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BIOTECHNOLOGY and BIOENGINEERING

Inducing Mechanism of Biological Phosphorus Removal Driven by the Aerobic/Extended-Idle Regime

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ABSTRACT: Recently, it was found that excess phosphorus (Pi) removal could be achieved in activated sludge with an aerobic/extended-idle (AEI) process. In this study, batch tests were performed to further reveal the inducing mechanism of Pi removal involved in the AEI process. Unlike the classical anaerobic/aerobic process where an anaerobic Pi release along with a significant polyhydroxyalkanoate (PHA) accumulation drives polyphosphate (poly-P) accumulating organisms (PAOs) to over-store Pi as poly-P, an idle Pi release accompanied by a low-idle PHA production, which is usually considered to be detrimental for biological Pi removal, was observed to induce some cells to effectively uptake Pi in excess of metabolic requirement in the AEI process. With the increase of idle Pi release, Pi removal efficiency linearly increased. The results also showed that a long idle period with a low level of intracellular glycogen could significantly increase Pi release contents, thus remarkably enhancing Pi removal performances. Fluorescence in situ hybridization analysis further revealed that activated sludge in the AEI process contained 37.6% of *Accumulibacter* (PAOs) and 28.2% of *Competibacter* and *Defluviicoccus*-related organisms (glycogen accumulating organisms). This study revealed an actually existent, yet previously unrecognized, inducing mechanism of poly-P accumulation, and this mechanism behind the AEI regime may provide a scientific basis for the development of an alternative strategy for Pi removal from wastewaters.

Biotechnol. Bioeng. 2012;109: 2798–2807.

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Contract grant sponsor: National Natural Science Foundation of China
Contract grant number: 51078128
Contract grant sponsor: Hunan Provincial Innovation Foundation for Postgraduate
Contract grant number: CX2009B079
Contract grant sponsor: China Postdoctoral Science Foundation
Contract grant number: 2012M510888
Contract grant sponsor: Zhejiang Provincial Natural Science Foundation of China
Contract grant number: Q12E080007
Additional supporting information may be found in the online version of this article.
Received 22 February 2012; Revision received 15 April 2012; Accepted 26 April 2012
Accepted manuscript online 7 May 2012;
Article first published online 16 May 2012 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.24543/abstract)
DOI 10.1002/bit.24543

KEYWORDS: biological phosphorus removal; polyphosphate; DAPI staining; polyphosphate accumulating organisms; aerobic/extended-idle regime

Introduction

Excessive phosphorus (Pi) discharged from municipal sewages and industrial wastewaters is one key reason for eutrophication, thus efficient and reliable methods of Pi removal are required urgently to reduce Pi levels in freshwater ecosystems. Classical enhanced biological phosphorus removal (EBPR) technology [i.e., anaerobic/oxic (A/O) process], which is based on the principle that the biomass needs to be repeatedly recycled through alternating anaerobic and aerobic stages (Ahn et al., 2007), is widely applied in full-scale wastewater treatment plants. Such a process is economical in the long-term and has a low environmental impact when successfully operated (Martín et al., 2006). However, the stability and reliability of A/O process have still been a problem by far. Even operated under seemingly favorable operational conditions, deteriorations and even failures in Pi removal performances have been widely observed due to external disturbances, such as high rainfall, excessive nitrate loading to the anaerobic reactor, or nutrient limitation (Oehmen et al., 2007), causing violations to discharge regulations. Especially in some volatile fatty acids (VFAs) insufficiency regions, such as South China, EBPR systems are more prone to failures (Crocetti et al., 2002; Mullan et al., 2006; Wong et al., 2004; Zhang and Chen, 2009). Consequently, periodic organic matter supplementation and/or chemical precipitant addition may be required to attain compliance in those cases, increasing operational costs.

Recently, it was reported that biological Pi removal could be achieved in a conventional-activated sludge (CAS) system using both glucose and acetate as the sole carbon source if the idle period is extended suitably (Wang et al., 2008, 2009a,b). Here we defined this operation as the aerobic/

extended-idle (AEI) regime. Compared with the conventional EBPR process, a strict anaerobic period was not conducted whereas an extended-idle zone (210–450 min) was operated between the decanting and the next aerobic phases. Although the extended-idle zone was also not aerated which caused this period to be anaerobic (Wang et al., 2008), mixture stirring did not require to be conducted during this phase, showing that the AEI process is simpler than the A/O process. Further, the cycle investigations showed that the external substrate deficiency idle zone seemed not to serve as the conventional anaerobic period where external carbon source was present. The facts suggested that a significant, yet previously unrecognized Pi removal mechanism might exist in the AEI regime, and this regime might provide an alternative approach to Pi removal. Nevertheless, our previous studies merely verified the existence of the novel phenomenon of Pi removal and proposed the probable relation between Pi uptake and energy storage formations. As yet, the inducing principle of polyphosphate (poly-P) accumulation in the AEI process has not been well comprehended. Therefore, if this process is used as a supplementary method to remove Pi in full-scale wastewater treatment plants, the following problems should be solved: How to stably induce a high Pi removal efficiency? What's the driving force of poly-P accumulating organisms (PAOs) to uptake excess Pi? Why can poly-P accumulation be achieved in such a process?

Since the AEI process seems to be different from the A/O process and PAOs involved in such processes have not been identified before, an anaerobic Pi release experiment with VFA addition and fluorescence in situ hybridization (FISH) analysis were first combined to identify whether poly-P containing cells cultured in the AEI regime showed a conventional PAO phenotype. Then, the effect of the idle time on Pi removal performance was explored, because the idle time configuration was the only seeming difference between the AEI and the CAS processes. To enhance wastewater removal capability, the CAS processes usually conduct a very short idle period (e.g., 30 min) and often have poor levels of Pi removal. In addition, it is known that biological phosphorus (P) removal is related to the transformations of intracellular polyhydroxyalkanoate (PHA) and glycogen (Mino et al., 1998). We further investigated the effects of PHA and glycogen levels and their periodic accumulations/degradations on Pi removal, especially examined the relationships among idle PHA/glycogen transformations, idle Pi release, and aerobic poly-P accumulation. Finally, the inducing metabolism of poly-P accumulation in the AEI regime was concluded.

Materials and Methods

Parent Sequencing Batch Reactor (SBR) Operation

Seed sludge was inoculated into a sequencing batch reactor (SBR) with a working volume of 60 L. The SBR was fed with acetate as the sole carbon source because it was the most common VFAs present in real septic domestic wastewaters (Chen et al., 2004). SBR cycle consisted of approximately 240-min aerobic period, 28-min settling, 2-min decanting, and 450-min idle periods. Forty liters of supernatant was discharged after settling period, and was replaced with 40-L synthetic medium (contained 500 mg $CH_3COONa\,L^{-1}$, 15 mg Pi L^{-1}, 120 mg $NH_4Cl\,L^{-1}$, 10 mg $MgSO_4{\cdot}7H_2O\,L^{-1}$, 5 mg $CaCl_2\,L^{-1}$, and 0.5 mL L^{-1} of a trace metals solution described in our previous publication (Wang et al., 2009b)) during the first 2 min of aerobic period. The dissolved oxygen (DO) and pH were controlled at 3 ± 0.2 mg L^{-1} and 7–8 during aerobic period. The sludge retention time (SRT) was maintained at approximately 20 days by withdrawing 3 L of the sludge mixture (once per day) from the reactor at the end of the aerobic period but before settling. Mixture stirring did not perform in the idle period of the parent SBR all the time.

Batch Experiments

Three types of batch experiments were carried out, and the common methods and materials were described below.

All batch experiments were carried out in identical reactors with working volumes of 1.5 L each. Seed sludge was withdrawn at the end of aerobiosis but before settling from the parent reactor. The compositions of synthetic media and operations in all reactors, unless otherwise described, were the same as those depicted in the parent reactor. When effluent Pi concentration reached stable levels, cycle studies were performed once per week to monitor the changes of PHA, glycogen, and Pi concentrations and to investigate the relationships among them. To sample conveniently, each of the reactors was mixed with a magnetic stirrer (150 rpm) except during settling and decanting periods when cycle studies were performed.

Anaerobic Pi Release Experiment With VFA Addition and Subsequent Aerobic Pi Uptake Experiment

To examine whether poly-P containing cells cultured in the AEI regime showed a conventional PAO phenotype, an anaerobic Pi release experiment with VFA addition as well as a subsequent aerobic Pi uptake experiment was conducted. Two reactors were operated. One received 1.5-L sludge sample withdrawn from the parent SBR, the other received 1.5-L activated sludge [with similar mix liquor volatile suspended solids (MLVSS) concentration] withdrawn at the end of aerobiosis from a parent A/O reactor which was operated in our laboratory, and set as a control. The parent A/O reactor cycle consisted of approximately a 120 min anaerobic period, a 180-min aerobic period, a 55-min settling period, 5-min decanting, and 120-min idle phases. The pH and DO during the aerobic period in the parent A/O reactor were the same as the parent AEI reactor, but the SRT was approximately maintained at 14 days. Both samples

were centrifuged (5,000 rpm for 5 min) and washed three times with tap water to remove the residual nitrate, PO_4-P and COD, and then resuspended in tap water with a final volume of 0.5 L. Both reactors were operated as the A/O regime which consisted of a 2-h anaerobic period, followed by a 3-h aerobic period according to the literatures (Oehmen et al., 2005; Tong and Chen, 2007). One liter of synthetic medium (the same as described above) was added into both reactors at the beginning of the anaerobic period. The experiment was conducted in triplicate.

Effect of Idle Time Configuration on Pi Removal Efficiency

To understand more clearly how idle time affected Pi removal, a batch experiment with 10 reactors was performed. Ten sludge samples (1.5 L each) from the parent SBR were transferred to 10 reactors, and the reactors were divided into two groups. Group one (reactors 1–5) was operated under 7.8 mM C [250 mg chemical oxygen demand (COD) L^{-1}] of influent acetate concentration, while group two (reactors 6–10) was conducted under 15.61 mM C (500 mg COD L^{-1}) of influent acetate concentration. From reactors 1 (6) to 5 (10), idle time was operated as 1.5, 3.5, 5.5, 7.5, and 9.5 h, respectively.

Effects of PHA and Glycogen Levels and Their Periodic Transformations on Pi Removal

External acetate concentrations can affect the levels of PHA and glycogen under the same operations. To explore the effects of PHA and glycogen levels and their periodic accumulations/degradations on Pi removal efficiency, we conducted a batch experiment with different influent acetate concentrations. Five reactors were operated, and a total of 7.5 L of activated sludge from the parent reactor was divided equally into five reactors as seed sludge. Influent acetate concentration in these reactors was as follows: 6.34 mM C (203 mg COD L^{-1}), 12.44 mM C (398 mg COD L^{-1}), 18.66 thinsp;mM C (597 mg COD L^{-1}), 24.88 mM C (796 mg COD L^{-1}), and 31.22 mM C (999 mg COD L^{-1}).

To further verify the effects of PHA and glycogen levels (especially, idle PHA and glycogen levels) on Pi removal performance and to eradicate potential difference due to acetate concentration, another batch test with different aerobic lengths was conducted because different aeration times could significantly influence the levels of idle initial PHA and glycogen under the same external substrate concentration. A total of 7.5 L of activated sludge mixture taken from the parent SBR was divided equally into five reactors, and influent acetate concentration in the five reactors was equal (28 mM C, 898 mg COD L^{-1}). For each cycle, the five reactors were aerated for 4, 5, 6, 7, and 8 h, respectively.

Analytical Methods

Sludge samples from the reactors were immediately filtered through a Whatmann GF/C glass microfiber filter (1.2 μm). The filtrate was analyzed for total phosphorus (TP), total organic carbon (TOC), and the filter was assayed for mix liquor suspended solids (MLSS), MLVSS, glycogen, PHA.

TP, MLVSS, and MLSS were measured according to Standard Methods (APHA, 1998), and TOC was determined using a TOC analyzer (ShimadzuTOC-500, Tokyo, Japan). The measurements of sludge glycogen, poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV), and poly-3-hydroxy-2-methylvalerate (PH2MV) were the same as described in our previous publication (Wang et al., 2009b). The total PHA was calculated as the sum of measured PHB, PHV, and PH2MV.

Intracellular poly-P granules were visualized by either Neisser staining or using the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI; Mullan et al., 2006). DAPI, which was used in this study, stains poly-P emitting a distinct bright white fluorescence (Liu et al., 2001). The stained samples were examined using a confocal scanning laser microscope (FV 500). For estimation of all the abundance of PAOs in sludge samples, visible cell counts of total and poly-P positive cells were taken, as previously used by Mullan et al. (2006). Cell counts were arithmetically averaged from five random fields at each test.

FISH analyses were the same as described by Nielsen et al. (1999) and Liu et al. (2001) with minor revisions. Sludge samples (taken from the reactors at the end of aerobic zone during steady operation period) were fixed in 4% formaldehyde for 20 h at 4°C, and then subjected to freeze-thaw treatment [five freeze (−80°C) and thaw (60°C) cycles] to improve the penetration of oligonucleotide probes according to the literatures (Liu et al., 2001; Sekiguchi et al., 1999). Cells were attached to poly-L-lysine coated slides and dehydrated by sequential washes in 50%, 80%, and 100% ethanol (3-min each). The hybridization and washing procedures were performed according to the method of Nielsen et al. (1999). Briefly, 20 μL of hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl (pH 7.2), 0.01% sodium dodecyl sulfate, 35% deionized formamide and 0.2 ng probes) was hybridized with the fixed samples, and then the slides were incubated at 46°C for 2 h, followed by a washing step at 48°C for 20 min in a washing buffer [20 mM Tris–HCl (pH 7.2), 70 mM NaCl, 5 mM EDTA and 0.01% SDS]. The washing buffer was removed by rinsing with sterile water and the slide was dried in air. The following oligonucleotide probes, EUBmix labeled with 5′FTTC (containing EUB338-I, EUB338-II, and EUB338-III, specific for most Bacteria), PAOmix labeled with 5′AMCA (containing PAO462, PAO651, and PAO846, specific for *Accumulibacter*), and GAOmix labeled with 5′Cy3 (containing GAOQ431, GAOQ989, and GB_G2, specific for *Candidatus Competibacter phosphates*; TFO-DF218, TFO-DF618, DF988, DF1020, specific for "*Defluviicoccus*"-related organisms) were used for hybridization and listed in

Supporting Information (SI) Table S1. For quantitative FISH analysis, at least 20 microscopic fields were analyzed for the hybridization of individual probes using a confocal scanning laser microscope (FV 500) with image database software (VideoTesT Album3.0). Each was expressed as a percentage of the total area fluorescing with the EUBmix probes.

Results

Performance of the Parent SBR

Effluent TP concentration reached stable level after 23 days operation. The data obtained in a cycle study (on day 46) during stable operation before the batch experiments was shown in Figure 1. The 0.68 mg L^{-1} of ammonia, 0.32 mg L^{-1} of nitrite, and 9.26 mg L^{-1} of nitrate were measured in the effluent, which implied that 67.5% of total nitrogen was removed. The results suggested aerobic denitrification occurred in the parent reactor. In the literature, some specific bacteria (e.g., *Nitrosospira* sp.) were also found to achieve denitrification under aerobic period with plenteous DO concentration (Shaw et al., 2006). Also from Figure 1, 2.86 mg L^{-1} of TP concentration was monitored at the end of the aeration, which indicated that about 32 mg COD was required to remove 1 mg Pi and 80.9% of TP removal efficiency was achieved. This Pi removal performance was comparable with the published data that acquired in the A/O process, also employing acetate as the sole carbon source (Oehmen et al., 2007; Tong and Chen, 2007). For example, 35 mg COD was consumed to remove 1 mg Pi in the A/O process by Tong and Chen (2007). DAPI staining revealed that the activated sludge

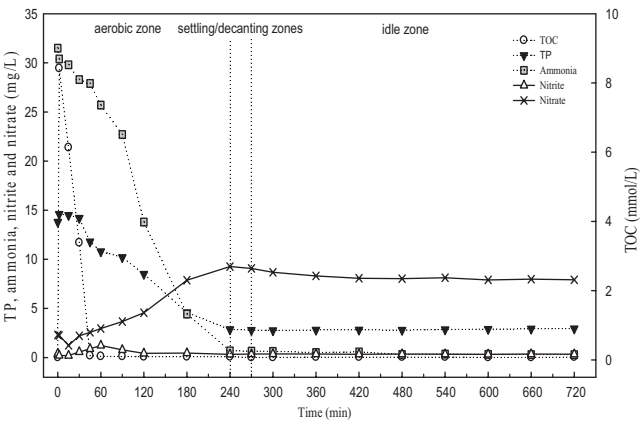

Figure 1. Variations of TP, TOC, ammonia, nitrite, and nitrate during one cycle in the parent SBR (day 46). Forty liters of synthetic wastewaters was fed to SBR during minutes 0–2, and data measured at minute 0 represented the values, which were obtained at the end of the last idle period. Here, the reactor was not mixed during the idle zone when cyclic studies were performed.

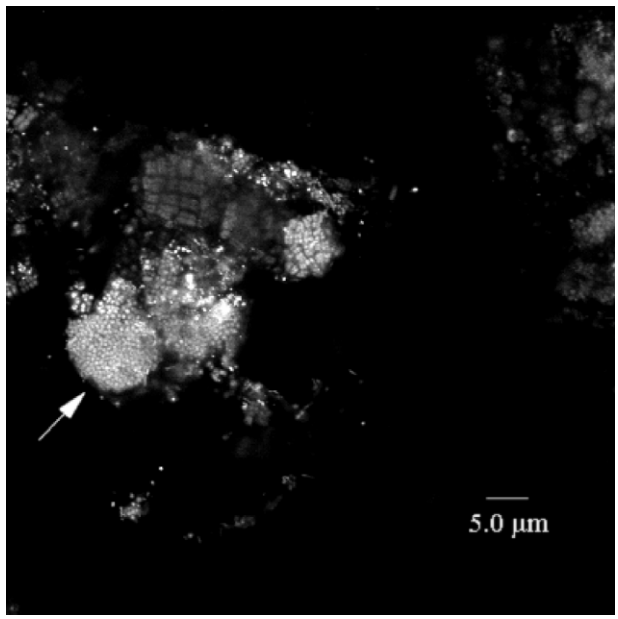

Figure 2. Micrographs of DAPI staining of the sludge samples taken from the parent reactor (sludge sample was obtained on day 46). Poly-P inclusions were found to be bright white with DAPI staining.

biomass contained a large number of cells with visible poly-P inclusions (43.6%, Fig. 2), further confirming the existence of the phenomenon of biological Pi removal in the parent SBR. The reasons for the AEI regime driving a considerable Pi removal efficiency would be discussed in the following text.

Poly-P Containing Cells Involved in the Parent SBR

An anaerobic Pi release experiment with VFA addition as well as a subsequent aerobic Pi uptake experiment using the sludge withdrawn from the parent SBR was carried out to examine whether poly-P containing cells cultured in the AEI regime showed a conventional PAO phenotype, and the results were compared with the data obtained in A/O cultured systems (this study and previous studies, Table I). Though the anaerobic and aerobic transformations detected in the AEI-sludge reactor were lower than those in the A/O-sludge reactor (this study) and previous studies, anaerobic Pi release coupled with PHAs accumulation and glycogen degradation occurred clearly. During the following aerobic phase, Pi uptake, PHAs utilization, and glycogen replenishment also took place concurrently. This behavior was very similar to the classical phenotype of conventional PAOs. FISH analysis further revealed that the parent SBR biomass contained plenty of *Accumulibacter* (PAOs) as well as *Competibacter*- and *Defluviicoccus*-related organisms (glycogen accumulating organisms, GAOs), which accounted for 37.6% and 28.2% of all bacteria in the sludge, respectively (Fig. 3). The composition of the remainder of

Table I. Comparison of the anaerobic and aerobic transformations between AEI cultured sludge with anaerobic VFA addition and A/O cultured sludge (this study and previous studies).

	Anaerobic transformations			Aerobic transformations	
Refs.	PHA-up/VFA (C-mol/C-mol)	Gly-de/VFA (C-mol/C-mol)	P-rel/VFA (P-mol/C-mol)	Gly-syn (mmol-C/g VSS)	P uptake rate (mmol-P/g VSS h)
AEI-sludge[a]	0.59 ± 0.05	0.19 ± 0.04	0.32 ± 0.05	1.03 ± 0.07	0.31 ± 0.04
A/O-sludge[a]	1.27 ± 0.06	0.44 ± 0.05	0.46 ± 0.07	1.77 ± 0.11	0.36 ± 0.04
Pijuan et al. (2009)	1.28	0.63	0.319	—	—
Oehmen et al. (2005)	1.22	0.33	0.42	—	—
Smolders et al. (1994)	1.22	—	0.50	—	—

[a]Data reported in this study are the averages and their standard deviations of triplicate studies.

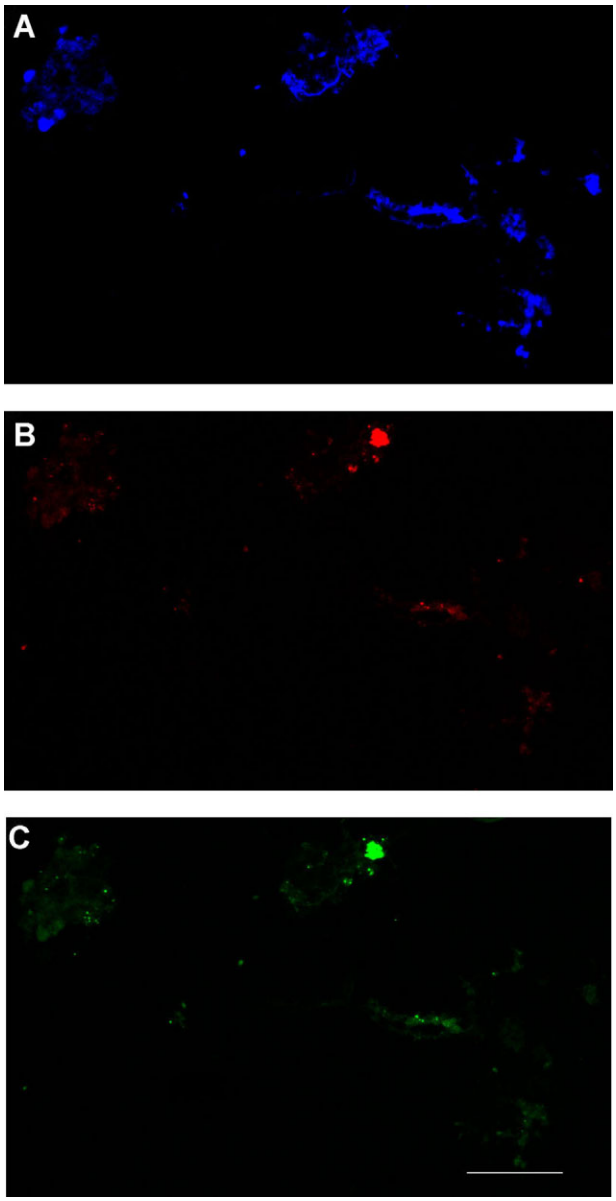

Figure 3. FISH micrographs of activated sludge (sample was obtained on day 46) hybridizing with PAOmix (blue, **A**), GAOmix (red, **B**), and EUBmix (green, **C**) probes specific for *Accumulibacter* (PAOs), *Competibacter*- and *Defluviicoccus*-related organisms (GAOs), and the dominant bacteria, respectively. Bar = 100 μm.

cells in the sludge is not well understood at present and ordinary heterotrophs and a small number of other PAOs and GAOs may be presented. The results showed that the conventional PAOs could thrive in the AEI process, and a defined anaerobic zone was not necessarily required for putative PAOs growth. In fact, other researchers have found putative PAOs can grow under a wide variety of environments (Ahn et al., 2007; Peterson et al., 2008; Silva et al., 2012).

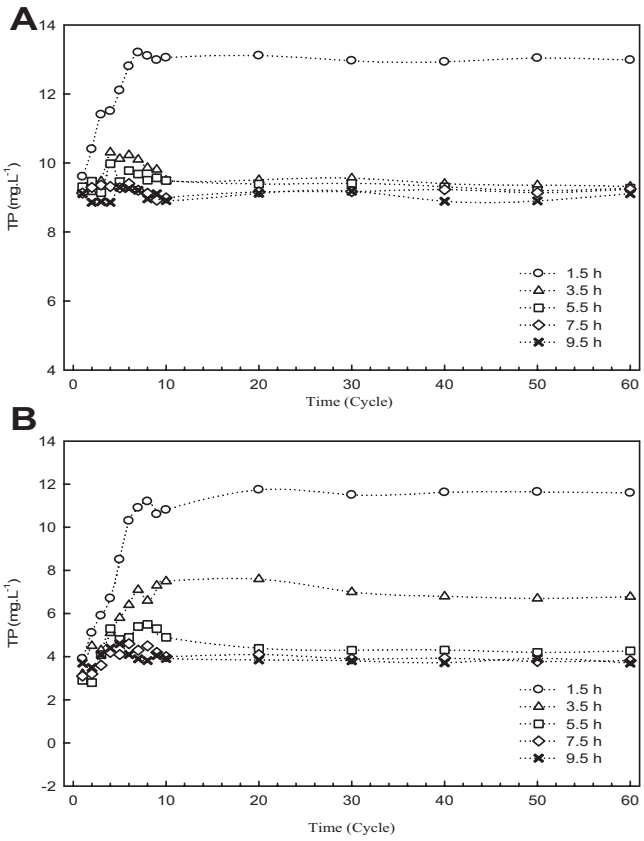

Figure 4. Effluent TP concentrations in the reactors under different idle times. **A**: 250 mg COD L^{-1} fed group; **B**: 500 mg COD L^{-1} fed group.

Effect of Idle Time Configuration on Pi Removal

Ten reactors were working in steady-state approximately after 30 cycles, displaying the effect of idle time on effluent TP concentration (Fig. 4). In both 250 and 500 mg COD L^{-1} fed groups, effluent TP concentration decreased obviously with the prolonging of the idle time, but inconspicuous decrease of effluent TP was monitored after 3.5 h (or 5.5 h) idle time in 250 (or 500) mg COD L^{-1} fed group. The cyclic changes of TOC, glycogen and PHA in the reactors with the shortest (1.5 h) and the longest (9.5 h) idle times under both levels of COD concentration were measured to obtain a deep understanding (SI Fig. S1). In 250 mg COD L^{-1} fed group, acetate was fully taken up during the feast. PHA and glycogen were accumulated meanwhile, but mainly degraded during the subsequent famine. Accordingly, negligible variations of PHA and glycogen were observed during the final idle period. In 500 mg COD L^{-1} fed group, PHA and glycogen were firstly synthesized, and then the accumulated PHA was degraded completely during the aerobic period. A fraction of the accumulated glycogen was consumed during the aerobic period while the remaining was utilized during the idle period. Simultaneously, a small amount of PHA formation (mostly measured as PHV, data not shown) was detected along with glycogen degradation, probably due to the transformation from glycogen to PHV. Lopez et al. (2006) and Lu et al. (2007) observed that glycogen could convert into PHV under long-time anaerobic starvation.

To exhibit in detail the influence of idle time configuration on Pi removal, Table II summarized the aerobic and idle transformations of TP, PHA, and glycogen in 10 reactors. In 250 mg COD L^{-1} fed group, 9.5-h idle time-operated reactor had the highest Pi removal performance. A slight decrease of Pi removal coupled with a simultaneously mild reduction of idle TP release was observed along with the idle time shortening except for 1.5-h idle period-operated reactor. Although $37.1 \pm 0.5\%$ of TP removal efficiency was only detected in the reactor with 3.5-h idle period, 3.16 ± 0.18 mg of TP removed per gram of MLVSS was measured, suggesting a considerable Pi removal performance. DAPI staining of the sludge samples taken from the 3.5-h idle time-operated reactor showed that the biomass contained an abundance of cells (32.6%) with visible poly-P inclusions (SI Fig. S2), further confirming this point. However, in the reactor with 1.5-h idle time, the Pi removal efficiency remarkably deteriorated to $13.5 \pm 1.1\%$ accompanied by a notable decrease of idle TP release. The values of effluent TP concentration (12.97 ± 0.16 mg L^{-1}) and TP removed per MLVSS (1.35 ± 0.13 mg g^{-1}) indicated the capability of P removal almost collapsed. Similar relationships among Pi removal performance, idle TP release and idle time configuration were observed in 500 mg COD L^{-1} fed group, suggesting that the length of idle time was quite important for the AEI process to achieve excess Pi removal. The reactors in latter group had greater Pi removal efficiencies than those in

Table II. TP removal performance and PHA and glycogen transformations under different idle time configurations*.

	Influent COD (mg L^{-1})									
	250					500				
Idle time configuration (h)	1.5	3.5	5.5	7.5	9.5	1.5	3.5	5.5	7.5	9.5
Effluent TP (mg L^{-1})	12.97 ± 0.16	9.43 ± 0.08	9.25 ± 0.14	9.17 ± 0.24	9.12 ± 0.18	11.51 ± 0.12	6.82 ± 0.18	4.29 ± 0.08	3.93 ± 0.22	3.85 ± 0.14
TP removed per MLVSS (mg g^{-1})	1.35 ± 0.13	3.16 ± 0.18	3.27 ± 0.22	3.46 ± 0.30	3.59 ± 0.28	1.21 ± 0.09	2.49 ± 0.17	3.13 ± 0.23	3.28 ± 0.22	3.34 ± 0.27
TP removal efficiency (%)	13.5 ± 1.1	37.1 ± 0.5	38.3 ± 0.9	38.3 ± 1.6	39.2 ± 1.2	23.3 ± 0.8	54.5 ± 1.2	71.4 ± 0.6	73.8 ± 1.5	74.3 ± 0.9
Idle TP release per MLVSS (mg g^{-1})	0.39 ± 0.07	2.18 ± 0.18	2.29 ± 0.19	2.31 ± 0.20	2.59 ± 0.14	0.31 ± 0.07	1.77 ± 0.13	2.23 ± 0.25	2.37 ± 0.19	2.43 ± 0.22
Aerobic PHA synthesis per MLVSS (mM C g^{-1})	2.81 ± 0.22	3.22 ± 0.11	3.29 ± 0.23	3.25 ± 0.19	3.41 ± 0.12	2.86 ± 0.12	3.03 ± 0.18	3.11 ± 0.14	3.42 ± 0.28	3.36 ± 0.17
Aerobic PHA degradation per MLVSS (mM C g^{-1})	2.77 ± 0.18	3.18 ± 0.26	3.43 ± 0.08	3.19 ± 0.16	3.36 ± 0.15	3.19 ± 0.07	3.55 ± 0.15	3.87 ± 0.24	4.15 ± 0.16	4.22 ± 0.21
Idle PHA degradation per MLVSS (mM C g^{-1})	0	-0.11 ± 0.04	0	0.04 ± 0.01	-0.12 ± 0.02	-0.44 ± 0.09	-0.62 ± 0.12	-0.84 ± 0.11	-0.87 ± 0.13	-0.92 ± 0.08
Aerobic glycogen synthesis per MLVSS (mM C g^{-1})	0.59 ± 0.17	0.72 ± 0.21	0.77 ± 0.11	0.97 ± 0.18	0.93 ± 0.14	1.03 ± 0.15	1.28 ± 0.17	1.53 ± 0.24	1.46 ± 0.14	1.63 ± 0.26
Aerobic glycogen degradation per MLVSS (mM C g^{-1})	0.55 ± 0.13	0.77 ± 0.16	0.71 ± 0.24	0.93 ± 0.16	0.98 ± 0.12	0.32 ± 0.14	0.36 ± 0.12	0.27 ± 0.08	0.31 ± 0.12	0.35 ± 0.06
Idle glycogen degradation per MLVSS (mM C g^{-1})	-0.06 ± 0.01	0	0.05 ± 0.01	0.06 ± 0.01	0.17 ± 0.04	0.68 ± 0.08	0.95 ± 0.14	1.22 ± 0.18	1.21 ± 0.16	1.32 ± 0.16

*Data reported are the averages and their standard deviations of 3 cyclic studies.

former, respectively, mostly due to the higher influent acetate concentrations. Oehmen et al. (2007) showed that a sufficient amount of VFAs has to be provided in order to achieve good Pi removal.

Effects of PHA and Glycogen Levels and Their Periodic Transformations on Pi Removal

Five reactors were working in stable operation approximately after 42 cycles. As expected, a higher influent COD resulted in more sludge PHA and glycogen contents under the same operational conditions (Table III). Aerobic initial PHA and glycogen increased from 1.43 ± 0.16 and 1.62 ± 0.12 mM C g^{-1} to 3.68 ± 0.20 and 4.54 ± 0.26 mM C g^{-1} with an increase in influent COD from 203 to 999 mg L^{-1}, respectively. It is well known that a higher influent COD can increase the biomass thereby enhancing the Pi required for the growth of ordinary heterotrophs, and a greater PHA content can provide more energy for Pi uptake and poly-P accumulation. However, the Pi removal capability per MLVSS was not improved but deteriorated distinctly with an increase in influent COD under seemingly favorable conditions as operated in the parent reactor. Especially in the 999 mg L^{-1} COD-fed reactor, 1.18 ± 0.08 mg TP was merely removed per gram of MLVSS despite $42.6 \pm 0.8\%$ of TP removal efficiency. DAPI staining showed that the sludge samples from the 999 mg L^{-1} COD reactor scarcely contained visible poly-P (SI Fig. S3), further demonstrated the deterioration in Pi removal performance. This deterioration could be well explained by the decrease of *Accumulibacter* (PAOs, 6.8% of all cells) and the increase of *Competibacter*- and *Defluviicoccus*-related organisms (GAOs, 73.5% of all cells, SI Fig. S4). Even in the highest Pi removal efficiency measured reactor (i.e., 597 mg L^{-1} COD reactor), TP removed per gram of MLVSS was still less than that in 203 and 398 mg L^{-1} COD-fed reactors.

From the data shown in Table III, we could acquire some relationships among Pi removal performance and idle transformations of PHA, glycogen, and poly-P. In the best Pi removal performance (per MLVSS) detected reactor (i.e., 203 mg L^{-1} reactor), the most poly-P degradation (i.e., idle TP release) was measured, whereas the least glycogen degradation and PHA formation were observed during the idle period. When influent COD increased, idle glycogen degradation and PHA accumulation increased consequently, but idle poly-P degradation and Pi removal capability decreased contrarily. Accordingly, TP removal level seemed to show some positive correlation with idle TP release, but negative correlation with idle glycogen degradation/PHA accumulation. The results obtained from the batch tests with different aerobic lengths (898 mg L^{-1} COD fed) also supported this deduction (SI Table S2). In the 4-h aerated reactor, Pi removal performance, levels of PHA and glycogen, and their cyclic transformations were very similar with those in the 999 mg L^{-1} COD-fed reactor formerly operated. When the aerobic time increased from 4 to 8 h, aerobic initial and end PHA/glycogen gradually decreased. Also, gradual reductions of idle glycogen degradation and PHA accumulation were observed, but both idle TP release and TP removal performance gradually increased.

As is well known, PHA is the key storage product for conventional A/O process, and more anaerobic PHA accumulation will provide more energy for subsequent Pi uptake and poly-P synthesis, thus causing a higher Pi removal performance. However, some negative correlation was observed between idle PHA formation and TP removal per unit MLVSS in two above batch tests (Fig. 5), suggesting that the idle period presented here was indeed different from the anaerobic period operated in the conventional A/O process, and a novel inducing mechanism of Pi removal might exist in the AEI process.

Table III. Transformations of PHA and glycogen and performance of TP removal under different COD in influent*.

	Influent COD (mg L^{-1})				
	203	398	597	796	999
Aerobic initial PHA per MLVSS (mM C g^{-1})	1.43 ± 0.16	1.68 ± 0.20	2.17 ± 0.18	3.12 ± 0.22	3.68 ± 0.20
Aerobic end PHA per MLVSS (mM C g^{-1})	1.37 ± 0.08	1.13 ± 0.09	1.21 ± 0.07	1.72 ± 0.16	2.18 ± 0.12
Idle end PHA per MLVSS (mM C g^{-1})	1.46 ± 0.10	1.76 ± 0.15	2.12 ± 0.14	3.26 ± 0.24	3.64 ± 0.16
Aerobic PHA synthesis per MLVSS (mM C g^{-1})	3.05 ± 0.15	3.52 ± 0.24	3.49 ± 0.16	3.35 ± 0.17	3.16 ± 0.12
Aerobic PHA degradation per MLVSS (mM C g^{-1})	3.11 ± 0.14	4.07 ± 0.26	4.45 ± 0.19	4.75 ± 0.11	4.66 ± 0.22
Idle PHA degradation per MLVSS (mM C g^{-1})	-0.09 ± 0.02	-0.63 ± 0.04	-0.91 ± 0.08	-1.54 ± 0.10	-1.46 ± 0.08
Aerobic initial glycogen per MLVSS (mM C g^{-1})	1.62 ± 0.12	2.38 ± 0.10	2.95 ± 0.17	3.77 ± 0.25	4.54 ± 0.26
Aerobic end glycogen per MLVSS (mM C g^{-1})	1.68 ± 0.14	3.19 ± 0.22	4.48 ± 0.28	6.18 ± 0.26	6.95 ± 0.29
Idle end glycogen per MLVSS (mM C g^{-1})	1.64 ± 0.08	2.23 ± 0.09	3.02 ± 0.15	3.85 ± 0.27	4.48 ± 0.20
Aerobic glycogen synthesis per MLVSS (mM C g^{-1})	0.53 ± 0.10	1.67 ± 0.18	2.36 ± 0.12	2.77 ± 0.19	2.63 ± 0.11
Aerobic glycogen degradation per MLVSS (mM C g^{-1})	0.47 ± 0.06	0.86 ± 0.12	0.83 ± 0.14	0.36 ± 0.06	0.22 ± 0.06
Idle glycogen degradation per MLVSS (mM C g^{-1})	0.04 ± 0.01	0.96 ± 0.06	1.46 ± 0.08	2.33 ± 0.15	2.47 ± 0.12
Effluent TP (mg L^{-1})	9.83 ± 0.11	5.15 ± 0.14	2.73 ± 0.08	7.21 ± 0.12	8.61 ± 0.14
TP removed per MLVSS (mg g^{-1})	3.51 ± 0.23	3.34 ± 0.28	2.91 ± 0.16	1.63 ± 0.12	1.18 ± 0.08
TP removal efficiency (%)	34.5 ± 0.7	65.7 ± 0.8	81.8 ± 0.5	51.9 ± 0.8	42.6 ± 0.8
Idle TP release per MLVSS (mg g^{-1})	2.46 ± 0.21	2.39 ± 0.18	1.99 ± 0.16	1.15 ± 0.11	0.81 ± 0.08

*Data reported are the averages and their standard deviations of 3 cyclic studies.

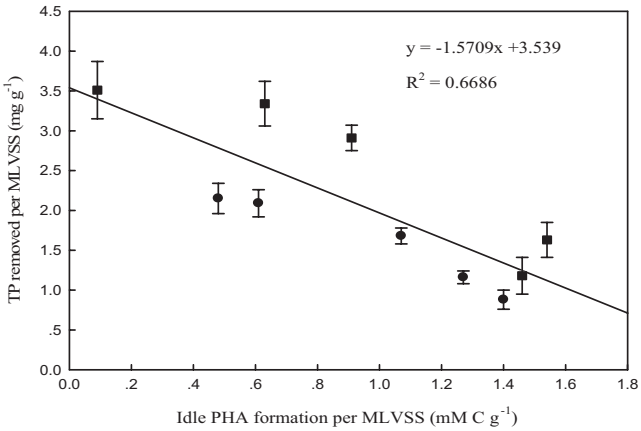

Figure 5. The correlation between idle PHA formation per MLVSS and TP removed per MLVSS. Square and circle represent the data obtained in batch tests of the effects of acetate concentration and aeration time on Pi removal performances, respectively. Error bars represent standard deviations of 3 cyclic studies.

The Inducing Mechanism of Pi Removal in the AEI Process

As is known, in the A/O process, poly-P will also hydrolyze to phosphate during settling/idle periods. This type of Pi release is detrimental for the conventional A/O system, and is usually called secondary Pi release. Accordingly, a very short idle period is generally conducted not only to enhance the wastewater removal capability but also to minimize the secondary Pi release. However, TP removal per MLVSS linearly increased with idle TP release in all above batch tests ($R^2 = 0.965$, Fig. 6), indicating an opposite fact that a more idle TP release would not deteriorate but benefit Pi removal, and idle TP release seemed to be the driving force for enhanced Pi removal in the AEI process. In the AEI systems with P removal well achieved, the ratio of TP removed/MLVSS was approximate 3.6 mg Pi g^{-1} of MLVSS. As comparisons, this value was usual around 3.0 mg Pi g^{-1} MLVSS in the conventional A/O process (Tong et al., 2007), and increased along with PAOs population (e.g., about 4.7 mg Pi g^{-1} MLVSS with 75% PAOs and 6.1 mg Pi g^{-1} MLVSS with 91% PAOs; Lu et al., 2006). This inducing mechanism could easily explain the reason for inconspicuous decrease of effluent TP after 3.5 h (or 5.5 h) idle time in 250 (or 500) mg COD L^{-1} fed group shown in Figure 4, because the variations of idle TP release in those reactors were slight (Table II). According to above batch studies, the levels of idle TP release can be affected by idle period length and intracellular PHA/glycogen contents (more accurately expressed as idle initial glycogen; $R^2 = 0.9379$, SI Fig. S5). Hence, to induce a high Pi removal performance, a relatively longer idle period with intracellular glycogen deficiency needs to be operated, as compared with that conducted in the conventional EBPR and CAS processes. For the most common domestic wastewater (COD $\leq$ 250 mg L^{-1}), 4-h aerobic and 3.5-h idle periods seem to be enough, but the operational condition still needs to be further optimized.

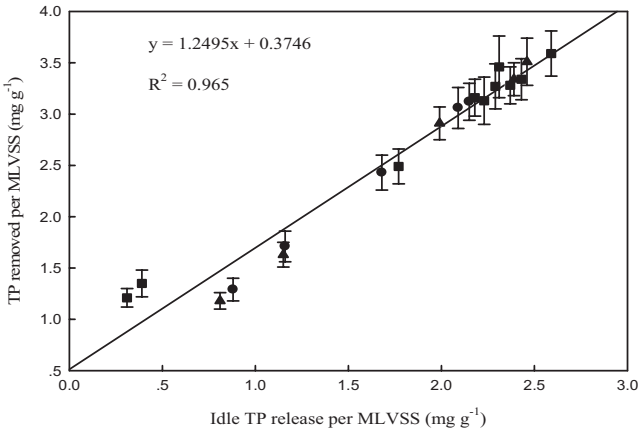

Figure 6. The relationship between idle TP release per MLVSS and TP removed per MLVSS in all batch tests. Square, triangle, and circle represent the data obtained in batch tests of the effects of idle time configuration, acetate concentration, and aeration time on Pi removal performances, respectively. Error bars represent standard deviations of 3 cyclic studies.

Discussion

The results show that idle Pi release, usually considered to be detrimental for the conventional A/O process, can drive PAOs to over-store Pi as intracellular poly-P in the AEI process without a defined anaerobic period. Vargas et al. (2009) also proved that P removal could be maintained for a long time without a specific anaerobic phase (under permanent aerobic conditions). However, idle period was not conducted and an obvious P release was observed during the substrate uptake period in their study, which were different from this study. Clearly, this inducing principle involved in the AEI process is different from all those of previous studies of P removal, it is therefore necessary to discuss why idle Pi release can induce PAOs to accumulate poly-P. In fact, the essential of biological P removal is to exploit the ability of certain microorganisms to accumulate P in excess of metabolic requirement and to store this intracellularly as poly-P (Mullan et al., 2006). This capability is largely dependent on the role of poly-P playing in their biochemical metabolism (de-Bashan and Bashan, 2004). If the cells' metabolic activities require poly-P to serve as one of the energy storages, P removal is achieved (for instance, the microbial community is enriched in PAOs); or else, P removal is failed (e.g., the sludge is enriched in GAOs). It is well known that PHA, glycogen, and poly-P are the three most common intracellular energy storages. Compared with the rapid aerobic degradation of PHA and glycogen, poly-P is hardly utilized under aerobic (even aerobic starvation) conditions, but easily degraded under anaerobic conditions

(Lopez et al., 2006). This can ensure that poly-P is kept at high levels even at the end of an aerobic starvation period, thus serving as the only suitable energy source in the extended-idle period. In the systems of this study where P removal is well achieved, external substrate, PHA, and glycogen consumptions do not occur in the idle period, but the relatively long idle period conducted in the AEI system force the microbes requiring more energy for bacterial maintenance than that in the CAS system. This energy requirement can be nicely meet by abundant idle poly-P degradation (i.e., idle Pi release), thereby enhancing the important significance of poly-P in PAOs metabolism. That is, the AEI regime can help the cells that are able to store poly-P to be dominant in the microbial ecosystem. Silva et al. (2012) also found that a defined anaerobic zone was not necessarily required for putative PAOs growth in membrane bioreactors, because poly-P storage might provide a selective advantage in fulfilling cell maintenance requirements in substrate-limited conditions.

As has been elucidated in the foregoing, Pi removal performance per unit MLVSS is negatively correlated with the level of idle initial glycogen (i.e., aerobic end glycogen). The higher idle initial glycogen remains, the lower Pi removal capability is observed. One might want to know why a higher idle initial glycogen results in a lower Pi removal performance. It has been reported that biological Pi removal is related to the transformations of intracellular PHAs and glycogen, higher glycogen transformation suggests that the GAOs metabolism might be activated (Mino et al., 1998). A high idle glycogen transformation indicated there was higher GAOs fraction in the sludge, thus causing a lower Pi removal performance. As glycogen can be rapidly utilized under anaerobic conditions, high idle initial glycogen will weaken the significance of poly-P in PAOs metabolism which may result in a shift from PAOs to GAOs. If a higher idle initial glycogen remains, more glycogen will be utilized, and then less energy will be required from poly-P degradation. This explanation is in agreement with the transformation data of idle glycogen degradation and idle TP release measured under different levels of idle initial glycogen (Table III, SI Table S2). Moreover, this explanation is also in accordance with the negative correlation between idle PHA formation and TP removal per unit MLVSS shown in Figure 5, if anaerobic conversion of glycogen to PHV occurs in the idle period (combination of glycolysis, conversion of pyruvate to both acetyl-CoA and propionyl-CoA, and PHV production, SI Fig. S6).

In addition, as shown in Figure 1, it was indicated that 32 mg COD was approximately required to remove 1 mg Pi in the parent reactor, which was comparable with the data that acquired in the A/O process. However, this value seemed to be around 40–50 mg COD mg^{-1} Pi in the systems of batch tests with high P removal (Fig. 4, Tables II and III), which was higher than that obtained in the Figure 1 and in the A/O process. Thus the question as to why the COD required for Pi removal reported in the batch tests are far from the ratio measured in the parent reactor needed to be discussed. As described in the Materials and Methods Section, temperature was not controlled in this study, and the data reported in Figure 1 (parent reactor) and the results shown in Figure 4, Tables II and III (batch tests) were not measured at the same day but detected during a relatively long period. One possible reason was that different temperature during this period might cause a shift in the microbial community from PAOs to GAOs, thereby increasing COD required for Pi removal. In the literatures, temperature was often reported to affect the PAOs–GAOs competition (Oehmen et al., 2007). Furthermore, it is well known that one factor influencing the PAO–GAO competition is the ratio of organic carbon to Pi in the influent, or the so-called influent COD/Pi ratio. This ratio in the parent reactor was also different from that in the batch experiments, hence an alternative explanation was that a lack or excess of influent COD in the batch tests might induce the excessive growth of GAOs, which increased the COD requirements for Pi removal. Considering that the value of COD required for Pi removal is an important index for application in full-scale wastewater treatment plants, it is therefore necessary to compare it in detailed between the AEI and the A/O processes using model substrates and real municipal wastewaters in future.

This study shows that a significant poly-P accumulation can be easily induced in an AEI process. From our current studies, this process behaves quite simply in operation, and may serve as an alternative option for Pi removal from wastewaters. The process still needs further examinations and optimizations, especially to evaluate its feasibility and reliability in a pilot-scale or full-scale study using real domestic wastewaters, and to optimize the aerobic and idle times, making it cheaper in construction and operation. Besides, the effects of operational parameters (like pH, temperature, and carbon sources, etc.) on Pi removal performances also need to be investigated.

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