Who eats what? Classifying microbial populations based on diurnal profiles of rRNA levels

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Abstract Identifying the relationships between various bacterial populations and the substrates they consume is central to the understanding of population dynamics and to the development of process control in activated sludge. However, linking a heterotrophic population to its activity in situ is difficult because ribosomal RNA (rRNA) techniques, while allowing the rapid identification of populations, provide little information about their heterotrophic activity. Activated sludge models describe biodegradation kinetics by classifying substrates into two types: readily and slowly degradable substrates. Assuming that bacterial populations specialize in degrading one type of substrate, their growth rate should be affected differently if the COD loading rate varies diurnally as for a municipal activated sludge system. Modeling results suggested that the growth rates of populations consuming readily degradable substrates vary according to variations in COD loading rate. On the other hand, the growth rates of populations consuming slowly degradable substrates do not change despite the variation in COD loading rate. Since the cellular rRNA level is positively correlated with the growth rate, we hypothesized that the rRNA levels of some populations in municipal activated sludge should increase throughout the day, while they should stay constant for other populations. This hypothesis was verified by monitoring the rRNA level of Acinetobacter (a model population consuming readily degradable substrates) and Gordonia (a model population consuming slowly degradable substrates) in the mixed liquor of a full-scale municipal activated sludge reactor for three weeks.

Keywords Activated sludge modeling; oligonucleotide probe hybridization; population dynamics; readily degradable substrate; rRNA; slowly degradable substrate

Introduction
The identification of substrates used by microbial populations is important when studying activated sludge population dynamics and system control. However, the level of precision used for the identification of a substrate consumed by a population of interest changes with the application. Sometimes it is important to know the exact identity of a substrate, whereas in other cases it may be sufficient to know in which class of compounds a substrate fits. Mathematical models developed for modeling activated sludge systems for the treatment of municipal wastewater (IWA Task Group on Mathematical Modeling, 2000) have defined two classes of substrates on the basis of their biodegradation kinetics: readily degradable substrates and slowly degradable substrates. This approach has been proven helpful in activated sludge system design and optimization. For example, selectors are effective in preventing the proliferation of organisms consuming readily degradable substrates, such as Sphaerotilus natans. However, selectors are ineffective in selecting against filamentous organisms consuming slowly degradable substrates such as Gordonia (formerly Nocardia) amarae and “Candidatus Microthrix parvicella” (hereinafter referred to as “M. parvicella”) (Jenkins et al., 1993). These observations were successfully modeled by assuming two classes of substrates categorized on the basis of their biodegradation kinetics (Kappeler and Gujer, 1994a,b,c).
Other applications require the exact identification of the compounds serving as substrates for a population of interest. New approaches, based on this exact identification, could be developed to solve foaming problems caused by *Gordonia amarae* and "*M. parvicella*" since current control methods (e.g. modification of reactor configuration) are inefficient. A possible approach would consist in mixing specific additives (e.g. an organism competing for the same substrate or a substrate-specific inhibitor) to the reactor influent. Microautoradiography has proven useful to link a population of interest to the consumption of specific chemical compounds. This approach demonstrated that "*M. parvicella*" present in activated sludge communities incorporates radioactive carbon from long chain fatty acids and lipids, suggesting the consumption of lipids in municipal wastewater treatment systems (Andreasen and Nielsen, 2000). While microautoradiography combined with fluorescence *in situ* hybridization (FISH) is an elegant approach to evaluate substrate consumption profiles in complex microbial communities such as activated sludge (Lee *et al*., 1999; Ouerney and Fuhrman, 1999; Cottrell and Kirchman, 2000), the substrates added are foreign to the activated sludge system and may not represent the natural food source of the organism. Furthermore, the number of potential substrates present in wastewater is so large that a comprehensive assessment is difficult. Therefore, it may be helpful to reduce the domain of substrates by determining a priori if a population of interest consumes readily degradable material (e.g. volatile fatty acids [VFA], sugars, amino acids) or slowly degradable material (e.g. lipids, starch, proteins).

The goal of the present study is to demonstrate the possibility of classifying various populations in activated sludge based on their substrate type (readily or slowly degradable). Mathematical models predict that the diurnal variation in loading rate observed in municipal systems impacts the metabolic activity of the microorganisms differently depending on the type of substrate present in the wastewater (Grady *et al*., 1999). Since cellular ribosomal RNA (rRNA) levels are positively correlated with growth rate (Schaechter *et al*., 1958; Herbert, 1961; Bremer and Dennis, 1996), we hypothesized that the impact of substrate fluctuation on the metabolic activity would be reflected in rRNA levels of the consuming population. Therefore, variation in rRNA abundance would serve as a criterion to classify the population based on their substrate source. To verify this hypothesis, we studied a municipal activated sludge system for three weeks by measuring the variations in chemical oxygen demand (COD) loading and in rRNA content for two populations, the genera *Gordonia* (a model population consuming slowly degradable substrates) and *Acinetobacter* (a model population consuming readily degradable substrates).
Materials and methods

Reactor and sampling

The activated sludge system of the Urbana-Champaign Sanitary District Northeast (UCSD-NE) wastewater treatment plant is a contact-stabilization system consisting of four aeration tanks (Figure 1) and four secondary clarifiers. Each tank is 7 m wide, 42.5 m long, and 5.5 m deep. The results of a tracer study indicated that the stabilization tank behaved as a single completely stirred tank reactor (CSTR), while each of the three contact tanks behaved as 2.5 CSTRs in series (Frigon and Raskin, unpublished data). During the study, the system was operated at an average temperature of 16°C with a hydraulic retention time (HRT) of six to eight hours and a solids retention time (SRT) of approximately four days.

The influent to the activated sludge reactor was sampled every two hours for a period of three weeks. The samples were preserved by adding sulfuric acid to reduce the pH below 2 and were kept at 4°C until analysis. The biomass was sampled every four days during the same period. Samples from each tank were collected at 6:00 h, 12:00 h, and 18:00 h. Samples for oxygen uptake rate (OUR) analyses were kept at the same temperature as the mixed liquor in the activated sludge reactor and were analyzed within an hour after sampling. Samples for molecular analyses were put on ice immediately after sampling, and were frozen at –80°C within two hours after sampling.

Chemical analysis

COD concentrations were determined using Hach vials according to the manufacturer’s instruction (Hach, Loveland, CO). Soluble fractions were prepared by filtering the wastewater through a 0.45 µm filter. The concentration of mixed liquor suspended solids (MLSS), the concentration of mixed liquor volatile suspended solids (MLVSS) and the OUR were determined according to Standard Methods (American Public Health Association, 1992).

Molecular analysis

The RNA from approximately 15 mg of suspended solids was extracted by a hot phenol-bead beating procedure (Stahl et al., 1988; Raskin et al., 1995). RNA was denatured with 2% glutaraldehyde for 10 min and applied to a Magnacharge nylon membrane (Osmonics Inc., Minnetonka, MN; (Raskin et al., 1994)). The membranes were hybridized overnight with 32P-labeled oligonucleotide probes at 40°C using the Sigma “Perfect HYB” buffer (Sigma, St. Louis, MO), washed twice for one hour at the same temperature using a sodium dodecyl sulfate–sodium citrate buffer, and washed for 30 min at a previously determined temperature (Raskin et al., 1994). The probes used in this study and the stringent wash conditions for the final 30-min wash are reported in Table 1. The signal obtained with each specific probe was normalized using the signal obtained with probe S-*-Univ-1390-a-A-18, which targets virtually all organisms.

Table 1 Probes utilized in this study and stringent wash temperatures

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Target group</th>
<th>Sequence</th>
<th>Reference</th>
<th>Wash temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-G-Acin-0659-a-A-24</td>
<td>genus Acinetobacter</td>
<td>CTGGAATTCTACCATCCTTCTCCCA</td>
<td>Oether et al., in prep</td>
<td>64</td>
</tr>
<tr>
<td>S-G-Gor-0596-a-A-22</td>
<td>genus Gordonia</td>
<td>TGCAGAATTTCACAGACGACGCC</td>
<td>de los Reyes et al., 1997</td>
<td>54</td>
</tr>
<tr>
<td>S-Myb-0736-b-A-22</td>
<td>mycolic acid containing Actinomycetes</td>
<td>CAGCGTGCGTTTACTTCCCCAGAG</td>
<td>de los Reyes et al., 1997</td>
<td>51</td>
</tr>
<tr>
<td>S-Univ-1390-a-A-18</td>
<td>virtually all organisms</td>
<td>GACCGGGCGGTGGTACC</td>
<td>Zheng et al., 1996</td>
<td>44</td>
</tr>
</tbody>
</table>
Modeling

Modeling simulations were performed using the program ASIM version 3.0 (Gujer and Larsen, 1995). The stabilization basin was assumed to be a single CSTR, while the three other tanks combined were modelled as five CSTRs in series even though a tracer study showed that each of the three tanks behaved as 2.5 CSTRs (the maximum number of CSTRs in series that can be used in ASIM is six). The biomass was assumed to behave according to the ASM1 model configuration (IWA Task Group on Mathematical Modeling, 2000) and kinetic parameters commonly used in the modeling of municipal wastewater treatment systems were employed (Grady et al., 1999; Table 2).

Statistical method

The experiment was designed as a repeated measures ANOVA with each biomass sampling day as an independent experimental unit. Calculations were performed with the procedure GLM of SAS/STAT version 6.12 for windows (SAS Institute Inc., 1997). An alpha level of 0.1 was adopted for all the statistical testing.

Results and discussion

Activated sludge loading

During a period of three weeks, the activated sludge reactor influent flow rate and total COD concentration were measured every two hours. The flow rate and the total COD concentration varied according to a typical diurnal profile (Figure 2). The soluble COD fraction in the influent to the activated sludge reactor, which should comprise the majority of the readily degradable COD (IWA Task Group on Mathematical Modeling, 2000), accounted for approximately 35% of the total COD at all times. The remaining 65% of the COD was assumed to be associated with particulate material and hence was considered slowly degradable. The fraction of inert material remains to be determined before a complete picture of the loading pattern can be ascertained, but 10% of the COD in each fraction was assumed to be inert for modeling purposes (IWA Task Group on Mathematical Modeling, 2000).

Modeling

Preliminary modeling of the UCSD-NE activated sludge system was performed to evaluate how the metabolic activities of two populations might vary along the length of the reactor and throughout a day. The modeling exercise did not aim to predict precisely the plant’s performance, but rather to show how populations consuming different types of substrates exhibit different growth activity profiles. The model predicted that the respiration rates of populations degrading readily degradable COD vary over time in the first contact tank (corresponding to sampling points 4, 5, and 6, Figure 1), but stay relatively constant in the other

Table 2 Parameters used in modeling of the UCSD-NE wastewater treatment plant activated sludge reactor

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>YH</td>
<td>mg biomass COD/mg COD removed</td>
<td>0.6</td>
</tr>
<tr>
<td>fD</td>
<td>mg COD debris/mg biomass COD</td>
<td>0.09</td>
</tr>
<tr>
<td>bH</td>
<td>d⁻¹</td>
<td>0.408</td>
</tr>
<tr>
<td>μH</td>
<td>d⁻¹</td>
<td>6</td>
</tr>
<tr>
<td>Ks</td>
<td>mg readily degradable COD/litre</td>
<td>20</td>
</tr>
<tr>
<td>K_{O2,H}</td>
<td>mg O₂/litre</td>
<td>0.1</td>
</tr>
<tr>
<td>kH</td>
<td>mg COD/(mg biomass COD × day)</td>
<td>2.21</td>
</tr>
<tr>
<td>kD</td>
<td>mg slowly degradable COD/mg biomass COD</td>
<td>0.15</td>
</tr>
<tr>
<td>HRT</td>
<td>d</td>
<td>0.3</td>
</tr>
<tr>
<td>SRT</td>
<td>d</td>
<td>4</td>
</tr>
</tbody>
</table>
contact tanks (corresponding to sampling points 7 and 8, Figure 1) and in the stabilization
tank (corresponding to sampling points 1, 2, and 3, Figure 1; Figure 3a). On the other hand,
the respiration rates of populations degrading slowly degradable COD stay relatively
constant over time, but vary more among the tanks (Figure 3b). A community degrading a
mixture of 31.5% readily degradable COD (SS), 58.5% slowly degradable COD (X₃),
3.5% inert soluble COD (SI), and 6.5% inert particulate COD (XI; similar to the influent of
the UCSD-NE plant) exhibits a respiration rate profile hybrid of the two previously men-
tioned profiles (Figure 3c).

Statistical approach

Analysis of variance (ANOVA) is commonly used to test the statistical significance of
changes observed in an experiment. Regular ANOVA requires each sample to be independ-
ent, but this requirement is violated when the samples are obtained closely in time and
space. The repeated measures ANOVA corrects for this lack of independence by evaluating
the variability of the average trend across independent experimental units (Diggle, 1988;
Dutilleul and Pinel-Alloul, 1996). A given effect (e.g. increase in rRNA during the course
of a day) is found significant if it is observed for a majority of the units sampled. On the
other hand, if the effect changes from one experimental unit to the next (e.g. upward trend
in rRNA level during one day, but downward trend during the next sampling day), the effect
is not significant. In contrast, regular ANOVA evaluates the variability of the average
response across experimental units.

The simplest definition of an experimental unit for this experiment would be to consider
each reactor of a set of several reactors operated side-by-side as an experimental unit.
However, since this approach was not possible with the current experiment because of the
possibility that identical populations (e.g. Acinetobacter) can have different functional
roles in different full-scale reactors, we defined each sampling day as an experimental unit.
All biomass samples taken during the same day were considered to be repeated measures in
time (time during the days) and space (length of the reactor).

This approach can be justified as follows. The lack of independence between successive
observations obtained over time can be estimated by calculating the correlation between
observations separated by a given temporal distance (Box et al., 1994). This correlation is
referred to as autocorrelation. Observations made with activated sludge biomass samples
likely are positively autocorrelated, meaning that observations obtained on a particular
day are more likely to be similar to observations taken the day before than to observations collected two days before. Indeed, the process of positive autocorrelation follows an exponential decrease proportional to the time delay between observations. Based on activated sludge reactor theory, we can evaluate the level of autocorrelation between successive sampling days, which are the experimental units of this experiment and are four days apart, by estimating how much of the biomass present on one sampling day would still be present the next sampling day. After one SRT (four days for the UCSD-NE activated sludge system), the theory predicts that approximately 65% of the solids would have turned over. Therefore, 35% of the biomass present on day one is likely to be present one SRT (four days) later, which corresponds to a level of autocorrelation of 0.35. By also considering an endogenous respiration rate of 0.0076 h⁻¹ (Grady et al., 1999), we estimate that at least 90% of the biomass would have turned over in four days, a level of autocorrelation of 0.1. Consequently, the sampling days can be considered independent from one another.

Figure 3 Metabolic activity profiles across the reactor and at different times of the day. (a, b, c) SOUR profiles at 6:00 h (---), 12:00 h (--), and 18:00 h (-----) as modeled for a reactor with six CSTRs in series (see text for the composition of the influent). a) SOUR considering only the soluble portion of the influent; b) SOUR considering only the particulate portion of the influent; c) SOUR considering the complete effluent. (d, e, f) Average profiles measured at the UCDS-NE plant at 6:00 h (●), 12:00 h (▲), and 18:00 h (▼). d) rRNA level of Acinetobacter; e) rRNA level of Gordonia; f) SOUR.
rRNA profiles
The contact-stabilization system of the UCSD-NE wastewater treatment plant was studied to confirm the presence of different growth activity profiles predicted by the preliminary modeling. The average SOUR profiles (Figure 3f) were similar to the profiles predicted by the simulation for the treatment of a S_C:S_I:X_C:X_I wastewater mixture of 31.5%:3.5%:58.5%:6.5% (Figure 3c). The concentration of RNA in the biomass did not change significantly within a day (P > 0.1) and remained constant at approximately 75 µg rRNA/mg MLVSS throughout the experimental period (data not shown). However, the rRNA level was reduced by approximately 5% (P < 0.1) near the point of entrance of the influent (sampling point 4 in Figure 1) compared to the rest of the reactor. A tracer study showed that the biomass leaving the stabilization tank was poorly mixed with the influent in this region of the reactor (Frigon and Raskin, unpublished results). If it is assumed that no rRNA is contributed by the influent solids and that the concentration of influent solids near the entrance is the same as in the influent, this 5% decrease in rRNA level can be explained by the introduction of influent solids in this region. This suggests that little rRNA is contributed by the incoming solids.

While the total rRNA level remained constant throughout the day, the proportion of *Acinetobacter* rRNA increased during the day (P < 0.1), but stayed stable across the reactor (P > 0.1; Figure 3e). Considering that the time scales of rRNA production and decay are much longer than the respiration rate time scale, it can be anticipated that the rRNA level does not sharply increase or decrease across the reactor, while it can be expected that the SOUR does change considerably across the reactor. On the other hand, the increase in rRNA during the day is consistent with the increase in SOUR during the day predicted by the model for a population consuming readily degradable substrate. Furthermore, the precursor RNA level of *Acinetobacter* significantly increased over the length of the reactor (P < 0.1) after exposure to incoming substrate (Figure 4). Although the increase was small, the upward trend was observed every day and for most of the sampling times, which explains the significance of this increase (see above). The precursor rRNA is an rRNA transcript that needs further processing to produce a mature ribosome (Srivastava and Schlessinger, 1990). Since the conversion of precursor rRNA to mature rRNA is a slower process than transcription, precursor rRNA accumulates during a metabolic up-shift such as the one observed when biomass is exposed to fresh wastewater influent (Oerther et al., 2000). These data are consistent with the view that *Acinetobacter*, which belongs to the gamma-subclass of the Proteobacteria, grows on readily degradable substrates, such as VFA (Fuhs and Chen, 1975; Wagner et al., 1993).
No significant changes (P>0.1) in the proportion of *Gordonia* rRNA were observed either during the day or throughout the reactor. The lack of change in the rRNA level of *Gordonia* throughout the day was confirmed by the constant rRNA level for the mycolic acid containing Actinomycetes, a group of bacteria comprising *Gordonia*, but with two to three times more rRNA in this reactor (data not shown). In the literature, it was suggested that *G. amarae* is one of the organisms consuming *in situ* fatty materials, which need to be hydrolyzed by the organism before consumption (Kappeler and Gujer, 1994a,b,c). The data presented here are consistent with the results of the preliminary modeling, which showed that the SOUR level of a population degrading slowly degradable substrate does not change throughout the day despite variation in COD loading.

**Conclusion**

In conclusion, this experiment shows that it is possible to classify bacterial populations on the basis of the type of substrate they consume (slowly or readily degradable) by using the rRNA temporal profile under diurnal loading variation as a classification criterion. This method offers a natural bridge between the kinetic description of activated sludge processes by mathematical models and the identification of bacterial populations provided by rRNA based analyses of microbial communities. Defining heterotrophic functions in terms of specific compounds consumed by populations would require the inclusion of tens or maybe hundreds of substrates and populations in an activated sludge model. The complexity of this approach would likely make it less precise than current approaches and therefore futile. Generally, a simple kinetic description of population growth and substrate utilization is sufficient to model the influence of reactor configuration on population selection. If kinetic selection is not effective, substrate specific solutions could be developed. Since degradation kinetics influence the rRNA levels, collecting this information would be helpful to reduce the domain of compounds that need to be evaluated when determining the substrates that support the growth of specific populations and therefore accelerate the elucidation of a compound-population association. Thus, this approach may be helpful in finding ways to eliminate particular populations (or reduce their abundance) from activated sludge systems or other bioreactors.

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