DISCUSSION

Of: **The Role of Molecular Methods in Evaluating Biological Treatment Processes,** B. E. Rittmann, **74,** 421 (2002).

R. Iranpour, F. Alatriste-Mondragon, S. V. Diaz-Perez, H. H. J. Cox

This paper, along with the accompanying editorial in the September/October 2002 issue, highlights a significant development that is now occurring in research in biological treatment processes for wastewater and other wastes. It summarizes a long line of important accomplishments by Professor Rittmann and his co-workers that should guide applications of molecular techniques in the future of the environmental engineering industry. As the authors note, treatment processes rely on the activity of microbial cultures; however, our knowledge of the organisms present in the cultures and their interactions is very minimal. Traditional methods for isolating and identifying organisms in a culture have now been superseded by new techniques, such as targeting the small subunits of ribosomal RNA with oligonucleotide probes.

The examples in this paper are primarily from studies of nitrifying bacteria, with a summary of the paper by Angenent et al. (2002), about an anaerobic methanogenic system. It is clear that these techniques open enormous opportunities for improved understanding, and perhaps also for some kinds of cost reduction, in anaerobic sludge digestion, which has been a major focus of our Applied Research Group at the City of Los Angeles (California) Bureau of Sanitation for several years (Iranpour et al., 2002a, 2002b, 2002c). In this discussion we identify several areas where molecular techniques might be beneficial and discuss existing limitations or questions about practical application of these techniques.

1. One obvious possibility is to identify organisms and their relative population densities in cultures living under various conditions or receiving different feedstocks. For example, at the Hyperion Treatment Plant we have recently acquired many empirical observations of temperature dependence in the composition of the biogas and biosolids produced by digestion at thermophilic temperatures. In particular, the concentration of odorous sulfur compounds rises rapidly with higher temperatures. This prevents using temperatures of approximately 53 °C or higher, which would be desirable when plants do not have enough digester volume to apply lower temperatures with longer holding times to satisfy alternative 1 (the time–temperature batch mode operation) of the Part 503 Biosolids Rule (U.S. EPA, 1993). Table 1 shows the situation at Hyperion when temperatures were raised to 56 to 57 °C to achieve Class A biosolids with a holding time of 16 hours. Obviously, changes in overall digester performance can ultimately be related to the densities and relative activities of microbial subpopulations in the digester. Determining the microbial ecology with molecular tools might provide a better understanding of digester performance than simply attributing poor digester performance to "process instability".
2. The City of Los Angeles Class A biosolids program has been conducted primarily because of a local regulatory mandate to disinfect biosolids to a standard slightly higher than the Class A standard defined by the U.S. Environmental Protection Agency regulations on sludge usage and disposal (Iranpour et al., 2003). One aspect of demonstrating compliance with the mandate thus consists of performing bioassays that show low densities of fecal coliform bacteria and densities of *Salmonella* species that are below the threshold of detectability that existed when the Part 503 Biosolids Rule was adopted. The other aspect is to use one of six alternative ways of demonstrating that other types of pathogens have also been killed; two of these alternatives consist of bioassays for enteric viruses and viable helminth ova. As the bacterial bioassays take several days, and the other bioassays not only are costly but also take several weeks, replacing any of them with faster or less costly tests using molecular methods would have great appeal. However, we are not sure that using oligonucleotide probes to detect nucleic acids (fluorescent in situ hybridization [FISH]) or nucleic acid amplification (polymerase chain reaction [PCR]) from the target species would be able to distinguish between living and dead organisms, since pathogenicity requires a cell to be viable.
3. In the long term, development of on-line technologies to measure the relative number and activity of microbial subpopulations or specific pathogenic strains is highly desirable and would provide a valuable asset to better and faster process control.

The above discussion presents several areas for use of molecular tools in the wastewater treatment plant, however, several questions remain to be answered. For example, how reliable are the molecular methods, regarding false positives, to differentiate between viable and dead cells? How might the use of oligonucleotide probes specific for *Salmonella* or helminth ova help to evaluate the disinfection efficiency of full-scale anaerobic digesters? In addition, quantification of pathogenic strains of enteric bacteria, such as *Escherichia coli* O157:H7, would be a valuable asset. Would it be possible to target nucleic acid sequences of specific enterotoxins? Would it be possible to standardize the molecular methods for replacement of the current traditional cultivation methods? How can matrix interferences be prevented, in particular in samples with a high-solids content, such as biosolids? Considering that knowledge of PCR amplification and FISH methodologies is needed, how easily could plant personnel be trained in these techniques?

Considerable research has been reported lately regarding molecular probing of a variety of anaerobic digestion systems. Oligonucleotide probes have been described for several groups of methanogens (Raskin et al., 1994). Detection and relative abundance of syntrophic bacteria in an anaerobic biogas reactor have

Table 1—Effect of temperature on performance of thermophilic digesters at Hyperion Treatment Plant (average data; two-stage digestion with 19 digesters; first stage continuous at hydraulic residence time of 9.9 days; second stage batch with holding for 16 hours).

	Digester temperature (°C)	
Parameter	**50**	**57**
VFA (mg/L)[a]	300	1000
VFA/alkalinity ratio	0.1	0.33
Volatile solids reduction (%)	60	60
Digester gas		
Methane (%)	65	65
Carbon dioxide (%)	35	35
Hydrogen sulfide (ppm_v)	80–190	120–170
Methyl mercaptan (ppm_v)	0–75	200–350
Dimethyl sulfide (ppm_v)	0–5	30–40

[a] VFA = volatile fatty acids.

been reported (Hansen et al., 1999). Changes in methanogenic populations in anaerobic digesters under different operating conditions have been described (Griffin et al., 1998; McMahon et al., 2001). Confocal microscopy and FISH have allowed studying the topographic location of methanogenic and syntrophic bacteria in anaerobic granules (Imachi et al., 2000; Rocheleau et al., 1999). What are the next research efforts to implement a molecular database for anaerobic digestion similar to the Activated Sludge Biomolecular Database? Is there already an organized effort to construct such a valuable tool?

Any comments that the author might offer about these topics would be greatly appreciated.

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R. Iranpour and H. H. J. Cox are with the Applied Research Group, City of Los Angeles, California, Bureau of Sanitation. F. Altatriste-Mondragon and S. V. Diaz-Perez are with the Department of Civil and Environmental Engineering and Department of Human Genetics, respectively, of the University of California, Los Angeles.

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Closure

B. E. Rittmann

The upbeat and forward-looking comments by Iranpour et al. highlight why the emerging tools from molecular biology hold great promise, but also "have a ways to go" before the promise is realized in practice. The first two examples given by the discussers point out the kind of high-level information that molecular tools need to provide about specialized microorganisms that may be only a small fraction of the biomass, but are essential to the success of the process.

In the first example, the buildup of sulfurous organic compounds in a thermophilic digester may come about because the higher temperature selects against key microorganisms that transform the organic sulfides to inorganic sulfide. Alternately, the buildup of organic sulfides may be caused by the selection of bacteria that transform organic sulfur from innocuous forms to the odorous forms. At least in principle, molecular techniques can be used to identify and track these types of specialized microorganisms, if they are at the root of the problem with organic sulfides.

In the second example, the discussers seek a rapid, reliable, and low-cost means to detect small numbers of pathogens in biosolids. Molecular methods directed towards the small subunit ribosomal RNA (SSU rRNA) should meet these criteria. Unlike DNA, rRNA disappears rapidly when a cell dies. Thus, the problem of false positives is minimized when rRNA is the target. Although still relatively new, commercial kits make it possible to do hybridization to the rRNA rapidly (about three hours), without specialized laboratory equipment other than an epifluorescent microscope, and for a modest cost per sample.

The third example and the final paragraphs of the Discussion pose many good questions about what can be targeted, reliability, and what to do next. The potential is great, and the field is still wide open. The following are some of my ideas of advances that seem feasible *and* are needed to put molecular methods into routine use:

- We need to make quantification of the results of molecular assays more accurate and reliable. For example, we need to be able to convert hybridization results, which are in terms of the mass of rRNA, to the amount of active biomass. We do not yet know how conversions differ for among microbial types or relevant physiological conditions of the microorganisms (such as specific growth rate). Quantification is more tenuous when amplification is required, but recent developments in quantitative PCR techniques offer promise (e.g., Dionisi et al., 2002).
- We need to be able to assay for many different microorganisms simultaneously. One-by-one measurement is too time-consuming and costly for routine use, unless we are interested in only one or two microorganisms. For environmental engineering applications, we usually need to track many organisms. Fortunately, DNA microarrays (e.g., Guschin et al., 1997; Koizumi et al., 2002; Loy et al., 2002) offer great promise for being able to do hundreds or even thousands of hybridizations at one time.
- We need to be able to measure targets that tell us about the function of the community, or what activities its members are carrying out. The best target may be the messenger RNA (mRNA), and tools are being developed for this target (e.g., Bustin, 2000). mRNA is not stable, which means that it must be rapidly transcribed to DNA before analysis, a step that adds complexity and may introduce biases. And, each cell could produce mRNA from hundreds or thousands of genes, which means that we need to be able to assay simultaneously for many different mRNA products. Microarray or fingerprinting techniques offer promise.
- We need to emphasize the connections between results obtained from molecular tools and the results obtained from measurements of system performance. The second theme of my paper was that molecular tools are complements to traditional measures, not substitutes. The City of Los Angeles should not and will not stop measuring all of the parameters shown in Table 1 (see Discussion by Iranpour et al.) once they have a wonderful molecular method to detect microorganisms implicated in the buildup of organic sulfides. Instead, they will be able to understand how temperature, community structure, and system performance interact so that they can achieve good performance all of the time. Then, they can have the complete picture of the microbial community, what it is doing, and how it can be controlled.

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