The microbiology of biological phosphorus removal in activated sludge systems

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Abstract

Activated sludge systems are designed and operated globally to remove phosphorus microbiologically, a process called enhanced biological phosphorus removal (EBPR). Yet little is still known about the ecology of EBPR processes, the microbes involved, their functions there and the possible reasons why they often perform unreliably. The application of rRNA-based methods to analyze EBPR community structure has changed dramatically our understanding of the microbial populations responsible for EBPR, but many substantial gaps in our knowledge of the population dynamics of EBPR and its underlying mechanisms remain. This review critically examines what we once thought we knew about the microbial ecology of EBPR, what we think we now know, and what still needs to be elucidated before these processes can be operated and controlled more reliably than is currently possible. It looks at the history of EBPR, the currently available biochemical models, the structure of the microbial communities found in EBPR systems, possible identities of the bacteria responsible, and the evidence why these systems might operate suboptimally. The review stresses the need to extend what have been predominantly laboratory-based studies to full-scale operating plants. It aims to encourage microbiologists and process engineers to collaborate more closely and to bring an interdisciplinary approach to bear on this complex ecosystem.

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Keywords: Activated sludge; Enhanced biological phosphorus removal; Phosphorus accumulating organism; ‘G-bacteria’; Glycogen accumulating organism; Rhodocyclus

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Abbreviations: CFB, Cytophaga-Flavobacterium-Bacteroides division; DAPI, diamidino phenyl indole; DGGE, denaturing gradient gel electrophoresis; DNPAO, denitrifying polyP accumulating organisms; EBPR, enhanced biological phosphorus removal; FISH, fluorescence in situ hybridization; GAO, glycogen accumulating organisms; MAR, microautoradiography; PAO, phosphorus accumulating bacteria; PCR, polymerase chain reaction; PHA, poly-β-hydroxyalkanoate; PHB, poly-β-hydroxybutyrate; polyP, polyphosphate; SBR, sequencing batch reactor; SSCP, single strand conformation polymorphism; UCT, University of Cape Town; VFA, volatile fatty acids

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1. Introduction

Eutrophication is a global problem, in which blooms of cyanobacteria and eukaryotic algae occur as a consequence of the breakdown of community homeostasis from continued pollution of oligotrophic aquatic bodies with nutrients like nitrogen (N) and phosphorus (P) at levels which exceed growth-limiting concentrations for these photosynthetic organisms [1,2]. Of these nutrients, P input is considered more critical [3,4] since many of these photosynthetic organisms [1,2]. Of these nutrients, P input is considered more critical [3,4] since many of these photosynthetic organisms [1,2].

Increasing evidence points to wastewater treatment plants as major point sources for both N and P, which in the latter case originates from fecal material, industrial and commercial sources and synthetic detergents and other cleaning products [5]. Conventional activated sludge plants designed principally to remove organic carbonaceous material produce treated effluents with high residual P levels, although some N removal does occur in these systems [3,6,11]. In the past 30 years many plants have been designed and built around the world to deliberately reduce not just organic carbon and N levels but also P, using microbially based methods [11–14]. This removal is achieved by encouraging the accumulation of P in bacterial cells in the form of polyphosphate (polyP) granules in excess of the levels normally required to satisfy the metabolic demand for growth, a storage process commonly referred to as ‘enhanced biological phosphorus removal’ (EBPR) [15,16]. This P uptake and polyP storage in organisms known as polyP accumulating organisms (PAO) occur under particular plant operational conditions that are reasonably well understood, even though how these conditions encourage the accumulation of polyP in PAO is not [17,18]. When the cells are removed from the process during sludge wasting then so is the P.

Since the initial reports of the EBPR phenomenon, many different full-scale plant configurations have been designed and built, but these have evolved in an empirical manner, with little or no real understanding of the microbiology involved. In the absence of this vital information, it is quite feasible that these plants may not be designed for, or operate under optimal conditions for EBPR. Even though they are used increasingly frequently to treat domestic waste around the world, especially in land locked communities discharging their wastes into environmentally sensitive receiving bodies of water, most still operate unreliably. Such unreliable performance persuaded the US Water Environment Research Foundation to fund projects...
in 2001 to investigate technology yielding consistently low P levels in treated effluents. Although P concentrations in the influent will vary with its source and the cultural behavior of individual societies, and especially whether low P detergents are used, most plant effluents need to satisfy discharge licence agreements and contain <1 mg l⁻¹ P [19]. Thus, chemical P removal by precipitation, with serious environmental problems of its own, is often necessarily incorporated to produce a treated effluent that meets the required standards [19], and these will probably become more stringent as governments become more environmentally sensitive and respond increasingly to lobbying from articulate environmental groups.

It is likely that EBPR technology will never fulfill its true potential until we have a more comprehensive understanding of the microbial ecology of P removal. Several reviews have already been written [14,17,20–22], but in the past few years the application of molecular RNA-based culture-independent methods [23–25] has had a profound impact on how we now view EBPR microbiology. Many of the questions raised in the earlier reviews concerning the identity of the putative PAO have been addressed [26], but there is still much to be learned even here. Therefore, it seems timely to look at the topic again to summarize our current state of understanding of EBPR microbiology and to speculate on what still needs to be understood if we are to design and run better EBPR systems in the future.

2. Some history of EBPR processes

Several reviews have discussed how EBPR technology developed from its accidental discovery [27] more than 40 years ago. In an often provocative review, the early events showing the biological basis for EBPR and the importance of the South African work by Barnard are dealt with chronologically [21]. Barnard, with others, was responsible for designing an evolving series of plant configurations for EBPR, many of which are still used [6,11,14]. Most of these evolved from his Bardenpho plant that was designed for more efficient N removal than existing configurations like the modified Ludzak–Ettinger configuration [28], but adding an anaerobic reactor to this system gave reliable P removal in the five-stage Bardenpho, or Phoredox, system. His subsequent work, and that of others (reviewed in [6,11,14]) suggested that the following operational conditions were needed for successful EBPR:

1. an anaerobic reactor which receives the initial influent containing the readily degradable carbon and energy sources,
2. a need to limit the concentration of nitrate entering this anaerobic zone by incorporating an anoxic zone in the design, since in its presence EBPR fails because the denitrifying bacteria were considered able to anaerobically respire and deplete the supply of organic substrates, making them no longer available for the PAO,
3. strictly maintaining anaerobic conditions in the anaerobic zone,
4. recycling the biomass through alternating anaerobic: aerobic conditions, although some of the first EBPR systems did not incorporate this design feature.

The reasons for these requirements and how critical they are to EBPR will be discussed later in this review, but they were developed in an empirical manner with no understanding of their microbiological bases, and this is still largely the case. Thus, like many other aspects of EBPR, our knowledge of the engineering requirements has advanced much more rapidly than our microbiological understanding. Since the five-stage Bardenpho process was introduced, many other configurations have been designed and built, in attempts to simplify plant configuration and decrease construction costs, and to minimize or eliminate the problems of nitrate recycling into the anaerobic zone [6,11,14]. These stimuli led to the design of the three-stage Bardenpho or Phoredox system, which can be considered the progenitor of most of the EBPR plants now used, like the Johannesburg and modified University of Cape Town (UCT) processes, which were both conceived to deal better with anaerobic nitrate recycling problems, and are described in detail elsewhere [6,11,14].

3. Which chemical transformations characterize EBPR biomass?

Many studies have described the gross chemical changes that occur during the anaerobic/aerobic cycling of the biomass in EBPR systems, and these have been critically reviewed [17,21]. Most have been carried out on biomass from laboratory scale reactors fed synthetic sewage and single carbon sources, where some control can be exercised over parameters like feed rates, hydraulic retention times and sludge age. So they operate under quite different conditions to those usually encountered in full-scale systems, especially in terms of the selective pressures brought to bear on the microbial populations [29,30], and have been criticized accordingly. However, the general agreement is that in the anaerobic zone, some bacteria very rapidly assimilate short chain volatile fatty acids (VFA) formed by the action of chemoorganotrophic fermentative bacteria [31]. These are not used to support an increase in cell growth rates, but instead are stored as poly-β-hydroxyalkanoates (PHA), whose chemical composition depends on the carbon source assimilated [32,33]. Hence with acetate assimilation, poly-β-hydroxybutyrate (PHB) is synthesized, while propionate leads to the production of mainly poly-β-hydroxyvalerate. Often mixtures of PHA are found in biomass from a single plant, although the identity of the PHA synthesizing bacteria in activated sludge is still poorly understood. During this anaerobic stage, biomass
PHA levels increase in parallel with the assimilation of acetate [34]. Intracellular polyP content falls and an increase in phosphate levels in the bulk liquid can be detected following its release from the biomass [35]. Then in the subsequent aerobic stage, PHA levels in the biomass fall concomitant with a decrease in phosphate levels in the bulk liquid and an increase in biomass P levels as polyP, which can be > 15% of cell dry weight [18]. If a biomass sample taken from the aerobic reactor is examined microscopically, cells staining for polyP are abundant, but no intracellular PHA can be detected by staining. On the other hand, cells in biomass from the anaerobic reactors stain for PHA but not polyP. These are common features of EBPR plants, although some show patterns of chemical changes quite different to these, as discussed below. Furthermore, the usual experience is that cells appearing as large coccobacilli in pairs showing these staining responses are commonly organized into large floc-associated clusters (Fig. 2), although they may sometimes be loosely dispersed throughout the biomass [18,36,37] for reasons not understood. It is assumed that these chemical changes are implemented only by the PAO arranged in this distinctive way. The consequence of PAO cell cluster organization on EBPR is not clear, although good EBPR performance is often associated with appearance of large tight clusters in the biomass.

4. The biochemistry of EBPR systems

4.1. EBPR metabolism with acetate as sole carbon source

Several empirical models have been described [16,17,38] to attempt to explain the chemical changes, mainly from data from studies analyzing community chemistry in response to acetate as the sole carbon source, and these models have been critically examined in earlier reviews [17,21]. Their main features are given in Fig. 1. Although there is general agreement in the interpretation of the major changes, the models differ in certain important aspects of EBPR. It should be emphasized that the chemical data upon which all the models are based are gross reflections of the overall activities of communities of unknown microbial nature, and so are impossible to interpret in terms of the activities of individual populations. The models also assume that the detected uptake and release of polyP, and PHA synthesis and reutilization all occur in a single population of PAO cells, all sharing the same metabolic attributes [39]. This may or may not be the case, but seems unlikely from what we now know about EBPR communities (see Section 9).

The models all explain successful EBPR as a consequence of the PAO achieving dominance under anaerobic/aerobic recycling conditions by having a selective advantage over the other bacterial populations present in their abilities to synthesize intracellular storage compounds under the ‘feast-famine’ conditions which characterize EBPR systems. Thus, under anaerobic conditions, the PAO are thought to rapidly assimilate organic substrates like acetate and use these to synthesize PHA using stored polyP as an energy source, and the orthophosphate generated from polyP degradation is released into the bulk liquid (Fig. 1). Then in the absence of any organic compounds in the aerobic zone, organisms with stored PHA are able to use these as carbon and energy sources to grow and to assimilate phosphate to synthesize polyP. Thus, PAO achieve dominance under the prevailing anaerobic: aerobic conditions because they alone can grow aerobically in the absence of any exogenous source of carbon and energy, by using the PHA accumulated anaerobically. The general view is that the PAO are probably better able to survive in ecosystems exposed to these ‘feast-famine’ regimes, with a capacity for rapid substrate assimilation and storing energy and carbon taking precedence over high growth rates [40,41]. Synthesis of PHA requires a source of reducing power, and the various models proposed differ in what is used for this. Suggestions were that the electrons required were derived from the anaerobic operation of the TCA cycle [38,42], although Mino et al. produced evidence [17,22] that glycogen, an intracellular...
lar storage compound synthesized aerobically by the PAO, was catabolized anaerobically to generate electrons for PHA synthesis (Fig. 1). Evidence in favor of a key role for glycogen in EBPR has increased since then and the Mino model is now preferred. However, it has been proposed that, as glycogen degradation alone would not satisfy the demands for reducing power for PHA synthesis, then the TCA cycle provides the remainder [43,44]. Glycogen is thought to play a key role in EBPR by regulating the redox balance of the PAO [17], which may be necessary for permitting the anaerobic assimilation and metabolism of the diverse range of readily biodegradable substrates encountered in full-scale systems (see below). Some evidence suggests that glycogen availability, and not intracellular polyP levels, may ultimately limit the ability of cells to assimilate substrates anaerobically under shock loading conditions [44,45].

A similar debate has surrounded the bioenergetics of anaerobic substrate assimilation and PHA synthesis in PAO. Early studies claimed that adenosine triphosphate (ATP) from polyP degradation was the only energy source used anaerobically by the PAO for substrate (i.e. acetate) assimilation, PHA synthesis and other biosynthetic reactions, and cell maintenance. However, glycogen catabolism is now also thought to provide ATP for PHA production, but how much energy is made available de-
pends on the pathway used for glycogen catabolism [17,44,45].

Chemical analysis of these enriched cultures can reveal the bulk chemical changes taking place but contributions from less dominant populations may be masked, and often interpretation of the data is difficult. In vivo nuclear magnetic resonance (NMR) studies have also been used to follow metabolic flux in mixed communities in response to the supply of particular substrates under specified conditions. This approach has allowed clarification of some of the confusions and contradictions arising from the data from the chemical studies, as well as pointing out how poorly understood the biochemistry of EBPR communities still is [43-45]. With NMR, Pereira et al. [43] clearly showed anaerobic operation of the TCA cycle in their biomass, and glycogen was confirmed as a source of reducing power for PHA synthesis, where glycogen appeared to be degraded via the Entner Doudoroff (ED) pathway. Importantly, both acetate and glycogen appeared to contribute to anaerobic PHA synthesis, and the PHA was then used aerobically for glycogen production [45].

Some data support these earlier interpretations. Thus, Hesselman et al. [44] provided evidence that the TCA cycle could act as a partial source of reducing power for PHA production, and that glycogen catabolism occurred via the ED pathway. However, some of their other data raise questions about many of the previous assumptions made about EBPR biochemistry, especially its bioenergetics. In particular, they suggested, on theoretical ground as well, that cells assimilated acetate not by active transport, as assumed earlier, but by passive or facilitated diffusion, requiring no expenditure of energy. Furthermore, acetate activation, the most energetically demanding step in anaerobic EBPR metabolism apparently occurs with no involvement of the enzyme acetate kinase, which had been assumed to be important in all earlier models [17]. Instead acetyl CoA synthetase was used.

A role was proposed for involvement of energy sources in anaerobic EBPR additional to those suggested in the earlier models. These included pyrophosphate, released by the action of the polyP degrading enzyme detectable at high levels of activity in their biomass, pyrophosphate: phosphoenolpyruvate:AMP phosphotransferase, participating in substrate level phosphorylation and the establishment of a proton motive force. In addition, efflux of MgHPO₄ was coupled to proton translocation via a secondary transporter system. As with many other similar studies using laboratory scale reactors, the ratio of anaerobic P released to acetate assimilated was not a constant. The literature suggests that this ratio depends on the mixed liquor pH affecting the energy demand for acetate transport [17], but Hesselman et al. [44] considered it as reflecting directly both the polyP and glycogen content of their biomass. It may also vary of course with different EBPR communities, not all behaving precisely as these biochemical models suggest they should. From their data, Hesselman et al. [44] proposed a modified biochemical model for EBPR to accommodate their ideas.

However, little experimental biochemical data are yet available to validate any of these empirical models, and even NMR data are unable to explain fully the behavior of the different communities, since the structure/function relationships of the populations involved are completely unknown. Thus, some of the differences in chemical performance of biomasses from the different studies may be explained by the presence of communities of quite different composition developing in reactors run under very similar conditions, a situation now documented in the literature [26,46]. It is strongly recommended that all future studies of this nature should involve parallel molecular microbial community studies if their purpose is to clarify and not to confuse.

4.2. EBPR metabolism with substrates other than acetate

The other difficulty with studies of this kind is that full-scale activated sludge systems deal with a much more diverse range of substrates than acetate or other short chain VFA, and yet relatively little is known about their influence on the chemical changes described above, although many organic compounds both singly and in combination appear to support EBPR [47,48], possibly by being converted to VFA first [15]. It has been suggested [49] that the chemical nature of the VFA supplied, by directly determining the amount and chemical composition of the PHA synthesized anaerobically, in turn determined aerobic P assimilation. However, once again no community analyses were carried out under the different feed conditions used and no convincing explanation for the results was provided.

It has been well documented in the literature that glucose as sole carbon source can lead to a failure of EBPR [50,51] and the possible microbiological reasons for why this might happen will be discussed later. However, biochemical models for EBPR with glucose have been proposed and supported by laboratory scale studies [52-56], which have convincingly shown that glucose may support stable EBPR in laboratory scale reactors. For example, from ¹³C-NMR data it has been suggested [55] that the lactic acid producing bacteria play a crucial role in the anaerobic zone. This population has been held responsible for anaerobic glucose assimilation leading to lactic acid production and eventually glycogen storage [55-57]. In the model described in [55] it was hypothesized that glycogen was transformed anaerobically into PHA by the PAO, while the lactic acid bacteria used glycogen to synthesize a lactate storage polymer. This model allowed P uptake aerobically by the PAO, as predicted by the models discussed above (Fig. 1). Unfortunately no microbiological evidence was provided to suggest whether the community structure would support the model, apart from scant morphological descriptions of the main bacteria seen in the
biomass [55]. A different model to explain EBPR with glucose was proposed in [56], which was much more similar mechanistically to the Mino and Comeau–Wentzel suggestions, but also with little or no direct microbiological or biochemical evidence to support it.

How EBPR biomass might be supported by amino acids as possible substrates has attracted surprisingly little interest since their presence in wastewater is certain; but both glutamate and aspartate as sole carbon sources could support EBPR in a laboratory scale reactor [57,58]. It was suggested that anaerobic substrate storage again occurred, but not all the glutamate used as substrate was stored as intracellular PHA. Instead, storage was thought to involve synthesis of some storage polypeptide yet to be chemically characterized and identified.

It is also clear that the existing biochemical models still fail to account for all the experimental data from studies of this kind. For example, biomass has been shown to assimilate substrates anaerobically [59] with neither polyP hydrolysis nor conversion of glycogen to PHA, and EBPR has also been achieved with glucose as carbon source, but in the absence of any anaerobic storage of PHA [60]. These outcomes emphasize how poorly we still understand the basic biochemistry of EBPR and how difficult it is to interpret such chemical trends in any comparative manner in the absence of any knowledge about the structure and function of the responsible microbial communities. It is probable that these models will remain speculative until the biochemistry of pure cultures of authentic PAO is examined under conditions that closely simulate those found in EBPR systems.

5. The nature and role of polyP storage

PolyP is a polyanionic polymer consisting of many orthophosphate monomers linked by phosphoanhydride bonds of high energy, and the level of its polymerization is known to vary between different organisms. All cells contain polyP, which suggests that this polymer is essential for cell function, although it may not be visualized or easily detected there [22,61]. Yet surprisingly little is known about its biochemistry, especially in EBPR biomass, where it can be readily detected as intracellular granules after staining with basic stains like methylene blue and toluidine blue. These granules stain pink with methylene blue, and so are referred to as metachromatic. DAPI (diamidino phenyl indole), which imparts a yellow fluorescence to polyP granules [62,63], has frequently been used to detect polyP in EBPR studies described below. Little recent work has been published on its organization and intracellular location in relationship to EBPR biomass. Earlier data convincingly showed that P in EBPR biomass is organized as intracellular polymeric polyP and is not chemically precipitated on the surface of cells as inorganic deposits or associated with extracellular polymeric substances associated with flocs [64]. Its organization inside PAO is less certain, with various claims supporting its location in the cytoplasm, the periplasm or associated with intracellular membranes [65–67], and possibly complexed with proteins and DNA and RNA [18,61]. These may reflect stages in its synthesis, but all may be possible if PAO contain several different populations, as now seems likely (see Section 10), and each stores polyP differently.

Several attempts have been made to extract this polyP to determine the distribution of chain lengths in EBPR sludge and to fractionate it to understand more about P flux during its anaerobic/aerobic release and uptake. For example, a high and a low molecular mass fraction was obtained by acid extraction, and it was proposed that the low molecular mass fraction was involved in anaerobic polyP degradation [68], results which disagree with those using different polyP characterization methods including 31P-NMR [69–71]. Other methods [72,73] have been used to fractionate polyP into short chain polyP (SCP), long chain polyP (LCP) and granular polyP from EBPR biomass. Evidence has been presented [74] to suggest that P was exchanged between these pools during EBPR and that most of this exchange in a UCT plant involved the LCP fraction. However, this pattern of biomass P flux was markedly influenced by plant configuration and in plants where nitrate was found in the anaerobic zone, intracellular P location shifted mainly to the SCP fraction. No explanation was given for these trends, but the study does illustrate the complexity of EBPR and reinforces the difficulties in interpreting data from mixed communities of unknown microbial status.

It is clear that polyP needs the presence of counter ions to stabilize it and neutralize the strong negative charge it carries. Energy dispersive X-ray spectroscopy has revealed the presence of Mg2+, Ca2+ and K+ in varying amounts depending on their availability in the bulk liquid, associated with polyP granules in EBPR biomass. Several studies [75–78] have suggested that Mg2+ ions but not Ca2+ are particularly important for stable EBPR, and increasing the available Mg2+ substantially increased the P removal capacity of biomass in a bench scale pilot plant [77]. These results are interesting but not easy to test with full-scale systems. However, evidence from some Australian EBPR plants that fail routinely during the summer implicates a fail in the subterranean water table and a measurable drop in the influent Mg2+ levels [79]. It also appears that Mg2+ and K+ are released and taken up simultaneously with P in EBPR systems, but Ca2+ is not [77], and that Ca2+, which is important in stabilizing polyP granules, may play no apparent role in anaerobic P release. Very similar results were obtained with pure cultures of Acinetobacter johnsonii strain 210A [80].

It is not clear what function(s) polyP performs in cells that accumulate it, but several functions have been proposed, supported by experimental data from pure cultures.
of bacteria, especially *Escherichia coli*, and are summarized in [22,61]. Known functions include acting as a source of ATP following the action of the enzymes polyP:AMP phosphotransferase and adenylyl kinase, by replacing ATP as a phosphate donor in the phosphorylation of sugars and a reserve of inorganic P, and as an intracellular buffer. By acting as a reservoir for Mg$^{2+}$ polyP may also serve to supply cells with Mg$^{2+}$, limiting conditions and to play a vital role in heavy metal (e.g. cadmium) tolerance by excreting the metal via a metal phosphate transport system [81]. PolyP synthesis in some bacteria occurs in response to nutritional and osmotic stress, and serves as a signal for production of stress proteins. It is also involved in regulating differentiation and morphogenesis in both eukaryotes and prokaryotes [61]. Whether these functions apply to PAO in EBPR systems is not clear, although polyP is assumed to act as an energy source for the anaerobic substrate assimilation and PHA synthesis, and convincing evidence is available that polyP may buffer EBPR biomass under alkaline conditions, regulating the intracellular pH of PAO by its degradation [82].

6. The microbiology of EBPR – the culture-dependent approach

Fuhs and Chen [15] were the first to isolate bacteria from biomass with a high P removal capacity and they identified their isolates as members of the genus *Acinetobacter* in the γ-Proteobacteria. For the next decade and more, these observations from culture-dependent techniques largely determined the course of EBPR microbiological research. Many other groups around the world also successfully isolated and grew bacteria from laboratory and full-scale EBPR plants using different selective media, and in nearly all cases the majority was identified as *Acinetobacter* spp. [83,84]. So the conclusion was enthusiastically reached that the PAO in EBPR systems were members of this genus. Micromanipulation [85] was used to try to culture the clustered cells from EBPR plants, believing these were certain to be authentic PAO, but very few clustered cells grew to give colonies. Of those that did grow, many did not identify as *Acinetobacter* spp. [86,87]. It has been suggested [18] that the clustered cells may have been representatives of bacterial genera that are not easily cultured. In retrospect, because of the prevailing belief that acinetobacters were the PAO, badly little effort was made to identify the other colonies that grew from the isolated clusters in these studies.

Considerable effort was also directed at resolving the taxonomy of these EBPR isolates of *Acinetobacter*. Several studies showed that although most belonged to *Acinetobacter junii*, *Acinetobacter lwofii* and especially *A. johnsonii*, for reasons that are still not clear [87-90], many could not be identified [91,92]. This partly reflected the identifi-

cation methods used, but the presence of many previously undescribed *Acinetobacter* spp. in activated sludge systems is now established [93,94]. Most of the pure cultures obtained from EBPR systems could synthesize polyP and PHA aerobically at high levels from acetate, although not all isolates synthesized both, and this ability was not associated with any particular species [18]. *A. johnsonii* strain 210A shared many of these features and its physiology and biochemistry have been examined most closely [22,89,95,96]. However, even though low levels of anaerobic P release were detectable in it and many other isolates, none of the isolates obtained then (or since) could assimilate acetate and synthesize PHA anaerobically concomitant with P release, as the biochemical models demand, even under conditions set up to simulate EBPR plants [97]. There are several explanations for such results [97]. These include the possibility that the biochemical models are incomplete or incorrect, that *Acinetobacter* behaves differently in pure culture than in EBPR systems, or that *Acinetobacter* is not a major PAO responsible for carrying out the chemical changes so characteristic of EBPR sludge.

The ability to synthesize intracellular polyP granules is widespread among bacteria [61], and not too surprisingly other Gram-negative and Gram-positive bacteria cultured from EBPR plants showing P removal have also been considered as potential PAO [98,99]. These include isolates of members of the actinobacterial genus *Tetrasphaera* [100,101]. Some filamentous bulking and foaming bacteria like *Microthrix parvicella* and *Nostocoida limicola* II, commonly seen in EBPR systems [102], both stain positively for polyP granules in pure culture and in situ [62,103,104]. However, in most cases there is no evidence that these and the other isolates behave according to the predictions of the models, because of lack of relevant information. The Gram-positive coccus *Mycrobatillus phosphovorus* that accumulates polyP to a very high level [105] conforms partially to the models by assimilating P aerobically and releasing it anaerobically. Yet *M. phosphovorus* cannot assimilate acetate anaerobically, although other substrates including glucose are used to synthesize polyglucose storage compounds. NMR studies have confirmed that the metabolism of *M. phosphovorus* is not that expected of a PAO [106]. Anaerobically assimilated glucose was not used for growth but instead was fermented to acetate, which was stored unmodified with no conversion to PHA, but then respired aerobically to support growth. Molecular studies carried out so far (see Section 9) would suggest that *M. phosphovorus* is not a dominating population in EBPR systems.

An EBPR isolate ‘identified’ as a *Lampropedia* sp. only from its morphological features [107], which incidentally were different to those usually associated with PAO in EBPR systems, came closer to fitting the expected metabolism of a PAO. It assimilated acetate anaerobically with corresponding PHA synthesis and P release, although the
acetate uptake to P release ratio, which is known to vary considerably in different EBPR biomasses [17,21,44], was lower than that predicted by the models. Disappointingly, no further data on its physiology or identity are available in the literature.

7. The microbiology of EBPR – the culture-independent approach: chemical markers

In our view the importance of obtaining pure cultures of EBPR bacteria cannot be overstated [108,109], because these are essential if any basic understanding of how these systems work and how they might then be manipulated is to be obtained. However, the disadvantages and inherent biases associated with analyzing natural microbial communities using culture-dependent methods, sometimes referred to as the ‘plate count anomaly’ have been discussed extensively in the literature [23,110], where the risk of ending up with what Amann and Ludwig [111] refer to as ‘laboratory weeds’ is well documented. Consequently culture-independent techniques have been applied to EBPR processes, and in almost every case the data obtained have raised further doubts about the importance of Acinetobacter spp. However, the evidence presented is not always convincing. For example, when fluorescent antibodies of an unknown specificity were prepared against Acinetobacter [112] their application to EBPR systems only revealed very small percentages (3%) of members of this genus. It could be argued that 3% of the total number of cells present still represents several million cells per gram of biomass. If all were accumulating polyP at the levels acinetobacters are known to be capable of, then they would represent a significant contribution to EBPR [29].

The other approach has been to use chemotaxonomic markers to indicate community composition, where the presence of certain cell components may indicate the presence and relative abundances of particular bacterial populations [113]. These are considered [114] to lack the biases inherent in the rRNA-based culture-independent methods discussed below. They all extrapolate laboratory data to activated sludge systems, by assuming that the chemical markers exploited are present in the same proportions and same populations in activated sludge as in the pure cultures from where the chemical data were originally obtained. Whether this extrapolation is justifiable in all cases is not entirely clear. However, diaminopropane was used as a marker for members of the genus Acinetobacter [115] and shown to be present in low amounts in biomass from full-scale plants with high P removal, although detectable at much higher levels in those with moderate EBPR capacity.

Where respiratory quinones have been used as markers with both laboratory scale and full-scale EBPR systems, most communities studied have given similar population trends. For example, ubiquinone Q-8, diagnostic of the β-Proteobacteria was the most abundant quinone in both EBPR and non-EBPR plant biomass samples from both full-scale and laboratory scale systems, and not Q-9 associated with the γ-Proteobacteria which include Acinetobacter spp. [114]. The Actinobacteria and α-Proteobacteria possessing ubiquinone Q-10 were also thought to be present in large numbers, and then the Gram-positive low mol% G+C bacteria, the Planctomycetes and the Cytophaga–Flexibacter–Bacteroides (CFB) division in smaller proportions. Very few (3%) of the Actinobacteria appeared to be M. phosphovorus. In fact there were only small differences in quinone profiles between the communities of the EBPR and non-EBPR systems analyzed, similar to other experiences [116]. This trend could be interpreted as meaning that only a small proportion of the total bacterial community is involved in EBPR. Similar ubiquinone profiles were reported in the EBPR biomass samples [117,118], again with Q-8 and Q-10 more dominant than Q-9. However, in the latter study, the levels of Q-9 increased and of Q-8 decreased in parallel with an increase in P removal, which suggested an increase in the cell numbers of the bacteria containing Q-9, i.e. the γ-Proteobacteria, associated with EBPR. Earlier ubiquinone Q-9 was shown to dominate in EBPR sludge [119], and so the evidence against a possible role for Acinetobacter under some circumstances from these data is still equivocal. Menaquinone profiles may also change during EBPR, and have been suggested as possible useful chemical indicators for monitoring P removal [120].

8. The microbiology of EBPR – the culture-independent approach: molecular microbial ecology of EBPR communities

It is platitudinous to state that the impact of applying molecular methods of analysis to activated sludge microbiology including EBPR in the past decade has been profound. As well as showing how unsatisfactory culture-dependent techniques are for providing a true representation of microbial community biodiversity, this approach in some cases has changed completely the way we now view processes like N removal [26,110]. Yet there still remains the challenge of using this information productively to help design and operate plants better [26,121]. The principles and problems associated with the basic methodology of the so-called 16S rRNA approach and its application to activated sludge have been reviewed, where some of the possible biases inherent in the steps of DNA extraction, polymerase chain reaction (PCR), including chimera formation and cloning are discussed critically [25,26,110,122]. Little information is available on which DNA extraction method for activated sludge samples is least subject to bias [26,110], but in many studies the numbers of sequences found for the Actinobacteria were much lower than the
numbers of these bacteria detected by methods like fluorescence in situ hybridization (FISH) described below, suggesting that their DNA was not as readily obtained as that from other bacteria (e.g. [123,124]). One sensible approach is to use a combination of different extraction procedures and pool the DNA obtained for analysis, as adopted in [30] where it was also shown from clone library analysis that each extraction method sampled a phylogenetically different community. Regardless, the biases together prevent the application of such methods to quantitative community analyses. They apply equally to techniques like denaturing gradient gel electrophoresis (DGGE) [125], single strand conformation polymorphism (SSCP) [126] and T-restriction fragment length polymorphism (T-RFLP) [127], all of which are molecular fingerprinting methods which have been applied to following changes in the compositions of activated sludge communities over time or in response to changing operational conditions [24,26,110].

Therefore, if semi-quantitative data are required, it is necessary to use techniques like FISH with fluorescently tagged 16S rRNA or 23S rRNA targeted probes [111, 128]. Probes can now be readily custom designed using software packages like ARB [26,111] for targeting populations at various phylogenetic levels from domain to species and below using 16S rRNA or 23S rRNA sequences derived from either pure cultures or cloned sequences. These techniques also have their limitations, which are discussed in [24,25,111,128], and all depend heavily on the sequence databases used for their design. It is now clear that many of the earlier probes designed from limited numbers of sequences are not specific for the organisms against which they were originally designed. These include the ALF1b and EUB338 probes [26,111, 129].

While revealing the population structure of activated sludge communities, by themselves these techniques tell us little about the functions of these populations in situ. This is critically important information if we hope to understand how these systems work. FISH in combination with microautoradiography (FISH/MAR) is one approach to obtaining this information, where specific populations can be identified with probing and their in situ physiology under various conditions determined by their abilities to metabolize individually supplied radioactively labeled substrates of interest [130,131]. The other approach is to analyze communities for detection of functional genes encoding enzymes important in metabolic processes of interest in activated sludge, like nitrification or denitrification [132–135]. As this methodology involves the same practical protocols of DNA extraction and PCR discussed above, it is also subject to the same biases, and the detection of a particular gene is not by itself evidence that the gene is being expressed in situ. Furthermore, it is only appropriate if each gene of interest is known together with its sequence.

9. What have molecular techniques revealed about the nature of EBPR microbial communities?

It is clear from the literature that almost all studies applying these molecular methods have used laboratory scale reactors often fed with artificial sewage, and have undertaken either FISH and/or clone library community analysis. These studies are summarized in Table 1. As pointed out [30], only two published studies have used a full cycle rRNA approach to full-scale plants [30,124], and neither were EBPR systems. The data invariably derive from analysis of a single sample of biomass, and very few studies have followed changes in population composition in response to changes in operational performances of EBPR systems. Where this was done using DGGE profiling, data were generated [136] suggesting that the community composition in the sequencing batch reactor (SBR) biomass studied became more highly specialized and less diverse as EBPR improved. All published studies have ignored the possible presence and importance of the Archaea in EBPR systems, although methanogenic bacteria were detected using DGGE in conventional aerated activated sludge plants [137].

9.1. Diversity of EBPR community populations

The data now available show that EBPR communities are phylogenetically very diverse (see [26,110]), as are the non-EBPR activated sludge communities that have been analyzed [26,30,110,124]. Furthermore, all the 16S rRNA clone library data suggest that most of the EBPR clones are very different to sequences from cultured bacteria, although different studies have often yielded clones with similar but rarely identical sequences to each other [26,123,124,138]. Clone library data have often differed from data from FISH analysis of the same communities, as mentioned above. For example, several studies with EBPR systems have detected a much higher proportion of the CFB division in their clone libraries than revealed by FISH analyses [116,123,138,139]. This may be because the probes presently available for the CFB group do not encompass all naturally occurring strains [140], but the dangers of using clone library data quantitatively have already been mentioned. These are common trends, and may reflect biases in DNA extraction efficiency, as may the differences in the levels of phylogenetic diversity detected in different activated sludge communities [110,126]. However, in one study [29] with FISH, the phylogenetic diversity of the EBPR communities also appeared to depend on the influent composition. Using the same DNA extraction method, a higher diversity, similar to that seen in full-scale EBPR systems, was detected in a SBR community receiving a complex feed containing several carbon sources, than in one being fed acetate alone. Thus, with acetate as sole carbon source, the community was dominated by β-Proteobacteria in the β-2 subclass and very few Actinobacteria
<table>
<thead>
<tr>
<th>Reactor configuration</th>
<th>Reactor feed</th>
<th>Methods of community analyses</th>
<th>Major populations detected ( &gt;10% of total)</th>
<th>PAO identified</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-scale municipal plant</td>
<td>complex synthetic sewage with acetate as carbon source</td>
<td>FISH</td>
<td>β-Proteobacteria (13%)</td>
<td>n.d.</td>
<td>[141]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate as carbon source</td>
<td>16S rRNA clone library</td>
<td>α-Proteobacteria (13%)</td>
<td>n.d.</td>
<td>[123]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic continuous flow</td>
<td>synthetic sewage with acetate as carbon source</td>
<td>16S rRNA clone library</td>
<td>β-Proteobacteria (33%) Planktonicetes (13%)</td>
<td>n.d.</td>
<td>[145]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate and peptone as carbon sources</td>
<td>FISH</td>
<td>Actinobacteria (37%) β-Proteobacteria (45%)</td>
<td>n.d.</td>
<td>[147]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate as carbon source</td>
<td>FISH</td>
<td>β-2-Proteobacteria (55%) Actinobacteria (35%)</td>
<td>n.d.</td>
<td>[142]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate as carbon source</td>
<td>16S rRNA clone library, FISH</td>
<td>Actinobacteria (10%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td></td>
<td>[29]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate as carbon source</td>
<td>complex synthetic sewage</td>
<td>β-Proteobacteria (34%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td></td>
<td>[29]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate and peptone as carbon sources</td>
<td>quinone profiles, DGGE and 16S rRNA clone library</td>
<td>β-Proteobacteria (n.d.) Actinobacteria (n.d.) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td>n.d.</td>
<td>[116]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate as carbon source</td>
<td>16S rRNA clone library, FISH</td>
<td>β-Proteobacteria (12%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td></td>
<td>[139]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate as carbon source</td>
<td>16S rRNA clone library, SSCP profiles</td>
<td>β-2-Proteobacteria (80%) Actinobacteria (28%) Terrabacter-like CFB group (14%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td></td>
<td>[138]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate as carbon source</td>
<td>16S rRNA clone library, SSCE profiles</td>
<td>β-Proteobacteria (17%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td></td>
<td>[62]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with acetate, propionate and peptone as carbon sources</td>
<td>16S rRNA clone library, DGGE, FISH</td>
<td>β-Proteobacteria (41%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td></td>
<td>[37]</td>
</tr>
<tr>
<td>Pilot plant (A/O), no N removal</td>
<td>municipal sewage with substrate supplementation</td>
<td>FISH/MAR</td>
<td>Actinobacteria (18%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’ but others suspected</td>
<td></td>
<td>[63]</td>
</tr>
<tr>
<td>Pilot plant (UCT) with N removal</td>
<td>complex municipal sewage with substrate supplementation</td>
<td>FISH/MAR</td>
<td>γ-Proteobacteria varied over time Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’ but others suspected</td>
<td></td>
<td>[37]</td>
</tr>
<tr>
<td>Laboratory scale anaerobic: aerobic SBR</td>
<td>synthetic sewage with mixture of VFA as carbon sources</td>
<td>FISH</td>
<td>β-Proteobacteria (25%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td></td>
<td>[156]</td>
</tr>
<tr>
<td>Full-scale (UCT)</td>
<td>complex municipal sewage</td>
<td>FISH</td>
<td>α-Proteobacteria (25%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td></td>
<td>[63]</td>
</tr>
<tr>
<td>Full-scale aerobic:anoxic (aerobic process with N removal)</td>
<td>complex municipal sewage</td>
<td>FISH</td>
<td>β-Proteobacteria (25%) Rhodocyclus-related organisms, Candidatus ‘Accumulibacter phosphatis’</td>
<td></td>
<td>[63]</td>
</tr>
</tbody>
</table>

n.d., no data.
were detected, while with a complex feed far more *Actinobacteria* and members of the CFB division were present and fewer *β-Proteobacteria* were detected.

9.2. Possible candidates for PAO

With these molecular methods Wagner et al. [141] were the first to raise further serious doubts about an important role for *Acinetobacter* spp. in EBPR systems, but left some questions unanswered. Using a genus-specific FISH probe ACA23a, they suggested that *Acinetobacter* spp. were unlikely to be PAO, because they represented <10% of the total bacteria present, and the β-Proteobacteria and *Actinobacteria* were the dominant groups detected. These results agree with the quinone profiling study [114]. However, no mention was made of the identity of the clustered cells, so diagnostic of EBPR systems (see above), and 10% of the total cell number may still represent enough cells to contribute markedly to P removal [29]. Furthermore, the ACA23a probe was designed from a database containing sequences almost exclusively from clinical isolates of *Acinetobacter*, and so any unsequenced environmental strains may not necessarily have responded to it. In fact, sequences of two activated sludge *Acinetobacter* spp. from a full-scale treatment plant described in [124] did not match with the ACA23a probe sequence, and two additional 16S rRNA targeted probes were described for this genus. However, when a range of activated sludge isolates representing several new *Acinetobacter* species [93,94] was tested against each of these probes, all responded to the ACA23a probe but only a few to the other probes reported in [124].

The general community population trends reported in [141] have been supported by much subsequent FISH work on EBPR systems, especially in their conclusions that *Acinetobacter* spp. are numerically minor populations [37,142–144]. However, the use of biomass samples obtained from different experimental systems in each of the individual studies has meant that no universal view on what might represent a typical EBPR community, and whether in fact it exists, is yet possible. While bacteria in the β-Proteobacteria consistently emerge as major populations, as they do with non-EBPR plant communities [26,30,110,141], the relative importance of the *Actinobacteria* varies considerably, from being the most dominant in a continuous laboratory scale reactor (e.g. [145]) to only being detected in very small numbers in an SBR (e.g. [123]). Such differences cannot always reflect DNA extraction biases because both these studies used essentially the same protocols [123,145]. Instead these data indicate that considerable variations may exist in EBPR community compositions under different operational conditions [29], and also that several different populations may be involved in EBPR. As mentioned above, of the *Actinobacteria* detected in EBPR systems, only very small numbers have ever responded to the FISH probe designed against *M. phosphovorus* [37,146].

Although similarities were seen between the EBPR and non-EBPR communities, Bond et al. [123] highlighted the increased presence of the *Rhodocyclus* group in clone libraries from their EBPR biomass. Without supplying any direct proof, they implied that these organisms might play a role in EBPR. Acinetobacters were again present in very low numbers. Later, using FISH they showed [147] that the β-Proteobacteria, especially members of the β-2 subclass, together with the *Acinetobacteria* dominated their EBPR community, adding further weight to the view that both of these may be involved in EBPR, and supporting data from other similar studies with FISH [141,148] and quinone profiling [114]. However, other FISH studies have suggested that the α-Proteobacteria, albeit detected with the ALF1b probe now known to be of dubious specificity [26], may be more important than the β-Proteobacteria in some EBPR systems [142,143], and DGGE failed to show the presence of any β-Proteobacteria in the SBR EBPR community examined [116].

It is also clear from clone library data that full-scale EBPR systems can contain large populations of phylogenetically diverse and novel ‘Chloroflexi’ or green non-sulfur bacteria [149], as apparently can conventional activated sludge processes [30]. These populations appear to be predominantly filamentous in morphology and some of the morphotypes seen in both studies using FISH probes designed against these clone sequences were novel, as well as being phylogenetically different to other filamentous ‘Chloroflexi’ reported previously in activated sludge like Eikelboom type 1851 [150] and *N. lunicola* (II?) [151]. The clone libraries from both communities contained predominantly the ‘Choroflexi’ 1 group but FISH analyses revealed more ‘Chloroflexi’ 3 group bacteria in the EBPR communities [152,153]. Whether these were accumulating polyP was not discussed, but other indirect evidence suggests that some ‘Chloroflexi’ may have this ability [142].

9.3. The case for Rhodocyclus-related organisms as PAO

Studies of the kind described above have been invaluable in helping us understand EBPR microbiology, but semi-quantitative community population analysis by itself will not allow a precise identification of the nature of the PAO. Elucidating structure-function relationships are required, where the in situ ability of specific populations to accumulate P can be resolved, using techniques like FISH/MAR [130,131] or FISH/histochemical staining [154]. In 1999 a link was first suggested between specific bacterial populations and their abilities to accumulate polyP in EBPR biomass [29]. Clone libraries were derived from biomass from an SBR fed acetate, using *Rhodocyclus*-specific primers. These were presumably selected because of the predominance of these bacteria in other EBPR clone libraries [123], and because the dominating β-Proteobacteria in FISH analysis were nearly all in the β-2 subgroup.
These clones revealed sequences closely related to members of the genus *Rhodocyclus*. FISH probing showed that these cells hybridizing with their RHC439 and RX991 probes designed against these sequences (see Table 2) had the same morphology as cells staining aerobically with DAPI, thus behaving in situ as the biochemical models required [29]. Simultaneous DAPI or Nile blue A staining with the probes and, crucially, these cells also contained polyP, as revealed directly by staining with methylene blue [139]. Furthermore, when these probes were applied to full-scale EBPR systems, a direct correlation between the P removal capacity and the numbers of *Rhodocyclus*-related organisms was demonstrated.

**Table 2**

Sequences and hybridization conditions for rRNA targeted probes for putative PAO and ‘G-Bacteria’/GAO in activated sludge

<table>
<thead>
<tr>
<th>Trivial probe name</th>
<th>Probe sequence (5'-3')</th>
<th>rRNA target site (E. coli numbering)</th>
<th>Reported specificity</th>
<th>Stringency: formamide concentration (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP2</td>
<td>GACCAAGCTTCTCTGACCCCG</td>
<td>16S rRNA (68-87)</td>
<td>M. phosphorus PAO??</td>
<td>10 [146]</td>
<td></td>
</tr>
<tr>
<td>RHC175</td>
<td>TGCTCAGAATATCGCAG</td>
<td>16S rRNA (175-192)</td>
<td>Rhodocyclus cluster</td>
<td>30 [29]</td>
<td></td>
</tr>
<tr>
<td>RHC439</td>
<td>CATTCGCTTCCCGCCGGA</td>
<td>16S rRNA (439-456)</td>
<td>Rhodocyclus cluster</td>
<td>30 [29]</td>
<td></td>
</tr>
<tr>
<td>PAO462</td>
<td>CGCTCATCTACGGGTTATTAAC</td>
<td>16S rRNA (462-485)</td>
<td>Candidatus ‘Accumulibacter phosphatis’</td>
<td>35 [139]</td>
<td></td>
</tr>
<tr>
<td>PAO651</td>
<td>CCTTCGCAAACCTCCAG</td>
<td>16S rRNA (651–668)</td>
<td>Candidatus ‘Accumulibacter phosphatis’</td>
<td>35 [139]</td>
<td></td>
</tr>
<tr>
<td>PAO846</td>
<td>GTTACACTCGCGACTAAAAGGC</td>
<td>16S rRNA (846-866)</td>
<td>Candidatus ‘Accumulibacter phosphatis’</td>
<td>35 [139]</td>
<td></td>
</tr>
<tr>
<td>PAO462b</td>
<td>CGCTCATCTACGGGTTATTAAC</td>
<td>16S rRNA (462–485)</td>
<td>Rhodocyclus temuis group</td>
<td>35 [63]</td>
<td></td>
</tr>
<tr>
<td>PAO846b</td>
<td>GTTACACTCGCGACTAAAAGGC</td>
<td>16S rRNA (846-866)</td>
<td>R. temuis group</td>
<td>35 [63]</td>
<td></td>
</tr>
<tr>
<td>AMAR839</td>
<td>TGGCGCGACGCAGCGAAAGCC</td>
<td>16S rRNA (839-860)</td>
<td>Amaniricus spp. ‘G-Bacteria’</td>
<td>20 [198]</td>
<td></td>
</tr>
<tr>
<td>DEF438</td>
<td>CGCTGAGACGACTGATGACC</td>
<td>16S rRNA (438-456)</td>
<td>Deflavibacillus varus ‘G-Bacteria’</td>
<td>20 [194]</td>
<td></td>
</tr>
<tr>
<td>MIC184</td>
<td>CATTCTCCAAGTCTCCAG</td>
<td>16S rRNA (438-456)</td>
<td>M. glycogenica GAO</td>
<td>20 [194]</td>
<td></td>
</tr>
<tr>
<td>KB5831</td>
<td>TTTACCTCCCGGACACAGAC</td>
<td>16S rRNA (438-460)</td>
<td>clone sbr-ge28</td>
<td>20 [194]</td>
<td></td>
</tr>
<tr>
<td>TET63</td>
<td>GTCCTGGGCTACGGCCTT</td>
<td>16S rRNA (63–80)</td>
<td>Tetrasphaera spp. ‘G-Bacteria’</td>
<td>20 [194]</td>
<td></td>
</tr>
<tr>
<td>actino_1011</td>
<td>TGGCGCGACGCAGCTT</td>
<td>16S rRNA (1011–1029)</td>
<td>T. elongata clones Ebpr19 and 20</td>
<td>30 [62]</td>
<td></td>
</tr>
<tr>
<td>GB</td>
<td>GGATCTCAGCGCCACTCATC</td>
<td>16S rRNA (612-628)</td>
<td>γ-Proteobacteria GAO?</td>
<td>35 (70) [199]</td>
<td></td>
</tr>
<tr>
<td>GB1 and 2</td>
<td>GCGCTGCAGCCCGACCTCC</td>
<td>16S rRNA (587–604)</td>
<td>γ-Proteobacteria GAO?</td>
<td>20 (23) [199]</td>
<td></td>
</tr>
<tr>
<td>GB_2</td>
<td>GCCATCGCTGCCTCCTGT</td>
<td>16S rRNA (64-81)</td>
<td>γ-Proteobacteria GAO?</td>
<td>n.d. [199]</td>
<td></td>
</tr>
<tr>
<td>GB_3</td>
<td>GCACTAACTGCACGCGT</td>
<td>16S rRNA (642-659)</td>
<td>γ-Proteobacteria GAO?</td>
<td>n.d. [199]</td>
<td></td>
</tr>
<tr>
<td>GB_4</td>
<td>GGCCTTCTGCGGCACGCT</td>
<td>16S rRNA (1020–1037)</td>
<td>γ-Proteobacteria GAO?</td>
<td>35 (40) [199]</td>
<td></td>
</tr>
<tr>
<td>GB_5</td>
<td>CTAGCAGGCCAGGGGCCC</td>
<td>16S rRNA (69–86)</td>
<td>γ-Proteobacteria GAO?</td>
<td>n.d. [199]</td>
<td></td>
</tr>
<tr>
<td>GB_7</td>
<td>CACTTCTGAGACACCTCC</td>
<td>16S rRNA (1000–1017)</td>
<td>γ-Proteobacteria GAO?</td>
<td>35 [199]</td>
<td></td>
</tr>
<tr>
<td>GAQQ431</td>
<td>TCCCGCGCTAAAGGCTT</td>
<td>16S rRNA (431–448)</td>
<td>Candidatus ‘Competibacter phosphatis’ GAO?</td>
<td>35 [200]</td>
<td></td>
</tr>
<tr>
<td>GAQQ893</td>
<td>TTCCCCGATGCAAGGCC</td>
<td>16S rRNA (989–1006)</td>
<td>Candidatus ‘Competibacter phosphatis’ GAO?</td>
<td>35 [200,228]</td>
<td></td>
</tr>
</tbody>
</table>

n.d., no data.

10. Structure:function relationships within EBPR communities

These two reports [29,139] have since led to intense research activity, often using a range of methods, to examine structure–function relationships in EBPR communities. All have been aimed at determining the importance of these *Rhodocyclus*-related bacteria as PAO. It is now clear from such studies that these bacteria are important populations in EBPR, and where their presence has been sought they have usually been found. Thus, *Rhodocyclus*-related organisms were detected in an EBPR SBR community by SSCP fingerprinting [138]. However, this story has not yet reached a wholly satisfactory conclusion, and much remains to be achieved before the ecology of the PAO is comprehensively understood.
10.1. Using DAPI staining to indicate PAO identity

Several studies have taken advantage of the ability of DAPI to impart bright yellow fluorescence to polyP [62, 84], so allowing the PAO to be detected by double staining with FISH, a technique not without its problems in some laboratories [29, 63, 136]. This approach was used to show sequences of many of the dominant bands obtained by DGGE profiling of EBPR SBR biomass were closely related to the Rhodocyclus-related clones obtained by [139], and cells responding to the probes were similar in morphology to the DAPI staining clustered cells. A cluster of PAO after simultaneous DAPI staining and FISH probing with the PAO462 probe, showing the presence of polyP in these cells, is presented in Fig. 3.

Alternatively, DAPI stained cells can be enriched after separation and recovery from most other non-fluorescing and hence non-polyP accumulating populations using flow cytometry [142]. These fractionated cells can then be sequenced to generate clone libraries and hence be identified. With this approach, Kawaharasaki et al. [142] showed that although the β-Proteobacteria from FISH analyses were the major populations in their biomass, very few fluoresced bright yellow, while almost all the α-Proteobacteria, and many CFB, both present as minor components of the community, did. No evidence for a role in EBPR for the Actinobacteria was found. Later, with the same methodology it was suggested that Rhodocyclus-related bacteria, although detected by DAPI staining as PAO, were not necessarily the dominant PAO in their community, and other bacteria may also be involved in EBPR [155].

10.2. Using FISH staining and FISH/MAR to indicate PAO identity

Combining FISH with DAPI and Sudan black staining for detection of polyP and PHA has revealed [62] that the Rhodocyclus-related PAO in their biomass accumulated both polyP aerobically and PHA anaerobically, while the actinobacterial N. limicola II [104] and Tetrasphaera elongata [101], also present in large numbers, accumulated polyP aerobically but not PHA in situ. However, in this biomass, no cell inclusions were detected in either the α-Proteobacteria or the CFB populations detected with FISH. Also with FISH/staining, [156] could show their Rhodocyclus-related organisms behaved according to the biochemical models in an EBPR SBR system, synthesizing PHA anaerobically and polyP aerobically. Staining methods together with FISH/MAR were employed on biomass from an SBR operating under conditions with high and low P removal [144]. The FISH analysis revealed that the numbers of β-Proteobacteria and Rhodocyclus-related organisms in the community corresponded directly to the level of P removal, and that most but not all the Rhodocyclus-related organisms, which appeared as pleomorphic cells mainly in clusters, assimilated acetate and synthesized PHA anaerobically. Aerobically, most of the cells responding to the Rhodocyclus probes assimilated 33P, exactly as the models require. FISH/MAR also demonstrated that some Actinobacteria could assimilate 33P, but these did not include the Tetrasphaera spp.

Very similar results were reported in [37] using FISH/MAR, where both the Actinobacteria and Rhodocyclus-related organisms were shown to accumulate P aerobically. Uneven acetate and 33P assimilation was also noted with the Rhodocyclus-related PAO, and it was proposed that either some of these cells are physiologically inactive in situ or there is considerable physiological variation among the populations detected by the probes used. It was also suggested [37] that not all clustered cells characteristic of EBPR biomass are necessarily Rhodocyclus-related PAO, and FISH showed that while large grape-like clustered cells were β-Proteobacteria, the irregular clusters contained...
Actinobacteria and the rounded clusters consisted of α-Proteobacteria. These interesting observations need to be confirmed with other biomass samples, preferably from full-scale plants. Furthermore, because the changes in population levels of Rhodocyclus-related PAO did not correspond to changes in the P removal capability of their plants, it was concluded that other bacteria are likely to be involved in EBPR.

10.3. Using differential centrifugation to indicate PAO identity

Another approach has been to separate and enrich the PAO by buoyant density centrifugation in Percoll, based on the assumption that PAO cells because of their intracellular polyP, PHA and glycogen granules have a higher density than cells with no inclusions [84]. These fractionated enriched PAO cells may then be analyzed phylogenetically. With this approach, sequence analyses of bands from DGGE fingerprints of 16S rDNA fragments in the enriched populations from laboratory and pilot scale EBPR systems, revealed most cells belonged to the γ-Proteobacteria [157]. The β-Proteobacteria were poorly represented. It is most unlikely that a complete separation of PAO is achievable by either centrifugation or flow cytometry used in the studies described above, bearing in mind how the biomass is aggregated into flocs, and so some caution is needed in interpreting quantitatively data from studies of this kind.

11. Studies with full-scale EBPR plants

Apart from the work by Crocetti et al. [139], most of the discussion so far has dealt with laboratory scale systems, and the role of Rhodocyclus-related PAO in full-scale EBPR systems has received comparatively little attention (Table 1). However, both flow cytometry of DAPI stained cells and buoyant density centrifugation have been applied to biomass samples from full-scale plants [63]. Rhodocyclus-related PAO were plentiful and so probably are important in full-scale EBPR. This may not always be the case and their influence on EBPR may depend on the plant configuration. Thus, in a UCT-configured plant, 73% of PAO, representing about 20% of the total bacterial population were Rhodocyclus-related organisms, and all were storing polyP [63]. However, in aerobic:anoxic EBPR plants only 26% of the PAO, representing about 6% of all cells, were Rhodocyclus-related organisms. One possible reason given for this lower figure was the absence of a strictly anaerobic zone in this plant. About 50% of these Rhodocyclus-related organisms contained no DAPI staining polyP, and thus probably played no role in EBPR, which is similar to the findings in [37]. In their generated clone libraries from the two plants, Zilles et al. [63] also showed that while most of the clones from the UCT plant clustered closely with Candidatus ‘Accumulibacter phosphatis’, the clones from the other plant formed an independent branch <97% similar to the others, and thus possibly were not representatives of Candidatus ‘Accumulibacter phosphatis’. Two additional probes (Table 2) were designed from these sequences, but have yet to be applied to other EBPR biomass samples to determine if these or other phylogenetically different Rhodocyclus-related organisms may be present.

A full-scale plant was also deliberately selected in [158] because of reservations the authors held about the validity of using laboratory scale systems for studies of this kind. The PAO detected using DAPI staining in their biomass were typical but very large clustered cells with an unusual response to the Gram stain, and after their reactions to exposure to a series of stains, antibiotics and enzymes, these cells were considered to possess properties not of bacteria but of eukaryotic yeasts. They were successfully cultured, but surprisingly no molecular or other methods were applied to disprove unequivocally their prokaryotic nature or to confirm they were eukaryotic cells. Therefore their nature remains unresolved and the skeptics unsilenced.

12. Denitrifying EBPR systems

Earlier in this review the importance of ensuring that no nitrate was allowed to enter the anaerobic zone to stable EBPR was mentioned, and the influence this requirement has had on the design of EBPR plants emphasized. The presence of nitrate is thought to provide denitrifying bacteria with an opportunity to remove the selective advantage the PAO have in these systems by out-competing them for the metabolizable substrates available there [159–163]. However, the inhibitory effect of nitrate may be more direct because nitric oxide, an intermediate in denitrification has been shown to prevent anaerobic P release in EBPR sludge by inhibiting the adenylate kinase (see Section 14) involved in polyP degradation [164].

However, it has frequently been demonstrated that in the absence of any exogenous carbon sources in the anaerobic zone, EBPR may in fact occur in the presence of nitrate. This is presumed to happen by organisms using intracellular storage compounds like PHA as carbon and energy sources to assimilate P and synthesize polyP, as PAO do, but using nitrate not oxygen as their terminal electron acceptor [17]. Consequently two groups of PAO are now recognized, the PAO and so-called denitrifying PAO or denitrifying polyP accumulating organisms (DNPAO) [165]. Some evidence is also available to suggest that nitrite may be used by DNPAO in P uptake [166,167].

The attractions of exploiting such populations in wastewater treatment include the possibility of achieving simultaneous removal of N and P, producing less sludge and with no need for aeration, having a process that is cheaper.
to run [161]. Furthermore, a lower requirement for metabolizable substrates than in conventional EBPR systems overcomes a major problem associated with treating municipal wastes using EBPR [21,161]. Some systems taking advantage of these have been conceived. For example, Bortone et al. [168] described the DEPHANOX process based on DNPAO activities, and with a separate aerobic reactor for nitrification, were able to achieve very high P uptake rates in their anoxic reactor. These benefits have not convinced all, and it has been argued strongly on theoretical grounds [169] that because of their unpredictable nature and reduction in EBPR capacity, denitrifying EBPR systems are not worth bothering with.

Even less is known about the community structure and identity of DNPAO than about the microbiology of anaerobic:aerobic EBPR systems discussed above, and very few microbiological studies on denitrifying EBPR systems have been reported. Culture-dependent techniques have been used to study communities in an anaerobic:anoxic SBR which stored P anoxically [170,171]. A range of Gram-positive and Gram-negative bacteria were isolated with techniques selective for denitrifying bacteria and some of these isolates could assimilate P aerobically in pure culture [170]. Later it was reported that two of the cultures obtained, Agrobacterium tumefaciens and Agaspirillum dispara, used both oxygen and nitrate as electron acceptors for P assimilation, but polyP production in these isolates was not reported [171]. The problems associated with using culture-dependent methods in studies of this kind have been discussed earlier, and molecular methods directed at detecting functional nirS or nirK genes were capable of both aerobic and anoxic EBPR, and two of these bands were recovered and sequenced. Both were β-Proteobacteria, closely related to Rhodocyclus and Dechloromonas, known also to appear frequently in clone libraries from aerobic:anaerobic EBPR type plants [26,111]. FISH probing showed the Rhodocyclus-related population was dominant in both communities but Dechloromonas was not. It would have been interesting to see what emerged if the PCR protocol had used primers specific for functional genes for denitrifying enzymes like the nirS and nirK genes [132,133], and FISH/MAR had been applied to these communities to assist in identifying the denitrifying populations in situ [167]. SSCP was used in [138] to follow the changes in microbial community structure following a shift from an anaerobic:aerobic EBPR system to an anaerobic:anoxic one. Because the profiles obtained were very similar in each, many of the populations there were thought to carry out EBPR under aerobic and anoxic conditions, agreeing with the conclusions of Ahn et al. [174].

13. Why do EBPR systems often behave unpredictably?

As already mentioned, both laboratory scale and full-scale EBPR systems are notorious for their unreliability. One of the main motivating factors for elucidating the microbiology of EBPR systems is to understand why this deterioration in performance occurs and how it can be remedied. There may be reasons other than microbiological ones for this, and the dependence of stable EBPR on a ready supply of Mg$^{2+}$ has already been discussed as one possible example. The other possibility, which has attracted considerable interest, is that under certain operating conditions the PAO community is harmed or disadvantaged in some way and so EBPR eventually deteriorates. There are many publications dealing with how certain operational conditions like over-aeration [175] and aerobic:anaerobic contact times [176,177] may affect EBPR performance. Unfortunately these are mainly of little more than anecdotal interest because they include no structure–function analyses of the changes that are thought to have taken place in the microbial community, and their general applicability to EBPR operation is unlikely.

13.1. Influence of influent substrate composition on EBPR performance?

Most attention has been focused on the impact of influent substrate composition, especially the presence of glucose on EBPR failure, as discussed below, although many reports of successful EBPR in the presence of glucose have appeared in the literature, as mentioned earlier [52,55,59,178,179], and empirical biochemical models have been
published outlining how this may occur (e.g. [55]). Unfortunately, these studies have provided no microbial community compositional data. Particular attention has been paid to the role, if any, in such EBPR failure of the so-called ‘G-Bacteria’. This term describes a morphotype of cocci, which often appear in activated sludge and are now known to be very diverse phylogenetically (see Fig. 2), containing both Gram-positive and Gram-negative members. Cells are distinctively arranged in tetrads or in clusters, as shown in Fig. 4, and have been referred to as tetrad-forming organisms (TFO) in [180]. Much of what is known about their taxonomy and physiology has been reviewed [181].

13.2. What are these ‘G-Bacteria’ and GAO?

First reported in activated sludge in [182], their possible involvement in EBPR failure came initially from the ideas of Cech and Hartman [50,51], who noticed large numbers of ‘G-Bacteria’ in a plant fed glucose and showing poor EBPR. They hypothesized from the chemical transformations taking place with their mixed enriched culture that these ‘G-Bacteria’ may out-compete the PAO in anaerobic/aerobic EBPR systems under some operational conditions. The ‘G-Bacteria’ were thought to achieve this by more effectively assimilating some substrates anaerobically, such as when the feed contained glucose as well as acetate. The hypothesis was that the ‘G-Bacteria’ assimilated glucose anaerobically better than the PAO and used it eventually for PHA production, which could then be metabolized under subsequent aerobic conditions for glycogen formation. Thus, these ‘G-Bacteria’ were selectively favored and became dominant populations, and as they now synthesized no polyP aerobically, then EBPR gradually diminished.

Similar experiments were carried out by us [33,183] and similar conclusions were reached. Anaerobic assimilation of glucose and other sugars could occur in the absence of any P release, consistent with polyP not being used as energy source for substrate uptake, and hence different to that proposed by the EBPR biochemical models. It was suggested that this reflected the dominance of a physiological group of cells using stored intracellular glycogen synthesized aerobically as the energy source for anaerobic substrate assimilation. Some of the bacteria that appeared as large cocci in their biomass samples were considered responsible for these chemical changes because of their dominance. These cells were described not in terms of a morphotype but as a phenotype, the so-called glycogen accumulating organism (GAO). This is a descriptive term that has been adopted in many later studies [183–186], but in a sense it is confusing because according to the EBPR biochemical models, the PAO also accumulate glycogen. It was suggested [183] that the chemical nature of the substrate was less important than the phosphorus/carbon (P/C) ratio of the feed in determining the prominence or otherwise of the GAO in EBPR communities, and at the appropriate value both the GAO and PAO could coexist there.

There is no question in our view that the GAO exists in EBPR plants as a group physiologically distinct from the PAO. However, what they are and whether the ‘G-Bacteria’, often seen microscopically to dominate the biomass in failing EBPR plants, are GAO is still largely unknown, since in no case have their abilities to accumulate glycogen in activated sludge been directly demonstrated [181]. Instead, much of the evidence for GAO having a functional role in EBPR failure comes from an interpretation of trends in chemical transformations using enriched mixed communities, similar to the development of biochemical models for EBPR described above, and are subject to the same criticisms. With just a few exceptions, these chemical changes alone have been used to interpret the likely population structures of these communities, and no accompanying detailed microbial community analyses have been performed. Consequently, contradictions and inconsistencies abound in the literature, and clear, generally applicable principles are hard to find among the plethora of data. Most of the studies have also been carried out in laboratory scale reactors using a synthetic sewage feed.

13.3. Evidence for a role for GAO/‘G-Bacteria’ in EBPR systems from mixed culture studies

Filipe et al. [187,188] have suggested that the pH of the anaerobic and aerobic zones is important in determining
whether PAO or GAO will dominate, and hence whether EBPR is successful. In the anaerobic zone, acetate uptake rates were considered to be adversely affected in GAO but not in PAO at high pH, while in the aerobic zones — although growth rates, PHA consumption rates and P uptake rates in GAO were unaffected by pH — they were markedly lowered for PAO at a lower pH (6.5). So, while the GAO were advantaged at low pH, the PAO out-competed the GAO at pH 7–7.5. Jeon et al. [178] also followed the influence of pH on EBPR in an SBR, and interpreted their chemical data as follows. At a controlled pH of 7, GAO dominated; with no control, the pH rose, the PAO took over and good EBPR was achieved. They also suggested that pH might be used to manipulate the competition between the two groups, but this would only be valuable if generally applicable to all EBPR systems. Unfortunately the opposite situation has been found [189], where a PAO metabolism dominated that of GAO at pH 7. Possible reasons for such disagreements are many, but one likely explanation is that the communities in each study were quite different. Whether this is the case is not known, but this example reveals the problems in usefully interpreting data of this type.

Similar studies have also suggested that plant operating temperatures can determine EBPR stability by affecting the balance between the PAO and GAO populations [190]. Thus, at 20°C the PAO were thought to dominate, while at 30°C GAO were considered more influential, and when this 30°C biomass was examined microscopically, many ‘G-Bacteria’ morphotypes were in fact visible. Ear-when this 30‡C biomass was examined microscopically, the balance between the PAO and GAO populations temperatures can determine EBPR stability by affecting data of this type.

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13.4. The identity of the ‘G-Bacteria’ and GAO: culture-dependent approaches

What is known about the microbial nature of the ‘G-Bacteria’ or GAO and their possible role in glucose-induced EBPR deterioration? It is now clear that cells fitting this morphotype description (Fig. 4) can be found, often in large numbers, in many anaerobic:aerobic EBPR plants [181]. These cells sometimes occur in communities in the absence of any evidence of EBPR failure [29,145] or where EBPR failure occurs in the absence of any glucose in the feed [37,138,139,192]. Glucose dosing of a reactor [37] neither led to this morphotype appearing in larger numbers in their biomass nor caused EBPR to fail, but instead glucose enhanced it. Hence the question is still unanswered. However, with a few exceptions, the ‘G-Bacteria’ in these plants have not been obtained in pure culture, and so we still know little about what they are or what they do there. Bearing in mind their known phylogenetic diversity [181], as shown in Fig. 2, even if these ‘G-Bacteria’ are not microscopically distinguishable from each other, their signifi-cance in plants may be different, and conclusions about performance of plants containing them based only on microscopie appearance of the biomass, should be treated cautiously until more is known about their identity and in situ physiology [181]. Isolates of the original ‘G-Bacteria’ morphotype seen by Cech and Hartman [51] were placed in a new genus Amaricoccus, in the α-Proteobacteria [193]. Since then other α- and β-proteobacterial, as well as several actinobacterial ‘G-Bacteria’ have been grown and the pure cultures char-acterized [181], yet their role in EBPR failure is also not clear. For example, while isolates of Amaricoccus exam-ined so far fail to synthesize polyP, they do produce gly-cogen aerobically [194], as expected of GAO, but no gly-cogen synthesis could be detected under anaerobic conditions. In addition, pure cultures of Amaricoccus ka-plicens could assimilate neither glucose nor acetate anaerobically, and PHA synthesis, while occurring aerobi-cally, did not take place anaerobically [195]. Thus, as with Acinetobacter and the EBPR biochemical models, Amaricoccus does not behave in pure culture as the models for GAO/G-G-Bacteria’ demand [50,51].

13.5. The identity of the ‘G-Bacteria’ and GAO: culture-independent approaches

Partly as a consequence of unsuccessfully trying to cul-ture these ‘G-Bacteria’, culture-independent methods have been applied in attempts to identify them in EBPR bio-mass. FISH could show that the abundant large cocco-bacilli seen in biomass with poor EBPR were members of the β-Proteobacteria, but were not identified further [147]. They were described as GAO, but with no biochemical basis. From the ideas in [50,51], it was proposed that several different bacterial populations might be involved in EBPR deterioration as no α-proteobacterial ‘G-Bacteria’ (i.e. Amaricoccus) could be detected by FISH in the bio-mass studied [147]. This idea receives some support from the data in [192], where members of the γ-Proteobacteria, identified from DGGE profiling of community DNA, were detected in a deteriorating EBPR plant. Similarly, SSCP analysis of a community with poor EBPR capacity de-tected γ-Proteobacteria with sequences almost identical [138] to those found by Nielsen et al. [192]. FISH probes designed against the partial sequences of these fragments
[192] imparted fluorescence to large coccoid cells seen dominating their biomass. These cells were similar in appearance to those reported in [29], which by FISH analyses were also members of the \( \gamma \)-Proteobacteria, although this community showed good EBPR performance, indicating that these populations may be common in most anaerobic:aerobic systems.

Similar results were reported in [62], where sequences from large cocci were also detected in clone libraries from an EBPR SBR. These were considered to represent two physiologically different populations with a shared morphology. FISH showed that cells responding to the probes in [192] and those designed using clone library sequences from this community (given in Table 2), were also large and coccoid in shape. It is unlikely that they are the same bacterial populations as those reported in [196] in an EBPR system fed glucose, or in [29] (see below). High numbers of tetrad-forming ‘G-Bacteria’ in a biomass also with good EBPR capacity [145] would add to the view that the presence of this morphotype does not necessarily indicate potential EBPR deterioration, or vice versa [197]. These ‘G-Bacteria’ were not identified precisely, but library analysis yielded a few clones similar to *Amaricoccus*, which is known from FISH analysis to be common in many full-scale plants [198]. Most were *Actinobacteria* closely related to *Tetrasphaera* spp. [145], reported to be capable of growing as tetrads and synthesizing polyP [100].

13.6. Structure:function studies with non-EBPR communities and the role of ‘G-Bacteria’ and GAO

As with the PAO, knowing the identity of these ‘G-Bacteria’ is not sufficient to understand their role in anaerobic:aerobic EBPR systems. For example, if the hypothesis that these may out-compete the PAO is to be tested, then the questions of which organic substrates they are able to assimilate anaerobically, and what intracellular storage compounds are synthesized in situ need to be addressed. A clearer picture of what they might be doing there comes from applying techniques like those described earlier for analyzing EBPR communities that reveal structure–function relationships of communities containing them. It is clear now that many can synthesize storage polymers (Table 3). For example, the large cocci described in [196], which were not probed by FISH, contained both polyP and glycogen granules from TEM studies, but whether any PHA was produced is not clear. FISH/staining showed that the \( \gamma \)-Proteobacteria seen in [29] produced PHA anaerobically but not aerobically, and no polyP was detected in cells exposed to aerobic conditions. It was suggested that these characteristics might mean that such bacteria could compete anaerobically with the PAO for acetate. Thus, they behaved in a similar way to the \( \gamma \)-Proteobacteria in [192] but not like the large cocci reported in [62], which could synthesize both PHA and polyP. Therefore it would seem that populations, all very closely related phylogenetically and often indistinguishable by FISH may behave physiologically quite differently to each other in activated sludge.

The work reported in [144,180,194] supports this. The tetrad-forming bacteria that dominated SBRs fed glucose and with no EBPR [180] were members of the \( \beta \)-Proteobacteria, \( \gamma \)-Proteobacteria and *Actinobacteria*, and many of these were distinctive large cocci. As well as phylogenetic diversity, they also demonstrated considerable in situ physiological variation in this morphotype. Thus, the \( \beta \)-Proteobacteria stained DAPI−ve and PHA +ve, which differs from the behavior of the cocci reported earlier by the same authors [62], while the \( \gamma \)-Proteobacteria were both DAPI and PHB−ve. Some *Actinobacteria*, also appearing as large cocci in tetrads, were both PHA and DAPI −ve or PHA −ve and DAPI +ve. In the latter case this implies an ability to synthesize polyP with no formation or degradation of PHA, which is not as the biochemical models predict.

None of these populations was identified further, but subsequent FISH analysis [199] using probes (Table 2) designed against clones from systems with good and poor EBPR capacity showed the \( \gamma \)-Proteobacteria were large cocoid/rod-shaped bacteria. These were widely distributed in both laboratory and full-scale EBPR as well as conventional systems. These seem to be very similar phylogenetically to the bacteria reported in [156,200] in both a laboratory scale SBR and full-scale systems, which were named *Candidatus* ‘Competibacter phosphatis’. These stained for PHA but not polyP, and were assumed to be GAO, although their ability to synthesize glycogen was not reported, but implied [186]. They also appear to be the same populations originally identified using FISH (Table 2) as \( \beta \)-Proteobacteria in [147], a confusion that may reflect the difficulties differentiating between members of these two divisions with the currently available FISH probes for them [199].

Several methods including FISH/MAR and FISH/staining were employed to try to understand the community structure and function in an anaerobic:aerobic SBR fed acetate and glucose, where the biomass contained a high glycogen level and was dominated by ‘G-Bacteria’ [194]. From FISH analysis, this community contained mostly large and small cocci belonging mainly to the \( \alpha \)-Proteobacteria, \( \gamma \)-Proteobacteria, *Actinobacteria* and the low mol% G+C Gram-positive bacteria. The latter consisted of both large cocci typical of ‘G-Bacteria’ in tetrads and small clustered cocci. These populations were held responsible for the production of considerable amounts of lactic acid, a feature of other SBRs fed glucose [52,55,59], and, like the PAO and ‘G-Bacteria’, were thought to be selectively advantaged by a glucose feed together with anaerobic:aerobic biomass cycling. Very few \( \beta \)-Proteobacteria could be detected in this community, and the \( \gamma \)-Proteobacteria seen there did not respond to the FISH probes in
<table>
<thead>
<tr>
<th>Organism/clone name</th>
<th>Phylogenetic affiliation</th>
<th>Cell morphology</th>
<th>Anaerobic substrate assimilation</th>
<th>Anaerobic Polymer synthesis</th>
<th>Aerobic Polymer synthase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. maricoccus spp.</td>
<td>Proteobacteria</td>
<td>cocci in clusters</td>
<td>glycogen, glucose and acetate in pure culture</td>
<td>+ve</td>
<td>n.d.</td>
<td>[184, 193, 195]</td>
</tr>
<tr>
<td>T. maritima</td>
<td>Actinobacteria</td>
<td>cocci in clusters</td>
<td>none in pure culture; acetate in situ</td>
<td>+ve</td>
<td>n.d.</td>
<td>[106]</td>
</tr>
<tr>
<td>M. phosphovorus</td>
<td>Actinobacteria</td>
<td>cocci in clusters</td>
<td>acetate in pure culture; glucose in pure culture</td>
<td>n.d.</td>
<td>+ve</td>
<td>[180]</td>
</tr>
<tr>
<td>M. glycogenica</td>
<td>Actinobacteria</td>
<td>large cocccoid rods</td>
<td>glucose in situ</td>
<td>+ve</td>
<td>n.d.</td>
<td>[194, 201]</td>
</tr>
<tr>
<td>Q. unnamed</td>
<td>Proteobacteria</td>
<td>cocci in sheets</td>
<td>acetate in situ</td>
<td>+ve</td>
<td>n.d.</td>
<td>[180]</td>
</tr>
<tr>
<td>T. F101</td>
<td>Actinobacteria</td>
<td>cocci in clusters</td>
<td>acetate and glucose in situ</td>
<td>+ve</td>
<td>n.d.</td>
<td>[194]</td>
</tr>
<tr>
<td>G. sbr-gs28</td>
<td>Actinobacteria</td>
<td>large cocccoid rods</td>
<td>glucose and acetate in situ</td>
<td>+ve</td>
<td>n.d.</td>
<td>[194]</td>
</tr>
<tr>
<td>Clone sbr-gs28</td>
<td>Actinobacteria</td>
<td>cocci in clusters</td>
<td>acetate and glucose in situ</td>
<td>+ve</td>
<td>n.d.</td>
<td>[194]</td>
</tr>
</tbody>
</table>

*Detected using staining.
*Determined by direct chemical analysis.
*Determined using MAR.

Considerable physiological variation was noted in the anaerobic substrate assimilation abilities of the ‘G-Bacteria’ belonging to some of these divisions [199]. Thus, not all of the α-Proteobacteria took up acetate or glucose, although almost all the γ-Proteobacteria assimilated acetate but not glucose. The low mol% G+C Gram-positive small cocci, assimilated only glucose, consistent with them being lactic acid producers, while the large cocci assimilated both acetate and glucose, as did M. glycogenica and those cocci with the sbr-gs28 clone sequence. All except the low mol% G+C Gram-positive bacteria and a few α-Proteobacteria synthesized PHB anaerobically, and none stored polyP aerobically. This evidence pointed to M. glycogenica as the true GAO in this community, especially since its ability to utilize glucose anaerobically for glycogen synthesis in pure culture is known [201].

A similar approach was used to examine structure–function relationships in an SBR reactor fed acetate as the sole carbon source over a range of phosphorus/carbon ratios where good or very poor EBPR was detected [144]. FISH analysis revealed a marked shift in community structure as EBPR capacity decreased. The community changed from one where the α-Proteobacteria and to a lesser extent the γ-Proteobacteria, both of which were predominantly the morphotype of ‘G-Bacteria’, gradually took over from the β-Proteobacteria and Rhodocyclus-related organisms as EBPR fell. No Acinetobacter was detected in any of these communities, and neither were any low mol% G+C Gram-positive bacteria, M. glycogenica [201] or clone sbr-gs28, populations that dominated a glucose fed SBR [194]. The increase in the proportions of the α-Proteobacteria paralleled the increases in biomass glycogen content, but again FISH probing (Table 2) could not detect A. maricoccus spp. in high numbers. FISH/MAR showed that both α- and β-Proteobacteria assimilated acetate anaerobically in situ but not P aerobically, and no anaerobic glucose assimilation was detected in this community. These Proteobacteria could not be identified further, but the data indicated that they were physiologically quite different to those detected in the SBR with a glucose feed described above. The data also confirmed that these ‘G-Bacteria’ morphotypes may exist in communities showing good
EBPR capacity, and that in the community with poor EBPR, glycogen formation using acetate was probably from the activities of the α-Proteobacteria, in the absence of M. glycogenica. What is known about anaerobic substrate assimilation and intracellular storage polymer synthesis from these different studies with the ‘G-bacteria’/GAO is summarized in Table 3.

More studies are needed where communities with well-defined chemical properties are analyzed in this way to clarify the considerable confusion about the nature of the ‘G-Bacteria’ and GAO that currently exists. It is important to identify more clearly the major functional populations responsible for the chemical transformations taking place, using the whole range of culture-independent methods now available, to design more specific FISH probes for their detection, and to examine their in situ physiology by MAR, before the important questions raised above can be answered. Then these techniques must be applied to full-scale systems to see whether the populations considered important from studies with laboratory scale systems, where the selective pressures are considerably different, are also important there.

14. How is EBPR biochemistry regulated?

We have made a strong case for the need to understand the structure and function of the microbial communities in these systems. Equally important is the need to understand how the biochemistry of EBPR is regulated. For example, what is known about the enzymes involved in the synthesis and degradation of polyP, PHA and glycogen in PAO, and what affects their synthesis and levels of activity? We know very little about this, and interpretation of what is known, like much of EBPR, assumes that all PAO behave identically, which seems increasingly unlikely, bearing in mind the evidence that PAO populations are probably phylogenetically diverse.

The enzymes that are thought to be involved in polyP metabolism and their known properties have been described [22,61], and some have been purified and characterized from several bacteria, including A. johnsonii [202–205]. However, evidence for their presence in activated sludge and thus their role in EBPR is less convincing. Of those thought to be involved in aerobic polyP synthesis, polyP glucokinase has been detected in isolates of polyP synthesizing coryneform bacteria from activated sludge [70], but its activity in activated sludge was not reported. Most interest has focused on polyP kinase (PPK) and its role in polyP synthesis in EBPR. It can be detected in pure cultures of many bacteria [61], but although very low levels of activity were claimed in activated sludge [206], other attempts [207] have been less successful. Yet using PCR and degenerate primers fragments of genes encoding for this enzyme were recovered from biomass from an EBPR SBR system [208], and some were related to the gene in Rhodocyclus-related organisms. The mRNA transcribed from it could also be detected. The enzyme was apparently associated with the particulate fraction of lysed sludge [208], which might explain the lack of success in detecting it in earlier studies. Furthermore, the requirement of this enzyme for Mg2+ could be one reason why this cation is essential for successful EBPR [77].

This enzymatic information is very important, since it should now provide the tools to help understand how PPK synthesis and expression in this PAO population is affected in situ by factors like plant operating conditions, and provide the means to monitor plant performance in a highly relevant way. More studies like this are needed to analyze for the presence of other ppk genes in samples, including those from full-scale EBPR plants. Some evidence is available on regulation of synthesis of this enzyme in pure cultures of Acinetobacter spp., where transcription of the ppk gene is apparently induced by Pi starvation, a condition which also induces the synthesis of an exopolyphosphatase, PPX, involved in polyP degradation [205]. Whether this is the situation in any EBPR PAO is not known, but linking synthesis of these two enzymes—one involved in polyP synthesis and the other in its degradation—was thought to explain how PAO store polyP aerobically to be used later anaerobically. The suggestion was made [205] that the Pi levels should receive more consideration as possible key regulators of EBPR, because of their ability to affect the expression of several enzymes that may be involved in polyP metabolism.

There is some evidence to suggest that the polyP:AMP phosphotransferase:adenylate kinase system is associated with anaerobic polyP degradation and ATP synthesis during EBPR. High levels of its activity were detected in an EBPR biomass [44], and it has been characterized in A. johnsonii strain 210A [202,203]. However, while levels of this enzyme corresponded well to polyP formation in some studies with Acinetobacter spp. [70,203,209], in others the correlation was poor [210]. Also, whether it is the only enzyme involved in polyP degradation in EBPR systems is not known. Studies similar to those carried out with PPK [208] are clearly required to search in activated sludge for gene fragments encoding for this and other enzymes thought to play a role in polyP metabolism. In the absence of any sequence information, the method using gene cassette PCR [211] to recover complete open reading frames from enriched EBPR communities may well provide vital information on which genes are involved.

Even less is known about the regulation of PHA and glycogen in EBPR communities, or the diversity of bacteria synthesizing these intracellular storage polymers (see above). Our current understanding of their biosynthesis comes from studies with pure cultures, but knowing now the sequences of some of the biosynthetic genes involved [212,213] should allow PCR-based methods to be tested and applied to EBPR communities. Other methods, like
gene cassette PCR [211] or RNA stable isotope probing [214–216] using $^{13}$C-labeled substrates like acetate or glucose, might help to reveal the identity of these active populations. Although, if these substrates are used mainly for storage and not for growth and DNA/RNA synthesis in the PAO, as is believed to be the case from the biochemical models, then this approach may not be appropriate. Alternatively, FISH/staining already described for PAO and GAO may also provide useful information. Some information from studies with pure cultures of *Acinetobacter* sp. is available on regulation of the synthesis and degradation of PHA [212,213]. Oxygen limitation is a key trigger in PHA formation in many bacteria, arising from inhibition of the TCA cycle and accumulation of acetyl CoA, which in turn stimulates enzymes involved in PHA synthesis [217]. The same regulatory process may occur in the anaerobic zone of EBPR systems. How glycogen synthesis and degradation are regulated and which enzymes are involved in EBPR is not understood at all.

15. Mathematical models for EBPR

Developing mathematical models to describe the behavior of activated sludge processes and to assist in process design, operation and control and education of engineers has attracted considerable interest. However, to define such models for a wide range of different system configurations operating under various conditions requires substantial information on the physiology of individual microbial populations responsible for performing key functions. Therefore an essential key to modeling EBPR is an understanding of the physiology of PAO.

Efforts to integrate PAO metabolism into activated sludge models began in the 1980s at the University of Cape Town [218], but their work was taken over by the IAWQ Task Group on Mathematical Modeling for Design and Operation of Biological Wastewater Treatment. This international group developed a more comprehensive EBPR model, the IAWQ Activated Sludge model No. 2 (ASM2) [219], in which anaerobic uptake of carbon sources and their storage as PHA were modeled mechanistically. A modified version, ASM2d [220], incorporated a capability for denitrification by the PAO (see Section 13). A totally different approach was adopted by the group at the Delft University of Technology [39,221,222]. Their EBPR model was a structured metabolic one, based on the stoichiometry and bioenergetics of all the known and postulated metabolic reactions underlying EBPR. Introducing stoichiometric and kinetic information for all the individual reactions markedly reduces the number of parameters to be determined, even though the necessary kinetic and stoichiometric information is not readily obtainable. However, this model is much more complex than the IAWQ versions. Consequently, the most widely accepted mathematical representation of EBPR is that adopted in ASM2 and ASM2d, where the basic stoichiometry is taken from what is known about PAO metabolism. Although glycogen clearly plays an important role in anaerobic substrate uptake and storage by PAO [17,34,223,224] its metabolism is not modeled in ASM2 or ASM2d, since the IAWQ group deliberately sought to keep both as simple as possible. They also take no account of possible ecological interactions between different populations in EBPR systems. Of special interest are the competitive interactions between the PAO and GAO/G-Bacteria’ discussed in Section 13. Acquiring such information would make a valuable contribution to EBPR modeling since deterioration of EBPR systems due to GAO proliferation could then be predicted [225]. On the other hand detailed information on the structure–function relationships of individual populations in EBPR plants may not necessarily lead to more precise prediction of process behavior. The complexity of such models may mean that estimating the essential parameters needed for such applications becomes a very difficult task. However, modelers would agree that increasing our understanding of the microbiological nature of EBPR would make a valuable contribution to improving interpretation of model predictions.

16. Future work

In this review we have tried to summarize the present ‘state of the art’ in progress towards understanding the microbiology of EBPR. We can now speculate on where research should be focused. The most pressing need is the same as that emphasized previously [17], and the priorities have changed little. The critical challenge is to obtain in pure culture as many PAO as possible. These will provide invaluable material for understanding their physiology and biochemistry [108,109]. The crucial question is how can we successfully achieve this. It is now clear that knowing the phylogeny of organism does not often help in predicting its physiology [26,149], as exemplified by *Rhodocyclus* spp., which are phototrophic bacteria and clearly quite different to the *Rhodocyclus*-related PAO, which are heterotrophs [29]. However, FISH/MAR might provide clues on the nutritional requirements of PAO [130] by allowing insight into their physiology in situ. Further useful information may come from screening either enriched PAO populations (obtained by flow cytometry of DAPI stained biomass, Percoll centrifugation) or micromanipulated PAO clusters through systems like BIOLOG, which reveal their substrate utilization patterns [226]. It may not be possible to grow these as axenic cultures but only as mixed communities, as was achieved with a four-organism consortium, and in which no *Rhodocyclus*-related organisms appeared to play a role in EBPR [227].

It is also very important to keep an open mind in EBPR...
studies and not to repeat the mistake, made with Acinetobacter for example, of becoming blinkered and concentrating all the effort on one population type, important though Rhodocyclus-related organisms increasingly appear to be. There is now considerable evidence that EBPR community structures in different systems may vary markedly. Equally, although the biochemical models are very helpful in suggesting how EBPR might work, we see no absolute necessity for an organism to behave as these models demand to be considered a PAO. It is possible for example that polyP synthesizing organisms like Acinetobacter may not play a primary role in EBPR, but be crucial in scavenging for P in the aerobic zone and, importantly, be responsible for producing an effluent of very low P concentration. Furthermore, the need for anaerobic-aerobic cycling for successful EBPR may not be essential. Indeed, the authors are aware of a stable fully aerobic EBPR system producing an effluent with very low P, in which incidentally Acinetobacter spp. (identified in situ by a genus-specific probe targeting the PHA synthase gene) appear to be a dominant population (R.C. Bayly and J.W. May, personal communication). We still need to understand how the activities of the PAO and their population densities might be affected by operational parameters. Although progress has been made in exploring a possible role of the GAO and ‘G-Bacteria’ in EBPR performance [186,200], many other possible detrimental biological influences including PAO-specific bacteriophages [228] have received little or no attention.

Hence, there is still much to be learned about EBPR and PAO and, as we are reminded [26], eventually the knowledge acquired should be useful in helping design and run these activated sludge systems better, and in responding to EBPR failure in a rational and scientific manner. Bioaugmentation, with appropriate application technology [229,230], is one example of how such information might be applied [26]. Although often tried in the past, this strategy usually fails with activated sludge because of lack of understanding of which organisms are responsible for the particular operational feature sought e.g. EBPR. Another suggestion is to exploit the microbiological knowledge of community structure and function to design plants which will stimulate increased species diversity among the PAO, creating greater stability and therefore less chance of plant failure [26].

These are for the future, but the application of community fingerprinting methods like DNA microarrays to a wide range of different activated sludge systems with their ability to detect many genes simultaneously [231,232] may greatly increase the rate at which the information necessary for these applications is acquired. This in turn will involve a close cooperation between microbiologists and engineers, professions not always in empathy in the past, but without such cooperation the efforts of both to develop EBPR systems that perform better and more reliably will not be realized.

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