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ENVIRONMENTAL BIOTECHNOLOGY

Impact of increasing NaCl concentrations on the performance and community composition of two anaerobic reactors

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Abstract The anaerobic treatment of saline effluents using halophilic and halotolerant microbial consortia is of major interest. Inhibition of anaerobic digestion is known to occur at high salt content. However, it seems that the suitable adaptation of an anaerobic sludge makes possible the treatment of saline wastewater. In this study, a non-saline anaerobic sludge was inoculated in two anaerobic batch reactors operating with a different substrate (distillery vinasse and ethanol) and subjected to increasing NaCl concentrations. The performance of the digesters appeared to be highly dependent on the nature of the substrate, and a similar level of inhibition (i.e. around 90% of the specific loading rate and specific methanogenic activity) was stated at 10 g l^{-1} of NaCl with distillery vinasse and 60 g l^{-1} of NaCl with ethanol. The characterization of the microflora and its adaptation to increasing NaCl conditions were also investigated using molecular tools based on the analysis of genomic 16S rDNA. The microbial communities revealed a high diversity that could be maintained in both reactors despite the increase in NaCl concentrations.

Keywords Anaerobic SBR · Halotolerance · Microbial community · 16S ribosomal RNA

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Introduction

The treatment of saline and hypersaline wastewater could represent as much as 5% of worldwide effluent treatment requirements. Anaerobic digestion is known to be inhibited by high salinity mainly due to the presence of cations. It has already been reported that a sodium concentration exceeding 10 g l^{-1} strongly inhibits methanogenesis (Gourdon et al. 1989; Kugelman and McCarty 1965; Rinzema et al. 1988). In spite of the obstacle created by high salinity, a certain number of processes have been used successfully for the anaerobic treatment of saline wastewater. Some of them used a halophilic inoculum (Aspé et al. 2001; Lefebvre et al. 2006a; Mosquera-Corral et al. 2001; Vidal et al. 1997), whereas others required the adaptation of a non-halophilic inoculum to increasing salt concentrations (Boardman et al. 1995; Gangagni Rao et al. 2005; Gebauer 2004; Guerrero et al. 1997; Habets et al. 1997; Omil et al. 1995; Rovirosa et al. 2004). From the point of view of microbial ecology, the adaptation of a non-saline sludge to high salinity implies the acclimation of halotolerant microorganisms to high salt content. Some studies suggest that this acclimation is possible, depending on the nature and the progressive adaptation of the sludge to high salinity (Feijoo et al. 1995; Omil et al. 1995). However, little is known regarding the dynamics and the diversity of a microbial ecosystem treating highly saline wastewater, although it has already been shown that the diversity of a salt-tolerant ecosystem treating hypersaline industrial wastewater could be similar to that of a non-salt-tolerant one (Lefebvre et al. 2004, 2006b).

In this study, a non-saline anaerobic sludge was inoculated in two anaerobic batch reactors, each operating with a different substrate (distillery vinasse and ethanol), as it has been shown that the performance of anaerobic

digestion under saline conditions strongly depends on the type of methanogenic substrate used (Feijoo et al. 1995). Both digesters were then submitted to NaCl concentrations increasing in stages. The aim of the current study was to obtain a better understanding of the effect of increasing NaCl concentrations on the performance and community composition of anaerobic digestion.

Materials and methods

Bioreactors

This study was conducted using two double-walled reactors of 5 l each and maintained at 37°C by a thermostatically regulated water bath. Peristaltic pumps were used for substrate filling and withdrawal, and mixing was ensured using magnetic stirrers. The biogas production rate was measured by an Aalborg mass flow meter 0–50 ml min^{-1} fitted with a 4–20 mA output. The "Modular SPC" software, developed at the Institut National de la Recherche Agronomique -Narbonne laboratory, was used for data acquisition.

Both reactors were inoculated with anaerobic sludge originating from an upflow anaerobic sludge blanket (UASB) reactor treating sugar wastewater and so not adapted to saline conditions. After initial washout and disaggregation, volatile suspended solids (VSS) concentrations stabilized around 8.2 and 7.6 g l^{-1} for the reactor, operating with distillery vinasse and ethanol, respectively.

Substrates

Two different substrates were used to feed the two anaerobic reactors, i.e. ethanol with a purity level of 95%, used as a simple substrate, and distillery vinasse, the residue of wine distillation, used as a complex substrate. The distillery vinasse originated from a distillery at Narbonne (France) and was characterized by a high organic concentration, with the soluble chemical oxygen demand (COD) varying between 28 and 35 g l^{-1} and a COD/N/P ratio of 200:3:1. However, in regard to the nitrogen and phosphorus requirements for anaerobic digestion, no deficiency was recorded (Henze and Harremoës 1983). In addition, the effluent was characterized by a mean suspended solids (SS) concentration of 1.2 g l^{-1}, 75% of which were volatile, thus showing that the SS were constituted mainly of organic matter. Finally, the pH averaged 3.5 due to the high organic acid content. It was therefore neutralized by 15 ml of NaOH (32%) per liter of wastewater before being introduced into the reactor to maintain reactor pH close to 7 at the end of the filling period. This resulted in an initial Na^+ concentration of 3 g l^{-1} in the reactor operated with distillery vinasse after it reached the steady-state conditions. This initial addition of Na^+ will not be mentioned any further in this paper, as the study focuses on the effect of added NaCl. Yet, it should be kept in mind that the addition of 1, 5 and 10 g l^{-1} of NaCl (i.e. 0.4, 2 and 4 g l^{-1} of Na^+) in the reactor operating with distillery vinasse resulted in a Na^+ concentration of 3.4, 5 and 7 g l^{-1}, respectively. This effluent was stored at 4°C throughout the experiment. For the complete characterization of distillery vinasse, we refer to Vlyssides et al. (2005).

To avoid any nutriment deficiency, both reactors initially received a dose in excess of 200 µl of a mineral complement (Al 11.25 g l^{-1}, B 0.858 g l^{-1}, Fe 9.925 g l^{-1}, Ni 6.5 g l^{-1}, Ba 1.61 g l^{-1}, Co 1.75 g l^{-1}, Mn 12.71 g l^{-1}, Zn 14.87 g l^{-1}, Sr 18.33 g l^{-1} and Cu 20.215 g l^{-1}), the dose of this complement prescribed for industrial purpose being 5 ml m^{-3}. In addition, the ethanol-fed reactor was supplemented in nitrogen and phosphorus, following Henze and Harremoës (1983). The concentrations of these nutrients were regularly checked throughout the experimental period to ensure that no deficiency could be stated at any time.

Chemical analysis

COD, SS and VSS were analysed following the analytical methods recommended by Association Française de Normalisation, the French Standards Authority (AFNOR 1997). Soluble COD analysis was preceded by a centrifugation step (15,000 rpm, 15 min). COD was determined by the closed reflux method, mercuric sulphate being used to eliminate the interference of chlorides when dosing COD, in accordance with Sawyer and McCarty, who reported that this interference could be eliminated as long as a 10:1 weight ratio of mercuric sulphate to chloride is maintained (Sawyer and McCarty 1967). Determination of volatile fatty acids (VFA) was done using a gas chromatograph fitted with a flame ionization detector (Chrompac CP 9000) and coupled with an integrator (Shimadzu CR 3A). The percentage of methane in the biogas was determined with a gas chromatograph (Shimadzu GC-8A), with argon as the carrier gas, equipped with a thermal conductivity detector and connected to an integrator (Shimadzu CR 3A).

Molecular analysis of the microbial community

For each level of NaCl, sludge samples were withdrawn from both reactors after stabilization of the performance, and DNA was extracted as described by Lefebvre et al. (2004). Amplification, cloning and sequencing of 16S rDNA were also realized according to the procedure described by Lefebvre et al. (2004). Sequences in 16S rDNA (of about 500 bp) were identified by comparison with sequences available in databases using the BLAST

program. The nucleotide sequence data reported in this work will appear in the GenBank nucleotide database under accession numbers AM117917 to AM117928.

Single Strand Conformation Polymorphism (SSCP) analyses were performed for the overall detection of microbial populations and for a study of their dynamics (Zumstein et al. 2000). SSCP analysis makes it possible to separate DNA fragments of a similar size according to their configuration (secondary structure). Targeting the 16S rDNA V3 region, which permits the phylogenetic discrimination of microbial species, enables the reactor microbial community to be monitored by one profile of peaks, each peak corresponding to a distinct sequence of the 16S rDNA V3 region, i.e. to one bacterium. The area under the peaks corresponds to the quantity of 16S rDNA sequence after polymerase chain reaction (PCR) amplification. The amplification of the V3 region of bacterial 16S rDNA PCR-SSCP was carried out following the procedure described by Lefebvre et al. (2004). To identify SSCP peaks of interest, 16S rDNA V3 from reactor samples were amplified and cloned into *Escherichia coli* as described by Lefebvre et al. (2004).

Results

Experimental strategy and working conditions of the reactors

The reactor fed with distillery vinasse was operated in sequencing batch reactor (SBR) mode with cycles including the following four discrete steps: rapid filling (1 min), reaction (variable in time), settling (60 min) and withdrawal (15 min). The volumetric exchange ratio (VER) of this reactor was 0.07. The reactor operated with ethanol received only 10 ml of substrate per cycle, hence had a very low VER (0.003). It was then operated in a fed-batch mode with no settling and no withdrawal. This was made possible because the volume added daily was compensated by evaporation. The operating conditions applied to both reactors ensured an initial S_o:X_o ratio ranging from 0.2 to 0.3 kg of COD kg^{-1} of VSS for the reactor fed with distillery vinasse and from 0.3 to 0.4 kg of COD kg^{-1} of VSS for the reactor fed with ethanol.

After seeding, both reactors were operated at zero NaCl concentration during 2 weeks, and after that they were operated at increasing NaCl concentrations for over 60 days for the reactor operating with distillery vinasse and 100 days for the reactor fed with ethanol. The comparison of the results obtained at each NaCl concentration permitted the determination of the steady-state conditions at each NaCl concentration, on the basis of at least three consecutive cycles showing similar performance in terms of biogas production rate, reaction time, COD removal, specific loading rate and methanogenic activity. A standard cycle could be determined this way for each level of NaCl concentration. The results are illustrated in Fig. 1 that shows the evolution of the specific methanogenic activity (expressed in ml CH_4 g^{-1} of VSS day^{-1}) for both reactors at each level of salinity. The number of cycles for which the reactors were allowed to run at each NaCl concentration are indicated in parentheses, and the closed symbols indicate the points that were selected for the determination of the so-called standard cycle. After determination of such a standard cycle at a given NaCl concentration, NaCl was added to the reactor at the end of the last cycle after

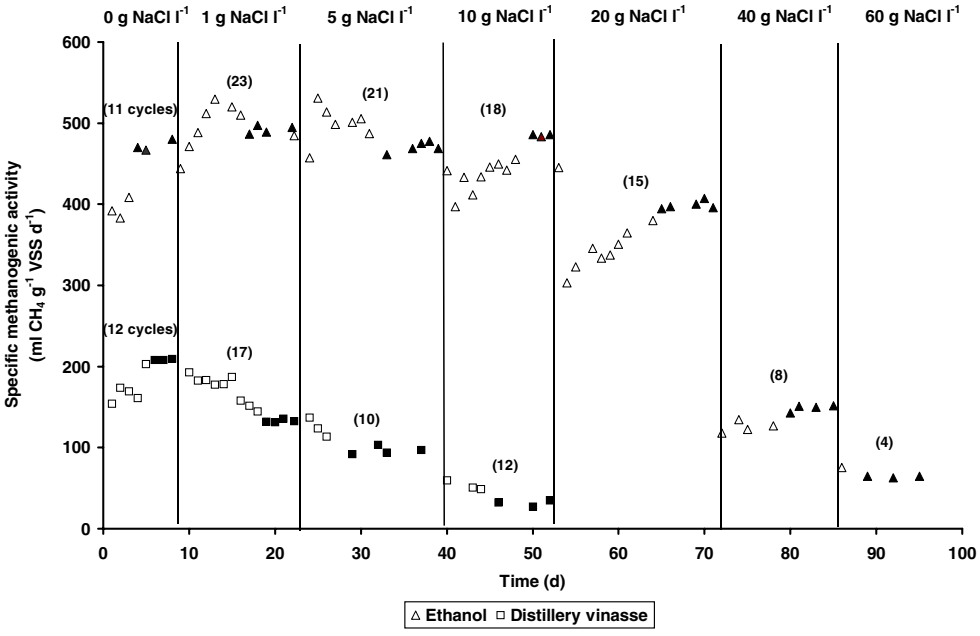

Fig. 1 Evolution of the specific methanogenic activity for two anaerobic reactors operating with a different substrate (distillery vinasse and ethanol) at different levels of salinity. The number of cycles for which the reactors were allowed to run at each NaCl concentration are indicated in *parentheses* and the *closed symbols* indicate the points that were selected for the determination of standard cycles

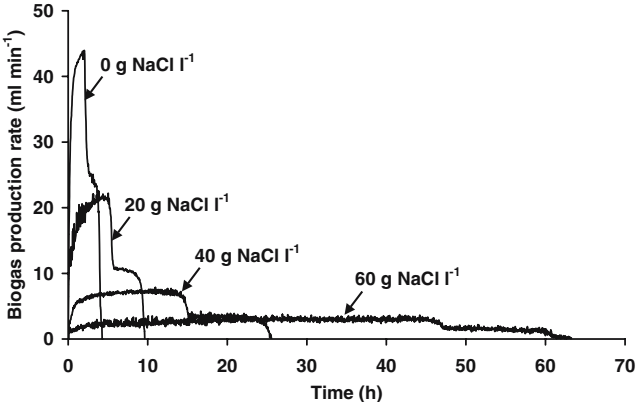

Fig. 2 Evolution of biogas production rate during standard cycles (reaction step) with increasing NaCl concentrations. Reactor operating with ethanol

withdrawal of the treated effluent to reach a higher NaCl concentration.

Evolution of biogas production rate during a cycle

Gas production from the reactor was measured online, and a biogas production rate profile proportional to the removal rate of organic matter was obtained for each cycle. An illustration of the standard biogas production rate profiles obtained with ethanol as a substrate at 0, 20, 40 and 60 g l^{-1} is shown in Fig. 2.

The rate of biogas production was maximal at the start of the cycle just after the feed period and then decreased with time, reaching very low levels at the end of the reaction stage, indicating very low metabolic activity. The reaction stage was considered as finished when the biogas production rate dropped below the minimum limit of 0.5 ml min^{-1} in our operating conditions, a level indicating that the added organic matter had been eliminated. At this point, the concentration of VFA was zero and soluble COD concentration at its lowest. Hence, the reaction step was not fixed in time but was adjusted, depending on the evolution of the biogas production rate.

Effect of increasing NaCl concentrations on the reactors' performance

Figure 2 shows that the main consequences of increasing NaCl concentrations were a reduction in the biogas production rate and an increase in the length of the cycles, indicating a slowdown of the microbial activity. Similar results were obtained with distillery vinasse as a substrate (data not shown). From the analysis of standard cycles obtained at each level of NaCl, it was possible to evaluate for each reactor the average length of the cycles, COD removal efficiency, specific and organic loading rates (SLR and OLR), as well as specific methanogenic activity (SMA), at each NaCl concentration. The results are compiled in Table 1. The reactor operating with distillery vinasse was already affected at a NaCl concentration of 1 g l^{-1}, whereas the reactor operating with ethanol could withstand higher NaCl concentrations. Indeed, with distillery wastewater, the addition of 1 g of NaCl l^{-1} was sufficient to reduce the SMA from 193 to 140 ml CH_4 g^{-1} VSS day^{-1} (27% reduction), and the reduction attained 88% when NaCl concentration reached 10 g l^{-1} (from 193 to 24 ml CH_4 g^{-1} VSS day^{-1}). With ethanol, the SMA decreased by 20% (from 494 to 396 ml CH_4 g^{-1} VSS day^{-1}) when NaCl concentration reached 20 g l^{-1} and by 88% for a NaCl concentration of 60 g l^{-1} (from 494 to 61 ml CH_4 g^{-1} VSS day^{-1}). Similar results were obtained using SLR as the criterion, both parameters being proportional (see Table 1).

Regarding COD removal, it should be kept in mind that the reaction time was adjusted depending on the biogas production rate to allow all the biodegradable fraction to be treated. Thus, the COD measured at the end of the cycle corresponded to the non- or very slowly biodegradable fraction of COD. The soluble COD removal efficiencies of the reactors averaged 93.5% and 99.9%, for distillery

Table 1 Average length of cycles, COD removal efficiency, specific and organic loading rates (SLR and OLR) and specific methanogenic activity (SMA) at different levels of NaCl concentration using distillery vinasse and ethanol as a substrate

NaCl (g l^{-1})	Length of cycles (h)		SLR (kg COD kg^{-1} VSS day^{-1})		OLR (kg COD m^{-3} day^{-1})		SMA (ml CH_4 g^{-1} VSS day^{-1})		Soluble COD removal (%)	
	vinasse	ethanol	vinasse	ethanol	vinasse	ethanol	vinasse	ethanol	vinasse	ethanol
0	10.7±0.1	4.5±0.2	0.50±0.08	1.38±0.07	4.93±0.47	17.03±1.56	193±4	494±14	94.3±0.8	99.9±0.1
1	16.8±0.2	4.3±0.3	0.39±0.03	1.41±0.09	3.28±0.99	17.68±0.75	140±10	524±21	93.7±2.2	99.9±0.1
5	24.5±1.2	5.7±0.1	0.26±0.02	1.27±0.07	1.86±0.20	13.59±2.42	94±7	498±7	93.4±0.9	99.9±0.1
10	106.1±15.1	7.8±0.3	0.06±0.01	1.19±0.09	0.50±0.50	9.80±0.57	24±6	480±25	93.0±1.4	99.9±0.1
20		9.5±0.3		0.99±0.09		8.08±0.75		396±15		99.9±0.1
40		28.5±2.1		0.37±0.05		2.70±0.21		151±1		99.9±0.1
60		66.4±4.5		0.15±0.02		1.16±0.18		61±6		99.9±0.1

vinasse and ethanol respectively, and remained constant throughout the experiment (see Table 1).

Finally, treated effluents showed high turbidity (data not shown). However, sludge volume indexes (SVI) remained constant around 60 ml g^{-1} of VSS for both reactors throughout the experiment, which indicated very good settling properties of the flocculating sludge. It should be reminded in this paper that SVI and turbidity are not necessarily related directly because they reflect different physical issues: SVI reflects the compactability of the flocculating biomass, whereas turbidity reflects the ability of dispersed organisms to flocculate (Ng et al. 2005). No massive lysis of biomass could be suspected at high NaCl concentrations, as effluent soluble COD and SS remained fairly constant.

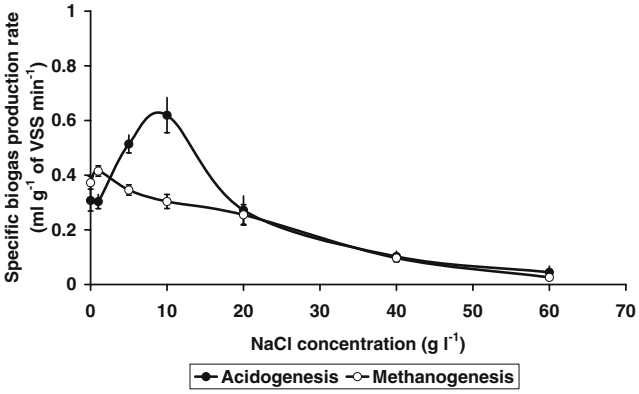

Fig. 3 Evolution of specific biogas production rate due to acidogenesis and methanogenesis with increasing NaCl concentrations using ethanol as a substrate

Effect of NaCl on acidogenesis and methanogenesis rates

Operating in batches enabled the determination of maximal biogas production rates during a cycle. With ethanol as a substrate, the cycles obtained showed two phases with a sharp drop in the biogas production rate indicating the separation between the two phases (Fig. 2). During the first phase, the biogas production rate was fairly constant and at its maximum. This phase ended with a sharp drop in this rate and the second phase was then characterized by a fairly constant but lower biogas production rate. It has already been demonstrated by Ruíz et al. (2002) that the first phase corresponded to the cumulative production of biogas by acidogenesis—which is the fastest reaction—and methanogenesis, whereas the second phase corresponded only to methanogenesis. This conclusion is supported by the evolution of VFA concentration and pH. Indeed, the first phase was characterized by increasing VFA concentration and decreasing pH. The pH increased regularly thereafter throughout the second phase, as VFA concentration slowly decreased to nil at the end of the cycle (data not shown).

For the reactor operating with ethanol, it was therefore possible to distinguish the biogas production rate induced by acidogenesis from that induced by methanogenesis, thanks to fairly constant values during each phase. The establishment of standard cycles for this reactor throughout the experiment enabled this distinction to be made for each level of NaCl. The effect of NaCl on the specific production of biogas by acidogenesis and methanogenesis is shown in Fig. 3. From this figure, it can be seen that, on the one hand, methanogenesis started to be inhibited by NaCl at a concentration of 5 g l^{-1}, the specific biogas production rate decreasing from 0.37 to 0.34 ml g^{-1} of VSS min^{-1} (a reduction of 7%). This inhibition then reached 93% (from 0.37 to 0.03 ml g^{-1} of VSS min^{-1}) at a NaCl concentration of 60 g l^{-1}. On the other hand, the specific biogas rate produced by acidogenesis first improved at low salt concentrations (0–10 g NaCl l^{-1}). Two hypotheses can explain this fact: either an enhancement of specific acidogenic bacterial activity or a rise in the concentration of acidogenic biomass. Thereafter, at NaCl concentrations of 20 g l^{-1} and higher, a significant reduction in acidogenesis was observed, reaching 11% (from 0.31 to 0.27 ml g^{-1} of VSS min^{-1}) and 85% (from 0.31 to 0.04 ml g^{-1} of VSS min^{-1}) at NaCl concentrations of 20 and 60 g l^{-1}, respectively.

Dynamics of microbial communities with increasing NaCl concentrations

The dynamics of microbial communities with increasing NaCl concentrations was monitored by PCR-SSCP. Four profiles related to the domain of Bacteria were compared for the reactor operating with distillery vinasse, out of which two are represented in Fig. 4. At 0 g l^{-1} of NaCl, the bacterial SSCP profile showed about 24 distinguishable peaks. The profiles obtained at 1, 5 and 10 g NaCl l^{-1} revealed similar diversity, with peaks appearing and others disappearing at each level of NaCl, indicating little but significant population shifts.

Seven profiles related to the domain of Bacteria were aligned for the reactor operating with ethanol, out of which two are represented in Fig. 5. At 0 g l^{-1} of NaCl, the bacterial SSCP profile showed about 17 distinguishable peaks, which is less than that of the profile obtained with the distillery vinasse, thus indicating less diversity. Later on, the profiles obtained at 1, 5, 10, 20, 40 and 60 g NaCl l^{-1} revealed similar diversity.

The SSCP profiles related to the domain of Archaea were very similar for both reactors, showing little diversity and two dominant peaks remaining throughout the experiment (data not shown). Eukaryota 16S rDNA amplification was also attempted using eukaryotic-specific primers and

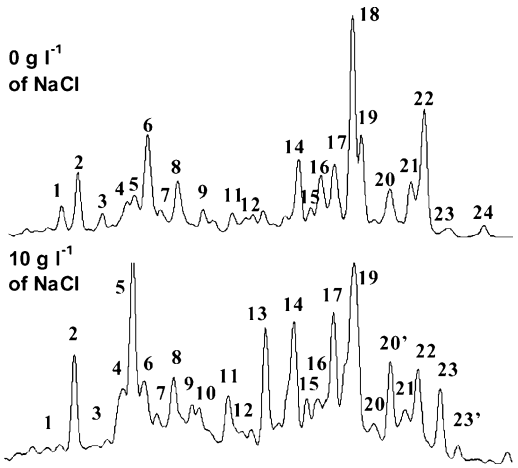

Fig. 4 SSCP peak patterns showing the dynamics of the microbial community in the reactor fed with distillery vinasse at different NaCl concentrations. The dominant peaks were *numbered* to facilitate their identification

various PCR conditions. However, the amplification failed, which indicates that Eukaryota, if present, were not major components of the autochthonous microbial community.

PCR-SSCP peak identification

Because the anaerobic sludge isolated from the reactor operating with ethanol displayed a smaller number of dominant peaks, dominant peak identification focused on it. Fifty bacterial clones were isolated from this reactor at 0 g l^{-1} of NaCl (clones MV1 to 50) and 50 at 60 g l^{-1} (clones MW1 to 50). All were analysed by SSCP and produced a discrete prominent peak that was aligned with the corresponding sludge profile. Only peaks of 5 MV

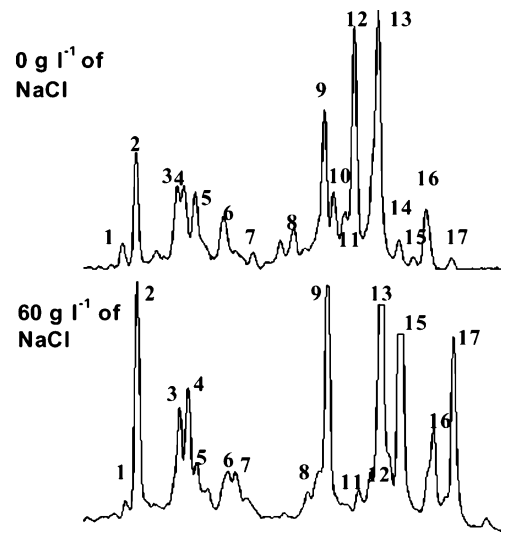

Fig. 5 SSCP peak patterns showing the dynamics of the microbial community in the reactor fed with ethanol at different NaCl concentrations. The dominant peaks were *numbered* to facilitate their identification

clones and 6 MW clones clearly co-migrated with detectable peaks from the corresponding sludge profiles. In addition, from the same reactor sludge fed with ethanol, ten archaeal clones were isolated at 0 g l^{-1} of NaCl (clones FL1 to 10) and analysed by SSCP. After alignment with the corresponding profile, nine clones co-migrated with the two dominant peaks, and one did not co-migrate with any peak, thus corresponding to an underlying archaeal species.

Finally, nine bacterial clones and three archaeal clones were sequenced, and the results are indicated in Table 2. The two dominant archaeal peaks corresponding to clones FL03 and FL04 were highly similar (≥99%) to *Methanosaeteta* sp. and *Methanobacterium beijingense*, and the underlying species (FL06) showed 99% similarity with the environmental clone LL25A1. Regarding Bacteria, all the identified peaks, except three, showed more than 95% similarity with environmental clones belonging to the *Deltaproteobacteria*, *Nitrospira* and *Synergistes* phyla. The remaining three presented between 90 and 95% similarity with environmental sequences belonging to the *Chloroflexi* and *Spirochaetes* phyla. All the Bacteria identified showed clear anaerobic characteristics, and most of them are known for their biodegradation capabilities.

Discussion

In this study, two reactors fed with distillery vinasse and ethanol, respectively, underwent a saline stress, increasing in stages. The experimental strategy involved the batch technology (SBR for vinasse and fed-batch for ethanol) that presented several advantages towards a continuous process. First, the batch method has already been shown to facilitate the adaptation of organisms to high salt concentrations (Kargi and Dincer 1996), and many SBRs could successfully treat saline wastewater (Lefebvre et al. 2004, 2005; Moon et al. 2003; Ng et al. 2005; Uygur and Kargi 2004; Woolard and Irvine 1995). Second, in our specific experiment, it enabled the determination of biogas production rate profiles during a cycle as well as the maximum biogas production rates with the two substrates used.

Although the experiments were not performed in duplicates, the reproducibility was excellent from one cycle to another after the steady-state conditions were reached at each level of salinity (see Fig. 1). The NaCl inhibition was quantified using SMA as a criterion, which was proportional to SLR, COD removal remaining constant over the experimental period for both reactors. In the reactor operating with distillery vinasse, the strongest perturbation was observed while increasing NaCl concentration from 5 to 10 g l^{-1}, which caused a reduction of 88% of the SMA

Table 2 Phylogenetic affiliation of 16S rDNA sequences

Name	Number of clones	Peak number	Phylum	% Identification	Closest microorganism or environmental clone (accession number)	Isolated from
FL03	2	–	*Euryarchaeota*	100	*Methanosaeta* sp. clone KB-1 (AY780568)	Chlorinated ethene-dechlorinating culture
FL04	2	–	*Euryarchaeota*	99	*Methanobacterium beijingense* strain 8-2 (AY350742)	Anaerobic bioreactor
FL06	1	–	*Euryarchaeota*	99	Clone LL25A1 (AJ745133)	Anoxic soil
MV01	1	13	*Deltaproteobacteria*	96	Clone SJA-111 (AJ009485)	Anaerobic microbial consortium
MV08	1	1	*Nitrospira*	99	Environmental clone (AB195896)	Anaerobic bioreactor
MV12	1	9	*Deltaproteobacteria*	99	Clone SHA-42 (AJ306771)	1,2-Dichloropropan dechlorinating culture
MV32	1	5	*Chloroflexi*	95	Clone B15 (AY426440)	Anaerobic bioreactor
MV43	1	12	*Deltaproteobacteria*	99	Clone E27 (AY426467)	Anaerobic bioreactor
MW13	1	4	*Synergistes*	100	Clone synarJD05 (AY654336)	Anaerobic bioreactor
MW14	1	14	*Spirochaetes*	90	–	–
MW41	1	16	*Firmicutes*	98	Clone B9 (AY426453)	Anaerobic bioreactor
MW48	1	7	*Chloroflexi*	91	–	–

Reactor operating with ethanol.

determined at zero NaCl concentration (from 193 to 24 ml CH_4 g^{-1} VSS day^{-1}). Regarding the reactor operating with ethanol, the most important disturbance occurred while increasing NaCl from 20 to 40 g l^{-1} and was characterized by a SMA reduction of 69% (from 494 to 151 ml CH_4 g^{-1} VSS day^{-1}). The clear conclusion is that the reactor operating with distillery vinasse appeared to be inhibited at a lower salt concentration than the reactor operating with ethanol. A similar inhibition level (i.e. 88% of SMA) was observed for the two reactors at final NaCl concentrations of 10 g l^{-1} with distillery vinasse and 60 g l^{-1} with ethanol. In the reactor operating with ethanol as sole source of carbon and energy, distinguishing acidogenesis from methanogenesis was possible. It could be seen clearly that methanogenesis started to be affected at a lower NaCl concentration (5 g l^{-1}) than acidogenesis (20 g l^{-1}).

The impact of NaCl thus was different according to the nature of the substrate: NaCl inhibition was observed at lower NaCl concentrations when using a complex substrate. This observation makes it possible to explain the difficulty encountered in the anaerobic biological treatment of complex saline effluents, which has frequently been noted in the literature. For instance, the limitation of the load applied to saline complex effluents was observed by Rovirosa et al. (2004) when treating complex piggery effluents. Indeed, at a salt concentration of 15 g l^{-1} (comparable to the maximum concentration tested for the reactor fed with distillery vinasse in our study) and using a down-flow anaerobic fixed bed reactor, a satisfactory soluble COD removal efficiency of 90% was stated by the authors only with a very low OLR of 0.5 kg COD m^{-3} day^{-1}. The anaerobic treatment of a fishmeal effluent (10 g Cl^- l^{-1}) (Guerrero et al. 1997) and of a saline fish farm effluent (10 g Na^+ l^{-1}; Gebauer 2004) was also limited in both cases to a low OLR (<3 kg COD m^{-3} day^{-1}) and a low specific loading rate (<0.1 kg COD kg^{-1} VSS day^{-1}). Finally, Lefebvre et al. (2006a,b) studied the anaerobic digestion of tannery soak liquor (71 g l^{-1} of NaCl) using UASB. A COD removal efficiency of 78% could be achieved provided OLR was maintained around 0.5 kg COD m^{-3} day^{-1}. These results are very similar to those obtained in this study. It seems, therefore, that the anaerobic treatment of complex saline effluents is only possible on condition there be a very low loading rate, which thus limits the industrial applicability of this type of treatment. The difficulty of treating complex effluents under saline conditions is likely to be related to the high number of intermediate stages required for their degradation: when the number of stages increases, the probability also increases that one of these stages may be inhibited by salt.

The choice of sludge appears to be a major factor in the effectiveness of biological treatment processes for saline wastewater, which raises the question of the adaptation of the micro-organisms involved in the purification of such wastewater. Molecular biology analyses (SSCP) made it possible to highlight changes in the reactor microflora in response to saline stress, with a view to correlating them with the performance of the reactors. Bacterial SSCP profiles obtained at 0 g l^{-1} of NaCl showed a higher diversity for the reactor operating with distillery vinasse (about 24 dominant peaks) than for the reactor operating with ethanol (17 dominant peaks). This could be a

consequence of the nature of the substrates: the distillery vinasse (complex substrate) might indeed require the intervention of a higher number of micro-organisms for its degradation, whereas the degradation of ethanol (simple substrate) may well require a lower number of Bacteria.

For the reactor operating with distillery vinasse, the increase in NaCl concentration caused notable changes in terms of activity as well as microbial diversity. On the contrary, the SSCP profiles of the reactor operating with ethanol were very similar for each level of NaCl, and according to Table 2, this bacterial community was found to be similar to that of conventional anaerobic reactors as described by Godon et al. (1997a,b). Even at high salt concentrations, the performance of these Bacteria was maintained, and they were severely affected only at NaCl concentrations higher than 20 g l^{-1}. Thus it can be concluded that, according to the nature of the substrate, the same sludge inoculated into two different reactors reacted in a different way to increasing salinity. The better performance obtained with ethanol could be explained by a better halotolerance of ethanol-degrading Bacteria.

The SSCP pattern obtained with ethanol as a substrate and the molecular inventory obtained on the same DNA gave two different representations of the ecosystem, as only 11 clones of the library out of 100 clearly co-migrated with detectable peaks from the corresponding sludge profiles. This shows that the underlying bacterial communities represented most of the diversity present in this reactor. This can also be explained by a differential PCR amplification between the primers used for the molecular inventory and those used for the SSCP analysis.

Lastly, it is worthy of note that the two dominant archaeal species identified in both reactors and close to *Methanosaeteta* sp. and *M. beijingense* were found in all the profiles whatever the salt concentration and thus appear to be halotolerant, a fact not previously recorded. As a conclusion, it can be said that the process performances stated in this study were made possible by the adaptation of halotolerant micro-organisms. Thus, increasing NaCl concentrations did not have a huge impact on the biomass composition but only on the micro-organisms' biodegradation rates.

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