Novel Applications of Molecular Biological and Microscopic Tools in Environmental Engineering

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ABSTRACT: Molecular biological methods offer flexible and powerful tools to environmental practitioners and researchers interested in studying environmental challenges in natural and engineered systems. In recent years, these techniques have allowed investigators to connect the fate, transport, and transformation of environmental chemical contaminants and pathogens with biological processes of functionally diverse microorganisms or microbial communities. Indeed, the boundaries of microbial ecosystems are constantly refined as researchers discover new links that extend beyond

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Bacteria to include Archaea and unicellular Eukarya. Quantitative polymerase chain reaction (qPCR) provides a rapid and sensitive approach to determine gene abundance and expression from a wide range of microorganisms from complex environments. Whole genome arrays (WGA) and functional gene arrays (FGA) are being used to elucidate transcriptional changes in response to environmental parameters. Antibiotic resistance profiling and microbial source tracking studies continue to benefit from the information provided by a molecular-based experimental design. Quantitative fluorescent in situ hybridization (qFISH) and next generation sequencing technologies are changing the way we view suspended solids in wastewater treatment. Innovative sensors are being developed that couple molecular biological, chemical, or physical properties to improve the sensitivity and specificity for intended targets. Thus, advanced molecular analysis complements conventional approaches to provide a more

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comprehensive understanding of microbes and microbial processes in the environment. The purpose of this review is to summarize high impact studies and novel applications of molecular methods for environmental engineering professionals and scientists.

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Optimization or Minor Modification of Existing Methods and Reviews

Limpiyakorn *et al.* (2013) reviewed studies suggesting the presence of ammonia-oxidizing archaea (AOA) in wastewater treatment plants. They reference a number of studies that have used molecular-based method to prove the presence of archael ammonia monooxygenase (*amoA*) genes at wastewater treatment plants, but point out that more advanced molecular techniques (such as MAR-FISH and stable isotope probing of nucleic acids) are needed to better understand the activity, function, abundance, and diversity of *amoA*-encoding archaea in wasterwater treatment plants.

Parawira (2012) reviewed research and applications of extracellular enzymes (particularly cellulases and lipases) in the pre-treatment of complex organic matter for anaerobic digestion. A number of studies were found in this review that demonstrated increased biogas production from complex organic matter, such as lignocellulosic material and sewage sludge, when pre-treated enzymatically with cellulases and cellulase-producing microorganisms. Similarly, pre-treatment of lipid-rich wastewater from food processing industries with lipases and lipase-producing microorganisms improved degradation of the waste sludge and an enhancement in methane production.

Syed and Yadav (2012) provided a review of their research on the P450 monooxygenases in the model white rot fungus *Phanerochaete chrysosporium*, which has been studied for decades for its ability to degrade lignin and a range of xenobiotic compounds. The degradation ability of *Phanerochaete chrysosporium* was originally attributed to its extracellular peroxidases. However, the whole genome sequence of this fungus revealed the presence of 149 P450 monooxygenase systems that are being studied and characterized for their biodegradative potential.

A review by Rosselló-Móra (2012) claims that the current methods used for taxonomic classification require significant laboratory experimentation, and generally will not produce interactive databases. According to the author, new high-throughput metabolomic technologies, such as ICR-FT and MALDI-TOF mass spectrometry methods, will open the door to the construction of metabolic databases for taxonomic purposes. It is to be foreseen that, in the future, taxonomists will benefit significantly from public databases speeding up the classification process. However, serious effort will be needed to harmonize them and to prevent inaccurate material.

In a web-alert titled “Annotation for environmental metagenomes,“ Wackett (2012) has enlisted

an annotated selection of World Wide Web sites relevant to the topics in environmental microbiology.

Behrens *et al.* (2012) review the technical capabilities and limitations of high-resolution secondary ion mass spectrometry (NanoSIMS) and scanning transmission (soft) X-ray microscopy (STXM) and give examples of their applications. Whereas NanoSIMS can be combined with isotope-labelling, thereby localizing the distribution of cellular activities (e.g. carbon/nitrogen fixation/turnover), STXM provides information on the location and chemical speciation of metabolites and products of redox reactions. The authors further propose that the combined use of both techniques and discuss the technical challenges of their joint application. Both techniques have the potential to enhance our understanding of cellular mechanisms and activities that contribute to microbially mediated processes, such as the biogeochemical cycling of elements, the transformation of contaminants and the precipitation of mineral phases.

Maurice and Turnbaugh (2013) discuss recent work that highlights the potential for assessing the human microbiome at a range of spatial scales, and for developing novel techniques that bridge multiple levels: for example, through the combination of single-cell methods and metagenomic sequencing. These studies promise to not only provide a much-needed epidemiological and ecological context for mechanistic studies of culturable and genetically tractable microorganisms, but may also lead to the discovery of fundamental rules that govern the assembly and function of host-associated microbial communities.

The suspended-growth and biofilms communities from an integrated fixed film activated sludge (IFAS) process were analyzed using denaturing gradient gel electrophoresis (DGGE) and FISH. Denitrifying bacteria were identified in the biofilm and species associated with the genera *Firmicutes* and *Pseudomonas* were implicated in organic degradation Li *et al.* (2012a).

A combination of DGGE, FISH and cloning and sequencing were used to explore the composition of sludge from a single-stage deammonification process. AOB associated with *Betaproteobacteria* were identified in the first 50-100 μm of the sludge granule and *Planctomycetes* were dominant at depth between 100- 200 μm Liu *et al.* (2013).

Qiu *et al.* (2012) evaluated the effects of alternative carbon substrates on the microbial community structure during anerobic debromination of polybrominated diphenyl ethers (PBDEs). DGGE was used to identify significant microbial community shifts associated with different electron donors and *Pseudomonas* spp. were found to be the dominant organism. In addition, microbial community structure was found to be correlated with the concentrations of PBDE breakdown products and total nitrogen. This study found that specific electron donors influence the biotransformation rates by changing the microbial community structures.

Guo and Zhang (2013) evaluated seven common DNA extraction kits based on yield, purity and sequencing results from activated sludge samples. They determined that bead-beating is a necessary step for DNA extraction from activated sludge. Among kits that had a bead-beating

step, they found that the FastDNA SPIN Kit for Soil extracted the most and purest DNA. Specifically, they discovered that kits without bead-beating yielded low amounts of DNA and under estimated Gram-positive *Actinobacteria*, *Nitrospirae*, *Chloroflexi*, and *Alphaproteobacteria*.

The bacterial community of wastewater effluent was studied to understand the interrelationships of antibiotic residues and antibiotic resistance genes with community dynamics in wastewater treatment Novo *et al.* (2013). DGGE was used to quantify changes in bacterial community with an emphasis on the effects of exposure to antibiotics. This bacterial community study provides a multivariate analysis into changes to community structure with plant operations and physciochemcial parameters.

Mielczarek *et al.* (2013) evaluated the bacterial population dynamics of an enhanced biological phosphorus removal process. This study incorporated quantitative fluorescent in situ hybridization (qFISH) to quantify population dynamics of polyphosphate-accumulating organisms (PAO) and glycogen-accumulating organisms (GAO) in different wastewater treatment plants. This study demonstrated that GAO were not the core species involved in EPBR communities.

Chen *et al.* (2013) studied the microbial community at a full-scale anoxic-aerobic-anoxic-aerobic (AOAO) process treating optoelectronic wastewater. PCR and DGGE were used to identifiy *Janthinobacterium* and *Nitrosospira* as dominant species in each of the AOAO process that was able to remove over 90% of both BOD and ammonia. This approach was also able to show that different strains of *Janthinobacterium* were dominant in different processes suggesting niche adaptation within the same species.

The temporal variability in bacterial diversity was studied in a free-chlorinated drinking water distribution system by McCoy and VanBriesen (2012). Bacterial populations were identified and quantified over several years using a host of molecular analyses including 16S rRNA gene clone libraries, qPCR, and DGGE. This study found that *alpha-proteobacteria* and *beta-proteobacteria* were most sensitive to seasonal shifts. The qPCR results demonstrated decreased *alpha-* and *beta-proteobacteria* in May and October suggesting that these shifts may be influenced by changes in chlorine dosing. This study shows the sensitivity of bacterial diversity analysis as potential indicators of disruptions to the water distribution system.

Belila *et al.* (2012) applied DGGE of PCR-amplified fragments of the 16S rRNA gene and of different functional genes involved in sulfur metabolism (*dsrB*, *aprA*, *pufM*) to study the microbial diversity and in particular the different functional groups of sulfur bacteria present in wastewater stabilization ponds in Tunisia. Although the dissimilatory sulfite reductase gene *dsrB* and the adenosine-5'-phosphate gene *aprA* are found in both sulfur-oxidizing bacteria and sulfate-reducing bacteria, the *dsrB* primers in this study only targeted sulfate-reducing bacteria. The *pufM* gene, which encodes that M subunit of the photosynthetic unit-forming gene of purple phototrophic bacteria, is not directly involved in sulfur oxidation processes, but allows the detection of purple

phototrophic bacteria which are able to oxidize sulfide. Therefore, a combination of 16S rRNA, *dsrB*, and *aprA* was used to describe the diversity of sulfate-reducing bacteria, while the combination of 16 rRNA, *aprA*, and *pufM* was used to identify the sulfur-oxidizing representatives present in the waste stabilization ponds.

Burns *et al.* (2012) studied the effectiveness of a passive flow sulfate-reducing bioreactor processing acid mine drainage generated from an abandoned coal mine. A microbial community analysis identified significant differences between pre-treated and post-treated water. Pre-treated samples were dominated by iron-oxidizing *Betaproteobacteria*, while sulfur-oxidizing *Epsilonproteobacteria* dominated the post-treated water. In addition, analysis of dissimilatory sulfite reductase genes (*dsrAB*) determined that there was limited diversity in the sulfate reducing microbial community.

Enhanced metals bioremediation was studied in *Cupriavidus metallidurans* CH34 Biondo *et al.* (2012). Biomolecular engineering was used to express a synthetic phytochelatin gene in this bacterium. The recombinant strain showed an increased ability to immobilize metal ions from the external medium when compared to the control strain for all divalent metals evaluated. This study demonstrates the advantages of biomolecular engineering to enhance and expand the ability of microorganisms for potential applications in bioremediation.

Heavner *et al.* (2013) used molecular biomarker data to construct a biokinetic model for PCE-dechlorination. 16S rRNA quantified by qPCR did not provide accurate model fits when feeding rates were higher demonstrating that biokinetic modeling using molecular biomarkers is negatively influenced by competitive inhibition. However, inclusion of an mRNA adjustment factor allows accurate modeling of dechlorination activity even under high feeding rates.

Biodegradation of polycyclic aromatic hydrocarbons was investigated in samples collected from the South Korean tidal flat impacted by an oil spill in 2007 Jin *et al.* (2012). This study used PCR, DGGE, and RT-PCR to identify *Alteromonas* as an active player in PAH biodegradation. The results of this study were used to show that Alteromonas is a native bacterium in the contaminated sea-tidal flats and these bacteria were adapted to colder climates as evidenced by their higher concentrations in the winter.

Zhu *et al.* (2012) investigated the expression of genes involved in energy conservation and community structure of attached and free acidophilic bacteria in chalcopyrite bioleaching. Quantitative real-time PCR revealed that sulfur oxidation genes of attached Acidithiobacillus ferrooxidans were up regulated, while ferrous iron oxidation were down-regulated compared with free Acidithiobacillus ferrooxidans due to the presence of elemental sulfur on the mineral surface. The community structures of free and attached bacteria were determined by DGGE-PCR amplification of 16S rRNA fragments, which showed the sulfur-oxidizing Acidithiobacillus thiooxidans dominating the attached bacteria while the ferrous-oxidizing Leptospirillum ferooxidans dominated the free bacteria.

Winkler *et al.* (2012a) used fluorescent in situ hybridization (FISH) and qPCR to analyze the nitrifying microbial community within aerobic granular sludge treatment systems and within one conventional system treating municipal wastewater. In acetate-fed aerobic granules, Nitrobacter was found to be the dominant NOB. The ratio of NOB to AOB in the aerobic granular sludge was elevated but not in the conventional systems. The elevated NOB/AOB ratio was supported by activity measurements which showed an approximately threefold higher nitrite oxidizing capacity than ammonium oxidizing capacity.

Mbadinga *et al.* (2012) screened genomic DNA from n-alkane-dependent thermophilic enrichment cultures from productions waters of a high-temperature petroleum reservoir for the presence of new alkylsuccinate synthase alpha-subunit (*assA*) homologues. Clone libraries were constructed from amplicons of *assA*, 16S rRNA, and *mcrA* (methyl-coenzyme M reductase subunit A) gene fragments. Sequencing analysis of the clones discovered the presence of new *assA* homologue sequences, as well as the presence of several bacteria related to Firmicutes, Thermodesulfobiaceae, Thermotagaceae, Nitrospriaceae, Dictyoogloceae, Candidate division OP8 , and Archaea related to uncultured members in the order Archaeoglobales and CO2-reducing methanogens.

Du *et al.* (2012) constructed a high-phosphate-accumulating microorganism by inserting a vector expressing a polyphosphate kinase (*ppk*) from *Microcystis aeruginosa* NIES843 in *Pseudomonas putida* KT2440. qPCR analysis of *ppk* confirmed that the transgenic bacteria, KTPPK, was maintained at a high density in a sequencing batch biofilm reactor, allowing for enhanced removal of phosphorous.

Gougoulias and Shaw (2012) evaluated the specificity of a 16S rRNA-targeted fluorescence in situ hybridization probe when applied to Lolium perenne rhizosphere soil. The PSE1284 probe, which targets Pseudomonads, was used in conjunction with flow cytometry for cell sorting. The 6-carboxyfluorescein (6-FAM)-PSE1284-hybridized population comprised 3.51±1.28% of the total population. Analysis of 16S rRNA gene libraries constructed from the cell-sorted recovered fluorescent populations (n=3), revealed that 98.5% were homologous to *Pseudomonas* spp. (68.7%) and *Burkholderia* spp. (29.8%). In silico sequence analysis justified the presence of *Burkholderia* spp. among the sorted cells. Rarefaction and DGGE analysis of the 16S rRNA sequences were used to evaluate the diversity of Pseudomonads.

Krishnamurthi and Chakrabarti (2013) revealed the diversity of Bacteria, using culture-dependent and culture-independent molecular approaches, and the diversity of Archaea (by only the latter approach) in a landfill in Chandigarth, India. Phenotypic characteristics as well as 16S rRNA gene sequence analysis of cultures isolated on a variety solid agar media were used to assess bacterial community structure. Culture-independent analysis of two 16S rRNA gene libraries was also performed. Both the culture and culture-independent techniques revealed that members of the phylum Firmicutes were extremely dominant in the landfill. The diversity of

Archaea determined by Archael-specific 16S rRNA primers was limited to members of two orders: Methanosarcinales and Methanomicrobiales.

Kojima *et al.* (2012) investigated the distribution of putative denitrifying, methane-oxidizing bacteria, related to *Cadidatus Methylomirablis oxyfera* of the candidate phylum NC10, in a mesotrophic freshwater lake (Lake Biwa, Japan). Sequence analysis of clone libraries of 16S rRNA genes and particulate methane monooxygene genes (*pmoA*) in the sediments of the lake contained sequences related to *M. oxyfera*. The presence of *M. oxyfera* and NC10 bacteria was also confirmed by catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH). Denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (qPCR) revealed a higher abundance of *M. oxyfera*-related phylotypes in the upper layers of the profundal sediment.

Lodders and Kampfer (2012) employed cultivation and cultivation-independent approaches to study the bacterial diversity in water-miscible metalworking fluid and water preparation basis samples from industrial plants in Germany, in order to assess the potential that aerosols produced from these liquids may contain bacteria and fungi that may pose a health risk to workers. Clone libraries of 16S rRNA genes of isolates and of DNA extracted directly from the fluids were sequenced. The metal working fluids showed high bacterial diversity that differed significantly from those found in the water preparation basis samples.

Valm *et al.* (2012) present the theory and guidelines for performing the novel technique CLASI-FISH (combinatorial labeling and spectral imaging – fluorescence in situ hybridization) to expand the number of taxa that can be distinguished in a single FISH experiment. The strategy of CLASI-FISH involves labeling a given type of microorganisms with two or more fluorophores. This technique relies on advancements in spectral imaging that allow the identification of fluorophores with highly overlapping excitation and emission spectra.

Kofoed *et al.* (2012) tested the potential of using mRNA-targeted FISH and microsensor measurements to link microenvironments to specific functional guild of bacteria. This technique was assessed on an artificial biofilm of the denitrifier *Pseudomonas stutzeri* by relating the expression of nitrite reductase (*nirS*) to oxygen concentrations along the thickness of the biofilm. A distinct zone of *nirS* transcript-containing cells was detected at the oxic-anoxic zone in the biofilm.

Aslan and Rose (2013) studied the host specificity of a novel gene marker from *Bacteroides thetaiotaomicron* as an indicator of sewage contamination. Animal fecal samples and wastewater effluents were analyzed for the presence and quantity of the alpha-1,6-mannanase gene using qPCR. The results of this study demonstrated high specificity with human fecal contamination for the alpha-1-6, mannanase gene compared to current human markers.

The ecologogical interactions for two dehalorespiring bacterial populations was studied to understand their survival and the biotransformation of chlorinated ethenes in the environment Lai and Becker (2013). This study specifically quantified target populations using qPCR in anaerobic continuous-flow

stirred rank reactors and determined inhibition effects of PCE and TCE on VC dechlorination. This inhibition was exacerbated when grown in mixed cultures. Furthermore, the abundance and survival of *Dehalobacter restrictus* and *Dehalococcoides mccartyi* were only accurately predicted after including the inhibition interactions into the model.

Li *et al.* (2012d) monitored the microbial community structure of fixed-bed biofilm system performing perchlorate removal under different backwashing procedures using clone libraries and qPCR targeting the 16S rRNA gene. They demonstrated that changes in backwash intensity exerted more of a disturbance on the microbial community structure, and subsequently the performance of the rector, than changes in backwash frequency.

The biotransformation kinetics of micropollutants in wastewater treatment plants (WWTP) was associated with WWTP operational parameters Helbling *et al.* (2012). This study found that micropollutant oxidation was associated with ammonia removal. Analysis of *amoA* transcripts by qPCR determined that archael, but not bacterial transcripts associate with both ammonia removal and micropollutant transformation rates. These results suggest that ammonia removal and *amoA* transcript abundance are potential predictors of micropollutant biotransformation.

Rikmann *et al.* (2012) investigated the treatment of wastewater via sulfate-reducing ammonium oxidation (SRAO). This study found that the SRAO process could be established in a moving bed biofilm reactor (MBBR) or an upflow anaerobic sludge blanket reactor, however this process was sensitive to temperature. PCR and DGGE were used to identify and compare important bacteria involved in the SRAO process established in both reactor types. Although the isolates were different in both reactor types, total nitrogen removal rates were similar.

Abanoz *et al.* (2012) studied enhanced ethanol production in potato-processing wastewater using genetically engineered *Escherichia coli*. This study focused on fermentation of glucose by a genetically engineered strain of *E. coli* (FBR5) that was previously modified to produce ethanol as its sole fermentation product and expresses the VHb gene that has been shown to improve growth. Their findings demonstrate that VHb expression in genetically modified organisms can lead to increased yields of biofuels.

Narciso-da-Rocha *et al.* (2013) used partial *rpoB* gene sequences to identify to the species level different *Acinetobacter* spp. isolated from a municipal drinking water treatment plant and from connected tap water based. They also used an assessment of partial sequences of three housekeeping genes (*rpoB*, *gyrB*, and *recA*) to determine interspecies variation among isolates. The characterization of *Acinetobacter* diversity in water at the species and subspecies level by gene sequence analysis, in conjunction with antibiotic resistance susceptibility testing of the isolates, provided evidence that tap water may represent a potential exposure route of clinically relevant *Acinetobacter* to humans.

Wang *et al.* (2012) tested the application of casein as a competitor in improving RNA recovery from soils, including Andosols (volcanic ash soil), for gene

expression studies. It was proved in this study that autoclaved casein aids in releasing rRNA and mRNA from soil particles and in inhibiting rRNA and mRNA attachment to soil particles by competing with them for binding sites.

Gallo *et al.* (2012) successfully expressed the *alkB* gene, encoding an alkane monoxygenase, from the actinomycete *Gordonia* sp. SoCg in the non-alkane-degrading actinomycete *Streptomyces coelicolor* M145. The resulting engineered strain, M145-AH, was capable of growth on n-hexadecane as a sole carbon source. Proteomics analysis was performed on strain M145-AH using 2D-DIGE (two-dimensional differential gel electrophoresis) followed by MALDI-TOF or nLC-ESI-LIT/MS/MS for identification of protein spots. Comparative proteomic profiling revealed many proteins and pathways involved in the growth on n-hexadecane, including the β-oxidation pathway and the tricarboxylic acid (TCA) pathway.

Aktan *et al.* (2012) studied the inhibitory effects of free ammonia on Anammox bacteria in a lab-scale upflow fixed-bed reactor. The results of this study showed that anammox communities remained active at free ammonia concentrations up to 150 mg/L with significant inhibition experienced when ammonia concentrations reached 190 mg/L. Indeed, fluorescent in situ hybridization analysis detected anammox isolates at all free ammonia concentrations evaluated. However, it was more difficult to find positive granules when free ammonia concentrations reached 190 mg/L. FISH analysis was able to determine sequences with over 99% similarity to *Candidatus Kuenenia stuttgartiensis* which is supported by similar studies where nitrite concentrations were in excess.

Jang *et al.* (2013) studied antibiotic-resistant pathogenic *E. coli* in the Yeongsan river basin to better understand the public health risk of these pathogens found in the environment. PCR was used to screen *E. coli* isolates for extended-spectrum β-lactamase (ESBL) genes. The results of this study found that 60% of the ESBL positives were likely related to one or both of the diarrheagenic and extraintestinal pathogenic *E. coli* pathotypes.

Liu *et al.* (2012) studied the abundance and distribution of antibiotic resistance genes from an antibiotic production wastewater treatment system. This study used qPCR to quantify 16 *tet* genes and 5 mobile elements. The results of this study demonstrate that *tet* genes are prevalent and abundant in treated effluents and that antiobiotic production wastewater may be a significant source of antibiotic resistance genes in the environment.

The relationship between chloroethene respiration and protein and mRNA biomarkers were evaluated by Rowe *et al.* (2012). This study found that transcript levels of TceA and HupL biomarkers were highly correlated with respiration rates. This study highlights the importance of biomarker analysis for contaminant biodegradation and further supports incorporation of molecular analyses in kinetic models.

The distribution of genetic markers for fecal pollution was evaluated in water, soil, and sediments receiving wastewater effluents using qPCR Eichmiller *et al.* (2013). Molecular marker concentrations in the wastewater

effluents were similar to that in the water column, however sand and sediment samples had the highest concentrations of markers. This finding suggests that sand and sediment are potential reservoirs of fecal pollution markers in bodies of water receiving wastewater effluents.

Garcia *et al.* (2013) used PCR of the 18S rRNA and ITS region to study the occurrence of free-living amoeba (FLA) and amoeba-associated bacteria found in reservoirs and water treatment plants. They found 77.1% of the water samples were positive for FLA. In addition, 88.4% of amoeba isolates contained at least 1 bacterial species analyzed suggesting that FLA may be a potential reservoir of pathogens.

Chen *et al.* (2012b)used PCR and qPCR to study the β-lactam antibiotic resistance (*bla*) genes in microorganisms collected from 6 different rivers in China. They found that all river samples contained *bla* genes. Analysis of the community plasmid metagenome (205 ampicillin-resistnat transformants) showed that *bla* genes were found in 27.3% of the antibiotic resistant clones. This study is significant because it illustrates the environmental prevalence of antibiotic resistance genes in Chinese rivers and suggests that synthetic plasmid vectors are a potentical source a source of antibiotic resistance.

Zhang *et al.* (2013) describe the biodegradation of benzene, toluene, ethylbenzene, and *o*-xylene (BTE(*o*-)X) by a novel bacterium, *Mycobacterium cosmeticum* byf-4. This organism was able to completely remove these compounds whether added individually or in mixtures. RT-PCR was used to determine the expression of a toluene dioxygenase gene that was potentially responsible for the initial catabolism of BTE(*o*-)X compounds.

The effects of biostimulants on 2,4,6-trinitrotoluene (TNT) biodegradation was studied by Fahrenfeld *et al.* (2013). Lactate, ethanol, and naturally occurring organic matter were used to biostimulate microcosms for TNT degradation. This study found that microcosms amended with lactated produced the highest rates of TNT transformation. PCR and DGGE analysis of microbial communities showed that each microcosm undergoing reductive TNT degradation had distinct microbial communities with *Pseudomons* sp. common to all conditions evaluated.

A gene analysis approach was used to evaluate enhanced reductive dechlorination of trichloroethylene (TCE) in a pilot-scale study Chiu *et al.* (2013). Remediation of TCE was enhanced through addition of brown sugar and activated sludge previously used to treat TCE contaminated wastewater. PCR analysis demonstrated the absence of *Dehalococcoides* sp., *vcrA* and *tceA* genes prior to injections followed by their positive detection in injection wells and downgradient after injection. This bioaugmentation and biostimulation effort resulted in up to 97% removal of TCE.

Factors that affect the cultivation of marine anammox bacteria, temperature and salinity, were investigated using fluorescence *in-situ* hybridization (FISH) Awata *et al.* (2012). Anammox activity was observed at temperature above 20 ^{o}C but little change in activity was observed with variation in salinity.

DNA stable isotope probing (DNA-SIP) combined with cloning and sequencing was used to identify microorganisms responsible for triclosan degradation Lolas *et al.* (2012). The degradation of triclosan by these microorganisms, related to *Methylobacillus*, was confirmed using microautoradiography FISH (MAR-FISH) through the uptake of C-14-labeled triclosan by these bacteria.

Bamboo charcoal was used to improve anammox enrichment in upflow anaerobic sludge blanket reactors Chen *et al.* (2012a). Spherical plastic, bamboo charcoal and a carrier-free control were evaluated for their influence on reactor start-up times. This study found that the addition of bamboo charcoal led to faster start-up times and significantly higher concentrations of Anammox bacteria as enumerated by qPCR.

Quantitative PCR is often used to monitor changes in microbial populations over time or the activity of specific subsets of a population. Populations of *Legionella pneumophila* and *Pseudomonas* species were monitored during the operation and shut down in the aeration ponds and air space above ta biological treatment process using qPCR. There was a notable decrease in *L. pneumophila* during the shut-down period Fykse *et al.* (2013). qPCR was also used to monitor the reduction of human viruses and F-specific coliphages in a full-scale wastewater treatment plant. The activated sludge process was responsible for the greatest reduction, though each of the ten virus type examined in this study were detected in the wastewater effluent Hata *et al.* (2013). Primers designed for the nitrous oxide reductase gene, *nosZ*, for bacteria related to *Firmicutes* and *Bacteroidetes* were used for qPCR to detect bacteria that are often missed when conventional Proteobacteria-based primers are used Jung *et al.* (2013).

Ikuma and Gunsch (2013) examined the gene expression effects of adding glucose to enhance phenotype functionality of transconjugants of the TOL plasmid. Specifically, they demonstrated that the addition 1 g/L of glucose enhanced toluene degradation rates and catechol 2,3-dioxygenase activity. In addition, they showed by RT-qPCR that the *xylE* gene, which encodes for a catechol 2-3 dioxygenase is up-regulated in transconjugants exposed to glucose and toluene compared to toluene alone. Gene expression of *xylE*, two regulatory genes (*xylS* and *xylR*) and *xylM* (xylene monooxygenase) were normalized to 16S rRNA.

New Methods to Detect 16S rRNA or 16S rDNA genes (Cloning, Hybridization, Fingerprinting)

qPCR assays have been developed for the specific identification of pathogens in mixed microbial environments. TaqMan assay protocols were developed for the identification of *Cryptosporidium* genera originating from fecal contamination Burnet *et al.* (2013). Another qPCR method was develop to minimize inhibition and monitor *Enterococcus* in recreational water samples, reducing false negative results Cao *et al.* (2012). Toxigenic *Microcystis* were detected in surface waters using a microwave-based pre-treatment for qPCR Michinaka *et al.* (2012).

Feng *et al.* (2012) developed a new 16S rRNA-based sandwich hybridization assay for detecting

Acidithiobacillus ferrooxidans, a Gram-negative, acidophilic, and chemolithotrophic bacterium, in bioleaching processes.

Gray and colleagues explored the effectiveness of commercially available assays for the preservation of DNA from environmental samples. They observed no significant difference between DNAgard^{TM}, RNAlater^{R}, DMSOEDTAsalt, FTA^{R} cards, and FTA Elute^{R} cards, though there was a uniform bias against GC-rich microorganisms Gray *et al.* (2013).

A new automated BOX-PCR and Pulsed-Field Gel Electrophoresis (PFGE) were used to subtype enterococcal isolates from broiler and layer litter and surface and groundwater. *E. faecalis* grouped primarily by source using BOX-PCR. Although enterococci from litter and water sources were grouped together using BOX-PCR and PFGE, isolates originating from water could not be definitively identified as originating from poultry litter. Jackson *et al.* (2012) report that automation of BOX-PCR amplicon separation and visualization increased the reproducibility and standardization of subtyping using this procedure.

Han and Gu (2013) describe the successful application of a newly reported 16S rRNA gene-based PCR primer set to detect anammox bacteria from four ecosystem samples, including sediments from marine, reservoir, mangrove wetland, and wastewater treatment plant sludge. This primer set showed the ability to amplify a much larger range of all reported anammox bacterial genera.

Sequential mRNA fluorescence in situ hybridization (mRNA FISH) and fluorescence-assisted cell sorting (SmRFF) was used by Mota *et al.* (2012) for the identification of nitrite-reducing bacteria in mixed microbial communities. An oligonucleotide probe labeled with horseradish peroxidase (HRP) was used to target mRNA of *nirS*, the gene that encodes nitrite reductase, the enzyme responsible for the dissimilatory reduction of nitrite to nitric oxide. Clones for *nirS* expression were constructed and used to provide proof of concept for the SmRFF method. Mota and coworkers believe that the molecular approach described can be useful as a tool to help address the longstanding challenge of linking function to identity in natural and engineered habitats.

Fu *et al.* (2012) state that existing methods, such as library cloning and screening, are too demanding or inefficient for high-throughput application to the wealth of genomic data being delivered by massively parallel sequencing. In their report Fu and coworkers describe direct DNA cloning based on the discovery that the full-length Rac prophage protein RecE and its partner RecT mediate highly efficient linear-linear homologous recombination mechanistically distinct from conventional recombineering mediated by Redαβ from lambda phage or truncated versions of RecET. The authors conclude that direct cloning with full-length RecE expands the DNA engineering toolbox and will facilitate bioprospecting for natural products.

Dolinšek *et al.* (2013) present a novel approach to deplete unwanted sequence types from complex nucleic acid mixtures prior to cloning and downstream analyses. This novel method employs catalytically active oligonucleotides containing locked nucleic acids

(LNAzymes) for the specific cleavage of selected RNA targets. When combined with in vitro transcription and reverse transcriptase PCR, this LNAzyme-based technique can be used with DNA or RNA extracts from microbial communities. The simultaneous application of more than one specific LNAzyme allows the concurrent depletion of different sequence types from the same nucleic acid preparation. This new method was evaluated with defined mixtures of cloned 16S rRNA genes and then used to identify accompanying bacteria in an enrichment culture dominated by the nitrite oxidizer "*Candidatus Nitrospira defluvii*." In silico analysis revealed that the majority of publicly deposited rRNA-targeted oligonucleotide probes may be used as specific LNAzymes with no or only minor sequence modifications. Authors claim that this efficient and cost-effective approach will greatly facilitate tasks such as the identification of microbial symbionts in nucleic acid preparations dominated by plastid or mitochondrial rRNA genes from eukaryotic hosts, the detection of contaminants in microbial cultures, and the analysis of rare organisms in microbial communities of highly uneven composition.

Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) were used to explore the bacterial and ciliate protozoan populations in metal contaminated biofilms in stream sediments, observing changes in community structure with the variety of metal contamination Ancion *et al.* (2013).

Ahmed *et al.* (2012) used PCR and biochemical fingerprinting to study the presence of toxin genes in *Escherichia coli* isolated from rainwater tanks from Southeast Queensland, Australia. This study evaluated different types of pathogenic *E. coli* and found that 59% of the rainwater tanks examined contained at least 1 of the 10 toxin genes evaluated. The *east1* and *cdtB* genes encode the enteroaggregative heat-stable enterotoxin and the cytolethal distending toxin genes, respectively, and were found to be the most prevalent in this study. Results of the biochemical phenotype (BPT) analysis found that *E. coli* from 6 water tanks were identical to BPTs of *E. coli* isolated from bird and possum fecal material suggesting that these animals may serve as a potential source of contamination.

Flavin-containing monooxygenases were studied in *Phanerochaete chrysosporium* and found to be responsible for the metabolism of phenolic compounds Nakamura *et al.* (2012). A combination of peptide mass fingerprinting and two-dimensional gel electrophoresis was used to isolate peptides that were differentially expressed in the presence of vanillin. Targeted proteins were cloned into *E. coli* and analyzed for their phenol hydroxylase activities. This study determined that one of the isolated flavin-containing monooxygenases (PcFMO1) showed catalytic activities against monocyclic phenols, suggesting that the ortho-cleavage pathway of phenolic compounds are associated with PcFMO1.

T-RFLP was combined with an analysis of the protein and polysaccharide content of EPS to investigate relationship of microbial communities and dewaterability of biomass from an activated sludge process Li *et al.* (2012c). Though significant relationships were not found between EPS composition and sludge dewaterability,

changes in microbial community population correlated with dewaterability.

Pervin *et al.* (2013) assessed the microbial communities in thermophilic (50-65ºC) and mesophilic (35ºC) biological pre-treatment reactors treating primary wastewater sludge before anaerobic digestion. Clone libraries of 16S rRNA gene sequences were constructed from samples collected over a 180 day period, in order to monitor the diversity and abundance of bacteria in the pre-treatment reactors. Over 90% of the sequences from the thermophilic pre-treament reactor were previously undetected, with members of the *Betaproteobacteria* dominating the community over the first half of the study. Terminal-restriction fragment length polymorphism (T-RFLP) revealed that the thermophilic reactor was dominated by phylotypes affiliated with the genera *Comamonas*, *Clostridium*, and *Lysobacter*, which are believed to have hydrolytic and fermentative abilities. The bacterial community in mesophilic pre-treatment reactor differed from the thermophilic reactor but both were relatively dynamic over the course of the study.

Brito *et al.* (2013) characterized the bacterial populations present in hyper-alkaline, hyper-saline industrial waste residue highly contaminated with chromium and iron using TRFLP and 16S rRNA gene library analyses. Pre-treatment of samples (i.e., washing them with phosphate buffer until the pH fell to 7, followed by crushing the sample in liquid N2) was found to be necessary to carry out these molecular analyses on the industrial waste residue.

An improved sequence-aided terminal restriction fragment length polymorphism (T-RFLP) was used by Vajna *et al.* (2012) to identify and (semi-)quantify the dominant bacteria of oyster mushroom substrate preparation. The main features of the improved T-RFLP data analysis were the alignment of chromatograms with variable clustering thresholds, the visualization of data matrix with principal component analysis ordination superimposed with cluster analysis, and the search for stage-specific peaks (bacterial taxa) with similarity percentage (analysis of similarity) analysis, followed by identification with clone libraries.

Haack *et al.* (2012) investigated the effects of antibiotics on groundwater microbial communities using a combination of viability stain, dose-response, and T-RFLP assays. Microbial communities that were acclimated (AC) or unacclimated (UAC) to the antibiotic sulfamethoxazole (SMX) were evaluated. The results of the study found that UAC communities exposed to SMX were the most affected and that antibiotic concentrations in the μg/L range may influence ecological function through changes in the composition of the microbial community.

Yuan *et al.* (2012) investigated the long-term impact of fertilizer on CO2 assimilation by bacterial communities in paddy soils via terminal restriction length polymorphism (TRFLP) and qPCR of the cbbL gene that encodes for the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). For TRFLP, 492-495 bp fragments of the *cbbL* gene from DNA extracted from soil samples were amplified using a forward primer labeled at the 5' end with 6-carboxy-fluorescein (FAM). The

fluorescently labeled PCR amplicons were digested with HhaI and analyzed using an automated sequencer. Clone libraries from the soils samples were constructed using the same primers for TRFLP without fluorescence labeling, in order to examine the phylogenetic diversity of cbbL gene sequences in rice paddy soils. Sequences of 308 clones containing cbbL gene fragments were deposited into GenBank under accession numbers HQ388500-HQ388807. The terminal restriction fragments (T-RFs) from the TRFLP profiles matched cbbL sequences corresponding to facultative autotrophic bacteria such as Rhodopseudomonas palustris, Bradyrhyzobium japonicum, and Ralstonia eutropha. The average relative abundance of *cbbL* T-RFs showed that community composition of autotrophic bacteria in fertilized soils were distinct from that in unfertilized soil. In addition, the abundance of *cbbL* gene (copies per g soil), determined by qPCR, and RubisCO enzyme activity were found to increase as the addition of fertilizer increased.

A new genetic fingerprinting method has been proposed. Long PCR-RFLP of the 16S-ITS-23S rRNA gene was validated by yielding a high-resolution signatures of over 900 samples Huguangyan Maar Lake in southern China Zeng *et al.* (2013).

Pinto and Love (2012) studied the effects of cadmium on the structure and function the activated sludge microbial community. This study found that the bacterial community briefly inhibited by cadmium perturbations, however the community was demonstrated resiliency and increased abundance and activity after washout of cadmium. Increased biomass activity was not resolved by differences in microbial community. It was determined that inhibition of protozoal grazing led to an increase in bacterial activity suggesting that microbial ecological studies may be required to expand beyond bacterial boundaries in bioengineered systems.

More efforts are being made to explore not only the bacterial populations but also the archeal and fungal communities. Single strand conformational polymorphism (SSCP) molecular profiles and Sanger sequencing were used to explore the bacterial and fungal community structure in sediments filtering urban water runoff, identifying the dominant bacteria are related to *Microcoleus vaginatus* Badin *et al.* (2012). Bengelsdorf *et al.* (2013) used 16S and 18S rRNA genes to identify the dominant bacterial, archael and eukaryotic communities in biogas-producing consortium with T-RFLP and sequencing. The dominant bacteria were associated with the phyla Bacteroidetes, Chloroflexus, and Firmicutes. Fungi from the genera *Saccharomyces* and *Mucor* were abundant Bengelsdorf *et al.* (2013). The spatial distribution of N-cycling microbial communities were explored using qPCR. Populations of bacterial and crenarchaea showed patchy distribution for nitrifying and denitrifying genes Correa-Galeote *et al.* (2013). A clone library and DGGE were prepared for bacterial and archaeal community structures of an anaerobic digester fed either cassava pulp, pig manure or a mixture. *Bacteriodetes* and *Clostridia* were the most dominant bacterial groups for all communities and *Methanosaeta* sp. were the dominant archaea, there were clear differences with substrate Panichnumsin *et al.* (2012).

Another study proposed new primers, fITS7, gITS7, and fITS9, for amplification of the internal transcribed spacer (ITS) of fungal species to prevent biases observed with the more commonly used ITS1f/ITS4 primers Ihrmark *et al.* (2012).

Fru *et al.* (2013) compared bacterial and archael populations in laboratory incubations of oil sand fluid fine tailings to those occurring in oil sand tailings ponds by analyzing TRFLP produced fragments using the ecological statistical software Primer E (version 6). This comparison was performed to determine whether the microbial communities encountered in laboratory systems were representative of field populations. The Primer E software allowed for clustering and non-metric multidimensional statistical analysis of the bacterial and archael populations in laboratory and field samples based on fragment sizes and fingerprints determined by TRFLP analysis.

Vitte *et al.* (2013) studied the dynamics of metabolically active bacterial communities and their involvement in polycyclic aromatic hydrocarbon degradation in industrial waste sludge. TRFLP targeting 16S rRNA gene was used to study changes in bacterial communities in bioreactors operated under oxic, anoxic, and anoxic/oxic oscillating conditions. TRFLP targeting ring hydroxylating dioxygenase transcripts was performed to assess the dynamics and diversity of metabolically active PAH-degrading bacterial communities. Clone libraries of cDNA of the ring hydroxylating dioxygenase transcripts were constructed for sequencing efforts aimed at identifying the phylogeny of metabolically active PAH-degrading bacteria present in the bioreactors.

Das *et al.* (2012) investigated the influence of silver nanoparticles on bacterial community structures in natural waters. PCR was used in combination with denaturing gradient gel electrophoresis (PCR-DGGE) and terminal restriction fragment length polymorphism (PCR-TRFLP) to evaluate changes in microbial community structures. This study demonstrated that exposure to silver nanoparticles resulted in either intolerant, impacted but recovering, tolerant, and stimulated phylotype responses from the microbial community.

New Microscopy-based Methods

Berk *et al.* (2012) developed a novel labeling strategy to visualize the extracellular matrix of developing biofilms using conventional and superresolution light microscopy. Modification of several polysaccharide matrix proteins was performed to allow fluorescent labeling of these proteins in vivo. This study reveals the roles of these proteins in biofilm formation.

Environmental scanning electron microscopy (ESEM) and confocal laser scanning microscopy in combination with fluorescent in situ hybridization (FISH) was used by Delatolla *et al.* (2012) to investigate the effects of 4 months of exposure to 4 °C on nitrifying biofilm and biomass. These molecular and microscopic methods were modified to minimize loss of mass and distortion of in situ perspectives.

Confocal microscopy continues to be a staple in measuring biofilm activity. The role of nitric oxide (NO) in biofilm development was explored using confocal laser microscopy in wild types and periplasmic nitrate reductase

mutant of *Azospirillum brasilense* Arruebarrena Di Palma *et al.* (2013).

Mu *et al.* (2012) evaluated the effects of zinc oxide nanoparticle shock loads to anaerobic granular sludge in biological wastewater treatment. Concentrations of over 100 mg/g-TSS were found to negatively impact the concentration of extracellular polymeric substances in the sludge. Multiple fluorescence labeling and confocal laser scanning microscopy showed that proteins were decreased by nearly 70% in the presence of high ZnO NPs.

Schmidt *et al.* (2012) developed a novel fluorescence in situ hybridization method, which combines epifluorescence and scanning electron microscopy (SEM), to identify single microbial cells.

Kim *et al.* (2012a) characterized methane-oxidizing biofilm microbial communities using a custom microarray, qPCR, and confocal microscopy. Biofilm communities were cultivated in the laboratory for 40 days before performing microscopy and DNA extraction. A custom microarray containing 59 oligonucleotide probes targeting the α-subunit of the particulate methane monooxygenase gene (*pmoA*) was used to assess the methanotrophic community structure differences between the methane-oxidizing biofilms and their suspended-growth inoculum source. Microarray results showed that type II methanotrophs dominated the methanotrophic communities in both the biofilms and the inoculum. qPCR analysis of the extracted DNA showed that the methanotrophs made up less than 1% of the methane-oxidizing consortia when comparing pmoA gene copy number to 16S rRNA gene copy number. FISH and confocal microscopy revealed that methanotrophs accounted for 4-9% of the biofilms, in terms of volume, with the relative abundance of methanotrophs peaking at a depth of about 5 μm, in biofilms averaging a thickness of 20 μm.

Immunofluorescent microscopy (IFM), flow cytometry (FCM), and PCR methods for the detection of *Giardia* spp. and *Cryptosporidium* spp. were studied for their efficacy in field deployment situations Keserue *et al.* (2012). This study determined that all methods performed consistently well at high oocyst concentrations with FCM and PCR being the fastest and IFM was the slowest. While all three methods were able to detect *Giardia* and *Cryptosporidium*, the PCR method was least sensitive. The results suggest FCM is a promising rapid detection method for oocysts in water.

New Genomic and Post-Genomic Methods

'Omic' methods continue to be popular for understanding global function of microbial systems. A combination of metagenomics and metaproteomics was used to identify the function of extracelluar polymeric substances (EPS) in an enhanced biological phosphorus removal (EBPR) process. Referencing the metaproteomic results with the metagenomic data from this microbial community, Albertsen *et al.* (2013) identified a variety of genes responsible for alginate production were associated with phylum Bacteriodetes. A metaproteomic approach was developed for a biogas-producing community from the agricultural biomass digester using nanoHPLC-MS Hanreich *et al.* (2012).

With the generation of large amount of sequence data for metagenomic analyses, some attention has been given to the analysis of these data sets. Bonilla-Rosso and colleagues Bonilla-Rosso *et al.* (2012) proposed new metrics for analysis of metagenomic data sets. They suggest that abundance matrices created from protein-coded marker genes are able to recreate the structure of microbial community more accurately than small subunit rRNA genes. Another study suggested a methods of determining metagenome size and structure of soil microbial communities, estimating 20,000 unique bacterial genomes in each of their experimental soil samples Frisli *et al.* (2013).

Li *et al.* (2012b) examined the bacterial community differences via pyrosequencing in two biotrickling filters, one filled with ceramic granules and the other with volcanic rocks, removing hydrogen sulfide. Fragments of 16S rRNA genes, containing the variable V4 and V5 regions, were amplified and pyrosequenced using a 454/Roche GS-FLX Titanium instrument. Pyrosequencing results indicated that members of *Thiomonas* dominated both biotrickling filters, but that the relative abundance of *Acidithiobacillus* was higher in the biotrickling filer filled with volcanic rocks. This higher abundance of *Acidithiobacillus* may contribute to this filter's better performance in removing H_2S under acidic conditions.

Douterelo *et al.* (2013) studied the effects of hydraulic flow regimes on the bacterial community of biofilms in an experimental drinking water distribution system. Pyrosequencing analysis of the 16S rRNA gene was used to quantify bacterial communities under each experimental condition. This research demonstrates an applied approach to understanding hydraulic flow regimes on bacterial communities in biofilms and bulk water of a water distribution system.

Pyrosequencing was used to identify culturable, antibiotic resistant microorganisms from wastewater treatment effluent. *Acrobacter spp.* and *E. Coli* were the predominant microorganisms recovered from ciprofloxacin and doxycycline amended agars Sigala and Unc (2013).

Ye and Zhang (2013) were able to successfully study the various classes of bacteria present in activated sludge, digestion sludge, influent, and effluent samples from a full scale municipal wastewater treatment plant in Hong Kong via the use of 454 pyrosequencing. The DNA of each sample collected was amplified with a set of primers targeting the hypervariable V4 region of the 16S rRNA gene.

Kim et al (2013) examined the bacterial communities present in a laboratory scale simulation of a landfill biocover using ribosomal tag pyrosequencing of extracted DNA and RNA. Specifically, the 340–805 region of the 16 S rRNA gene containing the V3 and V4 regions was amplified for pyrosequencing. The results from this study suggest that bacterial community analysis of DNA alone can lead to a significant underestimation of active members.

West *et al.* (2013) used a genus-wide microarray targeting *Dehalococcoides* to examine the transcriptional changes within an undefined microbial community during a reductive dechlorination feast and famine feeding cycle. The Affymetrix GeneChip microarray applied in this study

contained 4,744 probe sets that represent more than 6,000 ORFs from four published *Dehalococcoides* genomes (strain 195, VS, BAV1, and CBDB1) and 348 functional genes in other microorganisms common to reductive dechlorinating microbial communities.

Lu *et al.* (2012) studied the microbial functional gene diversity in a landfill leachate-contaminated aquifer using a comprehensive functional gene array. This comprehensive functional gene analysis identified several genes from the extracted groundwater microbial communities that were significantly influenced by different environmental parameters. Furthermore, this study determined that leachate from the landfill impacted the diversity, composition, structure, and function of groundwater microbial communities.

The microbial community structure, function, and metabolic pathways in activated sludge bioreactors were studied using Illumina Sequencing Ye *et al.* (2012). Ammonia-oxidizing bacteria were found to be more abundant than ammonia-oxidizing archaea in both reactors evaluated. Interestingly, the results suggest that metagenomic sequencing was a better approach than qPCR for the quantification of these microorganisms.

Microbial diversity was compared amongst small and large-scale activated sludge processes using barcoded pyrosequencing, revealing major classes of bacteria present as *Betaproteobacteria* and *Bacteroidetes* Ranasinghe *et al.* (2012).

Ziv-El *et al.* (2012a) examined the microbial community structure within a H_2-based membrane biofilm reactor for reductive dechlorination of chlorinated ethenes using 454 pyrosequencing and qPCR techniques. 454 pyrosequencing targeted the combined V2 and V3 regions of the 16S rRNA gene on one DNA sample from the liquid, using a mixture of two extracted liquid samples, and one from the biofilm, using a mixture of five extracted biofilm samples. qPCR assays for determining the abundance of specific bacteria used TaqMan primer and probe sets to target the 16S rRNA gene of bacteria, the 16S rRNA gene of *Geobacteraceae*, the 16S rRNA gene of *Dehalococcoides*, and three reductive dehalogenases of *Dehalococcoides*: *tceA*, *vcrA*, and *bvcA*. Reductive dehalogenase gene expression analysis applied the same qPCR assays on cDNA. SYBR Green qPCR assays were used to quantify the *mcrA* gene of methanogens and the formyltetrahydrofolate synthese gene of homoacetogen in DNA and cDNA. The 16S rRNA gene of acetoclastic methanogens was targeted using the family-specific Taqman primers and probe for *Methanoscarcineceae* and *Methanosaetaceae*. The molecular data demonstrated that *Dehaolococcoides* were abundant in membrane biofilm reactor, which also contained homoacetogens, *Geobacter*, and other *Proteobacteria*.

In a second study, Ziv-El *et al.* (2012b) used the same pyrosequencing and qPCR approach for determining trends in the community structure of TCE-decholorinating consortiums in fill-and-draw reactors. The results from this study demonstrated a direct correlation between microbial community function and structure as the TCE-pulsing rate was increased. This correlation allowed the application of an electron-balance analysis to predict the community

structure based on measured concentrations of produces and constant net yields for each microorganism.

Zheng *et al.* (2013) investigated the microbial community in anaerobic sludge fermentation reactors to determine optimal production of short-chain fatty acids (SCFAs). Using a combination of pyrosequencing and fluorescent in situ hybridization, this study determined that sludge fermentation at pH 10 led to increases in *Pseudomonas sp.* and *Alcaligenes sp.* which led to improved hydrolysis reactions. In addition, the high pH led to a decrease in methanogenic archaea and lower methanogenesis further improving the generation of SCFAs.

Detection Methods and Sensors

Pierce *et al.* (2012) developed the Plex-ID biosensor system, a platform that uses electrospray ionization mass spectroscopy (ESI-MS) to detect the base composition of short PCR amplicons. The assay was targeted to identify the common food-borne pathogens *Salmonella, Escherichia coli, Shigella,* and *Listeria monocytogenes*. This assay was tested to determine the scope of the assay's ability to detect and differentiate the enteric pathogens and to improve the reference database associated with the assay. More than 800 bacterial isolates of *S. enterica, E. coli,* and *Shigella* species were analyzed. Overall, 100% of *S. enterica*, 99% of *E. coli*, and 73% of *Shigella spp.* were detected using this assay. The results of this study suggest that this high-throughput method may be useful in clinical and regulatory laboratories testing for these pathogens.

Mota *et al.* (2013) developed a new method to quantitatively detect single walled carbon nanotubes (SWCNTs) in water by coupling single stranded DNA probes to magnetic fluorescent spheres. This method was able to specifically quantify SWNTs using an incubation time of 30 minutes. In a comparison to other methods for SWNT detection, the developed DNA probe with magnetic fluorescent spheres approach was as good as or better than the alternatives for the range of concentrations evaluated in this study.

Antimicrobial resistance is a persistent problem in the public health sphere. However, recent attempts to find effective substitutes to combat infections have been directed at identifying natural antimicrobial peptides in order to circumvent resistance to commercial antibiotics. A study reported by Lira *et al.* (2013) describes the development of synthetic peptides with antimicrobial activity, created in silico by site-directed mutation modeling using wild-type peptides as scaffolds for these mutations. Fragments of antimicrobial peptides were used for modeling with molecular modeling computational tools. To analyze these peptides, a decision tree model, which indicated the action range of peptides on the types of microorganisms on which they can exercise biological activity, was created. The decision tree model was processed using physicochemistry properties from known antimicrobial peptides available at the Antimicrobial Peptide Database (APD). The methods described in this work and the results obtained are useful for the identification and development of new compounds with

antimicrobial activity through the use of computational tools.

The activity of microbial population in a manure digester was monitored with a volatile fatty acids sensor. The sensor uses headspace chromatography to detect VFA and optimized biogas production through feed flow manipulations Boe and Angelidaki (2012).

Microsensors measuring hydrogen were combined with FISH and beta-imaging to understand methane production in anaerobic granules. Hydrogen production was associated with *Smithella spp.* and *Syntrophobacter spp.* between 100-200 μm in the granules and Archaea produce methane at depth greater than 200 μm Satoh *et al.* (2012).

The community isotope array (CIArray) provides sensitive detection of sub-populations within mixed communities. CIArray was demonstrated by identifying anoxic phenol-degrading microorganisms in a predenitrification activated sludge process. C-14 labeled phenol was introduced to the mixed community and DNA from microorganism that assimilated the C-14 hybrized to the CIArray. 'Heavy' DNA from the array was sequenced and were distantly related to Gamma-*proteobacteria* Tourlousse *et al.* (2013).

Ji *et al.* (2013) determined the carboxymethyl cellulose degradation efficiency, dominant microbial flora, eubacteria and archaebacteria characteristics, and expression levels of genes cel5A, cel6B, and bglC in an anaerobic/aerobic bio-reactor consisting of two-stage UASB (U1 and U2) and two-stage BAF (B1 and B2) in series. DNA was recovered from DGGE gels for PCR amplification using primers for the bacterial 16S rDNA V3 region with GC Clamp.

Wang and He (2012) devised a two-step denaturing gradient gel electrophoresis (2S-DGGE) method for obtaining full-length 16S rRNA gene sequences. 2S-DGGE involves seven steps: (1) amplification of partial 16S rRNA gene sequences [using primer 8FGC and 518R]; (2) separation of partial 16S rRNA gene fragments on DGGE gels, followed by excision, re-amplification, purification, and sequencing of bands of interest; (3) design of strain-specific forward primers targeting highly variable regions in V1, V2, or V3; (4) amplification of the remaining part of the 16S rRNA gene was carried out using the new strain-specific forward primers and universal bacterial reverse primers [1492R/1541R]; (5) PCR products from Step 4 were amplified using universal bacterial primer sets [519FGC and 926R]; (6) the PCR products from Step 5 were separated by DGGE to check for purity/diversity; (7) once DGGE gels showed single bands, the corresponding purified PCR products were sequenced and then nearly full-length 16S rRNA gene sequences were obtained by assembling sequences obtained in Step 2 and Step 7. The usefulness of this procedure for characterizing minor population was demonstrated on an artificial DNA sample, which was constructed to have less than 1% of 16S rRNA gene sequences from two Dehalococcoides and two Dehalobacter strains. The 2S-DGGE approach was also effectively used to phylogentically characterize a 1,2-dichloroethane dechlorinating consortium, as well as polybrominated diphenyl ether (PBDE) debrominating microcosms established with sediments and soils from different geographic locations.

Shi *et al.* (2012) developed a universal primer-multiplex PCR method (UP-MPCR) for detection of five significant enterotoxin genes (*toxA*, *phzM*, *lasB*, *ExoU*, and *ExoS*) and one internal control gene *ecfX* of *Pseudomonas aeruginosa* in drinking water and environmental samples. The UP-MPCR method employs a combination of compound specific primers and universal primers for PCR amplification of multiple targets. The compound specific primers contain specific primers at the 3' end and the universal primer sequence at the 5' end. In this study, a universal primer sequence was designed that did not match *P. aeruginosa* genomic DNA sequences. In the initial cycles of UP-MPCR, PCR amplifications are mainly with compound specific primers (in this case, targeting the enterotoxin and control genes). With the compound specific primers exhausted and amplified products containing the universal primer sequence now abundant, these amplicons are used as a template for universal primers. The use of universal primers in the UP-MPCR reduces the variation of amplification efficiency that is common when using different primers in multiplex PCR. The UP-MPCR method developed in this study was used to rapidly and sensitively type 214 drinking water and environmental isolates based on the presence of five *P. aeruginosa* enterotoxin genes.

Collado *et al.* (2013) investigated a proteomics approach to study bisphenol A and nonylphenol removal in activated sludge. The method developed in this study involves the application of a proteome map from *Sphingomonas*, a bacterium capable of degrading bisphenol A and nonylphenol. Application of the proteome approach to activated sludge samples demonstrated the ability of this method to determine the presence of specific enzymes in diverse environmental samples.

Silva *et al.* (2012) developed a strategy for protein enrichment and proteome characterization of extacellular polymeric substances from activated sludge. The strategy involved (1) acquisition of sludge biomass samples, (2) extraction of soluble and bound EPS by cation exchange resin, (3) concentration of the EPS samples via lyophilization, (4) precipitation of EPS proteins using acetone or tricholoroacetic acid, (5) separation of proteins by SDS-PAGE, and (6) protein identification and analysis by mass spectrometry (MALDI ToF-ToF). From activated sludge samples collected from a lab-scale membrane bioreactor, 25-32 and 17 proteins were identified in soluble and bound EPS, respectively.

Li *et al.* (2012e) incorporated proteomic analyses to study 17β-estradiol degradation by *Stenotrophomonas maltophilia* to identify potential metabolism pathways. Gene expression analysis and multidimensional LC proteomics were used to determine that *S. maltophilia* was able to convert E1 to tyrosine followed by protein biosynthesis. This study is one of the first to propose an E2 degradation pathway based on protein identification.

A new assay was develop to evaluate the bactericidal activity of chemical disinfectants based on the European Norm standard suspension and determining ATP content rather than using culture based techniques Aragones *et al.* (2012).

High performance liquid chromatography (HPLC) and quadrapole mass spectrometry (QMS) were paired with DNA stable isotope probing (DNA-SIP) to investigate the

metabolic function of *E. coli.* HPLC-MS is proposed as an alternative to the use of isotope ratio mass spectrometry often used for DNA-SIP assays Auclair *et al.* (2012).

Despite various efforts to develop tools to detect and compare the catabolic potential and activity for pollutant degradation in environmental samples, Vilchez-Vargas *et al.* (2013) believe that there is still a need for an open-source, curated and reliable array method. Vilchez-Vargas and co-workers in their research developed a custom array system including a novel normalization strategy that can be applied to any microarray design, allowing the calculation of the reliability of signals and make cross-experimental comparisons. Array probes, which are fully available to the scientific community, were designed from knowledge-based curated databases for key aromatic catabolic gene families and key alkane degradation genes. This design assigns signals to the respective protein subfamilies, thus directly inferring function and substrate specificity. Experimental procedures were optimized using DNA of four genome sequenced biodegradation strains and reliability of signals assessed through a novel normalization procedure, where a plasmid containing four artificial targets in increased copy numbers and co-amplified with the environmental DNA served as an internal calibration curve. The array system was applied to assess the catabolic gene landscape and transcriptome of aromatic contaminated environmental samples, confirming the abundance of catabolic gene subfamilies previously detected by functional metagenomics but also revealing the presence of previously undetected catabolic groups and specifically their expression under pollutant stress.

To give higher confidence in MALDI-TOF biomarker description Christie-Oleza *et al.* (2013) exploited information from proteins identified by shotgun nanoLC-MS/MS, consisting of the identification and quantification of low-molecular-weight proteins after SDS-PAGE, in-gel trypsin proteolysis and analysis of tryptic peptides. The authors also proposed the standardization of the inclusion of internal calibrants in the bacterial sample to improve the accuracy of the MALDI-TOF measurements. As an application of these biomarkers, Christie-Oleza and coworkers quickly screened 30 seawater bacterial isolates by MALDI-TOF and found one belonging to the *Ruegeria* genus, as further confirmed by 16S RNA sequencing. Due to its simplicity and effectiveness, this technique could be of immense value in monitoring bacteria in the environment in the near future.

Differences in solid retention time (SRT) of bacteria in flocculent and granular sludge were revealed by Winkler *et al.* (2012b). Microbial groups were quantified from reactor and effluent sludge to determine a specific SRT for each target group. This study was able to show that bacteria associated with the exterior of granules have a slightly lower SRT than those in the interior. This study presents the improved resolution that is achieved by complementing standard operational parameters with molecular methods in wastewater treatment.

Gilmore *et al.* (2013) demonstrated that partial nitrification and anaerobic ammonium oxidation was possible with continuous aeration in a membrane-aerated biofilm reactor. FISH was utilized to provide a spatial analysis of important microbial groups and determined that aerobic

ammonia-oxidizing bacteria were closest to the membrane while anaerobic ammonia-oxidizing bacteria were clustered further away.

DGGE was used to reveal the difference in microbial communities in activated sludge with an anaerobic side-stream reactor (ASSR) Kim *et al.* (2012b). Microbial communities were found to be very diverse and diversity was attributed to solids retention time as well as shifts between aerobic and anaerobic conditions. This study also showed that identical sludge produced distinct communities depending on subsequent processes.

The removal of indicator bacteria, coliphage and enteric viruses was determined for different treatment and disinfection strategies Francy *et al.* (2012). Membrane bioreactors were found to provide improved removal of microorganisms compared to conventional secondary treatment. This is translated in the disinfection results as samples from the MBR typically had a lower log-removal rate. Interestingly, adenovirus was detected in all post-preliminary samples and had very little log-removal during disinfection.

The interactions of Anammox and heterotrophic bacteria in biofilms were studied by Ni *et al.* (2012). FISH analysis was able to show that biofilms contained Anammox and heterotrophs at 77% and 23% of the total bacteria population, respectively. In addition, the investigators found that heterotrophs were able to use soluble microbial products and decay products for growth, however the relative abundance of heterotrophs and Anammox bacteria remained fairly stable.

Vuong *et al.* (2013) developed a novel fecal source tracking technique using a mitochondrial DNA microarray. This method relied on qPCR amplification of sequences that were universally present in target commercial and domestic animals commonly used in source tracking studies. This study determined that the new method was able to accurately detect fecal contamination when compared to currently accepted PCR methods.

Currently available molecular and serological methods for the sensitive and specific detection of *P. aeruginosa* from the environment are not satisfactory. To characterize populations of *P. aeruginosa*, Choi *et al.* (2013) successfully developed a novel and specific marker for a SYBR Green Direct qPCR assay for the species-specific detection of *P. aeruginosa*. Since the O-antigen acetylase gene of *P. aeruginosa* PA01 is structurally diverse among species, a primer set (PA431CF/R) was designed based on this gene.

The Listeria genus is considered as one of the most important foodborne pathogens to be transmitted by food and water. Barbau-Piednoir *et al.* (2013) propose a new system, the Combinatory SYBR®Green qPCR screening for foodborne pathogens (CoSYPS Path Food), which uses assays to target two levels of discrimination: the entire Listeria genus (except *Listeria grayi*) and *Listeria monocytogenes*. The 4 primer pairs used in this research were developed using a uniform primer design approach and were chosen in order to perform at the same PCR conditions, allowing the different assays to be performed as four simplex assays simultaneously, on the same plate.

New Data Analysis Techniques for Molecular Methods

Concern regarding the quality of qPCR analysis for the quantification of fecal indicator bacteria has launched an investigation into potential causes for interference and variation in results. Haugland *et al.* (2012) and Cao *et al.* (2013) evaluated a host of factors that could influence qPCR results or cause increased variation. These studies found that sample processing and interference controls are required to improve data quality because quantification was sensitive to many different factors.

Genome-scale metabolic modeling was developed to better understand the feast-famine cycles in activated-sludge systems. Genomic-scale metabolic modeling of storage metabolism for *Rhodococcus jostii* RHA1 and *E. Coli* K-12 were developed with the objective function of maximizing growth rates, minimizing biochemical fluxes and minimizing metabolic adjustment. When the glucose and acetate were the substrates for the feast-famine cycles, the model was in agreement with the experimental results Tajparast and Frigon (2013).

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