

Appendix 3

*High-throughput approaches to analyze waste biotreatment
in confined environments*

High-throughput approaches to analyze waste biotreatment in confined environments

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(2006) In E. Diaz (ed.), Microbial Biodegradation: Genomics and Molecular Biology.

¹ L. Eysers participated in the writing of this paper (description and use of microarrays) and reviewed it.

A3.1. Abstract

Sustainable development requires the promotion of environmental management and a constant search for new technologies to treat vast quantities of wastes generated by increasing anthropogenic activities. Biotreatment, the processing of wastes using living organisms, is an environmentally friendly, simple and cost-effective alternative to physico-chemical clean-up options. Confined environments, such as bioreactors, have been engineered to overcome the physical, chemical and biological limiting factors of biotreatment processes in highly controlled systems. The great versatility in the design of confined environments allows the treatment of a wide range of wastes under optimized conditions. In this chapter, we provide an overview of current genomic-driven strategies to analyze, monitor and assess biotreatment of different classes of wastes in various confined environments. This overview shows that to perform a correct assessment, it is necessary to consider various microorganisms having a variety of genomes and expressed transcripts and proteins. In addition, a great number of analyses are often required. Using traditional genomic techniques, such assessments are limited and time-consuming. On the other hand, several high-throughput techniques originally developed for medical studies hold great promise. We discuss in this chapter various high-throughput techniques and their possible -or already demonstrated- applications to assess biotreatment in confined environments.

A3.2. Introduction

In December 2005, the world's population reached the 6.5 billion mark, a billion more than 1993, and estimates for 2050 point to 9 billion inhabitants. In a world that grows so rapidly in population and in corresponding waste generation and individual consumption levels, the scientific community is facing major challenges owing to the increasingly stringent environmental legislation and our imperative of sustainability. The elimination of a wide range of inorganic and organic wastes is an absolute requirement to promote sustainable development with low environmental impact. Consistent with objectives defined in 1992 at the Earth Summit in Rio de Janeiro (Agenda 21), special topics have emerged in the field of environmental biotechnology through the development of green engineering and white (i.e., industrial) biotechnology. Among these technologies, biotreatment refers to biological processes that take advantage of the astonishing catabolic versatility of microorganisms to detoxify and/or convert wastes. This is clearly an interdisciplinary field, including subjects as varied as environmental law, engineering design or microbial genomics. In its scientific basis, there is a need to bridge the gap between constant advances in laboratory research and commercial field applications. Consequently, a crucial challenge in biotreatment research is to control and scale up key biodegradative processes from the laboratory to full-scale, consistent with the physical, chemical and biological reactions operating at volumes from that of the microbial cell ($0.1\text{--}2\ \mu\text{m}^3$ (Kubitschek and Friske, 1986)) to that of the waste to treat (Paerl and Steppe, 2003). Increasing system complexity often hinders the successful outcome of biodegradative processes, such as bioaugmentation where exogenous bacteria with catabolic capabilities are introduced to contaminated environments (El Fantroussi and Agathos, 2005). The use of bioreactors and other confined environments are necessary to better exploit and extrapolate our knowledge of microbial capabilities and their associated processes from bench-, to pilot-, and finally to full-scale systems. Confined environments offer conditions far more controllable and manageable than most open large-scale ecosystems, in terms of predictability, dynamics of catabolic microbial populations and process monitoring. For these reasons, and because a single configuration will never provide a universal solution, diverse confined environments have been engineered for the biological treatment of a variety of wastes (Table 1).

Table 1. Common confined environments and suitable waste characteristics

Confined environments	Examples of wastes treated	Demonstrated applications
Biological nutrient removal (BNR)-activated sludges (Suspended-growth biological treatment)	Biodegradable, organic wastewater constituents and inorganic fractions	WWTP ¹ (Lee et al., 2006b; Seviour et al., 2003)
Bioslurry treatment in a sequencing batch reactor (SBR), a continuous stirred tank reactor (CSTR) or lined lagoon engineered system	Hazardous wastes	SABRE (“Simplot Anaerobic Bioremediation” to treat TNT-contaminated soils (Lewis et al., 2004)); xenobiotic-contaminated ecosystems (Mohan et al., 2004)
Biological fluidized bed reactors	Biodegradable, organic wastewater constituents and nitrogen removal (nitrification/denitrification)	WWTP (Karapinar and Kargi, 1996; Mulder et al., 1995)
Biofilters and biotrickling filters	Odorous compounds and volatile organic compounds (VOCs) from contaminated gas stream	Waste air treatment at publicly owned wastewater treatment facilities (Iranpour et al., 2005)
Anaerobic digesters (e.g., fixed-bed reactors, upflow anaerobic sludge blanket (UASB) or expanded granular sludge bed (EGSB) reactors)	Solid wastes with pathogens contamination, high-strength ³ industrial wastewaters	Production of class A biosolids ² (Aitken et al., 2005), phenolic wastewater treatment (Veeresh et al., 2005) or selected high-strength wastewaters (Rajeshwari et al., 2000)
Trickling filters (Attached-growth biological treatment)	High BOD ⁴ loading (particularly shock loads from industrial effluents)	WWTP (Vesilind, 2003)

¹WWTP: wastewater treatment plant

²For biosolids, the regulation establishes two levels of quality with respect to pathogen densities (Class A and B)

³High-strength wastewaters are commonly defined as wastewater with a high content of COD (chemical oxygen demand) (e.g., COD > 1000 mg/l)

⁴BOD: biological oxygen demand

Understanding the physico-chemical characteristics of a waste and its environment are essential requirements to develop a biotreatment strategy. In addition, the successful implementation of a biotreatment process requires a detailed description of the microbial communities, major players in key physiological processes. As stated in a critical viewpoint

of leading experts in environmental biotechnology (Rittmann et al., 2006), understanding and exploiting a microbial community requires knowledge of its structure (i.e., who is there?), its phenotypic potential (i.e., what are the biocatalytic capabilities?), its function (i.e., which ones among those biocatalytic capabilities are realized?) and its interactions with its environment. The interactions between microbes in a community have the potential to alter their dynamics and the underlying microbial activities, such as catabolic reactions relevant to biotreatment operation. Therefore, knowledge of the ecophysiology of microbial communities and our capacity to provide a continuous picture of biological processes contribute to maintaining the stability and efficiency of waste biotreatment. For instance, the biotreatment of xenobiotic chemicals, such as nitroaromatic compounds, requires the maintenance of microbial communities with specific bacteria that possess particular catabolic traits (Stenuit et al., 2005). In the case of activated sludge wastewater treatment processes, it is essential to monitor the population of specific sludge bacteria and its dynamics to operate the process efficiently and prevent the uncontrolled overgrowth of filamentous bacteria that cause sludge bulking and foaming (Kim et al., 2004a). However, the lack of sensitive, fast (i.e., “complete in a single day”) methodologies for extensive characterization of microbial communities have until recently hampered our understanding and assessment of their catabolic activities. So far, this task has been mainly approached using time-consuming and limiting cultivation-based studies or traditional methods that provide non-discriminating information, e.g., biochemical oxygen demand (BOD) and volatile suspended solids (VSS). Therefore, it is difficult to perform corrections or adjustments to the treatment system when process stability changes occur. The development of molecular techniques based on the ribosomal RNA gene or rRNA itself has allowed the study of microbial diversity and community dynamics in fine detail. Several methods have been developed to provide a qualitative (e.g., denaturing gradient gel electrophoresis (DGGE)) and quantitative (e.g., dot blot and fluorescence *in situ* hybridization (FISH)) description of microbial communities. However, because they are time-consuming, these quantitative methodologies have been applied in few studies such as analysis of wastewater treatment plants (WWTPs) (Ginige et al., 2005). In addition, quantitative characterization is often limited to a small number of targeted microorganisms. To tackle this problem, high-throughput approaches are emerging. They offer the advantage of miniaturization, automation and massive parallelization of time-consuming steps, allowing the simultaneous “real-time” analysis of numerous samples with thousands of nucleic acids targeted. The increase in genome-wide information has spurred the development of high-throughput molecular techniques, which have been initially applied in the biomedical field.

However, an expanding sequencing infrastructure combined with rapidly dropping genome-sequencing costs have made available an increasing number of complete bacterial genomes relevant to the elimination of recalcitrant compounds (Kube et al., 2005b; Schneiker et al., 2006) or nutrients (Strous et al., 2006). In addition, the sequencing of community genomes derived from metagenomic approaches (Deutschbauer et al., 2006) provide information on model microbial consortia from various environments. This ever-increasing massive and extremely valuable genetic information has encouraged scientists to adapt and extend high-throughput genomic techniques to the field of environmental microbiology and waste biotreatment. Furthermore, the recent acquisition of genomic data sets directly from environmental samples makes possible the comprehensive application of postgenomic techniques, such as high-throughput functional analysis of metaproteomes (Ram et al., 2005b). In this chapter, we focus on major high-throughput molecular tools that can be applied or adapted to analyze waste biotreatment in confined environments. The first section provides an overview of methodological breakthroughs in high-throughput molecular techniques. In the second section, the applications of these techniques to analyze biotreatment of a wide spectrum of wastes (ranging from nutrients to recalcitrant organic compounds) are discussed. The last section addresses the special task of monitoring and elimination of pathogens in domestic wastewaters.

A3.3. High-throughput approaches as part of a management strategy for waste biotreatment

Figure 1 summarizes the different high-throughput approaches to analyze waste biotreatment and emphasizes the need to link microbial community structure with its function(s). The necessity to use novel molecular approaches is highlighted by the fact that, on average, about 99% of microbes living in natural environments are refractory to conventional cultivation. For instance, the genus *Pelagibacter* or the phylum *Acidobacteria* are environmentally abundant in the surface of the ocean and soil, respectively, but not enough members of these groups have been cultivated to unravel their physiological function. In wastewater reactors and other confined environments, microbial communities can be as complex as those present in natural environments. To gain insights into this tremendous “hidden reservoir” of microorganisms, functions and activities, culture-independent molecular techniques are needed. Microarrays, fingerprinting techniques, real-time PCR, metagenomics and metaproteomics are culture-independent methods fully compatible with a high-throughput design and setup. Moreover, biosensor arrays are also high-throughput techniques, providing a genetically focused approach different from traditional analytical procedures (e.g., high performance liquid chromatography and gas chromatography) to detect trace organic compounds. These techniques are discussed below.

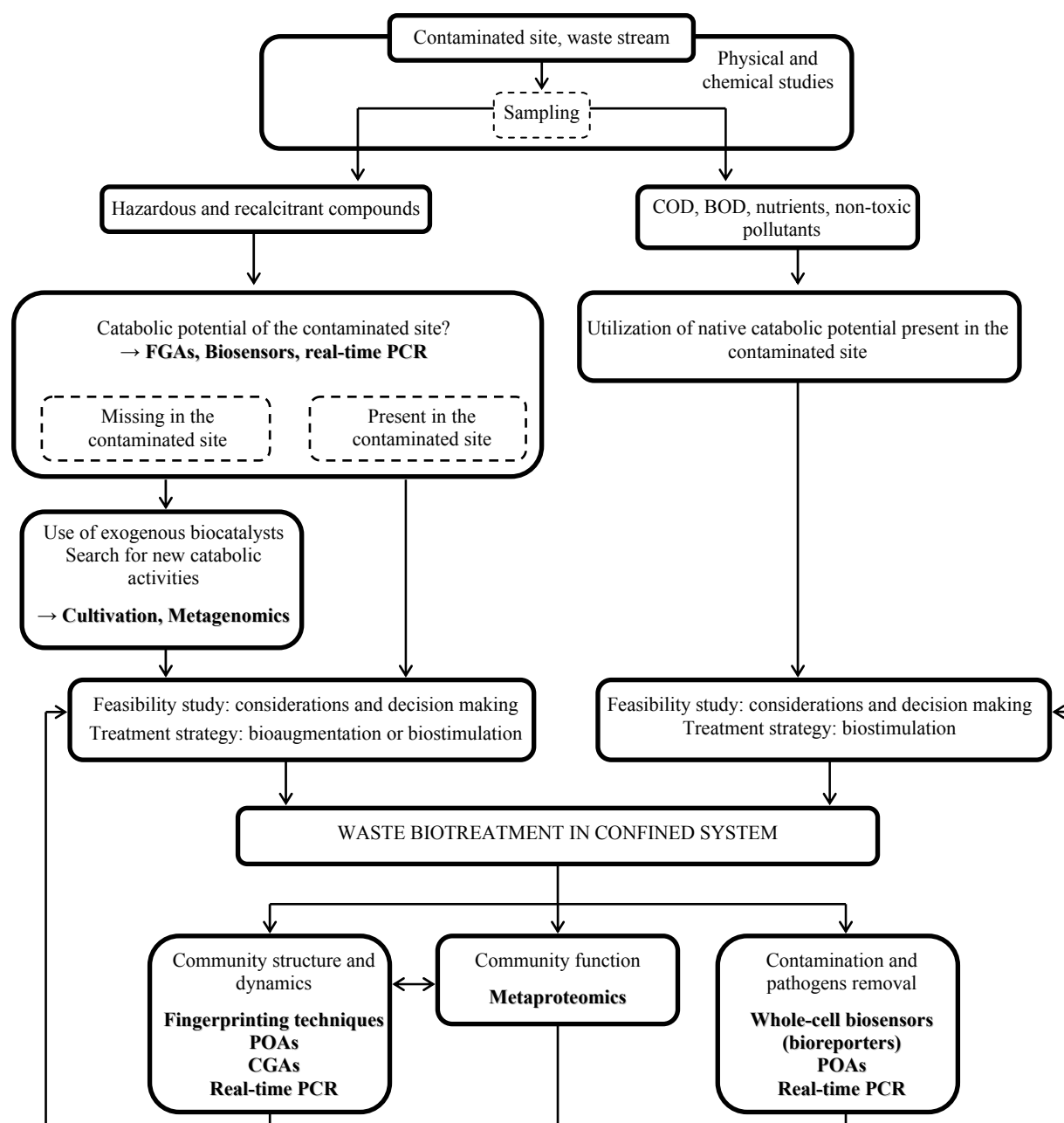


Figure 1. A holistic approach to waste biotreatment, with special emphasis on high-throughput approaches for its characterization and monitoring.

A3.3.1. DNA microarrays

Microarrays (or microchips) are based on the property of a single-stranded DNA or RNA molecule (“target molecule”) to hybridize to a complementary molecule (“probe”) attached to a solid support (Zhou, 2003). Compared to traditional nucleic acid membrane hybridization, microarrays present the advantage of miniaturization (thousands of probes can be spotted on a slide), high sensitivity and rapid (“real-time”) detection (Eyers et al., 2004b). There are fundamental differences between microarray technologies based on the immobilization technology used to attach the probes, length and nature of the probes, and synthesis and labeling of the targets (Nees and Woodworth, 2001). The choice between the different technologies is based on parameters such as cost, probe density, specificity, sensitivity and quantification. Probes are synthesized *in situ* (e.g., “GeneChip” arrays) or spotted directly onto the solid support. GeneChip arrays are synthesized using photolithographic masks. Probes are built by repeated cycles of light-activated reactions with nucleotide monomers. *In situ* synthesis can also be realized by electrochemical reactions. On the contrary, spotted microarrays require a preliminary *ex situ* synthesis. Spotted microarrays are of lower cost than *in situ* synthesized probe microarrays as they do not require highly sophisticated equipment to synthesize probe spots. In addition, they offer a much greater flexibility since probes can be easily spotted in-house at low cost. However, they are of lower density than *in situ* synthesized probe microarrays. Probe material consists in synthetic oligonucleotide sequences from 10- to 75-mer (this material is adapted for *in situ* synthesized and spotted microarrays) or DNA sequences such as PCR products, cDNAs or clone libraries (this material is adapted to spotted microarrays only). Longer probes offer the advantage of lower production cost and higher signal intensities. The great advantage of oligonucleotide probes is that they allow the study of probe-target specificity since mismatch probes can be designed and printed on the array. Therefore, sequence-specific signals can be distinguished from non-specific signals. Readout of the microarray is commonly achieved using a fluorescence signal. Fluorescent dyes can be enzymatically or chemically incorporated in the sample to be hybridized. In addition, radioactively labeled substrates can be used to provide detection of specifically labeled target molecules (i.e., the so-called “isotope arrays”) (Wagner et al., 2006).

In the field of environmental genomics, three major classes of microarrays have been developed based on the size and specificity of the probes: (i) phylogenetic oligonucleotide arrays (POAs) contain oligonucleotide probes targeting taxonomic genes (e.g., 16S rRNA

gene), (ii) functional gene arrays (FGAs) contain probes corresponding to genes encoding key enzymes involved in various ecological and environmental processes, and (iii) community genome arrays (CGAs) are constructed from whole genomic DNA of many individual pure cultures (Zhou, 2003). The design of probes for POA and FGA formats is based on sequences retrieved from databases. For this reason, it is not possible to have access to unknown phylogenetic affiliations and functional activities. To discover novel genes and operons responsible of a targeted physiological activity, clone libraries spotted on microarrays can be a very powerful tool to accomplish gene expression studies with environmental bacteria whose genome sequence is still unknown, avoiding the time and effort necessary for whole genome sequencing projects. For instance, a clone library of a specific microorganism can be printed on a microarray and hybridized with mRNA isolated prior and after exposure of this microorganism to a specific pollutant. Differences in hybridization patterns will allow the identification of clones harboring genes expressed (or repressed) in response to this pollutant. This methodology was used by Parro and Moreno-Paz (2003) to identify most of the genes involved in nitrogen fixation in *Leptospirillum ferrooxidans*. However, in their work, cultivation was used for construction of the genomic library. Alternatively, integrated approaches can be used by combining metagenomics (i.e., direct extraction and cloning of collective genomes from environmental samples (see the next section)) with microarrays. Metagenomic arrays (MGA) can be a potentially powerful technique because, unlike the other arrays, they contain probes produced directly from environmental DNA itself and can be applied with no prior sequence knowledge of the community (Gentry et al., 2006). For instance, the MGA was used as a high-throughput library screening technique by Sebat et al. (2003) using a cosmid library from a microcosm of groundwater. The authors hybridized the microarrays with cDNA of groundwater isolates and cDNA of the microcosm itself. Afterwards, the differences in hybridization patterns allowed the identification of clones in the library corresponding to uncultured members of the microcosm. Sequencing of these clones revealed ORF's assigned in several ecologically important processes including denitrification, hydrogen oxidation, and transposition. As stated by Gentry et al. (2006), the MGA technology is still in the early stages of development, but it holds great promise for environmental applications since the tremendous reservoir of unknown sequences in these environments is one of the major limitations of microarray analysis.

Environmental application of array technology poses great challenges in terms of specificity, sensitivity and quantification (Cook and Saylor, 2003). The specificity issue is critical,

especially for POA formats dealing with rRNA since it is highly conserved at the nucleotide sequence level and is present in every microorganism. In addition, the stable secondary structure of small-subunit rRNA has serious effects on hybridization specificity and sensitivity. To overcome the secondary structure problem, the target rRNA can be chemically fragmented (e.g., Kelly et al., 2002). The specificity problem can be resolved by using thermal dissociation studies. By linearly increasing the temperature of the microarray and recording signal intensities, dissociation curves are obtained. Several studies have shown that dissociation curves provide a useful tool to discriminate between perfect-match and single-mismatch probe-target duplexes (e.g., El Fantroussi et al., 2003). Another challenge is the sensitivity of microarrays. With environmental samples, the presence of various contaminants (e.g., humic acids) inhibits enzymatic reactions and generates a high signal background when environmental samples are hybridized to the microarray. Various protocols have been developed to extract RNA and DNA of sufficient purity from various environments. For instance, using bead-beating and phenol extraction, El Fantroussi et al. (2003) have shown that rRNA can be extracted from sediments and used for microarray hybridization studies. In addition, this study showed that sufficient rRNA was extracted without the need for amplification prior to hybridization. The extraction of mRNA from environmental samples is more challenging than the extraction of rRNA because the latter is present at higher quantities than mRNA. Reverse-transcription of mRNA is therefore a necessary step to achieve sufficient sensitivity. Another alternative consists in using extracted DNA and increasing the sensitivity of microarray detection by performing enzymatic amplification. However, when the extracted DNA contains various templates, PCR amplification has to be used with caution because of its associated bias and artifacts (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). Alternatively, whole genome amplification can be carried out with phi29 DNA polymerase and random exonuclease-resistant primers without thermal cycling. Dean et al. (2002) have shown that such amplification was surprisingly uniform across the genomic target, with the relative representation of different loci differing by less than 3-fold. In contrast, PCR-based whole genome amplification methods exhibited strong amplification bias ranging from 4 to 6 orders of magnitude. Wu et al. (2006) adapted this phi29 DNA polymerase based amplification method to analyze subnanogram quantities of microbial community DNAs and demonstrated representative amplification of individual genomes and its application for low-biomass microbial communities. Last but not least, quantification is a third challenge. With FGA and CGA formats, a linear relationship between signal intensity and DNA concentration was found over a fourfold concentration of DNA isolated from pure

cultures and mixed populations (Wu et al., 2001). In the case of POA formats, quantifying microbial populations is challenging because of cross-hybridizations that may occur when dealing with complex environmental samples containing perfect and mismatch targets in unknown abundance. Again, the use of dissociation curves holds great promise for correct quantification of environmental samples (e.g., Wick et al., 2006).

A3.3.2. Metagenomics

The ‘metagenome’ (i.e., the collective genomes of a given habitat) and its associated ‘-omics’ science (i.e., metagenomics: the culture-independent genomic analysis of entire microbial communities) provide direct access to the pool of environmental genomes and circumvent bottlenecks of lab-based cultivation of microbial species (Eyers et al., 2004b). In the broad sense of the term, metagenomics refers to the application of recent genomic techniques (e.g., large-scale sequencing, DNA microarrays, DNA shuffling (Boubakri et al., 2006) and stable isotope probing (Friedrich, 2006)) to the culture-independent study of microbial communities within the context of their natural environment. Metagenomics offers the possibility to retrieve unknown sequences or functions, whereas methods relying on PCR amplification, POA or FGA formats are based on prior knowledge of the sequence of genes obtained through the study of laboratory isolates. More specifically, metagenomics frequently refers to the construction of metagenomic libraries. It involves the same methodology as the one used to generate recombinant DNA libraries from individual microorganisms: (i) generation of DNA fragments of appropriate size, (ii) ligation of the fragments into an appropriate cloning vector (e.g., cosmid, fosmid or bacterial artificial chromosome (BAC) vectors), (iii) introduction of the recombinant vectors into a suitable bacterial cloning host, and (iv) screening of clones expressing particular proteins, harboring particular metabolic activities, or containing specific sequences (Daniel, 2005). However, metagenomics is faced with unique difficulties, as it is dealing with environmental samples. For instance, the isolation of genomic DNA from complex environmental matrices, such as sludge, wastewater, sediment or soil, is particularly challenging in terms of quality (e.g., inhibitory contaminants are frequently co-extracted), molecular size (e.g., DNA is often sheared to low-size fragments) and representation of all microbial genomes (e.g., size fractionation or experimental normalization are often necessary) (Cowan et al., 2005). Improvements in DNA extraction procedures and the recent developments of various commercial kits to generate complex DNA libraries have

allowed the construction of metagenomic libraries derived from diverse environmental samples, such as soils highly contaminated by 2,4,6-trinitrotoluene (TNT) (Stenuit et al., 2004).

Screening strategies for metagenomic libraries can be designed to explore functional and/or genetic diversity. In the function-driven analysis, clones of interest are screened or selected through the detection of heterologous expression of a desired trait. The functional-based screening has opened the doors to the discovery and characterization of new enzymes, antibiotics and other chemicals for biotechnological and therapeutic applications (Daniel, 2005). However, function-driven analysis is typically realized with low-throughput screens (i.e., each clone is tested separately for a desired feature, and detected, for example, by visual detection of growth inhibition of indicator bacteria) and selection procedures (i.e., only clones expressing specific genes grow, such as genes conferring resistance to antibiotics or genes involved in catabolic pathways allowing the use of xenobiotic compounds provided as sole carbon source) (Handelsman, 2005). Although selection presents an attractive way to detect rare positive clones among the entire clone pool (e.g., Henning et al., 2006), few functional activities with valuable biotechnological applications are compatible with selection strategies. Alternatively, high-throughput screens can be performed on the basis of phenotypes readily detectable (e.g., proteolytic activity tested for each clone and identified with visual detection on indicator plates by clearing zones around the clones (Daniel, 2005)). This screening is facilitated by automated colony picking, pipetting robotics, use of microtiter plates, informatics-assisted data management and sensitive assays targeting a broad range of biomolecules. However, the reported screening “hits” are often low (typically, less than 2 out of a total of 10,000 clones have industrially relevant enzymes and biocatalysts (Lorenz and Eck, 2005)). This is due to several constraints, such as the size of inserts (the insert has to contain the entire cluster of genes required for the function of interest), the effective heterologous expression of the cloned genes (with associated post-transcriptional and (post)-translational events) and the secretion of the foreign protein. In addition, false positive clones will occur when the host has unexpected physiological activities (e.g., the discovery of new catabolic pathways for the denitration of trinitroaromatic compounds using *E. coli* as a host is biased because *E. coli* can denitrate TNT (Stenuit et al., 2006)). Therefore, other approaches are necessary to increase the screening hit rates, such as (i) the use of different expression hosts, (ii) the use of diverse strategies to enrich/select for community genomes with desired traits before metagenomic library construction (for instance, a “labeled metagenome” can be

obtained from an environmental sample incubated in the presence of a [^{13}C]-substrate (Friedrich, 2006)), (iii) the use of liquid enrichment cultures for the screening of multiple clones simultaneously (this has also the advantage to select for metagenomic clones performing syntrophic catabolic reactions), and (iv) the development of novel high-throughput screening strategies. For instance, individual clones can be identified through promoter activity rather than phenotypic expression. Intracellular fluorescent biosensors (Handelsman, 2005; Williamson et al., 2005) or ‘substrate-induced gene expression screening’ (Uchiyama et al., 2005) are examples of possible applications. Positive clones can be detected by fluorescence microscopy or fluorescence-activated cell sorting, the latter allowing a throughput level of 10^9 events per day (Robertson and Steer, 2004).

In the sequence-driven analysis, clones of interest are identified based on the presence of conserved regions in microbial genes, such as phylogenetic markers (e.g., genes coding for 16S rRNA (e.g., Elshahed et al., 2005)) or conserved motifs in functional genes of environmentally relevant processes, such as those encoding oxygenases (e.g., Erwin et al., 2005). PCR and hybridization are commonly used for screening using suitable primers or target-specific probes, whose design is based on the information available in databases. Therefore, the sequence-driven analysis can only be applied for the identification of members of known gene families or novel variants of known functional classes of proteins (Daniel, 2005). Recently, large-scale shotgun sequencing of the entire metagenome has been used by several groups to characterize nutrient-poor habitats (e.g., acid mines and Sargasso Sea) and nutrient-rich environments (e.g., agricultural soil, deep-sea whale skeletons and deep-sea sediments) (reviewed in Deutschbauer et al., 2006). In contrast to metagenomic studies focusing on particular genes, pathways or organisms, this emerging approach consists in sequencing a given environment on the scale of the entire community by furnishing a global picture of the microbial ecosystem. The method involves the construction and random end-sequencing of small-DNA-insert libraries (~3-5 kbp), followed by the assembly of the subsequent sequences into contigs and scaffolds *in silico*. The reassembly of contigs from smaller sequence fragments is challenging because of the dynamics of environmental populations in terms of microbial species richness, evenness and intraspecific genetic heterogeneity (DeLong, 2005). Nonetheless, massive data have been accumulated by this shotgun sequencing approach. Tringe and Rubin (2005) described an interesting line of attack to exploit data through a ‘gene-centric’ approach in which each environment is characterized by a particular ‘fingerprint’ comprised of environmental gene tags (EGTs: short DNA

sequences from a microbial community that contain fragments of functional genes). In this way, comparative metagenomics has been successfully used to evaluate the differences and similarities in gene abundances in the EGT data of several terrestrial- and oceanic-environments. In addition, sequencing of large-DNA-insert libraries (e.g., DeLong et al., 2006) or large-DNA-insert libraries in combination with small-DNA-insert libraries (Hallam et al., 2004) can be used for a better characterization of natural ecosystems. As stated by DeLong (2005), a combination of these approaches could enhance the accuracy, coverage and reliability of genomics-based efforts to understand complex microbial communities. The pros and cons of these different sequencing methods (i.e., shotgun sequencing approach of small-DNA-insert libraries or sequencing of libraries constructed with large fragments) have been largely discussed by Whitaker and Banfield (2006). This large scale sequencing approach may be valuable in the quest to compare different environments, but it remains unclear whether such sequencing will bring insights on particular genes and associated functions.

A3.3.3. Metaproteomics

Because mRNA expression levels alone are unreliable indicators of the abundance of the corresponding proteins (e.g., the transcriptome does not account for post-transcriptional events), proteomics has a higher physiological relevance than genomics (Pradet-Balade et al., 2001). Recently, the term ‘metaproteome’ was proposed as the collective protein content of a given habitat (Rodriguez-Valera, 2004). Furthermore, its associated ‘-omics’ science (i.e., metaproteomics: the proteomic study of microbial communities) refers to the large-scale characterization of the entire protein content of environmental microbiota at a given time point (Wilmes and Bond, 2004). This approach has great potential for the functional analysis of microbial communities. Typically, complex mixtures of proteins from an environmental sample are separated with two-dimensional (2D) gel electrophoresis or high performance liquid chromatography. Following protein separation, fractions of interest (e.g., protein spots on a 2D gel) are analyzed by high-throughput mass spectrometry-based analytical platforms (Domon and Aebersold, 2006). Thanks to available relevant metagenomic sequence data, protein prediction and subsequent identification are greatly facilitated (Wilmes and Bond, 2006a). For instance, using the extensive dataset generated by large-scale shotgun sequencing from a natural biofilm of an acid mine drainage (AMD) (Tyson et al., 2004), a database of 12,148 predicted protein sequences was constructed (Ram et al., 2005b, supporting online

material). As expected, the correlation between protein expression profiles and the metabolic potential discovered using metagenomics was not always positive. Nevertheless, the AMD metaproteome contained 2033 proteins from the five most abundant species in the biofilm, including 48% of the predicted proteins from the dominant biofilm organism, *Leptospirillum* group II.

Although a few studies have been carried out (Wilmes and Bond, 2004; Ram et al., 2005b; Wilmes and Bond, 2006b), the metaproteomics is still in the prime of live. Extensive bioinformatics implementation is necessary to archive and analyze the increasing amount of data generated by metaproteomics (Wilke et al., 2003). In addition, successful application of metaproteomics relies on the development of an efficient protein extraction that ensures (i) a sufficient purity and (ii) a representative recovery of the total proteins within the natural microbial community (Maron et al., 2006). Finally, metaproteomics still faces great challenges in terms of statistical analysis and protein separation and identification.

A3.3.4. Fingerprinting techniques

Genetic fingerprinting techniques provide a specific pattern or profile of a given microbial community to evaluate its composition and dynamics. These techniques necessitate a preliminary community DNA extraction and PCR amplification of phylogenetic anchors using universal or specific primers (e.g., hypervariable or conserved region of the gene coding for 16S rRNA). Some of these fingerprinting techniques have the potential for high-throughput studies, such as terminal restriction fragment length polymorphism (T-RFLP) (Marsh et al., 2000). T-RFLP separates fragments obtained by enzymatic restriction of PCR amplicons according to their size. The use of labeled primers allows a rapid, automated and high-throughput detection (the output is digital) of polymorphic terminal fragments. The separated fragments are visualized by an automated DNA sequencer to provide a pattern of peaks on an electropherogram. In addition, the identification of T-RFLP peaks can be directly obtained by comparing them to databases (Marsh et al., 2000). Denaturing gradient gel electrophoresis (DGGE) is another approach relying on the separation of PCR amplicons based on their GC-content. This technique provides a pattern of bands on a gel and requires the cutting of bands out of the gel in order to sequence and identify them (Eyers et al., 2004a). DGGE can also accommodate a high-throughput format. Denaturing high performance liquid

chromatography (D-HPLC) separates DNA fragments using fast and repeatable reverse-phase ion-pair chromatography. The separation of PCR products in D-HPLC is based on the elution of partially melted DNA molecules according to the column temperature and their interactions with the positively charged ion pairing reagent and the cartridge matrix of the system (Barlaan et al., 2005). Thanks to automation, D-HPLC allows the separation of PCR amplicons within minutes. Then, the fragments can be collected at the end of the column for further automated sequencing. Thus, a high-throughput study of microbial community composition is possible.

Two tagging methods for high-throughput profiling of complex microbial communities have been recently published: serial analysis of ribosomal sequence tags (SARST) and single-point genome signature tags (SP-GSTs) (Kysela et al., 2005; van der Lelie et al., 2006). These techniques provide a fingerprint of microbial communities in the form of tag sequences. SARST relies on the generation of concatemers of PCR amplicons produced from the V1 (Neufeld et al., 2004) or V6 (Kysela et al., 2005) hypervariable regions of 16S rRNA genes. For instance, the SARST-V6 method is based on PCR amplification of ribosomal sequence tags (RSTs) from homologous V6 hypervariable regions of bacterial genes coding for 16S rRNA. Then, the amplicons are ligated to yield concatemers with multiple, serially arranged PCR products that are further cloned and sequenced to characterize microbial community composition (Kysela et al., 2005). In this way, multiple sequence tags from many different organisms are obtained simultaneously from a single sequencing reaction. However, the highly variable nature of the tag sequences and paucity of positions do not allow direct inference of phylogenetic frameworks (Sogin et al., 2006). A similar method based on the V1 hypervariable region of 16S rRNA genes (referred to as SARST-V1) has also been reported (Neufeld et al., 2004). Thereafter, Yu et al. (2006) described an improved version of the original SARST-V1 that significantly expedites the construction of RST clone libraries by eliminating the need for linkers and all the associated steps (i.e., enzymatic and purification steps needed to ensure head-to-tail ligation of RSTs for successful RST clone library screening and DNA sequencing). Nowadays, while the RSTs are expected to provide a lower phylogenetic resolution than longer 16S rDNA fragments or full-length gene sequences (Yu et al., 2006), this methodology holds great promise. For instance, the group of de Lorenzo (Neufeld et al., 2006) used a SARST method based on the V1 region for the construction of a sensitive and specific microarray (i.e., RST habitat-specific microarray). The probes of the microarray were designed to have maximum specificity for the most abundant phylotypes in the RST library derived from particular environments.

On the other hand, another high-throughput sequencing method has been developed on the basis of tags generated from various conserved bacterial genes of interest (van der Lelie et al., 2006). These are further concatemerized, cloned and sequenced. Remarkably, in addition to taxonomic identification (e.g., 16S rRNA gene as target), GSTs can provide specific functional information (e.g., a functional gene as target) to study changes in microbial community composition.

A3.3.5. Real-time PCR

The sensitivity of a microarray-based approach may be 100- to 10,000-fold lower than that of PCR, which might be problematic for sequences of poor abundance (Eyers et al., 2004b). A higher sensitivity is achieved by real-time PCR since its limit of quantification is typically 1-2 genome copies (Inglis and Kalischuk, 2004). Real-time PCR uses a fluorescent internal probe to obtain information on PCR product concentration. This technique allows the detection and quantification of specific groups of bacteria during PCR amplification (i.e., the target gene is amplified and the initial copy number of this target gene is quantified throughout the PCR process). Thus, the traditional time-consuming PCR amplicon detection steps (i.e., electrophoretic migration of DNA fragments and UV detection with the toxic ethidium bromide) are eliminated thanks to the detection of amplicons by real-time measurement of fluorescence signals. Therefore, real-time PCR is a high-throughput technique with high analytical sensitivity for the detection and quantification of specific genes present in low concentration in complex and variable DNA mixtures, such as those extracted from environmental samples (Harms et al., 2003). Recently, a quantitative fingerprinting method called real-time-T-RFLP has been developed. The method uses real-time PCR to provide quantitative information of the absolute numbers of copies of total 16S rRNA genes, in combination with T-RFLP analysis to give qualitative information of the relative distribution of each ribotype (Yu et al., 2005). An additional refinement of this method was realized by Yu and Chu (2005). The authors combined this integrated approach with the stable isotope probing technique (SIP) to develop a quantitative assay for concomitant identification and quantification of active microorganisms involved in a specific function in a given microbial community.

A3.3.6. High-throughput cultivation of microorganisms

Pure culture microbiology fell into disfavour among many microbial ecologists over the past two decades as an irrational exuberance about the power of environmental molecular analyses took hold. The reasons for this disfavour are the artefacts related to the enrichment procedure as it is known that most of the bacteria are reluctant to cultivation using traditional cultivation techniques (Torsvik and Ovreas, 2002). However, the recovery of isolates that are representative of the microbial community of contaminated environments can be invaluable because studying these isolates provides the opportunity to investigate not only their biodegradation reactions, but also other aspects of their physiology that are likely to control their growth and activity in contaminated environments. Novel and creative cultivation techniques were recently developed to provide access to the immense reservoir of untapped microbial diversity. These new techniques are commonly based on the use of poor nutrient media, and chemically defined medium approaching the composition found *in situ*. These techniques were also adapted to accommodate a high-throughput design. For instance, Cannon and Giovannoni (2002) developed high-throughput culturing procedures using dilution-to-extinction to isolate cultures in small volumes of low-nutrient media. The percentage of cells that could be cultured was several orders of magnitude higher than what could be typically achieved by classic growth experiments on agar plates. Another approach consists in the use of gel microdroplets to encapsulate single cells for massive parallel microbial cultivation under low nutrient flux conditions (reviewed by Keller and Zengler, 2004). To detect growth within the microcapsules and formation of microcolonies from single cells, flow cytometry can be used. Then, the microcolonies can be sorted individually into microtiter dishes.

A3.3.7. Whole-cell-based biosensors for environmental biomonitoring

A biosensor is a self-contained integrated device (all parts being packaged together) providing a concentration-dependent response in the presence of a chemical species using a biomolecular recognition element (e.g., microorganism) which is interfaced to a transduction element that converts the biomolecular recognition event into a useable output signal (Rogers, 2006). Biosensors are classified on the basis of the method of signal transduction or the

biorecognition element. For instance, a variety of whole-cell-based biosensors (namely bioreporters) has been widely used for environmental sensing and reporting (Harms et al., 2006). Basically, genetically engineered bacteria (GEMs) report the presence of an effector (e.g., a xenobiotic compound) *via* the production of an easily measurable reporter protein. The expression of the promoterless reporter gene linked to a particular promoter is triggered by the interaction between transcriptional regulators (activated by the target contaminant for example) and the promoter (e.g., promoter of the biodegradative routes for the contaminant of interest). The most frequently used reporter genes include *lacZ* (encoding β -galactosidase), *lux* (encoding bacterial luciferases) or *gfp* (encoding the *Aequorea victoria* green fluorescent protein). Several arguments have been proposed by Harms et al. (2006) for using bioreporters as an alternative to other common analytical techniques. Particularly, bioreporters can serve specifically for sensing the bioavailability of a particular contaminant, rather than its total quantity. Bioreporters target chemicals, stress or toxicity and can be specific (e.g., arsenic species (Stocker et al., 2003)), group-specific (e.g., endocrine disruptors (Hahn et al., 2006)) or semi-specific (e.g., reporter gene fused to a stress-responsive promoter (SOS or heat-shock response) (Sorensen et al., 2006)). Using different types of bioreporters and multi-well platform techniques (e.g., 384-well plates), an attractive alternative is the high-throughput biomonitoring of multiple contaminants (Gu et al., 2004). In addition, since bioreporters have the potential for miniaturization, rapid multi-target analysis can be obtained using chip-based bioreporters. For instance, the bioluminescent bioreporter integrated circuit is a whole-cell biochip that uses a complementary metal-oxide semiconductor photodetector for the detection of low-level bioluminescence induced in a *Pseudomonas fluorescens* sp. by the presence of naphthalene or salicylate (Bolton et al., 2002).

A3.4. Waste biotreatment in confined environments

There are several engineered systems to treat wastes using microorganisms. The characteristics of the waste(s) will determine the most appropriate system. For instance, a polluted air stream will be treated using a biotrickling filter (i.e., a reactor in which a contaminated gas stream passes through a reactor containing immobilized microorganisms on a solid support with a concurrent or countercurrent trickling of nutrient media). Hazardous solid wastes will be preferentially treated using a bioslurry reactor (i.e., a reactor design in which polluted sediments or soils are mixed with water and nutrients to create a slurry) or an anaerobic digester (Table 1). Other examples of confined environments are sewage treatment facilities and related wastewater treatment plants (WWTPs) that are used to treat easily biodegradable wastes and to control nutrients. Although these engineered environments vary considerably in their design, the workflow of treatment systems follows a common pattern as indicated in Figure 2. Before entering the system, waste characterization is an absolute prerequisite (Figure 2, phase A). During biotreatment, it is necessary to assess biological and physico-chemical processes, i.e., to understand how this “black box” operates (phase B). This helps to identify corrections or adjustments needed towards an efficient biodegradation. At the end of the process, characterization of the outflow is necessary in order to evaluate the performance of the treatment plant (phase C).

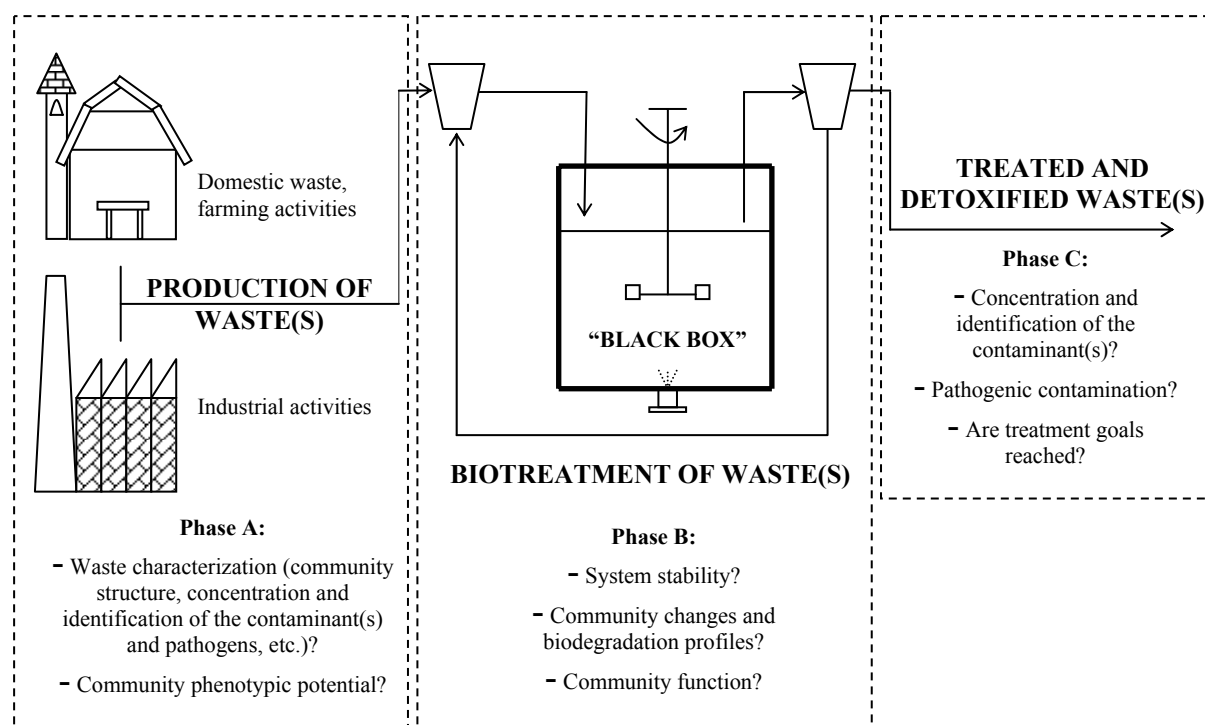


Figure 2. Waste biotreatment in confined environments.

A3.4.1. High-throughput options in wastewater treatment plants

In nature, the removal of organic matter from wastewaters is mediated by well-regulated aerobic and anaerobic biological processes. In contrast, many wastewater treatment plants face increasingly stringent effluent standards regarding specific contaminants such as nutrients (N and P) and sulfurous compounds that deteriorate the quality of inland and coastal waters (Vesilind, 2003). In particular, eutrophication is a global problem in which increased nutrient availability enhances the accumulation of microbial and algal biomass (Seviour et al., 2003). When the rates of photosynthetic production exceed the rates of consumption by higher-ranked trophic levels, organic matter accumulates in the form of 'blooms' of cyanobacteria and algae. Those are potentially toxic, malodorous and aesthetically detracting (Paerl and Steppe, 2003). As phosphorus input is the main limiting factor in eutrophication and nitrogen removal is one of the most important and costly elements in wastewater treatment, intensive research has led to novel biological technologies for nutrient removal (Wagner and Loy, 2002; Seviour et al., 2003; Khin and Annachhatre, 2004). The technology used to eliminate nutrients from wastewater relies on adjusting several parameters such as pH, $[O_2]$, E_h , hydraulic retention time or sludge age, to favor the activities of subpopulations relevant to N-removal, i.e., nitrification-denitrification (release of nitrogen as N_2) and biomass

production, and to P-removal, i.e., accumulation of polyphosphate (PO_3)_n in the biomass. For instance, nitrifying bacteria have slow growth rates and therefore need a hydraulic retention time higher than their generation time of 1-5 days to allow their survival at the treatment plant. Nitrification will be inefficient if sufficient oxygen is not provided (i.e., nitrification competes with organic matter oxidation), if pH and temperature are not within a specific value range, and if various inhibitors, such as heavy metals, are present (Kelly et al., 2005). Furthermore, P-removal mediated by polyphosphate-accumulating organisms (PAOs) will fail when competing glycogen-accumulating microorganisms (GAOs) proliferate. Therefore, in general, integrated systems for nutrient control require additional physico-chemical processes to produce effluent concentrations of less than 2 mg/l total nitrogen and less than 1 mg/l total phosphorus (Vesilind, 2003). The microbial population structure of a typical 'sludge' is difficult to predict (Bramucci and Nagarajan, 2006). Major differences in the microbial community structure of an activated sludge exist in function of geographic location (e.g., there are great differences between activated sludges in USA, Japan, Europe or Australia) and seasonal variation. In addition, bioreactors with different designs contain different microbial communities even when identical wastewaters are treated (Bramucci and Nagarajan, 2006). This has to be considered when a 'model community' of an activated sludge is described. Moreover, the structure and variation of microbial communities in the confines of wastewater treatment installations can give important clues about their functioning and dynamics. For instance, evidence for highly structured bacterial communities in different compartments of an activated sludge WWTP treating a phenol-laden effluent was provided by DGGE and community changes were closely reflected by FISH whole-cell targeting (Whiteley and Bailey, 2000).

These issues show that it is necessary to elucidate the composition of complex microbial communities. They also indicate the need for high-throughput culture-independent techniques that could rapidly detect and prevent unwanted changes within critical microbial subpopulation in WWTPs before serious problems arise. For instance, microarrays offer the possibility to assess the distribution, the relative abundance and the activity of nitrifying bacteria in a massively parallel rather than a serial manner (e.g., PCR, cloning and sequencing). Of course, it is also important to verify the good functioning with other objective assays, since in non-axenic environments, like activated sludge, community structure may change whereas the conversion remains stable.

Biological nitrogen removal processes

Numerous biological processes exist to metabolize inorganic nitrogen compounds (Khin and Annachhatre, 2004). Conventional biological removal of nitrogen is a two-step process involving nitrification followed by denitrification (Figure 3). Nitrification is the aerobic conversion of ammonia to nitrate *via* nitrite by obligate aerobic lithotrophic nitrifying bacteria, including ammonia-oxidizing (AOB) and nitrite-oxidizing (NOB) ones. This critical step is considered as the Achilles heel in many wastewater treatment schemes (Wagner and Loy, 2002). Denitrification is a dissimilatory process in which nitrate is used as an alternative terminal electron acceptor by denitrifiers, i.e., phylogenetically distinct groups of bacteria, *Archaea* and fungi, to form nitrogen gas and nitrous oxide. This denitrification step is somewhat more difficult to achieve than nitrification only, because it requires the presence of both a degradable carbon source (COD) and nitrate (Vesilind, 2003). Since the nitrogen in the influent of a wastewater treatment plant is present either in the form of urea (which is easily hydrolyzed to ammonia) or ammonium, the stability and activity of nitrifying bacterial communities are very important. In addition, as nitrification relies on microorganisms with low growth rates, it will result in a long start-up period and a slow recovery after an uncontrolled loss of biomass and activity.

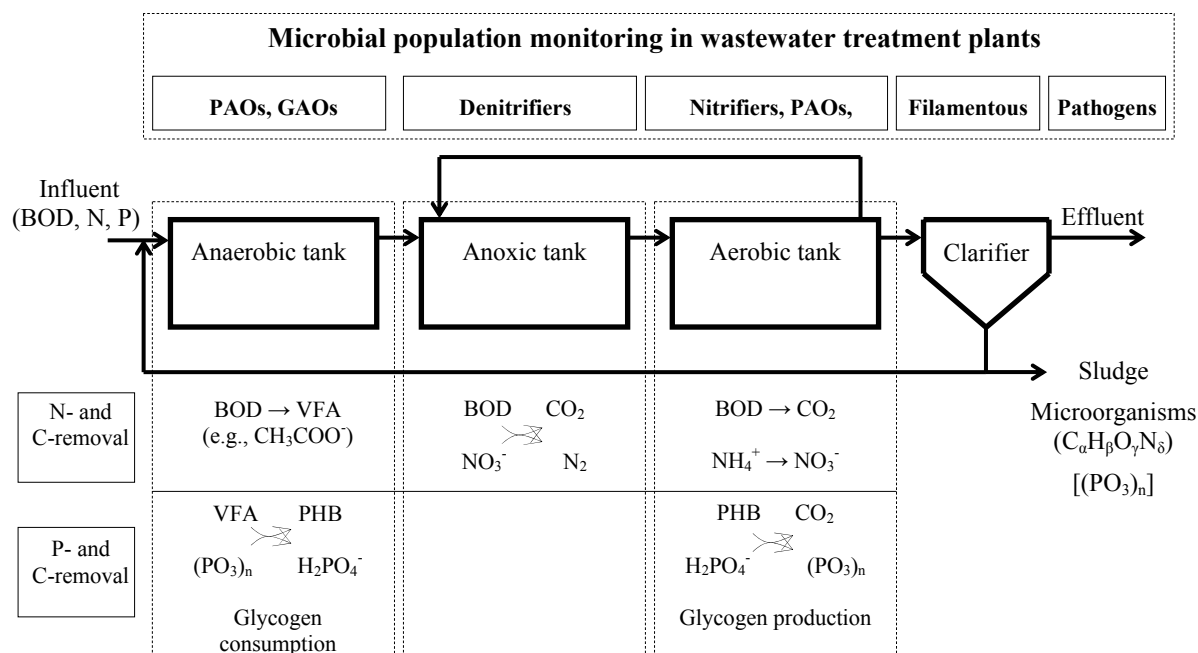


Figure 3. Typical three-stage anaerobic-anoxic-oxic (A²/O) process for simultaneous nitrogen and phosphorus removal. In the A²/O process, the return activated sludge (RAS), which contains nitrate, is recycled to the anaerobic zone. Because the introduction of nitrate into the anaerobic tank can deteriorate the biological P-removal process, the UCT (University of Cape Town, South Africa) process is used generally as an alternative for simultaneous N- and P-removal for relatively weakly loaded wastewaters. In the UCT process, the RAS is first introduced into the anoxic zone, after which the anoxic mixed-liquor is partly recycled to the anaerobic tank.

T-RFLP analysis has been used to investigate the stability of the bacterial community structure in response to changes in operational parameters of activated sludge systems (Saikaly et al., 2005). Terahara et al. (2004) successfully used the same method to monitor the microbial nitrifying population dynamics at the start-up stage of a wastewater treatment reactor. Similar goals have been achieved using real-time PCR, which allows precise enumeration of AOB and NOB populations (Harms et al., 2003; Limpiyakorn et al., 2005). For instance, real-time PCR standard curves obtained for *Nitrospira* 16S rRNA gene (NOB) and AOB 16S rRNA gene assays were log-linear over 6 orders of magnitude (Harms et al., 2003).

An interesting alternative for monitoring these communities is the use of POA formats. They have been applied in only few studies so far to analyze the microbial community structure of environmental samples. Urakawa et al. (2003) used a 18-mer oligonucleotide probe to target *Nitrosomonas* (AOB bacteria) and demonstrated that probe-target specificity could be

evaluated with thermal dissociation curves. Recently, a DNA microarray containing a set of oligonucleotide probes targeting the 16S rRNAs of several groups of nitrifying bacteria has been designed for the monitoring of wastewater treatment plant samples (Kelly et al., 2005). The authors demonstrated the direct detection of specific nitrifying bacteria (*Nitrosomonas* spp.) in an activated sludge treatment facility without the need for prior PCR-amplification (the latter may introduce bias towards quantitative assessment of microbial communities). This is a promising technology to monitor and control treatment systems susceptible to periodic failures. In addition, as nitrification is brought about by a limited number of phylogenetically related bacteria, 16S rRNA-based methods can be used to provide both structural and functional information (Wellington et al., 2003). For instance, an isotope array was used with a small POA format targeting AOB from nitrifying activated sludge samples that were incubated with [^{14}C]-bicarbonate (Adamczyk et al., 2003). CO_2 fixation activities of the AOB populations within the complex activated-sludge communities were detectable on the microarray by ^{14}C incorporation and were confirmed independently by FISH-MAR and by control experiments where the AOB activity was inhibited. Although this technique is not applicable routinely in a wastewater treatment plant, simultaneous monitoring of the community structure and specific substrate incorporation of model and complex microbial communities under particular conditions can be achieved. This provides new insights into key physiological interactions between the different members of complex communities (Wagner et al., 2006).

On the other hand, denitrification is achieved by numerous unrelated bacteria (as well as *Archaea* and fungi). Consequently, in order to characterize microbial populations performing denitrification, genes encoding denitrification enzymes are primary target of investigation (Wellington et al., 2003). The genetic reason for the conserved trait of ammonia oxidation in AOB is that the relevant genes are not subject to horizontal gene transfer, in contrast to denitrification genes (Philippot, 2002). Consequently, FGA formats have also been applied to monitor the functioning of environmental microbial communities (Wu et al., 2001; Zhou, 2003). Specific DNA microarrays have been targeted on functional genes involved in nitrogen cycling, such as genes encoding dissimilatory heme- and copper-containing nitrite reductase (*nirS* and *nirK*) and ammonia mono-oxygenase (*amoA*) (Wu et al., 2001; Taroncher-Oldenburg et al., 2003; Tiquia et al., 2004; Chung et al., 2005; Liebich et al., 2006).

Recently, metagenomic studies revealed that some *Archaea* have nitrification activities. Indeed, *Crenarchaeota* contain and express genes related to those of bacterial ammonia monooxygenases (Nicol and Schleper, 2006). This may be of considerable significance since *Crenarchaeota* constitute an important prokaryotic fraction of mesophilic marine and terrestrial environments. In addition, an isolated member of the abundant fraction of low-temperature marine *Crenarchaeota* (*Nitrosopumilus maritimus*) was also found to oxidize ammonia (Konneke et al., 2005). Using PCR primers targeting archaeal ammonia monooxygenase subunit A (*amoA*) genes, Park et al. (2006) reported molecular evidence that ammonia-oxidizing archaea (AOA) occur in activated sludge bioreactors. This study opens avenues to examine the relative abundance and activity of AOA in activated sludge environments and the relative contribution of AOB and AOA to ammonia oxidation. These findings complement the biochemistry and physiology of nitrification that was previously found to be exclusively the privilege of bacterial nitrifiers (Costa et al., 2006). They constitute a breakthrough in the global nitrogen cycle and expand our understanding of the microbial communities in WWTPs. Besides the extension of nitrification processes to the *Archaea*, a paradigm shift in the world of nitrogen cycling has been proposed by Costa et al. (2006) from the classic discovery of Winogradsky (two-step nitrification) to the possible existence of complete ammonium oxidation (i.e., Comammox: one-step nitrification) in biofilms and other microbial aggregates with low mixing of substrates and biomass.

The newly discovered anaerobic ammonium oxidation (i.e., Anammox: the oxidation of ammonium under anoxic conditions with nitrite as the electron acceptor and dinitrogen gas as the product) has promoted innovative and promising technologies for the removal of nitrogen from wastewater, over and above the classic consecutive steps of nitrification/denitrification (Mulder et al., 1995). Pure cultures of anammox organisms have not yet been isolated. However, using enrichment cultures with a density gradient centrifugation protocol, physical purification of anammox cells (> 99.5%) has been achieved (Jetten et al., 2005). Their extremely slow growth rate (doubling time of 10-14 days) is a significant roadblock. In confined environments, high sludge retention times are required to obtain a sufficient biomass concentration. Given these isolation and growth rate challenges, our knowledge of anammox bacteria, which belong to deep-branching *Planctomycetes*, is based mainly on rRNA approaches (Schmid et al., 2005). Molecular methods revealed that anammox bacteria can be abundant in WWTPs with nitrogen removal and anaerobic niches. However, we are still lacking a detailed understanding of the genetics and biochemistry of these microorganisms.

Recently, Strous et al. (2006) used a metagenomic approach to sequence the genome of the uncultured anammox bacterium *Kuenenia stuttgartiensis* from an anammox bioreactor. The almost complete 4.2-Mb genome of this organism contained more than 200 genes relevant to anammox catabolism and respiration. The authors confirmed the great versatility of anammox bacteria by demonstrating that *K. stuttgartiensis* was also able to respire both iron and manganese oxides (Strous et al., 2006). The genome of *K. stuttgartiensis* sheds new light on a major process of global nitrogen cycling since anammox accounts for up to 50% of oceanic nitrogen loss (Strous et al., 2006). This study is a starting point for implementing new ways of N-removal from wastewaters and confirms the indisputable value of high-throughput environmental genomics as a tool for identifying and monitoring bacteria relevant to biotreatment processes.

These discoveries in inorganic nitrogen metabolism and the corresponding consequences for the nitrogen cycle have given rise to novel biological nitrogen elimination technologies to complement conventional processes. A large-scale two-step process has been patented and implemented in the wastewater treatment plant of Rotterdam (Netherlands). The process combines partial nitrification (single reactor system for high rate ammonium removal over nitrite (SHARON)) followed by the anammox process (Khin and Annachhatre, 2004). This combination has been realized in a single reactor system to reduce operational costs through the decrease of both the energy (half of the aeration required for the nitrification) and space requirements. In this way, the completely autotrophic nitrogen removal over nitrite (CANON) process removes ammonium from wastewater in which *Nitrosomonas*-like aerobic bacteria (AOB) cohabitate with *Planctomycete*-like anaerobic ammonium-oxidizing bacteria in a single reactor under oxygen-limited conditions (< 0.5% air saturation to avoid inhibition of anammox bacteria) (Khin and Annachhatre, 2004). This process required no added COD because of its completely autotrophic character. It is therefore suitable for the treatment of wastewaters containing high concentration of ammonium but low content of organic carbon that cannot support the conventional nitrification/denitrification process. The widespread application of the anammox process is limited by the long start-up time needed because of the extremely slow growth rate and biomass yield of anammox bacteria. In addition to the on-line monitoring of chemical conditions within the reactor (e.g., O_2 and NO_2^-), the implementation of such a combined process requires an understanding of a complex microbial community during start-up and steady-state operation to prevent any deterioration of the anammox

population or ingrowth of nitrite oxidizers (Jetten et al., 2002). Moreover, the cooperation and stable interaction between the two groups of autotrophic microorganisms in the CANON reactor require a detailed control of the relative ratio of AOB and anammox bacteria. This can be achieved for instance by a quantitative fingerprinting method such as real-time T-RFLP (Yu et al., 2005). This can provide a fundamental basis for fine-tuning of operational parameters to enhance the nitrogen removal efficiency.

Biological phosphorus removal processes

In wastewater biotreatment units, most of the organically-bound phosphates and condensed phosphates (e.g., polyphosphates) are converted to inorganic orthophosphates ($[\text{PO}_4^{3-}]$, $[\text{HPO}_4^{2-}]$ and $[\text{H}_2\text{PO}_4^-]$). These must be removed before they are discharged in aquatic environments. The enhanced biological phosphorus removal (EBPR) process offers an attractive and environmentally friendly alternative to chemical precipitation using coagulants, such as lime and iron or aluminium salts. The EBPR technology consists in alternating an anaerobic zone in series with an aerobic one that is being selectively enriched for organisms able to take up and accumulate phosphorus at concentrations beyond stoichiometric requirements for normal cellular growth, i.e., the so-called polyphosphate accumulating organisms (PAOs) (Meyer et al., 2006). No PAOs have been isolated in pure culture so far. However, experimental evidence from laboratory- and pilot-scale conditions has established a P-removal pathway. In the anaerobic zone, the PAOs rapidly assimilate volatile fatty acids (VFA) that are stored as poly- β -hydroxyalkanoates (PHA), with the use of polyphosphates ($(\text{PO}_3)_n$) as energy source and the concomitant release of orthophosphates in the bulk liquid (Seviour et al., 2003). This storage is not accompanied by an increase in cell growth rates and the chemical composition of the stored PHA depends on the carbon source assimilated (e.g., storage of poly- β -hydroxybutyrate (PHB) through acetate assimilation) (Figure 3). Furthermore, the synthesis of poly- β -hydroxyalkanoates requires a source of reducing power typically provided by glycogen (Emmenden Meyerhof pathway (Martin et al., 2006)), an intracellular storage compound synthesized aerobically by the PAOs (Seviour et al., 2003). In the subsequent aerobic stage, the PAOs store orthophosphates in the form of polyphosphate granules and multiply using poly- β -hydroxyalkanoates as carbon and energy source (Seviour et al., 2003). Poly- β -hydroxyalkanoates are also used aerobically for glycogen production.

Finally, sludge removal in the aerobic zone leads to a net removal of phosphorus from the system (Figure 3).

Despite this biochemical understanding, EBPR processes fail frequently for unclear reasons. This unreliability may be due to the presence of glycogen-accumulating organisms (GAOs) that has a detrimental impact on the P-removal process (Meyer et al., 2006). Indeed, these GAOs can outcompete the PAOs for carbon utilization under anaerobic conditions. Although the GAOs perform similar carbon transformations as PAOs, they do not accumulate phosphorus in excess of their requirement for growth (Meyer et al., 2006).

Although the phenotype of the PAOs is well characterized, the fundamental microbiology and microbial ecology of the EBPR process are poorly understood. For instance, a good understanding of the genetics and biochemistry of the complete PHA cycle is still lacking. In this connection, Henne et al. (1999) and Wang et al. (2006) constructed metagenomic DNA libraries of microbial communities from soil and activated sludge. Using a selection strategy based on providing a PHA intermediate as sole carbon and energy source, the authors successfully isolated genes involved in PHA metabolism. Furthermore, a PAO was recently identified as a Gram-negative bacterium and was found to be related to *Rhodocyclus* of the β -*Proteobacteria* phylum (Crocetti et al., 2000). This microorganism was named ‘*Candidatus Accumulibacter phosphatis*’ (Hesseltmann et al., 1999). In addition, only a few GAOs have been identified, hampering a clear understanding of the observed EBPR deterioration events. Recent studies have shown the existence of two GAOs belonging to the so-called ‘G-bacteria’ (Meyer et al., 2006) in the α -*Proteobacteria* phylum.

In summary, the isolation of representative pure cultures that carry out key processes in N- or P-removal is a crucial step, but difficult to achieve using conventional culture-based techniques. So far, attempts to obtain pure cultures of both polyphosphate- and glycogen-accumulating organisms have failed, preventing a comprehensive understanding of their basic microbial physiology and biochemistry. This is also likely the case for other key physiological processes as only 27 out of 53 bacterial phyla contain cultivated organisms (Keller and Zengler, 2004). The previously described high-throughput cultivation-dependent and -independent techniques will greatly accelerate the isolation and deciphering of new microorganisms relevant to waste biotreatment. An obvious illustration is the above-mentioned sequencing of the uncultured anammox bacterium *Kuenenia stuttgartiensis* using a

metagenomic approach (Strous et al., 2006). In the same way, Martin et al. (Martin et al., 2006) presented a metagenomic analysis of two lab-scale EBPR sludges dominated by the uncultured bacterium '*Candidatus Accumulibacter phosphatis*'. They reported the complete genome sequence of this bacterium and its analysis provided insight into some aspects of energy and carbon metabolism that are central to its phosphate-removing functions. These include the presence of a high-affinity orthophosphate (P_i) transporter gene ensuring low P_i concentration in treated wastewater, the discovery of a cytochrome supporting an anaerobic functionality of the tricarboxylic acid (TCA) cycle, and the presence of a fumarate reductase that could account for the biosynthesis of polyhydroxyalkanoate (PHA) polyhydroxyvalerate, a biodegradable plastic substance (Mino and Satoh, 2006). Knowledge of the genome sequence will improve understanding of how EBPR systems work and provide engineers with the tools needed to optimize EBPR-based wastewater treatment. Another study mediated by Yeates and Blackall (Yeates and Blackall, 2006) reported the construction and analysis of a metagenomic library from an EBPR biomass. Clones were identified that contained DNA originating from '*Accumulibacter*' and '*Competibacter*'. These clones will be further investigated with additional sequencing to discover genetic information from these bacteria. In addition, Loy et al. (2005) have designed a phylogenetic oligonucleotide array to analyze the '*Rhodocyclales*'. This microarray contains 79 probes for the parallel detection of all bacteria of the order '*Rhodocyclales*' and constitutes a new promising step towards the monitoring of confined environments that display the PAO phenotype.

Besides genomics approaches, metaproteomics are useful to gain insights of P-removal processes. For instance, the entire proteome of a laboratory-scale activated sludge system optimized for the EBPR process was studied by Wilmes and Bond (2004). The authors isolated proteins using quadrupole time-of-flight mass spectrometry (Q-ToF MS) and *de novo* peptide sequencing followed by BLAST homology searching. The identified proteins likely stem from the dominant and uncultured *Rhodocyclus*-related PAOs. In addition, Wilmes and Bond (2004) investigated another metaproteomic strategy consisting in comparing peptide mass fingerprints of environmental samples (generated by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS)) with DNA sequences. However, this strategy was inappropriate because of the scarce genetic information currently available for EBPR organisms which are not yet sequenced. A direct link between peptide mass fingerprints and sequence information will now be feasible, thanks to the recent publication of the ~5.6-Mbp genome sequence of '*Candidatus Accumulibacter phosphatis*'

(Martin et al., 2006) and the ongoing sequencing project of a microbial community from a wastewater treatment plant (pending (December 2006), Community Sequencing Program, Sequencing Plans for 2007, DOE Joint Genome Institute, US Department of Energy, Office of Science, <http://www.jgi.doe.gov/sequencing/>). With these genetic blueprints of bacteria relevant to EBPR research, there will be no need for a time-consuming and expensive *de novo* peptide sequencing strategy (Wilmes and Bond, 2006a).

Going one step beyond, comparative metaproteomics has been successfully used by Wilmes and Bond (2006b) to evaluate the differences and similarities in metaproteomic maps between the anaerobic and aerobic zones of two distinct activated sludge systems characterized by a poorly- and by a well-performing EBPR process. Interestingly, different protein expression patterns were obtained. In the well-performing sludge, a balanced and stable protein expression during the anaerobic-aerobic sludge cycling was observed. This sludge was dominated by PAOs of the *Rhodocyclus* type. On the contrary, the poorly-performing sludge showed protein expression profiles alternating sharply during the anaerobic-aerobic sludge cycling.

Filamentous bacteria: bulking and foaming control in activated-sludge systems

The successful operation of the conventional activated sludge process is strongly dependent on the performance of a gravity secondary clarifier that separates biomass from the effluent thanks to the formation of healthy settleable flocs. However, unsatisfactory floc formation and settling occur frequently, resulting in a bulking sludge. This is caused by the uncontrolled overproliferation of filamentous bacteria (Martins et al., 2004). Because of their open or porous structure, the flocs do not settle properly leading to the release of organic material with the treated water and a drop in the final effluent quality. In addition, biomass loss in the effluent is also triggered off by biological foaming that forms a stable scum layer on the surface of aeration basins and secondary clarifiers (Rossetti et al., 2005). This foam is produced mainly by filamentous bacteria and contains floating biomass that prevents an effective solid-liquid separation. Therefore, it is of major interest for WWTP operators to rapidly detect the appearance of filamentous bacteria responsible for bulking and foaming. If such bacteria are present, treatment strategies can be used. These treatments range from

chlorination, ozonation, application of hydrogen peroxide to anti-foaming devices. The only known preventive engineering tool to maintain the correct balance between floc-forming bacteria and filamentous microorganisms is the use of selector reactors that precede the activated-sludge aeration tank. A selector is a small tank (20 to 60 min contact time) or a series of tanks in which is maintained a high substrate concentration (i.e., mixing of incoming wastewater with return sludge) that favors the growth of nonfilamentous organisms (Tchobanoglous et al., 2003). However, there exist conflicting results on their effectiveness (Martins et al., 2004). On the other hand, polyaluminium chloride is added as a precautionary measure to control specific filamentous bacteria responsible for bulking and foaming problems. However, this strategy is considered too expensive (Kaetzke et al., 2005). Therefore, identifying population shifts before the appearance of bulking and foaming events is particularly important. More specifically, high-throughput tools to detect filamentous bacteria are needed for a more proactive control instead of the commonly used feedback control (Martins et al., 2004).

Numerous filamentous bacteria involved in foaming and bulking have been identified. They include *Microthrix parvicella*, an unbranched filamentous bacterium belonging to the phylum *Actinobacteria* (Rossetti et al., 2005), and actinomycetes belonging to the genera *Mycobacterium*, *Gordonia*, *Nocardia*, *Tsukamurella* and *Rhodococcus* (Kim et al., 2004a). Real-time PCR has been successfully used by Kaetzke et al. (2005) for quantifying *Microthrix parvicella* 16S rRNA gene copies in activated sludge samples collected from WWTP with and without foaming problems. The level of *M. parvicella* 16S rRNA gene copies in the nonfoaming samples was below 3% of the total number of 16S rRNA gene copies and approximately one order higher for the foaming samples. The method may become an important component of a warning system for forthcoming bulking and foaming episodes. Kim et al. (2004a) designed a random genomic DNA microarray (i.e., without any sequence information) for *Gordonia amarae*, a bacterial strain involved in bulking and foaming, as well as *Zoogloea ramigera*, a strain known to form flocs, and *Mycobacterium peregrinum*, a bacterial pathogen. The authors demonstrated that this microarray could be used as a quality and operational control tool. Subsequently, the same authors constructed a more comprehensive microarray containing random genomic DNA probes generated from 13 different bacterial strains, for the purpose of multiple bacterial detection or bacterial community analysis (Kim et al., 2005).

A3.4.2. Biotreatment of hazardous and/or recalcitrant wastes in confined environments

There is an ever-increasing number of hazardous waste sites that pose risks to human health and the environment. For instance, the U.S. Environmental Protection Agency's Final National Priorities List counts 1,241 sites that must be urgently and completely treated (U.S. EPA, National Priorities List, December 2006, <http://www.epa.gov/superfund/sites/npl>). These hazardous wastes can be liquids, solids, contained gases, or sludge. These contaminants result from manufacturing activities such as waste streams contaminated by TNT (Stenuit et al., 2005) or commercial products like pesticides. Among these priority sites, the U.S. EPA identified 60 sites that are contaminated with petroleum hydrocarbons, 69 sites with nitroaromatic compounds (including 16 contaminated with TNT), 468 with pesticides, 749 sites with polycyclic aromatic hydrocarbons (PAHs) and 1,165 sites with metals. Contaminants in hazardous waste sites are natural compounds (e.g., paleogeochemical compounds such as fossil fuels or heavy metals found in minerals) augmented and further released by anthropogenic activities to concentrations toxic for living organisms. However, the majority of toxic and recalcitrant contaminants are synthetic chemicals not found in the environment (the so-called “xenobiotic” compounds). The term xenobiotic comes from the Greek “xenos”, foreign, and “bios”, life, and refers to chemically synthesized compounds containing structural elements or substituents that do not (or seldom) occur in nature. Therefore, their structure is not easily recognized by existing degradative enzymes and, as a consequence, they accumulate in the environment (Stenuit et al., 2005). Among xenobiotics, polyaromatic, chlorinated and nitroaromatic compounds have been shown to be toxic, mutagenic and carcinogenic for living organisms.

In contrast to the relatively innocuous waste streams containing nutrients, such as domestic wastewaters for which sewerage systems and biotreatment technologies exist, the biotreatment of wastes contaminated with noxious and recalcitrant compounds is more challenging. Such hazardous wastes need special biotreatment technologies. The heterogeneity of the waste in terms of origin, complex nature (e.g., presence of multiple contaminants) and toxicity requires case-specific management. As a result, this represents a niche for objective strategies and highly efficient methodologies of site characterization and contamination history extrapolation (environmental forensics). In addition, as recalcitrant compounds or highly toxic chemicals require more costly remedial approaches, the

apportionment of costs among potentially responsible parties has to be discussed with particular allocation models (Ram et al., 2005a).

Because hazardous compounds are often slowly biodegraded, the use of confined environments for their treatment guarantees more predictable end-points with minimal duration of key biological processes. In addition, confined environments offer the possibility for controlled application and safe containment of bioaugmentation when specific catabolic activities are needed (El Fantroussi and Agathos, 2005). Again, this is an emerging niche for reliable monitoring techniques.

High-throughput approaches to characterize contaminated sites

The characterization of a contaminated site involves a detailed understanding of its biological and physico-chemical components, such as the detection of specific phylogenetic groups relevant to biotreatment processes, the assessment of degradative capabilities of the native microflora or the detection of critical contaminants.

The application of DNA microarrays to environmental systems for the detection of specific organisms has been described by several researchers. For instance, Loy et al (2002) used a 16S rRNA gene-targeted oligonucleotide microarray to specifically detect all known lineages of sulfate-reducing prokaryotes. Environmental samples were hybridized to the microarray and the results obtained were consistent with other molecular techniques. In another study, Small et al. (2001) designed an oligonucleotide microarray for the direct detection (i.e., without the need for PCR amplification) of 16S rRNA extracted from soil samples. The authors were able to detect *Geobacter chapellei* directly from soil RNA extract without further purification or removal of soluble soil constituents. The detection sensitivity of *G. chapellei* in these soil extracts represented approximately 7.5×10^6 *Geobacter* cell equivalents of RNA. Direct use of soil RNA extracts for microarray hybridizations was also shown by El Fantroussi et al. (2003). These two studies reveal the potential of PAO formats for direct evaluation of microbial characterization of environmental samples. However, and as previously mentioned, specificity, sensitivity and quantification are three microarray issues in the context of environmental studies, and therefore in the context of microbial characterization of contaminated sites. For instance, Koizumi et al. (2002) designed species-

specific oligonucleotide probes based on the sequencing of dominant DGGE bands from toluene- and ethylbenzene-degrading consortia. The hybridization results obtained with the microarray and the different consortia were consistent with the DGGE results. The microarray was also used to analyze oil-contaminated sediments. However, hybridization signals were found with universal and eubacterial probes, but not with probes designed from DGGE bands. In such case, the whole genome amplification procedure previously described (Dean et al., 2002) might be considered. The specificity issue can be evaluated using thermal dissociation analysis. For example, Eysers et al. (2006) identified differences in hybridization signals and dissociation curves of group- and species-specific probes between an uncontaminated and a TNT-contaminated soil sample. The authors also found that generation of dissociation curves allowed the distinction between perfect-match and single mismatch probe-target duplexes. Given the potential of dissociation curves to evaluate the specificity of probe-target nucleic acids duplexes, they are now used by several research groups (e.g., Wick et al., 2006).

Detection of catabolic genes has also been achieved using microarrays. For instance, Rhee et al. (2004) designed a 50-mer-based oligonucleotide FGA format based on most of the 2,402 known genes and pathways involved in biodegradation and metal resistance. This array contained 1,662 unique and group-specific probes. The microarray was successfully used to study catabolic genes in naphthalene-amended enrichments and soil microcosms. Under the experimental conditions used, the microarray differentiated sequences having <88% similarity. Since probes were designed to have <85% sequence identity to any sequence found in the databases, cross-hybridizations were probably not a severe problem. In addition, a good linear relationship was found between signal intensities and DNA concentration of pure cultures and mixed DNA templates. However, the microarray failed to detect less abundant functional genes as it was only sensitive enough to detect dominant catabolic genes (the detection limit in the presence of background RNA corresponded to 1.3×10^7 cells). Dennis et al (2003) designed another FGA format using PCR products to target genes involved in the degradation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Induced 2,4-D catabolic genes were detected with a 2,4-D degrading strain in pure culture and in the presence of a constructed mixed microbial community. In this latter sample, detection of induced genes of the 2,4-D degrading strain was achieved with cell concentrations of 10^5 to 10^6 cells/ml against a background of 10^8 cells/ml. Besides 2,4-D, the induction of resin acid catabolic genes (*tdtA*, *tdtB* and *tdtL*) was detected in sludge-fed pulp exposed to the dehydroabietic acid resin, but there was no increase of the regulatory gene *tdtR*. Therefore, this versatile microarray was a

useful tool for the detection of bacterial gene expression in wastewater and other complex environments. However, the authors mentioned that the number of probes printed was limited because of the need to obtain PCR products from cloned genes or bacterial isolates (Dennis et al., 2003). In order to print a higher number of probes, they suggested the use of oligonucleotide probes. In addition, they proposed an alternative to the use of known sequences by considering clone libraries obtained from DNA or cDNA from a specific environment (such as a wastewater treatment system) instead of oligonucleotides or PCR products (Dennis et al., 2003).

In addition to the detection of particular microorganisms in contaminated environments, the responsiveness of these microorganisms to the presence of pollutants can be used to detect and monitor those pollutants. In particular, such responsiveness can be analyzed using microarrays. For instance, Reichert and Menzel (2005) used a whole genome microarray of the soil nematode *Caenorhabditis elegans* to study the effect of five xenobiotics on gene expression profiles. The authors found that several genes were down- and up-regulated in response to the different xenobiotics. This study opened the doors to the use of gene expression levels for the development of a xenobiotic detection and monitoring system. Kim et al. (2004b) evaluated the potential toxicity of unknown chemicals in burned ash with a yeast microarray. They identified changes in expression level of genes associated to energy/metabolism, stress response and detoxification. The toxicities were caused by reactive oxygen species, metals and other xenobiotics. Compared to classical toxicity tests based on the response of a specific (micro)organism to a single xenobiotic, the microarray approach offers the opportunity to rapidly screen a large number of different xenobiotics at the same time.

Another high-throughput alternative for the detection and monitoring of pollutants are whole-cell-based biosensors. Tecon et al. (2006) designed a phenanthrene bioreporter through the transcriptional fusion between the *phnS* promoter region (a regulatory gene of the *phn* operon involved in phenanthrene catabolism) and the bioreporter gene *gfp*. The fusion was introduced into a broad-host range plasmid which was transferred into *Burkholderia* sp. strain RP007. The results showed that the bioreporter acted as an appropriate sensor to study phenanthrene bioavailability. Another application example was provided by Toba and Hay (2005). The authors used a *lux*-based bioreporter as a simple, low cost and rapid procedure for the detection of 2,4-D in soil. In order to overcome some of the limitations associated with the

inoculation and monitoring of bioreporters directly in soil, the authors spotted bioreporter expressing cells onto glass microfibre filter discs to easily retrieve them after soil exposure (Toba and Hay, 2005). The response of this disc-fixed cell bioreporter was dependent on the concentration of 2,4-D spiked in the soil. In another study, Sanseverino et al. (2005) developed an estrogen-inducible bacterial *lux*-based bioluminescent reporter in *Saccharomyces cerevisiae* for chemical sensing and environmental assessment of estrogen disruptor activity. The authors observed bioluminescence within as little as 1 h. Therefore, this bioreporter fills the niche for rapid, high-throughput screening of estrogenic compounds. Hahn et al. (2006) used another yeast bioreporter, *Arxula adeninivorans* strain LS3, for the detection of estrogenic activity in wastewater. In one of the constructs, a detection limit of 5 ng l⁻¹ for 17 β -estradiol was achieved. Recently, two formats of bioreporters (chip or 384-well plate format) were constructed (Lee et al., 2005). The arrays contained inducible bioluminescent *E. coli* bacteria with 20 different *lux*-fused promoters for the detection and the classification of various types of toxicity (e.g., superoxide damage in the presence of paraquat or DNA damage in the presence of mitomycin C). Cell responses could be measured with a cooled charge-coupled device camera within 2 h. Therefore, this is a fast, portable and economical high-throughput biosensor system for detecting environmental pollutants (Lee et al., 2005).

High-throughput approaches to discover new catabolic activities

The degradation pathways of many xenobiotics remain to a large extent poorly characterized, if not totally unknown. Therefore, they need to be discovered. This will promote new biotreatment processes to completely mineralize various recalcitrant pollutants.

The identification of new catabolic operons has been investigated in various environments using metagenomic strategies. For instance, Robertson et al. (2004) identified 137 unique nitrilases suited for industrial biocatalysis applications through functional screening of hundreds of environmental DNA libraries. Nitrilases are likely involved in detoxification processes, by hydrolyzing nitriles to their corresponding carboxylic acids. This reaction releases ammonia and was used as a screening strategy to select clones expressing nitrilases (i.e., clones converting nitriles grew when nitriles were provided as sole N-source). In another study, a metagenomic investigation of a microbial mat from the anoxic seafloor of the

Black Sea has provided the first evidence for the presence of a catabolic gene cluster for anaerobic benzoate and hydroxybenzoate degradation in such environments (Kube et al., 2005a). However, as previously mentioned, a limitation of the use of metagenomic libraries lies in the poor screening hits of positive clones performing a particular catabolic reaction. Currently, several authors have proposed a screening/selection procedure based on genetic reporters rather than on direct enzymatic screening or DNA sequence similarity (Uchiyama et al., 2005; Mohn et al., 2006). Using the “substrate-induced gene expression screening”, Uchiyama et al. (2005) isolated aromatic-hydrocarbon catabolic operon fragments from a metagenome library constructed from groundwater microbial flora. The authors isolated 33 positive clones induced by benzoate and two positive clones induced by naphthalene from a total of 152,000 clones. Nevertheless, the concept of guilt-by-association (i.e., regulatory genes lie adjacent to catabolic genes) exploited in this screening has some limitations (reviewed in de Lorenzo, 2005). To overcome these limitations, de Lorenzo and colleagues (Mohn et al., 2006) exploited the ability of the *product* of a desired reaction (rather than the xenobiotic itself) to activate a bacterial transcriptional regulator. In turn, the regulator subsequently induced its associated promoter linked to a reporter gene. This strategy translates the detection of a particular reaction into upregulation of a reporter gene (e.g., the σ^{54} -dependent transcriptional regulator XylR and the *xylR* promoter (*Pu*) fused with *gfp*). The XylR protein, belongs to the NtrC family of prokaryotic enhancer binding proteins (EBPs), is a well-characterized effector-binding activator of an operon for catabolism of toluene and *m*-xylene. Abril et al. (1989) had shown that the XylR protein exhibited broad effector specificity (from toluene to dichlorotoluene isomers). In this way, Mohn et al. (2006) illustrate the generation of a genetic trap from mutated effector-responsive transcriptional regulators in order to reveal the catalytic potential of any cloned DNA segment, e.g., the conversion of lindane (γ -hexachlorocyclohexane) to 1,2,4-trichlorobenzene. To this end, the sequence of the regulator XylR was modified by DNA shuffling and mutagenic PCR to optimally respond to 1,2,4-trichlorobenzene. The authors demonstrated that the genetic trap could be easily optimized *à la carte* for various targeted transformations. Subsequently, the authors successfully derived *E. coli* strains that constitutively expressed the modified XylR variant, named XylR5, and had *lacZ* under control of the *Pu* promoter. The resulting strain was transformed with a *linA*⁺ clone (expressing a lindane dehydrochlorinase) and responded positively in a lindane concentration-dependent manner to a robotic β -galactosidase assay. Since aerobic biodegradation of recalcitrant and xenobiotic compounds is often regulated by effector-binding proteins that respond to diverse chemical structures (Mohn et al., 2006), this

à la carte host strategy is a very promising and elegant approach to discover new catabolic activities in metagenomic libraries. In addition, several effector-binding transcriptional factors of many known regulatory systems can be used to identify biodegradation pathways of unusual C-sources (Galvao and de Lorenzo, 2006). For instance, the transcriptional regulator DntR is an effector-binding activator of an operon for the 2,4-dinitrotoluene catabolic pathway (Johnson et al., 2002; Johnson and Spain, 2003; Smirnova et al., 2004). This catabolic operon encodes a unique 2,4-dinitrotoluene catabolic pathway that uses specific oxygenases for oxidative denitration and subsequent ring-fission. The DntR protein has been used in a prototype bioreporter for the detection of dinitrotoluene in contaminated soil and groundwater (Smirnova et al., 2004). DntR mutants with enhanced specificity for dinitrotoluenes were designed to optimize the sensitivity of the biosensor (Smirnova et al., 2004). The biosensor consisted in a plasmid in which the expression of a reporter gene (*gfp*) was placed under the control of the promoter (P_{DNT}) and the transcriptional regulator DntR (Smirnova et al., 2004). For instance, such an approach can be used to screen metagenomic libraries for new TNT catabolic pathways. Indeed, an obvious critical step in the mineralization of TNT is its preliminary denitration to dinitrotoluene compounds (Stenuit et al., 2005). Therefore, based on the genetic construction described by Smirnova et al. (2004) combined with flow cytometry and cell sorting, the translation of TNT denitration into fluorescent phenotypes represents a promising high-throughput screening in comparison to the detection of the release of nitrite by colorimetry.

Recently, Boubakri et al. (2006) described a metagenomic DNA shuffling process by combining a mutation generator process by protein engineering and the high potential of metagenomic DNA for the creation of novel genes capable of degrading pollutants. Using this strategy, the authors successfully constructed the *linA* gene using the metagenome of a lindane-free soil as a donor of *linA*-like fragments.

Finally, the bacterial genomes sequenced so far are valuable resources to study catabolic pathways of xenobiotics. For instance, Kube et al. (2005b) used the available genome sequence of *Dehalococcoides* strain 195 to study the catabolic pathway of *Dehalococcoides* sp. strain CBDB1, a bacterium able to metabolize many of the most toxic and persistent chlorinated aromatics and aliphatics by reductive dechlorination. This approach led to the discovering of new genes with putative reductive dehalogenase functions. It is expected that, for example, the large-scale sequencing of a terephthalate-degrading microbial community (in

production (December 2006), Community Sequencing Program, Sequencing Plans for 2006, DOE Joint Genome Institute) and a dechlorinating community (pending (December 2006), Community Sequencing Program, Sequencing Plans for 2007, DOE Joint Genome Institute) will provide novel biocatalytic activities and valuable information to understand microbial communities with catabolic activities towards xenobiotics. However, additional studies will be required to determine the function of the sequenced genes that will be discovered under such sequencing projects.

High-throughput approaches to analyze and monitor the biotreatment of hazardous and/or recalcitrant wastes

Monitoring is crucial for the evaluation of the biodegradation performance in a waste biotreatment process. In addition, high-throughput and cost-effective biomonitoring tools are essential to link, in real-time, operational parameters reflecting physico-chemical conditions with microbial stability (or resilience) to maintain optimal biodegradation performance continuously. For instance, a microarray could be used to correlate gene expression patterns to phenotypic properties in order to monitor the efficiency of treatment systems.

In particular, validation of the successful outcome of a bioaugmentation program should be based not only on the quantitative performance of the site (kinetics and extent of biodegradation of the target pollutant) but also on the detection and tracking of the seeded microorganisms and on the tagging of the transferred genes (El Fantroussi and Agathos, 2005). By looking at previous attempts to demonstrate the potential for bioaugmentation in soils, one can see that it has resulted in successes and failures. The difficulty in implementing a successful bioaugmentation process lies in appropriate assessment of several parameters: bioavailability of the pollutants, survival, activity and transport of the added microorganisms, and general environmental conditions. The indigenous community is often responsible for the rapid decline of exogenously added microorganisms because they compete for nutrients and electron acceptors. Therefore, for increased survival and long-term activity of exogenously added microorganisms, these should fill a metabolic niche that is not currently used by indigenous bacteria (El Fantroussi and Agathos, 2005). Another bioaugmentation strategy relies on the use of indigenous bacteria themselves. For instance, a selected consortium composed of four bacteria frequently found in waste metal-working fluids led to a more

effective treatment than uncharacterized communities (van der Gast et al., 2004). Alternatively, improved biodegradation capabilities can be expected by transferring catabolic genes from a donor strain to the already fit indigenous microflora (El Fantroussi and Agathos, 2005). Whatever the strategy used, microbial communities and catabolic genes have to be identified and tracked. This can be accomplished for instance by using microarrays to provide information of microbial diversity and abundance, and of catabolic activities.

A3.4.3. High-throughput approaches to control biofilters and biotrickling filters for the removal of organic compounds and odorous compounds

The removal of volatile organic compounds (VOC) and odorous compounds from gaseous wastes is recognized as of great importance by wastewater treatment plants and other processing facilities. This is underscored by increasingly stringent environmental legislation, odor nuisance complaints from neighboring communities and the potential toxicity and carcinogenicity of volatile organic compounds (Iranpour et al., 2005). These compounds include chlorinated chemicals (e.g., trichloroethylene) or petroleum-originated compounds, especially BTEX (benzene, toluene, ethylbenzene and xylene isomers). Additional odorous chemicals include nitrogen (e.g., NH_3) and sulfur species (e.g., H_2S). Biofiltration and biotrickling filtration are known as the most common biological waste air treatment technologies. Biofilters are reactors in which a humid polluted air stream is passed through a porous packed bed (generally peat or compost mixture) on which a mixed culture of pollutant-degrading organisms is naturally immobilized (Deshusses, 1997). Biotrickling filters work in a similar manner as biofilters, except that an aqueous mineral nutrient solution is trickled over the packed bed which is made of inorganic materials (e.g., plastic rings, lava rocks, structured packing) (Cox and Deshusses, 2002). A wider range of pollutants can be potentially treated in biotrickling filters than biofilters. This is due to the fact that environmental conditions are better controlled in biotrickling filters and potentially toxic dead-end metabolites can be purged out of the system *via* the free liquid (Cox and Deshusses, 1998). These filters can be inoculated with pure cultures (either wild-type or genetically engineered microorganisms), consortia, or complex microbial communities (e.g., activated sludge, the packing material itself, such as compost, and so on). Therefore, in addition to operating conditions, there is a need to study the microbial ecology of biotrickling filters and biofilters in order to better optimize the performance of such biological treatment systems (Malhautier et al., 2005). No

high-throughput monitoring of these filters has been reported so far. However, several authors have studied the microbial community composition and dynamics of such ecosystems. Using a common fingerprinting technique, single-strand conformation polymorphism (SSCP), Khammar et al. (2005) investigated the relationship between the structure of microbial communities and the performance of VOC removal in peat-packed columns seeded with two different inocula (activated sludge from an urban or a mixed urban-industrial wastewater treatment plant). The authors observed a vertical structure of microbial communities correlated with the vertical distribution of biodegradation activity patterns in biofilters. This particular distribution must be taken into account when biofilters are inoperative (Khammar et al., 2005). Using DGGE, Cai et al. (2006) observed that the structure of the microbial community in a biofilter was modified when different volatile organic compounds (i.e., methyl ethyl ketone, toluene, methyl isobutyl ketone and styrene) were provided. These results show the importance of understanding the link between microbial community composition and structure to optimize biofiltration processes. In this respect, high-throughput monitoring approaches will be useful tools to identify, in real-time, community changes leading to inefficient biofilter systems.

A3.5. High-throughput techniques for the monitoring of pathogens – the case of bacterial contamination of domestic wastewater

Water is a universal source and vehicle for bacterial, viral and protozoan pathogens, many of which represent major environmental threats to human health (e.g., Straub and Chandler, 2003). In an increasing number of countries, the population is concentrated in the cities, where outbreaks of pathogens are generally due to safety failures in the drinking water supply. Contamination of drinking and recreational water often results from discharges of wastewater treatment facilities or overflowing sanitary systems. Therefore, the development of an efficient monitoring of pathogens in wastewater is necessary. This remains a challenging task for several reasons. When dealing with bacterial contamination, the nature of the pathogen has to be considered. In the United States, important pathogens today include *Salmonella* (responsible for salmonellosis), *Shigella* (responsible for shigellosis, also called bacillary dysentery), the hepatitis virus, *Entamoeba histolytica* (responsible for amoebic dysentery), *Giardia lamblia* (a flagellated protozoan responsible for giardiasis, formerly known as “beaver disease”), *Cryptosporidium* (an enteric protozoan responsible for cryptosporidiosis), and the deadly *E. coli* H57 strain (Vesilind, 2003). A specific detection procedure must be addressed to each pathogen. In addition to the nature of the pathogen, its concentration is of significant importance since it can be so low that its detection will be virtually impossible (Vesilind, 2003). Conventional techniques to detect pathogens are time-consuming, laborious and biased because they are culture-dependent. Because it is impossible to monitor all pathogenic organisms by conventional techniques, the bacteriological water quality is assessed by the detection of indicators of fecal contamination, such as coliforms or enterococci. These are not pathogens but reflect a fecal contamination and therefore a potential risk for human health. The concept of such “indicators” itself is questionable, as many indicator numbers do not correlate well with pathogen levels, especially in the effluents of WWTPs (Harwood et al., 2005). Therefore, the development of fast and accurate tools for the detection of waterborne pathogenic organisms is a necessity.

Microarray technology has been proposed as a promising alternative for monitoring known and emerging pathogens in the environment. Several microarray prototypes for detection of environmental pathogens in municipal wastewater have been designed with probes targeting

phylogenetic markers, such as 16S, 18S or 23S rRNA genes (Behr et al., 2000; Lemarchand et al., 2005; Maynard et al., 2005; Lee et al., 2006a) or virulence genes (Lee et al., 2006a). Rapid progress is being made in clinical microbial diagnostic microarrays (Bodrossy and Sessitsch, 2004), including the detection of pathogenic viruses (Wang et al., 2002). These techniques could be transferable to environmental samples/settings. Better or entirely new strategies are required in parallel with microarray development for simultaneous recovery of multiple pathogen groups with very different physical characteristics (viruses, bacteria, and protozoa) and density levels, for efficient concentration of large volumes of water in order to detect low target densities, and for better removal of co-concentrated inhibitors from the samples. Moreover, as for any microarray application, a very solid bioinformatics background is needed to extract the meaningful information from the vast amount of data generated. Finally, validation of diagnostic accuracy often remains an unresolved question in microarray technology, as it is time-consuming and requires “gold standards” for meaningful comparisons of the data (Call, 2005). Despite these technical challenges, microarray technology has the highest potential of any other technique for further development of a universal, standardized pathogen detection and monitoring system for water quality assessment (Straub and Chandler, 2003).

Finally, an interesting example of domestic wastewater reuse in closed loop is the ongoing research at NASA and ESA (European Space Agency) on sustainable life support of long-duration human space missions based on bioregenerative water reclamation and solid-waste management (Mergeay, 2006). Development of such life support systems is inspired by Earth's natural waste and water cycles. Although space technology may seem very far from terrestrial needs of millions of people for reliable water and sanitation installations, there have been several examples of successful technology transfer from aerospace to earth-based uses in the field of wastewater treatment, like the Biostyr process (wastewater nitrification-denitrification), the biomass sensor (biological activity determination), or the BIOMET process (heavy metal management). ESA is currently developing a biological engineering approach called MELISSA (Hendrickx et al., 2006), whose water recycling component is being tested at Concordia Station, Antarctica. This compact bioregenerative system is based on the conversion of human waste products to edible biomass by several interconnected biological compartments, whose degradation/conversion efficiency and stability need to be accurately monitored. In this prospect, high throughput methods, including microarrays, provide a reliable tool to track microbial and chemical contaminants (fecal pathogens,

pharmaceuticals like hormones, and so on) and to monitor the response of the system to destabilizing factors.

A3.6. Conclusions

The establishment of an optimal waste biotreatment process requires multidisciplinary efforts and an effective cooperation among all responsible parties to integrate the biological and physico-chemical processes relevant to biotreatment in the most cost-effective configuration. As stated in this chapter, microbial communities and their biochemical potentialities are key driving forces behind an effective biological waste treatment. The use of confined environments for biotreatment allows a better control and maintenance of catabolic activities through the improvement of the stability or resilience of microbial communities, i.e., the capability of microbial communities to respond and adapt to environmental changes. This requires an understanding of the microbiology of key microorganisms, of the detailed performance of microbial communities and of the relationship between microbial community structure and function. For specific hazardous and recalcitrant wastes, bioaugmentation may provide a valuable strategy (El Fantroussi and Agathos, 2005). In particular, the discovery of new catabolic activities is of the greatest importance to enable or accelerate the biotreatment process. In the case of more ‘innocuous’ compounds, protecting the plant from biological process deterioration is a more sustainable strategy than a bioaugmentation approach.

For these purposes, high-throughput genomic tools provide extensive and valuable information. Although these particular genomic tools present some economic constraints (e.g., the need for sophisticated equipment and specialized scientific staff) and their application is still in the cradle, we have provided an overview of their potential to: (i) discover catabolic activities, (ii) assess (qualitatively and/or quantitatively) microbial community composition, (iii) identify the biodegradation capabilities and the function of a specific microbial community, (iv) relate microbial community analysis to the metabolic function of specific groups of bacteria, (v) monitor in “real-time” the performance of a biotreatment plant, and (vi) design new confined environments for waste biotreatment.

In summary, for optimal management of confined biotreatment, the combination of the holistic and single cell approach is primordial, as is the combination of ‘-omics’ approaches between them and in concert with other microbial ecology methods.

A3.7. References

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