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RESEARCH ARTICLE

Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA)

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Summary Amplified ribosomal DNA restriction analysis (ARDRA) is a simple method based on restriction endonuclease digestion of the amplified bacterial 16S rDNA. In this study we have evaluated the suitability of this method to detect differences in activated sludge bacterial communities fed on domestic or industrial wastewater, and subject to different operational conditions. The ability of ARDRA to detect these differences has been tested in modified Ludzack-Ettinger (MLE) configurations. Samples from three activated sludge wastewater treatment plants (WWTPs) with the MLE configuration were collected for both oxic and anoxic reactors, and ARDRA patterns using double enzyme digestions *Alu*I+*Msp*I were obtained. A matrix of Dice similarity coefficients was calculated and used to compare these restriction patterns. Differences in the community structure due to influent characteristics and temperature could be observed, but not between the oxic and anoxic reactors of each of the three MLE configurations. Other possible applications of ARDRA for detecting and monitoring changes in activated sludge systems are also discussed.

Key words Amplified ribosomal DNA restriction analysis (ARDRA) · Wastewater treatment plants (WWTPs) · 16S ribosomal DNA · Activated sludge · Dice similarity coefficient

Introduction

Many problems found in wastewater treatment plants (WWTPs) that perform biological removal of pollutants are due to alterations in the microbial communities involved. These alterations are caused by changes in influent characteristics and operating conditions. The study of their influence on the microbial communities of WWTPs can provide useful information to solve these problems. Plate counting and most probable number (MPN) techniques have been used for the study of microbial communities in mixed culture systems. However, less than 1% of microorganisms in the environment can be usually cultivated by standard techniques because culture techniques fail to reproduce in artificial media the niches of many microorganisms found in high-diversity environments such as activated sludge [13]. Recoveries from activated sludge, even with optimized media, can be as low as 5% [17]. Traditional thinking, based on viable counts of bacteria, suggests that the bulk of bacterial biomass in activated sludge is near death. However, by probing with fluorescent-labelled oligonucleotides, we can see that up to 90% of the biomass present in activated sludge can be metabolically active [17]. Thus, the recent development of molecular biology techniques, which do not rely on cultivation methods, allows microbial ecologists to reveal inhabitants of natural microbial communities which have not yet been cultured [7, 13, 15]. As a result, these techniques are now widely applied to characterize microbial community structures in different environments such as biological wastewater systems [6, 12, 14, 15].

Two of these techniques, cloning and sequencing, allow us to determine which microorganisms are present in the community, but they are time-consuming. Hybridization and probing are faster, but require a sufficient knowledge of the community to choose the appropriate target sequences [2]. In this study, another molecular biology technique, the amplified ribosomal DNA restriction analysis (ARDRA), is applied to activated sludge samples. Even faster than hybridization and probing, ARDRA has been used in the analysis of mixed bacterial populations from different environments [1, 8, 10]. Although ARDRA gives little or no information about the type of microorganisms present in the sample, it can be used for a

quick assessment of genotypic changes in the community over time, or to compare communities subject to different environmental conditions.

Materials and methods

Description of the WWTP configurations The activated sludge samples were collected from the oxic and the anoxic reactors of three WWTPs located in Girona, Spain. The WWTP of Vidreres-Sils (D1) has an anoxic tank (D1-an) of 350 m^3, and an oxic tank (D1-ox) of 1500 m^3, arranged in a modified Ludzack-Ettinger (MLE) configuration. It treats about 1500 m^3/day of domestic wastewater. The WWTP of Ripoll (D2) was built to operate as an Orbal but, due to the low influent load, only the two inner channels are in use. Of these two, the outer one (4500 m^3, designated as D2-an) operates under anoxic conditions, while the other (3500 m^3, designated as D2-ox) is under oxic conditions. Internal recycling from the inner to the outer channel allows the system to work in MLE configuration. The WWTP of Ripoll treats approximately 9000 m^3/day of domestic wastewater. Finally, the third WWTP studied is a pilot plant that treats industrial wastewater from a food-processing factory. It has an oxic reactor of 3.5 l (designated as IN-ox), and an anoxic reactor of 2.2 l (designated as IN-an) in an MLE configuration. The influent flow to the pilot plant is 0.83 l/day.

Sampling and analytical determinations Samples of the inlet and the outlet of each reactor were collected in order to determine influent characteristics and reactor efficiencies. The conservation of the samples, their processing and all analytical measurements were carried out according to APHA [3]. Activated sludge samples of 50 ml for DNA extraction were collected in sterile Falcon tubes and frozen at –20ºC until processing.

16S rDNA amplification DNA extraction was carried out in Nalgene sterile tubes by the phenol-chloroform method described by Moore [9]. DNA was purified with Bio-Spin chromatography columns (Bio-Rad, Hercules, CA, USA) to eliminate proteins and nucleotides. PCR amplification of 16S rDNA, using the primer set of fD1 and rP1, was performed as described previously [18]. PCR products were purified using the QIAquick PCR Purification Kit (Quiagen, Valencia, CA, USA).

rDNA restriction fragments separation by electrophoresis Double restriction endonuclease digestions were performed for every sample with *Alu*I+*Msp*I (Promega, Madison, WI, USA) as described previously [1]. Restriction enzymes were chosen on the basis of their high average number of restriction sites per taxon [11]. Separation of digested products in poly-acrilamide gels were performed as described elsewhere [1, 8]. The gel was digitallized using a scanner AGFA Arcus II and the images were contrasted using the NIH Image Program 1.59 (National Institutes of Health, Bethesda, MD, USA).

Data analysis The patterns of each sample were compared by identifying, from different samples, fragments of identical size in the same digestion. Pairwise comparations of the band patterns were manually performed, and a presence/absence matrix was constructed. In this way, the Dice similarity coefficient [16] was obtained for every pair of samples, enabling us to generate a similarity dendrogram. The data were computed by using the SPSS program version for Macintosh 4.0.

Results and Discussion

Influent characteristics, operational parameters and removal efficiencies Table 1 summarizes the principal influent characteristics and operational parameters for each of the three WWTPs. The nitrite and nitrate concentrations in the influents were always under 1 ppm N. The chemical oxygen demand (COD) removal efficiencies of all WWTPs were over 85%. The nitrification efficiencies were 83% and 94% for reactors D1 and IN, respectively, while reactor D2 showed complete nitrification. Reactors D1 and D2 had denitrification efficiencies of 16% and 64%, respectively. Reactor IN also achieved complete denitrification.

Table 1 Principal characteristics and operational parameters of the treatment plants studied: D1, urban Vidreres-Sils wastewater; D2, urban Ripoll wastewater; IN, industrial wastewater

Operational parameter	Reactor		
	D1	D2	IN
sCOD (mg/l)	144	157	8539
pCOD (mg/l)	96	149	1138
$N\text{-}NH_4^+$ (mg/l)	11	18	655
sNorg (mg/l N)	2	3	327
pNorg (mg/l N)	<1	8	–
$MLSS_{anoxic\ reactor}$ (mg/l)	1600	3048	7080
$MLSS_{oxic\ reactor}$ (mg/l)	1600	4082	4080
$\%VSS_{anoxic\ reactor}$	50%	47%	49%
$\%VSS_{oxic\ reactor}$	49%	48%	71%
$\theta_{anoxic\ reactor}$ (h)	20.3	5.6	63.4
$\theta_{oxic\ reactor}$ (h)	15.7	24	100.8
θ_c (day)	>30	22	>30
external recycle (%)	75	290	150
internal recycle (%)	200	290	1000
$T_{mixed\ liquor}$ (°C)	10	12	38

s: soluble, p: particulate, θ: sludge residence time
COD, chemical oxygen demand
MLSS, mixed liquor suspended solids
VSS, volatile suspended solids

Differences between industrial and domestic WWTP communities The restriction patterns obtained by electrophoresis are shown in Fig. 1. The differences between restriction patterns in all the communities subject to domestic wastewater are smaller than those between domestic wastewater WWTPs and those treating the industrial wastewater, as reflected in the dendrogram and in the Dice similarity coefficient matrix shown

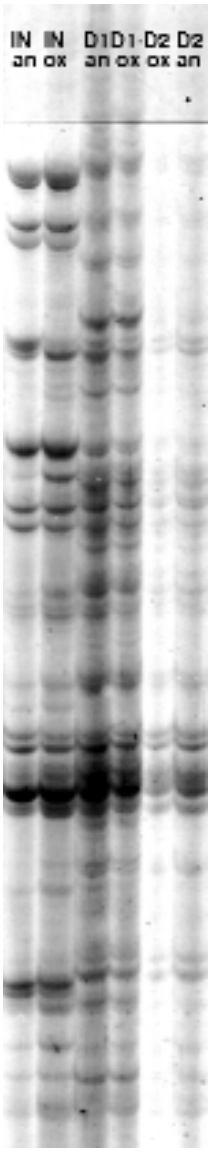
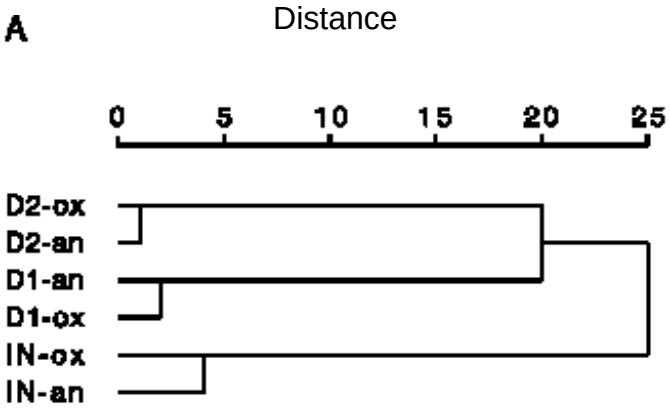


Fig. 1 Polyacrilamide gel showing *Alu*I+*Msp*I digestions of the whole eubacterial communities from the six reactors studied: D1-an, Vidreres-Sils wastewater anoxic reactor; D1-ox, Vidreres-Sils wastewater oxic reactor; D2-an, Ripoll wastewater anoxic reactor; D2-ox, Ripoll wastewater oxic reactor; IN-an, industrial wastewater anoxic reactor; IN-ox, industrial wastewater oxic reactor

in Fig. 2. Two main factors can explain these results. First, industrial WWTP communities are subject to much higher COD, ammonia and organic nitrogen inputs than the domestic WWTP communities, as shown in Table 1. Besides, the temperature was higher in the industrial wastewater reactors (38ºC) than in all the domestic wastewater reactors, where it remained between 10 and 12ºC (Table 1).

Differences between the oxic and anoxic reactors in MLE configurations No significant differences have been observed between the restriction patterns of oxic and anoxic reactors of all the systems studied, as shown in the dendrogram (Fig. 2A). The absence of differences between the patterns does not ensure that the composition of the communities is exactly the same. However, significative composition changes in the community should be detected with the restriction enzymes used [11]. Previous works demonstrated that double restriction endonuclease digestions are sensitive enough to detect important composition changes in the community [1, 8, 11]. The absence of differences between the patterns of the oxic and the anoxic reactors leads to the conclusion that there were probably no significant changes between the microbial communities of the two reactors. Similar conclusions were drawn by Ehlers and Cloete [5] by using protein fingerprints to evaluate the differences between the microbial community structures among P-removing, non-P-removing and N-removing systems. Thus, the similarity of endonuclease restriction patterns among the samples agrees with the high similarity of protein fingerprints in bacterial communities of different activated sludge systems. Given the residence times and the internal recycle values of the systems studied, the generation times of the microorganisms are probably too long to observe significant differences in community composition among the anoxic and oxic reactors. Therefore, the aerobic and anaerobic populations apparently do not have enough time to change while inside the oxic or anoxic reactors, and therefore they merely coexist. Despite the absence

A



B

Variable	D1-an	D1-ox	D2-ox	D2-an	IN-ox
D1-ox	.9778				
D2-ox	.7458	.7586			
D2-an	.7458	.7586	.9971		
IN-ox	.6766	.6909	.7246	.7246	
IN-an	.7170	.7306	.7273	.7273	.9524

Fig. 2 Differences in restriction patterns. (A) Dendrogram of eubacterial 16S rDNA-ARDRA similarities obtained by digestion with *Alu*I+*Msp*I. (B) Dice similarity coefficient matrix. Reactors D1-an, D1-ox, D2-an, D2-ox, IN-an and IN-ox, as in Fig. 1

of changes in community composition, there are probably differences in the microbial activity developed in the oxic and anoxic reactors. In each of these, only the part of the community able to grow under the conditions found in the reactor is active, whereas the rest is not able to develop activity until it reaches the other reactor. Facultative anaerobic bacteria could be active in both oxic and anoxic reactors. Further work, using specific rRNA-targeted probes, will be necessary to determine the metabolic activity of a given group of microorganisms in each of the reactors.

Concluding remarks

ARDRA is able to detect differences between activated sludge communities from industrial and domestic wastewater treatment plants, and these differences could be due to influent composition and temperature. However, differences in the community compositions of the anoxic and oxic reactors of each of the three MLE configurations studied have not been observed. Before this study, ARDRA had only been applied in raw sewage samples to detect the presence of rotaviruses by comparing the pattern of the samples with known viral patterns [4, 14]. ARDRA is a promising first approach in the evaluation of the changes in activated sludge communities of WWTPs caused by modifications in influent composition, temperature and other operational conditions. However, the effects that changes or perturbations have on a system can only be detected as long as they cause changes in community composition. Further studies will be required to evaluate the effect of other parameters on the activated sludge microbial communities, as well as to see how sensitive is ARDRA to them.

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