MICROBIAL PROCESSES AND PRACTICAL GUIDANCE FOR ON-SITE ASSESSMENT

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Abstract

The microbial physiology of wastewater treatment can be summarised under five main headings: Oxidation, Hydrolysis, Acid Fermentation, Obligate Hydrogen Proton Production and Methanogenesis. These basic microbial processes facilitate the decomposition of organic waste, the reduction of Biochemical Oxygen Demand (BOD), the generation of noxious gases and contribute to the reduction of the pathogenic load in effluent streams.

Emerging molecular techniques have shown that we can only culture a small percentage of the bacteria identified by molecular sequences and since many new bacteria are being found, it is unlikely that a thorough understanding of microbial ecology will be had within the near future. Wastewater microbiology continues to be one area in which much progress has been made. The benefits of the recent revolution in molecular biology are becoming readily available and this provides wastewater microbiologist with access to techniques that can be used to quickly elucidate microbial community biodiversity and structure. It is however, unlikely that molecular techniques will be routinely applied in the wastewater industry in the immediate future.

This paper contrasts the interesting dichotomy between the ability to measure molecular microbial ecology by culture independent methods and the manipulation of the microbial ecology of on-site wastewater treatment systems for sustaining system performance and maintaining public health. Based on microbial ecology, suggestions are made for suitable on-site assessment of organic and bacteriological loads.

Keywords

bCOD, DNA microarray, microbial ecology, 16s rRNA, RFLP, SOUR,

1 The Genetic Diversity of Microbial Communities in Wastewater

Historically, microbiologists focused on medically and agriculturally important microbes utilizing culture dependant and biochemical techniques. We now know the vast majority of naturally occurring microbes are unculturable by conventional methods (Bergquist and Saul 1996). The recognition of Archaea as distinct life forms by Woese and co-workers using molecular techniques (Woese and Fox1977) has been hailed as one of the most significant developments in the history of microbiology. These molecular studies, based on genetic distances and signature sequences in the 16S ribosomal RNA (rRNA) gene revealed a phylogenetic tree of procaryotic and eukaryotic life based on three distinct groups or 'Domains' (Woese 1987). The Archaebacteria are more widely distributed and are common co-inhabitants with bacteria, protists and nematodes in composting biomass and wastewater. The major constituents of the microbial communities in wastewater are Eubacteria. Archaea, including methanogenic organisms, are common in anaerobic sludge environments. The Eucarya, present in wastewater and activated sludges include Protista, protozoa, nematodes, and fungi. The Eubacteria support a community of eucaryotic organisms that graze on them in these mesophilic wastewater environments.

2 Understanding the Diversity of Genomes in Microbial Communities

Hybridisation of DNA extracted from whole communities compared with that obtained from individual species, or other communities, first allowed the determination of the presence or absence of defined taxa. Based on DNA hybridisation studies it was estimated that there are at least 4000 separate microbial genomes per gram of Norwegian Forest soil (Torsvik *et al.* 1990). If Torsvik's data reflects the general extent of biodiversity and each genome represents an individual species, the number of species in a single soil sample exceeds the number of all described procaryotic species (Blackall 2000). Such analysis suggests an inherent rich genetic diversity in wastewater and the environment generally.

A major review of the role and value of molecular techniques in understanding wastewater treatment processes is given by Amann *et al.*, (1998) and further expanded by Blackall (2000).

3 Microbial Metabolism in Wastewater

The food and other material we ingest are subjected to both acid hydrolysis and enzymatic digestion. It passes through our intestine in a relatively short period (6 – 28 hours) under substantially anaerobic conditions. Any waste we eliminate already has both undigested and pre-digested components that are readily available for physiochemical or biological oxidation. This biologically active waste is subsequently combined with urine (which is considered sterile when it leaves the body), and greywater. This mixture provides the substrate for a wide variety of naturally occurring microorganisms. The continued digestion of waste by microbes that either leave the body or enter the waste stream from other sources remains the (principal) mechanism by which the BOD is reduced.

Physiological studies of mixed microbial communities in waste have been proceeding for some time and hence they are generally well understood. They form the basis of our understanding of the five principal modes of the microbial breakdown of organic matter. However, unculturable microbial candidates, identified by molecular techniques based on the possession of functional genes, and other major organisms involved in the biogeochemical transformations of organic waste have only recently been identified (Blackall, 2000).

The basic microbiological processes which facilitate the decomposition of organic waste, the reduction of BOD, the generation of non-toxic and other gases coupled with the reduction of the pathogenic load in wastewater treatment processes include:

3.1 Oxidation

When wastewater enters the treatment process the resulting turbulence impacts on both the existing microbial community and the readily oxidisable (chemically reduced) high BOD (i.e., 300-600 mg BOD /litre) substrates. This process is further enhanced by any secondary treatment process such as aeration or trickling filters. Any free available oxygen (the maximum available dissolved oxygen approximates only 8mg/l) is quickly used up in the process. The presence of aerobic organisms in wastewater, whilst preferable (because of their higher rate and quality of 'treatment') must be facilitated by secondary treatment processes. Aerobic microbes are generally a minor component of septic systems. Free oxygen is also toxic to some fastidious anaerobic microorganisms (such as methanogens), which need to form complex associations with other facultative anaerobes around particulate matter in order to achieve their niche in the community.

3.2 Hydrolysis

The most common microbial process resulting in the breakdown of organic matter is hydrolysis of waste compounds facilitated by microbial exoenzymes including amylases, proteases, lipases and ureases. This results in the breakdown of relatively large molecules into smaller ones that may diffuse through the cell membranes of microorganisms and become utilised in fermentation.

3.3 Acid Fermentation

The products of hydrolysis are further broken down by a rich variety of fermentation pathways inside bacteria producing smaller, often volatile organic carbonaceous substances (VOCs). These are the source of many odours from treatment plants. A byproduct of fermentation is the increase in hydrogen ion concentration or acidity. Thus, the term acid fermentation is used to describe this major process in the microbial digestion of organic material in the wastewater.

3.4 Obligate Hydrogen Proton (OHP) Production

In the presence of increasing acidity, another fastidious group of OHP microbes further break down any intermediate organic compounds into smaller molecules such as acetate, lactate and formate. They often use inorganic sources of oxygen from nitrates, sulphates or iron oxides in their metabolism and produce carbon dioxide as a waste product.

3.5 Methanogenesis

Methanogens are a fastidious physiological group requiring acetate or formate as their sole energy source and a ready supply of free hydrogen ion to produce methane. Methanogen physiology is such that it only occurs in a near neutral pH environment. The methanogens are usually embedded in metabolically structured granular formations and are active in the anaerobic sludge that accumulates in primary tanks and in the water-saturated zones of drainfields.

Therefore, wastewater microbial communities are composed of five basic trophic levels. The concerted action by microbes of fermentation, acetogenic hydrogen production and methanogenesis are required for the partial to complete degradation of organic compounds to methane and carbon dioxide. Disturbances in microbial populations at one trophic level in these systems affect the entire community and cause imbalances in performance or failures of on-site systems. This phenomenon is regularly observed in the field and has been demonstrated in a methanogenic bioreactor's performance by the accumulation of intermediates, pH changes or reduced efficiency (Schink 1988).

4 Ecological Balances in On-Site Wastewater Systems

On-site wastewater treatment relies on the growth of microbial consortia on the organic matter and inorganic nutrients in the influent wastewater. These materials are eventually transformed by the microbial ecosystem into innocuous compounds that may accumulate in the treatment system but can be either discharged into (i) the atmosphere if they are volatile, (ii) into a holding soil or water body, (iii) to land or landfill as sludge or (iv) they can be recycled as biosolids. In some cases when the chemical transformation into simpler molecules has not occurred completely (e.g. halogenated organics), the pollutant may be complexed onto or contained within the microbial biomass. Disturbances are caused by various substrates such as bleach, caustic substances, washing powders, surface-active ingredients or cleaning agents, heavy metals and the use of antibiotics and chemotherapeutic agents by the occupants.

5 Ecosystem Complexity

Our normal understanding of an ecological community is limited, even on a macroscale, for at this level we observe a myriad of complex and diverse associations between its members. A 'simple' climax plant community, for example, appears to be uniform to the eye from a distance but once below the canopy, integrative structure is apparent. Integrative structure goes beyond a holistic view of a community physiology and partitions it into functional entities.

An illustration of the integrative diversity that is apparent in a wastewater microbial community is found in the study by Juretschko *et al.* (1998). This study of a wastewater stream used Fluorescent In-Situ Hybridisation (FISH) to demonstrate that eubacterial nitrifiers are clustered and that the ammonia oxidiser clusters are closely juxtaposed to the nitrite oxidiser clusters. These microbes have a mutualistic association and rely each others byproducts of nitrogen metabolism; they structure their populations together in close association. This means that the microbial community within wastewater has its nitrogen metabolism more highly structured rather than being just randomly distributed throughout the wastewater.

Microbial communities therefore are complex assemblages of populations. Each community is usually characterised by diverse phylogenies and a hierarchy of physiologies. Generally, it was thought that to characterize microbial communities they needed to be brought into cultivation but it is now accepted that well over 90% of the members of an environmental microbial community cannot be cultured (Bergquist and Saul 1996).

The dynamics of microbial communities are determined by the interaction of environmental variation and general biotic properties, including the genetic potential of the populations in the community. This interaction can occur on both spatial and temporal scales. Hence, the expectation is that community dynamics will be complex.

Maurer (1987) states that previous theoretical approaches to community dynamics have assumed linear, near equilibrium states with the alternative approach assuming the community dynamics are the result of the balance between energy use by the community and its tendency to move towards thermodynamic equilibrium. In the latter case, this would lead to the extinction of all species in the community as all the available energy is dissipated. Because this balance will be imprecise and microbial communities are generally not closed systems; community dynamics are expected to be oscillatory. If a "standing crop" of microbial biomass became established it must contain representatives from each physiological niche depending upon the available nutrients. These representatives may be in a reproductive viable, dormant or spore form but one generalised community structure would dominate a balanced wastewater treatment stage.

A generalised community structure common among on-site treatment system designs would allow molecular comparisons to be made. Generally whole communities can be compared or individual taxons or species can be analysed by Amplified Ribosomal DNA Restriction Analysis (ARDRA) of PCR products of the 16S rRNA gene (see figure 1).

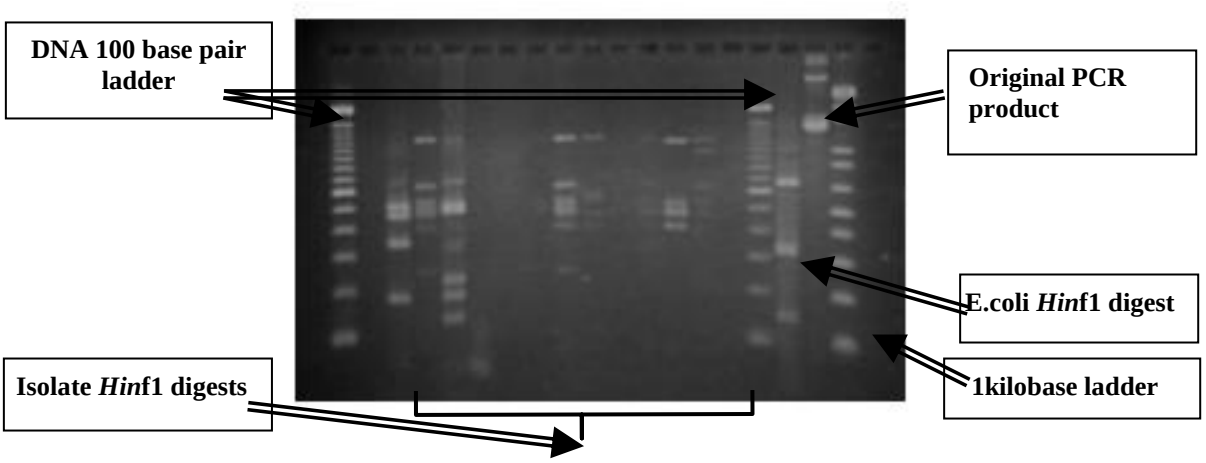
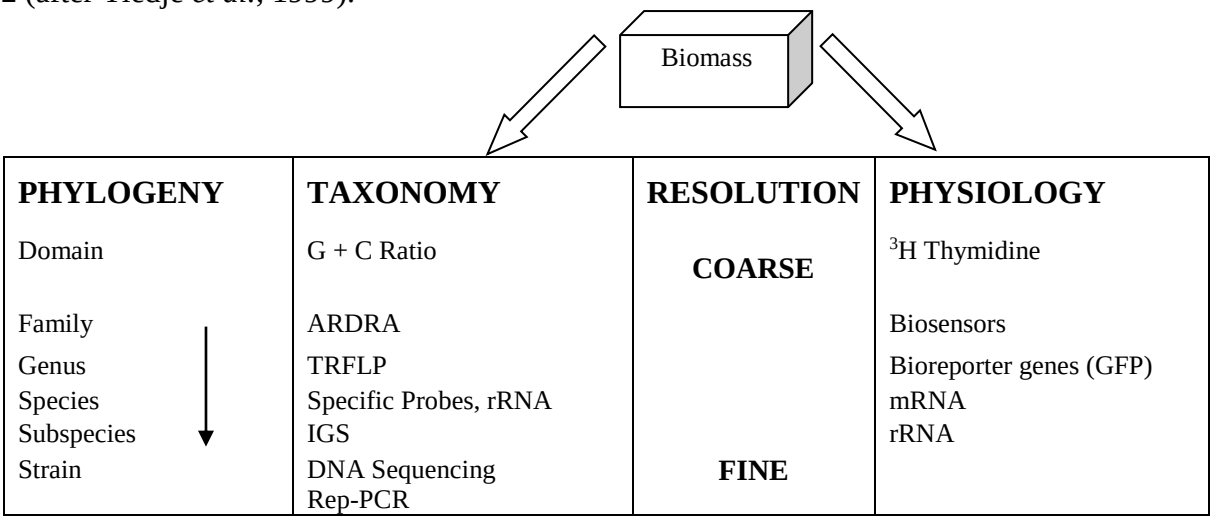


Figure 1. *Hin* f1 ARDRA of isolated species from wastewater

An alternative approach to the analysis of 16S rRNA gene PCR is to use the 16S–23S rRNA intergenic spacer (IGS) between the *rrn* (small sub unit) and *rrl* (large sub unit*)* genes (Ranjard et al 2000) complete sequencing of the 16S rRNA gene or other targeted genes if physiological and lower divisional analysis is required. Taxonomic analysis now requires that a 95% - 100% rRNA gene homology is needed to consider two organisms to belong to the same species. The framework of molecular methods available provides different levels of resolution and penetration with regard to community structure and activity is shown in figure 2 (after Tiedje *et al.*, 1999).



*modified from Tiedje *et al.*, (1999)

Figure 2: Microbial Community Analysis by Culture Independent Molecular Methods*

A microbial community is defined as an assemblage of populations within a community that tend to interact with other microbes within that community and not with microbial populations in other communities (Swift 1984). In other words the microbial communities which arise in any given wastewater treatment stage or process are unique in that they consist of a number of populations of microorganisms which exist in situ. The microbial community that assemble in evapotranspiration trenches, for example, arrive as components of the wastewater stream or by other means such as vectors, spores, or are already present in the soil. Generally, these microbes are endemic to the region and many are cosmopolitan species. They form a complex structured microbial community that reflects the directional movement of water and nutrients. The biomat that forms on the edges of the trench is a prime example of the highly complex nature of the microbial community as the medium moves from the

aggregate fill through to the soil medium. We can expect the communities within separate compartments of an on-site wastewater treatment plant will related but dominated by a few major physiologically important taxa. This may give rise to a distinct genetic DNA fingerprint (see figure 1) shown by distinct bands in the gel as opposed to a smear of DNA in a highly complex community.

A recent addition to microbial community analysis is terminal restriction fragment length polymorphisms (T-RFLP) (see Fig 3). This approach permits the rapid profiling of a microbial community. Briefly, 16S rRNA genes are PCR amplified from community DNA directly extracted from the environment of interest. The forward primer is labelled at the 5' terminus with a fluorescent dye that allows the investigator to track only the terminal fragment of a restriction digest. Generally, a single species will contribute a uniquely sized terminal fragment although several species, often closely related, may have terminal fragments of identical size, (http://www.cme.msu.edu/RDP/trflp/). T-RFLP is both sensitive and specific and can identify slight changes in the structure of microbial communities over time. This molecular approach has demonstrated the ability to rapidly detect and identify microorganisms unique to experimental treatments and provides a means to fingerprint microbial communities in the biosystems being developed at NASA for optimising advanced life support systems (Kerkhof *et al.*, 2000).

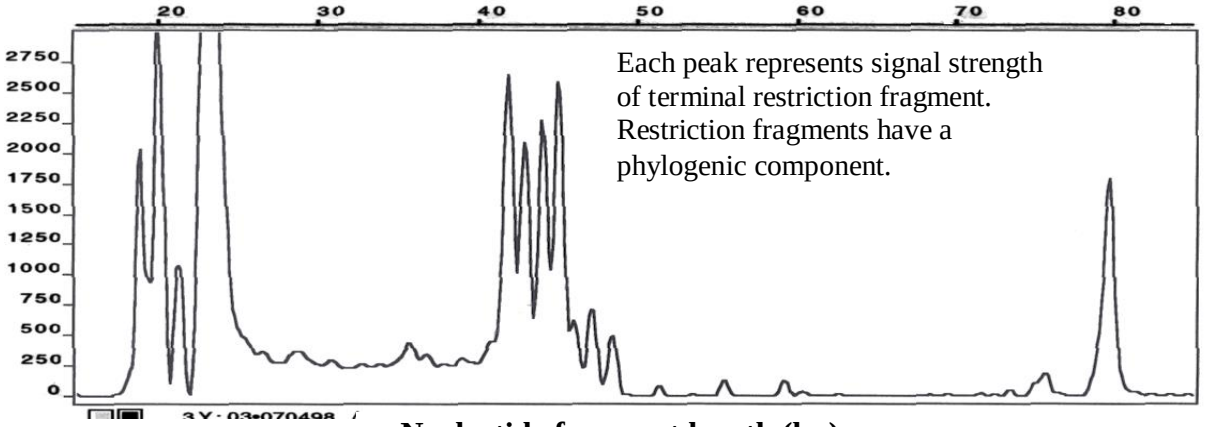


Figure 3: T-RFLP of a typical wastewater community

7. Recommendations

There are at present no regulatory requirements for measuring microbiological or oxygen demanding loads discharging from on-site wastewater treatment plants. However under ANZS1547: 2000, local regulatory authorities have the power to require regular prescribed inspections of on-site systems and culture based microbial assays may be required. These laboratory-based tests remain expensive for the consumer. Other tests that give much the same level of specificity can be applied routinely in the field. One of the best indicators for a microbial load is the presence of particulate matter since microbes have a preference for adhering to particulate matter since it provides a surface upon which the microbes have close access to energy providing substrates either from biomass or from other microbial byproducts of metabolism. If treatment is efficient, there are minimal particulates in the final effluent. Final effluents with high levels of particulate matter will clog drainfields. Therefore, assays based on turbidity, or a solid settling index of final effluent, such as a modified (i.e. by facilitating sedimentation with alum) sludge volume index at 30 minutes (SV30) can provide valuable information on the performance of on-site systems and potential risks to public health. Anecdotal evidence indicates the presence of flocs in the final effluent of on-site

systems, after the addition of alum, will indicate a high carry over of suspended solids and associated bacterial biomass.

A Standard Oxygen Uptake Rate (SOUR), at 30 minutes, for final on-site effluent can be correlated with BOD and is easy to do using Chemetrics™ comparisons. When the standard oxygen curve for BOD is analysed the uptake rate of oxygen by the wastewater in a SOUR assay can be multiplied by the initial rate divided by the five-day rate then extrapolated to the five-day rate to give an on-site BOD (Equation 1).

$$BOD_{on\text{-}site} = (\text{DO time zero (10)} - \text{DO time 30min}) \times 240 \times (\text{Slope average } BOD_5 / \text{slope initial } BOD_5) \quad \textbf{(Equation1)}$$

Table 1 shows a SOUR comparison of treated and untreated waste from four rural townships.

Table 1. A Comparison of SOUR with BOD_5 (Correlation 0.96)

SITE	BOD_5	SOUR
Branxton Raw	205	10
Cessnock Raw	148	9
Farley Raw	99	8
Kurri Raw	137	9
Branxton Clarifier	3	0
Cessnock Humus	24	1
Farley Clarifier	3	0
Kurri Clarifier	21	1

Alternatively, a modified *biological* Chemical Oxygen Demand (bCOD) can be performed to give similar information. These assays can reflect the efficiency of treatment in both septic and aerated systems.

8. Discussion

The use of molecular methods in wastewater research has allowed the description of new species of bacteria for nitrification (Burrell *et al.*, 1998, Mobarry *et al.*, 1996) and the identification of phosphate accumulating organisms (PAOs) in Enhanced Biological Phosphorus Removal (EBPR) treatment plants (Bond *et al.*, 1999; Nielsen *et al.*, 1999). It is most likely that the organisms responsible for these biotransformations in on-site systems are very closely related. Such progress in the development of molecular methods will allow us, in principle, to determine not only the taxonomic relatedness of any isolate to previously described species but also the genomic variety of microbial communities from one on-site system to the next.

The wastewater industry however, requires time-efficient, reliable, low cost and reproducible methods to assess microbial populations to assess public health risks and the efficiency of essential microbial processes (nitrification, denitrification, phosphate accumulation, methanogenesis, etc). Molecular methods are not yet routinely available. They will eventually be utilised in industry in the form of DNA microarray technology. DNA microarrays typically consist of up to 20,000 individual spots, which each contain millions of copies of a specific genetic probe. DNA microarrays can be used to detect pathogenic bacteria and virus and/or specific physiological markers). The technology of DNA microarrays will eventually be modified for use in the field (Bavykin, 2001 #102). At present, quality control and therefore reliability of a direct complex analysis of mixed microbial communities in wastewater is difficult to achieve and maintain. In the meantime simplified assays based on similar approaches that are used in municipal wastewater treatment facilities need to be adopted for on-site microbiological analysis.

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