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Characterization of phosphorus removal bacteria in $(AO)^2$ SBR system by using different electron acceptors

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Abstract: Characteristics of phosphorus removal bacteria were investigated by using three different types of electron acceptors, as well as the positive role of nitrite in phosphorus removal process. An $(AO)^2$ SBR (anaerobic-aerobic-anoxic-aerobic sequencing batch reactor) was thereby employed to enrich denitrifying phosphorus removal bacteria for simultaneously removing phosphorus and nitrogen via anoxic phosphorus uptake. Ammonium oxidation was controlled at the first phase of the nitrification process. Nitrite-inhibition batch tests illustrated that nitrite was not an inhibitor to phosphorus uptake process, but served as an alternative electron acceptor to nitrate and oxygen if the concentration was under the inhibition level of 40mg $NO_2-N \cdot L^{-1}$. It implied that in addition to the two well-accepted groups of phosphorus removal bacterium (one can only utilize oxygen as electron acceptor, P_1, while the other can use both oxygen and nitrate as electron acceptor, P_2), a new group of phosphorus removal bacterium P_3, which could use oxygen, nitrate and nitrite as electron acceptor to take up phosphorus were identified in the test system. To understand $(AO)^2$ SBR sludge better, the relative population of the different bacteria in this system, plus another A/O SBR sludge (seed sludge) were respectively estimated by the phosphorus uptake batch tests with either oxygen or nitrate or nitrite as electron acceptor. The results demonstrated that phosphorus removal capability of $(AO)^2$ SBR sludge had a little degradation after A/O sludge was cultivated in the $(AO)^2$ mode over a long period of time. However, denitrifying phosphorus removal bacteria (P_2 and P_3) was significantly enriched showed by the relative population of the three types of bacteria, which implied that energy for aeration and COD consumption could be reduced in theory.

Key words: phosphorus removal bacteria; electron acceptor; nitrite; nitrate; oxygen; $(AO)^2$ SBR

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The enhanced biological phosphorus removal (EBPR) process is an economical and environmentally friendly technology for removing phosphorus from wastewater, where the phosphorus removal bacteria is the key functional organism [1]. According to the original consensus concerning phosphorus removal bacteria metabolism, intermediate products of biological nitrogen removal process (nitrite/nitrate) were detrimental to phosphorus release for their prior sequestration of substrate by non-polyP heterotrophs, and consequently reduced the availability of organics for phosphorus removal bacteria. The implied assumption is that phosphorus removal bacteria lacked the ability to denitrify and, hence could only grow and accumulate phosphate in aerobic conditions. However, recent studies illustrated that at least a fraction of phosphorus removal bacteria could accumulate phosphate under anoxic conditions, capable of utilizing either oxygen or nitrate as an electron acceptor (denitrifying phosphorus removal bacteria), where it performed a biological metabolism based on intracellular PHB and glycogen similar to aerobic phosphorus removal bacteria[2-4]. Such an efficient usage of the same organics for both nitrogen and phosphorus removal is significant since organics availability is often a limiting factor in EBPR processes in most countries [5].

On the theory, nitrite, the same as nitrate, can also play a role as an electron acceptor for denitrifying phosphorus removal bacteria. In fact, no clear answer regarding the effect of nitrite on anoxic phosphorus uptake has been reported in literatures [2,3,6]. Comeau et al. believed that anoxic phosphorus uptake did not occur with nitrite as electron acceptor, but in his research only one concentration (10mg $NO_2-N \cdot L^{-1}$) was examined [6]. Whereas, according to the studies of Meinhold et al. and Lee et al., low concentration of nitrite of 10mg $NO_2-N \cdot L^{-1}$ did not inhibit anoxic phosphorus uptake [2,3]. Therefore, it is interesting to investigate the role of nitrite in EBPR process.

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In this study, an anaerobic-aerobic-anoxic-aerobic SBR [$(AO)^2$SBR] for simultaneous phosphorus and nitrogen removal through anoxic phosphate uptake via nitrite was employed to enrich and investigate the phosphorus removal bacteria. During the experiment, ammonium oxidation was controlled at the first phase of nitrification (i. e., nitrosification) in aerobic phase to enhance the accumulation of nitrite and therefore induce the occurrence of denitrifying phosphorus removal bacteria using nitrite as electron acceptor to take up phosphorus.

1 Materials and Methods

1.1 Experimental Equipment

A cylindrical vessel with a 7 L effective volume was used for the $(AO)^2$ SBR which was operated in a fill-and-draw mode, comprised of 2 h anaerobic, 1.5 h aerobic, 2.5 h anoxic, 1 h aerobic and 1 h settling and fill/draw stages. In each operational cycle, 4 L of clarified supernatant was replaced by raw wastewater during the fill/draw stage. Sludge retention time (SRT) was maintained at 20 d by wasting mixed liquid at the end of aerobic stage. For achieving nitrosification in the aerobic phase, the temperature was controlled at 32 ℃ with a thermostat and pH 7.5 ~ 8.0 by addition of 0.5 N NaOH/0.5 N HCl solution. The seed activated sludge was taken from another lab-scale nutrient removal system, which employed an (A/O) SBR process (anaerobic/aerobic) and had a good phosphorus and nitrogen removal performance.

The test wastewater was collected from a septic tank in the 2nd campus of Harbin Institute of Technology (HIT) and its characteristics are summarized in Tab. 1.

Tab. 1 The quality of the test wastewater (mg/L)

	SS	COD	BOD_5	NH_3-N	TN	TP
Range	32 ~ 152	187 ~ 327	89 ~ 165	19.8 ~ 39.5	22.6 ~ 45.6	4.4 ~ 6.1
Mean	76	248	108	30.3	37.8	4.7

1.2 Experimental Procedure

1.2.1 Nitrite inhibition batch tests

Recent studies have verified that although nitrite may not be as good as nitrate in replacing oxygen as electron acceptor, it is feasible to use nitrite as electron acceptor so long as its concentration does not exceed the inhibition level. However, the level is always variable and dependent on the type of sludge and operation condition[2,3,7]. Here, sludge harvested from the $(AO)^2$ SBR was firstly kept anaerobic in a glass vessel for 3h phosphorus release after sufficient carbon source (HAc) was added. Upon completion of phosphorus release, the mixed liquid was washed with distilled water to exhaust the residual HAc in the bulk. In this manner, the disturbance of denitrification by heterotrophs on anoxic phosphorus uptake by poly - P organisms could be remarkably restrained. Subsequently, the sludge was divided into three identical parts and exposed respectively to nitrite with three different concentrations (20, 40, 60 mg $NO_2-N\cdot L^{-1}$). Nitrite was intermittently dosed to balance the consumed amounts with the extension of the tests.

1.2.2 Phosphorus uptake batch tests

The design of the phosphorus uptake batch tests was similar to that of nitrite inhibition ones. The different was that the three parts of P-released sludge were exposed to three different electron acceptors: nitrite (anoxic 1), nitrate (anoxic 2) and oxygen (aerobic). The amount of nitrite dosed in anoxic 1 was determined based on the results of nitrite inhibition batch tests, while nitrate was added according to Wachtmeister et al. [4]. As for aerobic conditions, dissolved oxygen concentration (DO) was kept upon 3 mg/L. With the process of the test, phosphorus uptake capacities and specific uptake rates were both measured.

1.3 Analytical Procedure

Water quality items were detected using the standard methods issued by the National Environmental Protection Agency (NEPA) of China. pH was measured using glass electrodes connected to a P^{H}S - 3C pH meter. DO was detected with a YSI (MODEL 50B) dissolved oxygen meter.

2 Results and Discussion

2.1 The Fate of Nitrite and Phosphate in $(AO)^2$ SBR

Fig. 1 shows the change of PO_4-P, NO_3-N and NO_2-N concentrations in the effluent along with PO_4-P concentration at the end of anaerobic and anoxic phases during the operation period of SBR. As seen from the figure, the concentration of PO_4-P at the end of the anoxic stage (PO_4-P_{anoxic}) decreased step by step, with a corresponding declination of $PO_4-P_{efflu.}$ and $NO_2-N_{efflu.}$, which indicated a gradual enhancement of denitrifying phosphorus removal bacteria with the prolongation of the experiment.

In Fig. 2, typical profiles of NO_2-N, NO_3-N, PO_4-P, NH_3-N and COD concentrations in an operational cycle under the pseudo-steady state were presented. In the anaerobic phase, phosphate was released at the cost of COD consumption and was taken up luxuriously by phosphorus removal bacteria in the subsequent aerobic phase, which was well consistent with the wide-accepted theory of its metabolism. Meanwhile, it is interesting to note that the decrease of phosphate continued in the subsequent anoxic phase accompanied by a reduction of nitrite concentration, but the curve assumed a relatively lower rate. It implied that nitrite virtually acted as an electron acceptor during anoxic phosphate uptake process instead of nitrate and oxygen. Hu et al. believed that the kinetics behaviors of phosphate release and uptake under anoxic conditions were determined by the available organics[9]. When short-chain fatty acids were not available, nitrate/nitrite was not preferred to be as electron acceptor for non-polyP heterotrophs but for poly-P organisms to perform anoxic phosphate uptake. Accumulated nitrite from partial nitrification process (10 mg $NO_2-N \cdot L^{-1}$) and low amounts of the available organics (30 mg COD $\cdot L^{-1}$, mostly hard - degraded) at the beginning of anoxic phase were postulated beneficial for denitrifying phosphorus removal bacteria to utilize nitrite to generate energy for anoxic growth and phosphate uptake.

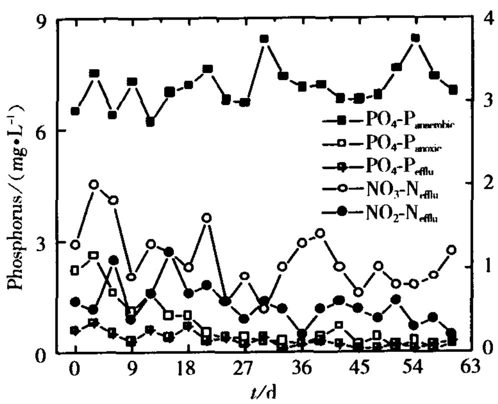

Fig. 1 Daily variation of P and N in the effluent and P at the end of anaerobic and anoxic phases

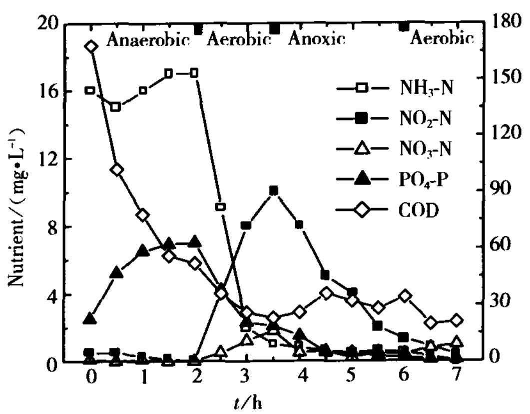

Fig. 2 Typical profiles of COD, PO_4-P, NH_3-N, NO_2-N and NO_3-N in the $(AO)^2$ SBR

It thereby affirmed the existence of a new group of phosphorus removal bacteria in the system, which could take up phosphorus using nitrite as an electron acceptor besides nitrate and oxygen. That is to say, three groups of phosphorus removal bacteria together contributed to the total phosphorus removal in the $(AO)^2$ SBR system. They were (1) those capable of utilizing only oxygen as electron acceptor, P_1; (2) those capable of utilizing both oxygen and nitrate as electron acceptor, P_2; (3) those capable of utilizing oxygen, nitrate and nitrite as electron acceptor, P_3. The details about the relative contribution is further elucidated in the following section.

2.2 Inhibition Effect of Nitrite on Phosphorus Uptake

According to the previous section, nitrite-inhibition batch tests with different concentrations (20, 40, 60 mg $NO_2-N \cdot L^{-1}$) were carried out to determine the inhibition level of nitrite on the $(AO)^2$ SBR sludge. As shown in Fig. 2, the profiles of phosphorus declined at the same rate when nitrite concentration was 20 mg $N \cdot L^{-1}$ and 40 mg $N \cdot L^{-1}$. Whereas, 60 mg $N \cdot L^{-1}$ presented a noticeable inhibition effect, as indicated by the much smaller slope of the phosphorus profile. It was thereby drawn that at least 40 mg $NO_2-N \cdot L^{-1}$ was not detrimental to anoxic phosphorus uptake, and in comparison with other reported inhibition levels it was a much broader range [2,3], which was possibly relevant to the operational condition of the $(AO)^2$ SBR process. The sludge in this system was periodically exposed to a rather higher nitrite concentration that was formed by nitritation process, and it consequently induced an enrichment of the specific denitrifying phosphorus removal bacteria (or enzyme) that could denitrify nitrite at a rather high concentration. Owing to the fact, one may conclude that nitrite would not inhibit biological phosphorus removal in domestic sewage treatment plants, where nitrite concentration is typically much lower than 40 mg $NO_2-N \cdot L^{-1}$.

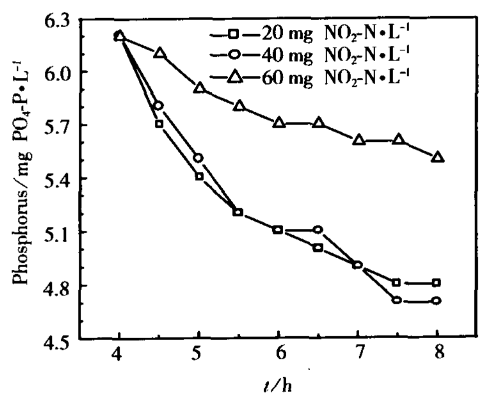

Fig. 3 Nitrite inhibition batch tests (VSS = 2.1 g $\cdot L^{-1}$)

2.3 Phosphorus Uptake Batch Tests

For characterizing the different types of phosphorus removal bacteria, three different electron acceptors of nitrite, nitrate and oxygen were applied to take up phosphorus in separate reactors. The initial concentrations of nitrite and nitrate were 40 mg $NO_2-N \cdot L^{-1}$ and 35mg $NO_3-N \cdot L^{-1}$ respectively based on the results of nitrite inhibition tests and Wachtmeister et al.[4]. The results are shown in Fig. 4, based on which, total phosphorus uptake capacity and maximum specific phosphorus uptake rate were calculated as well, as listed in Tab. 2. It was found that sludge had the highest total phosphorus uptake capacity and most fast specific phosphorus uptake rate under aerobic conditions, meanwhile the least uptake amounts and uptake rate occurred under anoxic 1 conditions. It was owing to the fact that oxygen was a common electron acceptor for P_1, P_2 and P_3. Therefore, aeration is an appropriate way for phosphorus uptake in terms of phosphorus uptake efficiency. However, a few publications have revealed that much more carbohydrate would be synthe-sized when oxygen as an electron acceptor than when chemically bond oxygen as the acceptor, and this in turn deteriorated the removal of phosphorus [1,2,8]. In viewpoint of this, denitrifying phosphorus removal bacteria takes the advantage over the aerobic one, and attracts the interest of environmental researchers.

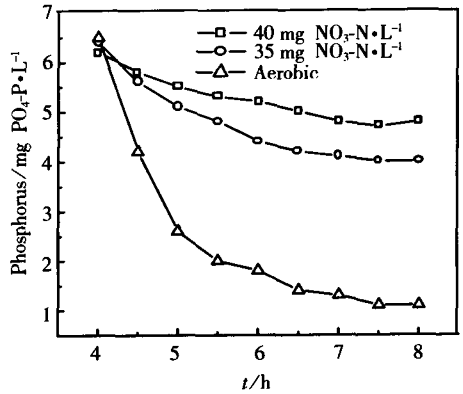

Fig. 4 Phosphorus uptake batch tests with different electron acceptors (VSS = 2.1 g · L^{-1})

Tab. 2 Summary of parameters in the phosphorus uptake tests

	Anoxic 1 (nitrite)		Anoxic 2 (nitrate)		Aerobic (oxygen)	
	$(AO)^2$SBR sludge	A/O SBR sludge	$(AO)^2$SBR sludge	A/O SBR sludge	$(AO)^2$SBR sludge	A/O SBR sludge
Concentration of electron acceptors/mg $NO_x-N \cdot L^{-1}$	40	10	35	35	-	-
Total phosphorus uptake/mg P · (gVSS)$^{-1}$	0.8	0	1.3	0.6	2.8	4.2
Maxim phosphorus uptake rate/mg P · (gVSS · h)$^{-1}$	0.4	-	0.8	-	2.0	-

Through the above illustration, three types of phosphorus removal bacteria were present in $(AO)^2$ SBR system, but the relative contribution of P_1, P_2 and P_3 to total phosphorus removal is still to be evaluated. Hu et al. announced that it could be estimated by comparing the total phosphorus uptake capacity under anoxic 1, anoxic 2 and aerobic conditions [7]. The detailed calculations are expressed as follows:

$$P_1/P(\%) = (M_1 - M_2)/M_1 \times 100\%,$$
$$P_2/P(\%) = (M_2 - M_3)/M_1 \times 100\%,$$
$$P_3/P(\%) = M_3/M_1 \times 100\% = 1 - (P_1/P + P_2/P).$$

Where P is the total phosphorus removal population, M_1 is the total amount of phosphorus uptake with oxygen (mg P/g VSS), M_2 and M_3 are that with nitrate (anoxic 2) and nitrite (anoxic 1) in respect.

The results after calculation are tabulated in Tab. 3. From the table, it was found that P_1 was the predominant group of phosphorus removal bactria in the $(AO)^2$ SBR sludge, which occupied over a half of the total populations. While P_3 also play an important role in phosphorus removal of the system, and the ratio of P_3/P was near to 30%, which in theory and practice upgraded the simultaneous removal of phosphorus under the conditions of nitrite accumulation in the system. On the other hand, the values of P_3/P obtained by this characterization method were generally 14 ~ 18% lower than those obtained by using nitrate alone as electron acceptor (the method of Wachtmeister et al. [4]). This observation suggested that using nitrite could provide a more realistic estimation of P_3 population by excluding prokaryotes (P_2) as denitrifying phosphorus removal bacteria.

Tab. 3 Percentages of P_1, P_2 and P_3 vs. total phosphorus removal population

P_1/P (%)		P_2/P (%)		P_3/P (%)	
$(AO)^2$ SBR sludge	A/O SBR sludge	$(AO)^2$ SBR sludge	A/O SBR sludge	$(AO)^2$ SBR sludge	A/O SBR sludge
54	86	18	14	28	0

To understand the characteristic of $(AO)^2$ SBR sludge better, similar batch tests were conducted with the seed activated sludge (A/O sludge). Knowing the existence of a ceiling nitrite concentration, a relatively low nitrite amount was used in the anoxic 1 batch test (see Tab. 2) to avoid the influence of nitrite inhibition. The results are also summarized in Tabs. 2 and 3. As seen from Tab. 2, $(AO)^2$ SBR and A/O SBR sludge had much different phosphorus uptake capacities under aerobic conditions, and it meant that the capability of phosphorus removal had a little degradation after the A/O sludge was cultivated in the $(AO)^2$ mode over a long period of time. However, the decrease capability seemed to be recovered under anoxic conditions since phosphorus could be taken up by P_2 and P_3 in $(AO)^2$ SBR sludge. From Tab. 3, P_2 combined with P_3 (denitrifying phosphorus removal bacteria) played an equal role in removing phosphorus in relation to P_1 (aerobic phosphorus removal bacteria), which was indicated by the ratio of $(P_2 + P_3)/P_1$ (0.85). However, with regard to A/O SBR sludge, there was no or little P_3 or P_2, and as a result, only two groups of phosphorus removal bacteria ascribed the phosphorus removal in the system. It was therefore presumed that the differences between the two types of sludge probably came from the different operation conditions. In general, the merit of having a long term $(AO)^2$ cultivation environment could attain a higher percentage of P_2 and P_3 in the system, and it hence led to more savings in terms of energy for aeration, COD supply and less sludge disposal [5].

3 Conclusions

1) Nitrite was an alternative electron acceptor to nitrate or oxygen for denitrifying phosphorus removal bacteria to take up phosphorus, if nitrite concentration was below its inhibition level. As for the $(AO)^2$ SBR sludge, the inhibition concentration was at least 40mg in terms of nitrite nitrogen.

2) The coexistence of three groups of phosphorus removal bacteria in $(AO)^2$ SBR system was affirmed by phosphorus uptake batch tests: one was capable of utilizing only oxygen as electron acceptor (P_1), the second was capable of utilizing both oxygen and nitrate as electron acceptors (P_2), and the third was capable of utilizing oxygen, nitrate and nitrite as electron acceptors (P_3).

3) The relative populations of the three groups of phosphorus removal bacteria were quantified by using different electron acceptors. By comparison between $(AO)^2$ SBR sludge and A/O SBR sludge, it was found that there was no or little P_3 or P_2 bacteria (denitrifying phosphorus removal bacteria) in A/O SBR sludge, whereas denitrifying phosphorus removal bacteria in $(AO)^2$ SBR system played the same role in phosphorus removal as the aerobic phosphorus removal bacteria did. It suggested that energy for aeration and COD could be saved up by cultivating sludge in an $(AO)^2$ SBR system.

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