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Current Opinion in Biotechnology

Resource recovery from wastewater: application of meta-omics to phosphorus and carbon management

Christopher M Sales[1] and Patrick KH Lee[2]

A growing trend at wastewater treatment plants is the recovery of resources and energy from wastewater. Enhanced biological phosphorus removal and anaerobic digestion are two established biotechnology approaches for the recovery of phosphorus and carbon, respectively. Meta-omics approaches (meta-genomics, transcriptomics, proteomics, and metabolomics) are providing novel biological insights into these complex biological systems. In particular, genome-centric metagenomics analyses are revealing the function and physiology of individual community members. Querying transcripts, proteins and metabolites are emerging techniques that can inform the cellular responses under different conditions. Overall, meta-omics approaches are shedding light into complex microbial communities once regarded as 'blackboxes', but challenges remain to integrate information from meta-omics into engineering design and operation guidelines.

Addresses
[1] Department of Civil, Architectural and Environmental Engineering, Drexel University, Philadelphia, PA 19104, USA
[2] School of Energy and Environment, City University of Hong Kong, Kowloon Tong, Hong Kong Special Administrative Region

Corresponding authors: Sales, Christopher M (chris.sales@drexel.edu) and Lee, Patrick KH (patrick.kh.lee@cityu.edu.hk)

Current Opinion in Biotechnology 2015, **33**:260–267

This review comes from a themed issue on **Environmental biotechnology**

Edited by **Spiros N Agathos** and **Nico Boon**

http://dx.doi.org/10.1016/j.copbio.2015.03.003

Introduction

A paradigm shift is emerging at wastewater treatment plants (WWTPs) where wastewater is no longer just a problem, requiring treatment for safe disposal, but also a resource [1,2]. Wastewater is rich in organic carbon, nitrogen and phosphorus that can be recovered as valuable and useful energy and/or nutrient sources. This approach has the potential to transform WWTPs from energy consumers to producers [3], which in turn allows them to lower their carbon emissions and reduce their contributions to climate change. Furthermore, the recovered nutrients using this approach can become a greener alternative for the agricultural industry that is tasked with feeding an increasing global population. The key to transforming WWTPs into waste-to-resource facilities is improving our understanding of the physical, chemical, and biological processes of established and emerging technologies.

Ammonia (the main nitrogen constituent in wastewater) has traditionally been converted to dinitrogen gas through the dissimilatory biological processes of nitrification and denitrification. A newer, alternative nitrogen removal process that is gaining interest is anaerobic (or anoxic) ammonium oxidation (ANAMMOX) [4]; however, much like nitrification and denitrification, ANAMMOX cannot be used to recover a nitrogen resource. Recent work has demonstrated a three-step process, referred to as Coupled Aerobic-anoxic Nitrous Decomposition Operation (CANDO), capable of energy recovery from reduced nitrogen species by conversion to nitrous oxide gas, a strong oxidant that can be used for the combustion of fuels, that is, methane from biogas [5]. In addition, recovery of nitrogen as a protein source in animal feed via biological assimilation by algae has been proposed [6]; however, further studies are required to assess potential human and animal risks from the use of animal feed produced from wastewater.

The recovery of phosphorus through sequential anaerobic/aerobic processes in enhanced biological phosphorus removal (EBPR) systems and the transformation of organic carbon to methane through anaerobic processes in digesters, unlike the nitrogen recovery processes mentioned above, are two established biotechnological approaches [7], and thus are discussed in more detail in this review. Although both of these bioprocesses involve microbial communities containing a diverse and complex group of microorganisms, phosphorus accumulation is mainly carried out by a small group of phosphate-accumulating organisms (PAOs) within the community [8], while anaerobic digestion requires synergistic interactions between different populations to convert the organic carbon to methane as the final product [9].

Propelled by advancements and affordability in sequencing technologies [10], as well as improvements in mass spectrometry methods [11], it is now feasible to investigate, down to the molecular level, the underlying microbiology of complex microbial communities in these bioprocesses. Specifically, techniques are being developed and applied to various ecosystems to probe their genetic potential (metagenomics), the regulation of the expression of genes (metatranscriptomics) and proteins

(metaproteomics), and the presence of metabolites and metabolic pathways (meta-metabolomics or community metabolomics) [12,13]. To date, all these methods, except community metabolomics, have been applied to some degree to the study of wastewater treatment biotechnology. In this review, we will focus on how meta-omics approaches have been applied to transform our understanding of and provide novel biological insights on resource recovery in wastewater treatment, and briefly discuss current limitations and avenues for its growth.

Enhanced biological phosphorus removal and recovery

In recent years, there has been significant interest in large-scale phosphorus recovery from domestic wastewater. However, due to its low concentration in wastewater ($<$10 mg P L^{-1}) [14], it is necessary to concentrate phosphorus to make recovery feasible. Although EBPR was originally developed to prevent eutrophication by producing effluents with low levels of phosphorus, it can also be a means to concentrate phosphorus for its recovery and reuse [14]. The PAOs in EBPR systems can take up excess phosphorus under alternating anaerobic and aerobic/anoxic conditions, producing sludge that contains 15–20% phosphorus by dry weight. Although the first biological model for EBPR was proposed by Fuhs and Chen in 1975 [15] and the discovery of laboratory-scale EBPR reactors being enriched by a group of organisms, now known as *Candidatus Accumulibacter phosphatis* [16,17], to date, isolation of a pure culture of a PAO has not been accomplished.

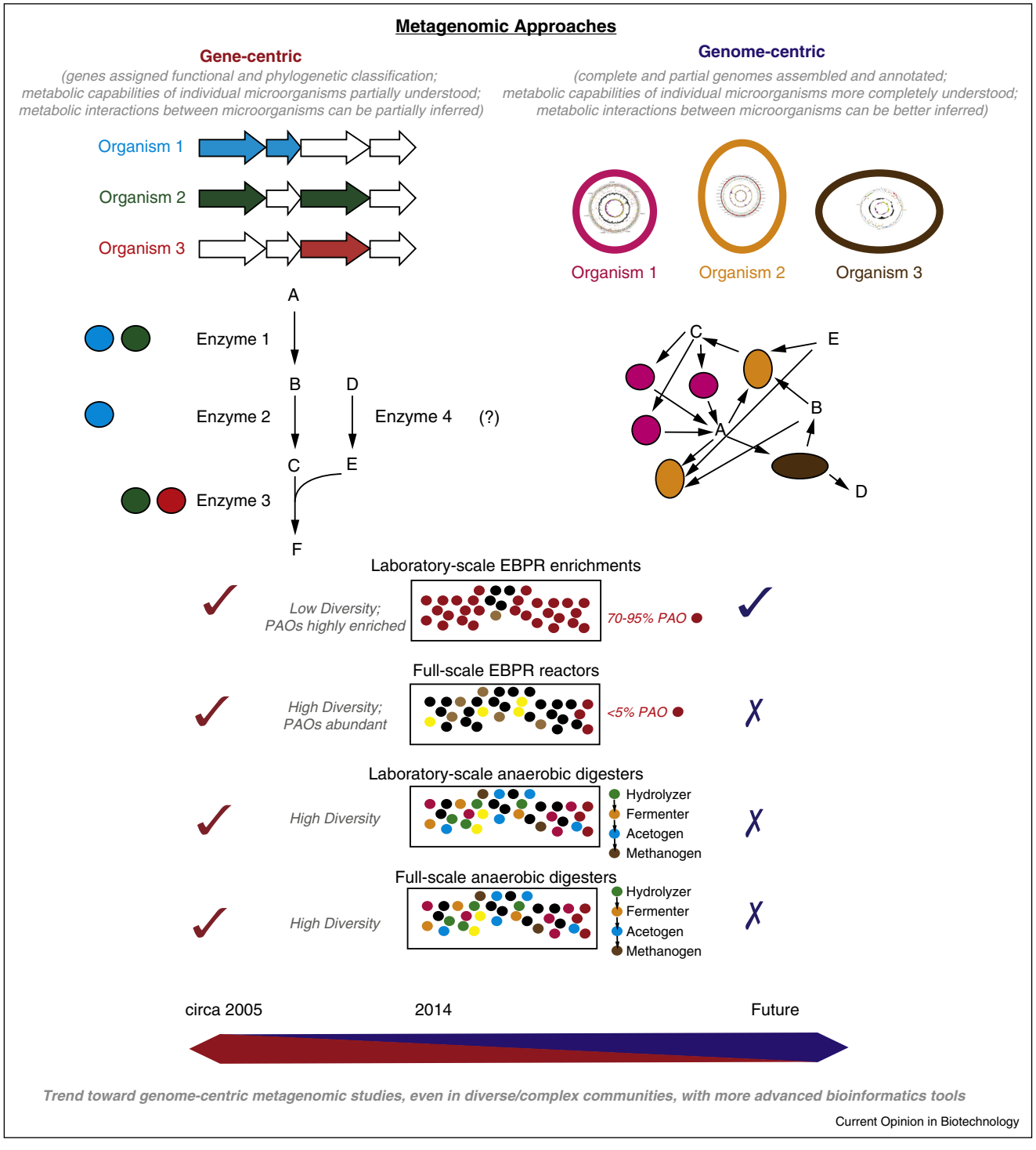
Despite the elusiveness of isolating an axenic culture of *A. phosphatis* [18], meta-omics studies, of primarily laboratory-scale reactors, over the last decade have enabled insights into the phylogenetic and functional diversity of PAOs. The ability to highly enrich laboratory-scale reactors with PAOs has allowed the EBPR process to be a model test-bed to apply meta-omics analyses, especially metagenomics, with the main objective to reconstruct genomes (Figure 1). In fact, the first metagenomics study of an environmental biotechnology process to recover a draft genome — in this case, of *A. phosphatis* UW-1 — was performed on EBPR laboratory-scale reactors [19]. Although this pioneering study employed Sanger sequencing that produced a relatively low amount of sequencing data compared to next-generation sequencing methods, such as 454 Life Sciences and illumina (Solexa), this study provided a comprehensive metabolic construction of pathways used by *A. phosphatis* during anaerobic and aerobic phases of the EBPR cycle [19] and laid the foundation for subsequent meta-omics analyses of EBPR.

Phylogenetic studies, largely based on the polyphosphate kinase *ppk1* gene, have revealed that the candidate genus of Accumulibacter is split into two major clades (types I and II), which are further subdivided into types IA-IE and IIA-IIF, respectively (as referred to in [20•,21••]). The phylogenetic diversity of the genus Accumulibacter based on *ppk1* has been thought to contribute to the phenotypic differences observed in different enrichment cultures, such as their differences in ability to denitrify and utilize different carbon sources. Until recently, our genetic understanding of PAOs has been limited to *A. phosphatis* UW-1, a member of Clade IIA. However, recent metagenomic investigations by Flowers *et al.* [20•], Skennerton *et al.* [21••], and Mao *et al.* [22•], all of which employed next generation sequencing platforms for deep sequencing of laboratory enrichment cultures, have immensely expanded our knowledge of the genetic diversity of the genus Accumulibacter by producing ten additional draft genomes: one of *A. phosphatis* UW-2 from Clade IA [20•]; three from Clade IIC, three from Clade IIF, one from Clade IA, and one from Clade IC [21••]; and one of *Accumulibacter* spp. strain HKU-1 from Class IB [22•]. Although comparative genomics inferred genetic differences among the Accumulibacter clades, it is important to note that many of these recently generated genomes are incomplete (ranging in completeness from 74 to 99%), and thus discrepancies in gene contents reported in these studies (e.g., presence and absence of nitrogen and carbon fixation genes and differences in denitrification pathways) may change as these genomes are finished (Table 1). Despite the incompleteness of the genomes, these metagenomic studies reveal metabolic differences among clades (e.g., the ability of some to utilize ethanol as a carbon source) that can inform potential strategies for enriching particular types within EBPR reactors [21••]. The Flowers *et al.* study [20•] also highlighted differences in phage resistance between the genomes of UW-1 and UW-2, based on their Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) content which could be used to assess and control populations of Accumulibacter in EBPR systems.

Metagenomics has also been applied to study the microbial community of a full-scale EBPR plant [23]. Results from this full-scale reactor revealed the presence of a complex, diverse microbial community, in which Accumulibacter only accounted for less than 5% of all bacteria based on quantitative fluorescence *in situ* hybridization (FISH) [23]. At the time the full-scale reactor study was published, the only reference genome available was that of UW-1 and, thus, reads assigned to Accumulibacter were only compared relative to UW-1 (type IIA), and not to the recently sequenced representatives of the other clades. One major difference between metagenomics of the full-scale plant and the laboratory-scale enrichment reactors was the inability to reconstruct genomes *de novo* from the full-scale reactor, which was likely due to the higher level diversity of the microbial community and current limitations in bioinformatic methods.

The genes and genomes from metagenomic sequencing represent metabolic potentials and form the foundation to

Figure 1



Comparison of gene-centric and genome-centric analysis of metagenomics sequencing data. In the gene-centric approach, reads or contigs from assembled reads can be assigned functional and phylogenetic classifications, which allow for partial metabolic pathway reconstruction. In the genome-centric approach, complete or partial genomes are reconstructed, allowing for function and physiology of individual members of the microbial community and their interactions to be studied. The gene-centric approach has been applied to both EBPR and anaerobic digestion, independent of the level of diversity and complexity of the microbial community. To date, the genome-centric approach has only been successfully applied to enriched lab-scale EBPR systems.

Table 1

Major characteristics of the genomes of *Candidatus* Accumulibacter phosphatis strains. Genes that are 'Present' in a genome are colored green, while those 'Not Determined' for a particular genome are shown in yellow. Gene name abbreviations in the table and the enzyme they encode for are: *narGHIJ*, nitrate reductase; *napABCDFGH*, periplasmic nitrate reductase; *nirBD*, nitrite reductase; *norZ*, nitric oxide reductase; *nosDFLZ*, nitrous oxide reductase; *nif*, nitrogenase; *rnfABCDEG*, nitrogenase electron transport complex.

Strain	Clade (based on *ppk1*)	Genome size [Mbp]	Genome completeness	Denitrification					Nitrogen fixation		Carbon fixation	Ethanol metabolism	Anaerobic reducing power		Ref
				narGHIJ [$NO_3^- \rightarrow NO_2^-$]	*napABCDFGH*[c] [$NO_3^- \rightarrow NO_2^-$]	*nirBD* [$NO_2^- \rightarrow NO$]	*norZ* [$NO \rightarrow N_2O$]	*nosDFLZ* [$N_2O \rightarrow N_2$]	*nif*	*rnfABCDEG*	Calvin cycle	Acetaldehyde dehydrogenase	Novel cytochrome b/b6[f]	Split TCA cycle	
BA-93	IA	4.62	99.53%[a]	Absent	Present	Present	Present	Present	Present	Present	Present	Absent	Present	Present	[21••]
UW-2	IA	4.5	80.00–90.00%[b]	Absent	Present	Present	Present	Present	Present	Present	Present	Absent	Present	Present	[20•]
HKU-1	IB	3.6	89%[c]	Absent	Present	Present	ND[d,e]	Present	Present	ND[d]	Present	ND[d]	Present	ND[d]	[22•]
BA-92	IC	4.94	92.74%[a]	Absent	Present	Present	Present	Absent	Absent	Present	Present	Absent	Present	Present	[21••]
UW-1	IIA	5.6 ± 0.2	97.2%[a]	Absent	Present	Present	Present	Present	Present	Present	Present	Absent	Present	Present	[19]
BA-91	IIC	4.32	74.41%[a]	Present	Absent	Present	Absent	Absent	Present	Present	Present	Absent	Present	Present	[21••]
SK-01	IIC	4.53	94.26%[a]	Present	Absent	Present	Absent	Absent	Present	Present	Present	Absent	Present	Present	[21••]
SK-02	IIC	4.35	97.17%[a]	Present	Absent	Present	Absent	Absent	Present	Present	Present	Absent	Present	Present	[21••]
BA-94	IIF	3.09	75.82%[a]	Absent	Present	Present	Absent	Absent	Present	Present	Present	Absent	Present	Present	[21••]
SK-11	IIF	4.7	76.18%[a]	Absent	Present	Present	Absent	Present	Present	Present	Present	Present	Present	Present	[21••]
SK-12	IIF	4.44	93.12%[a]	Absent	Present	Present	Absent	Present	Present	Present	Present	Present	Present	Present	[21••]

[a] Based on 182 essential genes, see Supplementary Table 2 for Ref. [19].
[b] See Ref. [22•].
[c] For UW-1 and UW-2, *napC* homologue was found in a locus separate from *napDAGHBF*. However, *napC* was not found in strain HKU-1.
[d] ND: Not Determined. Sequences for HKU-1 were not available online at time of publication so presence of genes in HKU-1 genome could not be determined. However, norQ, which encodes a subunit of a nitric oxide reductase, was identified in HKU-1 contigs according to Ref. [22•].
[e] *norQ*, which encodes a subunit of a nitric oxide reductase, was identified in HKU-1 contigs according to Ref. [22•].
[f] Novel cytochrome b/b6 described in Ref. [19].

examine when and under what conditions certain genes are expressed.

Both metatranscriptomics [24] and metaproteomics [25] analyses have been applied to laboratory-scale EBPR reactors. Only single time points from the anaerobic and aerobic phases were sampled from a single reactor for each study. Although the original intention was to compare differential expression of genes and proteins in both phases, the results from these studies primarily served to close knowledge gaps that remained after the draft genome of UW-1 was published [19]. However, aided by the recent availability of many Accumulibacter genomes, future metatranscriptomics, metaproteomics, and meta-metabolomics investigations should elucidate additional biological insights about this organism under different conditions.

Anaerobic digestion to recover carbon for energy generation

Besides phosphorus, typical municipal wastewater contains 500 mg/L of chemical oxygen demand (COD) [3], primarily in the form of organic carbon. This organic carbon is commonly transformed to carbon dioxide (CO_2) in aerobic activated sludge processes. The energy demand of aeration for activated sludge processes is the main reason why WWTPs are significant energy consumers. An estimated 1.93 kWh/m^3 from wastewater could potentially be recovered as energy from COD (based on its complete oxidation to CO_2) [3]. In a growing trend to shift their roles as energy consumers to producers, many WWTPs are making a strong push to adopt anaerobic digestion as a treatment process to catalyze the transformation of organic carbons to methane for heat or electricity generation [3]. Conventional anaerobic digestion processes are well-suited for treating high-strength wastewater and concentrated biomass from activated sludge processes [3], while the newly developed anaerobic fluidized membrane bioreactors have shown promise for low-strength wastewater [26].

The increasing importance of anaerobic digestion as a source of clean energy has attracted strong interest in elucidating the biological mechanisms of this process. Anaerobic digestion generally consists of four main processes: hydrolysis, fermentation, acetogenesis, and methanogenesis [9]. As a large number of microbes play a role in the process, meta-omics methods are applied to investigate the overall microbial community. This is evidenced by the utilization of amplicon sequencing of the bacterial and archaeal 16S rRNA genes with high-throughput sequencing platforms at the genomic level to prospect diversity, community structure and key populations in full-scale digesters [27•]. The substantial sequencing depth gathered from pyrosequencing has also enabled detailed microbial ecology questions to be addressed, such as illustrating that the communities in replicate laboratory anaerobic digesters were assembled via deterministic processes [28••], demonstrating that syntrophic populations were resilient and rebounded following short-term perturbations of the sludge loading rate (F:M ratio) and the addition of starved biomass in full-scale systems [29], and identifying the key syntrophs and methanogens associated with acid degradation in long-term enrichment cultures [30]. Beyond the 16S rRNA gene as a marker, amplicon sequencing of the methyl coenzyme M reductase (*mcrA*) gene, encoding the enzyme that catalyzes the terminal step in methanogenesis, has also been used to specifically probe the diversity and structure of the methanogens [31,32]. High-throughput sequencing of the archaeal 16S rRNA gene and *mcrA* gene in parallel revealed that the two genes produced different taxonomic profiles of full-scale anaerobic digesters with greater methanogen richness detected using the *mcrA* gene and potential novel methanogens could be identified via the *mcrA* gene [32].

Although amplicon pyrosequencing provides in-depth details on the presence and diversity of microorganisms in a digester, the metabolic functions of the populations can be better deciphered via shot-gun metagenomics sequencing. Thus far, a number of studies have investigated the metagenomes of anaerobic digestion in laboratory-scale [33–36] and full-scale systems [37–41]. Since earlier metagenomic studies mostly employed the 454 Life Sciences platform, the sequencing depth achieved was relatively shallow compared to the higher throughput illumina platforms. Hence, a gene-centric approach was typically employed to analyze the gene functions present along with recruitment against sequenced isolate genomes (Figure 1). Thus far, unlike the genome-centric approach in the studies of EBPR, *de novo* genome assembly has yet to be extensively applied in anaerobic digestion metagenomic studies. However, as advanced bioinformatics tools designed to reconstruct genomes from metagenomics data, such as GroopM [42], Metawatt [43] and MaxBin [44], become available, it will soon be possible to understand how specific populations within a complex community, such as in an anaerobic digester, interact and contribute to specific functions or niches and, ultimately, to the overall process.

In recent years, metatranscriptomic [45] and metaproteomic [46,47] analyses have been applied to study gene expression in anaerobic digesters. One of the inherent challenges in metatranscriptomic study is extracting high-quality RNA. Stark *et al.* [48] recently showed that extraction methods influenced the representation of particular microorganisms in a sample and likely the interpretation of metatranscriptomic results from complex digester samples. Despite technical issues that have to be further refined, metatranscriptomic analysis have provided novel biological insights. For example, Rotaru *et al.* [49••] used RNA-sequencing to demonstrate direct

interspecies electron transfer to *Methanosaeta* as a new mechanism for methane generation during anaerobic digestion. Another method to probe gene expression is to analyze the gene products expressed as proteins by metaproteomics. Metaproteomic analyses have been driven by advances in analytical detection [11] and the quality of metagenomic data, which form the library for mass spectrometry searches. Similar to metatranscriptomics, metaproteomic analysis has also facilitated novel discoveries, such as the recent identification of *Coprothermobacter proteolyticus* strains as scavengers and/or predators performing proteolysis and fermentation during thermophilic cellulose anaerobic digestion in microcosms [50].

Conclusion

As the world's non-renewable resources continue to be consumed, there is a strong need to tap into wastewater as a resource. Many full-scale anaerobic digesters and EBPR systems are already in operation globally but detailed knowledge of the biological reactions in these systems is often lacking due to their complexity. However, advanced meta-omics methods have begun to shed light into these 'blackboxes' as it is now possible to determine the metabolic potentials of the microbes and evaluate the conditions in which certain biological functions are executed.

At the moment, meta-omics methods are mainly applied as research tools to collect fundamental biological information. As the arrays of meta-omics methods improve further and more data is generated with these techniques, having a systems biology view of biological processes for resource recovery will soon be possible. The challenge, however, is to integrate the wealth of biological data into engineering designs and operation guidelines that will allow us to systematically improve resource recovery biologically rather than through 'trial and error' on 'black boxes'. For example, as illustrated in EBPR, different enrichment conditions have resulted in different PAO populations, suggesting that the composition of the input stream, process design and operation directly influence the microbiology, and, consequently, the effectiveness of resource recovery. Information from meta-omics studies can also lead to the development of diagnostic tools based on biomarkers (e.g., gene probes, expression probes, metabolites) that can tell us whether a key population is present/absent or whether genes in key enzymatic pathways are expressed or not. The incorporation of biological and ecological theories into the design and modeling of biological systems was described as the next frontier in engineering biological processes over a decade ago [51]. To date, many models of biological systems, such as the International Water Association (IWA) Anaerobic Digestion Model Number 1 (ADM1) [52], based on a set of physico-chemical and biochemical reactions, have yet to incorporate biological theories and information into them. Now as meta-omics methods for producing and analyzing large amounts of biological data improve, more efficient and sustainable technologies for resource recovery from wastewater that are designed and operated based on biological theories and the utilization of meta-omics data will likely emerge in the near future.

Acknowledgements

PKHL was supported by grants from the Research Grants Council of Hong Kong through projects #116111 and #116413. CMS was supported by seed funding awarded by the AJ Drexel Institute for Energy and the Environment. PKHL thank Mingwei Cai for his assistance.

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