

Modeling and Monitoring of Microbial Diversity in Ecosystems - Application to Biological Wastewater Treatment Processes

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Abstract: Key microbiological conversion processes do not result from the work of a single bacterial species but are performed by a wide variety of bacteria. Up till now, this microbial diversity is usually not tracked during reactor operation and mostly neglected in mathematical models. Nevertheless, experimental evidence is available that different process conditions may favor the selection of different types of bacteria, modifying the microbial diversity and consequently the behavior of the ecosystem. This contribution assesses microbial diversity in biological wastewater treatment systems by means of two case studies: nitrification and anaerobic digestion, experimental data being provided for the nitrification process. Situation where a toxicant is present is carefully analyzed for anaerobic digestion. In both cases, the potential of process control to optimize microbial populations is discussed and it is highlighted that frequent changing of operating conditions can impact favorably to overall stability of the processes.

1. INTRODUCTION

Biological wastewater treatment nowadays is considered as a proven technology. Different processes can be applied for the removal of organic substrate (quantified in terms of COD, i.e. chemical oxygen demand) and nutrients such as nitrogen (N) and phosphorus (P). These processes rely on distinct biological conversion reactions, carried out by different types of bacteria. Besides, microbial diversity can also be distinguished within the microbial communities responsible for a certain function.

For instance, the nitrification reaction, which plays a central role in biological nitrogen removal, consists of two subsequent steps, carried out by two types of bacteria: ammonium oxidizing bacteria (AOB) convert ammonium nitrite, while nitrite oxidizing bacteria (NOB) perform subsequent oxidation to nitrate. Nevertheless: within these functional groups, different species can be distinguished, like *Nitrosomonas* and *Nitrospira* for AOB, while *Nitrobacter* and *Nitrospira* are typical NOB.

Another wastewater treatment process which will be dealt with in this contribution is anaerobic digestion. Its main advantages lie in the possibility to convert even slowly degradable COD and at the same time produce methane gas which can be further used for energy recovery or even electricity production. For complete conversion of complex organic material to methane, five groups of bacteria are required: acidogenic bacteria, propionate and butyrate-utilizing acetogens, as well as H₂- and acetate-utilizing methanogens. These bacteria must work syntrophically, as they are linked physiologically, kinetically, and thermodynamically (Sekiguchi *et al.*, 2004). In comparison to nitrification, anaerobic digestion is a complex process, involving many different bacteria which interact through a

network of reactions, which is still not completely understood.

In general, anaerobic reactors are affected by external changes, although the severity of the effect is dependent on the type, magnitude, duration and frequency of the imposed changes (Leitão *et al.*, 2006). Typical responses indicating reactor failure include a decrease in performance, accumulation of reaction intermediates such as volatile fatty acids (VFAs), drop in pH and alkalinity, change in biogas production rates and compositions, sludge washout and shifts in microbial community structure.

The availability of new molecular biological tools for studying microbial communities in bioreactors and other engineered systems without cultivation, has resulted in remarkable insights linking microbial diversity and dynamics to process stability. Fernandez *et al.* (1999) monitored the community dynamics of *Bacteria* and *Archaea* in a functionally stable, continuously mixed methanogenic reactor, fed with glucose, over a 605 day period. Even though the reactor maintained constant pH and COD removal during this period, they found differences in the levels of diversity and dynamics between the Bacterial and Archaeal domains, indicating that functional stability does not imply community stability, i.e. levels of individual populations fluctuate in a functionally stable community. Similar results were observed in another methanogenic reactor system, a fluidized bed reactor fed with vinasse (wine distillation waste) in which the biomass was immobilized on powder from porous volcanic stone (Zumstein *et al.*, 2000).

Another aspect concerns the effect of operational disturbances on the underlying microbial community. Fernandez *et al.* (2000) experimentally investigated the effect of substrate loading shocks on population dynamics. For

continuously mixed methanogenic reactors that maintained two different communities, they found that the less stable community structure resulted in more stable functioning. These results were attributed to the substrate processing structure that was developed in each reactor type prior to perturbation: substrate processing through parallel pathways was associated with a functionally more stable (resilient) system, in contrast to serial processing of substrate.

An important outcome of these and other experiments is the realization that population diversity alone does not drive ecosystem stability. The positive relationship between the presence of multiple pathways towards a product (parallel processing of substrate) and functional stability parallels theoretical concepts in higher ecological organization (Peterson *et al.*, 1998). Ecosystem stability is not the outcome of population diversity as such, but of functional redundancy, which is ensured by the presence of a reservoir of species able to perform the same ecological function. Recognizing the diversity and the links within each key functional group of a system can lead to better ways to model diversity and functioning, and can help to improve process stability (Watanabe *et al.*, 2002).

It is our belief that the engineering of wastewater treatment systems would be improved if one could predict and manipulate the associated microbial diversity. Mathematical models in which data on micro-scale molecular diversity has been incorporated to more closely represent wastewater treatment processes, can provide a useful tool to reach this goal. Such models can be used to gain insight in the influence of process conditions on the selection of certain types of bacteria. In a later stage, these models can also be used to develop efficient control strategies adapted to model-based population optimisation. In this contribution, this approach is demonstrated for two different wastewater treatment applications.

2. MICROBIAL COMPETITION IN NITRIFYING BIOFILM REACTORS

2.1 Materials and methods

A first case study considers experimental data from two inverse turbulent bed reactors (ITBRs). In this type of reactors, biomass is grown on low density particles, fluidised by an upward current of gas. The reactors were filled with Extendsphere™ particles as solid carrier material. Biological ammonium oxidation was carried out in two ITBRs, only differing in their solid hold-up ratio, i.e. the ratio of static to expanded bed height: 0.1 (reactor R10) and 0.3 (reactor R30) (Bernet *et al.*, 2004). Synthetic wastewater and containing 250 mgN L⁻¹ as ammonium sulfate was supplied at a constant flow rate of 0.3 L h⁻¹. Temperature was maintained at 30°C, pH was controlled at 7.5. The airflow rate was kept constant at 30 L h⁻¹. Nitrate, nitrite and ammonium were analyzed by an ion chromatography system (DIONEX 100) using conductivity detection. Bacterial communities were monitored by total DNA extraction and 16S rDNA-targeted PCR-SSCP (single strand conformation polymorphism) (Dabert *et al.*, 2001).

2.2 Experimental observations

The two ITBRs showed a different nitrifying performance, both from a macroscopic and microbiological point of view (Bernet *et al.*, 2004). The reactor R30 (highest support concentration) accumulated nitrite whereas R10 produced only nitrate as a final nitrification product. The comparison of microbial communities in both reactors after 4 months of operation (Fig. 1) was in agreement with this result: the same population of nitrite-oxidizing *Nitrospira* (NOB) was present in both reactors but in very low proportion in R30 compared with R10. The major ammonium-oxidizer was different in both reactors, *Nitrosomonas europaea* (AOB1) in R30 and *Nitrosomonas* sp. (AOB2) in R10.

The question arises how the reactors' solid hold-up, being the only operating parameter different between both reactors, can act upon nitrifying activity and on the major ammonium oxidizer present? Note that the different solid hold-up of the reactors R10 and R30 results in different liquid volumes (1.27 L and 1.1 L respectively), leading to different ammonium loading rates (1420 and 1640 gN m⁻³ d⁻¹ respectively). The 15% higher loading rate in R30 compared to R10, for the same aeration flow rate, results in a lower oxygen: ammonium influent ratio in R30. The latter has likely caused oxygen depletion in R30, on its turn causing nitrite accumulation. It is postulated that the difference in the major ammonium oxidizer is also due to a selection pressure driven by the different oxygen concentration.

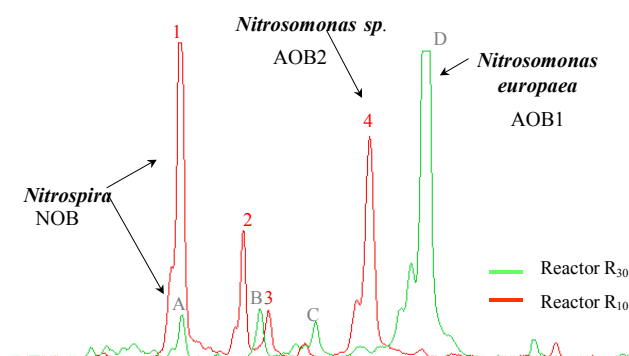


Fig. 1. Comparison of bacterial SSCP profiles from reactors R10 and R30 after start-up period (4 months of operation) with identification of nitrifying populations (Bernet *et al.*, 2004). The reactor R30 profile has been artificially increased to be able to detect the presence of peak A.

2.3 Reactor model

In order to describe the observed experimental behavior, a 0-dimensional biofilm model has been set up to describe the behavior of soluble components (ammonium, nitrite and nitrate) as well as biomass. The oxygen concentration is assumed constant in each of the reactors. The terminology '0-dimensional' indicates that a homogenous distribution of all components throughout the biofilm reactor has been assumed. Biomass retention in the reactor has been modelled in a simplified way, equivalent to the one suggested in the ADM1 report (Batstone *et al.*, 2002). The nitrifying

population considered consists of two ammonium oxidizing species and one nitrite oxidizing species, with respective concentrations X_{AOB1} , X_{AOB2} and X_{NOB} . In this way, the model contains the same number of nitrifying species as observed experimentally. The first ammonium oxidizing species (AOB1) was assumed to have a higher maximum growth rate than the second one (AOB2), which in turn had a higher oxygen affinity. Biomass detachment and biomass decay are assumed proportional to biomass concentrations. More details on the resulting model can be found in Volcke *et al.* (submitted).

2.4 Dynamic simulation results

Fig. 2. displays the simulated behaviour of the R30 and R10 reactors. The oxygen level in the reactor has been set to $0.2 \text{ gO}_2 \text{ m}^{-3}$ for R30 and to $3 \text{ gO}_2 \text{ m}^{-3}$ for R10. Precise oxygen measurements had not been recorded during experiments, but it was verified that the oxygen level in reactor R30 was indeed limiting and that this was not the case in reactor R10. The initial conditions are the same for both reactors. The simulation results agree with the experimental observations: nitrite accumulates in R30 while complete oxidation to nitrate is achieved in R10; the dominating microbial populations correspond to the ones in Fig. 1.

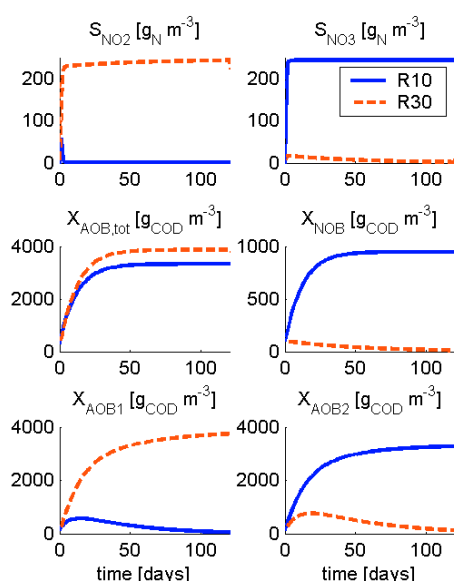


Fig. 2. Simulated behaviour of the R10 and R30 reactors : concentrations of ammonium (S_{NH}), nitrite (S_{NO2}), nitrate (S_{NO3}), total ammonium oxidizers ($X_{AOB,tot}$), individual nitrite oxidizers (X_{AOB1} and X_{AOB2}) as well as nitrite oxidizers (X_{NOB}).

The survival of only the ammonium oxidizer AOB1 at low oxygen concentrations can describe the occurrence of *Nitrosomonas europaea* in the R30 reactor. For high oxygen concentrations, as prevailing in reactor R10 due to the lower load, both AOB2 (*Nitrosomonas* sp.) and NOB (*Nitrospira*) colonize the reactor. Note the different timescales at which different phenomena take place: soluble component concentrations display fast changes, while total biomass concentrations take longer to reach their steady state.

Dynamics resulting from interspecies competition are even slower: individual AOB1 and AOB2 have not completely reached steady state values even after 4 months.

As a possible control strategy to maintain the two different types of AOB in the reactor, one could opt to switch the oxygen concentration in the reactor between two levels, e.g. by controlling the oxygen level between large boundaries rather than on a strict set point.

3. MICROBIAL DIVERSITY IN ANAEROBIC DIGESTION

3.1 Modelling diversity in anaerobic digestion

The IWA Anaerobic Digestion Model No. 1 (ADM1, Batstone *et al.*, 2002) was modified to handle microbial diversity (Ramirez and Steyer, 2008). The simulation software package MATLABTM/ Simulink was used to study the relationship between reactor performance and microbial community structure.

In the traditional ADM1 model, one microbial population is associated to each reaction. Seven main groups of microorganisms are represented, corresponding to the degradation of sugar, amino acids, LCFA, valerate and butyrate, propionate, acetate and hydrogen, each group of microorganisms having specific kinetic parameters. The microorganisms corresponding to the first five conversions are classified as bacteria, the ones corresponding to the latter two as archaea.

In order to account for microbial diversity, the traditional ADM1 model was extended in such a way that 10 different species were associated to each degradation reaction. For each species, the associated kinetic parameters were randomly chosen among 2 sets, normally distributed on each side of the kinetic parameters used to simulate ADM1 (Fig. 3). These sets were centered on 0.6 and 1.4 times the values used in ADM1 ($\pm 10\%$) in order to simulate two distinct populations of each reaction. In the following, this extended ADM1 model will be called ADM1_10. In order to maintain comparable conditions for simulations, the initial biomass concentrations in ADM1 will be distributed equally among the corresponding microbial populations in ADM1_10.

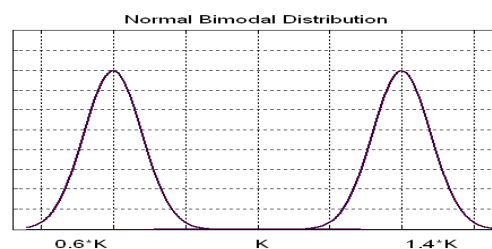


Fig. 3. Kinetic parameters in ADM1_10 (Ramirez and Steyer, 2008).

All inhibitions from ADM1 were kept in the model but an additional specific toxicant inhibition was added. No precise definition was here chosen for the toxicant since it was assumed to affect all microbial populations and modelled as a

non-competitive inhibition factor added to all substrate uptake rates:

$$I_{tox} = \frac{1}{1 + \frac{S_1}{K_1}} \quad (1)$$

where S_1 is the toxicant concentration and K_1 the inhibition constant. In the following, S_1 was simulated as a pulse signal and the K_1 mean value was arbitrarily chosen equal to 8 kgCOD/m³. In line with the choice of the kinetic parameters of ADM1_10, the values of the inhibition factors were randomly chosen for each biomass from a uniform distribution within two sets of mean values: 5 kgCOD/m³ and 11 kgCOD/m³, to represent the fact that some microbial populations (in this case the latter) can be more tolerant than the global biomass represented in ADM1.

The resulting model was applied to simulate the behavior of four identical continuous stirred tank reactor (CSTR) configurations with a fixed headspace volume of 20 L, and nominal reactor size of 948 L, at mesophilic temperature (35°C), with identical inoculum. The composition of the simulated influent was based on the characterisation of vinasses from local wineries in the area of Narbonne, France (see Ramirez and Steyer, 2008, for details).

Traditional performance parameters such as biogas production, VFAs concentration and removal soluble COD were used to evaluate CSTR's performance. Abundance Biomass Curves comparison and Simpson's diversity index (Magurran 2005) were used to describe the microbial community structure. In order to quantify microbial diversity, the Simpson diversity index (D) was calculated as follows:

$$D = \frac{1}{\sum_{j=1}^N p_i^2} \quad (2)$$

The ratios p_i have been calculated by dividing the biomass concentration of each species in a given family (*Bacteria* and *Archaea*) by the total biomass concentration at a given time instant.

3.2 Continuous versus pulsed loading rate operation

The response of two CSTRs with identical inoculums was simulated for constant and pulsed organic loading rate (OLR) operation, respectively. In the following, these reactors will be called R1 and R3 respectively. R1 was operated at a hydraulic retention time (HRT) of 2.5 days and fed with vinasses with total COD of 15 kg COD/m³. R3 was operated with a HRT between 0 and 2.5 days and the multiple-pulse OLR consisted of five sequential pulses with a duration of 5 days with 5 days between pulses, and amplitude twice the constant OLR, in such a way that the average organic loading rates for the perturbation cycle for all reactors were equal (Fig. 4). A P-controller was implemented in the model to maintain the reactor pH above a lower limit of 6.9, in order to

avoid pH inhibition of aceticlastic methanogenesis. Fig. 4 presents the simulation results over a period of 50 days.

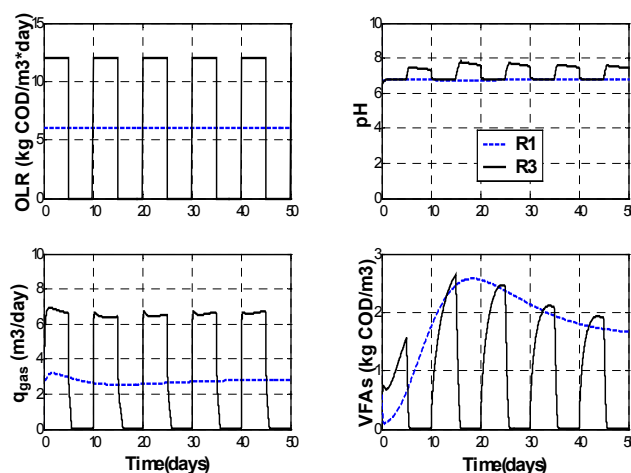


Fig.4. ADM1_10 predicted VFAs concentrations, Biogas production rate, pH and OLR of R1 and R3 feed with vinasses, for constant and pulsed OLR respectively.

Every incremental increase in OLR during pulsed operation caused inhibition in the reactor performance during a short period after the loading. This may be attributed to the increase in the substrate concentration to be converted, which requires sufficient acclimatization period for native microflora to sustain to the changed environmental condition of the system. During each substrate shock load, the model showed increases in effluent VFA and soluble COD (not shown, but mainly consisting of VFAs) while the gas production increased but the methane content decreased. Nevertheless, the reactor always recovered to its normal performance within the next cycle, meaning that the shock were not too severe and assimilated by the reactor "buffer" for load capacity.

Considering the averaged behaviour over the cycles, R3 performs better than R1: it has a higher soluble removal efficiency, a higher gas production (33.8 m³ in R3 versus 27.4 m³ in R1), for a lower mean concentration of accumulated VFAs. Another difference from these two reactors lies in the biomass evolutions. Fig. 5 displays the dynamic evolution of acetate degraders together with Abundance Biomass Curves and Simpson's diversity index for the *Bacteria* and *Archaea* domains, corresponding to the operation of R1 and R3. Similar results were obtained for all degraders but they are not shown due to space limitation. The R3 microbial community appears to be more diverse, with higher temporal variations. In contrast, the R1 microbial community appears more homogeneous with less diversity in the *Bacteria* and *Archaea* domains.

Summarizing, the pulsed OLR reactor (R3) displays a better performance than the one with constant OLR (R1), despite having a more diverse and less stable microbial community.

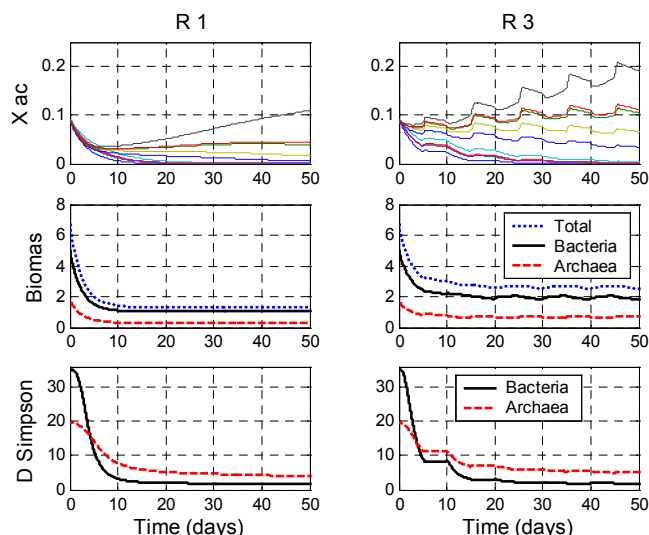


Fig. 5. ADM1_10 simulated biomass behaviour of R1 and R3 fed with vinasses, under constant and pulsed OLRs.

3.3 Effect of a toxicant pulse

The response of two CSTRs, with the same constant and pulsed loading rate operation, has now been simulated to a pulse toxicant concentration applied at day 12.5 for 2.5 days with amplitude 50 kg COD/m³. In the following these reactors will be called R2 and R4, respectively. Fig. 6 shows the simulated behavior of both reactors, over 50 days.

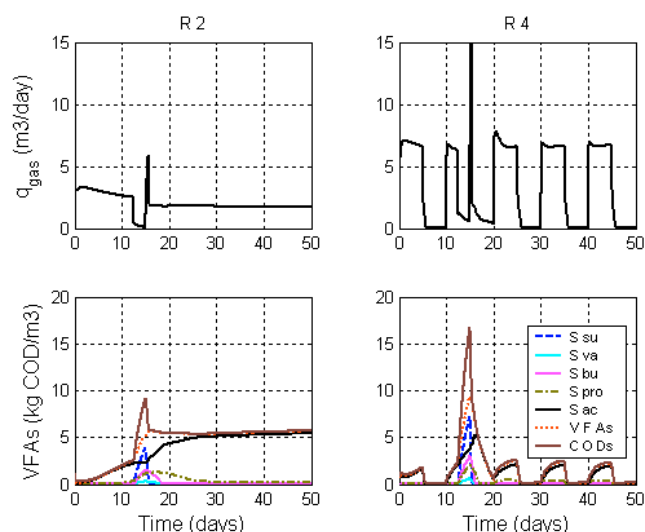


Fig. 6. ADM1_10 predicted VFAs, CODs and intermediate products concentrations and Biogas production rate of R2 and R4 reactors, under step input and pulsed OLR with a 2.5 day toxicant pulse applied at day 12.5.

Upon toxicant addition, the concentration of VFAs (and consequently COD) increases rapidly in both reactors. This is associated with a pH decrease (not shown). In R4, VFA and COD concentrations decrease to their pre-perturbed values once the toxicant has been removed, while the new steady state values in R2 are higher than the pre-toxicant ones. The gas production displays a similar behaviour: it decreases in both reactors upon toxicant addition, then recovers to its pre-

toxicant value in R4, while staying at a lower value than before the shock in R2. From the third cycle on, the gas produced per cycle was 34.5 m³ for R4 and 20.7 m³ for R2.

As in the previous section, the main difference between both reactors lies in the biomass evolution. Fig. 7 shows the dynamic evolution of acetate degraders together with Abundance Biomass Curves and Simpson's diversity index evolution for the *Bacteria* and *Archaea* domain. The rapid accumulation of VFAs in both reactors (Fig. 6) results from a clear decrease in the activity of acetate-utilizing methanogens (Fig. 7) and acetogens. Fermentative bacteria were also affected by the toxicant substrate perturbation. The H₂-utilizing methanogens appeared to be less affected by the substrate perturbation, furthermore no significant accumulation of H₂ was observed during the entire experiment (results not shown).

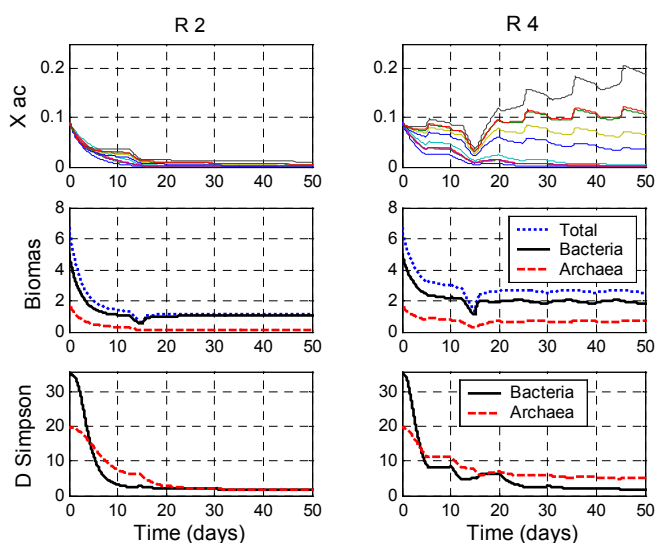


Fig. 7. ADM1_10 predicted biomass behaviour R2 and R4 feed with vinasses, under constant and pulsed OLR with a toxicant pulse applied at day 12.5.

The observed changes in individual volatile fatty acids concentrations (Fig. 6) indicate that all the major groups were impacted by the toxicant perturbation. Sugar accumulation points out that the fermentative bacteria were also affected by the toxic substrate perturbation. The most evident sign of this was the dramatic change in the products of sugar fermentation, and this may contribute to explain the ability of R4 to adapt to the toxic substrate perturbation. The microbial community in the reactor R4 appeared to be more diverse, with high temporal variations. In contrast, the microbial community in the reactor R2 appeared more homogeneous with significantly less diversity mainly in Bacteria's domain.

Summarising, the pulsed OLR reactor (R4) has better performance than the constant OLR (R2) towards toxicant addition, despite that its microbial community was more diverse and less stable. The main difference between these communities is that R4 microbial community was able to return to the pre-toxicant conditions, while this was not the case for the one in R2. The simulation results thus indicate that the reactor with a less stable community but with higher diversity was more functionally stable towards pulsed

toxicant disturbances. The differences found between the fermentation pathways of accumulated sugar during the toxicant disturbance period suggest that the R4 community structure was more flexible than the R2 one.

In order to further emphasize the influence of microbial diversity in response to toxicant pulse, Figure 8 shows simulations results with ADM1 and ADM1_10 under pulse feeding regime. As it can be seen, the diversity of ADM1_10 is higher than ADM1 for both domains: *Bacteria* and *Archaea* and although the biomass was able to recover in both models, higher biogas production and lower VFAs accumulation is obtained with ADM1_10, demonstrating a better tolerance to the toxicant pulse.

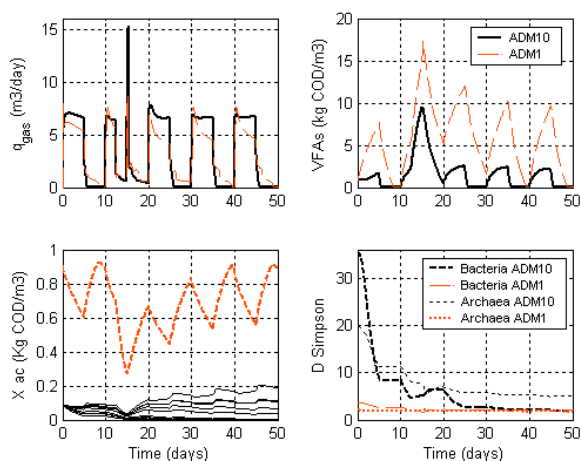


Fig. 8. ADM1 (thin dotted lines) and ADM1_10 (thick continuous lines) simulations: Comparison of VFAs and acetate degraders concentrations, biogas production rate and Simpson diversity indices under pulsed OLR with a 2.5 day toxicant pulse applied at day 12.5.

4. CONCLUSIONS

Enhancing system heterogeneity by fostering the right populations can be achieved in several ways. One way is to take advantage of spatial effects, as in granulation or biofilm development or membrane reactors, which can be effective due to the different location of different populations and functions in these systems. Another possibility to be explored is to introduce heterogeneity at temporal scale, e.g. by means of providing substrate pulses to encourage the growth of desired microorganisms, as suggested in this paper. The development of communities that are more resilient in the long term due to the pulse disturbances has been demonstrated in other ecosystems, and the stability developed therein is the result of heterogeneity operating in both temporal and spatial scales (Bengtsson *et al.*, 2002).

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