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Effect of carbon sources on phosphorus forms distribution and functional bacteria metabolic pathway in anoxic-aerobic mainstream nitrogen removal process

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ABSTRACT

In order to analyze the influence mechanism of different carbon sources on non-traditional wastewater phosphorus removal technology, the effects of sodium acetate and starch on the distribution of phosphorous forms in an anoxic-aerobic mainstream nitrogen removal process were investigated. The result showed that total phosphorus content in the sludge of the two systems was similar (51.47 and 48.15 mg/g). Intracellular and extracellular organic phosphorus accounted for a high proportion in system I (fed by sodium acetate), while the proportion of inorganic phosphorus (IP) content was higher in system II (fed by starch). For IP, both systems have a relatively high proportion of non-apatite inorganic phosphorus content in the cells, and relatively high apatite inorganic phosphorus content in the extracellular polymeric substance (EPS). Then the microbial community in the sludge was analyzed. The study revealed *Thauera* was the dominant group of bacteria in both the reactors, the abundance of which was 11.56% and 12.37%, respectively. Besides, *unclassified_Rhodocyclaceae* was also the dominant bacterial community in system I, *Tetrasphaera* and *Opitutus* was also the dominant bacterial community in system II. Furthermore, the metabolic pathways differences of functional bacteria were analyzed. In system II, Starch was hydrolyzed to glucose and was further fermented to produce volatile fatty acids. The adenosine triphosphate produced by fermentation and glycogen aerobic decomposition was used to absorb phosphorus. Therefore, the whole anoxic-aerobic operation cycle presented phosphorus absorption state. In system I, nitrate inhibited the hydrolysis process of intracellular phosphorus particles, leading to phosphorus release first and then phosphorus absorption in the anoxic phase. The energy of microbial metabolism and anoxic phosphorus absorption came from glycogen decomposition. Finally, a more suitable phosphorus metabolism model for biological nitrogen removal system was summarized, which expanded the understanding of biological phosphorus removal.

Keywords: Carbon sources; Anoxic-aerobic process; Phosphorus forms distribution; Functional bacteria; Metabolic model

1. Introduction

For urban domestic sewage with low nitrogen and phosphorus content, anoxic-aerobic process can realize efficient simultaneous nitrogen and phosphorus removal, therefore it has attracted significant attention during the past two decades [1–4]. There are various and complex types of organic matter in domestic sewage as carbon sources, which are mainly composed of carbohydrate, protein and fat macromolecular organic matter. Carbon sources are the important driving factors changing microbial community structure and thus affecting the biological phosphorus removal performance. To date,

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many studies focused on the impact of the carbon source type on the phosphorus formation and distribution in bacteria and extracellular polymeric substance (EPS) in enhanced biological phosphorus removal (EBPR) process [5–7]. Studies have found that starch, as the only carbon source in anoxic/aerobic sequencing batch reactor (SBR), can induce mechanisms different from traditional EBPR [8–10]. Phosphorus forms distribution and migration in the anoxic-aerobic process fed by different carbon sources is still not clear. Though the effect of carbon sources on the microbial community diversity in the sludge was investigated [11–13], the genetic influence of different carbon sources on carbohydrate metabolism in microorganisms has also been rarely explored. Therefore, it is necessary to systematically study the influence of external carbon source types on the biological phosphorus removal efficiency of anoxic-aerobic nitrogen removal system, and accurately locate the role of EPS in biological phosphorus removal. Furthermore, the relationship between carbon source, EPS and the formation of microbial community structure needs to be deeply studied.

Hence, we applied sodium acetate and starch as sole carbon source respectively for the operation of anoxic-aerobic SBR system. The main objectives are: (i) to reveal the effects of carbon sources on the distribution of phosphorus forms in the EPS and bacteria; (ii) to study the microbial diversity in the SBRs to identify the functional microbial species; (iii) to establish phosphorus metabolism model for biological nitrogen removal system; (iv) to better understand and master the mechanism of biological phosphorus removal, and lay a theoretical foundation for revealing and developing new biological phosphorus removal methods.

2. Material and methods

2.1. Setup of reactors and operation

In this study, alternate anoxic/aerobic process (A/O) was selected as the typical wastewater removal process for analysis. Two parallel cylindrical SBRs were employed with a height to diameter ratio of 1.8 (height 40 cm, diameter 22 cm), the working volume of 12 L and a volume exchange ratio of 50%, as shown in Fig. 1. Reactors were operated by a programmable logic controller (PLC). The inlet pH value was controlled between 7.0 and 7.5, the aerobic segment dissolved oxygen was between 2.0 and 2.5 mg/L and the initial sludge concentration of the system was about 3,000 mg/L.

A/O system was operated with four 6 h cycles per day. Each cycle included 75 min of anoxic period, 255 min of aerobic period, 30 min of settling, decanting, and idle. The sludge retention time was controlled at about 20 d. It took about three months before stable nitrogen and phosphorus removal was achieved in the two SBRs. Thereafter, the experiments reported below were conducted.

2.2. Synthetic wastewater

The activated sludge used was taken from A^2/O process of a wastewater treatment plant in Tianjin. Sodium acetate and starch (400 mg/L as chemical oxygen demand, COD basis) were used as sole carbon source respectively (system I and system II), KH_2PO_4 (8 mg/L as PO_4^{3-}–P basis), NH_4Cl (10 mg/L as NH_4^+–N basis) and KNO_3 (20 mg/L as NO_3^-–N basis) were used as phosphorus source and nitrogen source in the synthetic wastewater. An additional amount of $CaCl_2$, $MgSO_4$ and trace element solution was added in the synthetic wastewater, and the content composition was the same as described by Li et al. [14].

2.3. High-throughput sequencing

16S rRNA high-throughput sequencing was carried out using the Illumina MiSeq system (Illumina MiSeq, USA) at Sangon Biotech (Shanghai) Co., Ltd. A detailed description of this experimental procedure can be seen in Li et al. [14].

2.4. Conventional analytical method

COD, NH_4^+–N, NO_3–N, NO_2–N, PO_4^{3-}–P, total nitrogen, total phosphorus (TP) and mixed liquid suspension solid were determined according to the standard method [15]. The phosphorus forms in sludge and bacterial cells were measured by European Standard SMT Protocol [16]. EPS extraction was carried out by ultrasonic-cation exchange resin method [17]. Polysaccharide (PS) were determined by anthrone colorimetry, while protein (PN) was determined by coomassie bright blue colorimetry [18,19]. Humic acid (HA) was determined by modified Folin–Lowry method [20].

3. Results and discussion

3.1. Phosphorus removal characteristics in two SBRs

As shown in Fig. 1, the stable effluent PO_4^{3-}–P content in system I and system II were about 0.49 and 0.78 mg/L, the removal rate both reached 90%, which indicated that effective biological phosphorus removal was achieved in the mainstream denitrification anoxic-aerobic process. However, system II fed by starch required a longer acclimation time.

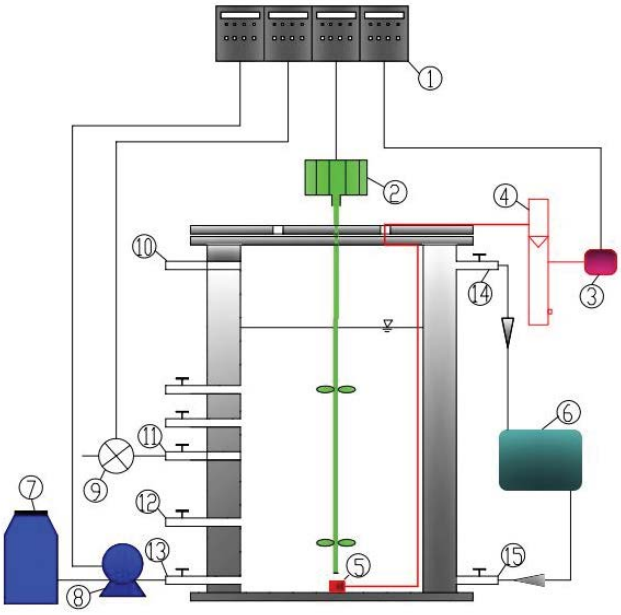

Fig. 1. Schematic diagram of the SBRs.

In system I, a phosphorus removal mode was manifested in which the anoxic phase phosphorus release first and then phosphorus absorption, and aerobic phosphorus absorption (as shown in Fig. 2). Meanwhile glycogen degradation was obvious at the later anoxic phase, which was speculated that the anoxic absorption of phosphate was the demand for phosphate in the degradation of intracellular glycogen, and the phosphate in the liquid was transported to the interior of the cell through the phosphor carrier protein, which was consistent with the results of previous studies [21,22].

In system II, no phosphorus release occurred during the whole cycle, the content of glycogen increased in the anoxic phase and decreased in the aerobic phase. The accumulation of glycogen under anoxic phase provided energy for phosphorus removal in aerobic phase. System I mainly relied on aerobic phosphorus absorption, while system II mainly relied on anoxic phosphorus removal, with a proportion of 71.68%.

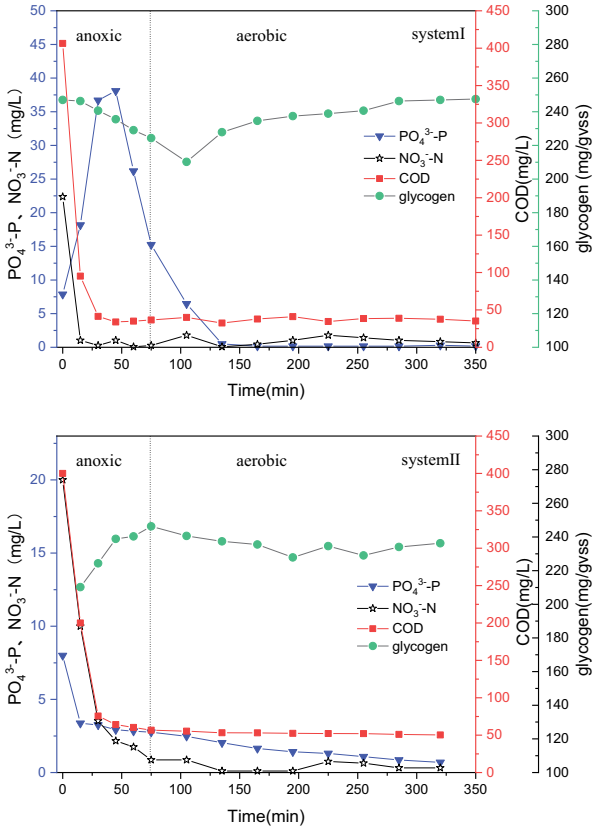

Fig. 2. Variation of pollutants in typical cycle of two SBRs.

3.2. Intracellular and extracellular phosphorus forms distribution

As shown in Tables 1 and 2, it can be seen that TP concentration in the sludge floc fed with two carbon sources varied slightly. However, the contents of inorganic phosphorus (IP), organic phosphorus (OP), apatite inorganic phosphorus (AP) and non-apatite inorganic phosphorus (NAIP) in bacterial cells and in EPS varied significantly. OP accounted for the largest proportion in extracellular and intracellular total phosphorus fed by sodium acetate. On the contrary, IP accounted for the largest proportion in extracellular and intracellular total phosphorus fed by starch. Under two carbon sources, NAIP was the main component of IP in bacterial cells and AP was the main component of IP in EPS. Studies have shown that the higher the proportion of IP to TP, the higher the metabolic activity of phosphorus accumulating bacteria (PAOs), the better the effluent TP effect. The increase of NAIP content indicated that the microorganisms related to phosphorus removal, such as PAOs, occupied a dominant position in the biochemical system and has a good metabolic level [23]. This was relevant to the microbial population structure and metabolic mode.

Tables 1 and 2 also show the percentages of each form of extracellular phosphorus to that of in sludge floc fed with two carbon sources. The proportion of TP were 28.60% and 21.52%, the proportion of OP were 24.56% and 10.26%, and the proportion of IP were 35.19% and 24.06%. The proportion of NAIP were 14.90% and 10.92%, and the proportion of AP were 91.91% and 42.58%, which indicated that two carbon sources were both beneficial to the absorption and binding of AP by EPS, but unfavorable to the absorption of NAIP. AP was Ca^{2+} bound phosphorus and strongly adsorbed phosphorus, which was more easily adsorbed by cations in EPS, formed precipitation and stored in EPS.

3.3. Distribution characteristics of dominant and functional genera in two systems

Compared the two systems as shown in Table 3, the Chao index and Ace index of system I were lower than those of system II, indicating that the microbial flora abundance of system I was lower than that of system II, starch as carbon source would increase the microbial community abundance. The Shannon index of system I was higher than that of system II, while the Simpson index was lower than that of system II, indicating that the microbial population

Table 1
Content variation of different-form phosphorus of the sludge in system I

Sample	Total phosphorus	OP	IP	NAIP	AP
			w (mg/g)		
P content in sludge floc	51.47 ± 1.86	31.92 ± 0.44	19.55 ± 0.34	14.48 ± 0.29	5.07 ± 0.12
Intracellular P content	36.75 ± 0.72	24.08 ± 0.51	12.67 ± 0.23	12.26 ± 0.63	0.41 ± 0.01
Extracellular P content	14.72 ± 0.21	7.84 ± 0.17	6.88 ± 0.03	2.22 ± 0.03	4.66 ± 0.12
P_{EPS}/P_{floc}	28.60%	24.56%	35.19%	14.90%	91.91%

Note: P_{EPS}/P_{floc} represented the proportion of various phosphorus forms in the EPS to that in the sludge floc.

diversity of system I was higher than that of system II. The results showed that although the abundance of microbial community was low, the diversity of microbial population was high in the anoxic-aerobic nitrogen removal system based on starch as carbon source.

Fig. 3 shows that the distribution of dominant microbial species in activated sludge samples of different influents. *Thauera* was less affected by the type of carbon source, the abundance was slightly higher in the starch group than in the sodium acetate group. Besides, the dominant bacteria in system I were *unclassified_Rhodocyclaceae* (9.89%), *Chryseolinea* (3.41%) and *Terrimonas* (2.59%). *Simplicispira* (25.45%), *Tetrasphaera* (5.76%), *unclassified_Sphingobacteriales* (5.66%), *Opitutus* (5.56%) and *Hydrogenophaga* (3.53%) were the top five genera in system II. As denitrifying bacteria, the high abundance of *Simplicispira* was often detected when the nitrogen removal system used macromolecular organics as carbon sources [24,25].

Tetrasphaera and *Opitutus* belong to fermentation bacteria, which used starch for fermentation metabolism, and adenosine triphosphate (ATP) produced by this metabolic pathway can meet the needs of glycogen synthesis and cell maintenance, and even promote the absorption of PO_4^{3-}. *Tetrasphaera* and *Opitutus* have synergistic metabolism with other bacteria, which jointly improve the phosphorus removal rate of the system. The microbial community structure basically verified our speculation in the former analysis of Section 3.2 – Intracellular and extracellular phosphorus

Table 2
Content variation of different-form phosphorus of the sludge in system II

Sample	Total phosphorus	OP	IP	NAIP	AP
			w (mg/g)		
P content in sludge floc	48.15 ± 1.44	8.87 ± 0.12	39.28 ± 0.61	22.98 ± 0.61	16.3 ± 0.32
Intracellular P content	37.79 ± 0.86	7.96 ± 0.17	29.83 ± 0.49	20.47 ± 0.31	9.36 ± 0.12
Extracellular P content	10.36 ± 0.13	0.91 ± 0.02	9.45 ± 0.11	2.51 ± 0.12	6.94 ± 0.06
P_{EPS}/P_{floc}	21.52%	10.26%	24.06%	10.92%	42.58%

Note: P_{EPS}/P_{floc} represented the proportion of various phosphorus forms in the EPS to that in the sludge floc.

Table 3
Analysis of Alpha diversity in different sludge samples

Sample	Number	OTUs	Shannon	Chao	Ace	Simpson	Shannoneven	Coverage
System I	47481	371	4.23	371	371	0.04	0.72	1.00
System II	34843	596	3.76	773	778	0.09	0.59	0.99

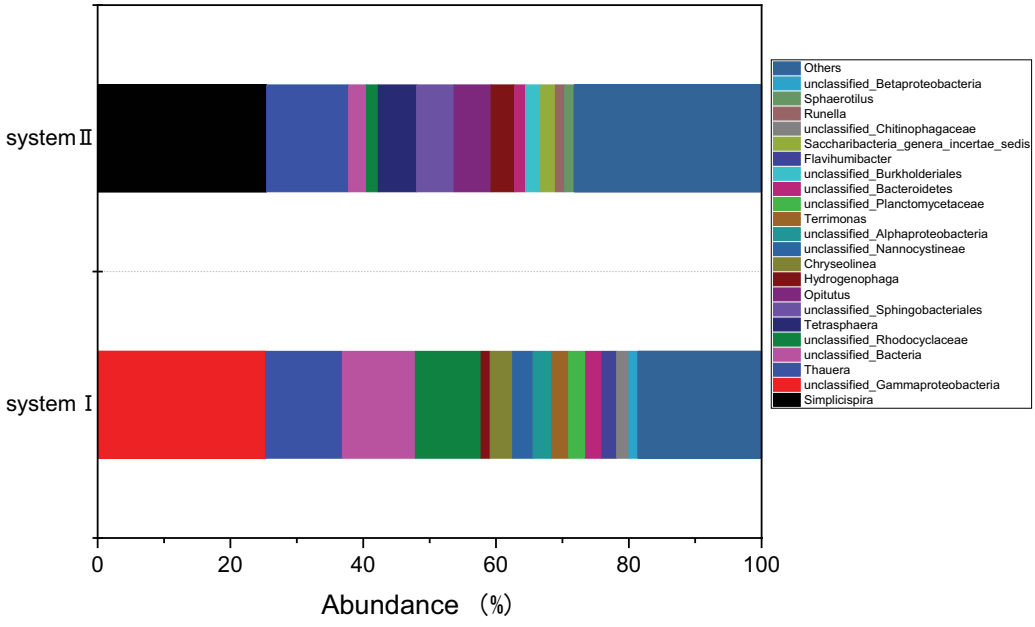

Fig. 3. Relative abundance of functional bacteria genera in two SBRs.

forms distribution. Thus, metabolic pathways of dominant and functional genera fed by different carbon sources needed further analysis and research.

3.4. Metabolic pathway analysis in Kyoto Encyclopedia of Genes and Genomes under different processes

As shown in Fig. 4, starch promoted membrane transport, carbohydrate metabolism, amino acid metabolism, signal transduction, xenobiotics biodegradation and translation. The most obvious difference of function genes contents was found in "Membrane Transport" of "Environmental Information Processing", which accounted for 9.49% and 11.06% in system I and system II respectively. The cellular homeostasis of an organism mainly depends on the transport of specific molecules on the cell membrane [26].

Further analysis based on the carbohydrate metabolism function genes in samples from the two systems is presented in Fig. 4. Glycolysis is an important part of carbohydrate metabolism. The gene abundance of glycolysis/gluconeogenesis in system I and system II was 0.91% and 1.03%, respectively. For citrate cycle (TCA cycle), the abundance of functional genes showed the same trend, accounting for 0.78% and 0.85%, respectively. The functional gene abundance of starch and sucrose metabolism was 0.46% and 0.52%, respectively. These data was consistent with the experimental results in Section 3.1 – Phosphorus removal characteristics in two SBRs. Fermentation bacteria dominated in the starch system, more organic acids were produced by starch decomposition during anoxic stage, which lead to more glycogen synthesized. Meanwhile more glycogen was decomposed in the aerobic stage, so higher gene abundance of glycolysis/gluconeogenesis and starch metabolism was found.

3.5. Phosphorus metabolism model for biological nitrogen removal system

As shown in Fig. 5, the phosphorus metabolic mechanism of anoxic-aerobic nitrogen removal system was summarized by analyzing the microbial flora structure and metabolic pathway under different carbon sources.

Phosphorus metabolism fed by sodium acetate was as follows: the traditional PAOs anaerobic released phosphorus, while the denitrifying phosphorus accumulating bacteria (DPAOs) absorbed phosphorus. The releasing phosphorus amount of the PAOs early in the anoxic period was greater than the absorbing phosphorus amount of the DPAOs, while the trend was opposite later in the anoxic period, showing the overall phosphorus absorbing state. The reason was that nitrate inhibited the hydrolysis process of intracellular phosphorus particles of phosphorus accumulating

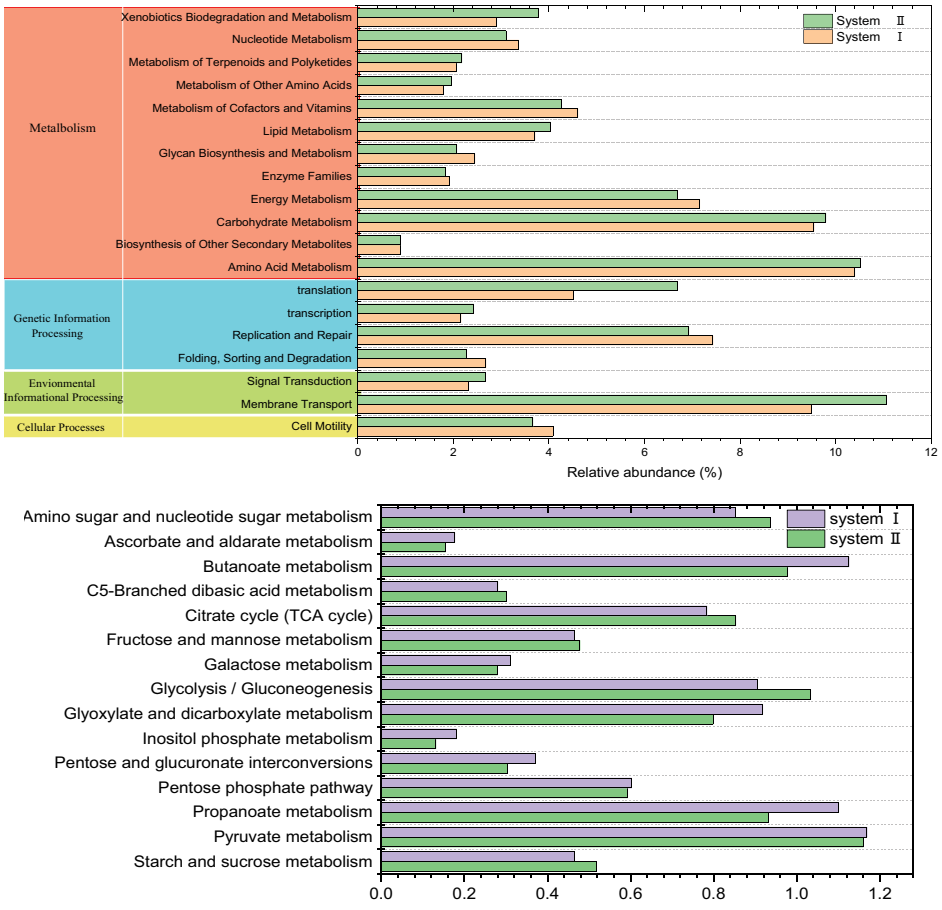

Fig. 4. Heatmap of number of genes annotated to the third level of Kyoto Encyclopedia of Genes and Genomes.

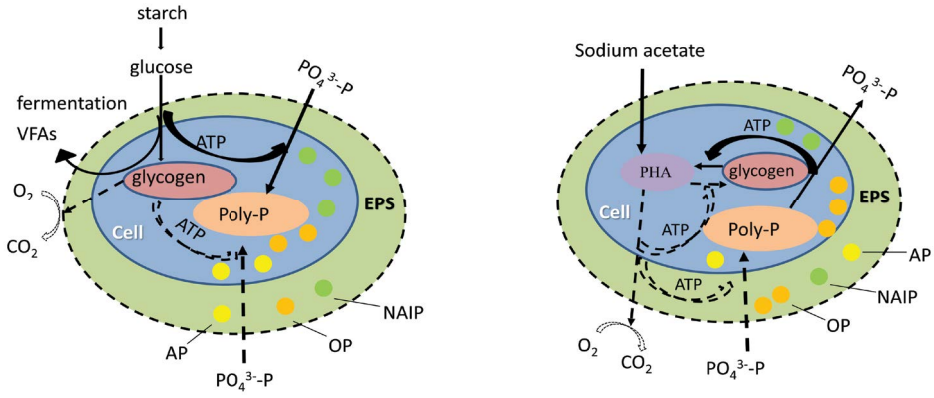

Fig. 5. Phosphorus metabolic mechanism of anoxic-aerobic nitrogen removal system.

microorganisms, so that ATP released by phosphorus hydrolysis could not be utilized. The energy of microbial metabolism and anoxic phosphorus absorption came from glycogen decomposition.

The phosphorous metabolism of starch as carbon source was different. First, starch in the anoxic phase was hydrolyzed to glucose by hydrolase before entering cells. Then, it was further fermented to produce volatile fatty acids, which was further utilized by other bacteria. The ATP produced by fermentation was used to absorb phosphorus, and the ATP produced by the aerobic decomposition of glycogen was also used to absorb phosphorus. Therefore, the whole anoxic-aerobic operation cycle presented phosphorus absorption state.

4. Conclusion

This study explored the influences of different carbon sources on the anoxic-aerobic process with respect to phosphorus removal mechanism. Efficient phosphorus removal was both achieved in the two systems. The phosphorus removal was accomplished mainly by aerobic phosphorus uptake of traditional PAOs in system I fed by sodium acetate, while the phosphorus removal was accomplished by anoxic phosphorus uptake of denitrifying phosphate accumulating organisms and fermentative PAOs in the system II fed by starch.

The type of carbon sources affected the structure and metabolism of microbial population. *unclassified_ Rhodocyclaceae* was the dominant genus for phosphorus removal in system I, while *Tetrasphaera* and *Opitutus* was the dominant genus in system II. Thus metabolic pathways of dominant and functional genera fed by different carbon sources resulted in obvious variations of phosphorus removal characteristics and phosphorus form distribution in bacterial cells and EPS.

The phosphorus metabolic model was established to gain an insight into the ideal metabolic strategy of the microbial community. This study proved the efficiency of the anoxic-aerobic process for biological phosphorus removal as an alternative system with higher ease of operation.

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