart from being a quite uncertain-content measure, it can only be used when wastewater are being treated but not with solid wastes, otherwise the particulate component of the substrate itself are counted as biomass.

Nevertheless, it is important to point out that our quantitative interpretation of DGGE profile is based on an assumption which can be subjected to conceptual limits and potential methodological biases, both implying that one DGGE band does not always correspond to one single ribotype and that relative band intensity is not always related to ribotype concentration. Conceptually, a single species can harbor multiple operon copies, either with different sequences (resulting in multiple bands) or with the same sequence (resulting in different band intensity than species with single operon copy). It has to be also assumed that each cell has the same dry weight. In addition, methodological biases can be introduced at various steps of the experiment: selective DNA extraction, preferential PCR amplification, DGGE detection limit and saturation (impairing the detection of minority species), and DGGE co-migration (resulting in an artifactual unique band for several different species) (Muyzer & Smalla 1998). The qualitative, rather than quantitative nature of such molecular tools is an important concern

for relating microbiological data to process modelling (Harmand *et al.* 2008). However DGGE still remains convenient to compare the dynamics of major populations in a large number of samples undergoing the same methodological treatment and, as previously reported, DGGE fingerprintings can be revisited by simulation and used as a tool to measure microbial diversity (Loisel *et al.* 2006).

## Model parameter determination and calibration

For parameter identification purposes, and taking into account the experimental data, only the three dominant ribotypes were considered: $X_2$, $X_3$ and $X_4$. All the other ribotypes were pooled in a sole group ($X^*$). Figure 3(a) presents the chemostat-model fit with the actual experimental data of total biomass and substrate concentration, from which the yield coefficient ($y$) could be estimated from Equation (4). Knowing that the applied dilution rate was $2\,d^{-1}$ and the glucose concentration of the inlet was $5\,g\,L^{-1}$, the estimated value of the biomass-substrate yield was $0.3\,g_{Biomass}\,g^{-1}_{Glucose}$. As observed, the model is able to reproduce the global trend of both the substrate and the biomass concentration during this initial period of the reactor operation. The yield obtained in this continuous study is higher than those reported in batch conditions (Fernández *et al.* 2011; Infantes *et al.* 2012). Kinetic parameters obtained in batch conditions are usually lower than those obtained in continuous mode, with similar inoculum and substrate, which is explained by the different dynamics that occurs in both operation modes (Batstone *et al.* 2008).

Figure 3(b) presents the multi-species chemostat-model fit for each ribotype concentration, from which the individual growth parameters could be identified from Equations (1) and (2) (see Table 1). The $X_4$ ribotype was the most abundant during the first 3 days but its abundance decreased after day 4. Its kinetic parameters (high affinity constant) are characteristic of a slow competitor (Table 1). $X_4$ was quickly out-competed by $X_3$ whose abundance increased from day 4 and became dominant from day 5. The $X_2$ ribotype is never in an advantaged position compared to the others. The model predicted in an acceptable way the evolution of each ribotype concentration. Selection of $X_3$ ribotype observed on DGGE profiles may be explained by its growth advantage over possible competitors, conferred by a significantly higher substrate affinity (lower $K$ constant) compared with $X_4$ and $X_2$. In regard to the parameters values (Table 1), in general the $\mu_1^{max}$ obtained in this study are higher than the ones reported using the same substrate in batch conditions (Fernández *et al.* 2011). $K$s values are in agreement with those found using the same substrate (Sharma & Li 2009). However the $X_3$ parameters values are similar to a study by Nath *et al.* (2006), reported for *E. clocae*, a bacterium that can produce hydrogen at a substantially high rate (the maximum specific growth rate was $0.398\,h^{-1}$ and $K$s $5.5\,gL^{-1}$ with glucose).

However, due to the small amount of samples, these results can only be considered as a preliminary evaluation of the application of the procedure in model identification. It is also necessary to identify dominant ribotypes as representative species and to use the pure cultures to validate the kinetic constants obtained and to take into consideration

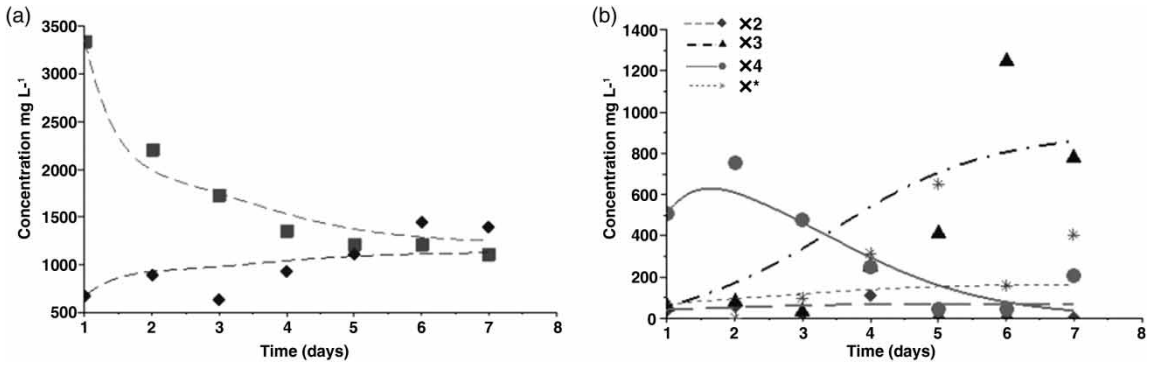


**Figure 3** | (a) Evolution of substrate concentration (squares) and biomass concentration (diamonds) during the first 7 days of reactor operation. (b) Evolution of ribotype concentrations during the first 7 days of reactor operation.

**Table 1** | Estimation of maximal growth rate ($\mu^{max}$) and affinity constant (*Ks*) of the four main groups of ribotypes identified from DGGE profiles

| Ribotype | Parameters | |
|---|---|---|
| | $\mu^{max}$ ($h^{-1}$) | *Ks* ($g\ L^{-1}$) |
| X* | 0.225 | 5.5 |
| $X_2$ | 0.68 | 18 |
| $X_3$ | 0.45 | 5.8 |
| $X_4$ | 0.45 | 10 |

that the behaviour of a species can be different from the behaviour of a whole.

## CONCLUSION

A novel procedure that combines common experimental measurements and molecular biology technique, in this case the DGGE, with image analysis allow us to count with a quantitative approximation of the most important microbial species of a hydrogen bioreactor. The proposed method has allowed fitting the results with a simple chemostat model based on the assumptions that each species has the substrate yield and their specific growth rate follow a Monod function. Because the growth curves that have been identified intersect (more precisely, the set of non-dominant species is expected to be less efficient under larger dilution rate), further experimental investigations with other dilution rates and more measurements are necessary to validate thoroughly the assumptions.

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