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Review

A critical review on the effect of different carbon sources on EBPR: Revaluation of performance and applications

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ABSTRACT

Enhanced biological phosphorus removal (EBPR), as one of the most sustainable and economical technologies for efficient P removal from wastewater, is widely applied in full-scale wastewater treatment plants (WWTPs). The types of carbon sources exert key effects on the performance of EBPR, resulting in different microbial communities. Polyphosphate accumulating organisms (PAO) contribute to P removal. However, the dominance of glycogen accumulating organisms (GAO) under certain carbon source can outcompete PAO, leading to reduced stability of EBPR and even system failure. A key aspect in the choice of carbon source is the potential to alter PAO/GAO ratio and thus the EBPR performance. The advanced investigations show more versatile metabolic pathways of various putative PAO (e.g. *Tetrasphaera*, *Dechloromonas*, *Thiothrix*) with different carbon source strategies, which could be beneficial for the full-scale WWTPs to increase the resistance to unstable environment. This review carefully re-evaluates the application of different carbon sources (sole, multiple or complex carbon sources) in the field of EBPR in recent years, with special emphasis on the fermentation products from wastewater and waste solids as an additional carbon source by different strategies. The application of waste fermentation as additional carbon source not only shows successful system performance, but also avoids the need for commercial carbon source input and alleviates the waste disposal problem, which could be a promising development trend in view of the insufficient COD of raw wastewater as well as the environmental pressure problem.

1. Introduction

The overload of phosphorus (P) contained in the wastewater could lead to eutrophication, which is harmful to the aquatic environment [1]. Enhanced biological phosphorus removal (EBPR) has been proposed as one of the most efficient and sustainable processes for treating wastewater containing P in full-scale wastewater treatment plants (WWTPs). During the EBPR process, polyphosphate accumulating organisms (PAO) are considered as functional bacteria for P and COD removal. The most reported lineages of PAO in full-scale EBPR systems include the β-*proteobacterial Candidatus* Accumulibacter (generally referred as *Accumulibacter*), *Tetrasphaera, Dechloromonas*, *Thiothrix* and *Comamonadaceae* [2–6].

In the traditional perspectives, PAO can uptake organic matter (mainly volatile fatty acids, VFA) and store them as polyhydroxyalkanoate (PHA) under anaerobic conditions. The energy required is supported by the hydrolysis of glycogen and intracellular polyphosphate (poly-P). Under anoxic/aerobic conditions, PHA is oxidized and energy is obtained for the replenishment of glycogen and poly-P as well as for biomass growth. Then, provided P uptake is higher than P release, net P accumulation is observed, and P is removed from the wastewater and most of it leaves the plant as part of the waste sludge.

Anaerobic substrate uptake is an energy-intensive process and not all organic compounds are suitable for uptake under these conditions. Short-chain organic compounds often require less energy to be transported through the membrane. Thus, organic substrates play an essential role in EBPR performance during PAO metabolism [7]. Most of the lab-scale experiments reported are conducted with VFA (e.g. acetic and propionic acids) and this has led to *Accumulibacter*-enriched sludge [8,9]. However, recent microbiological advances on EBPR have identified some other PAO-relative bacteria at full-scale WWTPs that show more versatile metabolic pathways under a wider range of potential carbon sources [10–14]. For example, *Thiothrix* or *Tetrasphaera* showed

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the classical P-release/P-uptake phenotype of PAO with carbon sources other than VFA and without PHA synthesis [4,10,12,15]. However, it is still unknown for the carbon storage molecules in *Tetrasphaera* [16].

The nature of the carbon source is not only relevant for promoting EBPR or for selecting a certain type of PAO, but it is also a key agent in the competition between PAO and their competitors: glycogen-accumulating organisms (GAO) (e.g. *Candidatus* Competibacter, *Propionivibrio*, *Defluviicoccus*). Both show anaerobic VFA uptake and PHA storage, which is consumed aerobically for biomass growth and glycogen replenishment. However, GAO break down their stored glycogen to generate the energy needed for VFA uptake instead of using poly-P (as PAO). The main difference is therefore in the type of intracellular storage: poly-P for PAO and glycogen for GAO [17]. Successful EBPR performance is characterised by a PAO-enriched sludge with an efficient carbon utilisation, as GAO outcompeting PAO would lead to EBPR failure. This competition is heavily dependent on the operational conditions and on the quantity/biodegradability of the carbon source [17–20]. GAO proliferation was observed with overload of VFA [7] and some certain carbon sources (e.g. glucose, starch and methanol) [21–25]. On the contrary, PAO-enrichment has been promoted by propionate [26,27], butyrate [28,29], glucose [15], amino acids [30,31] or mixture of carbon sources [32–34].

The carbon source can also affect the proliferation of denitrifying PAO (DPAO), which are able to simultaneously remove nitrogen (N) and P by using nitrate/nitrite as electron acceptors for PHA oxidation [35–37]. DPAO are reported to be favoured by fermentation products of solid wastes [38–40].

Experimental reports on the utilization of different carbon sources for EBPR must be taken with care for two reasons. On the one hand, EBPR-based experiments are never conducted with pure cultures and, hence, when using complex sources other than VFA, the possibility of flanking species fermenting the complex substrates into VFA and PAO living off these fermentation products must be considered. Predicting the type of fermentative bacteria and the fermentation products is not a straightforward issue, and it is difficult to estimate EBPR performance under different complex substrates. This issue is particularly significant when dealing with low COD/N/P wastewaters. Adding a commercial organic compound to provide the required electron donor is possible but inefficient from an economic and sustainability point of view. A more sustainable solution would be adding a suitable organic waste such as fermented products of waste sludge. The solid waste is normally pretreated under chemical (alkaline, acid), thermal conditions for fermentation [41–43], and it can be fermented by novel configurations implemented (i.e. side-stream sludge fermenter) or *Tetrasphaera* without pretreatment [44–46].

On the other hand, many reports on the utilization of carbon sources are based on batch tests with bio-P biomass fed for a long time with common substrates. An efficient/unsuccessful utilization of a certain substrate using batch or first-time experiments may result in the opposite results as the case of a stepwise replacement of the primary carbon source for the targeted substrate. Then, the history of biomass is very relevant when analysing these experiments.

This work reviews the opportunities of different carbon sources (sole, multiple or the fermentation products from wastewater and waste solids) on EBPR performance (Fig. 1), the dominant microbial communities and the metabolic pathways of PAO and GAO. The article is structured as follows: section 2 describes the application of VFA, section 3 presents fermentable substrates such as glycerol or amino acids, section 4 describes the possibilities of organic waste, and finally section 5 systematically evaluates and provides guidance and perspectives on their use and discusses the microbial communities involved.

2. VFA as carbon source for EBPR

VFA are clearly the most studied substrate for PAO. This section describes firstly the use of the most common ones (acetate and propionate) and then others for which there is less experience (butyrate and valerate).

2.1. Acetate and propionate

Acetate and propionate are the two most common substrates present in real domestic wastewater and the most prevailing carbon sources in the EBPR process, typically accounting for 60–80 % of the total VFA [27]. Much research has been conducted to understand the EBPR performance of acetate and propionate fed systems and their effect on the metabolic pathways of PAO and GAO and their relative abundance (Tables 1 and 2). In general, both can induce stable EBPR performance either by individual or mixed application [27,47–49], or by alternating acetate and propionate addition [50,51].

The different metabolic pathways of PAO and GAO with acetate and propionate have been described by metabolic models and validated experimentally [47,52–57] (Table 1). The anaerobic P/C ratio (i.e. the molar ratio of P release to C-mol of VFA uptake) is an indicator of the PAO activity. Using acetate as carbon source, the theoretical PAO P/C ratio is 0.5 [52]. However, there is a wide range of experimentally reported ranges for PAO cultures under different conditions (Table 1): 0.08–0.8 molP/molC. Lower values than the theoretical one can be easily explained by the presence of other anaerobic carbon scavengers, such as GAO [53], while values higher than 0.5 are more difficult to understand from a theoretical point of view, but could be related to the presence of additional carbon sources, such as fermentation products, or to the presence of other PAO with different metabolic pathways. Other ratios (Table 1) can also be used to investigate the enrichment of PAO/GAO in EBPR sludge such as the anaerobic PHA/VFA ratio (PHA formation to VFA uptake) and the Gly/VFA ratio (glycogen degradation to VFA uptake), and the aerobic Gly/PHA ratio (glycogen formation to PHA degradation). Increased glycogen utilisation can be a good indicator of predominance of GAO metabolism [53,58,59]. Please note that in this review it is assumed that these metabolisms are due to traditional PAO and GAO, as there is still no consensus on the intracellular metabolites of *Tetrasphaera*.

The amount and distribution of PHA depends on the carbon source used. Poly-β-hydroxybutyrate (PHB) is the dominant PHA fraction (60 %-100 %) in acetate-fed systems whereas poly-β-hydroxyvalerate (PHV) (35–85 %) and poly-β-hydroxy-2-methylvalerate (PH2MV) (40 %-60 %) are most promoted when propionate is used [7,26,52,60,61].

The role of the carbon sources when selecting PAO/GAO can be affected by temperature. At T > 25 °C, GAO has been reported as a major threat to EBPR, regardless of the carbon source [62–65]. However, recent studies have shown that successful lab/full-scale EBPR performance can be maintained with PAO enrichment, regardless of the VFA used at T > 25 °C [9,54,66,67], and some studies even mention that acetate could be a preferable option to propionate for PAO enrichment [68,69].

Propionate is suggested to be a more preferable carbon source than acetate in view of PAO enrichment over GAO [56,70–72]: the P/C is lower than that of acetate (experimental and theoretical ratios) (Table 1) and, thus, less energy is required by PAO to uptake propionate [70,73]. Some other studies, specifically for granular sludge EBPR, have found that acetate showed better EBPR performance [27,74]. In particular, Cai et al. [74] reported larger and more stable granules and higher bioavailable P content in acetate-fed granules than those fed with propionate. Wang et al. [27] found that successful P and COD removal efficiency in granular SBR could be maintained with both VFA, but PAO were favoured over GAO with acetate, whereas a mixed PAO/GAO culture was found when propionate was used.

Another key aspect in the PAO/GAO competition based on acetate and propionate as carbon sources is the concentration. Lower VFA concentrations may favour PAO over GAO and, thus, working in continuous stirred tank reactors could be a strategy to select PAO and/or to recover PAO activity [75,76]. In fact, the presence of GAO has been

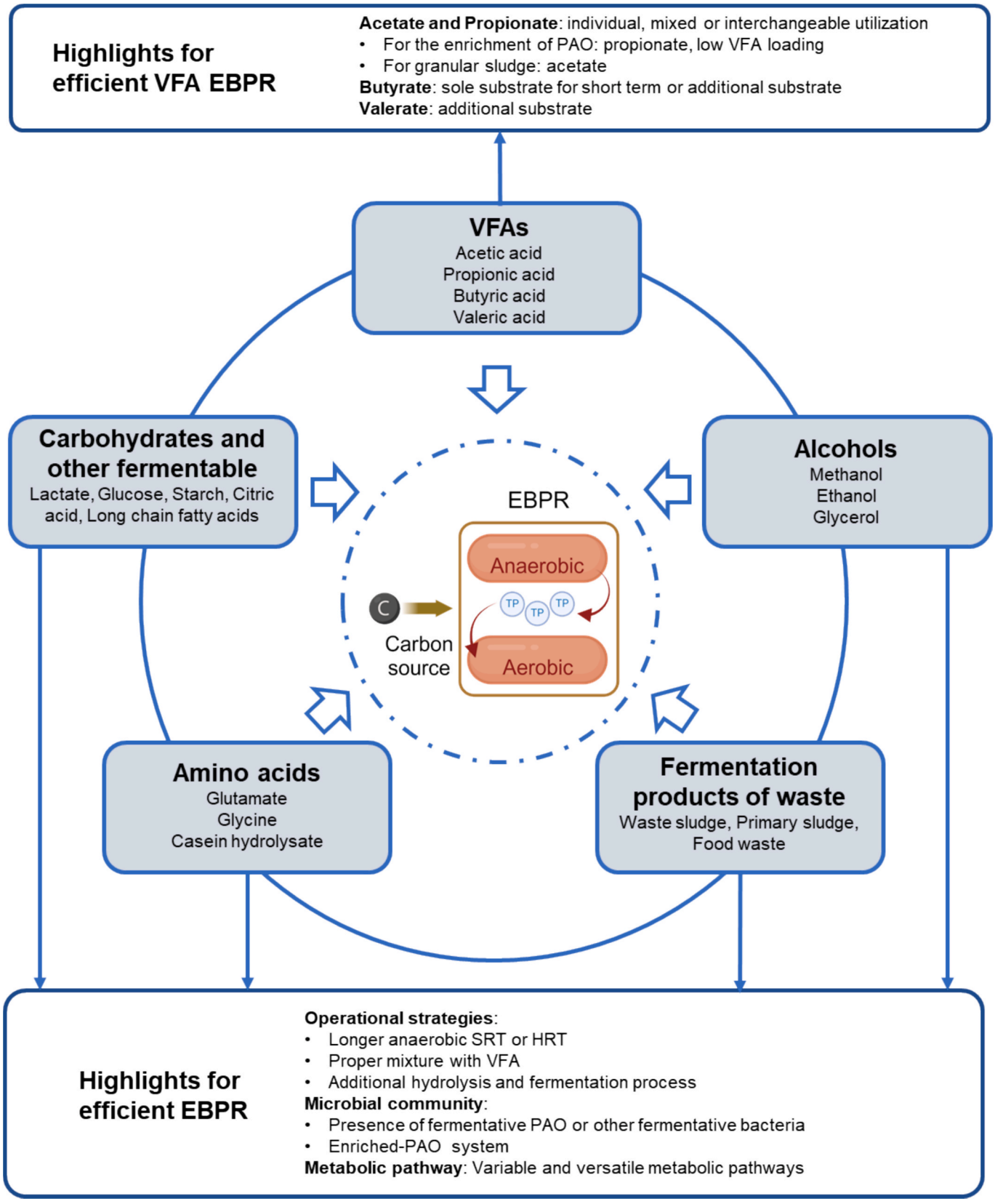

Fig. 1. Diverse carbon sources utilization for efficient EBPR.

Table 1
Summary of stoichiometric ratios of carbon transformation during the anaerobic and aerobic phases with VFA as carbon source.

Carbon source (with the ratio based on COD quantity)	Anaerobic VFA uptake (mmol C/g VSS/h)	P/C (mol/mol)	PHA/VFA (mol C/mol C)	Gly/VFA (mol C/mol C)	Aerobic P uptake rate (mmol/g VSS/h)	P/PHA (mol/mol C)	Gly/PHA (mol C/mol C)	References
Acetate	–	0.45–0.73	0.62–1.48	0.08–0.50	0.23–0.48	–	–	[7]
Acetate model	7.5	0.50	1.33	0.5	–	–	–	[52]
Acetate [a]	–	0.08–0.8	0.8–2.0	0.3–1.1	–	0.4–0.7	0.4–0.6	[53]
Acetate [b]	–	0–0.02	1.5–2.0	1.0–1.3	–	–	0.65	[53]
Propionate	–	0.23–0.44	0.52–1.39	0.08–0.50	0.41–0.72	–	–	[7]
Propionate model	–	0.42	1.22	0.33	–	–	–	[56]
Acetate	–	0.60	PHA/C 0.41 PHB/C 0.35 PHV/C 0.06	0.41	–	–	–	[27][T1]
Acetate	4.96	0.82	PHA/C 1.15 PHB/C 0.96 PHV/C 0.19	0.41	0.71	0.98	0.28	[68][T2]
Propionate	4.74	0.65	PHA/C 1.19 PHB/C 0.05 PHV/C 0.71 PH2MV/C 0.43	0.30	0.51	0.84	0.26	[68] [T2]
Acetate [c]	–	0.35–0.66	0.63–0.78	0.58–0.64	–	0.57–1.21	0.17–0.26	[67] [T2]
Propionate [c]	–	0.38–0.60	0.56–0.61	0.48–0.55	–	0.31–1.06	0.35–0.52	[67] [T2]
Acetate	–	–	PHB (88–94 %) [e] PHV (10–11 %)	–	–	–	–	[61]
Propionate	–	–	PH2MV (30–58 %) [e] PHV (36–63 %)	–	–	–	–	[61]
Acetate [a]	4.92	0.51–0.71	PHA/C 1.54 PHB/C 1.33 PHV/C 0.21	–	0.67–0.89	0.76–0.90	0.22–0.37	[83] [T2]
Propionate [a]	4.50	0.45–0.65	PHA/C 1.34 PHV/C 0.46 PH2MV/C 0.88	–	0.41–0.60	0.63–0.80	0.30–0.52	[83] [T2]
Acetate or propionate [d]	6.13–9.17	0.47–0.57	PHA/C 1.21–1.57 PHB/C 0–1.21 PHV/C 0.21–0.54 PH2MV/C 0–0.88	0.26–0.42	0.36–0.65	–	0.27–0.98	[83] [T2]
Butyrate (or isobutyrate) [d]	0.5–0.85	0.33–0.44	PHA/C 0.74–0.88 PHB/C 0.57–0.76 PHV/C 0.06–0.2 PH2MV/C 0.04–0.06	0.23–0.40	0.3–0.58	–	0–0.53	[83] [T2]
Valerate [d]	0.33–0.36	0.39–0.42	PHA/C 1.35–1.47 PHB/C 0.04–0.11 PHV/C 1.08 PH2MV/C 0.16–0.34	0.33–0.74	0.31–0.36	–	0.26–0.44	[83] [T2]
Mixture of four VFA [d]	5.84–9.62	0.37–0.63	PHA/C 0.88–1.42 PHB/C 0.34–0.67 PHV/C 0.35–0.76 PH2MV/C 0.05–0.15	0.16–0.36	0.37–0.47	–	0.18–0.29	[83] [T2]
Acetate [f]	–	0.1–0.14	–	–	0.119	–	–	[118]
Butyrate [d]	–	0.17–0.22	0.46–0.92[g]	0.43–0.49	–	–	–	[60]
Butyrate	–	0.20–0.80	–	–	–	–	–	[28]
Acetate [a]	–	0.58	PHA/C 1.05 PHB/C 0.83 PHV/C 0.22	0.28	2.58	–	0.33	[32] [T2]
Acetate: butyrate 1:1 [a]	–	0.74	PHA/C 1.04 PHB/C 0.73 PHV/C 0.13 PH2MV/C 0.08 PHH/C 0.12[g]	0.13	1.12	–	0.17	[32] [T2]
Butyrate [a]	–	0.59	PHA/C 0.69 PHB/C 0.52 PHH/C 0.17[g]	0.15	0.80	0.76	0.24	[32] [T2g]
Acetate [b]	–	–	PHA/C 2.45 PHB/C 1.55 PHV/C 0.90	1.23	–	–	0.55	[32] [T2]
Acetate: butyrate 1:1[b]	–	–	PHA/C 1.98 PHB/C 0.81 PHV/C 0.58 PHH/C 0.59[g]	0.72	–	–	0.34	[32] [T2]

(*continued on next page*)

Table 1 (*continued*)

Carbon source (with the ratio based on COD quantity)	Anaerobic VFA uptake (mmol C/g VSS/h)	P/C (mol/mol)	PHA/VFA (mol C/mol C)	Gly/VFA (mol C/mol C)	Aerobic P uptake rate (mmol/g VSS/h)	P/PHA (mol/mol C)	Gly/PHA (mol C/mol C)	References
Butyrate [b]	–	–	PHA/C 0.99 PHB/C 0.24 PHV/C 0.40 PHH/C 0.35	0.48	–	–	0.54	[32] [T2]

a. with enriched PAO.
b. with enriched GAO.
c. diverse PAO in full scale.
d. sporadic dosing to enriched-PAO.
e. relative terms are expressed as percentages.
f. with enriched clade-4 *Tetrasphaera*.
g. when butyrate was used as substrate, a novel PHA monomer was synthesised, and [60] indicated it was an unknown composition with quantified PHB + PHV = 95 %.
[T1] low down to 10 ℃.
[T2] high at 30 or 35 ℃.

identified as an indicator of the excess of COD load for EBPR [9,54]. PAO has been hypothesised to have a lower decay rate than GAO to describe this phenomenon [77], based on the preferential use for maintenance of PHA by PAO rather than glycogen by GAO. Finally, excessive COD loading, i.e. potential COD transfer to the aerobic phase, has been reported to promote the proliferation of filamentous bacteria, leading to sludge bulking problems and hindering EBPR performance [78–80]. P removal efficiency was found to decrease from 97 % to 50 % [78] when doubling the COD (propionate) load due to settling issues (49 % of *Thiothrix* detected and *Rhodocyclaeae* decreasing from 38 % to less than 3 %). Improved EBPR performance, *Accumulibacter* abundance (> 30 %) and decay of *Thiothrix* were observed when the normal load was restored. Similarly, Haaksman et al. [80] reported that over dosage of acetate led to the loss of P removal and deterioration of sludge settleability in EBPR-granules system, and a relatively lower dosage of acetate ratio (about 4 mg COD/gVSS/h) was recommended for full P removal and good sludge shape. Qiu et al. [54] pointed out that PAO could outcompete GAO under high temperatures (30 and 35 ℃) with a low COD load of a mixture of acetate and propionate.

Table 2 shows the microbial communities under different carbon sources. The results of Wang et al. [27] showed different microbial communities depending on the carbon source fed: PAO (*Rhodocyclaceae*) was around 32 % and 72 % in acetate and propionate fed systems respectively in family level. In addition, *Dechloromonas* (owning the ability to use nitrate as an electron acceptor for P uptake) was detected to be much more enriched in propionate-fed systems (61 %) than in acetate-fed systems (1 %) at genus level. Similarly, Zhang et al. [49] showed that changing the carbon source from acetate to the coexistence of acetate and propionate (with a ratio of 1:1) allowed optimal P and N removal (91 % and 85 % respectively), and increased the percentage of the main responsible DPAO *Dechloromonas* from 1.5 % to 4.8 %. The bacterial community responsible for the DPAO process was *Accumulibacter, Acinetobacter, Dechloromonas* and *Pseudomonas* with a percentage of 14 %-29 %, to the detriment of *Competibacter* and *Defluviicoccus*.

2.2. Butyrate and valerate

In addition to acetic and propionic acids, butyric and valeric acids are the major VFA species present in wastewater: they can account for 20–40 % of the total VFA in the anaerobic fermentation liquor of waste sludge [29,81]. Butyrate can be used as sole carbon source to drive EBPR but long-term P removal has not been shown to be feasible [28,82] (Fig. 1). Butyrate has lower P activity when compared to acetate and propionate in an *Accumulibacter*-enriched sludge [28,48,70] due to a slow butyrate uptake rate. A wide range of P/C ratio was reported (0.2–0.8) [28] during the first experiments but, in the long-term, P removal deteriorated after 6 weeks even though *Accumulibacter* (50 %) and *Defluviicoccus* (16 %) were favoured versus *Competibacter* (2 %) (Table 2). The failure was observed in the second phase of aerobic P uptake, probably due to a decrease in internal PHA levels. However, Wang et al. [32] showed successful and stable EBPR with butyrate as sole carbon source for more than 2 months and at a temperature around 30 °C, although they also observed that the total amount of PHA decreased when the carbon source was changed from acetate to butyrate. The same carbon source switch was conducted in a GAO-enriched system, and it was observed that butyrate could be detrimental to GAO metabolism and, in turn, favour PAO. Finally, the relative abundance of PAO species in the PAO-enriched SBR system experienced substantial changes (Table 2): *Accumulibacter* decreased from 37 % to 14 % and *Rhodocyclaceae* increased from 2 % to 15 %. In the GAO-enriched system, a reduction in microbial diversity and in the GAO percentage (from 27 % to 6 %) was observed and *Zoogloea* was favoured (from 0.2 % to 38 %). Butyrate was also tested as an additional carbon source (to VFA or glucose) to improve EBPR performance with successful results [29,83], and *Rhodocyclus* related bacteria and *Actinobacteria* as putative PAO were favoured (Table 2). In addition, fermentation liquid containing abundant butyrate has shown stable EBPR with the dominated clade IIF in *Accumulibacter* enrichment culture under saline conditions [84]. For the composition of PHA (Table 1), Pijuan et al. [60] showed 47 % of PHB, 49 % of PHV and 4 % of PH2MV and an unidentified monomer. In this sense, the novel PHA fraction poly- β-hydroxyhexanoate (PHH) was reported [32], with the percentage of 28 % and 35 % in PAO-enriched and GAO-enriched systems when butyrate was used as sole carbon source. The report by Meng et al. [84] also proved the presence of PHH and indicated PHH as the optimal glycogen transformer in *Accumulibacter* clade IIF enriched culture.

To the best of authors' knowledge, there is no research about valerate as sole carbon source for EBPR. The application of valerate as additional carbon source to glucose showed successful P removal and an enrichment of PAO (13 % of *Rhodocyclus*-related bacteria and 12 % of *Actinobacteria*) (Table 2). However, butyrate was more preferred to valerate and led to a much higher P removal ability and P content in the sludge [29]. The ratio of P/C showed a relatively lower value compared with other three VFA (Table 1) [60,83], and the relative PHA compositions were mainly PHV and PH2MV.

3. The application of fermentable carbon sources

Despite the suitability of VFA for EBPR [17], easily fermentable carbon sources such as glucose, starch, lactate, ethanol, amino acids (e.g. glutamate, casein hydrolysate, casamino acid) have been proposed as potential alternative carbon sources for EBPR.

Table 2
Summary of microbial communities under different carbon sources.

Type of carbon source	Carbon source	Microbial communities	References
VFA	Acetate	*Rhodocyclaceae* (32 %), genus *Dechloromonas* (1 %) [i]	[27]
	Propionate	*Rhodocyclaceae* (72 %), genus *Dechloromonas* (61 %) [i]	[27]
	Acetate [a]	Favours *Accumulibacter* IIC, *Accumulibacter* clade IIF	[68,69]
	Acetate [a]	*Accumulibacter* (64 %), *Defluviicoccus* (6 %), *Competibacter* (1 %) [h]	[83]
	Propionate [a]	*Accumulibacter* (52 %), *Defluviicoccus* (8 %), no *Competibacter* [h]	[83]
	Acetate [b]	*Thiothrix* (49 %), *Rhodocyclaeae* (3 %) [i]	[78]
	Acetate [c]	*Accumulibacter* (> 30 %), *Thiothrix* (17 %) [i]	[78]
	Acetate and propionate[d]	Favours *Accumulibacter, Acinetobacter, Dechloromonas* and *Pseudomonas*, less of *Competibacter* and *Defluviicoccus*	[49]
	Butyrate	*Accumulibacter* (50 %), *Defluviicoccus* (16 %), *Competibacter* (2 %) [h]	[28]
	Acetate changed to butyrate [e]	*Accumulibacter* decreased from 37 % to 14 %, and *Rhodocyclaceae* increased from 2 % to 15 % [i]	[32]
	Acetate changed to butyrate [f]	*Competibacter* reduced from 27 % to 6 %, *Zoogloea* increased from 0.2 % to 38 % [i]	[32]
	Butyrate: glucose 1:1	*Rhodocyclus*- related bacteria (17.5 %), *Actinobacteria* (1.4 %) [i]	[29]
	Valerate: glucose 1:1	*Rhodocyclus*- related bacteria (12.6 %), *Actinobacteria* (0.9 %) [i]	[29]
Fermentable carbon sources	Lactate	*Accumulibacter* and *Tetrasphaera*	[85]
	Glucose	Favours lactic acid producing organism and PAO	[178]
	Glucose	Favours *Competibacter*	[26,50,86]
	Glucose	Favours GAO-*Saccharimonadaceae* and other GAO, but no *Competibacter*	[90]
	Glucose	Decreased PAO, favours *Nitrospira*	[91]
	Glucose: acetate 1:1	Favours *Tetrasphaera* and *Microlunatus phosphovorus*	[33]
	Glucose	Favours *Tetrasphaera*	[15]
	Glucose	*Tetrasphaera elongata*	[164]
	Starch	Favours filamentous bacteria *Thiothrix* (4 %) [i]	[92]
	Starch	Favours lactic acid producing organism and PAO	[98]
	Long chain fatty acids	Favour filamentous bacteria *Microthrix parvicella*	[92]
Alcohols	Methanol [g]	PAO MIX (11 %), GAO MIX (5 %), DFI and II (7 %) [h]	[25]
	Acetate	PAO clades: *Thauera* (14 %), *Hyphomicrobium* (10 %), *Pseudomonas* (9 %) and *Hydrogenophaga* (4 %) [i]	[122]
	Ethanol	PAO clades: *Acidovorax* (14 %) and *Thaurea* (7 %) [i]	[122]
	Acetate	PAO 35 %, GAO 13 % [h]	[34]
	Acetate: glycerol 1:1	PAO 40 %, GAO 10 % [h]	[34]
	Glycerol	PAO 27 %, GAO 16 % [h]	[34]
	Acetate	PAO 32 %, GAO 14 % [h]	[110]
	Acetate: glycerol 1:1	PAO 40 %, GAO 10 % [h]	[110]
	Glycerol	PAO 28 %, GAO 25 % [h]	[110]
	Glycerol	*Tetrasphaera elongata*	[164]
	Glycerol	*Accumulibacter* (55 %), *Tessaracoccus* (7 %) *and Micropruina* (5 %) [j]	[116]
Amino acids	Glutamate	Favours Family *Comamonadaceae* (16 %), *Accumulibacter* (8 %), genus *Thiothrix* (37 %) [i]	[4]
	Glutamate	Favours *Actinobacterial* PAO	[130–132]
	Glutamate	Favours *Tetrasphaera*	[15]
	Casein hydrolysate	*Accumulibacter* (22 %) and *Tetrasphaera* (70 %) [h]	[126]
	Acetate: Casein hydrolysate 4:0	*Accumulibacter* (22.4 %) and fermentative PAO (*Comamonadaceae* and *Saprospiraceae*) (9.9 %) [i]	[134]
	Acetate: Casein hydrolysate 1:1	*Accumulibacter* (11.6 %) and fermentative PAO (19.5 %) [i]	[134]
	Acetate: Casein hydrolysate 0:4	*Accumulibacter* (4.9 %) and fermentative PAO (17.3 %) [i]	[134]

a. with temperature around 30 ℃.
b. 200 mg COD/L.
c. 400 mg COD/L.
d. with denitrifying P removal.
e. with enriched PAO.
f. with enriched GAO.
g. with coexistence of PAO and methanol degraders.
h. percentages obtained by fluorescence in situ hybridization (FISH).
i. percentages obtained by 16S rRNA sequencing.
j. percentages obtained by Metagenome sequencing.

3.1. Lactate

Lactic acid, which is produced by the fermentation of carbohydrates, has not been shown to lead to successful EBPR. In a lab-scale EBPR-SBR system [85], an EBPR failure was shown when the feed was switched from a mixture of acetate/propionate/lactate to lactate as the sole carbon source. The dominant metabolism shifted from a PAO to a GAO metabolism. Glycogen consumption and the percentage of PHV formation almost doubled (Table 3). The storage of PHA from lactate by *Accumulibacter* and *Tetrasphaera* did not seem to require poly-P hydrolysis and, thus, the PAO phenotype was lost. EBPR activity could only be triggered by fermentation of lactate to VFA (acetic acid or propionic acid). Sludge bulking problems have also been reported [86,87] when switching from acetate to lactate/acetate. On the other hand, diverse system performances were exhibited under the co-presence of lactate and acetate as carbon source with an enriched culture of *Accumulibacter* clade IIC [88]. The uptake of lactate was inhibited because both substrates shared the same transporter. However, lactate and succinate could be assimilated simultaneously.

3.2. Glucose

Glucose is a common substance in domestic wastewater that has also been reported to lead to deterioration of EBPR [55,68,86,89] due to the proliferation of GAO [26,50,86]. The common knowledge states that glucose cannot be used directly by PAO and that flanking species should transform glucose to produce pyruvate by glycolysis. In this sense, Zengin et al. [86] initially observed P removal in a glucose-fed SBR system (about 30 days), because lactic acid bacteria produced lactic acid that could be used under anaerobic conditions for PHA storage. System failure occurred on day 29 due to the significant increase in glycogen consumption and hence GAO proliferation. Similarly, five times higher glycogen with glucose than with acetate was reported [22]. Glucose favouring GAO in EBPR systems was in most cases confirmed by the presence of *Competibacter* (Table 2). However, Dockx et al. [90] showed

Table 3
Summary of stoichiometric ratios of carbon transformation during the anaerobic and aerobic phases with diverse carbon sources except for VFA.

Carbon source	Anaerobic				Aerobic			References
	Carbon uptake (mmol C /g VSS/h)	P/C (mol/ mol)	PHA/C (mol C/mol C)	Gly/C (mol C/ mol C)	P uptake rate (mmol/ g VSS/h)	P/PHA (mol/ mol C)	Gly/PHA (mol C/ mol C)	
Mixture of acetate, propionate and lactate	3.06	0.70	PHA/C 0.9 PHB/C 0.5 PHV/C 0.4	0.18	1.06	–	–	[85]
Lactate	2.71	0.11	PHA/C 0.76 PHB/C 0.1 PHV/C 0.66	0.47	0.21			[85]
Glucose [a]	1.32–4.68	0.05–0.12	PHA/C 0.36–0.44 PHB 30–44 % [f] PHV 56–70 %	0.12–0.28[b]	–	–	–	[60]
Acetate	–	0.17–0.31		19[c]	–	–	25[c]	[22]
Glucose	–	0.06–0.21	–	19[c]	–	–	45[c]	[22]
Glucose	–	0.0059	–	0.128	0.19	–	–	[50]
Glucose [i]	–	0.09–0.13	–	–	0.003	–	–	[118]
Starch	–	−0.08 [d]	0.03	2.64[b]	–	41	68 (1.36/ 0.02)	[98]
Methanol	–	0.38–0.54 [e]	PHB 8 %[f] PHV 92 %	–	–	0.2	0	[120]
Ethanol	–	0.2–0.4	PHB 18 % [f] PHV 82 %	0.8	0.05–0.22	0.2	–	[25]
Glycerol	–	0.22	PHA/C 0.31 PHB 26 % [f] PHV 45 % PH2MV 29 %	0.25	–	–	–	[112]
Glycerol [i]	–	0.14–0.24	–	–	0.02–0.03	–	–	[118]
Glycerol	–	0.23	PHA/C 0.97 PHB 0 PHV 53 % PH2MV 44 %	0.27	–	–	–	[116]
Acetate	–	0.38	PHA/C 1.21 PHB 95 % [f] PHV 3 % PH2MV 2 %	0.42	–	–	–	[34]
Acetate:glycerol 1:1	–	0.64	PHA/C 1.35 PHB 60 % [f] PHV 30 % PH2MV 10 %	0.34	–	–	–	[34]
Glycerol	–	0.24	PHA/C 1.03 PHB 30 % [f] PHV 55 % PH2MV 15 %	0.49	–	–	–	[34]
Crude glycerol	–	0.3	PHA/C 0.43 PHB 40 % [f] PHV 60 %	–	–	–	–	[101]
Long chain fatty acids	–	0–0.4	–	–	–	–	–	[101]
Glutamate	–	0.2–0.7	PHA/C 0–0.6 PHB 6 % [f] PHV 47 % PH2MV 35 % PH2MB 12 %	0.5–1.2	–	–	–	[130]
Glutamate	–	0.21	PHA/C 0.07 PHB 71 % [f] PHV 29 %	0.01[b]	–	–	–	[4]
Glycine [h]	–	0	PHA/C 0.12 PHB 17 % [f] PHV 75 % PH2MV 8 %	0.56	–	–	–	[126]
Casein hydrolysate	–	0.35	PHA/C 0.15 PHB 20 % [f] PHV 60 % PH2MV 20 %	0.38	1.76[g]	2.23	1.84	[126]

a. sporadic dosing to enriched PAO in VFA-fed system.
b. glycogen was synthesised, not degraded.
c. the percentage of glycogen content in the biomass at the end of anaerobic phase, and the same in acetate-fed system.
d. indicates P uptake, rather than common P release.
e. with the environment of coexistence of PAO and methanol degraders.
f. the relative percentages under successful EBPR performance.
g. P uptake with the unit of mmolP/L.

h. sporadic dosage to enriched PAO and *Tetrasphaera* in casein hydrolysate-fed system.
i. with enriched clade-4 *Tetrasphaera*.

that the enrichment of GAO-*Saccharimonadaceae* and other GAO increased with glucose over *Competibacter*.

The extra addition of glucose to an EBPR system also decreased its microbial diversity [91]. A glucose-amended feed induced the lowest microbial diversity compared to acetate and ethanol, as well as the worst P and N removal and granule formation (< 1mm) [21]. In Yazıcı and Kılıç [22], changing the carbon source from acetate to glucose had no significant effect on the settleability of biomass in a SBR system, but decreased P release and uptake ratios compared to acetate-fed systems. A decreasing trend in the P/C ratio was observed from 0.21 to 0.06 (Table 3). However, some recent research has shown that glucose could be effectively used as carbon source for EBPR under certain conditions. For instance, glucose could act as a proper carbon source for successful simultaneous P and N removal (more than 90 % and 70 % respectively) and good sludge settleability in an anoxic/oxic SBR system, although not as good as that of acetate as carbon source [92]. Similarly, simultaneous P and N removal was improved at high organic loading (53–88 of the influent C/P ratio) in the anoxic (anaerobic) /oxic mode plant with glucose and other carbon sources [93]. Recent investigations have shown that the acetate/glucose (1:1)-driven EBPR system with the enriched *Accumulibacter* (around 65 %) can efficiently use glucose as the sole carbon source. They proposed that *Accumulibacter* takes up glucose directly and stores it primarily as glycogen, with ATP provided by the hydrolysis of poly-P under anaerobic conditions, and secondarily as PHA by balancing ATP utilization (glycogen generation) and PHA storage [94].

Glucose as a supplemental carbon source was reported to result in successful P removal [95]. In addition, it was shown by Xie et al. [33] that the mixture of glucose and acetate as carbon sources (with a ratio of 1:1) exhibited the highest P removal (96.3 %) in EBPR-SBR systems compared to other different molar percentages of acetate and glucose. *Tetrasphaera*-related PAO, *Microlunatus phosphovorus* and another isolated PAO candidate were identified as the main functional P removal bacteria in these experiments. *Microlunatus* was considered to be a fermentative PAO [9,96].

The reported unfavourable effect of glucose as sole carbon source for *Accumulibacter* may limit its application in some cases. However, *Tetrasphaera*, another putative PAO favoured by glucose [15], may offer a possibility for its wide application due to the high percentage detected in many WWTPs, which will be discussed below.

3.3. Starch

Starch, as a polymer of glucose, is a common compound in wastewater that has been shown to be detrimental to EBPR as carbon source [23,97] and to promote sludge bulking [92]. EBPR efficiency was shown lower (77 %) with a 1:1 mixture of starch and acetate than with acetate as sole carbon source (94 %) in an anaerobic/aerobic SBR [24], because the internal amount of PHA decreased, limiting anaerobic P uptake. In an anoxic-aerobic SBR system with starch as sole carbon source [98], 80 % of P removal was achieved, reporting a novel P removal process without P release, more glycogen accumulation and less PHA. They proposed that starch was fermented to lactic acid and that lactic acid utilisation was responsible for most of the P removal in the anoxic phase.

3.4. Citric acid

Citric acid is an essential intermediate substance for the TCA cycle. It leads to the increase of ATP in the cells under aerobic conditions and enhances poly-P accumulation [99]. The feasibility of citric acid as carbon source to perform EBPR was demonstrated with a biofilm SBR operated in anaerobic/aerobic mode, and longer aerobic phase improved P removal [100].

3.5. Long chain fatty acids

The use of long chain fatty acid (LCFA) as carbon source to drive EBPR is possible, as it has been shown to have no inhibition/toxicity on PAO. However, long-term LCFA-fed EBPR is not feasible due to sludge bulking problems. In this sense, a mixture of VFA (acetic acid and propionic acid) and LCFA (half of myristic and half of palmitic acid) with a ratio of 2:3 showed successful P release and uptake performance with a P/C ratio between 0.1 and 0.4 [101]. However, failure of P removal and a decrease in PAO activity were observed when LCFA was used as the sole carbon source. The reason for this was hypothesised to be the adsorption of LCFA on the surface of PAO, which increased the hydrophobicity of the biomass and substrate and caused sludge bulking. EBPR was recovered by returning to VFA, increasing the P/C ratio from 0 to 0.3. The combination of oleic acid (the most prevalent LCFA in the composition of wastewater) and acetic acid under different ratios was also studied [102]. The best total N (TN) and total P (TP) removal efficiency (about 70 % and 96 %, respectively) was obtained with a ratio of acetate to oleic acid of 4:6. The increasing percentage of oleic acid resulted in sludge bulking problems due to the proliferation of the typical filamentous bacteria *Microthrix parvicella* [103,104]. Similarly, Tween 80 (a water-soluble emulsifier that contains oleic acid) could be used as carbon source with about 50 % of P removal [92]. Tween 80 favoured the production of extracellular polymeric substances (EPS) and the proliferation of *Microthrix parvicella*. In short, LCFA could be used as a supplementary carbon source for EBPR but with a low ratio of LCFA/VFA, as it can lead to sludge bulking problems.

3.6. Glycerol

Glycerol fermentation to VFA and its subsequent biological utilisation is a promising way to convert glycerol into a resource rather than a waste of biodiesel fuel production [105–109]. In Yang et al. [34], glycerol was used as an additional carbon source to acetate (1:1 ratio) and improved EBPR with P removal efficiency of about 96 % in a lab-scale A/O SBR compared to pure acetate (90 %) and pure glycerol (31 %). The combination of acetate and glycerol also favoured the percentage of PAO, decreased the percentage of GAO and increased the amount of PHA synthesis (Tables 2 and 3). The results of Zhao et al. [110] showed that EBPR performance decreased from 97 % with a mixture of acetate and glycerol to 58 % with glycerol only, and the corresponding percentage of PAO decreased from 40 % to 28 % and GAO inversely increased to 10 % to 25 %. Glycerol dosage was also proved to enhance denitrifying P removal when treating real wastewater in an A_2O system [111], and fermentative bacteria degraded glycerol to carbon sources for DPAO. The fermentative bacteria increased from 8 % to 18 % with the increased glycerol dosage. As a result, it was economically and technically feasible to apply glycerol as an additional carbon source for nutrient removal.

However, the use of pure glycerol as the sole carbon source has led to EBPR failure. Pure glycerol resulted in less PHA synthesis and therefore less energy available for subsequent P uptake [34,110,112]. The most common explanation was insufficient anaerobic fermentation time, since two sequential anaerobic processes were required: fermentation of glycerol to VFA and VFA utilisation by PAO [112–115]. Therefore, allowing a higher anaerobic HRT or adding a side-stream reactor to ferment glycerol to VFA could be a solution to avoid the EBPR deterioration when using pure glycerol. In a single-sludge anaerobic/aerobic SBR, a promising EBPR performance was shown with glycerol as the sole carbon source under sufficient anaerobic HRT (4 h) [112], and a high ratio of P/C (0.22) was obtained. With more and more research on granular sludge, the glycerol-driven EBPR showed successful P removal with the help of glycerol fermenters to 1,3-propanediol and PAO [116].

Two five-stage Bardenpho biological nutrient removal (BNR) pilot-scale systems coupled with a side-stream fermenter were proposed to treat raw wastewater [113], and a substantial concentration of VFA was obtained by the co-fermentation of glycerol and primary solids (around 2500 mg COD/L). Similar P removal efficiency (> 80 %) was obtained by adding glycerol to the anoxic reactor or to the side stream fermenter.

Crude glycerol (a mixture of methanol, LCFA and salts) was also tested as a sole carbon source and maintained successful EBPR in the medium term, but not in the long-term [101]. They argued that the complex carbon compounds can be degraded to VFA by flanking species, but crude glycerol with high content of LCFA deteriorated PAO activity and led to the collapse of EBPR. Crude glycerol can also be used to improve P and N removal in an A_2O system and to mitigate the detrimental effect of the presence of anaerobic nitrate and nitrite [117].

Glycerol might favour PAO over GAO. In particular, the dominant GAO *Defluviicoccus*, and *Competibacter* were rarely detected because propionic acid is reported as the main fermentation product of glycerol, and *Competibacter* can only assimilate acetic acid. Glycerol-driven EBPR may stimulate the interaction between fermentative bacteria and PAO. The increase of PAO and fermentative bacteria was witnessed with the increase dosage of glycerol [111]. The domination of glycerol fermenters (*Tessaracoccus* and *Micropruina*) and *Accumulibacter* was reported [116] (Table 2). For the PHA production, PHV appeared to be the main species (45 %-60 %) [34,101,112,116,118] (Table 3).

3.7. Alcohols. Methanol and ethanol

Methanol and ethanol are economical alcohols that are already used to supplement organic matter in COD limited wastewaters to enhance denitrification. Therefore, if added to the anaerobic phase, they could also enhance EBPR, albeit indirectly by improving denitrification. Long-term successful application of methanol as sole carbon source hasn't been reported yet, but the improvement of P removal and N removal efficiency with methanol as a supplemental carbon source was shown by Xu et al. [38]. However, in most cases, methanol as a sole carbon source has been reported to be unsuitable for EBPR [7,119]. The addition of methanol to the EBPR system was shown to be detrimental to the stability of the system as the methanol couldn't be directly degraded by PAO [25,120,121]. However, Tayà et al. [25] succeeded in obtaining methanol-based EBPR by using a sludge containing PAO and methanol-fermenters. A mid-term EBPR performance (about 35 days) was sustained with a P/C ratio of about 0.38–0.54. Methanol was fermented to acetic acid and PAO lived on this VFA. PAO (11 %) showed a relatively high percentage compared to GAO (5 %), DFI and II faded to a negligible amount regardless of the high initial percentage (7 %) in the PAO-enriched inoculum (Table 2).

Ethanol has been reported to be an efficient additional carbon source for EBPR in the long-term [119,120]. Compared to VFA, ethanol improved granule stability more than acetate as the sole carbon source in an EBPR-SBR [21], but acetate acted as better carbon source than ethanol in terms of N and P removal. Similar and successful P and N removal efficiencies (more than 80 %) were shown with acetate and ethanol as individual carbon sources in a moving bed biofilm reactor [122], and they reported that the addition of ethanol reinforced the functional PAO: *Thauera* (14 %), *Hyphomicrobium* (10 %), *Pseudomonas* (9 %) and *Hydrogenophaga* (4 %), whereas acetate as the sole carbon source selected PAO clades such as *Acidovorax* (14 %) and *Thaurea* (7 %) (Table 2). *Accumulibacter* taxa from clade IIF were suggested to convert ethanol to acetate to enhance EBPR [123], as this clade contained acetaldehyde dehydrogenase, which may generate acetate as the final step in ethanol degradation. As a result, the fermentation of both alcohols resulted in the production of acetate. A high percentage of PHB (80–90 %) in PHA with methanol or ethanol as carbon source was reported [120] (Table 3), which was similar to that of acetate.

3.8. Amino acids

The proteins may account for more than 50 % of all the organic substances in real wastewater [124]. The hydrolysates of protein, amino acids, have been reported as promising carbon sources to induce EBPR [30,125,126], and amino acid favoured the proliferation of *Accumulibacter*, *Thiothrix* and *Tetrasphaera* [3,4,8,15,126–129].

3.8.1. Glutamate

Glutamate as a common amino acid has been proved to support EBPR and to favour a variety of PAO clades (Table 2) [4,129,130]. It was shown that glutamate could boost EBPR with the range of P/C ratio of 0.2–0.7 [130], and *Actinobacterial* PAO were favoured in the glutamate-fed system. However, the levels of internal PHA decreased over time and EBPR activity was lost in the long-term. Glutamate contains a high fraction of N, which is released into the medium during glutamate fermentation. Thus, Rey-Martínez et al. [4] showed successful P and N removal with glutamate as the sole carbon and nitrogen source in an anaerobic/anoxic/oxic continuous pilot system. They also observed low PHA storage (with a PHA/C ratio of about 0.07) and anaerobic glycogen storage rather than consumption (Table 3). They suggested the possibility of carbon storage routes other than the involvement of PHA and glycogen. The sludge was initially enriched in *Accumulibacter* and glutamate addition promoted the growth of *Thiothrix* and the family *Comamonadaceae*. They indicated that *Thiothrix* has the ability to store poly-P with glutamate involvement but without PHA synthesis, *Comamonadaceae* was confirmed to degrade glutamate and denitrify, and the mechanisms for P uptake were unclear. In other studies [130,131], *Actinobacterial* PAO were favoured in the glutamate system, and were shown to assimilate glutamate and poly-P simultaneously, but without storing PHA [132]. It has also been shown that the acetate-fed sludge enriched *Accumulibacter* can metabolise glutamate without the formation of PHA [30]. PHA composition using glutamate was reported as PHB (70 %) and PHV (20 %) [4], while PHV (47 %), PH2MV (35 %) and PH2MB (12 %) were reported by Zengin et al. [130].

3.8.2. Glycine

The results reported on glycine for EBPR are also inconclusive. On the one hand, glycine was shown to induce the highest P release in batch tests with 11 different amino acids [31] using sludge from full-scale WWTPs. On the other hand, glycine was found to induce P release without efficient uptake during the anaerobic phase [30,12]. Thus, PAO could release P in the presence of glycine (with a high P/C ratio of 0.87–5.20) but could not uptake P during the aerobic phase. Therefore, glycine cannot be considered as an effective carbon source for EBPR systems. However, the unique characteristic of glycine to induce P release without cellular uptake could provide possibilities to recover P from P-enriched waste sludge.

On the other hand, glycine might be an effective carbon source for the specific genus of *Tetrasphaera*, but not for *Accumulibacter*. Marques et al. [126] observed no P release with glycine as carbon source in an enriched *Accumulibacter* and *Tetrasphaera*-culture. P release occurred only after complete consumption of glycine, suggesting that P release was not positive relatively to glycine uptake. They further showed that the energy for *Tetrasphaera* to take up P anaerobically came from glycine fermentation (as well as other carbon source such as glucose, glutamate and aspartate).

3.8.3. Mixtures of amino acids

The mixture of amino acids and other carbon sources may pose different effects on the performance of the system and on the microbial communities. Casein hydrolysate, as a mixture of amino acids and peptides, was used as sole carbon source with an enriched culture of *Tetrasphaera* and *Accumulibacter* [126], and more than 99 % P removal was observed. More than 90 % of *Tetrasphaera* cells were shown to be responsible for amino acid consumption and participated in about 80 %

of P removal. However, *Accumulibacter* was likely to survive only on the fermentation products such as acetate and propionate. The use of casein hydrolysate as sole carbon source effectively allowed successful EBPR with enriched *Tetrasphaera* of 91 % [133], and glycine and glutamate were intracellular metabolites. The mixture of acetate and casein hydrolysate was investigated [134]. The best system performance was achieved with dual PAO symbiosis of Ca. *Accumulibacter* and fermentative PAO, including *Comamonadaceae* and *Saprospiraceae* when the ratio of acetate to casein hydrolysate was 1:1.

Energy for anaerobic P release was provided by the fermentation of casein hydrolysate to VFA. Amino acids, sugars and some small amines were stored as intracellular substances to provide energy for aerobic metabolites. The PHA compositions were PHB (20 %), PHV (60 %) and PH2MV (20 %) (Table 3), which is more similar to a propionate-fed system with the PHV as the most dominant PHA [126].

In a similar study [135], replacing the carbon source from VFA to a mixture of amino acids, VFA and glucose had little impact on P and N removal and sludge settleability, but did affect the microbial communities, with *Actinobacteria* becoming the dominant bacteria. In another study [30], a mixture of acetate and amino acids as carbon source was proposed to save more than 17 % of energy compared to the single carbon source due to the flexibility of the metabolic pathway of *Accumulibacter* under different carbon sources. In Close at al. [10], a highly enriched *Tetrasphaera* (95 %) with amino acids as sole carbon source showed lower P removal (72 %) compared to a sludge containing *Tetrasphaera* and *Accumulibacter* (> 99 %).

4. The application of wastes to carbon sources

The dosage of an external carbon source is not economically and environmentally feasible for full-scale application. Therefore, environmentally friendly and economical carbon sources have attracted research attention. Carbon sources derived from waste materials, such as organic waste or waste sludge from side-stream, mainstream, primary sludge, are now being studied. The overall perspective of fermentation productes from wastes as carbon source for EBPR is shown in Fig. 2.

4.1. Pre-treated sludge and food waste

Sludge disposal is a major concern for many WWTPs. The digestion or fermentation of waste sludge not only alleviates this issue but can also be an efficient way of producing VFA. Soluble products from waste fermentation are mainly short-chain fatty acids with two to five carbon atoms, which can be used directly as carbon source for many bioprocesses [136,137]. Pretreatment methods are usually applied to increase the VFA yield, such as alkaline treatment [138–141], acid treatment [41,138], thermophilic operation [142], microwave-H_2O_2 [38] and mechanical disintegration [143].

Waste sludge fermentation products were shown to be used as carbon source to improve N and P removal in municipal wastewater with low C/N ratio [144], and the reduced sludge discharge was 44–52 %. The products of alkaline fermentation of waste sludge as sole carbon source demonstrated a higher nutrient removal efficiency (about 97 %) than that of acetic acid (about 75 %) at the same COD level [40]. The reason for the improvement in nutrient removal could probably be the

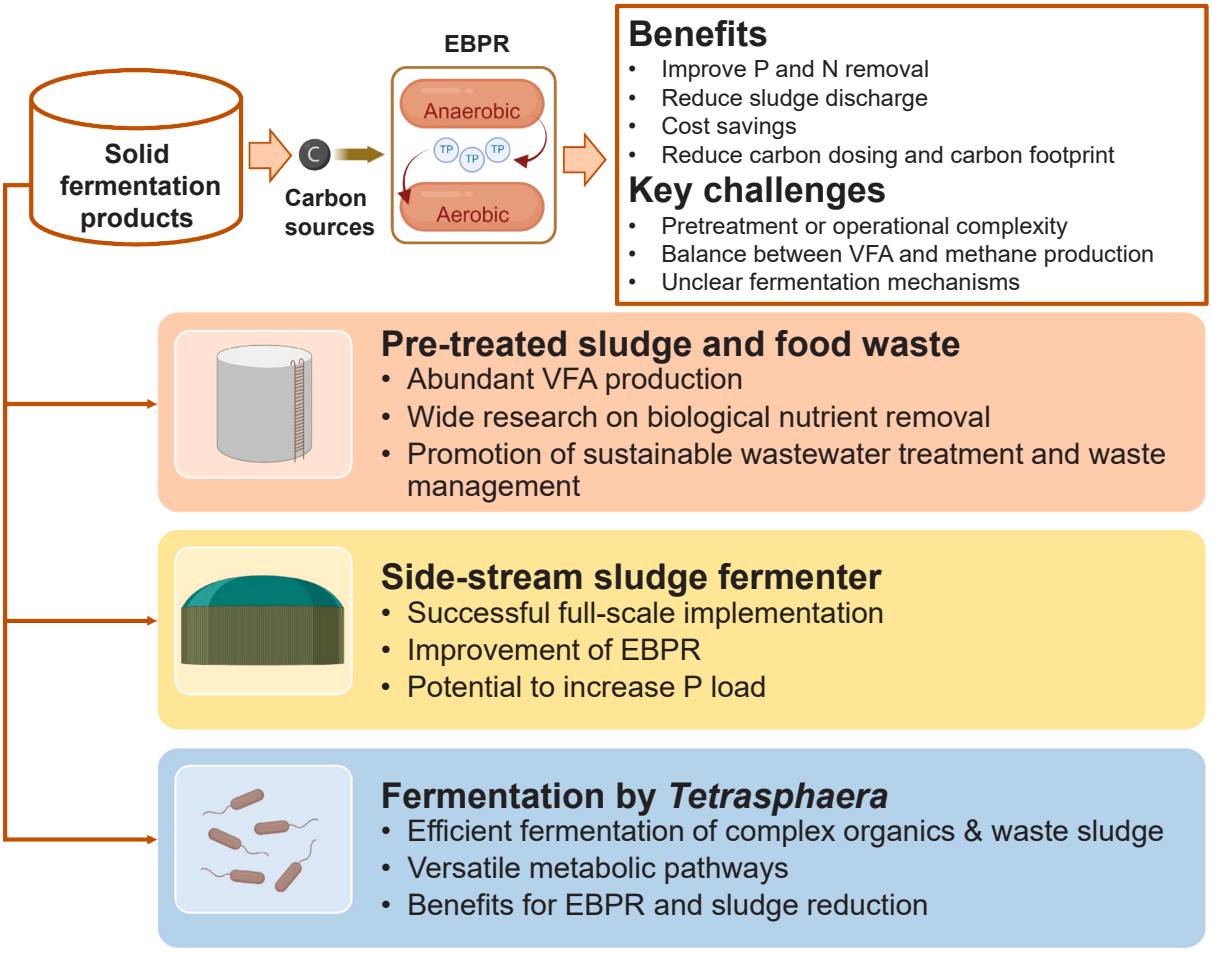

Fig. 2. Solid fermentation productions as carbon sources for EBPR.

dominant percentage of DPAO with 36 % (61 % of total PAO), much higher than in the acetic acid-fed system (3 %) (Table 4). In Zaman et al. [145], more than 90 % of P removal and about 70 % of N removal were also obtained with the effluent from the alkaline fermentation of solids from primary settling tank as carbon source. However, synthetic VFA showed an advantage in nutrient removal and P release and uptake rate compared to the fermentation liquid due to the other carbon sources except the VFA contained in fermentation liquid.

Anaerobic alkaline fermentation liquid used as carbon source to improve BNR and sludge disposal process has been applied in pilot and full-scale processes [138,146]. In Gao et al. [138], an alkaline continuous fermentation process was applied at pilot-scale system to provide additional VFA to an A_2O system for domestic wastewater treatment. They showed that TP and TN removal were about 90 % and 80 %, respectively, and simultaneously 42 % of waste sludge was reduced. In Liu et al. [146], a full-scale WWTP was operated as an A_2O process and the WWTP waste sludge was fermented to VFA as an additional COD. TP and TN removal were about 90 % and 73 % respectively, and 54 % of the sludge was reduced.

Fe-enhanced primary sedimentation sludge is an efficient way to convert solids into VFA through acidogenic sludge fermentation [41]. It was applied in an SBR system to provide COD [147]. The removal efficiencies of TP and TN reached 89 % and 83 % treating the raw wastewater without any additional COD dosage, which showed extensive improvement (with P and N removal efficiency increased by 65 % and 50 %) compared to conventional operation (Table 4).

Similar to the waste sludge, effluent from fermentation of food waste is enriched in VFA, alcohols and lactic acid and can therefore be used as carbon source to enhance nutrient removal [148–150]. Anaerobic thermophilic food waste fermentation resulted in P and N removal about 98 % and 90 % respectively in a lab-scale SBR system [142]. The EBPR performance increased due to the enrichment of *Rhodocyclacea* (7 %) (Table 4). Tang et al. [151] also showed successful P (90 %) and N (more than 80 %) removal when feeding the system with mesophilic fermentation of food waste (Table 4). The improvement in the biological nutrient performance was also found by alkaline fermentation products containing acetic acid and propionic acid (with ratio about 1:1) under optimal conditions [152]. Rather than using VFA produced from food waste, the use of food waste hydrolysate showed more advantages because the abundant content of sugars, amino acids and glycerol from the food waste hydrolysate exhibited more economic and operational benefits than VFA [153,154]. They showed that the food waste hydrolysate not only improved the removal efficiency of TN and P compared to glucose, but also didn't affect the effluent quality.

4.2. Waste sludge from a side-stream sludge fermenter

A recent proposal to reuse the surplus carbon generated in the plant is the integration of a side-stream sludge fermenter (SSSF), where part of the return activated sludge (4–30 %) is hydrolysed and fermented to provide VFA to the system without any pretreatment [45,155–157]. The integration of SSSF and conventional EBPR process (S2EBPR) has been extensively investigated in about 80 full-scale WWTPs worldwide [158–162]. Compared to conventional EBPR configurations, the S2EBPR configuration was shown to improve EBPR and system stability [45,163], and the ratios of anaerobic P/C and aerobic P/PHA in S2EBPR were 2–3 times those of A_2O [155] (Table 5).

4.3. Waste sludge by Tetrasphaera fermentation

Tetrasphaera are reported to have the ability to ferment complex organics such as amino acids and glucose [3,4,8,15,126–129]. The high amount of proteins and carbohydrates (30–40 % of total COD) contained in the waste [138,144] allows *Tetrasphaera* to ferment them and generate an effluent suitable for its application into EBPR [11,13,31,46]. In Fan et al. [46], a lab-scale SBR- *Tetrasphaera* operated in anaerobic–aerobic mode with the only carbon source provided by the waste sludge from the other parent SBR system. Enriched *Tetrasphaera* (91.9 %) was observed in the SBR- *Tetrasphaera* system, although no *Tetrasphaera* was detected from the parent system. Successful P removal (no P

Table 4
System performance and relative microbial communities of solids as carbon source with different pretreatment.

Biosolids type	Pretreatment	Carbon source and wastewater	Configuration and scale	P removal efficiency (%)	N removal efficiency (%)	Microbial community	References
Primary sedimentation sludge	Fe-based chemically enhanced pretreatment	Fermentation liquid + municipal wastewater	SBR 24L	89	83	–	[147]
Waste sludge	Alkaline fermentation (pH = 10)	Fermentation liquid + municipal wastewater	AOA-SBR 11.5 L	99	89	*Accumulibacter* 4 %	[144]
	Alkaline fermentation (pH = 10)	Fermentation liquid + synthetic wastewater	SBR 4L	98	99	PAO 59 % DPAO 36 % GAO 3 %	[40]
		Acetic acid + synthetic wastewater	SBR 4L	73	79	PAO 37 % DPAO 3 % GAO 11 %	[40]
	Alkaline fermentation (pH = 10)	Fermentation liquid + municipal wastewater	Pilot-scale A_2O 55L	90	80	–	[138]
	Thermal-alkaline fermentation (pH = 10–11)	Fermentation liquid + municipal wastewater	Full-scale A_2O with 40,000 m^3/d wastewater handling capacity	90	73	–	[146]
Kitchen wastewater	–	Acetic acid + domestic wastewater	Full scale A_2O with 25,000 m^3/d wastewater handling capacity	88	70	–	[152]
	Alkaline fermentation (pH = 8)	Fermentation liquid + municipal wastewater	Full scale A_2O with 25,000 m^3/d wastewater handling capacity	95	78	–	[152]
Food waste	Thermophilic fermentation (55 ℃)	Fermentation liquid + domestic wastewater	5L SBR	98	90	*Rhodocyclaceae* 7 %	[142]
	Mesophilic acidogenic fermentation (pH = 4)	Fermentation liquid + domestic wastewater	5L SBR	90	> 80	*Accumulibacter* 0.6 % *Rhodocyclaceae* 5.6 %	[151]

Percentage of the microbial community obtained by 16S rRNA sequencing.

Table 5
Summary of stoichiometric ratios of carbon transformation during the anaerobic and aerobic phases with waste sludge as carbon source by SSSF or by *Tetrasphaera*-enriched culture (modified from [155]).

Configuration	Carbon source and wastewater	P/C (mol/mol)	PHA/C (mol C/mol C)	Gly/C (mol C/mol C)	P uptake rate (mmol/g VSS h)	P/PHA (mol P/mol C)	Gly/PHA (mol C/mol C)	References
Full-scale A_2O	Municipal wastewater	0.22	0.64	0.16	0.07	0.32	0.55	[155] [a]
Full-scale S2EBPR	Fermentation liquid + municipal wastewater	0.45	0.50	0.22	0.14	0.97	0.61	[155] [a]
Continuous anaerobic/aerobic/anoxic system (10*L*) with enriched-*Tetrasphaera*	Fermentation liquid + municipal wastewater	0.26	0.36	0.34	–	0.99	–	[13] [a]

a. acetate sporadic dosage to the sludge from the system.

detected in the effluent) and sludge reduction (44 %) were obtained simultaneously, and it should also be noted that there was no pretreatment with the waste sludge. Compared to traditional sludge fermentation, the slowly biodegradable organics (e.g. amino acids and soluble microbial by-product) from the waste sludge in the *Tetrasphaera*-dominated reactor experienced better hydrolysis and acidification and further to VFA, and the final VFA concentration was 5.46 times that of the traditional sludge. In terms of metabolism of *Tetrasphaera*, they proposed that SBR-*Tetrasphaera* relied on amino acids as energy source for anaerobic storage and aerobic consumption in EBPR process, rather than glycogen and PHA, and glutamate was the most crucial intracellular substance for metabolites of *Tetrasphaera*. Other intracellular substances have been proposed [118] with the enrichment of clade-4 *Tetrasphaera* culture. Anaerobic PHA accumulation and a small fraction of PHA and higher glycogen consumption were observed with acetate as carbon source, which means that PHA could be possible intracellular substances. However, with glycerol and glucose as carbon sources, glycogen accumulation and degradation patterns were found. Further, both showed lower aerobic P uptake than that of acetate, and the reason was the additional energy supplied by PHA hydrolysis for P uptake when acetate was used carbon source (Table 1&3). However, the energy supply with glycerol or glucose as carbon source was only from glycogen. He at al. [164] showed that *Tetrasphaera* elongata can successfully uptake phosphorus regardless of the C/P ratio, and it was interesting that glycogen consumption and glucose uptake occurred simultaneously under aerobic condition, rather than the traditional view of anaerobic uptake of carbon source.

The operation of this system under continuous anaerobic/aerobic/anoxic conditions was investigated in their posterior work [13]. The only carbon source was provided by in-situ fermentation of waste sludge by *Tetrasphaera* through a prolonged anaerobic phase (increasing HRT from 2 h to 15 h) to treat low COD/N ratio real domestic wastewater for EBPR and partial nitrification. P removal was maintained at 100 % and sludge discharged was reduced by 61.9 % due to sludge fermentation. The abundance of *Tetrasphaera* accounted for 31.2 % and 72.8 % at genus and transcriptional level, respectively. The prolonged anaerobic HRT for sludge fermentation favoured more *Tetrasphaera* to outcompete *Accumulibacter* and further improve fermentation for VFA production, and it also benefited more stable partial nitrification.

The ability of *Tetrasphaera* to ferment on waste as well implies a variety of metabolic pathways. Fan et al. [13] reported the ratios of PHA/C and P/PHA were 0.26 and 0.99, and suggested *Tetrasphaera* did not depend on PHA for intracellular carbon storage and P removal (Table 5). Further investigation is needed due to the lack of consensus on the energy storage substances and intermediate metabolites [165].

5. Discussions and remarks

5.1. EBPR performance under sole carbon source

A general view of the availability of diverse carbon sources for efficient EBPR performance and the future challenges are shown in Figs. 1 and 3. Acetate and propionate are still the most common carbon sources for EBPR, particularly at lab scale, and a moderate concentration of VFA ensures successful system performance [9,54]. Other VFA (butyric and valeric acids) appear to be more suitable as additional carbon source to ensure stable EBPR [28,29,82]. Lactate, glucose and starch can induce EBPR, but it is controversial whether they support successful and long-term EBPR [22,86,93,98]. However, glucose allows successful EBPR in enriched-*Tetrasphaera* culture [15]. LCFA as sole carbon source could lead to a decrease of PAO activity and proliferation of filamentous bacteria, resulting in failure of P removal [101,103].

Methanol as sole carbon source generally leads to the failure of P removal, but ethanol has been reported to be directly assimilated and allow successful EBPR [7,119]. Pure glycerol leads to reduced PHA synthesis and hence EBPR failure [34,110,112]. Glutamate has been proved to support EBPR, but glycine is unlikely to be suitable for EBPR.

On the other hand, the complex carbon sources (carbohydrate (e.g. glucose, starch), LCFA, methanol, glycerol, protein (amino acids)) could act as a complementary carbon source for EBPR, as they are present in high proportions in real wastewater. In addition, as an alternative strategy, the hydrolysis and fermentation process of the complex carbon sources to VFA is proposed to take full advantage of these substances in real wastewater. For example, the fermentation of glucose and amino acids by *Tetrasphaera,* the fermentation of starch to lactic acid, the acidification of methanol to acetic acid, and the longer anaerobic fermentation time for glycerol, for some amino acids or LCFA to VFA.

5.2. EBPR performance under mixed substrate strategies

EBPR performance is strongly influenced by the different feeding strategies of carbon substrates. A mixture of carbon sources improves EBPR performance to some extent although the predominant position is VFA. Some complex carbon sources (e.g. carbohydrate as glucose or starch, methanol or LCFA), which often lead to unstable EBPR performance as individual carbon source, have been shown to be good candidates as complementary carbon source to improve EBPR and N removal [21–25]. These carbon compounds can be used as carbon sources when properly fermented to VFA as explained above, but the main issue is that they promote the growth of flanking filamentous species that lead to bulking issues and thus decrease system performance. However, when combined with other VFA, the growth of undesired microorganisms can be mitigated allowing these compounds to be used effectively as electron donors. For example, the mixture of acetate and glycerol [34] or the mixture of acetate and glucose [33] even showed higher EBPR performance than pure acetate. A mixture of amino acids and acetate can save more than 17 % energy compared to a single carbon source due to the more flexible metabolic pathway of *Accumulibacter* with different carbon sources [30]. In the case of casein hydrolysate, successful EBPR performance depends on the contribution of fermentative PAO such as enriched-*Tetrasphaera*, *Comamonadaceae* or *Saprospiraceae* cells [133,134].

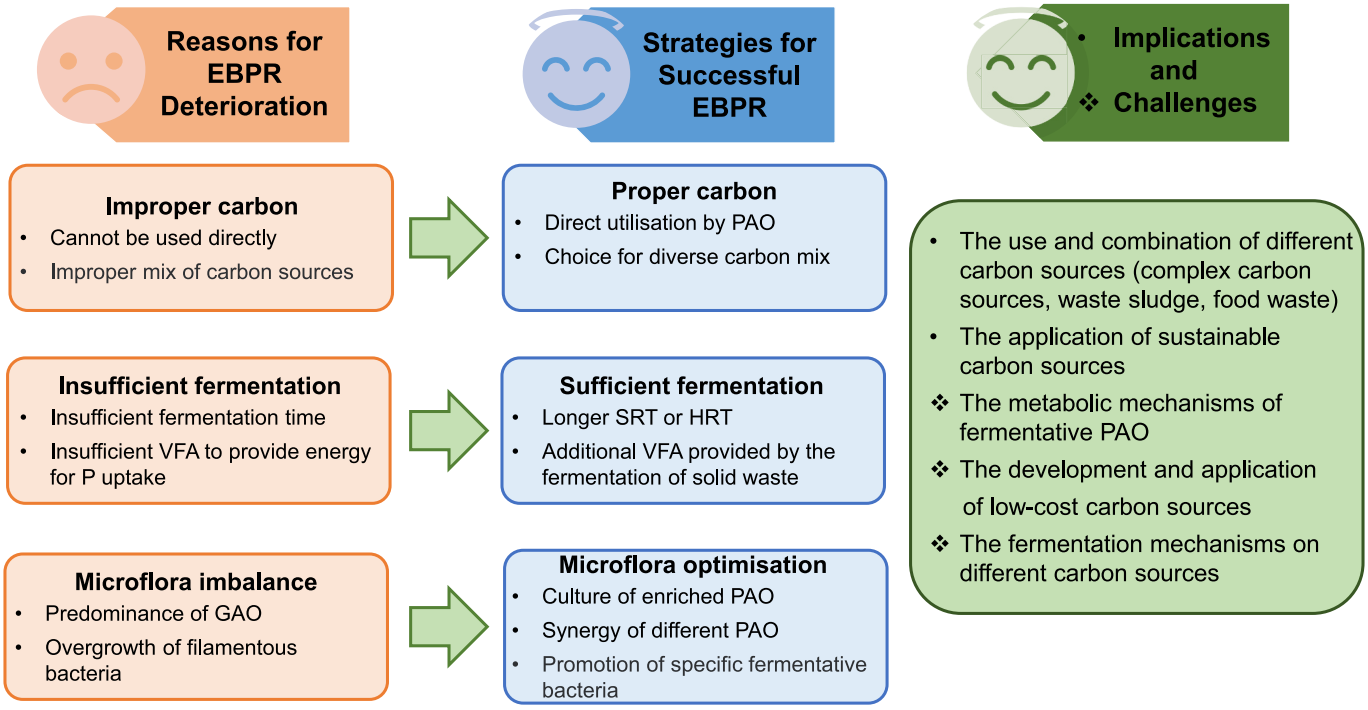

Fig. 3. The overall perspectives on the utilization of diverse carbon sources for EBPR.

5.3. Novel alternatives of carbon sources from the fermentation of solid wastes

The fermentation products of waste sludge and food have provided very good experimental results to enhance biological P and N removal and overcome potential C limitations in full-scale systems, showing promising benefits for application [40,143,152].

1. Potential COD production from waste sludge fermentation could be obtained for EBPR and N removal process. The concentration of soluble COD (COD_S) from the waste sludge fermentation is reported to be in the range of 1000–6000 mg/L depending on the operating conditions [39,40,144], and the concentration of short-chain fatty acids may account for half of the COD_S. In Liu et al. [146], the COD_S of the waste sludge fermentation liquor was reported to be about 30000 mg/L, and the VFA obtained was about 5000 mg/L (Table 6). In the case of food waste, much higher COD_S was detected (40000–50000 mg/L) as well as a high concentration of VFA, about 8600 and 30000 mg/L [151,152].
2. The other advantage of applying waste sludge fermentation could be the reduction of sludge discharge. The reported percentage for sludge discharge reduction is in the range of 40–55 % in lab or full-scale configurations [138,144,146].
3. Potential economic savings can be derived from the significant COD production. The total annual cost savings could be 1270,350 USD per year [147], considering the cost reduction benefiting from the saving of acetate dosage and electricity for aeration (for the treatment capacity of 100,000 m^3/d), and the net profit for VFA production reached to 9.12 USD/m^3 due to the unnecessary addition of commercial carbon source [146].

Table 6
Summary of the COD concentrations from the fermentation liquid.

Biosolids type	COD_S (mg/L)	VFA (mg/L)	References
Waste sludge	~3400	~1500[a]	[39]
	~3100	~1600[a]	[144]
	~5700	~1700	[40]
	~1300[b]	~330[b]	[138]
	~30000	~5000	[146]
Kitchen wastewater	~44000	~30000[c]	[152]
Food waste	~48200	~8600	[151]

a. short-chain fatty acids.
b. below pH = 10.
c. 67 % of the VFA obtained under the optimal conditions (pH = 8, HRT = 6d).

There is no doubt that the use of waste sludge reduces the sludge discharge and leads to significant economic savings and to a lower carbon footprint of the plant [13,143,145,147]. However, the potential disadvantage that can't be ignored is that the physical or chemical pretreatment process to obtain more suitable COD compositions for promoting EBPR will lead to additional costs in terms of electrical energy input and environmental threat due to the reagents addition. The COD compositions are reported to be dependent on the disintegration time [143], which means that the additional energy input for the sludge disintegration should be considered, and the additional cost could be due to the high temperature, pressure and pH [145,166]. Too high pH would also result in high pH in the effluent [167].

The above disadvantages may limit its feasibility. As a complementary solution, the application of biological fermentation to waste sludge avoids the additional use of chemicals and energy in full-scale WWTPs by introducing waste sludge into an SSSF. Secondly, it could also be a means of fermenting some complex carbon sources (e.g. carbohydrate, glycerol, protein or long-chain fatty acids) into VFA for EBPR and N removal. Apart from that, the fermentation ability of *Tetrasphaera* on waste sludge exerted more efficient VFA production than traditional sludge, and successful P and N removal could be achieved for treating real wastewater without the additional carbon source, which showed extensive advantages over the *Accumulibacter* dominated system demanding dosage of external carbon source [13,46].

However, a potential disadvantage of integrating an SSSF is that the fermentation of SSSF produces not only VFA, but also a significant amount of P in the EBPR system. As a result, the additional P load may pose a threat to plant performance because not all of the incoming P could always be removed due to the continuous input of high

concentrations of P from the SSSF effluent into the system. In a full-scale S2EBPR with 6 % of RAS to SSSF, 2.8 mg/L of P was reported in the effluent due to this effect [160]. Nevertheless, the SSSF can also be a good point to integrate P-recovery strategies [168]. Secondly, the additional COD production is at the expense of producing less purge for digestion, the likelihood of potential biogas production must be compromised due to the large biochemical methane potential of anaerobic digestion to recover the chemical energy [169–172], but it is independent of the strategy (with or without pretreatment) for the solid waste fermentation.

5.4. Microbial communities and metabolic pathways under different substrates

The carbon source favours certain PAO among all the putative PAO known. Most lab-scale studies have been conducted with acetate or propionate-based influents and this has led to the proliferation of *Accumulibacter*-the most common PAO of full-scale WWTPs. Apart from that, *Accumulibacter*-enriched sludge has also been reported to proliferate under butyrate, lactate, glucose, glutamate or casein hydrolysate as individual or supplemental carbon source, as well as the fermentation liquid (with pretreatment system or the integration of SSSF) as carbon source to support efficient EBPR.

Recent microbiological advances in full-scale systems fed with real wastewater have shown the synergistic relationship to perform biological P removal between many other different microbial consortia. For example, *Tetrasphaera*-related bacteria have been reported to assimilate glucose, amino acids (e.g. glutamate, glycine, aspartate, casein hydrolysate), and to ferment waste sludge into VFA for use by *Accumulibacter*. It has been speculated that they may play a more important role than *Accumulibacter*-PAO due to their high abundance and diversity in full-scale EBPR plants and the metabolic pathway they have developed [30,67,125,127]. The cooperation between *Accumulibacter* and other fermentative bacteria also occurred, for example, the appearance of lactic acid bacteria or glycerol fermenters with the lactate, glucose and glycerol.

In addition, some genera of *Dechloromonas,* as DPAO, have been shown to appear in high proportions in some successful EBPR systems with VFA, or the mixture of amino acids and VFA as carbon sources and store them as PHA [27,49,165,173]. *Dechloromonas* has also been shown to be enriched in the fermentation of waste sludge system for P and N removal, which could reduce the need for aeration for aerobic P uptake [39]. In fact, the proliferation of DPAO by the fermentation of the sludge is reported to improve the removal of P and N, which may show great potential in full-scale WWTPs. Organisms in the *Rhodocyclaceae* are known to be involved in P removal in full-scale WWTPs [155,174], and it was reported to hold an abundant percentage in lab-scale VFA-fed system (even to 72 %), as well as the food waste fermentation liquid-fed system (7 %).

The different uses of carbon source also determine the dominant microbial community when it comes to the competition between PAO and GAO for the substrate [17,174]. The glycogen consumption and regeneration are highly related to the activity of GAO [175,176]. Lower VFA concentrations may favour PAO over GAO, but overload dosage of VFA may favour the proliferation of filamentous bacteria [76,78–80]. Temperature is also a sensitive parameter that may influence this competition. VFA could favour the proliferation of *Accumulibacter* under high temperature conditions, and *Dechloromonas* in particular could be strongly favoured by propionate [27,54,177]. Butyrate also favours PAO more than GAO in warm climates [32]. In fact, butyrate has also been shown to select more *Accumulibacter* than *Defluviicoccus* or *Competibacter* [28]. Glycerol as carbon source selected more *Defluviicoccus* [112], but Yang et al. [34] showed that glycerol favoured more PAO than GAO. Zhang et al. [49] claimed that the coexistence of acetate and propionate (with a ratio of 1:1) favoured denitrifying P removal due to the transformation of PHB and PHV. *Accumulibacter, Acinetobacter, Dechloromonas* and *Pseudomonas* were the most enriched clades, outcompeting *Competibacter* and *Defluviicoccus*.

Another important aspect is the change in the microbial distribution of the community when the carbon source is replaced. Switching from acetate to butyrate resulted in a gradual increase of *Rhodocyclaceae* and a decrease in *Accumulibacter* and GAO, with a floc-forming specie *Zoogloea* taking the dominant position [32]. The increased ratio of Gly/VFA (and thus GAO) was reported when the carbon source was a mixture of acetate and glycerol at a ratio of less than 1:1 [34]. Glycerol as the sole carbon source resulted in the highest GAO abundance. Similarly, five times higher of the consumption of glycogen was found after changing the carbon source from acetate to glucose [22], which could be an indicator of the conversion of a PAO-enriched sludge to GAO. Although GAO were apparently considered as competitors of PAO in EBPR, their coexistence was not shown to threaten the system performance [9], and variable metabolic pathways of different biomass under different carbon sources could allow them to sustain the complex environments and facilitate more robust EBPR.

Taken together, future investigations on the metabolic mechanisms of fermentative PAO and other bacteria and the fermentation mechanisms under different carbon sources are of great importance for the development of advanced microbial technology and the application of sustainable carbon sources.

6. Conclusions

This review systematically evaluated the effects of carbon source on EBPR systems, especially the carbon utilisation strategies and the current developing trend due to the deficient COD in the influent. According to the above mentioned studies, acetate and propionate are still the most crucial and efficient substrates to promote the *Accumulibacter*-enriched sludge and ensure successful EBPR, and a moderate load of VFA is necessary to favour PAO. More complex substances (e.g. methanol, glycerol, lactate, starch, LCFA…) that are not directly degraded by PAO as sole carbon source may lead to unstable performance and even the system failure. The longer fermentation time of the complex carbon sources to VFA or the mixture of these carbon sources with VFA can support the successful lab-scale EBPR performance. In addition, the recent detection of other PAO-clades opens the door to more diverse carbon utilisation. The fermentative PAO- *Tetrasphaera* can ferment glucose, some amino acids and waste sludge, and the VFA from the fermentation productions can be assimilated by *Accumulibacter*. Using the fermentation products from the waste as carbon source has been a popular and environmentally friendly solution for the EBPR process, which can not only lead to lower carbon footprint but also reduce the large amount of sludge discharge. The full-scale applications have shown the efficient P and N removal performance with the utilization of the fermentation products (with abundant VFA) from waste sludge or food waste by some pretreatment strategies (mostly with alkaline pretreatment) and the waste sludge by SSSF. The SSSF can also be a device for fermenting some complex carbon sources from the wastewater to VFA, faced with the problem of VFA deficiency in real wastewater. Apart from this, using the fermentation ability of *Tetrasphaera* and other fermentative bacteria to ferment complex carbon sources and waste sludge as carbon sources may be a promising way forward for future full-scale WWTPs.

CRediT authorship contribution statement

Congcong Zhang: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Albert Guisasola:** Writing – review & editing, Supervision, Resources, Formal analysis, Data curation, Conceptualization. **Juan Antonio Baeza:** Writing – review & editing, Supervision, Resources, Formal analysis, Data curation, Conceptuaization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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