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Molecular and Phenotypic Characterization of the Microbial Communities in Two Pulp and Paper Wastewater Treatment Systems

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Abstract

Phylogenetic hybridization and phenotypic fingerprinting were applied to the analysis of bacterial communities in wastewater treatment systems. These approaches were aimed at (i) developing monitoring tools able to foresee operational problems, and (ii) providing the rationale to optimize the operation of bioreactors. The work presented is intended to first describe the community found in two reactors treating pulp and paper mill effluent, and second evaluate the possibilities of these techniques with respect to the development of new monitoring tools.

Phylogenetic membrane hybridization showed that the bacterial communities were dominated by Alpha and Beta Proteobacteria, a structure probably linked to the low F:M ratio. Other important factors determining the community structure were the proportion of COD in the high molecular weight fraction, the sludge age, phosphate addition, and the concentration of specific compounds (alcohols, phenols, volatile fatty acids) in the influent. The community structure partly determined the sludge characteristics demonstrating its potential value in the assessment of reactor performance. The results obtained by phylogenetic membrane hybridization suggest that the probes used in a monitoring tool would not need to be targeted to the species level to provide relevant information. However, they also suggest that the technique is more sensitive to changes in population density as opposed to changes in bacterial metabolism.

Phenotypic fingerprinting measured a smaller difference between the communities of the two reactors studied than what was measured by phylogenetic membrane hybridization. However, differences in heterotrophic activities observed between the two communities were linked to differences in influent composition.

Résumé

Les techniques d'hybridation phylogénétique et de profilage phénotypique furent appliquées à l'analyse des communautés bactériennes dans les systèmes de traitement des eaux usées. Ces approches expérimentales ont pour double but (i) de développer des outils capables de prévoir les problèmes d'opération et (ii) de fournir les bases théoriques pour l'optimisation des bioréacteurs. Le travail présenté dans ce mémoire a comme premier objectif de décrire les communautés bactériennes de deux réacteurs traitant des effluents d'usine de pâtes et papiers; et comme second objectif d'évaluer les possibilités des techniques en ce qui concerne le développement de nouveaux outils permettant le suivi des réacteurs.

L'hybridation phylogénétique sur membrane a démontré que les communautés bactériennes étaient dominées par les Alpha et les Beta Protéobactéries, une structure probablement reliée au faible ratio F:M. Les autres facteurs importants dans la détermination des structures des communautés étaient: la proportion de DCO dans la fraction à poids moléclaire élevé des composées, l'âge de boue, l'addition de phosphate, et la concentration de certains composés (alcools, phenols et acides gras volatiles). La structure phylogénétique de la communauté déterminait partiellement les caractéristiques des boues démontrant ainsi son lien avec la performance du réacteur. Les résultats obtenus par hybridation phylogénétique suggèrent que les sondes utilisées pour le suivi des réacteurs n'auraient pas besoin d'être dirigées au niveau de l'espèce pour fournir des informations pertinentes. Cependant, ils suggèrent aussi que la technique est plus sensible aux changements dans la densité des populations bactériennes plutôt qu'aux changements dans le métabolisme bactérien.

Le profilage phénotypique des communautés mesura une différence plus petite entre les comunautés des deux réacteurs de l'étude comparativement à la différence mesurée par hybridation phylogénétique. Cependant, les différences observées dans les activités hétérotrophiques des deux réacteurs étaient reliées aux différences dans la composition des eaux usées traitées.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ALF16	Probe targeted at the Alpha Proteobacteria
AOX	Adsorbable Organic Halides
BET23	Probe targeted at the Beta Proteobacteria
BKME	Bleached Kraft Mill Effluent
BOD_5	Biochemical Oxygen Demand after incubation for five days at 20°C
CFC16	Probe targeted at the *Cytophaga-Flavobacterium* Cluster
COD	Chemical Oxygen Demand
CV	Coefficient of Variation
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl Pyrocarbonate
DO	Dissolved Oxygen
EC(alf)16	Probe targeted at the same site as probe ALF16 but on *E. coli* 16S rRNA
EC(cfc)16	Probe targeted at the same site as probe CFC16 but on *E. coli* 16S rRNA
ETC	Electron Transport Chain
EUB16	Probe targeted at the Small (16S) Subunit rRNA of Bacteria
EUB23	Probe targeted at the Large (23S) Subunit rRNA of Bacteria
F:M ratio	Food to Microorganism ratio
GAM23	Probe targeted at the Gamma Proteobacteria
GPB16	Probe targeted at the Gram Positive Bacteria
HGC23	Probe targeted at the Gram Positive High GC Bacteria
LC_{50}	Lethal Concentration (50%): Concentration at which 50% of the test organisms die
LV	Latent Variable or Eigenvector of regressors
MLSS	Mixed Liquor Suspended Solids
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PLS	Partial Least-Squares Regression

PRESS	Predicted Residual Estimated Sum of Squares
r	Pearson correlation coefficient
R^2	Coefficient of determination
RAS	Returned Activated Sludge
rDNA	rRNA Sequence on the genome
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation
SOUR	Specific Oxygen Uptake Rate
SVI	Sludge Volume Index
TSS	Total Suspended Solids
UNI16	Probe targeted at the Small (16S or 18S) Subunit rRNA of all Organisms
UNI23	Probe targeted at the Large (23S or 25S) Subunit rRNA of all Organisms
VFA	Volatile Fatty Acid
VIP	Variable Importance in Projection
WAS	Wasted Activated Sludge

Chapter 1. Introduction

1.1 Context

The field of wastewater treatment was primarily developed by engineers. Since the beginning, about a century ago, the water specialists tried to build devices and reactors with increasing efficiency of removal of biochemical oxygen demand (BOD), pathogens and toxicants. In the 1930's the role of bacteria was acknowledged, but it took another thirty years to recognize the contribution of numerous microbial genera to the treatment systems (Dias and Bhat 1964; Pike and Carrington 1972). Over the last 20 to 30 years, this man-made ecosystem composed of bacteria and protozoa became the subject of increasing interest by microbiologists. The importance of understanding the ecological interactions occurring in this community in order to maintain the optimum performance of these systems is now recognized. This understanding should also provide the rationale for the design of treatment systems with enhanced performance.

In Quebec and Ontario, environmental regulations concerning mill effluents in the pulp and paper industry became stricter at the end of 1995 and the beginning of 1996, respectively. For example, the provincial governments enforced daily and monthly limits of discharge in receiving waters for BOD, total suspended solids (TSS), pH, total phosphorus, chloroform, toluene, phenol and toxicity. This forced pulp and paper mills to install wastewater treatment systems.

The challenge with these newly installed systems is to keep them optimally performing, which is strongly dependent on the ecological balance of the community. For example, an overgrowth of filamentous bacteria leads to bulking and foaming, and ultimately causes an increase in BOD and TSS discharges. Similarly, systems are dependent on the activity of adsorbable organic halide (AOX) degraders and resin acid degraders to remove these toxicants.

Classical strategies of process control which are currently used involve the monitoring of biomass activity and performance. Periodic microscopic examinations are also performed to control the floc structure as well as the density of filaments. However, all the information concerning the structure of the bacterial community is not used.

Classically, two approaches have been used in ecology to describe communities. They are referred to as taxonomic and functional analysis. The former classifies organisms based on their phylogenetic relatedness and the "taxon" used can be defined at any phylogenetic level. The latter classifies the organisms in terms of their function in the ecosystem. It is important to keep in mind that taxons of the same phylogenetic level never overlap, while representatives of a single functional group can be found in many taxons.

In the field of microbial ecology, information about microbial communities was obtained by the use of various cultivation media. The functionality of the bacteria recovered was related to the medium used. Alternatively, non-selective media were used and isolated bacteria were identified to obtain the taxonomic structure of the communities. Since these methods require bacterial growth, the time requirements are too great for process control. In addition, they are also inaccurate because some cultivation techniques will preferentially enrich specific bacteria (Wagner et al. 1993). To circumvent these problems, Biolog microtiter plates were recently used to obtain heterotrophic functional data on wastewater treatment communities (Victorio et al. 1996). To obtain taxonomic community structures, molecular biology techniques were developed (Wagner et al. 1993; Wagner et al. 1994a; Manz et al. 1994; Kämpfer et al. 1996; De Los Reyes et al. 1997; Snaidr et al. 1997) taking advantage of the classification of organisms based on their ribosomal RNA sequence (Woese 1987). From the taxonomic works, the following prediction was made:

> "Population shifts could serve as early indicators for upcoming malfunctions so that corrective measurements could be made in time."
>
> Wagner et al. 1993.

In spite of these developments, no study concerning the variation over time in bacterial community structure as determined by phylogenetic hybridization has been published to date. Without that information the above prediction remains hypothetical. Therefore, there is a need to assess these changes and verify the validity of Wagner and co-workers' affirmation. This information would also be useful in the identification of potential areas of development for the new techniques.

1.2 Objectives

Based on the context elaborated above, the objectives of the project are defined below:

1- To evaluate the taxonomic community structure as a source of information to diagnose wastewater treatment systems.

2- To evaluate the precision of the phylogenetic membrane hybridization assay (see Section 1.3), identify parameters affecting it and propose improvements.

3- To characterize the bacterial community and its variation over one year in a pulp and paper wastewater treatment system, using the techniques of phylogenetic membrane hybridization and phenotypic fingerprinting.

4- To determine the main process variables that influence changes in the taxonomic bacterial community structure and to model these changes based on the process variables.

5- To compare taxonomic data (obtained by phylogenetic membrane hybridization) to heterotrophic functional data (obtained by phenotypic fingerprinting).

1.3 Approaches

Two strategies can be used in taxonomic analyses. The first one is a bottom-up approach. In this approach, specific species or genera (great phylogenetic depth) are chosen and detected in the bacterial community. In the second approach, called top-down, phyla[1] (shallow phylogenetic depth) are detected in the bacterial community. The phyla with positive results may be investigated in more detail in subsequent studies. Since the taxonomic information on bacterial community of pulp and paper wastewater treatment system is limited, a top down approach was chosen.

The technique selected to obtain the taxonomic bacterial community structure was the phylogenetic membrane hybridization. By this technique, total community RNA is extracted, bound to a membrane and hybridized with 16S and 23S rRNA targeted probes. The bacterial communities of two different reactors treating pulp and paper effluent were then monitored over a one year period using this technique. This partially achieved objective three.

The precision required in an analysis is always dependent on the level of signal of the phenomenon measured. Therefore, to achieve objective two, the level of variation in the community structure obtained by phylogenetic membrane hybridization for a single sample was determined and compared to the level of variation over one year.

To achieve objective four, forty-three process variables were identified for their possible interaction with the bacterial community. Changes in the taxonomic community structure were modeled on changes observed in some process variables. Performance variables such as outgoing COD, sludge volume index (SVI) and specific oxygen uptake rates (SOUR) were also modeled using the taxonomic community data. These modeling exercises were used to achieve objective one.

In the pulp and paper industry, there is interest in development of community diagnosis tools based on phenotypic fingerprinting. Also, this technique provides heterotrophic functional information on the community which is not necessarily linked to the taxonomic structure (Buyer and Drinkwater 1997). Therefore, samples were also analyzed

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[1]In this thesis, the nomenclature proposed by the Oligonucleotide Database Project (Alm et al. 1996) is followed to describe the main bacterial lineages even if the terms kingdom Borneman and Triplett 1997), phylum (Ludwig et al. 1997) and class (González and Moran 1997) were used in the literature.

by phenotypic fingerprinting and these data were then compared with the taxonomic structure to provide insight on the relative nature of both types of data.

Chapter 2. Literature Review

2.1 Introduction to wastewater treatment

2.1.1 History and objectives of wastewater treatment

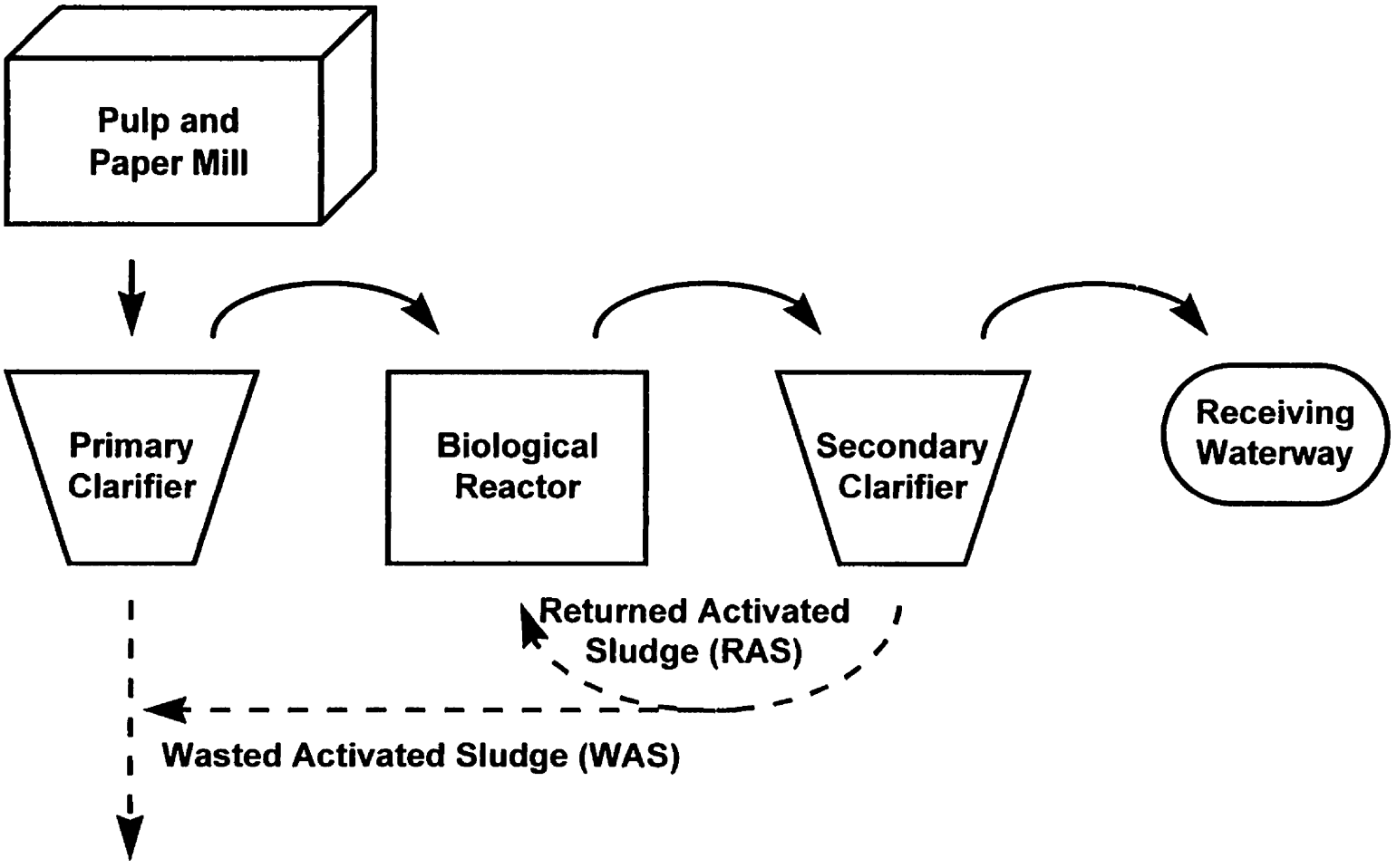
At the beginning of the 19th century, many major cities were facing epidemics of various diseases. As drinking water was thought to be the source of infectious agents, James Simpson had the idea around 1830 to treat it by sand filtration (Sterritt and Lester 1988). Twenty years later, John Snow suggested that most of the microbiological contamination of the water supply originated from cesspits and burial grounds (Sterritt and Lester 1988). Hence, the importance of treating the sewage water was also recognized.

By 1860, sewage treatment systems consisting of preliminary sedimentation of coarse material followed by irrigation on agricultural land were installed; the idea of trickling filters was born. However, stagnation in the holding reservoir created odour problems which were solved by blowing air into the water. During this aeration, organic precipitates were accumulating in tanks. In 1914, Ardern and Lockett discovered that when these deposits were reintroduced in another batch of sewage before aeration the oxidation proceeded much faster. With subsequent reintroductions, they obtained a dense humus-like material that they called "activated sludge" (Sterritt and Lester 1988). Today, almost all the wastewater treatment systems in North America are biological. They are still grouped in two major families: fixed growth (e.g., trickling filters, rotating biological contactors) and suspended growth (e.g., activated sludge, aerated lagoons) reactors (Nathanson 1997).

2.1.2 Engineering of wastewater treatment

Since the focus of this work was activated sludge reactors, this literature review will focus on suspended growth, and mainly activated sludge, systems (Fig. 1.1).

Figure 2.1. Flow diagram of an activated sludge treatment system. The solid lines represent the flow of wastewater and the dashed lines represent the flow of sludge.


Pulp and
Paper Mill
Primary
Clarifier
Biological
Reactor
Secondary
Clarifier
Receiving
Waterway
Returned Activated
Sludge (RAS)
Wasted Activated Sludge (WAS)

In general, two major units constitute a wastewater treatment facility. The first unit mechanically removes material from the wastewater. Often, a bar screen extracts the coarse debris, whereas the smaller particles are sedimented in a primary clarifier. This primary clarification usually removes 60% of incoming suspended solids and 35% of incoming BOD in municipal systems and produces the primary sludge (Nathanson 1997).

The second unit is the biological reactor. In the activated sludge process, biomass concentration is equal to 2000 to 4000 mg of biosolids per litre and the wastewater has a short residence time (hydraulic retention time: HRT) of about six hours (Ramalho 1983). This reactor is followed by a secondary clarifier in which the biomass (secondary sludge) is sedimented and recycled to the reactor. The excess biomass is removed, dehydrated and later disposed (Ramalho 1983).

For the aerated lagoon process, the biomass concentration is usually relatively low, around 80 to 200 mg of biosolids per litre. On the other hand, the HRT is extended to several days (Ramalho 1983). There is usually no secondary clarifier following this reactor, however, a quiescent zone to sediment the biomass is allowed to develop near the outlet. This creates an anaerobic zone at the bottom which promotes partial digestion of the sludge (Nathanson 1997).

2.2 Microbial community analysis

The main tasks of ecologists are to survey the populations present in an environment and to identify the factors impacting on the community. For microbial populations to be surveyed, they must be detected by some means. However, one needs to define the populations of interest before selecting the tools to use. As mentioned in section 1.1 above, the two underlying principles of functional and taxonomic analysis can be used (Martinez 1996). The former was pioneered by Sergei N. Winogradsky and Martinus W. Beijerink who developed selective media while studying biogeochemical cycles (Prescott et al. 1996). A modern approach to functional analysis is to use the Biolog system, which was originally developed for identification of pure cultures (Bochner 1989), but has been adapted to total community analysis (Garland and Mills 1991).

Taxonomic analysis is based on the identification of bacteria. Since the beginning of the century, this identification relied on phenotypic characteristics as described in *Bergey's*

Manual of Systematic Bacteriology, first published in 1923. However, this method of identification requires isolation of the organisms present in an ecosystem. This represents one of the greatest limitations since it was shown that any culture medium preferentially selects for certain species therefore biasing the observed structure of the community (Wagner et al. 1993).

Recently, the sequence analysis of universally common genes such as the ribosomal RNA genes made it possible to move away from the phenotypic classification and introduced phylogeny to microbiology (Woese 1987; Olsen and Woese 1993). Taking advantage of these findings, molecular tools were adapted to detect bacteria without culturing them creating the new field of molecular microbial ecology (Akkermans et al. 1995). For completeness, let us mention that besides their fundamental contribution to taxonomic analysis, molecular techniques also have application in a functional context. These techniques are discussed in the following section.

2.2.1 Phenotypic fingerprinting

Phenotypic fingerprinting is the terminology developed to describe the technique using the Biolog microtiter plate to obtain a heterotrophic functional profile of a bacterial community. The plate developed by Biolog Inc. at the end of the 1980s revolutionized the field of functional analysis because of its rapidity and the large number (95) of carbon compounds used (Bochner 1989). Biolog based its technology on the reduction of the soluble and colourless tetrazolium violet to an insoluble purple formazan by the electron transport chain (ETC). The carbon compound in a specific well, if utilized, provides the reducing power to the ETC and enables colour development.

Garland and Mills (1991) were the first to use this test panel to characterize heterotrophic communities in soil, hydroponic and freshwater environments. For instance, they were able to differentiate various habitats based on the fingerprint of the inhabiting microbial community. Knight and co-workers (1997) used the technique to study the change in a soil community amended with heavy metals. They showed that even when the microbial biomass level was not affected by metal contamination, changes in total activity and pattern of carbon utilization still occurred.

Phenotypic fingerprinting was introduced to the study of pulp and paper wastewater treatment microbial communities by the team of the Pulp and Paper Centre at University of Toronto (Fulthorpe et al. 1993; Fulthorpe and Allen 1994; Victorio et al. 1996). Fulthorpe and Allen (1993) used the technology to screen an isolate collection based on the ability of the isolates to utilize chloroaromatics. On the other hand, Victorio and co-workers (1996) used the technology to produce true community phenotypic fingerprints. This last study demonstrated that the "Biolog GN" plate was able to differentiate between bacterial communities treating municipal and pulp and paper wastewater. Their work also suggested that the technique could be used to detect the steady state of a reactor as well as its process upsets.

Although attractive, the technique also has some limitations and deficiencies. In soil environments, the reproducibility seems to be low (Knight et al. 1997) and the fingerprints are very sensitive to cell density (Kidd Haack et al. 1995). The detection system may also have variable toxic effects on the bacteria present in a community. At least one tetrazolium salt (CTC) was found to be toxic to some members of natural communities (Ullrich et al. 1996).

Other limitations are related to the interpretation of the heterotrophic profile. First, it was thought that the method was cultivation independent. However, it was demonstrated that the organisms grow inside the well (Smalla et al. 1998) and that fast growers might be advantaged (Verschuere et al. 1997). Second, it is usually hard to relate differences in heterotrophic activity profiles to functional differences (Bossio and Scow 1995; Garland et al. 1997). Third, it was suggested from work on artificial communities that the technique, although based on phenotypic characters, could be more sensitive to the taxonomic structure of the community (Garland et al. 1997). However, this claim was not substantiated when taxonomic structures and phenotypic fingerprints were compared for natural communities (Buyer and Drinkwater 1997; Palojärvi et al. 1997; Lawley and Bell 1998).

2.2.2 Molecular techniques

The adaptation of molecular tools to the analysis of bacterial communities created the field of Molecular Microbial Ecology. These tools can be divided into two main families. The first family uses the polymerase chain reaction (PCR) technology whereas the second one uses hybridization technologies. These methods are reviewed in the following sections.

2.2.2.1 PCR-based methods

Since its invention by Kary B. Mullis (Saiki et al. 1985; Mullis and Faloona 1987), PCR has revolutionized biology. It is now used in microbial ecology to study the various components of a community. In functional analysis, PCR is used to amplify genes of interest. One of the most interesting applications is the use of reverse transcriptase-PCR (RT-PCR). By this technique, the mRNAs of a specific gene present in a community are converted to DNA. This specific fragment of cDNA is then amplified and analysed. For example, this technique was used to determine the level of expression of the *dmpN* gene (coding for phenol hydroxylase) in a biological reactor treating phenol (Selvaratnam et al. 1995).

The primary application of PCR in taxonomic analysis is to assist in cloning of rDNA fragments from the community. The fragments amplified by universal primers are then cloned and analysed by a restriction enzyme defining operational taxonomic unit (e.g., Moyer et al. 1994) or by sequencing (e.g., Borneman and Triplett 1997). This is useful to obtain a global picture of the taxonomic diversity present in a habitat. This kind of global survey is extremely important since the amount of information on global microbial diversity is very limited (Amann et al. 1995; Palleroni 1997; Dykhuizen 1998).

Such cloning studies are tedious and labour-intensive. Therefore, at least three alternative strategies were developed to do the analysis without cloning the PCR products. The first one is known under the acronym ARDRA, which stands for amplified rDNA restriction analysis (Massol-Deya et al. 1995). By this technique, the PCR products are digested with a restriction enzyme and resolved by agarose electrophoresis. However, since a single rDNA fragment can produce multiple bands, this technique does not provide information on the genotypic diversity present.

The second strategy involves the use of denaturing gradient gel electrophoresis (DGGE) to resolve the fragments amplified by PCR. As they migrate through the gel, the PCR fragments melt according to their sequence which reduces their mobility. Therefore, fragments of the same size can form bands at various levels in the gel and the number of fragments is a certain measure of the genotypic diversity of the community. This technique was used to study the microbial community in the water column of a Danish fjord. rDNA and rRNA molecules were PCR amplified and, since fragments were present in the rRNA sample and not in the rDNA, it was concluded that a numerically small population of very active bacteria was present at the thermohalocline (Ramsing et al. 1996).

The third strategy is called terminal restriction fragment length polymorphism. In this technique either the 5' or the 3' primer is radioactively labelled before PCR. The amplified products are then digested by a restriction enzyme and resolved in a sequencing gel. Liu and co-workers (1997b) used it to compare the communities found in aquifers, municipal activated sludge systems and termite guts. They found that the aquifer and activated sludge communities were similar, whereas the termite gut community was significantly different from the two others.

Even if they are powerful, PCR-based techniques require great care when used since biases may be introduced at every step. In the first step of nucleic acid extraction the difficulty in achieving uniform cell lysis is a possible bias (Picard et al. 1992). The PCR step itself can introduce various biases such as the production of chimeric fragments (Meyerhans et al. 1990; Cariello et al. 1991; Liesack et al. 1991) and the unbalanced representation of genotypes present (Krawiec and Riley 1990; Reysenback et al. 1992; Cole and Saint-Girons 1994; Farrelly et al. 1995; Wilson and Blitchington 1996). Because of all these biases, the PCR-based methods should not be used to determine *in situ* microbial community structure (Snaidr et al. 1997).

2.2.2.2 Hybridization-based methods

Two distinct hybridization methods are available. First, hybridization could be performed on membrane-bound extracted nucleic acid. Second, hybridization could be directly performed on the sample and visualized under the microscope. The techniques are called respectively membrane and *in situ* hybridization. In this study, membrane hybridization was used.

The hybridization-based methods can be used in both functional and taxonomic analyses, and the probes can be targeted to either DNA or RNA (Akkermans et al. 1995). Since the work presented here mainly used the phylogenetic membrane hybridization technique and since the relevant studies are reviewed in section 2.3, this section will only focus on the interpretation of the quantitative taxonomic data that has been developed in the literature.

Before discussing the interpretation, the methods will be described briefly. During membrane hybridization, the bulk community nucleic acids (DNA or RNA) are extracted and immobilized on a membrane. If the extract is to be submitted to many probes, replica membranes are prepared (Stahl et al. 1988; Raskin et al. 1997). The membranes are then hybridized with either radioactively- or nonradioctively-labelled probes targeted to the rRNA or the rDNA and the signal is quantified. The assay is then standardized by relating the specific population probe signal to a universal or eubacterial probe (Stahl 1995).

In *in situ* hybridization, the nucleic acids are not extracted. Cells are fixed and permeabilized to allow the probes to enter the cell freely. In fact various permeabilization methods are necessary to ensure permeabilization of all types of bacterial cells (Roller et al. 1994; De Los Reyes et al. 1997). Fluorescently-labelled probes confer fluorescence on the hybridized bacteria, which are then visualized microscopically. The standardization is similar to membrane hybridization. A universal or a eubacterial probe is used in conjunction with a population specific probe. The general probe identifies the permeabilized bacteria or the total community individuals (Wagner et al. 1993). The specific population data are expressed as a ratio of this total community individual count. In addition, a fluorescent DNA stain such as DAPI (4',6-diamidino-2-phenylindole) is used to determine the degree of permeabilization in the community (Wagner et al. 1993).

For the interpretation, let us first discuss the simplest case: *in situ* hybridization. Because there is a visual inspection of the bacterial community, this technique informs on the number of individuals, their morphology and their spatial location (Amann 1995). Based on these data, it is possible to express the population density in terms of individuals, and also in terms of biomass. In addition, the technique may be complemented by a quantification of the hybridization signal for each individual, providing information on the rRNA content of this individual (Wallner et al. 1995). Therefore, it becomes possible to determine the specific activity of a population on a biomass basis, data more appropriate to incorporate into wastewater treatment models.

On the other hand, membrane hybridization data cannot be directly related to biomass since genome copies, cell size and RNA content vary greatly according to the species and the specific growth rate (Krawiec and Riley 1990; Cole and Saint-Girons 1994). For instance, when membrane and *in situ* hybridization were compared, the former had the tendency to detect proportionally more Beta Proteobacteria. This bias correlated well with the larger size of the bacteria of this group as observed microscopically (Wagner et al. 1993). Therefore, to draw a proper conclusion about the biomass density, one would need to know the size of the individual cells in the specific population as well as their specific rRNA content (Stahl et al. 1988; Wagner et al. 1993). However, since the rRNA content varies with the activity of an individual, the population structure as determined by membrane hybridization is often considered the metabolic contribution of the population of interest to the total activity of the community (Stahl et al. 1988; Raskin et al. 1997).

2.3 Microbial communities of wastewater treatment systems

In the pulp and paper industry, calls are constantly made to continuously improve the performance of wastewater treatment systems (Munkittrick et al. 1997). For this industry, objectives like mill closure are particularly challenging for treatment facilities. Also challenging is the permanent optimum operation of the reactor. Since the microbial community is central to the function of wastewater treatment systems, the development of powerful tools for community analysis as well as the understanding of the community dynamics is critical to its proper manipulation.

In that context, four important areas concerning the treatment of wastewater in pulp and paper are reviewed here. The microbial diversity section reviews the most pertinent works for the research presented in this thesis The other sections present areas of prime interest for the industry. Contrasting with the microbial diversity section, the investigations primarily used classical culture techniques. However, it is possible to see how a molecular approach could be used to complement these early studies. For the most part, they also represent the only information available on the microbial community of wastewater treatment systems in the pulp and paper industry.

2.3.1 The microbial diversity

Before any in-depth analysis of a microbial ecosystem, it is important to investigate the diversity of microorganisms present. This is essential to relate organisms to the various processes observed in the ecosystem. This diversity survey can be defined in terms of taxonomy or functions. These two complementary approaches were used to study wastewater treatment systems. In this section, knowledge about the bacterial diversity in wastewater obtained by classical or molecular methods is reviewed.

2.3.1.1 Taxonomic and functional diversity of culturable bacteria

2.3.1.1.1 Municipal wastewater treatment systems

The first taxonomic studies suggested that a single genus was responsible for the consumption of BOD. *Zoogloea* was thought to be the only genus able to form floc under aerobic conditions, hence the only genus positively selected in treatment systems (Butterfield 1935). However, subsequent studies demonstrated that many organisms can flocculate in presence of oxygen (Tischer et al. 1962; Dias and Bhat 1964). Results from the culture studies suggested that the treatment system communities were dominated by Gram negative organisms of the genera *Achromobacter, Aerobacter, Alcaligenes, Comamonas, Flavobacterium, Pseudomonas and Zoogloea.* The Gram positive genera present were *Bacillus, Brevibacterium, Corynebacterium* (Dias and Bhat 1964; Pike and Carrington 1972; Sterritt and Lester 1988).

Besides this taxonomic information, some aspects of functional diversity were also studied. In 1972, heterotrophic functionality was introduced to wastewater treatment analysis (Pike and Carrington 1972). Various growth media were used to enumerate populations of single carbon source utilizers from laboratory scale reactors. This result showed the influence of the sludge age on the abundance of citrate utilizers, gluconate utilizers and starch hydrolysers.

2.3.1.1.2 Pulp and paper wastewater treatment systems

In terms of taxonomy, the results for pulp and paper seemed to be similar to what was observed in municipal systems. Fulthorpe and co-workers (1993) reported that 80% of their isolates from a lagoon system treating bleached kraft mill effluent (BKME) were Gram negative organisms. The analysis of their isolate collection showed the presence of the genera *Acidovorax*, *Acinetobacter*, *Ancylobacter*, *Comamonas*, *Klebsiella*, *Methylobacterium* and *Pseudomonas*. *Klebsiella* is probably constantly seeded in reactors since it is very abundant in pulp and paper effluent (Liss and Allen 1992). *Azotobacter* was reported in lagoon systems and was found to be present in the wood room effluent of a Canadian pulp mill (Knowles et al. 1974; Bruce and Clark 1994; Clark et al. 1997). It is thought that, in pulp and paper lagoon systems, *Klebsiella* and *Azotobacter* are responsible for dinitrogen fixation (Bruce and Clark 1994; Clark et al. 1997).

Liss and Allen (1992) also studied a lagoon treating BKME, but used a functional approach. It was found that the obligate anaerobes constituted 40% of the culturable bacteria (on plate count agar with 30% mill effluent) in deposited sludge as opposed to 10% in the water column. Also, the psychrophiles (growth at 10°C) increased by two orders of magnitude over the winter months. Finally, a reduction of the culturable population of both anaerobes (40%) and aerobes (65%) was observed when softwood was used to produce the pulp instead of hardwood. These observations suggested that some of the changes observed in the performance of the reactor were related to the change in bacterial populations.

Using phenotypic fingerprinting it was found that pulp and paper activated sludge communities had a greater ability to consume cellobiose, malonic acid, uronic acid, bromosuccinic acid and chlorolignins compared to communities in municipal systems.

Meanwhile, the latter metabolized dextrin, τ-hydroxybutyric acid, succinamic acid and amino acid to a greater extent. These characteristics were useful in differentiating the communities of both types of systems (Victorio et al. 1996).

2.3.1.2 Taxonomic diversity as observed by molecular methods

In the field of wastewater treatment, molecular tools were first used in a taxonomic approach to survey the bacterial diversity. Since it has long been recognized that the proportion of culturable bacteria is relatively low (Pike and Carrington 1972; Wagner et al. 1993; Kämpfer et al. 1996), the first works done used *in situ* hybridization assessed the validity of the data obtained by cultivation. It was found that culture methods tended to preferentially select for members of the Gamma Proteobacteria (Wagner et al. 1993; Wagner et al. 1994b; Manz et al. 1994; Kämpfer et al. 1996) which invalidated any taxonomic population structure obtained by cultivation. In fact, German municipal wastewater treatment systems analysed by *in situ* hybridization were dominated by Proteobacteria (60-75%). Within this phylum, the Beta subphylum represented about 40% of the bacteria, whereas the Alpha and the Gamma subphyla usually represented 10-25% (Wagner et al. 1993; Wagner et al. 1994b; Manz et al. 1994; Kämpfer et al. 1996). By cultivation, the community dominancy had been attributed to the population of Gamma Proteobacteria.

Phylogenetic hybridization is used to give a less biased view of the community structure. However, it is restricted to the groups that are already represented in culture collections. To identify new rRNA genotypes, PCR-based amplification and cloning was used. Two studies found groups that were not originally thought to be present in activated sludge. Bond and co-workers (1995) cloned sequences affiliated to *Rhodocyclus* and planctomycetes from a laboratory scale activated sludge sequencing batch reactor, while, Snaidr and co-workers (1997) cloned sequences also affiliated to the planctomycetes and to the genera *Clostridium*, *Eubacterium*, and *Acrobacter*. The last genus is part of the Epsilon Subgroup of the Proteobacteria and include human pathogens. The presence in the community of sequences belonging to that group was confirmed by *in situ* hybridization. This is a good example of a "full-cycle rRNA analysis" through which a sequence is isolated and its presence is confirmed in the original community (Amann et al. 1995). This is in fact an actualization of Koch's postulates in the context of molecular microbial ecology.

Even if all these techniques are extremely useful to gain new insights in wastewater treatment systems, isolation of bacteria is still imperative. Our knowledge of microbial biodiversity is still very limited and one cannot hope to resolve an ecological system without studying the physiology of its constituents (Amann et al. 1995; Palleroni 1997).

2.3.2 Filamentous bulking and foaming

Central to the design of activated sludge systems is the optimum contact of the biomass with the wastewater and their separation in the secondary clarifier. Changes in the physico-mechanical properties of the floc may affect these two processes. These changes give rise to two types of problems: bulking and foaming. Bulking, characterized by poor sludge settlability, is of primary interest since 40-50% of all activated sludge plants suffer from it periodically (Blackbeard et al. 1986). Similarly, it was reported that foaming, characterized by the formation of a thick chocolate-like layer at the surface of the reactor, had affected 66% of USA activated sludge plants surveyed in 1990 (Pitt and Jenkins 1990) and 51% of the Australian plants (Seviour et al. 1990). From an economic and an environmental point of view, understanding the biological basis of these two problems is very desirable.

2.3.2.1 Filamentous bulking

Overproduction of exopolysaccharides or the proliferation of filamentous bacteria above a critical population density increases the buoyancy of the floc which settles slower (Jenkins et al. 1993). This condition of the sludge is called bulking due to the increased volume of "settled" sludge in the settlability test (i.e., the sludge volume index or SVI). In the clarifier, the settled sludge does not thicken (compact) well at the bottom and this last unit gets overloaded, increasing TSS discharge. Since exopolysaccharide or slime bulking is mainly due to unbalanced growth caused by specific environmental conditions such as high C:N ratio, this section will only focus on filamentous bulking.

From a biological point of view, the understanding of filamentous bulking started with Eikelboom's description of the organisms involved (1975). He constructed an identification key of various filaments present in activated sludge based on their

microscopically observable characteristics. Twenty-six types of filamentous bacteria were identified, but a vast majority of them remain to be taxonomically described (Wagner et al. 1994a; Blackall et al. 1996).

The use of Eikelboom's identification key had the advantage of facilitating the description of bulking events. It made it possible to study the kinetics of the organisms in cause and link it with the environmental conditions. Eventually, it was suggested that the filaments causing bulking can be classified in four groups: aerobic bulking (*Spherotilus natans*, Type 021N, Type 0961, etc.), low F:M ratio bulking (*Microthrix parvicella*, Type 0092, Type 0041, etc.), nutrient deficiency bulking (Type 021N and *Thiothrix*, etc.), high organic acid bulking (*Thiothrix*, Type 021N, *Nostocoida limicola*, etc.) (Wanner and Grau 1989; Jenkins et al. 1993).

In pulp and paper systems, bulking is mainly due (68%) to high VFA concentration (Richard 1997). However, this finding contradicts a survey done by the same author ten years ago which showed that low F:M ratio bulking (49%) was the main cause (Richard 1991; Richard 1997). The difference was interpreted by the author to be due to the increased use of readily biodegradable additives such as starch which are easily fermented before the wastewater treatment system. The second main cause of bulking (13%) is nutrient deficiency. This is usually related to the difficulties involved in optimizing nutrient addition, especially phosphorus, to pulp and paper effluent (Richard and Cummins 1997).

Although the development of identification keys was useful in studying bulking in activated sludge systems, there are general limitations to this approach. First, the physiological characteristics of the filamentous bacteria involved are variable, and depend on the environmental conditions (Wagner et al. 1994a). At times, this makes the identification very difficult, even by highly trained personnel. Second, nonfilamentous growth has also been described for some of the filaments such as *Helascominobacter hydrosis* (van Veen et al. 1973), *Spherotilus natans* (Mulder and Deinema 1992) and *Microthrix parvicella* (Foot et al. 1992). Their detection prior to the onset of bulking could lead to a better understanding of the cause and an earlier correction of the system operation. Here, molecular approaches could be beneficial in both increasing the accuracy of identification and detecting unusual growth forms (Wagner et al. 1994a).

Molecular studies of the filamentous bacteria also showed that some types were highly polyphyletic and the subgroups greatly differed in their growth requirements. For example, Type 1863 was first thought to be related to *Acinetobacter* spp., a member of the Gamma Proteobacteria subphylum (Wagner et al. 1994a; Rossetti et al. 1996; Blackall et al. 1996). However, a culture of properly identified Type 1863 was isolated by micro manipulation from an Australian plant and was found, on the basis of the 16S rRNA gene sequence, to be related to the *Cytophaga-Flavobacterium* phylum (Blackall et al 1996) These findings demonstrated that a molecular approach is becoming very important to fully resolve the complexity of bulking problems.

2.3.2.2 Biological foaming of activated sludge

Biological foaming is characterised by the accumulation of a thick chocolate-like foam at the surface of activated sludge reactors. This foam is stabilized by the presence of certain filamentous bacteria that tend to float. It causes reduction in treatment efficiency since it impairs proper contact of the biomass and the wastewater and can cause an increase in total suspended solids (TSS) discharged to the receiving water if it reaches the clarifier. In addition, for municipal systems, it may be hazardous for the health of the human population living in the vicinity of reactors since floating pathogens can be windblown (Blackall et al. 1988).

The biological foaming of activated sludge is usually produced by various Gram positive bacteria. The main species reported is *Gordonna amarae* (formerly *Nocardia amarae*; Klatte et al. 1994; Ruimy et al. 1994) and *Microthrix parvicella*. However, other genera that have been reported to cause foaming include *Nocardia*, *Mycobacterium*, *Rhodococcus*, and *Tsukamurella* (Blackall et al. 1989; Seviour et al. 1990; Jenkins et al. 1993). The common property of these genera is the presence of mycolic acid in their cell wall, making it hydrophobic (Minnikin 1982). It is thought that in sufficient numbers the filaments increase the hydrophobicity of the floc enough to attract it to the air-water interface of the bubbles introduced for aeration (van Niekerk et al. 1987). The floc is then driven to the surface of the reactor. Indeed, microscopic studies have shown that a majority of the

filaments are transferred to the foam during an upset (Pitt and Jenkins 1990). A membrane hybridization study arrived at the same conclusion showing that the mixed liquor contained 9% of *Gordonna* rRNA whereas the foam contained 13% (De Los Reyes et al. 1997). Still not understood are all the factors involved in the positive selection of foam-causing filamentous bacteria. It seems that method of water withdrawal, the temperature (above 20 °C) and sludge age (above six days) are all important (Pitt and Jenkins 1990; Cha et al. 1992).

2.3.3 AOX removal

AOX is the acronym for Adsorbable Organic Halides, a general measurement of the chlorinated organic compounds found dissolved in water. As they were implicated in pulp and paper effluent toxicity (Luthe et al. 1992; Leach and Thakore 1975), they should be specifically removed from the wastewaters. These compounds are primarily produced in the chlorine bleaching stage of the kraft pulp production. Therefore, the change from elemental chlorine as bleaching agent to chlorine dioxide and hydrogen peroxide helped to reduce the discharge of AOX. However, mills often rely on the performance of their wastewater treatment system for the final removal which ranges from 15 to 70% (Stuthridge and McFarlane 1994). Unfortunately, variable removal rates are achieved. Better control over the microbiology and the mechanisms involved should increase the reliability and the performance of treatment systems.

From a process point of view, hydraulic retention time is the only operation variable having a significant impact on AOX removal (Hall and Randle 1994). However, process modifications have been proposed to increase removal. For example, pH prehydrolysis was found to increase the AOX removal by 13% (Zheng and Allen 1997) by chemical reorganization of poorly degradable congeners into more degradable ones (Zheng and Allen 1996; Zheng and Allen 1997). Another proposed process modification is to retain (20 days) a portion of the returned activated sludge (RAS) before its reintroduction into the reactor. It was demonstrated that this strategy could increase the removal of AOX by 10%. The formation of sulfide and the increase in density of facultative organisms were correlated with

the increased removal. It was suggested that a dual mechanism by which chemical dehalogenation as well as the presence of a more actively dehalogenating population might be at work (Liu et al. 1997a).

Various bacteria have been found to be able to dehalogenate (for recent reviews, see Mohn and Tiedje 1992, and Copley 1997). Organisms indigenous to BKME treatment systems from the genera *Aeromonas*, *Ancylobacter*, *Chryseomonas*, *Flavimonas*, *Methylobacterium* and *Pseudomonas* have been associated with this function (Fulthorpe and Allen 1995; Buitrón and González 1996). However, it seems that the properties of isolates fall short at explaining the capacity of activated sludge at mineralizing chloroorganics. Usually, activated sludge has degradation rates of chlorophenols an order of magnitude higher than those of the rate of pure cultures (Buitrón and González 1996). It appears also that the substrate range does not always correlate with the ability of an organism to be effective *in situ*. *Methylobacterium* CP13 had a smaller chlorophenol range than *Pseudomonas* P1, but was more efficient at removing AOX from mill effluents (Fulthorpe and Allen 1995). Taken together, these findings suggest that either some indigenous AOX degraders have not yet been isolated, important microbial interactions in the activated sludge consortia were lacking in pure cultures or co-metabolism is also important for degradation. *In situ* studies using molecular technologies could be useful at resolving some of these possibilities.

For the *in situ* study of chlorophenol degraders, molecular strategies have only been used in functional analysis. One of the best examples of such studies was the use of reverse transcriptase PCR (RT-PCR) to characterize the transcription of *dmpN* gene (coding for phenol hydroxylase) *in situ*. It was shown that the transcription was dependent on the phenol and oxygen concentration (Selvaratnam et al. 1995). In a sequencing batch reactor, the maximum transcription occurred immediately after the start of the aeration period. This result also suggested that reactor operations can be used to manipulate the physiology of the organisms involved.

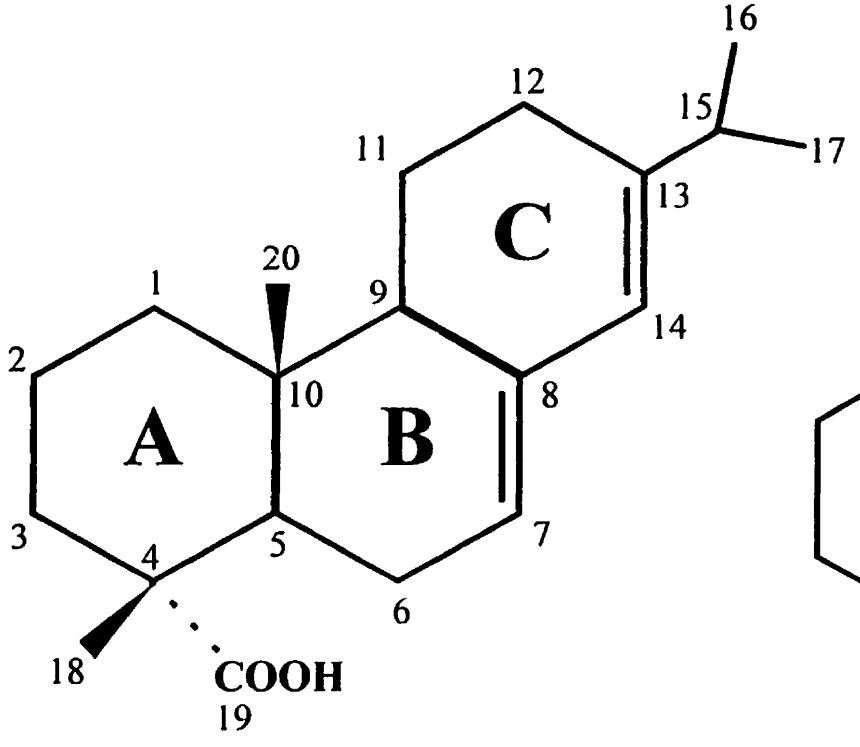
2.3.4 Resin acid removal

Resin acids are wood extractives mainly found in coniferous trees (Fengel and Wegner 1985). They are tricyclic diterpenes and are usually classified in two categories: the abietanes and the pimaranes. Abietanes are compounds with an isopropyl substituent at C-13, whereas pimaranes have both methyl and vinyl substituents at C-13 (Fig. 2.2; (Liss et al. 1997). They are very acutely toxic compounds with LC_{50} to rainbow trout of 0.4 to 1.7 ppm at pH 7 (Lee et al. 1990). Their concentration in softwood pulp mill effluent can range from 1000 ppm for kraft and sulfite mills to 10,000 ppm for mechanical mills (Liss et al. 1997); therefore, approximately 100% removal efficiency is necessary.

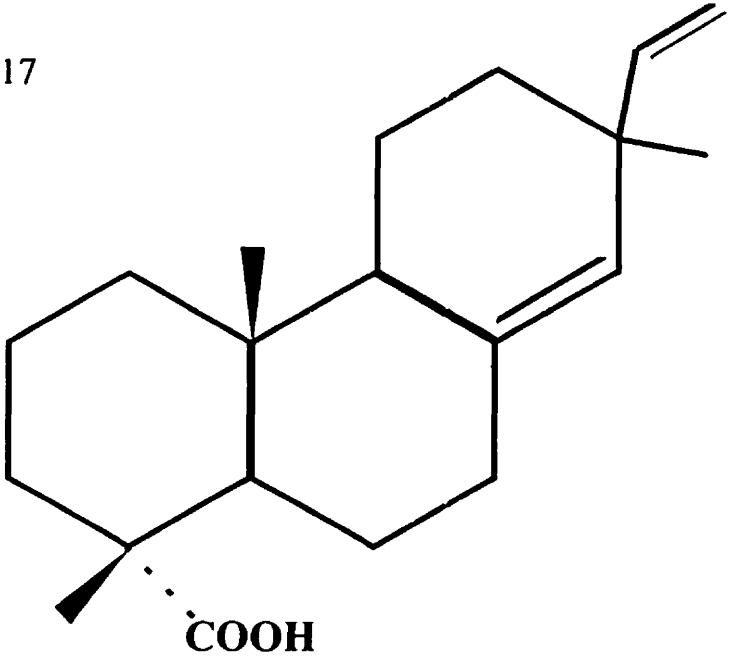
Whereas biosorption and air oxidation are removal mechanisms of resin acids, the major process is biological oxidation (Liss et al. 1997). Although aerobic treatment facilities usually achieve 80 to 100% removals, lower degradation efficiencies are encountered. This encouraged the recent efforts to gain insight into the diversity of organisms able to utilize resin acids. These works showed that resin acid degradation is shared by phylogenetically distant organisms. *Alcaligenes xylosoxidans*, *Comamonas testosteroni* (Morgan and Wyndham 1996), *Sphingomonas* sp., and *Zooglea ramigera* (Mohn 1995) have been isolated on abietic acid. Pimaranes are usually the most recalcitrant group of resin acids in pulp and paper wastewaters, and this is consistent with the failure to isolate organisms that are able to utilize these compounds as sole carbon and energy source.

Other studies demonstrated that resin acid dregradation is not a constitutive physiological state. Glycerol-grown cultures exhibited a lag period of two to four hours before starting to degrade DHA, and *de novo* protein synthesis was required as demonstrated by the lack of degradation if tetracycline was added at the same time as DHA (Bicho et al. 1995). In addition, presence of sugars in the growth medium may enhance degradation in some cases (Mohn 1995).

Figure 2.2. Chemical structure of the (a) abietanes and (b) the pymaranes.



a) ABIETIC ACID



b) PIMARIC ACID

Even with this phylogenetically diverse collection of isolates, Mohn (1995) reported $1.1x10^6$ propagules/mL and a degradation rate of 6 µmol/mg of protein/hour, numbers falling short at explaining all the resin acid removal in the system under study. A molecular approach aiming at measuring the *in situ* activity and abundance of resin acid degrading organisms is being developed to resolve this discrepancy (Muttray and Mohn 1998b). However, it is also necessary to have more protocols used for the isolation of novel bacteria, especially candidate pymarane degraders.

2.4 Conclusion

This review clearly shows the wide phylogenetic diversity found in wastewater treatment systems. It also shows the link between this diversity and the possible occurrence of problem situations. However, the lack of unbiased and quick methods to assess the microbial community made it impossible to incorporate these data in reactor control and diagnosis. The recent development of molecular microbial ecology and phenotypic fingerprinting resulted in new relevant information on *in situ* microbial communities. This information is a good complement to the previous culture studies. Because of the rapidity of obtaining the community information, it is possible to foresee reactor monitoring tools that would include them. However, temporal data on the variation in microbial communities are still lacking. These are very important in the development of these tools since they would be the comparative baselines to any diagnosis strategy. This is the basis of the work described in this thesis.

Chapter 3. Materials and Methods

3.1 Sampling

For molecular analysis, the biomass of the wastewater treatment systems was sampled once a week every Wednesday morning for one year. A 10 mL sample of mixed liquor was flash frozen in liquid nitrogen, transported to the laboratory in liquid nitrogen and kept at -70°C until analysis. For phenotypic fingerprinting, a 200 mL sample of mixed liquor was obtained on certain Wednesdays and transported on ice to the laboratory and analyzed within 24 hours.

For the biochemical analysis of the wastewater, 24 hour composite-samples (sampled every 15 min.) of influent and effluent were taken at 8 a.m. the same day as the biomass was sampled. The samples were transported on ice to the laboratory and were kept either at 4°C if the samples were to be analyzed within 5 days or at -20°C if they were to be analyzed later. The wastewater samples taken for VFA, phenol and alcohol analysis were preserved by adding few drops of 40% NaOH to 50 mL of sample.

3.2 Wastewater analysis

3.2.1 BOD_5

BOD_5 was determined according to the method of Greenberg and co-workers (1992). The wastewater was diluted in BOD buffer (65 μM KH_2PO_4, 125 μM K_2HPO_4, 148 μM Na_2HPO_4, 32 μM NH_4Cl, 91 μM $MgSO_4 \cdot 7\ H_2O$, 1.5 μM $FeCl_3 \cdot 6\ H_2O$, 364 μM $CaCl_2$) such that, after five days, the dissolved oxygen (DO) would be at least 1 mg/L and the DO uptake would be at least 2 mg/L. A BOD bottle was filled with wastewater/buffer mixture, inoculated with approximately 250 μg of solids from Reactor 1 (see Chapter 4), and the DO was read immediately using a YSA model 54A DO meter. After five days of incubation at 20°C, the DO was read again. The DO uptake was finally expressed for the undiluted wastewater.

3.2.2 COD

COD was determined by the Hach Dichromate COD Reagent Low Range (0-150 mg/L) or High Range (0-1500 mg/L) kits according to the manufacturer's instructions (Hach Company). A 2 mL aliquot of wastewater sample was introduced in a COD tube which was then digested for 2 hours at 150°C and cooled to room temperature. The COD was determined by analysis with a Hach model DR2000 spectrophotometer at wavelengths of 420 nm (Low Range) or 620 nm (High Range)

3.2.3 COD fractions

Four COD fractions (Particulate COD, COD > 1000 MW, COD 500-1000 MW, COD < 500 MW) were determined. Total COD was defined as the COD of the unfiltered sample, and the soluble COD was defined as the COD of the sample after filtration through a 0.45 μm filter (soluble fraction). Particulate COD was obtained by differentiating the total COD and the soluble COD.

To obtain the molecular weight fractions, the MPS (Amicon Inc.) system was used to ultrafilter 1 mL of the soluble wastewater fraction 45 min. at 1000 × g in a Sorval model SS-34 rotor (Dupont Instrument Inc.). A YC05 membrane (Amicon Inc.) was used to obtain the fraction smaller than 500 MW and a YM1 membrane (Amicon Inc.) was used to obtain the fraction smaller than 1000 MW. The COD of these two fractions was determined with Hach Dichromate COD Reagent Low Range kit (see section 3.2.2). The COD > 1000 MW was obtained by subtracting the COD less than 1000 MW from the soluble COD. COD 500-1000 MW was obtained by subtracting the COD smaller than 500 MW from the COD less than 1000 MW. All analyses were performed with two independent replicates.

3.2.4 VFA and alcohol determination

The concentrations of 5 volatile fatty acids (VFA) and 4 alcohols (Table 3.1) were determined as described below. The concentrations were converted to BOD_5 equivalents (Pitter and Chudoba 1990) and the total concentration of VFA-BOD_5 and alcohol-BOD_5 were reported.

A 5 mL aliquot of sample wastewater was spiked with 1-Butanol (internal standard) and acidified to pH 2-3 with 6 N HCl. The mixture was then centrifuged at 4000 × g for 10 min. to remove particulate matter. The clear supernatant was then analyzed by gas chromatography using a model HP5890 GC (Hewlett-Packard Company) equipped with a DB-WAX column (J&W Scientific) and an FID detector.

Table 3.1. Phenolic compounds, alcohols and VFA determined in wastewater by chromatography and their BOD_5 equivalence.

Compounds	BOD_5 equivalents[1] (g BOD_5/g)	Compounds	BOD_5 equivalents[1] (g BOD_5/g)
Phenolic Compounds		Alcohols	
Acetosyringone	0.97	Methanol	0.85
Acetovannilone	0.97	Ethanol	1.27
o-Cresol	1.70		
m-Cresol	1.70	VFA	
p-Cresol	1.44	Acetic Acid	0.65
Dihydroconiferyl Alcohol	1.22	Butyric Acid	0.89
4-Ethylguaiacol	0.94	Formic Acid	0.20
Guaiacol	1.40	Isovaleric Acid	1.29
4-Hydroxybenhaldehyde	1.08	Propionic Acid	0.93
4-Methylguaiacol	0.90		
Phenol	1.81		
Syringaldehyde	1.02		
Syringol	0.97		
Vanillin	1.02		
Vanillyl Alcohol	1.02		

[1] (Pitter and Chudoba 1990)

3.2.5 Phenols determination

The concentration of 15 phenolic compounds (Table 3.1) was determined by the method described below. The concentrations were converted to BOD_5 equivalents (Pitter and Chudoba 1990) and the total concentration of phenolic BOD_5 was reported.

A 5 mL aliquot of wastewater sample was buffered with 0.5 mL of aqueous sodium bicarbonate (1 M) and spiked with 4-isopropylphenol and hexachlorobenzene as surrogate recovery standard and internal standard, respectively. The phenols were then derivatized with 10% pentachlorobenzoyl chloride in toluene to form the corresponding esters. Pyridine was added to catalyze the reaction. After 10 min. reaction time, the sample was extracted with hexane (5 min. shaking). The hexane layer was extracted with 1 M NaOH to remove the excess reagent. The hexane extract was analyzed by gas chromatography using a model HP5890 GC (Hewlett-Packard Company) equipped with a DB-1 column (J&W Scientific) and an ECD detector.

3.2.6 Chloroform determination

The chloroform concentration was determined according to the U.S.EPA Protocol 8260B (U.S. Environmental Protection Agency 1996). A 5 mL aliquot of wastewater sample was spiked with surrogate standards (toluene, 4-bromofluorobenzene and 1,2-dichloroethane) and internal standards (bromochloromethane and 1,4-difluorobenzene), and introduced in a model HP7695 purge and trap concentrator equipped with a tenax/silica/charcoal trap (Hewlett-Packard Company). The sample was purged with helium (35 mL/min.) for 11 min. at 30°C. The trapped compounds were desorbed at 255°C for 2 min. and analyzed by gas chromatography/mass spectrometry using a model HP5890 GC equipped with a HP-VOC column (Hewlett-Packard Company) and a model HP5972A mass spectrometer (Hewlett-Packard Company).

3.2.7 Residual nutrient concentration

To determine the residual ammonia concentration, a 100 mL aliquot of wastewater sample was adjusted to pH 10 and the ammonia was measured with an Orion model EA940 electrometer equipped with an Orion model 93-12 ammonia selective electrode.

Residual phosphate concentration was determined by the Hach Reactive Phosphorus Test 'N Tube kit according to the manufacturer's instructions (Hach Company). A 5 mL aliquot of wastewater sample was acidified with 2 mL 5.25 N of H_2SO_4 and potassium persulfate (as provided in the kit) was added. After a 2 min. reaction time, 2 mL of 5 N NaOH and ascorbic acid (as provided in the kit) were added to the solution. The concentration of phosphate was determined at a wavelength of 890 nm with a Hach model DR2000 spectrophotometer.

3.2.8 Suspended solid concentration

A 100 mL aliquot of mixed liquor was filtered on a preweighed Whatman 934/AH glass fiber filter. The filter was dried at 105°C for 1 hour and cooled to ambient temperature in a desiccator before weighing. The suspended solid concentration was calculated from the difference in weight of the filter before and after filtration. Analyses were performed in duplicate.

3.2.9 Specific oxygen uptake rate

Specific oxygen uptake rate (SOUR) was determined according to the method of Greenberg and co-workers (1992) immediately after sampling of mixed liquor. A BOD bottle was filled with mixed liquor aerated by 1 min. shaking in a flask. Dissolved oxygen (DO) was read every 30 sec. for 10 min. using a YSA model 54A DO meter. The SOUR was obtained by dividing the slope of oxygen uptake by the mixed liquor suspended solid concentration.

3.2.10 Sludge volume index

Sludge volume index (SVI) was determined according to the method of Greenberg and co-workers (1992) immediately after sampling. A Nalgene Settlometer (2 L) was filled with mixed liquor. The solids were allowed to settle for 30 min. before the sludge volume was read. SVI was obtained by dividing the sludge volume by the suspended solid concentration.

3.2.11 Sludge level in clarifier

The sludge level in the clarifiers was determined every Wednesday morning at the same time with a Sludge Judge (Wheaton Inc.). The Sludge Judge was introduced vertically into the clarifier from the surface to the bottom. Precautions were taken so the rotating arms were at a right angle to the location where the sludge level was taken.

3.2.12 On-line probes

Influent flow, returned activated sludge (RAS) flow, wasted activated sludge flow (WAS), temperature, pH, and conductivity were recorded on-line by specific probes. The data were extracted from the mill recording system by averaging the value from 8 a.m. the day before biomass sampling until 8 a.m. the day of sampling.

Chloride tank level was also recorded on-line. The level was recorded at 8 a.m. daily. If the tank level decreased at least one day during the week prior to the biomass sampling, it was concluded that the biomass had been chlorinated and a "1" was reported in the variable chlorination, otherwise a "0" was reported.

Ammonia and ammonium polyphosphate (phosphorus source) tank levels were also recorded on-line. Nutrient addition was calculated from the difference of the level at 8 a.m. the day before biomass sampling and the level at 8 a.m. the day of sampling.

3.2.13 Calculated parameters

Four parameters describing the reactor conditions were calculated based on equations described below.

3.2.13.1 Hydraulic retention time

The hydraulic retention time (HRT) is defined as the time that the wastewater passes into the bioreactor (Jenkins et al 1993) However, the classical expression of the HRT does not take into account the effect of recirculating the wastewater to the reactor via the RAS flow. The HRT formula below corrected this inconsistency.

$$HRT = V \cdot \left(\frac{1}{Q_i} - \left(\frac{Q_r}{Q_r + Q_i} \right)^2 \right)$$

where V is the volume of the reactor, Q_i is the influent flow and Q_r is the RAS flow.

3.2.13.2 Recirculation time

The average time beween two successive introductions of a single floc (recirculation time) depends on the influent flow, the RAS flow and the sludge settling velocity. The equation below defines the recirculation time (RT)

$$RT = \frac{M_c}{Q_r} \cdot S_r$$

where M_c is the mass of sludge in the clarifiers and S_r is the solid concentration in the RAS line.

3.2.13.3 Sludge age

Sludge age is traditionally calculated based on the mass of sludge in the system and the wasting rate at the time of calculation. Therefore, the calculated sludge age can vary by more than one day in 24 hours, and does not represent the true age of the sludge in the

reactor. It is important to have an accurate and reliable measurement of sludge age because it is a relevant parameter affecting the physiology of the bacteria (Jenkins et al. 1993). Vaccari and co-workers (1988) proposed an algorithm to calculate the sludge age that takes into account the sludge production and is therefore a more accurate representation of the true age of the sludge in the reactor. The algorithm first calculates the net sludge production rate (K) and the average sludge production rate (P) since the last time the sludge age was calculated.

$$K = \frac{M - M_0}{t}$$

$$P = K + W$$

where M is the actual mass of sludge in the treatment system, M_0 is the mass of sludge in the treatment system the last time the sludge was calculated, t is the time since the sludge age was last calculated, and W is the mass of sludge wasted or lost in the effluent per unit of time since the sludge age was last calculated.

From these initial calculations, four cases may arise and the proper equation of the algorithm is selected to calculate the sludge age (A).

Case 1: No sludge was wasted or lost over the period t ($W=0$).

$$A = \frac{(A_0 + t)\cdot M_0}{M} + \left(1 - \frac{M_0}{M}\right) \cdot \frac{t}{2}$$

Case 2: There was no net sludge production ($W=P$).

$$A = \frac{M_0}{W} + \left(A_0 - \frac{M_0}{W}\right)^{\left(-\frac{W\cdot t}{M_0}\right)}$$

Case 3: Exactly twice as much sludge is wasted as was produced ($W=2P$).

$$A = A_0 \cdot \left(\frac{M}{M_0}\right) - \left(t + \frac{M_0}{K}\right) \cdot \ln\left(\frac{M_0}{M}\right)$$

Case 4: If none of the above conditions hold, the following equation is used.

$$A = \left(A_0 - \frac{M_0}{P+K}\right) \cdot \left(\frac{M}{M_0}\right)^{\left(-\frac{P}{K}\right)} + \frac{M}{P+K}$$

3.3 RNA probing and quantification

RNA probing and quantification was performed in four steps. First, the RNA was extracted and the integrity was evaluated by agarose gel electrophoresis. Second, replica membranes were produced by blotting the community RNA on a nylon membrane along with positive control reference RNA. Third, the membranes were hybridized with population-specific probes and eubacterial probes (Table 3.2). Fourth, the radioactive hybridization signal was detected by autoradiography, digitized and quantified by densitometry. The water used to make the solutions was always distilled and deionized water treated overnight with DEPC at 37°C and autoclaved. The glassware used to manipulate solutions in contact with RNA was baked overnight at 180°C. Pipette tips and microcentrifuge tubes were kept RNAse free as much as possible.

3.3.1 Culture conditions

The control organisms (Table 3.3) were cultured in TY broth (0.5% tryptone, 0.3% yeast extract and 0.044% $CaCl_2 \cdot 2\ H_2O$) at either 30°C or 37°C. The organisms were harvested in mid log phase at an optical density at 600 nm of 0.5 and were kept at −70°C until RNA extraction.

3.3.2 RNA extraction

A 10 mL aliquot of mixed liquor (2000-5000 mg/L of solids) or 50 mL of pure culture was centrifuged at 12,000 × g in a Sorval model RC-5B centrifuge (Dupont Instrument Inc.) for 10 min. at 4°C. The biosolids were resuspended in 1 mL of Trizol Reagent (Life Technologies Inc.; Isothiocyanate-phenol buffer) and introduced in a 2 mL vial with 1 g of glass-beads (100 μm; washed three times with nitric acid and baked overnight at 180°C).

The vial was beaten twice for 2 min. in a model 2876 beater (Bronwill Scientific Inc.) and centrifuged for 2 min. in an Eppendorf microcentrifuge. The supernatant was removed to a microcentrifuge tube. Fresh Trizol (1 mL) was introduced into the vial, and the biosolids and the vial was beaten once more for 2 min.. After centrifugation, the supernatant (Trizol) was pooled with the first portion and 0.2 volume of chloroform was added to separate the aqueous and organic phases and precipitate the proteins. The mixture was centrifuged for 20 min. and the aqueous phase was transferred to a new microcentrifuge tube RNA was precipitated by adding 0.6 volume of isopropanol and incubating on ice for 15 min.. RNA was pelleted by centrifugation for 20 min., washed with 70% ethanol and air-dried. The RNA was dissolved in water and kept frozen at −70°C until analysis.

3.3.3 Gel electrophoresis

Agarose gels were used to analyze the quality of extracted RNA. The gels were made up of TAE buffer (40 mM Tris-acetate and 1 mM EDTA) and 0.8% agarose and were subjected to an electrical field of 80 V (Sambrook et al. 1989). Since deteriorated RNA (sheared or enzymatically degraded) can affect the accuracy of quantitative membrane hybridization (Raskin et al. 1996; see also Appendix II), only intact RNA samples (i.e., samples with definite bands and 23S:16S bands ratio equal to 2) were used for quantification.

3.3.4 RNA blotting

The RNA blotting protocol was modified after Stahl and Amann (1991) and Raskin and co-workers (1996). The RNA samples (100 μg/mL) were denatured by adding 3 volumes of 5% glutaraldehyde in 50 mM sodium phosphate (pH 7.0) and incubating the mixture 10 min. at 65°C. The denatured RNA samples were then diluted about 50 times in dye/poly A blotting solution (1 μg/mL of polyadelinic acid and 0.0002% bromophenol blue). This last solution (100 μL) was slot blotted in triplicate on a positively-charged nylon membrane (Biorad Zeta-Probe GT membrane) using the Biodot SF vacuum blotter (Biorad Inc.). Replica membranes were produced if many probes were used for hybridization. The nucleic acids were fixed to the membrane by exposure to 120 mJ of UV in a Stratalinker model 1800 UV-crosslinker (Stratagene Inc.).

3.3.5 5'-End probe labeling

The 5'-end of the oligonucleotide probes were labeled with ^{33}P. The probes (10 pmol), T4-polynucleotide kinase (4 U) and [γ-^{33}P]ATP (10 pmol) were diluted in 10X polynucleotide kinase buffer (100 mM $MgCl_2$, 50 mM dithiothreitol, 700 mM Tris-HCl, pH 7.6). The final reaction volume was 15 µL. The reaction mixture was first incubated 30 min. at 37°C, then 10 min. at 75°C. For hybridization, the reaction mixture was completely transferred to 5 mL of hybridization buffer to give a final probe concentration of 2 pmol/mL.

Table 3.2. Probes used in this study.

Probe Name[1]	Abreviated Name	Target Group	Probe Sequence (5' to 3')[2]	References
S-*-Univ-0519-a-A-18	UNI16	Universal	GWATTACCGCGGCKGCTG	(Giovannoni et al.1988)
L-*-Univ-2576-a-A-20	UNI23	Universal	CGACGTTYTAAACCCAGCTC	(Amann et al. 1995)
S-D-Bact-0338-a-A-18	EUB16	Eubacteria	GCTGCCTCCCGTAGGAGT	(Amann et al. 1990)
L-D-Bact-1933-a-A-19	EUB23	Eubacteria	ACCCGACAAGGAATTTCGC	(Amann et al. 1995)
S-Sc-aProt-0019-a-A-17	ALF16	Alpha Proteobacteria	CGTTCGYTCTGAGCCAG	(Manz et al. 1992)
L-Sc-bProt-1027-a-A-17	BET23	Beta Proteobacteria	GCCTTCCCACTTCGTTT	(Manz et al. 1992)
L-Sc-gProt-1027-a-A-17	GAM23	Gamma Proteobacteria	GCCTTCCCACATCGTTT	(Manz et al. 1992)
S-Sc-Cfc-0319-a-A-18	CFC16	*Cytophaga-Flavobacterium* cluster	TGGTCCGTGTCTCAGTAC	(Manz et al. 1996)
S-P-Grps-1200-a-A-13	GPB16	Gram-positive Bacteria	AAGGGGCATGATG	(Fry et al. 1997)
L-P-Grps-1901-a-A-18	HGC23	Gram-positive Bacteria with high GC content	TATAGTTACCACCGCCGT	(Roller et al. 1994)
S-*-Ecol-0019-a-A-17	EC(alf)16	*Escherichia coli*	CGTTCAATCTGAGCCAT	This Study
S-*-Ecol-0319-a-A-18	EC(cfc)16	*Escherichia coli*	TGGACCGTGTCTCAGTTC	This Study

[1] Probe names follow the convention of the Oligonucleotide Database Project (ODP; Alm et al. 1996) which is S or L for small or large subsunit rRNA as the target; letter(s) designating the taxonomic level target D-Domain, K-Kindom, P-Phylum, Sc-Subclass, O-Order, F-Family, G-Genus, S-Species, Ss-Subspecies, St-Strain, *-Undefined taxon; letters designating the target group of the probe; nucleotide position (*E. coli* numbering) in the target where 3' end of the antisense probe binds; letter designating the version of the probe (a, version 1; b, version 2; etc.); A or S indicating whether the probe is identical to the antisense (template) strand or to the sense (nontemplate) strand; number indicating the length in nucleotide of the probe.

[2] K= G or T; W = A or T; Y = C or T

3.3.6 Hybridization and washing

A total of eleven probes were used in this work (Table 3.2). The hybridization protocol was modified after Manz and co-workers (1992) and Raskin and co-workers (1996). The membranes were introduced in hybridization bottles model HB-OV-MB (Hybaid Inc.) with 5 mL of hybridization buffer (0.9 M NaCl, 10X Denhardt's solution, 5 mM EDTA, 0.5% (W/V) SDS, 100 ug/mL sonicated salmon sperm DNA, 50 mM $NaHPO_3$, varied concentration of formamide, pH 7.2). After a prehybridization of 1 hour at 46°C in a model Micro-4 hybridization oven (Hybaid Inc.), radioactively-labeled oligonucleotide probes were added to the hybridization buffer to a final concentration of 2 pmol/mL and the membranes were hybridized at the same temperature for 12 hours. Probing stringency was introduced in both the hybridization and washing steps by varying the formamide concentration in the hybridization buffer and the NaCl concentration in the washing buffer (Table 3.3). After hybridization, the membranes were washed 15 min. at 48°C in the same bottle three times with 15 mL of washing buffer (20 mM TRIS-HCl, 5 mM EDTA, 0.01% SDS, varied concentration of NaCl, pH 8.0).

3.3.7 Detection and quantification

The radioactive probe signal was detected by exposure of the hybridized membrane to a Biomax MR autoradiographic film (Eastman Kodak Inc.) to obtain a signal within the dynamic range of the film. The films were then digitalized with the model Gel Print 2000i digitalizing system (Genomic Solutions Inc.) and the signals were quantified by densitometry using Scion Image for Windows, a public domain software developed by the National Institute of Health (USA) and available on the Internet at http://rsb.info.nih.gov/nih-image/. The probe signal was the average signal obtained from the three replicates on the membrane. The relative target rRNA abundance was obtained by dividing a specific probe signal by the signal of the eubacterial probe targeted to the same rRNA subunit and correcting for difference in signal of the two probes for the positive control rRNA.

Table 3.3. Control organisms and hybridization conditions used in this study.

Probe Name	Target Group	Control Organisms[1]	Hybridization Conditions[2]	
			Formamide (%)	NaCl (mM)
S-*-Univ-0519-a-A-18	Universal	All Eubacteria *Trametes versicolor* ATCC 20869	30	60
L-*-Univ-2576-a-A-20	Universal	All Eubacteria *Trametes versicolor* ATCC 20869	15	250
S-D-Bact-0338-a-A-18	Eubacteria	All Eubacteria	25	100
L-D-Bact-1933-a-A-19	Eubacteria	All Eubacteria	15	250
S-Sc-aProt-0019-a-A-17	Alpha Proteobacteria	*Sinorhizobium meliloti* Rm1021*[3] *Methylosinus tricosporium* OB3b	20	160
L-Sc-bProt-1027-a-A-17	Beta Proteobacteria	*Ralstonia eutropha* ATCC 17699* *Comamonas testosteroni* ATCC 11996	35	40
L-Sc-gProt-1027-a-A-17	Gamma Proteobacteria	*Escherichia coli* DH5α* *Pseudomonas putida* ATCC 17485	35	40
S-Sc-Cfc-0319-a-A-18	*Cytophaga-Flavobacterium* cluster	*Cytophaga johnsonae* ATCC 29589*	35	40
S-P-Grps-1200-a-A-13	Gram-positive Bacteria	*Staphylococcus epidermidis* ATCC 12228 *Arthrobacter* sp. ATCC 21920*	10	380
L-P-Grps-1901-a-A-18	Gram-positive Bacteria with high GC content	*Arthrobacter* sp. ATCC 21920*	25	100
S-*-Ecol-0019-a-A-17	*Escherichia coli*	NA[4]	15	250
S-*-Ecol-0319-a-A-18	*Escherichia coli*	NA	35	40

[1] Organisms used to test the specificity of the probes.
[2] Concentration of formamide in the hybridization buffer and NaCl in the wash buffer (see text).
[3] * indicates the organisms used for positive control in the quantitative membrane hybridization assay.
[4] NA: Not Applicable.

3.4 Phenotypic fingerprinting

An aliquot of mixed liquor (100 mL) was centrifuged 15 min. at 8000 × g. The biosolids were resuspended in 100 mL of sterile saline (0.8% NaCl) and blended for 1 min. The large debris were removed by centrifuging the mixture at 350 × g for 5 min. in model GLC-2 bench-top centrifuge (Dupont Instrument Inc.) and the supernatant was kept in a sterile flask. The debris were resuspended in saline, blended, centrifuged again and the supernatant was pooled with the previous one. The biomass in the pooled supernatants was washed three times by centrifuging 15 min. at 8000 × g and resuspending in 100 mL saline. The biomass was resuspended in phosphate buffered (0.1 M potassium phosphate) saline, dispersed by a cell homogenizer and the optical density (OD) at 600 nm was adjusted to 0.3. The Biolog GN (Table 3.4) plate was inoculated with 150 µL and incubated in the dark at 30°C. After 24 hours, the plates were read at 595 nm in a Biorad model 3550-UV microplate reader.

3.5 Statistical analysis

All the statistical analyses were performed with SAS/STAT ver. 6.12 for Windows (SAS Institute Inc. 1989) and SAS/ETS ver. 6.12 for Windows (SAS Institute Inc. 1993). Principal components analysis (Rencher 1995) was carried out on the correlation matrix using the PRINCOMP procedure of SAS/STAT. The scores were standardized to the unit variance prior to plotting. Correlations were calculated using the CORR procedure of SAS/STAT.

Empirical modeling was performed in two steps. First, autocorrelations and cross-correlations (Box et al. 1994) were calculated using the ARIMA procedure of SAS/ETS. Second, from the sample cross-correlation estimates, partial least-squares regression (PLS) models were fitted using the singular vector decomposition (SVD) algorithm (Manne 1987; Höskuldsson 1988) of the PLS procedure of SAS/STAT. This regression method was chosen because of its robustness with respect to colenearity of the data and high ratio of the number of variables to the number of observations. The number of latent variables (orthogonal projections of independent variables) to be used was determined by the leave-one-out cross-validation procedure, using the minimum predicted residual sum of squares (PRESS; Shao

1993). The final model was obtained by iteratively eliminating variables with a variable importance in the projection (VIP) criterion smaller than 0.8 (Wold 1994).

Table 3.4. Compounds in the Biolog GN plates ordered according to their location in the plate.

#	Compounds	#	Compounds	#	Compounds
1	α-Cyclodextrin	33	Xylitol	65	L-Alanine
2	Dextrin	34	Methyl pyruvate	66	L-Alanyl-glycine
3	Glycogen	35	Mono-methyl succinate	67	L-Asparagine
4	Tween 40	36	Acetic acid	68	L-Aspartic acid
5	Tween 80	37	cis-Aconitic acid	69	L-Glutamic acid
6	N-Acetyl-D-galactosamine	38	Citric acid	70	Glycyl-L-aspartic acid
7	N-Acetyl-D-glucosamine	39	Formic acid	71	Glycyl-L-glutamic acid
8	Adonitol	40	D-Galactonic acid lactone	72	L-Histidine
9	L-Arabinose	41	D-Galacturonic acid	73	Hydroxy-L-proline
10	D-Arabitol	42	D-Gluconic acid	74	L-Leucine
11	Cellobiose	43	D-Glucosaminic acid	75	L-Ornithine
12	i-Erythritol	44	D-Glucuronic acid	76	L-Phenylalanine
13	D-Fructose	45	α-Hydroxybutyric acid	77	L-Proline
14	L-Fucose	46	β-Hydroxybutyric acid	78	L-Pyroglutamic acid
15	D-Galactose	47	γ-Hydroxybutyric acid	79	D-Serine
16	Gentiobiose	48	p-Hydroxyphenylacetic acid	80	L-Serine
17	α-D-Glucose	49	Itaconic acid	81	L-Threonine
18	m-Inositol	50	α-Ketobutyric acid	82	D,L-Carnitine
19	α-D-Lactose	51	α-Ketoglutaric acid	83	γ-Aminobutyric acid
20	Lactulose	52	α-Ketovaleric acid	84	Urocanic acid
21	Maltose	53	D,L-Lactic acid	85	Inosine
22	D-Mannitol	54	Malonic acid	86	Uridine
23	D-Mannose	55	Propionic acid	87	Thymidine
24	D-Melibiose	56	Quinic acid	88	Phenylethylamine
25	β-Methyl-D-glucoside	57	D-Saccharic acid	89	Putrescine
26	D-Psicose	58	Sebacic acid	90	2-Aminoethanol
27	D-Raffinose	59	Succinic acid	91	2,3-Butanediol
28	L-Rhamnose	60	Bromosuccinic acid	92	Glycerol
29	D-Sorbitol	61	Succinamic acid	93	D,L-α-Glycerol-phosphate
30	Sucrose	62	Glucuronamide	94	Glucose-1-phosphate
31	D-Trehalose	63	Alaninamide	95	Glucose-6-phosphate
32	Turanose	64	D-Alanine		

Chapter 4. Results

The primary objective of the study is to evaluate, from an industry point of view, the utility of phylogenetically characterizing bacterial communities to control and diagnose wastewater treatment systems. First, it was necessary to follow the bacterial community in time and demonstrate that the community profile was not random, but related to changes in reactor conditions. Second, the relationship between the community profile and the performance of the reactor had to be made. Third, the technique was also compared to other community characterization techniques (phenotypic fingerprinting) that are currently being developed.

The results are presented in three sections. Section 1 describes the monitoring and behaviours of two bioreactors treating pulp mill effluent from the Domtar Cornwall facility. Reactor 1, the main bioreactor, was followed for thirteen months. Reactor 2, a pilot bioreactor, was followed for five months. Section 2 describes the weekly sampling of biomass in the two reactors. Total community RNA was extracted and the abundance of five phylogenetically-defined bacterial populations was determined by membrane hybridization. Section 3 presents phenotypic fingerprints of the bacterial communities obtained at seven different dates for Reactor 1 and four different dates for Reactor 2.

Principal component analysis (PCA) and partial least squares (PLS) regression were used for exploratory purposes, in order to investigate the relationships between reactor conditions, phylogenetic community structure and phenotypic fingerprint. First, PCA was used to visualize the differences between the two reactors according to the type of data. Second, the bioreactor conditions and behavior data, and the probe responses were used as potential explanatory variables to build empirical regression models with the PLS method. The statistical analyses and the relevant raw data are presented in Section 4.

4.1 Conditions and behaviors of the two Cornwall reactors

4.1.1 Reactor 1

Reactor 1 is a 25,000 m^3 reactor treating about 100,000 m^3 per day of integrated hardwood bleached kraft pulp and paper mill effluent (reactor influent). It is divided into four cells. During the time of the study, the reactor was operated in step-feed mode, meaning that all the biomass was returned to the first cell, but the influent was equally partitioned between the first and the third cell. A total of forty-three variables were used to monitor the reactor during the time of the study (Table 4.1).

The first group of variables described the characteristics of the influent wastewater. The wastewater entering Reactor 1 had a usual COD:BOD_5 ratio of about three (Table 4.1). Alcohols (90% methanol and 10% ethanol) and VFA (73% acetate, 23% formate and 4% propionate) composed a total of 45% of the BOD_5 in equal proportions. The COD was mainly composed of high molecular weight and low molecular weight soluble compounds.

The second group of variables described the operation of the bioreactor. They represent factors that can be manipulated by the operators. Reactor 1 was operated at an F:M ratio (BOD_5) of 0.28 Kg/Kg/day and a sludge age of 11 days on average (Table 4.1). The yearly average temperature of Reactor 1 was 30 °C (Table 4.1). From mid December until the end of March, the temperature averaged 25 °C, whereas from mid June until mid September, the temperature averaged 36 °C (data not shown). Between these periods, a steady increase (March to June) or decrease (September to December) was observed. Ammonia and phosphate were added to the system in a target BOD:N:P ratio of 100:5:1. However, the mill usually added slightly more ammonia and slightly less phosphate. The average observed ratio over the year of this study was 100:5.8:0.73 (Table 4.1).

Table 4.1. Variables [1] describing influent characteristics, bioreactor operation, sludge characteristics and the final effluent characteristics for the two Cornwall reactors.

Variables	Units	Reactor 1		Reactor 2	
		Average	SD[2]	Average	SD
Influent Characteristics					
COD	mg/L	640	141	6287	1843
BOD_5	mg/L	211	49	1122	482
COD:BOD_5		3.07	0.36	5.98	1.30
Alcohols (BOD_5)[3]	mg/L	43.8	13.8	1.4	1.3
VFA (BOD_5)[3]	mg/L	47.6	19.1	326.5	296.1
Phenols (BOD_5)[3]	mg/L	3.7	1.6	N.A.[4]	
Particulate COD	% Total COD	17	8	N.A.	
COD > 1000 MW	% Total COD	47	8	N.A.	
COD 500-1000 MW	% Total COD	12	5	N.A.	
COD < 500 MW	% Total COD	25	7	N.A.	
TSS	mg/L	59	26	N.A.	
Conductivity	uS/cm	1435	207	N.A.	
Absorbance (280 nm)		2.17	0.65	N.A.	
Bioreactor Operation					
Temperature	°C	30.8	4.7	29.2	3.0
pH	pH	7.5	0.6	8.7	0.2
F:M Ratio (BOD_5)[5]	Kg/Kg/Day	0.28	0.08	0.27	0.12
F:M Ratio (CODr)[6]	Kg/Kg/Day	0.60	0.15	0.84	0.65
Sludge Age	Day	11.3	2.7	44.9	13.3
Hydraulic Retention Time	hour	5.4	0.4	37.1	9.9
Recirculation Time[7]	hour	6.8	1.7	N.A.	
Chlorination[8]	Day	0.32	N.A.	N.A.	

[1] Average of one observation per week during the time of the study
[2] SD: Standard Deviation
[3] Alcohol, VFA and Phenol concentrations were determined by gas chromatography and converted to BOD_5 equivalent prior to the summation (See Chapter 3).
[4] N.A.: Not Available
[5] The sludge concentration is expressed in terms of MLSS through the table
[6] CODr: COD removed
[7] Recirculation Time: Average time period between two successive introductions of a floc in the first cell of the reactor
[8] Chlorination is a binary variable of value 1 when the reactor was chlorinated during the week before sampling. The fraction represents the proportion of weeks where chlorination was applied.

Table 4.1 (continued). Variables describing influent characteristics, bioreactor operation, sludge characteristics and the final effluent characteristics for the two Cornwall reactors.

Variables	Units	Reactor 1		Reactor 2	
		Average	SD	Average	SD
Bioreactor Operation (continued)					
Dissolved O_2 (Cell 1)	mg/L	2.39	1.81	3.59	2.05
Dissolved O_2 (Cell 2)	mg/L	2.62	1.35	3.64	1.73
Dissolved O_2 (Cell 3)	mg/L	1.76	1.11	N.A.	
Dissolved O_2 (Cell 4)	mg/L	2.24	1.52	N.A.	
Average added NH_3-N[9]	mg/L	12.3	3.3	11.0	27.7
BOD_5:N[10]	mg : mg	5.7	2.1	N.A.	
Average added PO_4^{3-}-P[9]	mg/L	1.5	0.5	7.2	6.3
BOD_5:P[10]	mg : mg	0.73	0.21	N.A.	
COD removal	%	71.4	5.7	45.6	16.7
BOD_5 removal	%	96.3	1.7	93.4	47.8
TSS removal	%	63.4	37.1	N.A.	
Sludge Characteristics					
SOUR 1[11]	mg O_2/g/hour	8.8	2.7	13.4	6.3
SOUR 2[12]	mg O_2/g/hour	8.6	3.3	8.0	3.8
SOUR 1:SOUR 2		1.1	0.3	1.5	0.6
Sludge level in clarifier	Feet	1.76	0.73	1.47	0.68
SVI	ml/g	80	26	141	61
Effluent Characteristics					
COD	mg/L	186	66	3421	1507
BOD_5	mg/L	8	5	74	44
TSS	mg/L	18	8	87	43
Chloroform	µg/L	2.0	1.2	N.A.	
NH_3-N in last cell	mg/L	1.6	1.7	8.6	17.5
PO_4^{3-}-P in last cell	mg/L	0.4	0.2	5.4	5.5

[9] Average added nutrient = Mass of nutrient added in one day divided by the flow.
[10] BOD_5 is fixed to 100 mg and the nutrient is expressed accordingly.
[11] SOUR 1: SOUR in the first cell of the reactor
[12] SOUR 2: SOUR in the last cell of the reactor

The third group of variables describes the sludge. The SOUR, a measure of the respiration activity, averaged 8.8 mg O_2/g/hour in the first cell and 8.6 mg O_2/g/hour in the last cell. However, the SOUR difference between cells is greater than what is suggested by the above averages since the SOUR 1:SOUR 2 ratio averaged 1.1 (Table 4.1). The SVI averaged 80 mL/g during the time of the study (Table 4.1) which is well below the bulking level of 150-200 mL/g often reported for systems experiencing bulking (Jenkins et al. 1993). In fact, bulking was not observed in Reactor 1 during the course of the study even if filaments of Type 021N, 0914 and *Thiothrix* were observed (data not shown).

The fourth group of variables described the treatment system effluent. The averaged BOD_5 and TSS were respectively 8 and 18 mg/L. The chloroform concentration average 2 µg/L. All these data were well under Ontario limits for 1996-97.

4.1.2 Reactor 2

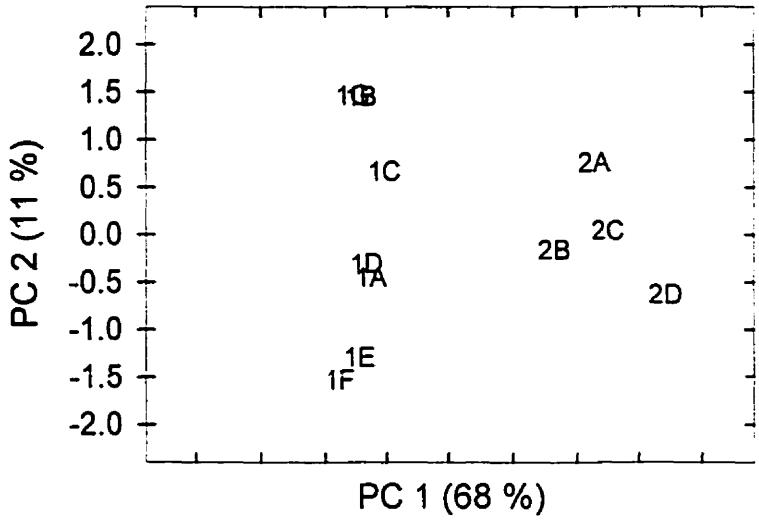
Reactor 2 is a 5 m^3 pilot reactor treating a high COD charged pulp mill effluent which was pretreated anaerobically. The reactor is divided in two cells of equal volume. During the course of the study, it was operated in plug-flow mode, meaning that all the influent entered the first cell. Twenty-seven variables were used to monitor the reactor (Table 4.1).

The influent of Reactor 2 differed from the influent of Reactor 1, having a much higher COD concentration, and an unusually high COD:BOD_5 ratio (Table 4.1). The alcohol concentration was much lower than in Reactor 1 influent, but the VFA concentration was higher. A strong sulfurous odour was also detected. These characteristics were probably due to the anaerobic pretreatment of the wastewater.

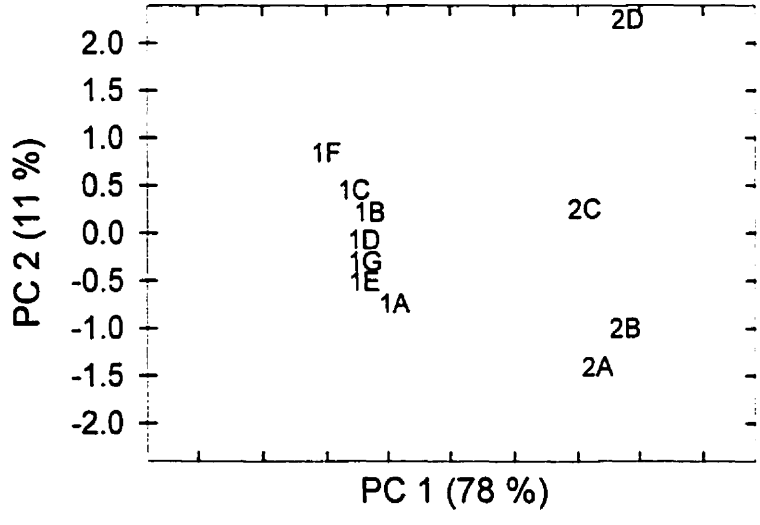
The operation of Reactor 2 also differed from the operation of Reactor 1. The operating pH of 8.7, the average sludge age of 45 days and the average hydraulic retention time (HRT) of 37 hours were all unusually high for such a conventional activated sludge system (Table 4.1).

Figure 4.1. Standardized scores biplot of PCA for (a) influent characteristics and reactor operation, (b) phylogenetic community structure, and (c) phenotypic fingerprint. Symbols designate the reactor (1: Reactor 1; 2: Reactor 2) and the dates (A, B, C, D: February; E, F, G: August). The variability accounted for by the PC is indicated in brackets.

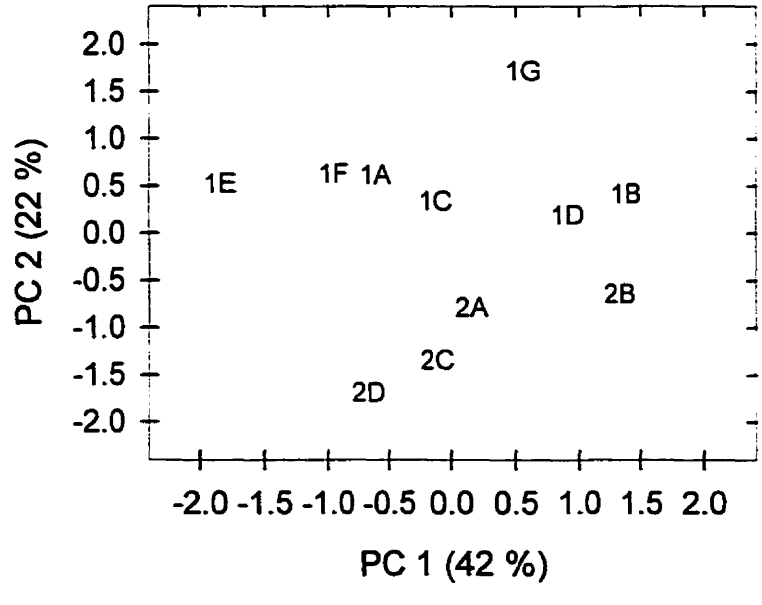
a) Influent Characteristics and Reactor Operation



b) Phylogenetic Community Stucture



c) Phenotypic Fingerprints



The sludge characteristics showed a SOUR in the first cell much higher than in Reactor 1. This higher SOUR was probably due to the presence of hydrogen sulfide in the influent. The SVI was also higher even if filaments were not observed in this reactor during the course of the study. The reason was not identified.

The characteristics of the effluent showed slightly worse performance of Reactor 2 as compared to Reactor 1. The BOD_5 and the TSS were 74 mg/L and 87 mg/L, respectively. The average ammonia and phosphate residual concentrations were also higher in Reactor 2 effluent (Table 4.1).

For most of the variables used to describe them, Reactor 1 and 2 are very different (Table 4.1). The differences in influent and operation can be quickly visualized in the Fig. 4.1a. The dates were included in the analysis only if phenotypic fingerprinting was performed. The first principal component clearly differentiated the two reactors.

4.2 Phylogenetic community structure

4.2.1 Reactor 1

The phylogenetic community structure was determined using five probes (ALF16: Alpha Proteobacteria, BET23: Beta Proteobacteria, GAM23: Gamma Proteobacteria, CFC16: *Cytophaga-Flavobacterium* Cluster, GPB16: Gram Positive Bacteria) and followed for 13 months (Fig. 4.2). The averages probe response during that period showed that probes ALF16 and BET23 targeted the most abundant fractions of rRNA in the bacterial community (Fig. 4.3). In absolute terms, the response of these two probes also varied the most (Fig. 4.3). Probe GAM23 targeted a population of intermediate rRNA abundance, whereas probes CFC16 and GPB16 targeted to populations of low rRNA abundance.

Probe responses varied independently from each other, except for three statistically significant correlations (Table 4.2). Negative correlations were obtained between probe GAM23 and probes ALF16 and BET23, suggesting competition between the population targeted by probe GAM 23 and the populations targeted by probes ALF16 and BET23. A positive correlation was observed between probes CFC16 and GPB16.

Table 4.2. Matrix of correlations among the probe responses for Reactor 1 (above diagonal) and Reactor 2 (below diagonal).

Probes	ALF16	BET23	GAM23	CFC16	GPB16
ALF16	–	-0.05	-0.46 *[1]	0.05	0.26
BET23	0.21	–	-0.32 *	0.04	0.05
GAM23	-0.10	-0.30	–	-0.14	-0.23
CFC16	0.47 *	0.07	0.30	–	0.31 *
GPB16	-0.17	-0.20	0.22	0.01	–

[1] *: Indicates significant correlations ($p < 0.05$).

Figure 4.2. Time profile over 13 months of the phylogenetic community structure of Reactor 1 as determined by membrane hybridization with probes ALF16 (▲), BET23 (▼), GAM23 (◆), CFC16 (●) and GPB16(■). Ticks are set at a scale of one week.

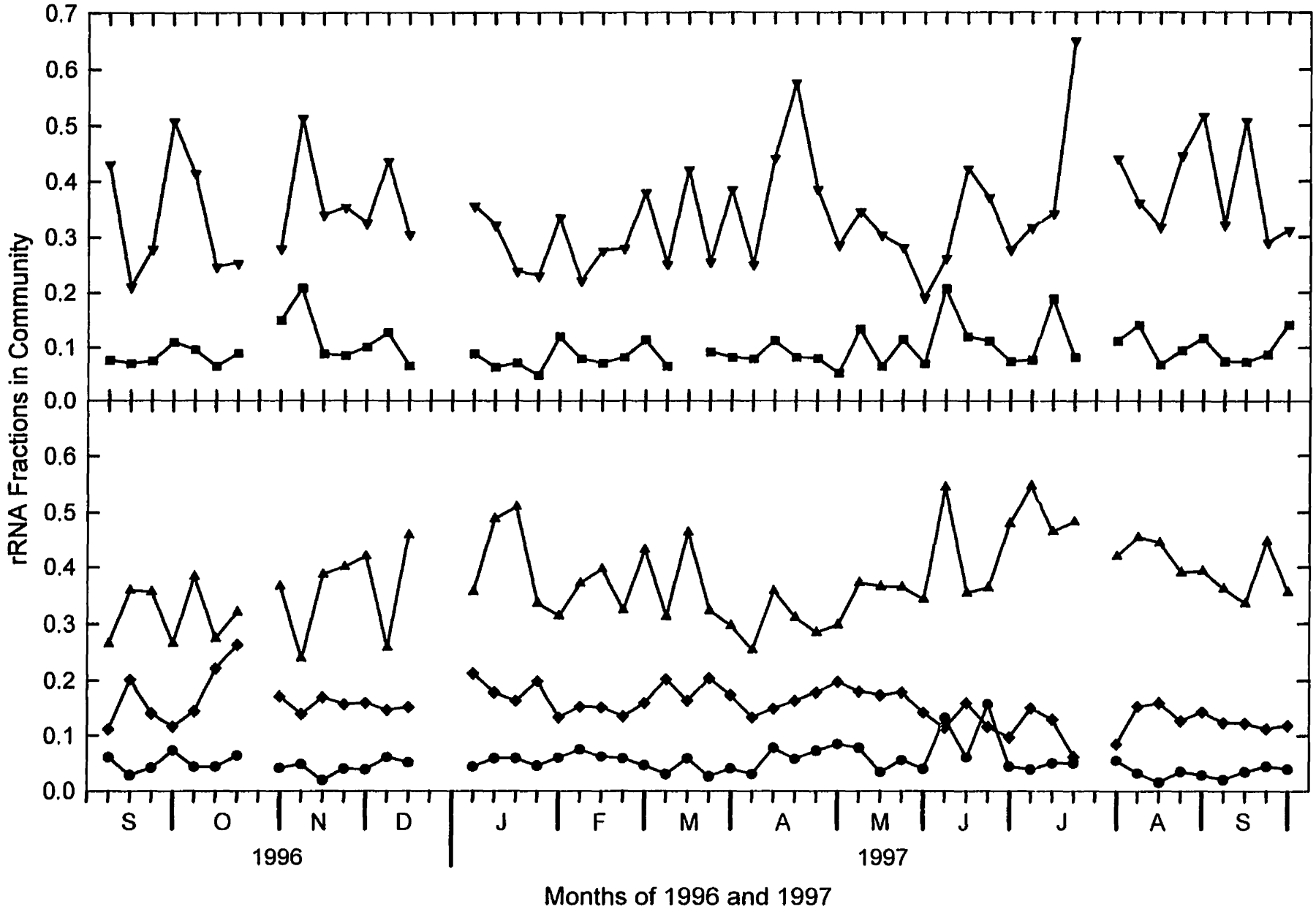
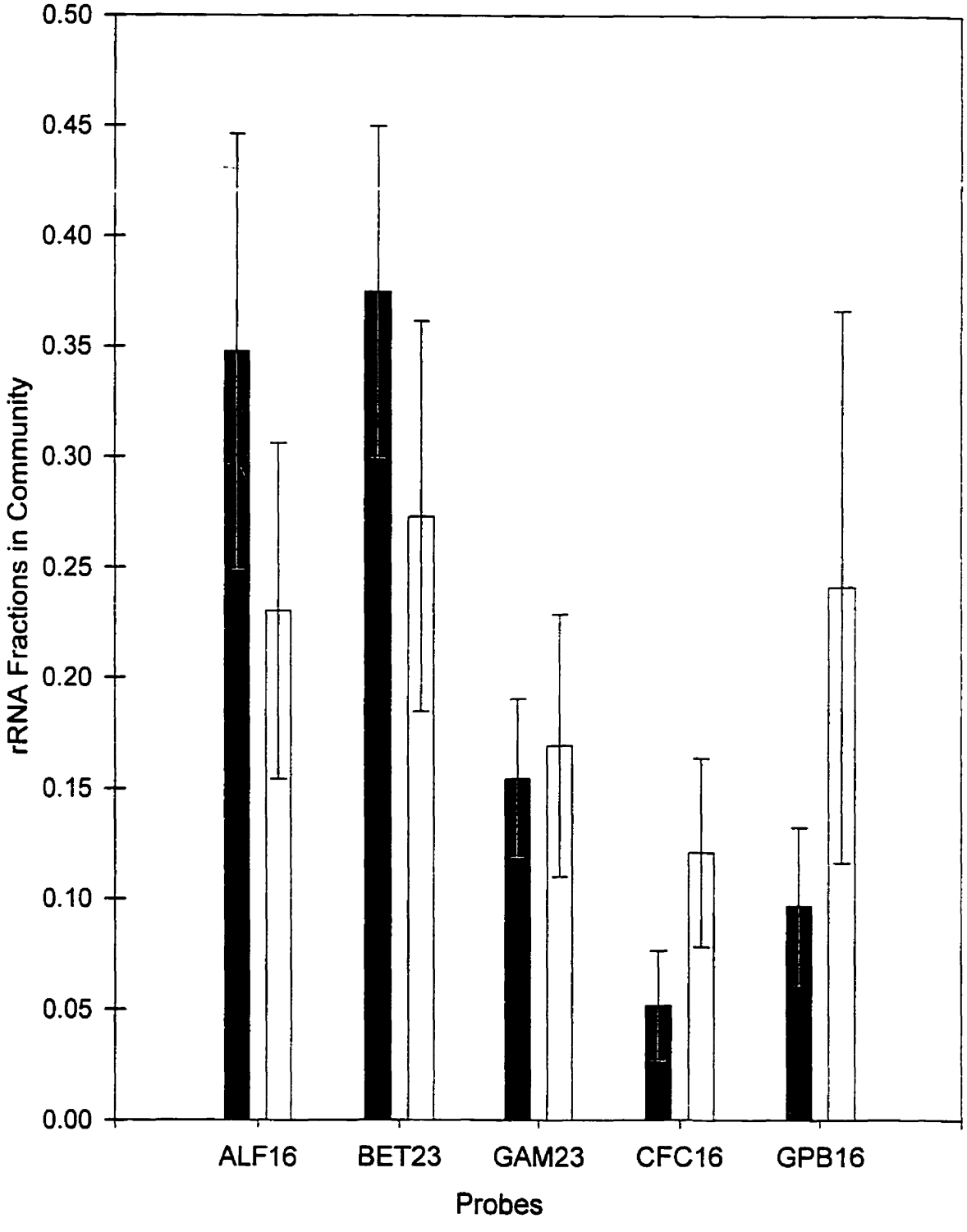

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
rRNA Fractions in Community
S
O
N
D
J
F
M
A
M
J
J
A
S
1996
1997
Months of 1996 and 1997

Figure 4.3. Average phylogenetic community structure as determined by membrane hybridization for Reactor 1 () and Reactor 2 (). Error bars indicate the temporal standard deviation (Reactor 1 n = 52; Reactor 2 n = 20).


0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
rRNA Fractions in Community
ALF16
BET23
GAM23
CFC16
GPB16
Probes

4.2.2 Reactor 2

In Reactor 2, the phylogenetic community structure was followed for 5 months (Fig. 4.4). The community structure in this reactor was much different from Reactor 1 (Fig. 4.3). The proportion of rRNA targeted by probes ALF16 and BET23 was lower in the community of Reactor 2, while the populations targeted by probes GAM23, CFC16 and GPB16 were more abundant. The response of probe GPB16 varied the most. These differences in phylogenetic bacterial community structure between Reactors 1 and 2 (Fig 4.3) are also apparent in the coordinate plot of the principal component analysis (Fig. 4.1b). The correlation analysis between the probe responses showed only one significant correlation (Table 4.2). The correlation coefficient between the response of probes ALF16 and CFC16 was 0.47 suggesting a mutualistic interaction.

4.2.3 Presence of Gram positive bacteria with high GC content

To investigate further the phylogenetic affiliation of the rRNA hybridizing to probe GPB16, the first community RNA sample of each month was hybridized with probe HGC23 (Fig. 4.5). Only a weak hybridization signal, probably due to non-specific binding, was obtained when probe HGC23 was used to hybridize RNA extracted from activated sludge from the two reactors. It was possible to detect 10^{10} cells of *Arthrobacter* sp., a species within the Gram positive high GC cluster, amended to about 60 mg of activated sludge before extraction (Fig. 4.5). Assuming a concentration of 10^{10} bacterial cells/mg of activated sludge, this indicates a detection limit of approximately 1%.

Figure 4.4. Time profile over 5 months of the phylogenetic community structure of Reactor 2 as determined by membrane hybridization with probes ALF16 (▲), BET23 (▼), GAM23 (◆), CFC16 (●) and GPB16(■). Ticks are set at a scale of one week.

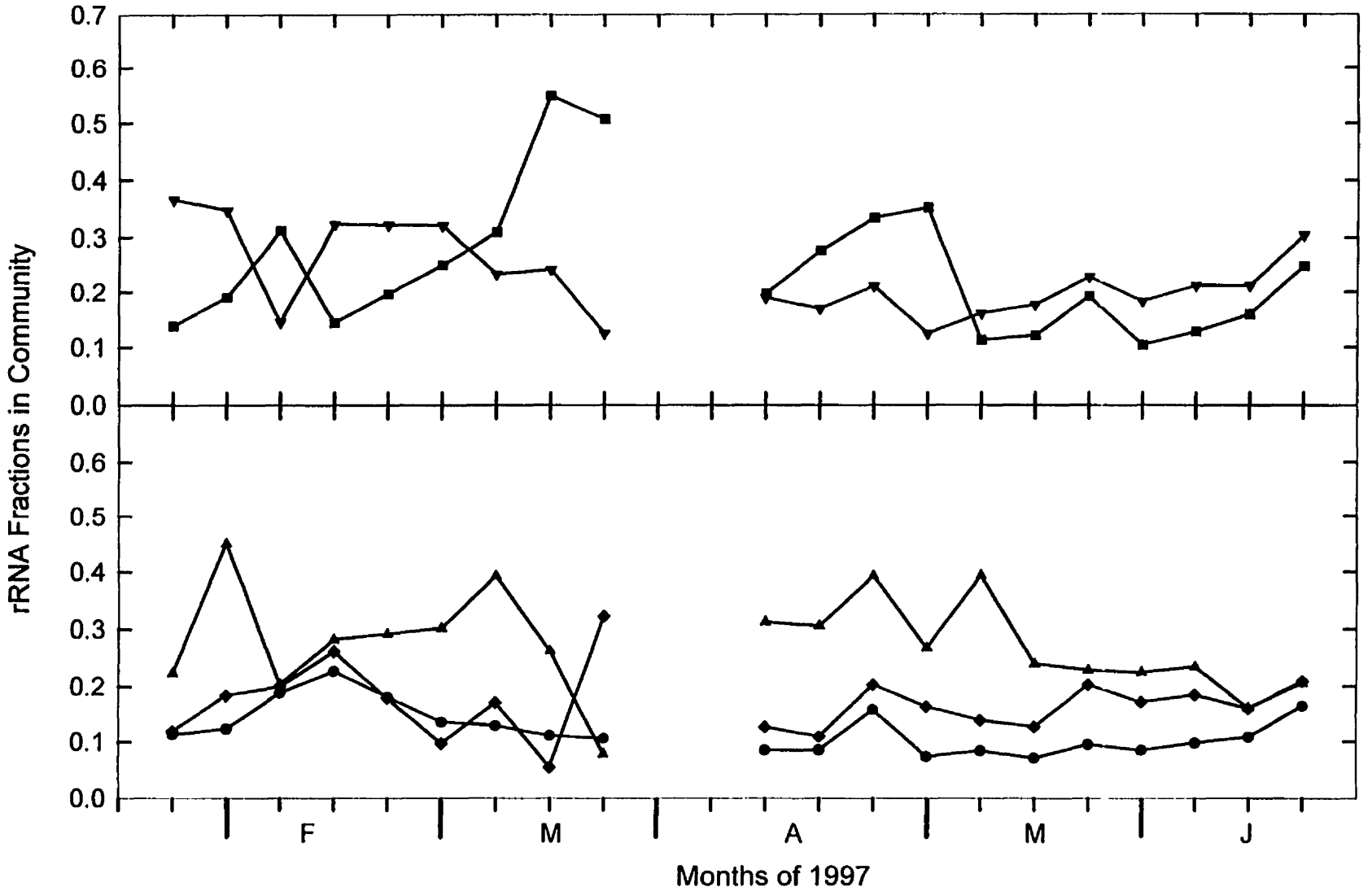
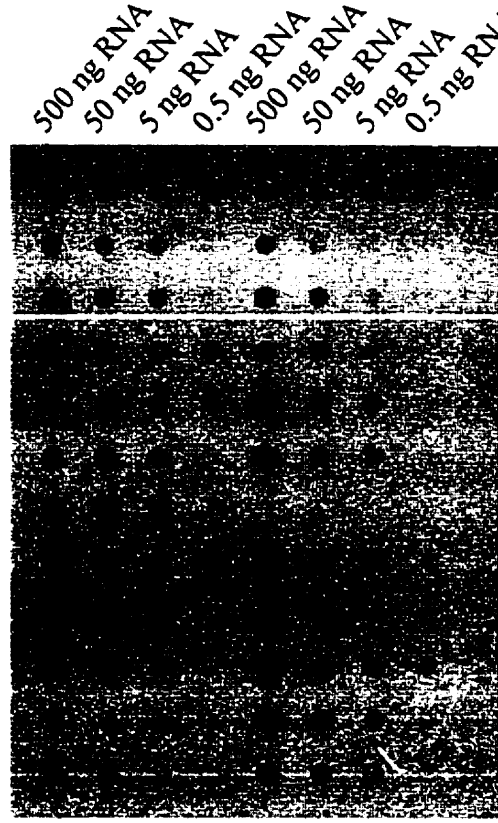

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
rRNA Fractions in Community
F
M
A
M
J
Months of 1997

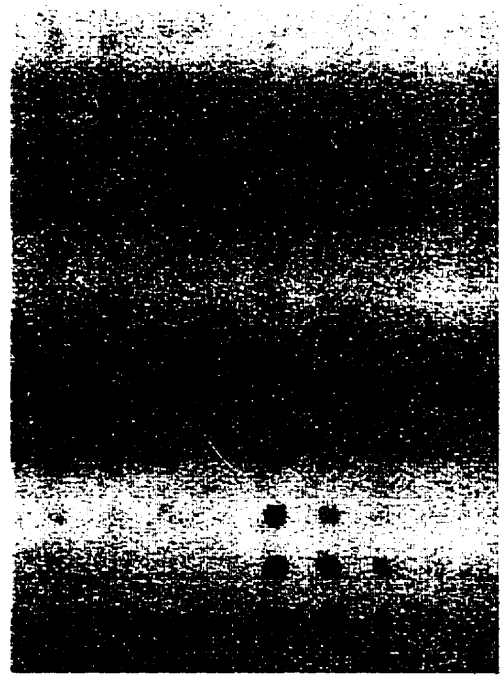
Figure 4.5. Autoradiogram of blot of total community RNA from Reactor 1 and Reactor 2 hybridized with (a) probe EUB23 and (b) HGC23. *Arthrobacter sp.* cells were added to activated sludge sample of October 1996 from Reactor 1 before RNA extraction (cells + sludge).

a) Probe EUB23



Reactor 1	Reactor 2
September 1996	January 1997
October 1996	February 1997
November 1996	March 1997
December 1996	April 1997
January 1997	June 1997
February 1997	July 1997
March 1997	*Arthrobacter sp.*
April 1997	Pure culture
May 1997	10^{9} cells + sludge
June 1997	10^{10} cells + sludge
July 1997	10^{11} cells + sludge
August 1997	10^{12} cells + sludge

b) Probe HGC23



Reactor 1	Reactor 2
September 1996	January 1997
October 1996	February 1997
November 1996	March 1997
December 1996	April 1997
January 1997	June 1997
February 1997	July 1997
March 1997	*Arthrobacter sp.*
April 1997	Pure culture
May 1997	10^{9} cells + sludge
June 1997	10^{10} cells + sludge
July 1997	10^{11} cells + sludge
August 1997	10^{12} cells + sludge

4.3 Community phenotypic fingerprinting

The two reactors were compared with respect to their phenotypic potential using the Biolog GN plate. Phenotypic fingerprints of Reactor 1 were taken seven times, four times during the cold season (February 1997) and three times during the warm season (August 1997). Reactor 2 was analyzed four times in February 1997. The phenotypic fingerprints of the two reactors are shown in Fig. 4.6.

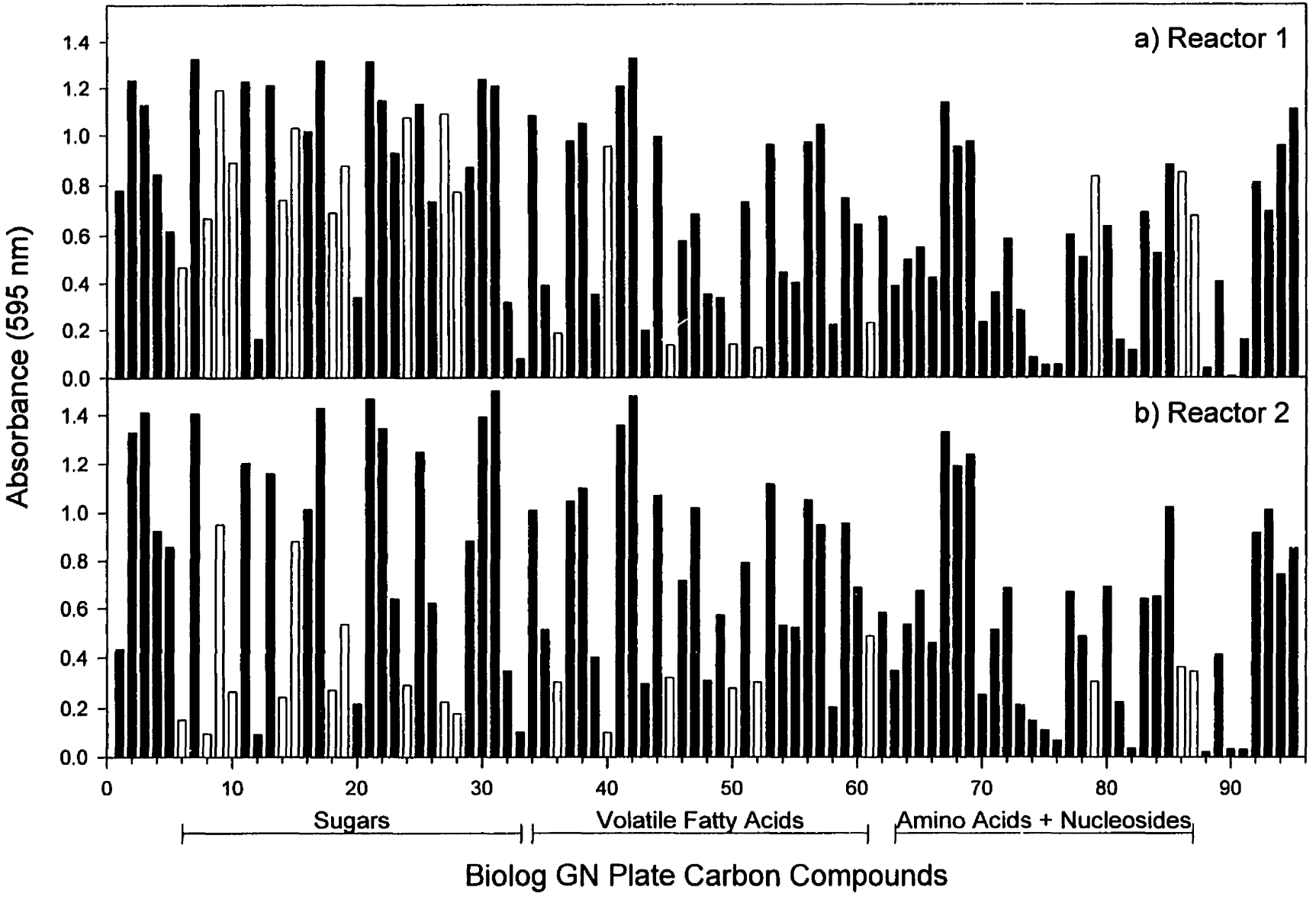
To assess the differences between the two reactors, a principal component analysis was performed on the profiles. Fig. 4.1c shows the biplot of the first two principal components of this analysis. Whereas the first principal component (PC 1) did not contrast the two reactors, the second principal component (PC 2) provided the highest scores for the samples from Reactor 1. However, the distance between the two reactor samples even with respect to PC 2 is small.

The carbon compounds related to the difference observed in the PC 2 of the principal component analysis of the phenotypic fingerprints (Fig. 4.1c) were identified by correlating the PC 2 scores and the compound responses. It was found that the community of Reactor 1 was relatively more active toward certain sugars, whereas the community of Reactor 2 was relatively more active toward VFA (Table 4.3). However, the difference in activity was greater for the sugars (Fig. 4.6) compared to the difference in activity toward the VFA.

Table 4.3. Phenotypic differences between the Reactors 1 and 2 bacterial communities as the carbon compound response correlated with PC 2 (Fig. 4.1c).

Carbon Compounds	Community Greater Activity	
	Reactor 1	Reactor 2
Sugars		
N-acetyl-D-glucosamine	+	
adonitol	+	
L-arabinose	+	
D-arabitol	+	
L-fucose	+	
D-galactose	+	
m-inositol	+	
α-D-lactose	+	
D-melibiose	+	
D-raffinose	+	
L-rhamnose	+	
Volatile fatty acids		
acetic acid		+
D-galactonic acid lactone	+	
α-hydroxybutyric acid		+
α-ketobutyric acid		+
α-ketovaleric acid		+
succinamic acid		+
Amino acids and Nucleosides		
D-serine	+	
uridine	+	
thymidine	+	

Figure 4.6. Average phenotypic fingerprint for the community of Reactor 1 (n=9) and the community of Reactor 2 (n=4). The numbers identifying the carbon compounds (x-axis) are the same as in Table 3.2. White bars indicate the carbon compounds correlated with PC 2 (Fig 4.1c; Table 4.3).


a) Reactor 1
b) Reactor 2
Absorbance (595 nm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
10
20
30
40
50
60
70
80
90
Sugars
Volatile Fatty Acids
Amino Acids + Nucleosides
Biolog GN Plate Carbon Compounds

4.4 Evaluation of phylogenetic community structure data

The information provided by the phylogenetic community structure as determined by membrane hybridization was evaluated using three criteria: (i) its precision, (ii) its level of determination by the reactor conditions, that is whether the probe responses vary randomly over time or they are related to changes in reactor, and (iii) the information it provided on the sludge and effluent characteristics. The first criterion was analyzed by comparing the average coefficient of variation among replicates and the average coefficient of variation in time The second and third criteria were analyzed by building regression models of the phylogenetic community structures on the reactor conditions and behavior, using the PLS method.

4.4.1 Precision of the membrane hybridization assay

An important factor to consider in the development of a new monitoring tool is its precision. To evaluate the precision of membrane hybridization among replicates, five activated sludge samples were taken at the same time and their RNA was extracted in parallel. The nucleic acid samples were then probed independently. The coefficient of variation (CV) was determined for each probe. The average CV and their standard deviation among the probes are reported in Table 4.4. This procedure was performed twice during the course of this one year study.

Similarly, to quantify the temporal variability, the average CV was computed using the weekly phylogenetic community structures. To compute this CV, five equidistant temporal points were considered around the time at which the replication studies were performed. More than one scale was used to select the time points of the temporal data points (Table 4.4) since scale can affect the variability observed (Dutilleul 1998).

Table 4.4. Variability of phylogenetic membrane hybridization at various scales using five probes.

Scale of Variability[1]	First Half of the Year (10/23/96)			Second Half of the Year (3/12/97)		
	Average CV [2]	CV Standard Error	Theoretical R^2	Average CV [2]	CV Standard Error	Theoretical R^2
Replicas	14.2	2.1	N.A.[3]	19.7	5.7	N.A.
Every day	N.D.[3]	N.D.	N.D.	29.7	7.0	0.56
Every 7 days	25.3	3.4	0.69	24.0	3.2	0.33
Every 14 days	24.8	5.2	0.67	21.0	7.6	0.12
Every 28 days	21.6	2.3	0.57	24.0	5.7	0.33

[1] One measurement was taken five times at the scale indicated. The range of the smaller scale falls always at the middle of the range of the larger scale.
[2] Average coefficient of variation for probes ALF16, BET23, GAM23, CFC16 and GPB16.
[3] N.A.: Not applicable, N.D.: Not determined

The average CV for the replication study ranged between 14 and 20% (Table 4.4). On the other hand, the temporal average CV was found to vary between 21 and 30%. The temporal variability observed did not seem to be affected by the scale used, except at the daily scale for which variability appeared slightly higher than the others (Table 4.4).

The following ratio:

$$\frac{\text{Temporal Variability - Among Replicates Variability}}{\text{Temporal Variability}}$$

is defined as the theoretical R^2. It could be associated with the signal-to-noise ratio. In this study, the theoretical R^2 was found to be between 0.3 and 0.7.

4.4.2 Regression models of Reactor 1

Regression modeling had three objectives. It was used to determine (i) if the phylogenetic community structure varied randomly, (ii) the most important reactor parameters influencing the community structure, (iii) if the community structure could be related to reactor performance.

4.4.2.1 Phylogenetic community structure

The modeling of the variation of phylogenetic community structure was performed in three steps. First, all the variables were submitted to autocorrelation analysis. Second, cross-correlation analysis was carried out between the variables describing reactor conditions (influent characteristics and reactor operation) and the probe responses phylogenetically describing the bacterial populations. Third, the probe responses were regressed on the reactor condition variables using the PLS method.

Before submitting the data to regression analysis, the variables were analyzed for autocorrelation. Only four variables (Temperature, Average added PO_4^{3-}-P, COD > 1000 MW and COD < 500 MW) were found to be autocorrelated, but the maximum autocorrelation coefficient was 0.65. All the other variables were not found to be autocorrelated (data not shown).

When the PLS regression was applied directly to the variables without further analysis, the cross-validation criterion (minimum in PRESS) used to determine the number of latent variables (LV) to use in the model indicated that no LV should be included in the model (i.e., no model was obtained). This first regression attempt may have failed because possible delayed effects of the reactor conditions on the bacterial community structure were not considered. To detect them, cross-correlations between reactor conditions variables and probe responses were performed. Significant cross-correlations at various time delays (lag[2]) were observed between bacterial population and reactor data (data not shown). However, in

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[2]The lag period is defined as being the time elapsed between the collection of two successive samples. In our case, a sample was taken every seven days. Therefore, the first lag equals seven days (i.e., sampling time unit).

the case of bulking, a bacterial community can take up to three sludge ages (4.9 lags) to reintroduce steady state (Palm et al. 1980). On that basis, cross-correlations at lags greater that 5 were not considered. With this procedure, significant cross-correlations at lags 0, 2, and 3 were detected (Table 4.5a).

When the results from the cross-correlation analysis were considered, the cross-validation criterion indicated that four LV should be included in the model (Table 4.5). The average R^2 of the model was then 0.53. Interestingly, the R^2 is higher for probes ALF16, BET23 and GAM23 and lower for the two other probes (Table 4.5b). Ordering the data by importance of variables (i.e., VIP criterion) showed that the model was mainly dependent on lagged variables, most of them by two or three sampling time unit (Table 4.5b). Among the first ten variables, only one was simultaneous (lag 0) and among those with a VIP greater than one, only 28% of them were simultaneous.

The most important variables in the PLS model were: high molecular weigth COD (COD > 1000 MW), particulate COD, average added phosphate, sludge age and alcohol concentration. Among the variables ranked relatively low were temperature and the average added ammonia. The pH and VFA concentration are totally absent from the final model

4.4.2.2 Sludge and effluent characteristics

To evaluate the diagnosis potential of the information provided by the phylogenetic community structure, the sludge and effluent characteristics were regressed on the probe responses the reactor condition variables. Regression models for the sludge characteristics are reported in Table 4.6. Those for effluent characteristics are reported in Table 4.7.

Table 4.5a. PLS regression[1] of probe responses on the influent characteristics and operation parameters for Reactor 1: importance of each parameter.

Rank	Model Variables	Lag	VIP[2]
Variables with VIP > 1 correlated with community at lag 2 or 3			
1	COD > 1000 MW	3	1.50
2	Particulate COD	3	1.48
4	Average added PO_4^{3-}-P	2	1.39
5	Sludge Age	3	1.29
6	COD 500-1000 MW	3	1.28
7	Alcohols (BOD_5)	3	1.15
8	Absorbance (280 nm)	3	1.15
9	Conductivity	2	1.14
10	BOD_5	2	1.13
11	F:M Ratio (BOD_5)	2	1.10
13	Phenols (BOD_5)	3	1.08
15	BOD_5:N	2	1.06
17	Temperature	2	1.02
Variables with VIP > 1 correlated with community at lag 0			
3	Average added PO_4^{3-}-P	0	1.41
12	Dissolved O_2 (Cell 4)	0	1.09
14	Conductivity	0	1.08
16	BOD_5:N	0	1.04
18	Dissolved O_2 (Cell 2)	0	1.01
Variables with VIP < 1 correlated with community at lag 2 or 3			
19	Average added NH_3-N	2	0.99
20	COD < 500 MW	3	0.95
21	BOD_5:P	2	0.94
Variables with VIP < 1 correlated with community at lag 0			
22	Dissolved O_2 (Cell 3)	0	0.92
23	TSS	0	0.91
24	BOD_5:P	0	0.85
25	Dissolved O_2 (Cell 1)	0	0.81

[1] Regression model characteristics
Number of latent variables: 4
Portion of variation extracted from independent variables: 57.5%
Average model R^2: 0.53

[2] VIP: Variable Importance in the Projection

Table 4.5b. PLS regression of probe responses on the influent characteristics and operation parameters for Reactor 1: regression coefficients[1] for each parameter.

Model Variables	ALF16	BET23	GAM23	CFC16	GPB16
Model R^2	0.66	0.53	0.61	0.36	0.48
Variables with VIP > 1 correlated with community at lag 2 or 3					
COD > 1000 MW	11	11	-25	-9	-9
Particulate COD	-21	-11	20	11	11
Average added PO_4^{3-}-P	-9	-13	17	-5	-11
Sludge Age	10	0	-5	-15	-20
COD 500-1000 MW	-7	6	-6	9	13
Alcohols (BOD_5)	-14	-8	13	4	3
Absorbance (280 nm)	-13	-4	5	7	8
Conductivity	-6	-5	3	-2	-4
BOD_5	-8	-5	3	0	-2
F:M Ratio (BOD_5)	-4	-4	1	-3	-5
Phenols (BOD_5)	-3	6	-10	4	6
BOD_5:N	-8	-2	0	4	4
Temperature	11	12	-20	0	3
Variables with VIP > 1 correlated with community at lag 0					
Average added PO_4^{3-}-P	-11	-13	22	-5	-10
Dissolved O_2 (Cell 4)	-4	-9	12	0	-3
Conductivity	1	-2	2	-8	-11
BOD_5:N	-10	-5	10	2	1
Dissolved O_2 (Cell 2)	-2	-7	7	-1	-4
Variables with VIP < 1 correlated with community at lag 2 or 3					
Average added NH_3-N	4	-4	2	-10	-15
COD < 500 MW	-6	-8	13	-4	-8
BOD_5:P	3	6	-14	0	2
Variables with VIP < 1 correlated with community at lag 0					
Dissolved O_2 (Cell 3)	-1	-5	5	-1	-3
TSS	6	2	-7	-9	-12
BOD_5:P	-5	2	0	3	4
Dissolved O_2 (Cell 1)	4	-2	-1	-3	-4

[1] Coefficients are multiplied by 100.

The regression models describing the SOUR and the SVI had an R^2 of 0.4 and 0.7, respectively. Among all the explanatory variables included in these regressions, probes ALF16 and GAM23 ranked among the first ten with respect to their VIP (Table 4.6) indicating a relationship between community structure and sludge characteristics. For SOUR, this relationship is understandable since different bacteria consume or are inhibited by different compounds which influence their respiration rate (e.g., Goodall and Peretti 1998; Brown et al. 1990; Volskay and Grady 1990).

Table 4.6. Importance of variables in PLS regression models describing sludge characteristics based on influent chracteristics, reactor operation and probe responses in Reactor 1.

PLS Regressions			Influent and Operation Variables[1]			Probes		
LV[2]	Variability Extracted[3]	R^2	Rank	Variables	VIP[4]	Rank	Variables	VIP
SOUR 1, SOUR 2 and SOUR 1/SOUR 2								
2	24.9%	0.42	1	Recirculation Time	2.7	7	ALF16	1.4
			2	F:M Ratio (BOD_5)	1.8	8	GAM23	1.4
			3	Dissolved O_2 (Cell 4)	1.7	15	CFC16	1.1
			4	Alcohols (BOD_5)	1.6	18	BET23	1.0
			5	BOD_5	1.6	30	GPB16	0.6
SVI and Sludge Level in Clarifier								
3	31.3%	0.71	1	Recirculation Time	1.8	4	GAM23	1.6
			2	F:M Ratio (BOD_5)	1.7	6	ALF16	1.5
			3	Chlorination	1.6	21	BET23	1.0
			5	Particulate COD	1.6	27	GPB16	0.8
			7	BOD_5	1.5	29	CFC16	0.6

[1] Variables with the five highest VIP of this category are reported.
[2] LV: Number of Latent Variables used.
[3] Percentage of variability extracted from the independent variables by the LV.
[4] VIP: Variable Importance in the Projection.

The chlorination, recirculation time, and probe GAM23 had positive regression coefficients relating them to SVI (data not shown). As shown in Fig. 4.7, the SVI curve followed up to a certain degree ($r = 0.41$) the response of probe GAM23 probably due to the pressure on filamentous bacteria of within the Gamma Proteobacteria lineage. However, such a causal interpretation should not be applied to chlorination and recirculation time. Local SVI peaks prompted chlorination (Fig. 4.7). Similarly, because of reduced thickening, an increase in SVI increased recirculation time. Therefore, the last two variables do not influence SVI, but they are rather influenced by it (see the conceptual model in Chapter 5).

The R^2 of the models describing the effluent characteristics ranged from 0.5 to 0.9 (Table 4.7). Contrary to the regression models describing the sludge characteristics, no probe response was among the ten most important variables in these models (Table 4.7). The lower ranking of the probe responses was also generally true for the sludge characteristics indicating that neither the sludge characteristics nor phylogenetic community structure provided with relevant information about the effluent. This is probably an effect of the near steady state of the reactor and of the control of SVI by chlorination which prevents an increase in TSS in the effluent.

Figure 4.7. Variation in Reactor 1 of probe GAM23 response (◆), SVI (○) and chlorination (▭) during 13 months in 1996 and 1997.

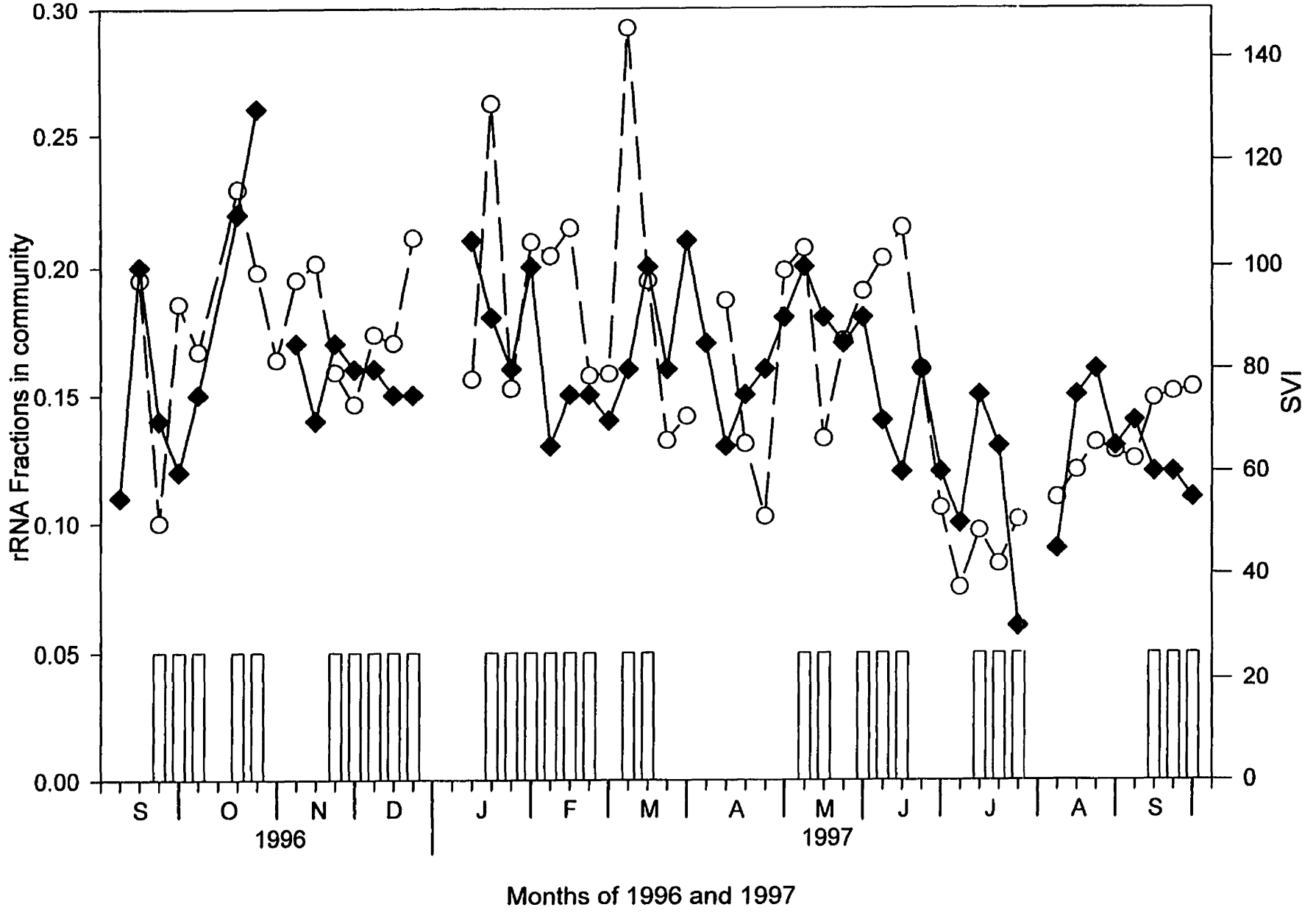

0.30
0.25
0.20
0.15
0.10
0.05
0.00
rRNA Fractions in community
140
120
100
80
60
40
20
0
SVI
S
O
N
D
J
F
M
A
M
J
J
A
S
1996
1997
Months of 1996 and 1997

Table 4.7. Importance of variables in PLS regression models describing effluent characteristics based on influent chracteristics, reactor operation, probe responses and sludge characteristics in Reactor 1.

PLS Regression			Influent and Operation Variables[1]			Probes			Sludge Characteristics		
LV[2]	Variability Extracted[3]	R^2	Rank	Variables	VIP[4]	Rank	Variables	VIP	Rank	Variables	VIP
BOD and TSS											
3	31.1 %	0.62	1	COD < 500 MW	2.0	12	BET23	1.2	19	SVI	0.9
			2	BOD_5	1.8	17	GPB16	1.0	23	SOUR 1	0.9
			3	COD	1.7	27	ALF16	0.7	26	SOUR 2	0.8
			4	Absorbance (280 nm)	1.6	29	CFC16	0.7	38	SOUR 1/SOUR 2	0.5
			5	Conductivity	1.5	34	GAM23	0.6			
COD											
2	26.0 %	0.86	1	COD	2.3	15	BET23	0.9	13	SVI	1.0
			2	BOD_5	2.1	22	GAM23	0.7	19	SOUR 2	0.9
			3	Absorbance (280 nm)	2.0	23	ALF16	0.7	21	SOUR 1	0.8
			4	Phenols (BOD_5)	2.0	25	GPB16	0.6	30	SOUR 1/SOUR 2	0.4
			5	Conductivity	1.8	37	CFC16	0.2			
Chloroform											
2	23.9 %	0.50	1	Temperature	2.3	13	GAM23	1.4	4	SVI	1.8
			2	COD 500-1000 MW	2.2	22	GPB16	1.1	20	SOUR 1	1.1
			3	VFA (BOD_5)	1.9	23	BET23	1.0	28	SOUR 2	0.8
			5	Phenol (BOD_5)	1.7	25	ALF16	1.0	30	SOUR 1/SOUR 2	0.6
			6	BOD_5	1.7	39	CFC16	0.1			

[1] Variables with the five highest VIP of this category are reported.
[2] LV: Number of Latent Variables used.
[3] Percentage of variability extracted from the independent variables by the LV.
[4] VIP: Variable Importance in the Projection.

4.4.3 Regression model of Reactor 2 phylogenetic community structure

Fourteen variables describing the conditions of Reactor 2 were investigated for their influence on the phylogenetic bacterial community structure. A cross-correlation analysis was performed and variables were found to be correlated at either lag 0 or lag 1. The probe responses were regressed on these variables using the PLS method.

The model explaining the phylogenetic community structure of Reactor 2 had an R^2 of 0.46 (Table 4.8). Probes GPB16 and ALF16 had the highest level of determination with an R^2 of 0.61 and 0.58, respectively. On the other hand, the performance of the model in explaining the response of probe BET23 is poor with an R^2 of 0.27.

According to the model, the VFA concentration, the phosphate concentration in the last cell and the sludge age are the most important explanatory variables. Their time curve corresponded with the response of probe GPB16 (Fig. 4.8), which is the probe response with the highest level of determination.

Table 4.8. PLS regression[1] of probe responses on the influent characteristics and operation parameters for Reactor 2.

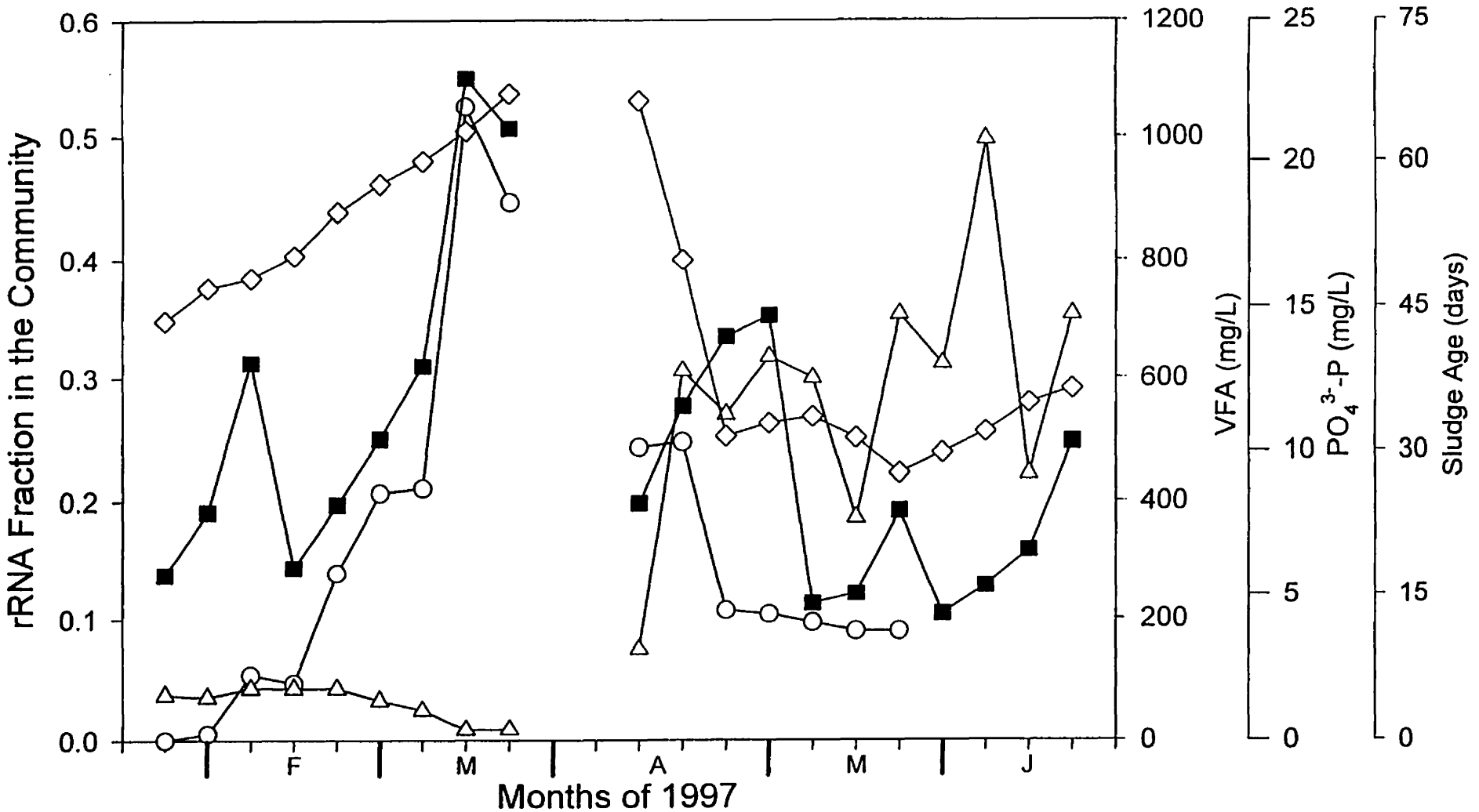
Rank	Model Variables	Lag	VIP[2]	Regression Coefficients[3]				
				ALF16	BET23	GAM23	CFC16	GPB16
	Model R^2			0.58	0.27	0.44	0.41	0.61
1	VFA	0	1.27	-1	-3	-21	-3	26
2	Alcohols	0	1.08	-3	-3	-18	-4	21
3	PO_4^{3-}-P (last cell)	0	1.04	-16	1	4	-13	-10
4	Sludge Age	1	1.04	14	-2	-9	11	16
5	COD	0	0.95	-10	-2	-11	-10	11
6	Dissolved O_2 (first cell)	0	0.86	11	1	8	10	-7
7	F:M ratio (CODr)	1	0.84	12	1	6	10	-4

[1] Regression model characteristics
Number of latent variables: 2
Portion of variation extracted from independent variables: 81.0%
Average model R^2: 0.46

[2] VIP: Variable Importance in the Projection

[3] Regression Coefficients were multiplied by 100

Figure 4.8. Variation in Reactor 2 of probe GPB16 response (■), VFA concentration (○), phosphate concentration the in last cell (△) and sludge age (◇) during 5 months in 1997.


rRNA Fraction in the Community
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1200
1000
800
600
400
200
0
VFA (mg/L)
25
20
15
10
5
0
PO_4^{3-}-P (mg/L)
75
60
45
30
15
0
Sludge Age (days)
F
M
A
M
J
Months of 1997

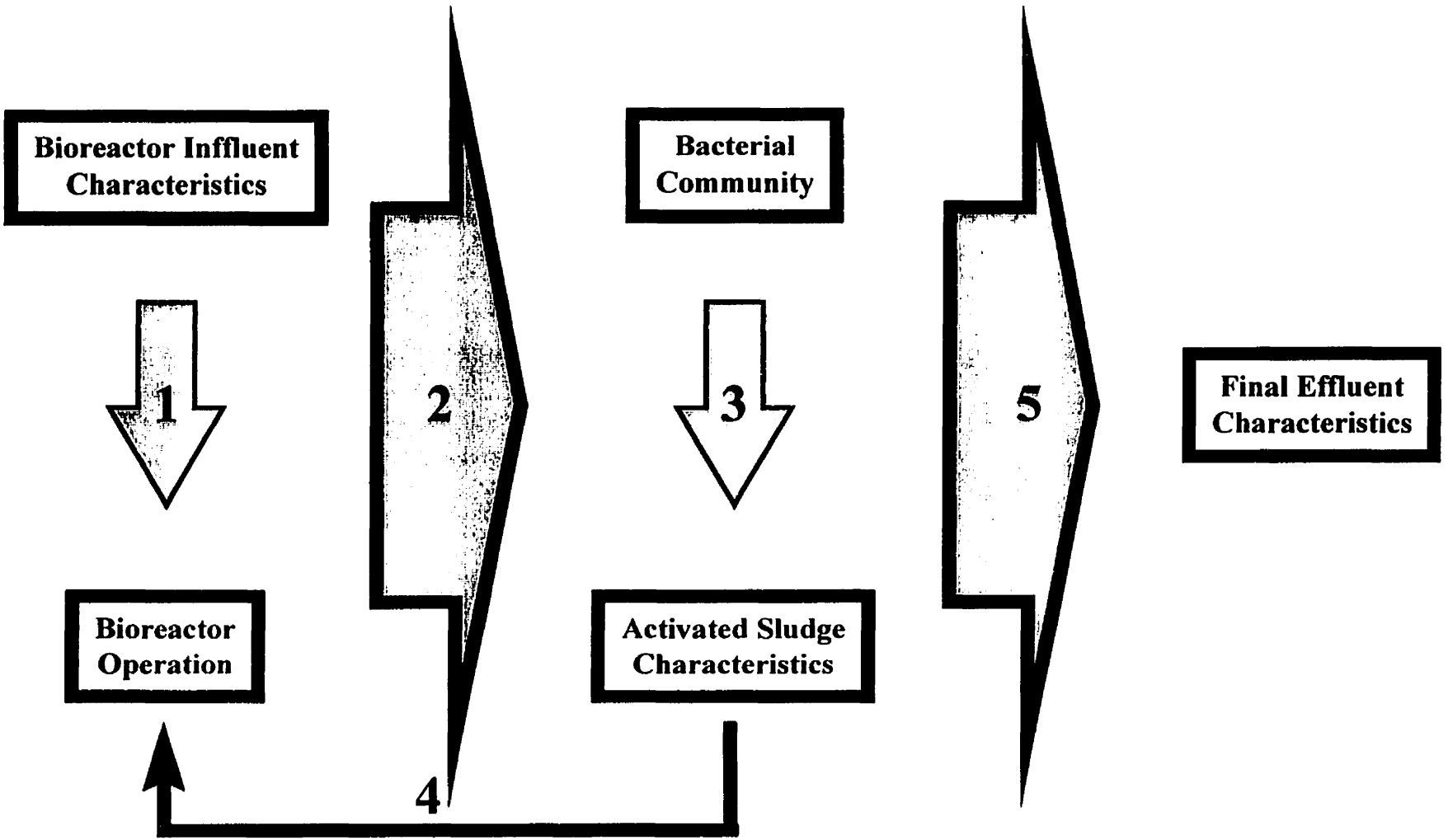
Chapter 5. Discussion

5.1 Conceptual model of wastewater treatment systems

To investigate the inter-influence of reactor variables and the phylogenetic community structure, empirical regression models were built. The analysis was based on a theoretical understanding of wastewater treatment systems described in the conceptual model presented in Fig. 5.1. Before discussing the results, this conceptual model will be introduced.

The primary factors impacting on the bioreactor are the influent characteristics. The entire operation of the reactor is dependent on variables such as the BOD_5 loading. For example, the BOD_5 loading will influence the rate of nutrient addition to maintain a constant residual concentration (Fig. 5.1, Arrow 1). Together, the influent characteristics and the reactor operation change the microbial community and the sludge characteristics (Fig 5.1, Arrow 2). For example, methanol would enrich for methylotrophic bacteria and ionic strength would affect the floc stability (Zita and Hermansson 1994). The microbial community itself changes the floc properties (Fig 5.1, Arrow 3). For example, an overgrowth of filamentous bacteria leads to bulking. The sludge characteristics also influence also the operation (Fig 5.1, Arrow 4). For example, during a bulking event, chlorine is often added to kill the filaments. Because they extend outside the floc, the filamentous bacteria are more affected by chlorine than the other bacteria embedded in the floc (Jenkins et al. 1993). Together (Fig. 5.1, Arrow 5), these parameters determine the characteristics of the effluent at the outlet of the treatment system.

Figure 5.1. Conceptual model of the influences in a biological wastewater treatment system.


Bioreactor Inffluent Characteristics
Bacterial Community
1
2
3
5
Final Effluent Characteristics
Bioreactor Operation
Activated Sludge Characteristics
4

5.2 Phylogenetic community structure

A theory in microbial ecology suggests that microorganisms, like macro organisms, maximize their growth rate (r-strategists) or their energy conservation (K-strategists), but not both (Andrews and Harris 1986). Zavarzin and co-workers (1991) suggested that the Proteobacteria, which have probably evolved in evolutionary pockets, may have taxonomically differentiated according to this biological theory. They proposed that the Gamma Proteobacteria tend to be more r-strategists (fast growers with low substrate affinity), whereas the Alpha and the Beta Proteobacteria tend to be more K-strategists (slow growers with high substrate affinity). It seems that this understanding of the Proteobacteria may explain the phylogenetic community structure observed in Reactors 1 and 2. As discussed above, the low F:M ratios of the two reactors would primarily select for K-strategic microorganisms, or the Alpha and Beta Proteobacteria according to Zavarzin and co-workers (1991) proposal. Dominance of Alpha and Beta Proteobacteria in the two reactors (Fig. 4.3) agrees with the Zavarzin theory. These results are also in agreement with the bacterial community structure reported for a German municipal system with a low F:M ratio of 0.1 kg/kg/day (Wagner et al. 1993).

Bacterial rRNA affiliated to the Gram positive bacteria with high GC content was not detected in the systems studied (Fig. 4.4) even if about 10% of community rRNA in Reactor 1 and 25% in Reactor 2 were affiliated to the Gram positive bacteria. This result contrasts with *in situ* hybridization studies of municipal wastewater treatment systems reporting 10 to 35% of Gram positive bacteria with high GC content (Manz et al. 1994; Wagner et al. 1994b; Kämpfer et al. 1996; Snaidr et al. 1997). On the other hand, two independent cloning studies of municipal systems did not report any clones belonging to this group (Bond et al. 1995; Snaidr et al. 1997), even if they had been observed by *in situ* hybridization (Snaidr et al. 1997). The authors commented that their nucleic acid extraction techniques could have had a low yield for Gram positive bacteria with a high GC (Snaidr et al. 1997). This possibility seems unlikely in our study for two reasons. First, the addition of *Arthrobacter* sp. cells to activated sludge before extraction made it possible to detect a signal from probe HGC23 (Fig. 4.4). Second, similar RNA extraction techniques were utilized by another laboratory

to succesfully extract RNA from bacteria belonging to the Gram positive high GC lineage in activated sludge (De Los Reyes et al. 1997). Therefore, the relative abundance of Gram positive bacteria with a high GC was probably low. However, to further substantiate this hypothesis, a study by *in situ* hybridization would be necessary.

5.3 Evaluation of the phylogenetic community structure

5.3.1 Precision of the membrane hybridization assay

As mentioned, the first important parameter to evaluate in developing a new tool is its precision. Two definitions can be used for precision. First, the precision is defined by the variability of a measurement among replicates and represents the reliability of the technique. Second, the precision is defined in relation to the amplitude of the phenomenon to measure. It delimits the minimum amplitude of the phenomenon that would be detected by the technique. The signal-to-noise ratio is used to investigate this relationship.

In our study, the average CV among replicates was found to range between 14 and 20% (Table 4.4). For environmental samples, this is usually considered as acceptable. Wallner and co-workers (1995) reported an average CV of 20% when working with the same probes by *in situ* hybridization on activated sludge samples. Similar variability was also reported with membrane hybridization (Raskin et al. 1996). Thus, the technical precision encountered in this study corresponded well with other studies. The origin of this variability among replicates was not specifically investigated in this study. It could have been sample heterogeneity or analytical variability. However, since 60 mg (at least 10,000 flocs) of activated sludge were used to extract rRNA and since a cell of the reactor studied is considered completely mixed, it seems unlikely that sample heterogeneity was the major source of variability with respect to replication.

The precision of the membrane hybridization assay compares favorably with that reported by other laboratories. However, this precision has to be evaluated in terms of the expected signal. The theoretical R^2 between 0.3 and 0.7 (Table 4.4) showed a relatively low signal-to-noise ratio (R^2 ranges from 0 to 1). These figures could be seen as lower boundaries since Reactor 1 did not experience any major disturbances at the time of sampling

for the replication studies (10/23/96 and 3/12/97), which minimize the temporal variability of the microbial community structure. However, since activated sludge reactors are usually maintained at near steady state and the temporal variability of the microbial community structure would be expected to be low, the low theoretical R^2 observed suggests that, in order to be useful, the precision of the community structure evaluation has to be improved. A possible improvement would be increasing the sampling effort. This would require molecular techniques demanding fewer manipulations since they are the limiting factor of the sampling effort (Raskin et al. 1997). The use of flow cytometry with *in situ* hybridization (Wallner et al. 1995) or the development of multiple probe microchips (Guschin et al. 1997) represent steps in this direction.

5.3.2 Validity of the phylogenetic community structure information

The average level of determination (R^2) obtained by the model describing the microbial community of Reactor 1 was 0.53. This R^2 may seem low, but it is common in modeling of environmental data (e.g., Payette et al. 1996). It is also in the range predicted by the variability analysis (see section 4.4.1). Thus, the community structure as determined by membrane hybridization is not randomly distributed, but is determined by the reactor conditions. It is therefore a source of information on the bioreactor.

From an operational point of view, the quality of the effluent and the performance of the bioreactors are important parameters. It is therefore very important to evaluate the phylogenetic community structure data in these terms. In a diagnosis strategy, the community structure should provide valuable information to maintain optimal bioreactors performance. In the conceptual model (Fig. 5.1), the performance of the system is associated with the quality of the effluent and the sludge characteristics since their deterioration will lead to degradation of the effluent quality. Although it was not possible to detect any relationship with the effluent characteristics (Table 4.7), the model describing the sludge characteristics suggests they were determined in part by the phylogenetic community structure since two probe responses ranked among the ten most important variables (Table 4.6). Therefore, foreseeing the evolution of the bacterial community would help predict the

evolution of sludge characteristics even for common variables such as SOUR and SVI. These two lines of evidence demonstrate that the phylogenetic community structure is valuable in a bioreactor monitoring strategy.

5.3.3 Phylogenetic depth required

In developing molecular tools to diagnose wastewater treatment systems, it is important to determine the phylogenetic depth to which the probes should be targeted. This has technical and economical implications. A technique targeted at too shallow a level (e.g., phylum) may be compromised in resolution, whereas a technique targeted at too deep a level (species) would be prohibitively expensive due to the large number of probes required. The work presented here does not claim to resolve this question, but it gives certain indications.

Interestingly, the highest R^2 values were obtained for the Alpha, Beta and Gamma Proteobacteria (Table 4.5b). They are the three most abundant populations in the community of Reactor 1 (Fig. 4.3). This suggests that the coarse description of these populations at the phylum level is able to detect the major events occurring in the community. This may contrast with the high phylogenetic diversity observed within the Beta 2 Proteobacteria in activated sludge floc by *in situ* hybridization (Amann et al. 1996). Such diversity implies a high enough variability within the Beta Proteobacteria to render the changes at the phylum level random. However, the diversity within the Beta 2 Proteobacteria was visualized on bacteria sharing various combinations of three probe target sites presupposing their close phylogenetic relatedness. Since closely related organisms may share similar metabolisms (Stackebrandt 1991), it is probable that these genotypically diverse bacteria could have been phenotypically similar and would therefore react similarly to the surrounding environment. Therefore, a description of the bacterial community at the species or the subspecies level may not be necessary to develop an effective molecular tool to monitor it.

The SVI model is a good example of diagnostic information provided by the phylogenetic community structure. SVI was positively influenced by the Gamma Proteobacteria population. Identification of the filaments in Reactor 1 during 1996 and 1997 revealed the presence of *Thiothrix* sp. and Type 021N which are members of the Gamma

Proteobacteria lineage (Wagner et al. 1994a). This explains the relationship between the response of probe GAM23 and the SVI (Fig. 4.7). Although the relationship could have been stronger with probes targeted directly to the rRNA of the filamentous bacteria, this result indicates that hybridization at the species level is not absolutely necessary to obtain relevant information on the community since the probe used here are targeted at the phylum level.

Although the results suggest that probes targeted to a very deep phylogenetic level are not necessary to develop an effective monitoring tool, the probes used in this study do not allow the straightforward interpretation which would be possible at deeper levels. A compromise strategy would be to identify significant phylogenetic levels for monitoring. For example, the filaments Type 021N and *Thiothrix* sp. are phylogenetically closely-related (Wagner et al. 1994a). A single probe detecting them should be enough for proper diagnosis. In addition, a nested approach in which probes are targeted to different levels might be envisioned (Wagner et al. 1996).

5.3.4 Foreseeing operational problems

The striking observation about the model of the microbial community of Reactor 1 is the higher importance of lagged variables (lag 2 or 3) over simultaneous variables (lag 0). How can this be interpreted? The classical interpretation of the phylogenetic membrane hybridization is that the relative abundance in rRNA of a given population is a function of the density of this population and its cellular rRNA content (related to its metabolism). Therefore, the phylogenetic membrane hybridization measures the relative metabolic contribution of a population to the community (Stahl et al. 1988).

Many environmental conditions can affect the cell rRNA content such as its specific growth rate (Herbert 1961), the growth temperature (Veldkamp 1976) and nutrient concentration (Minkevich et al. 1988). These factors change the rRNA content very rapidly, in the order of a few hours. At the scale of our sampling (i.e., every week), changes occurring over a few hours would be perceived simultaneously (lag 0) in the data. Therefore, simultaneous variables are probably factors affecting the rRNA content of a cell.

On the other hand, changes in population densities should occur over a much longer time. For a reactor with a sludge age of 11 days, these changes should occur in at least one sludge age (1.6 lag). Therefore, variables lagged by two or three sampling time unit are probably related to changes in the bacterial density of bacterial populations. This interpretation could also explain why no important variables were found to be lagged one sampling time unit.

Based on the previous interpretations, the model suggests that, at the scale of sampling and the average sludge age of Reactor 1, the phylogenetic membrane hybridization mainly detected changes in population densities. This conclusion slightly contrasts with the classical interpretation. However, the cellular rRNA content in the natural environment is probably more constant than in pure culture. On a cell mass basis, the rRNA content observed in pure culture can increase by 10 to 50 fold from resting to exponentially growing cells (Herbert 1961). However, a recent study of a resin acid degrader showed that the change in cellular rRNA upon exposure to resin acids was five times lower if the organism was in activated sludge compared to pure culture (Muttray and Mohn 1998a). In another study, a culture of *Nitrosomonas europaea* completely inhibited for nitrification by Nitrapyrin maintained its rRNA content for at least a few days after the addition of the inhibitor (Wagner et al. 1996). These observations substantiate the validity of the conclusion that phylogenetic membrane hybridization could be more sensitive to changes in bacterial density than changes in rRNA content.

This conclusion is critical for understanding the capabilities of the phylogenetic membrane hybridization in a monitoring and diagnosis strategy. Since most conventional activated sludge bioreactors are operated at sludge ages of between five to ten days (Jenkins et al. 1993), and since a sampling scale smaller than a few days might not be possible economically, the study of Reactor 1 was probably close to the real monitoring. Therefore, generalizing the results obtained, it may be that phylogenetic membrane hybridization cannot be predictive. The ability to monitor a variety of populations is certainly an advantage over the current methods. However, the suggestion of Wagner and co-workers (1993) that molecular tools could foresee operational problems does not seem to apply to the technique used in this study.

To foresee operational problems, a technique should foresee changes in community structure. Therefore, because the density of a population is the balance between its net specific growth rate and the sludge age of the reactor at steady state, the molecular tool should inform on the metabolic status of the organisms present in the reactor. Since *in situ* hybridization can measure independently the cellular rRNA and the bacterial density, this technique seems more promising if foreseeing operational problems is desired.

5.4 Major factors influencing the phylogenetic community structure

5.4.1 Reactor 1

The model describing Reactor 1 provided information on the relative importance of the reactor conditions at influencing the phylogenetic community structure. Mechanisms mediating the effects of the most influential variables are discussed here. The variable with the highest VIP was high molecular weight COD (COD > 1000 MW). This is surprising since this fraction is usually not consumed (Jokela et al. 1993; Bullock et al. 1996). However, it could make unavailable to the biomass degradable compounds. In pulp and paper effluent, the COD higher than 1000 MW is mainly composed of aggregates of lower molecular weight compounds (Jokela and Salkinoja-Salonen 1992). Aggregating degradable compounds could somehow hide them from bacterial consumption. Since the aggregation ability is a function of the chemical structure of the compound, it is not constant from compound to compound and it can drive the selection of certain populations.

The variable with the second highest VIP was Particulate COD. A positive regression coefficient related this variable and the Gamma Proteobacteria. This relationship could describe seeding of the reactor since Gamma Proteobacteria like *Klebsiella* sp. were found to be abundant in the wastewater of pulp and paper mills (Liss and Allen 1992). To further substantiate this hypothesis, the particulate matter in the reactor influent should be hybridized. The results from the model would predict the predominance of Gamma Proteobacteria.

Sludge age was the variable with the fourth highest VIP. The presence of sludge age among the most influential variables is not surprising. Certain treatment systems such as nitrifying bioreactors require a strict range of sludge age to operate (Jenkins et al. 1993). The growth rate also affects the competition between organisms. For example, the acetate uptake rate of *Zoogloea ramigera* and filament Type 021N is a function of their growth rate. This change in uptake rate was one of the factors affecting the competition between these two organisms in a chemostat (van Niekerk et al. 1988).

The average added phosphate was the only variable simultaneously affecting the phylogenetic community structure that ranked in the ten most influential variables. This suggests that (i) the biomass was limited by the phosphate addition and (ii) the phosphate concentration directly affect the cellular rRNA content of the microorganisms. Phosphorus limitation has been shown to occur in pulp and paper wastewater treatment systems with residual concentration as high as 4 mg/L in the effluent (Richard and Cummins 1997). In Reactor 1, the average residual of 0.4 mg/L and the average BOD:P ratio of 0.7 (Table 4.1) were consistent with phosphorus limitation.

When nitrogen is the limiting nutrient in dual-substrate limited conditions, an increase in nitrogen concentration causes an increase in cellular rRNA content (Minkevich et al. 1988). Recently, a similar relationship was found for a strain of *Acinetobacter johnsonii* in carbon-phosphorus dual-substrate limited conditions (Frank et al. 1998). Because all the filtered (0.45 μm) BOD_5 (data not shown) and 96% of the total BOD_5 was consumed in Reactor 1, the biomass is in dual substrate limitation which could explain the model. In such conditions, change in phosphate concentration would change the cellular rRNA content of the bacteria.

Temperature ranked seventeenth in the model with respect to VIP. This contrasts with observations made in other pulp and paper wastewater treatment systems. Difference in the abundance of psychrophiles (growth at 10°C) between the warm and the cold seasons was reported for a lagoon treating bleached kraft mill effluent (Liss and Allen 1992). In the same system, PCR amplification revealed the presence of the genes *dhlB* (coding for haloalkanoic acid dehalogenase) and *mmoX* (coding for soluble methane monooxygenase)

during the warm season, but not during the cold season (Fortin et al. 1998). These observations suggest a shift in the bacterial community structure. This shift was not apparent in the data presented here (Fig. 4.2), which is consistent with the regression model. Changes may have occurred at phylogenetic depth deeper than the phylum explaining the lack of their detection. Alternatively, the changes in temperature of Reactor 1 between the cold and the warm seasons may have not been enough to prompt a drastic change in phylogenetic community structure. The lagoon system previously mentioned experienced a temperature of 25°C during the warm season and 13°C during the cold season. The average temperature of the cold season is at the low end of the mesophylic spectrum (Prescott et al. 1996). On the other hand, the temperature observed for Reactor 1 (35°C during the warm season, 25°C during the cold season) were well in the range tolerated by mesophiles. Therefore, drastic changes in the phylogenetic community structure might not have occurred between the warm and the cold seasons.

5.4.2 Similarities between the two models describing community structure

The regression models describing the phylogenetic community structure of Reactors 1 and 2 showed similarities in the important variables chosen to build the models. First similarity, the sludge age is ranked fifth in the model of Reactor 1 and fourth in the model of Reactor 2. These two independent results with sludge age suggest that it plays a major role in the selection of the populations in the bacterial community. This is probably due to the influence of the growth rate of bacteria on their competitive ability (van Niekerk et al. 1988). It is also consistent with the importance of the sludge age in the occurrence of filamentous bulking and foaming (Jenkins et al. 1993).

Second similarity, the concentration of certain specific compounds seems important in the two models. The alcohol concentration ranked seventh in the model of Reactor 1 (Table 4.5a) and the VFA concentration ranked first in the model of Reactor 2 (Table 4.8). In Reactor 2, it seems that the VFA concentration was responsible for the high increase in relative abundance of Gram positive bacteria in February and March (Fig. 4.8) suggesting that they might be the primary consumers in this system at least at high concentration. The importance of easily degradable compounds in determining the phylogenetic community structure would have to be considered in designing a monitoring and diagnosis strategy.

Third similarity, the phosphate concentration ranked third in the two models. This suggests that the two reactors were phosphate limited at least for part of the study. If the supply of phosphate is sufficient, its concentration would not have influenced the bacterial community. Molecular techniques could help in optimizing nutrient addition. For example, the high importance of phosphorus addition in the model explaining the community structure of Reactor 1 remained even if were included in the model only the observations where 1.8 mg-P/L added to the system (data not shown). This suggests that for a proper operation of the system, at least 1.8 mg-P/L should be added.

5.5 Phenotypic fingerprinting and contrast with phylogenetic hybridization

Besides the taxonomic molecular approach used in this study, other methods are being developed to use data information on the microbial communities within treatment systems in a monitoring and diagnosis strategy. One of these techniques is the phenotypic fingerprinting with Biolog microtiter plates. Because the two techniques are developed in parallel for pulp and paper bioreactors, it is important to determine the relative quality of the information provided by phylogenetic hybridization and phenotypic fingerprinting. However, the nature of the information provided by the Biolog assay is still unresolved. While it was argued that phenotypic fingerprinting may be more sensitive to changes in taxonomic structure (Garland et al. 1997), it was found that the technique would miss differences between bacterial communities detected by fatty acid determination (taxonomic analysis; Buyer and Drinkwater 1997; Palojärvi et al. 1997).

Based on the conditions of the reactors, the two bacterial communities should be different (see section 4.1). This difference is obvious when the phylogenetic community structures of the two reactors are compared (Fig. 4.1b). However, the PCA of the phenotypic fingerprints did not show the same difference (Fig. 4.1c). While the PC 1 did not differentiate the communities of the two reactors, the average distance between them in terms of PC 2 was small suggesting they were similar with respect to their heterotrophic potential. The difference between phenotypic fingerprinting and phylogenetic membrane hybridization is consistent with the findings obtained by fatty acid analysis (Buyer and Drinkwater 1997; Palojärvi et al. 1997). It also reinforces the interpretation that Biolog assay is a measure of the community heterotrophic potential.

The interpretation above is also in agreement with the kind of differences found between the two communities. The responses of a total of 20 compounds were significantly correlated with the PC 2 (Table 4.3). The community of Reactor 1 was relatively more active toward certain sugars compared to the community of Reactor 2. In contrast, the community of Reactor 2 was relatively more activity toward certain VFA. These differences in activity are consistent with the anaerobic pretreatment of the wastewater flowing into Reactor 2 and its higher VFA concentration.

The difference in distance between the communities of the two reactors as detected by phylogenetic hybridization and phenotypic fingerprinting suggests that the information obtained by the two techniques is different. Eventually, the two techniques could be complementary in a monitoring and diagnosis strategy. For example, difference in filamentous bacteria could be detected by phylogenetic hybridization whereas difference in activity toward specific compounds such as resin acids could be detected by phenotypic fingerprinting.

Chapter 6. Conclusion

The primary objective of this work was to evaluate the phylogenetic community structure as a source of information to monitor and diagnose wastewater treatment systems. Two lines of evidence demonstrate the validity of the approach. First, it was shown that the response obtained by phylogenetic membrane hybridization was not random. Second, the phylogenetic community structure determined the sludge characteristics. Since deterioration of sludge characteristics such as SVI leads to deterioration of reactor performance, changes in the community structure should predict operational problems.

The results presented in this thesis suggest that the molecular tool does not need to be targeted at a very deep phylogenetic level. A description at the phylum level was enough to obtain a high level of determination for at least some populations in the two reactors, indicating that major events in these populations were observed. Phylogenetic membrane hybridization was also able to detect the impact of the filamentous bacteria that are members of the Gamma Proteobacteria on SVI. However, the phylogenetic level used in this study does not allow the straightforward interpretation which would be possible at deeper levels.

Despite these positive results concerning phylogenetic membrane hybridization, the technique has some weaknesses. One of them is its precision. If the technique is to be employed in monitoring bioreactors, it should be able to detect phenomena with relatively low amplitude, because these reactors are operated in a near steady state. The low signal-to-noise ratio obtained in this study means that the precision should be improved to maximize the accuracy and the rapidity of the interpretation. Since these factors have impact on the rapidity of implementation of corrective measurements, they are very important.

A second weakness of phylogenetic membrane hybridization is its inability to foresee operational problems. Even if the results showed that the technique was able to provide information on the sludge characteristics, they also suggested that the technique tended to detect primarily changes in population densities rather than changes in their metabolisms. Therefore, the technique monitored the bacterial community in real time and would not inform on its future development. A technique able to analyze population densities and metabolism independently might be able to predict the development of the population.

The secondary objective of this work was to characterize the bacterial community in a pulp and paper wastewater treatment system with phylogenetic membrane hybridization and to determine the main process variables affecting it. The results showed the predominance of the Alpha and Beta Proteobacteria in two distinct wastewater treatment systems probably due to their low F:M ratios. The presence of Gram positive bacteria was also observed, but they were not associated with the high GC lineage as for other systems. The rest of the community was composed of bacteria members of the Gamma Proteobacteria and the *Cytophaga-Flavobacterium* lineages.

It was found that the main process variables affecting the bacterial community were high molecular weight COD, particulate COD, phosphorus addition, sludge age and the concentration of specific compounds (Alcohol and VFA). The importance of the first two was surprising and further study should be conducted to fully understand their impact on the bacterial community. The importance of phosphorus addition indicated that the community was limited by this nutrient. The importance of sludge age was expected since it is already known that certain system conditions such as nitrification and bulking occur at specific sludge ages. The importance of the concentration on specific compounds was also expected. It suggests that the approach of the study could be used to gain insight on the degradation of organic compounds *in situ*.

The tertiary objective of this thesis was to characterize the bacterial community with phenotypic fingerprinting and compare this technique with phylogenetic membrane hybridization. The results substantiated the interpretation that phenotypic fingerprinting measures heterotrophic potential. They also suggested that the difference in heterotrophic potential observed between the two bacterial communities were linked to the anaerobic pretreatment of the wastewater flowing in Reactor 2. Because phenotypic fingerprinting measures heterotrophic potential and phylogenetic membrane hybridization measures taxonomic community structure, the two techniques will probably be complementary in a monitoring and diagnosis strategy.

Although phylogenetic membrane hybridization is unlikely to be useful in predicting operational problems, it can generally be concluded that the phylogenetic community structure is a promising source of information to monitor, diagnose and optimize biological wastewater treatment systems.

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Appendix I. Study of Probes Specificity

Introduction

The first step in developing a quantitative membrane hybridization assay is to optimize the hybridization conditions in order to minimize the background hybridization signal from organisms outside the target group. The hybridization conditions were optimized and the specificity of the probes was then evaluated by hybridizing to a collection of 10 organisms. Only the data concerning the specificity of the probes are reported here since the optimization data are similar to those previously published (Manz et al. 1992; Wagner et al. 1993; Fry et al. 1997; Roller et al. 1994; Manz et al. 1996).

Materials and methods

The dissociation curves of the probes were evaluated as proposed by Manz and co-workers (1992). Briefly, the formamide concentration in the hybridization buffer was increased incrementally by 5% from 0 to 50% and the washing buffer was adjusted to the same stringency as the hybridization buffer by varying the monovalent salt concentration. The conditions at which 50% of the probe-target duplex remained intact (temperature of dissociation: T_d) were considered optimum (Stahl and Amann 1991). The hybridization and wash conditions as well as the optimum formamide and monovalent salt concentrations are described in Chapter 3. The specificity of the probes at their optimum hybridization and wash conditions was evaluated by hybridizing each probe to a collection of rRNA samples extracted from ten phylogenetically diverse organisms. The hybridization signal was quantified as described in Chapter 3.

Results

Although the universal probes (UNI16, UNI23) hybridized to all the organisms in the collection, both produced variable signals with different species (Fig I.1). The bacterial probes (EUB16, EUB23) hybridized with constant specific signal to all bacterial species tested and did not react with the non-target eukaryal rRNA (Fig I.1). The specificity of the probes used in this work was relatively high. However, probes GPB16 and ALF16 were found to have 4.2 and 3.6% average non-target signal, which were the highest of all the probes tested (Table I.1).

Table I.1. Level of non-target signal for each probe for quantitative membrane hybridization.

Probe	Target Group	Non-Target Signal[1] (%)	
		Mean	Range
EUB16	Eubacteria	1.4[2]	ND[3]
EUB23	Eubacteria	2.3[2]	ND
ALF16	Alpha Proteobacteria	3.6	1.4 - 5.6
BET23	Beta Proteobacteria	0.4	0.0[4] - 3.0
GAM23	Gamma Proteobacteria	0.9	0.0 - 3.8
CFC16	*Cytophaga-Flavobacterium* cluster	1.9	0.9 - 4.7
GPB16	Gram positive bacteria	4.2	1.2 - 8.8
HGC23	Gram positive bacteria with high GC	1.7	0.5 - 5.6

[1] Percentages expressed in relation to of the target signal.
[2] Determined only for *Trametes versicolor* ATCC 20869.
[3] ND: Not determined.
[4] 0% refers to values < smaller than the 0.5% detection limit.

Discussion

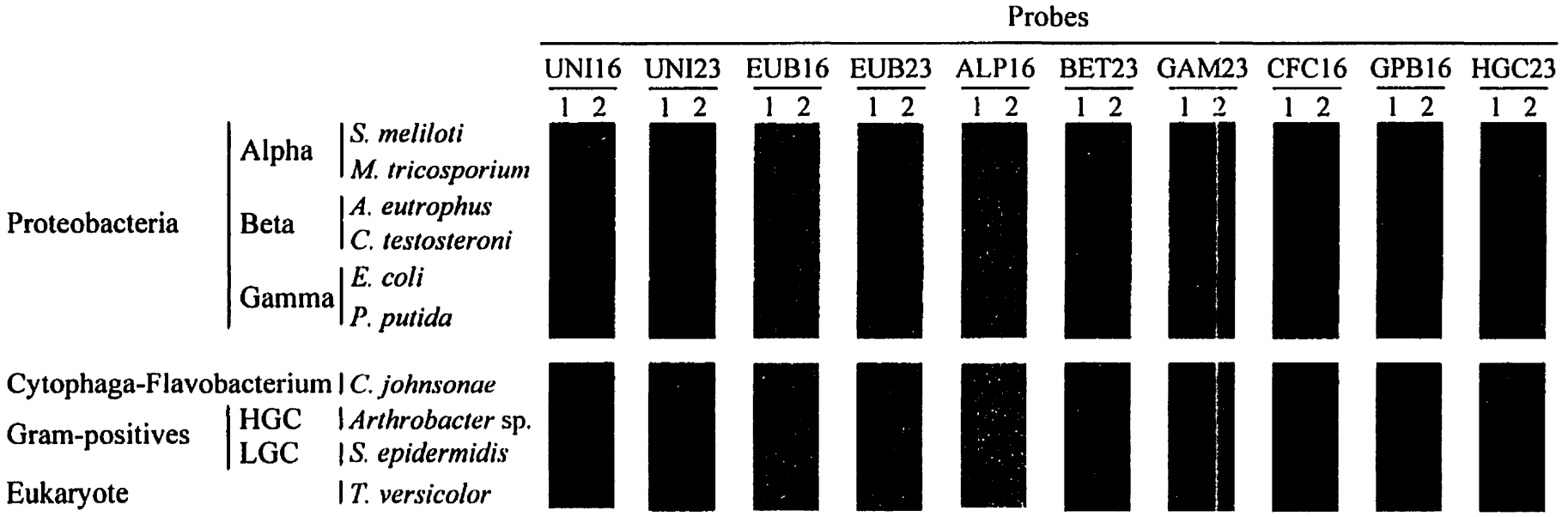
In phylogenetic hybridization, a certain level of non-target hybridization signal is often reported (Manz et al. 1992; Reyes et al. 1997). However, T_d is theoretically the point in a hybridization curve of a probe that maximizes the Target/Non-Target signal ratio (Stahl and Amann 1991). Even if the hybridization conditions used here are similar to the ones originally used with the probes (Manz et al. 1992; Wagner et al. 1993; Fry et al. 1997; Roller et al. 1994; Manz et al. 1996), no data were published concerning their level of cross-reaction with non-target sites, except for probes BET23 and GAM23. These probes have the same target site and differ by only one nucleotide. The strategy used to increase the discriminatory ability between these two probes is to label one of them and use the other as a competitor (Manz et al. 1992). In our work, this succeeded in keeping the non-target signal below 4% of the target signal (Fig I.1 and Table I.1). These data are similar to the ones reported by Manz and co-workers (1992).

Probes GPB16 and ALF16 showed the highest level of non-target signal since they hybridized with all the other rRNA to a detectable level. This could be due to the lack of mismatch between the probes and the non-target organisms' hybridization site. For example, *Comamonas testosteroni*, *Staphylococcus epidermidis* and *Arthrobacter* sp. have a maximum of 2 mismatches within probe ALF16 (Maidak et al. 1994). Of these mismatches at least one is a non-canonical base pair (G:U). In addition, because of their low GC contents and their short lengths, the optimum hybridization conditions correspond to a concentration of formamide of 10% for probe GPB16 and 20% for probe ALF16. These conditions are among the least stringent among the probes tested here. Nevertheless, probes GPB16 and ALF16 detected their target groups unambiguously since the non-target signal was less than 6% and 9% respectively (Fig. I.1).

Conclusions

Most of the time, cross-hybridization signal was observed for each of the probes. However, the average cross-hybridization signal was less than 5% of the target signal. This should not extensively impair the precision of the measurements by quantitative membrane hybridization.

Figure I.1. Autoradiogram of dot blotted control RNA for the probe specificity study. 150 ng (lanes 1) and 15 ng (lanes 2) of total RNA extract were blotted on the membrane and then hybridized with one of the probes. Probes and organisms are described in Table 3.3.


Probes
UNI16
UNI23
EUB16
EUB23
ALP16
BET23
GAM23
CFC16
GPB16
HGC23
1 2
Proteobacteria
Alpha
S. meliloti
M. tricosporium
Beta
A. eutrophus
C. testosteroni
Gamma
E. coli
P. putida
Cytophaga-Flavobacterium
C. johnsonae
Gram-positives
HGC
Arthrobacter sp.
LGC
S. epidermidis
Eukaryote
T. versicolor

Appendix II. RNA Extraction Optimization

Introduction

When nucleic acids are extracted from bacterial cells, the yield is never 100%. However, to determine community structure through quantitative membrane hybridization, one must assume that yield is constant from one bacterial group to the next (Stahl 1995). This assumption is more likely to be true if the yield is maximized. Due to the physico-chemical properties of each sampling environment and due to the microorganisms present, maximization has to be done for each environment (Stahl 1995; Ward et al. 1995; Raskin et al. 1997).

The extraction method affects both the yield and the quality of the extracted nucleic acids (Ward et al. 1995). The literature recommendations concerning the quality of nucleic acids for quantitative membrane hybridization are often confusing. Whereas some researchers do not stress the importance of working with intact rRNA (Wagner et al. 1993; Ward et al. 1995) others argue that deteriorated rRNA (enzymatically degraded or sheared) is completely unsuitable for quantitative membrane hybridization (Raskin et al. 1997). This confusion arises from the lack of information concerning site specific sensitivity to deterioration. There is also limited information on the impact of rRNA quality on the determination of community structure.

We have investigated the effects of four mechanical lysis methods on rRNA quality and the measured community structure. The sensitivity of probe specific hybridization signal to rRNA shearing was also investigated.

Materials and methods

Total community nucleic acid extraction

An aliquot of 10 mL of mixed liquor was centrifuged and the solids resuspended in 1 mL of Trizol (Life Technologies Inc.) as described in Chapter 3. The mixture was subjected to four mechanical lysis methods at various intensities. In the first method, the activated sludge was slurried in a cell homogenizer. In the second method, the sludge was sonicated at an intensity of 350 μm using a Vibracell 250 (Sonic Materials Inc) equipped with a microprobe (model V1A). The samples were sonicated either (i) 3 × 15 sec. or (ii) 6 × 15 sec. with 30 sec. of chilling on ice between pulses. The third method was similar to the second, except that 1 g of 100 μm glass-beads (prepared as described in Chapter 3) was added to the buffer prior to sonication. The fourth method was by bead-beating as describe in Chapter 3. The sludge was bead-beaten for either 2 × 2 min., 2 × 2 min. with Trizol being changed between beatings and pulled after (See Chapter 3), or 3 × 2 min. with Trizol replaced between beating 2 and 3. Following the extraction protocol, the nucleic acids were dissolved in the same volume of buffer and three independent replicate extractions were done. Nucleic acid quality was evaluated by agarose gel electrophoresis as described in Chapter 3. Hybridization conditions, signal quantification and community structure determination were also described in Chapter 3.

Hybridization susceptibility to rRNA shearing

To test the hybridization susceptibility to rRNA shearing, a sample of rRNA was sonicated at various levels, blotted on a nylon membrane, hybridized, and the signal quantitated. The comparative probe hybridization response was used to determine the relative probe susceptibility to shearing.

Specifically, a mixture of pure equimolar 16S and 23S rRNA from *E. coli* MRE600 (Boehringer Mannheim) was sonicated at an intensity of 470 μm for 15 sec., 30 sec., or 120 sec.. A non-sonicated control was also used and all conditions were done in triplicate. The samples were blotted, hybridized, and the signal quantified as described in Chapter 3. The probes used in both experiments are listed in Table II.1.

Table II.1. Probes used in the rRNA extraction and the rRNA shearing experiments.

Probe Name	rRNA Extraction	rRNA Shearing
UNI16		X
UNI23		X
EUB16	X	X
EUB23	X	X
ALF16	X	
BET23	X	
GAM23	X	X
CFC16	X	
EC(alf)16		X
EC(alf)23		X

Statistical analysis

The ANOVA and the Duncan multiple-stage pairwise comparison (Miller 1981) were done using the procedure GLM of SAS/STAT v. 6.12 for Windows (Sas Institute Inc. 1989).

Results

Nucleic acid extraction

Analysis by agarose gel electrophoresis revealed that the various mechanical lysis methods differentially affected yield and quality of extracted nucleic acids (Fig. II.1). High molecular weight DNA was observed only in the bead-beaten extracts (Fig II.1a). The rRNA from the bead-beaten extracts as well as from the cell homogenizer extracts seemed less degraded than the sonicated extracts, since the rRNA appeared to be of higher molecular weight and the 23S/16S rRNA band ratio appeared to be closer to the theoretical 2/1 (Fig.

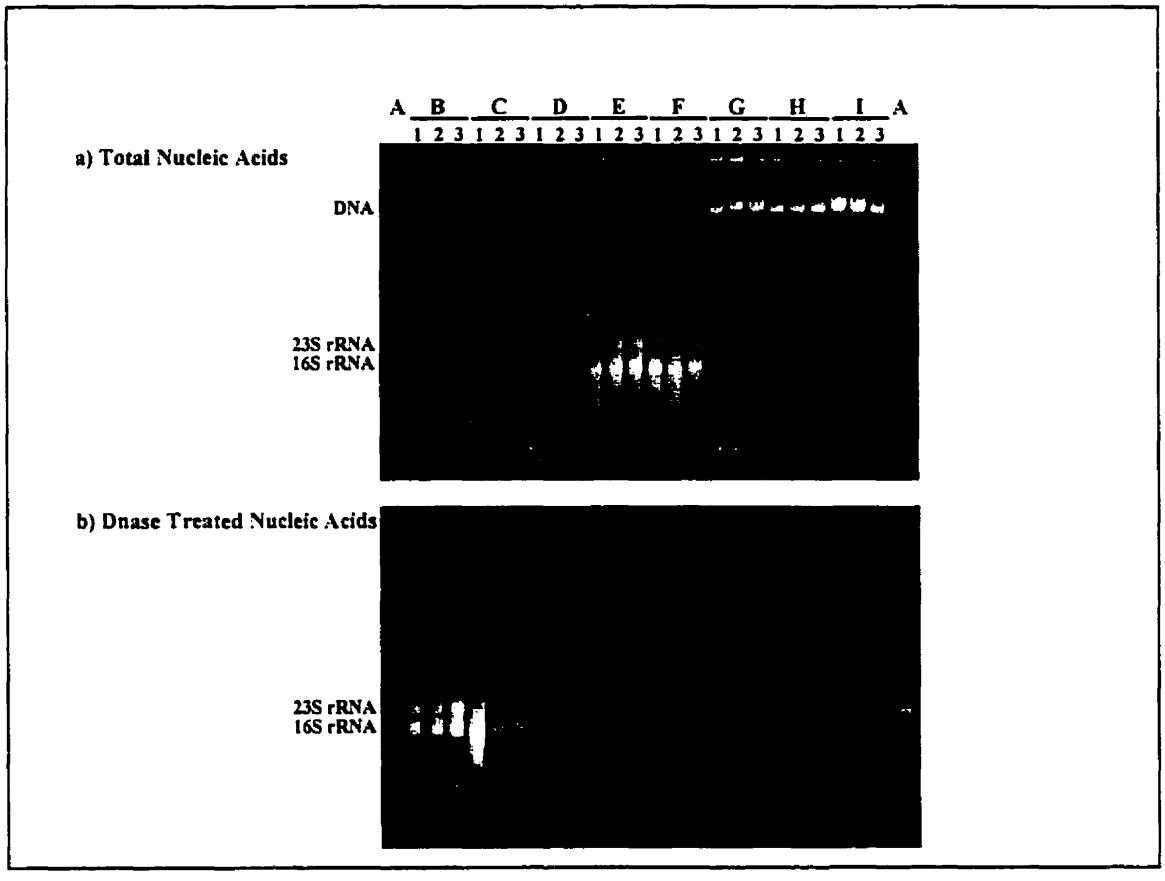
II.1b). Yield was evaluated by visual inspection of the agarose gels (Fig II.1a) and by comparing the total 16S or 23S hybridization signal of the extract as determined with probes EUB16 and EUB23. It was observed that sonication consistently gave the highest yield of RNA; however, this technique also had the most variable yields (Table II.2).

Table II.2. Total 16S and 23S hybridization signal extracted from activated sludge with trizol in conjunction with four mechanical lysis methods at various intensities.

Mechanical Lysis Method	Total 16S Signal[1]	Total 23S Signal[1]
Slurrying in Cell Homogenizer	1.0 ± 0.0[2]	1.0 ± 0.0
Bead Beating		
4 min.	1.6 ± 0.5	1.8 ± 0.5
4 min. with Trizol Replaced	1.6 ± 0.4	1.9 ± 0.3
6 min. with Trizol Replaced	2.3 ± 0.4	1.9 ± 0.4
Sonication		
45 sec.	14.0 ± 6.4	5.6 ± 3.1
90 sec.	6.5 ± 2.6	3.6 ± 0.4
45 sec. with Glass Beads	11.0 ± 0.4	9.5 ± 0.3
90 sec. with Glass Beads	13.1 ± 2.3	11.8 ± 2.6

[1] Total Signals are expressed relative to the signal obtained by slurrying in the cell homogenizer.
[2] ± standard error (n=3).

Figure II.1 Electrophoresis agarose gel of total community nucleic acids (a) and RNA (b) extracted from activated sludge with four mechanical lysis methods at various intensities. Gel (a) was loaded with equal volume and Gel (b) was loaded with equal rRNA mass. Lanes A: rRNA controls; Lanes B: Slurried in a cell homogenizer; Lanes C: Sonicated 45 sec.; Lanes D: Sonicated 90 sec.; Lanes E: Sonicated 45 sec. with glass-beads; Lanes F: Sonicated 90 sec. with glass beads; Lanes G: Bead-beaten for 4 min.; Lanes H: Bead-beaten for 4 min. with Trizol replaced; Lanes I: Bead-beaten for 6 min. with Trizol replaced; Lane numbers (1,2,3) represent triplicates.


A B C D E F G H I A
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
a) Total Nucleic Acids
DNA
23S rRNA
16S rRNA
b) Dnase Treated Nucleic Acids
23S rRNA
16S rRNA

An unexpected result was obtained when the community structure was determined. Whereas the four probes gave a community structure totalling about 100% for the cell homogenizer and bead-beating extracts, the sonicated extracts resulted in community structures totalling from 130% to 150% (Table II.3). This difference appeared to be mainly due to a change in the signal obtained with probe ALF16 since its signal is about 75% for the sonicated extract and 40% for the others.

Table II.3. Average population density determined by four probes on activated sludge samples independently extracted by four mechanical lysis methods at different intensities.

Mechanical Lysis Method	ALF16 (%)	BET23 (%)	GAM23 (%)	CFC16 (%)	Total [1] (%)
Slurrying in Cell Homogenizer	39.4 ± 7.8[2]	28.2 ± 3.9	23.2 ± 0.1	4.8 ± 0.6	95.6 ± 12.5
Bead Beating					
4 min.	45.1 ± 11.0	32.9 ± 0.9	23.5 ± 0.8	2.8 ± 0.7	104.2 ± 13.4
4 min. with Trizol Replaced	44.5 ± 7.5	33.8 ± 1.9	22.6 ± 0.6	3.7 ± 0.9	104.6 ± 10.9
6 min. with Trizol Replaced	39.3 ± 6.7	33.6 ± 2.9	26.6 ± 0.2	4.4 ± 0.3	103.9 ± 9.9
Sonication					
45 sec.	76.5 ± 11.4	37.8 ± 5.1	19.1 ± 2.6	5.7 ± 0.4	139.1 ± 19.5
90 sec.	80.1 ± 10.5	41.0 ± 2.3	21.9 ± 3.6	6.3 ± 0.7	149.3 ± 17.1
45 sec. with Glass Beads	81.4 ± 6.7	41.0 ± 3.8	14.7 ± 0.8	4.9 ± 0.6	141.9 ± 12.0
90 sec. with Glass Beads	74.6 ± 11.4	31.4 ± 2.4	17.2 ± 1.0	4.8 ± 0.6	128.0 ± 15.3

[1] Summation of the four probes.
[2] ± standard error

Susceptibility of hybridization to rRNA shearing

Four levels of rRNA shearing, from "intact" to "completely sheared" rRNA (Fig. II.3) were used to describe the influence of rRNA shearing in the determination of the community structure after nucleic acid extraction by various lysis methods. It was observed that the hybridization signals from different probes were not affected the same way (Fig. II.4). Duncan multiple-stage pairwise comparison was used to compare the hybridization results after sonication of the sample for 120 sec. (Table II.4). By this technique, it was possible to

distinguish three shearing susceptibility groups (low, moderate, high) for the seven target sites tested. The first group has a low susceptibility to rRNA shearing. It is comprised of a single probe, EC(alf)16 which only lost 8% of signal as compared to the unsonicated-control rRNA (Table. II.3). The second group has moderate susceptibility to rRNA shearing. Probes UNI16, GAM23, and UNI23 are part of that group. On average, they lost 30% of the unsonicated-control signal. The third group has a high suscpetibility to rRNA shearing. Probes EUB16, EUB23, EC(cfc)16 constitute this group which lost, on average, 48% of their unsonicated-control signal (Table II.4).

Table II.4. *E. coli* rRNA hybridization signal after 120 sec. of sonication relative to unsonicated rRNA and shearing susceptibility grouping.

Shearing Susceptibility[1]	Probe Names	Signal[2]	Pairwise Comparison[3]		Unpaired Nucleotides[4]	
					Total	Consecutive
Low	EC(alf)16	0.92	A		4	2
Moderate	UNI23	0.72	B		7	3
	GAM23	0.71	B		6	2
	UNI16	0.65	B	C	6	3
High	EC(cfc)16	0.56	D	C	12	10
	EUB16	0.54	D	E	7	4
	EUB23	0.45		E	13	13

[1] Site shearing susceptibility group according to the pairwise comparison.
[2] Average relative hybridization signal (unsonicated rRNA equals 1).
[3] Duncan multiple-stage pairwise comparison of averages (n=3, α=0.05; Miller, 1981). Averages with the same letter are not significantly different from each other.
[4] The rRNA secondary structures were obtaines from (Gutell et al. 1993) and (Gutell 1994).

Figure II.2. Equimolar 16S and 23S *E. coli* rRNA sonicated for three different times (15 sec., 30 sec., 120 sec.). Lane numbers (1,2,3) represent replicates.

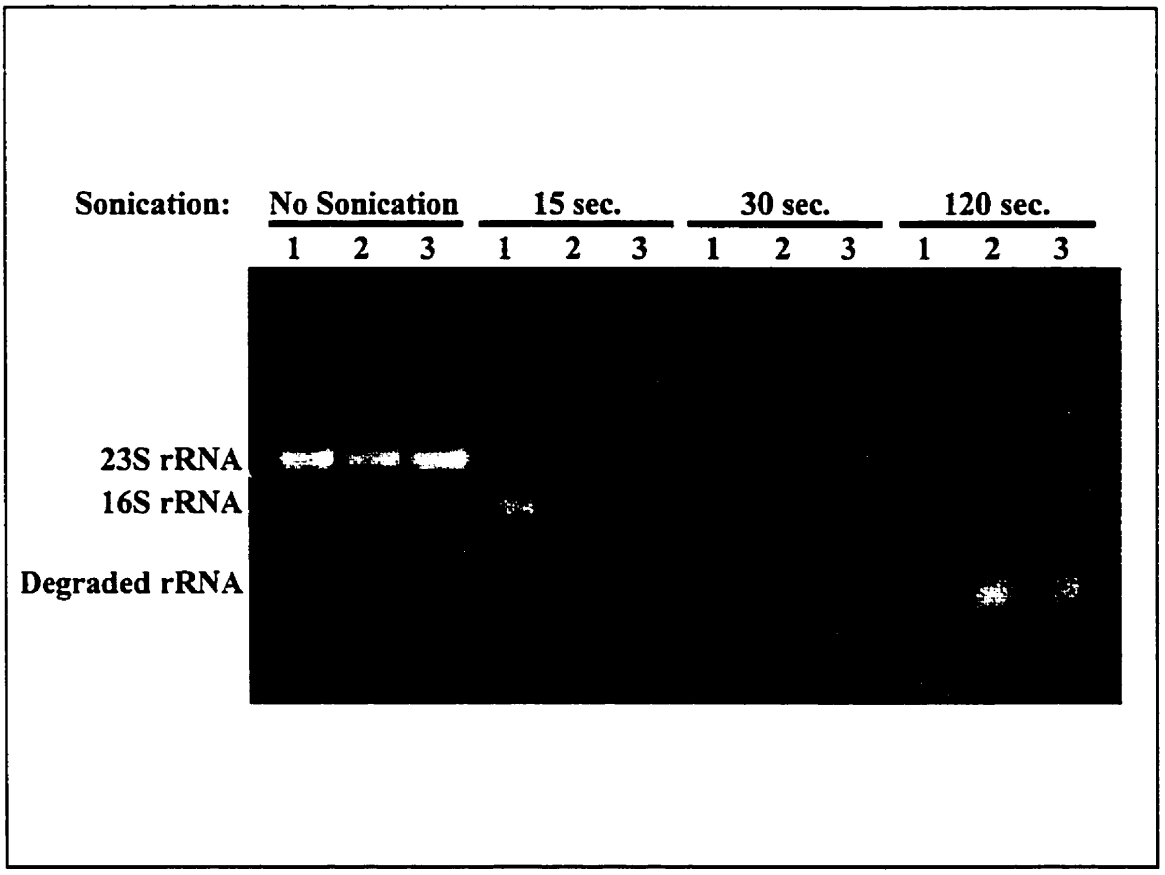
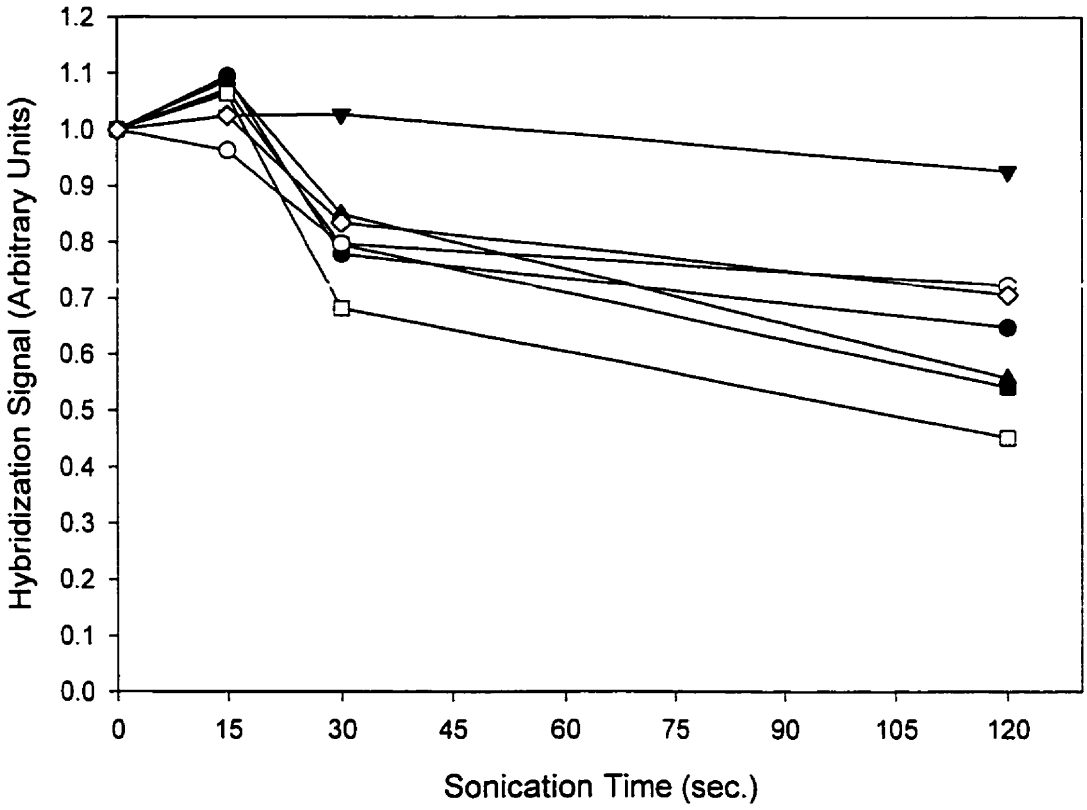

Sonication:
No Sonication
15 sec.
30 sec.
120 sec.
1 2 3 1 2 3 1 2 3 1 2 3
23S rRNA
16S rRNA
Degraded rRNA

Figure II.3. Specific membrane hybridization signal in function of the sonication time for probes targetted to the 16S rRNA (solid symbols) and 23S rRNA (open symbols) hybridized to an equimolar mixture of 16S and 23S *E. coli* rRNA. Arbitrary Units are defined such that no sonication equals 1. Probes: EC(alf)16 (▼), EC(cfc)16 (▲), EUB16 (■), UNI16 (●), EUB23 (□), GAM23 (◇), UNI23 (△).


Hybridization Signal (Arbitrary Units)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
15
30
45
60
75
90
105
120
Sonication Time (sec.)

Discussion

Two criteria must be considered when optimizing rRNA extraction from environmental samples. First, to maximize the yield, and second to make sure that the extraction does not bias the community structure, due to either differential lysis or rRNA deterioration (Stahl 1995; Ward et al. 1995; Raskin et al. 1997).

In comparing the four mechanical lysis methods, differences were found in the rRNA yield, the rRNA quality and the measured community structure Sonication gave the highest rRNA yield, but the lowest rRNA quality. The yield was also highly variable. Since the rRNA yield was determined by hybridization with probe EUB16 and EUB23, the high variability of the yield is consistent with the high shearing susceptibility of the sites targeted by these probes. It seemed that the addition of beads to the sonicated medium did not alter the effects of sonication on the yield and the quality of RNA extracted. On the other hand, lysis by slurrying in a cell homogenizer and bead-beating extracted RNA of similar quality. However, the latter technique gave slightly higher yields. The various bead-beating protocols did not have a large effect on yield.

The measured community structure was different for the sonicated sample as compared to the other methods. The proportion of probe ALF16 (Target: Alpha Proteobacteria) is similar for the slurried and the bead-beaten RNA extracts. However, it is much higher for the sonicated ones. Two possibilities can explain the data. The first one is that sonication extracted more Alpha Proteobacteria rRNA. Since the sonicated RNA extracts are also the most deteriorated, the second possibility is that the site targeted by probe ALF16 is less susceptible to degradation than are the other sites. The unrealistically high total community percentage for the sonicated extracts would argue against the first possibility since the abundance of the other populations did not change. Therefore, these data suggest that probe ALF16 is indeed less susceptible to rRNA shearing. Raskin and co-workers (1997) suggested that the eubacterial target could be more susceptible to enzymatic degradation. Unfortunately, direct study concerning the variation of susceptibility to degradation through the rRNA molecules was not presented.

To investigate these hypotheses further, the susceptibility of probe specific hybridization signal to sonication was investigated. It was observed that while probe EC(alf)16 (Same target site as probe ALF16) only lost 8% its hybridization signal with 120 sec. of sonication, the other probes lost on average 31% and 48% for the moderately and highly susceptible ones, respectively. Therefore, the site targetted by probe ALF16 is less sensitive to rRNA shearing than are the other sites. This argues for a bias of the observed community structures due to rRNA shearing. It also demonstrates that deteriorated rRNA is completely unsuitable for quantitative membrane hybridization.

On average, 30 to 50% of the hybridization signal was lost by most of the probes. Many possibilities exist to explain this lost of hybridization signal. Probably some secondary and tertiary motifs are more sensitive to degradation. If a probe overlaps a "hot-spot" of breakage, the signal from that probe will be preferentially lost. On the other hand, it is possible that smaller fragments do not bind as efficiently to the blotting membrane. However, the fragments generated by 120 sec. of sonication seemed to be relatively homogeneous (Fig II.3). Since probe ALF16 did not lose more than 8% of its hybridization signal, loss of binding efficiency to the blotting membrane seem unlikely. Therefore, the loss of signal is probably due to overlapping breakage "hot-spots".

Raskin and co workers (1997) hypothesised that the single stranded portion of rRNA might be more susceptible to enzymatic degradation. Three groups of site susceptibility to rRNA shearing were identified with the probes tested here. The following observations were made by observing the secondary structure of the small subunit and large subunit rRNA molecules (Gutell et al. 1993; Gutell 1994). The low susceptible EC(alf)16 target had four unpaired nucleotides. The moderately susceptible UNI16, GAM23, and UNI23 targets have four to eight unpaired nucleotides and a maximum of three consecutives. The most susceptible EUB16, EUB23, EC(cfc)16 targets have seven to thirteen unpaired nucleotides and four to thirteen consecutive ones . This suggests that the single stranded portion of rRNA molecule might also be more susceptible to shearing, since the less susceptible sites have fewer unpaired nucleotides than the more susceptible ones. However, it is important to keep in mind the three dimensional models of rRNA do not represent static interactions,

but a variety of bases that pair, or not, depending on the biochemical environment (Gutell et al. 1994). They map possible base interactions that do not necessarily occur simultaneously. The interactions may also change inside and outside the ribosome (Moazed et al. 1986). Therefore, the study presented here on the shearing susceptibility of various sites is only an approximation of what can happen during the extraction of the RNA from the sludge using sonication.

Conclusions

It was shown that the site targeted by probe ALF16 is much less susceptible to RNA shearing than the sites targeted by the other probes tested. Therefore, sonication biases the measured community structure because it does not shear rRNA completely randomly. rRNA extracted by bead-beating or by slurrying in cell homogenization did not exhibit the same bias with respect to community structure. In addition, the community structure determined after extraction by these two methods was similar. Therefore, it seems that the bias is either the same or absent for these methods. Since bead-beating had a higher rRNA yield, it was the method of choice.

Appendix III. Optimization of Phenotypic Fingerprinting

Introduction

Two factors should be considered in order to functionaly characterize a bacterial community on the basis of its utilization of carbon sources: (i) the lag time and (ii) the consumption rate (Hackett and Griffiths 1997; Lawley and Bell 1998). The determination of these factors requires multiple measurements over several days, and in a pulp and paper mill, this is not practical because of human resource considerations (Victorio et al. 1996). An alternative approach is to obtain a single point measurement that somehow combines the lag period and the consumption rate. The critical step of this approach is to determine the optimal time of incubation prior to that measurement. The data concerning the optimization of the incubation time for two reactors are reported. The average well colour development (AWCD) as well as the power of resolution between two *a priori* different communities were considered in this optimization.

Materials and methods

Microbial cells were extracted from three independent samples of mixed liquor taken the same day from each of the two Cornwall reactors (Reactor 1 and 2), described in Chapter 3. The extracted cells were washed and resuspended in phosphate buffer (see Chapter 3). The optical density (absorbance at 600 nm) of the extracts were adjusted to 0.3. Two Biolog GN plates (Biolog Inc.) were inoculated (150 µL of cell suspension per well) with each extract to evaluate the reproducibility of incubation. The plates were incubated in the dark at 30 °C and then read five times using a microplate reader as described in Chapter 3 after (i) 8 h, (ii) 12 h, (iii) 24 h, (iv) 36 h, and (v) 72 h.

Cluster analysis was performed by the procedure CLUSTER of SAS/STAT v. 6.12 for Windows (Sas Institute Inc. 1989). The average linkage method was used, but no difference in the clustering was observed when other methods were used.

Results

The operation parameters are very different between Reactor 1 and Reactor 2. These differences were reported in Chapter 4. Differences between the two reactors were observed in operation pH, the sludge age, the COD/BOD ratio, and the VFA and alcohol concentrations (Table 4.1).

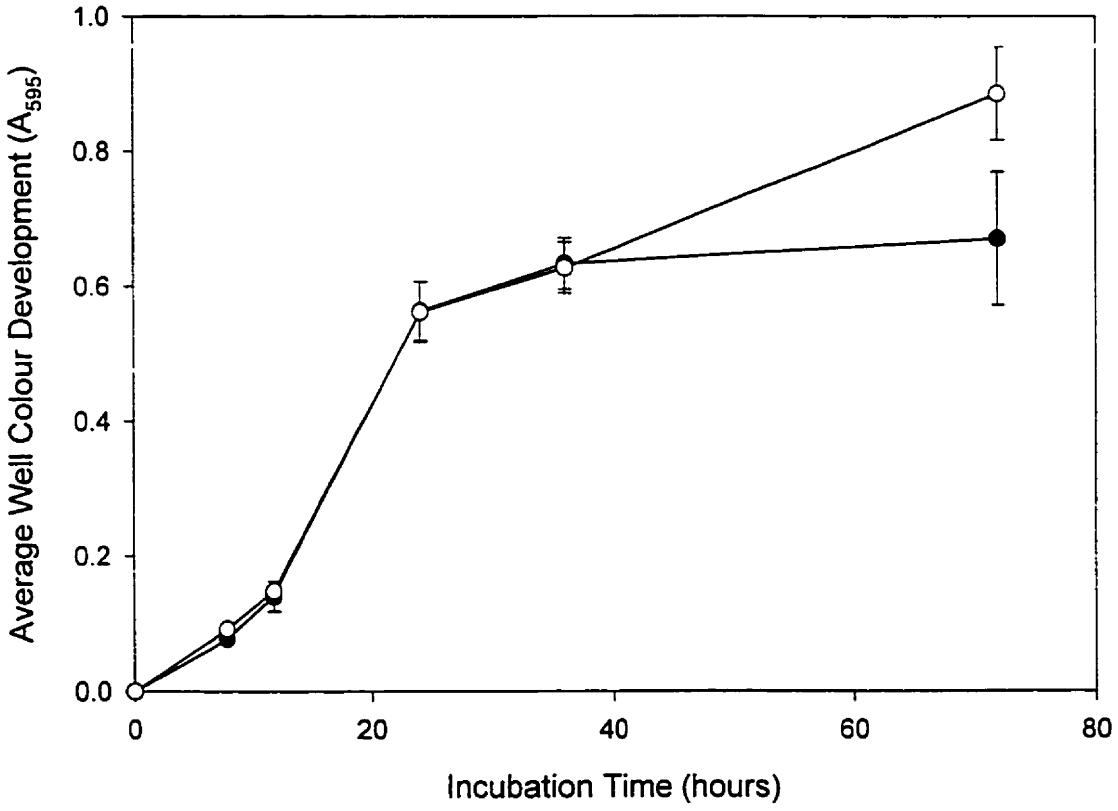
The AWCD was found to follow a sigmoid curve over time. For the samples from Reactors 1 and 2, this sigmoid curve started to plateau after approximately 24 h incubation (Fig. III.1). The discriminating power of the technique was also found to be maximum early in this plateau. After 24 h of incubation, the cluster analysis was able to group all the samples from the same reactor on the same main branch (Fig. III.2). This was also observed after 36 h of incubation. On the other hand, when the plates were incubated only 8 or 12 h, or when the incubation was prolonged to 72 h, the clusters did not agree with the *a priori* differences between the communities.

Discussion

As presented in Chapter 4, the two reactors are operated very differently. The mechanical operation, the wastewater to be treated, as well as the pH are examples of these differences (Table 4.1). Because of these factors, the two communities were assumed to be functionnally different with respect to their heterotrophic activity. This difference was also substantiated by differences observed in the taxonomic community structure (Chapter 4). However, care should be taken in interpreting the taxonomic differences since no unifying phenotypic characteristic was found to explain the phylogeny of the proteobacteria at the highest levels (phylum and subphylum; Zavarzin et al. 1991).

Based on this *a priori* difference, the phenotypic fingerprinting should be able to differentiate the two communities if the plates are incubated for an optimal time. Therefore, this criterion was used to determine the optimum incubation time. All the samples from the same reactor are grouped on the same branch of the dendrogram after 24 and 36 hours of incubation (Fig. III.2). Because we wanted to detect heterotrophic activities as close as possible to the *in situ* ones, the shortest incubation time was selected (Konopka et al. 1998)

Figure III.1. Average Well Colour Development versus time for Biolog GN plates inoculated with bacterial communities from the Reactor 1 (●) and Reactor 2 (□). Error bars represent standard errors (n=3).


1.0
0.8
0.6
0.4
0.2
0.0
Average Well Colour Development (A_{595})
0
20
40
60
80
Incubation Time (hours)

The concept of AWCD was proposed by Garland and Mills (1991). The sigmoid behaviour of the AWCD over time found with the samples from the two Cornwall Reactors is in agreement with the one reported for aquatic and soil samples by Garland and Mills (1991). From Fig. III.1, it can be seen that the AWCD reached a plateau level at about 24 hours. This means that most of the activity to be expressed was detected by 24 hours.

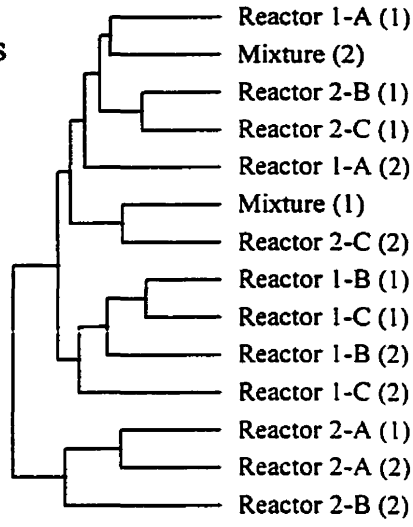
The results from the cluster analysis and the AWCD suggest that the optimum incubation time for our system is 24 hours. This is comparable to the incubation time of 18 hours reported by Victorio and co-workers (1996) for samples from pulp and paper wastewater treatment systems. On the dendrogram derived from the data obtained after 24 hours incubation (Fig. III.2c), most of the replicated plates clustered together. This suggests that the reproducibility is relatively good for replicate plating. This is in contradiction with the result reported by Knight and co-workers (1997) for soil samples. However, soil has a more heterogeneous distribution of heterotrophic organisms as compared to pulp and paper activated sludge which may explain this difference in reproducibility between our study and Knight's.

Conclusion

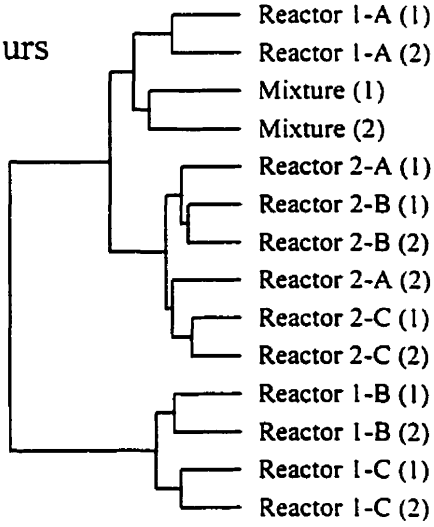
It is possible to find phenotypic fingerprint differences between the two Cornwall reactors. These differences agree with differences in reactor operations and in taxonomic community structure. Using a single point measurement, functional differences appeared after 24 hours of incubation. This incubation time also corresponded to a plateau in AWCD. It is therefore concluded that the optimum incubation time is 24 hours.

Figure III.2. Dendrograms of average linkage cluster analysis for Biolog GN plates inoculated with bacterial communities from Cornwall Reactors 1 and 2 as well as a Mixture of the two following incubation for (a) 8 h, (b) 12 h, (c) 24 h, (d) 36 h, and (e) 72 h. Letters (A, B, C) represent samples prepared independently and numbers (1,2) represent plates inoculated with the same sample.

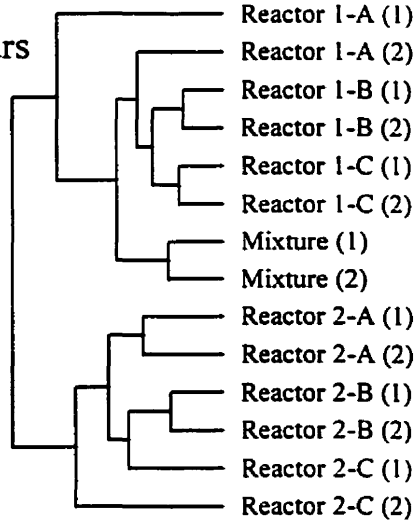
a) 8 hours



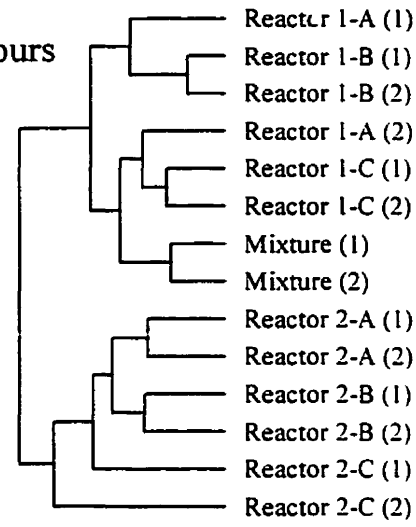
b) 12 hours



c) 24 hours



d) 36 hours



e) 72 hours