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Free nitrous acid inhibition on carbon storage microorganisms: accumulated inhibitory effects and recoverability

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Abstract

Recent research has shed light on utilization of carbon storage microorganisms in the A-stage of AB process for higher methane generation and resource recovery potential. Typically, organic matters are entrapped into biomass in the A-stage and subsequently channeled to the anaerobic digester for energy/resource recovery. In the following B-stage, nitrite shortcut strategy is often implemented to achieve low energy nitrogen removal. In this study, an enriched glycogen accumulating organism (GAO) culture was deployed as the A-stage carbon storage microorganisms to enhance the removal of soluble COD. This study aimed (1) to address the challenge arising from incidental nitrite leakage into the A-stage tank, leading to free nitrous acid (FNA) inhibition; and (2) to evaluate the continued (henceforth referred to as 'accumulated')

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inhibitory effects on GAOs' carbon metabolisms under the subsequent FNA-free condition. Upon FNA exposure, dynamics in carbon storage mechanisms were obtained and could be linked to higher cellular energy expenditure for detoxification activity. The inhibition on carbon transformation, however, was found to be reversible, suggesting the robustness of GAO towards FNA inhibition and its potential application in the nitrite-shortcut AB process.

Keywords AB process, carbon storage, free nitrous acid, glycogen accumulating organism, nitrite shortcut

1. Introduction

The AB process has received considerable attention lately due to concern over energy consumption in wastewater treatment. The AB process is a two-stage approach with an extremely high loaded carbon capture stage (A-stage), which is subsequently followed by a low loaded biological stage (B-stage) and so ensuring removal of dissolved organics and ammonia. In the recent years, there has been interest in application of the nitrite shortcut strategy for nitrogen removal in the B-stage due to its potential carbon and energy savings [1, 2]. The entrapped organics (energy) during the A-stage is recovered from the solids train containing thickened sludge through biogas generation in the anaerobic digester located downstream.

It was proposed that entrapment of organics in the A-stage mainly involves three mechanisms: (i) fast microbial growth, (ii) bioflocculation and enmeshment (physico-chemical adsorption by cell surface typically known as biosorption or extracellular polymeric substances (EPS) entrapment), and (iii) bioaccumulation into the cell [3-6]. It is generally assumed that under non-substrate

limiting condition, the A-stage microorganisms take up readily biodegradable COD in excess of and at a faster rate than its consumption for cell growth and subsequently store it as intracellular storage products, such as poly-hydroxyalkanoates (PHAs) or glycogen [2, 7]. Upon depletion of the influent COD, the microorganisms would in turn utilize the stored substrate as carbon and energy sources. On the other hand, the particulate and colloidal fractions of the COD are removed via surface sorption and EPS entrapment [8]. Among the above mechanisms, microbial storage is considered important for the following reasons: (1) lower aeration energy requirement than mineralization; and (2) the ability to recover not only energy but also material resources. Indeed, a recent report had suggested that approximately 66.3% of the overall carbon removal capacity during A-stage was attributable to intracellular carbon storage as PHA [4]. In addition, higher methane generation was obtained from anaerobic digestion using PHA accumulating sludge as substrate as compared to the excess activated sludge [9, 10]. Glycogen accumulating organisms (GAOs) are among the major groups of microorganisms highly capable for carbon removal via internal storage mechanism in the form of PHAs. GAOs phenotype anaerobically takes up soluble carbons for storage as PHA by utilizing intracellular glycogen as a source of energy. In the subsequent aerobic phase, PHA is used for active biomass production, glycogen replenishment and cellular maintenance. For the selection and acclimation of GAOs in the A-stage reactor, subdivision into anaerobic and aerobic zones is therefore required.

The suspended solids concentration of the mixed liquor in the A-stage reactor is maintained by returning sludge from the end of A- and B-stage [11]. A side stream sludge breeder tank may also be designed as an additional biomass source. In the effort to reduce energy expenditure and greenhouse gas emission from wastewater treatment, removal of nitrogen via partial nitrification

has been increasingly applied in the following B-stage. In this configuration, an incidental nitrite leakage to A-stage with the recycle flow is likely to take place as a result of nitrite accumulation in B-stage. The accumulation of nitrite is often intensified during an upset process or a process during start-up period and by the high-strength ammonia stream of reject water originating from dewatering activity of digested sludge which is typically returned to the mainstream bioreactor as well as the infiltration of industrial wastewater [12]. Such leakage would cause inhibition on carbon storage microorganisms and lead to the deterioration of the A-stage performance.

Various researchers have reported the accumulation of nitrite or nitrous acid (FNA or HNO_2) inhibits various microorganisms existing in the wastewater treatment system, such as ammonia and nitrite oxidising bacteria (AOB and NOB) [13, 14], denitrifiers [15], poly-phosphate accumulating organisms (PAOs) [16, 17], and GAOs [18]. It has then been reported that FNA, instead of nitrite, is the key factor responsible for the inhibition [19]. While FNA inhibition on carbon uptake by GAO has been reported elsewhere [20], there are no reports on the recovery of GAO after FNA inhibition to date. It is indeed crucial to investigate the response of GAO after inhibition, so that long-term performance can be predicted. Furthermore, in order to address the incidental nitrite leakage as a possible challenge in the implementation of GAO as a supplemental factor responsible for enhanced sCOD removal in the A-stage of AB process and to study the post-inhibition recovery, a series of experiments have been carried out. The objectives of this study included: (1) to investigate the accumulated effect of FNA on carbon uptake and transformation by the GAO-acclimated sludge; and (2) to evaluate the recovery of GAO metabolisms in the subsequent FNA-free condition.

2. Materials and methods

2.1 Sludge acclimatization

GAOs were cultivated in a laboratory-scale sequencing batch reactor (SBR) under alternating anaerobic-aerobic conditions. Inoculum was taken from a conventional WWTP in Singapore. The SBR had a working volume of 4 L and was operated with a cyclic time of 4 h consisting of 70 min anaerobic, 160 min aerobic, and 10 min wasting, settling and decanting periods. To maintain anaerobic and aerobic conditions, nitrogen gas and air were sparged intermittently through the mixed liquor at a flow rate of 1.0 L/min during the respective periods. One litre of synthetic wastewater containing acetate as the sole carbon source, ammonium, and microelements was fed into the SBR in the first 10 min of the anaerobic period, resulting in a hydraulic retention time of 16 h. The initial COD and ammonium concentrations were 200 mg COD/L and 10 mg NH_4^+-N/L, respectively. The microelements solution was prepared in accordance with Smolders et al. [21]. 5 mg/L ATU (allylthiourea) was applied to inhibit nitrification. The solid retention time (SRT) was maintained at 8 d by wasting 83 mL of mixed liquor at the end of each cycle. The reactor was operated in a temperature-controlled room at 24 ± 1 °C. The pH was controlled during both the anaerobic and aerobic phases at a range of 7.0-7.3 by dosing 0.5 M HCl and 0.5 M NaOH as required. Weekly cycle study was performed to monitor the SBR performance. After the SBR had reached steady-state condition, as indicated by stable treatment performance and biomass concentration (4.4 ± 0.3 g MLVSS/L), sludge was withdrawn at the end of the cycle and used for batch experiments.

2.2 Batch experiments

A series of batch experiments were carried out with GAO sludge to investigate the accumulated effects of FNA on the metabolisms of GAO as well as the recoverability from the inhibition in the subsequent FNA-free cycles. Due to the absence of nitrification during acclimation period, the GAO culture used in this study was not adapted to nitrite/FNA. The sludge was withdrawn from the SBR at the cycle end and subsequently washed with phosphate buffer to remove any residual nutrient. The washed sludge was then distributed into 250 mL batch reactors where a 2-h anaerobic condition was first applied. N_2 gas was sparged through the headspace to ensure anaerobic conditions. Acetate and nitrite stock solutions were then injected, resulting in initial acetate concentration of 150 mg COD/L and the initial nitrite concentrations are as presented in Table 1. The FNA concentration was calculated using $S_{N-NO_2}/(K_a * 10^{pH})$, with the K_a value determined using $e^{-2300/(273+T)}$ for any given temperature T (°C) [13]. The control test was carried out in the absence of nitrite.

2 mL mixed liquor samples were taken at 15-minute intervals using a syringe and immediately filtered through disposable Millipore filter units (0.45 μm pore size) for the analyses of acetate and nitrite. Solid samples (10 mL mixed liquor) for the analyses of PHA and glycogen were taken every 30 min, while 10 mL mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) samples were taken at the end of the batch experiment.

At the end of the anaerobic inhibition period, the sludge was washed with phosphate buffer to remove the remaining COD and nitrite. An hour of aerobic phase was then initiated by the injection of ammonium and ATU at the concentrations of 10 mg NH_4^+-N/L and 5 mg ATU/L, respectively. Dissolved oxygen concentration was kept at atmospheric saturation by a continuous

air supply. Due to the absence of nitrification, the consumption of ammonium could be attributed to bacterial growth [22]. 1 mL liquid samples for the analysis of ammonia were taken at 30-min intervals, while 10 mL solid samples for the analyses of PHA and glycogen were withdrawn at the beginning and end of the aerobic phase. The rates of anaerobic acetate uptake, PHA production and glycogen consumption, as well as aerobic ammonium consumption, PHA degradation and glycogen production were determined from the measured acetate, ammonium, PHA and glycogen profiles using linear regression.

Subsequently, an anaerobic recovery phase was applied in all tests for 2 h with the injection of acetate at 150 mg COD/L. In this phase, liquid and solid samples were taken at the same intervals as in the aerobic recovery phase. During tests 7, 10, 16 and 17 (Table 1), three consecutive recovery cycles were applied in order to further examine the extent of recoverability of GAO's metabolisms. At the end of the third recovery cycle, approximately 80 mL mixed liquor was left in the batch reactor.

2.3 Chemical and microbial analyses

2.3.1 Chemical analyses

Ammonia nitrogen (NH_4^+-N) and nitrite nitrogen (NO_2^--N) concentrations were analyzed in accordance with Standard Methods [23]. Acetate was measured with an Agilent GC equipped with a DB-FFAP 15m×0.53mm×1.0μm (length×ID×film) capillary column. The injector block and flame ionization detector (FID) were operated at 250°C and 300°C, respectively. High purity helium was used as carrier gas at a constant pressure of 103 kPa. 0.9 mL of the filtered sample was transferred into a GC vial to which 0.1mL of formic acid was added. For the analysis, a

159 volume of 1μL of sample was injected in splitless mode. MLSS and MLVSS were determined in
160 accordance with Standard Methods. The biomass concentration was obtained by subtracting
161 PHA and glycogen from the MLVSS value.
162
163 PHA analysis was conducted after Oehmen et al. [24] to determine poly-β-hydroxybutyrate
164 (PHB) and poly-β-hydroxyvalerate (PHV). Approximately 20 mg of freeze-dried biomass and
165 PHB/V standards (Aldrich, USA) were put into screw-topped glass tubes. For calibration of the
166 method, the standard consisting of 3-hydroxybutyric acid and 3-hydroxyvaleric acid copolymer
167 (88% and 12% wt) was used. The tubes were heated at 100°C for 20 hours after being suspended
168 in 2 mL methanol acidified with 3% H_2SO_4 and 2 mL chloroform. After cooling, 1 mL Milli-Q
169 water was added and the sample was mixed by vortexing. When the phases were separated,
170 around 1 mL of the bottom organic layer was transferred to the GC vials for analysis. The
171 quantification of PHA was performed by GC using column DB-5ms 30 m × 0.25 mm × 0.25 μm
172 (length × ID × film). High purity helium was used as carrier gas at 138 kPa. 3 μL of sample
173 volume was injected in split mode. Both injector port and FID detector were operated at 250°C.
174
175 Cellular glycogen in the mixed liquor was measured according to a method by Bond et al. [25].
176 Glycogen was extracted from lyophilized sludge samples which were previously fixed by
177 formaldehyde. The glycogen sample was subsequently broken down to glucose by digestion at
178 105°C for 6 hours in screw-topped glass tubes. After cooling and centrifugation at 5000 rpm for
179 5 min, the supernatant was used in the colorimetric carbohydrate assay for glucose analysis. For
180 the assay, 0.8 mL of anthrone reagent (0.125% anthrone in 95% sulphuric acid) was slowly
181 added into the tube and mixed by inversion. The tube was heated to 100°C using dry bath for 14

182 min and then put in the 4°C fridge for 5 min cooling. The mixture was then transferred into a
183 cuvette and allowed to reach room temperature before being measured using spectrophotometer.
184
185 **2.3.2 Microbial analysis**
186 The microbial community composition was determined by fluorescence *in situ* hybridization
187 (FISH) which was performed after a method described by Amann [26]. The presence of
188 "*Candidatus* Competibacter phosphatis" or *Competibacter* was detected using Cy5-labelled
189 GAOMIX probes (consisting of equal amounts of probes GAOQ431 and GAOQ989) [27]. Cy5-
190 labelled DF1MIX comprising equal amounts of probes DEF218 and DEF618 were used to target
191 *Defluviicoccus vanus* cluster 1-related bacteria or α-GAO [28]. Cy5-labelled DF2MIX,
192 comprising equal amount of probes DF988 and DF1020 plus helper probes H966 and H1038,
193 was used for detecting *Defluviicoccus vanus* cluster 2-related GAOs [29]. All of GAOMIX,
194 DF1MIX and DF2MIX probes were against FITC-labeled EUBMIX probes. Probe hybridisation
195 took place at 46°C for 1.5 h followed by washing and 10 min incubation in the washing buffer
196 which was previously warmed at 48°C. FISH preparations were visualized with a confocal laser
197 scanning microscope (LSM 510 Meta, Zeiss) with Plan-Apochromat ×63 oil (NA1.4) objective.
198 The area containing cells targeted by the Cy3- or Cy5-labelled specific probes (PAOMIX,
199 GAOMIX, or α–GAO) was quantified as a percentage of the area of Cy5 or FITC labeled
200 bacteria probe (EUBMIX) within each image using a pixel counting program. The reported
201 quantification results are mean percentages obtained from 30 images analyzed along with the
202 standard errors. The standard error of the mean (SE_{mean}) was calculated as the standard deviation
203 of the percentage area divided by the square root of the number of FISH images.
204

205 ## 3. Results and discussion

206 ### 3.1 Enrichment of GAO and application in AB-process configuration

207 The SBR served as the source of GAO culture and was operated in steady state for more than
208 three months before the experiments were conducted. FISH result showed that *Competibacter*
209 was present with an abundance of 90 ± 5%. The abundance of *D.vanus*-cluster 1 was less than
210 1% whereas the cluster 2 was not detected. The population showed a typical phenotype for GAO
211 throughout the SBR operation. During the anaerobic phase, the uptake of organic carbon
212 substrate took place with concurrent intracellular PHA production and glycogen degradation.
213 Under the subsequent aerobic period, PHA mineralization and bacterial growth were observed
214 while the intracellular glycogen was replenished. The organic carbon was completely taken up
215 from the bulk liquid within the first 20 min of the anaerobic period. Given the fact that HRT of
216 the A-stage tank was typically short, e.g. 0.5 h as reported by Wett et al. [30], the fast kinetics of
217 organic carbon entrapment by GAOs notably suggested it feasible to implement in the A-stage of
218 the AB process. The 8-d SRT used in the parent SBR is the typical SRT for GAO enrichment. In
219 view of the short-SRT characteristic of the A-stage, a side stream sludge breeder tank is
220 proposed to maintain sufficient biomass in the A-reactor.
221
222 In a configuration where partial nitrification is applied in B-stage, an upset process or a process
223 during start-up period would result in a considerably high concentration of nitrite in the recycle
224 stream to the A-stage reactor. Furthermore, nitrite concentration in the mainstream bioreactor(s)
225 may be intensified by the receipt of high-strength ammonia stream of reject water. In one of our
226 unpublished studies, nitrite was observed to accumulate to more than 50 mg N/L in a municipal
227 wastewater treatment system, due to high ammonia concentration in the influent that was caused

228 by infiltration of industrial wastewater. Such extreme situation can seriously affect system
229 performance by inhibiting a broad range of microbes. This formed a major reason to look into
230 recovery mechanisms after inhibition.
231
232 **3.2 Effects of FNA on anaerobic carbon uptake rate by GAOs**
233 The impacts of FNA on carbon uptake of GAO as a result of nitrite leakage into the A-stage unit
234 are shown in Figure 1. The inhibition level was denoted as the percentage of the biomass-
235 normalized rate obtained in the control reactor, in which the maximum rate of the activity was
236 obtained. No nitrite consumption was observed in this study. The acetate uptake rate was
237 decreased as the applied initial FNA concentration increased. The acetate uptake rate decreased
238 sharply in the FNA concentration range of 0.00005-0.02 mg HNO_2-N/L, with 50% inhibition
239 occurred at FNA concentration of approximately 0.0075 mg HNO_2-N/L (equivalent to 32.5 mg
240 NO_2^--N/L at pH 7.0). However, the acetate uptake was not completely inhibited even at a higher
241 FNA concentration range (0.02-0.08 mg HNO_2-N/L) and the activity remained at approximately
242 30% of the control test. This suggested the robustness of GAOs as a carbon-channeling medium
243 from the primary wastewater treatment step to the anaerobic digester.
244
245 The effects of pH on GAOs' carbon metabolisms have been presented by Filipe et al. [31]. This
246 study, on the other hand, aimed to investigate the impacts of FNA on GAOs' carbon
247 metabolisms with the focus of recovery from the inhibition. Therefore, pH and nitrite
248 concentration were systematically varied in order to obtain various FNA concentrations. This
249 design method has been widely used in many research works in the field. It has also been proved
250 that neither pH nor nitrite alone is the inhibitor while the combination of these two factors –

FNA is the true inhibitor. Based on findings reported in Filipe et al. [31], applying different pH values for control reactors (without nitrite addition) in experiments involving GAO culture would cause inconsistency in the supposed baseline itself and thus would affect the relative activities for other experimental reactors.

3.3 Recovery

3.3.1 Accumulated inhibitory effects on the subsequent aerobic metabolism

At the end of the anaerobic tests, FNA/nitrite was removed from the bulk liquid and an aerobic condition was subsequently applied to assess the accumulated effects of FNA on GAO aerobic metabolism. Figures 2a, 2b, and 2c showed FNA dependency of the aerobic growth, PHA degradation and glycogen replenishment, respectively, indicating that FNA inhibition on GAOs under the previous anaerobic condition continued to the aerobic period. Bacterial growth was inhibited by 50% at initial FNA concentration of approximately 0.01 mg HNO_2-N/L (equivalent to 43 mg NO_2^--N/L at pH 7.0) and completely stopped at initial FNA concentrations above 0.025 mg HNO_2-N/L. The accumulation of nitrite at 43 mg NO_2^--N/L is indeed possible in the wastewater treatment plant treating medium- to high-strength wastewater, especially with the return stream of the ammonia-rich reject water. In one of the local WWTPs treating domestic wastewater in Singapore, the average influent ammonia is around 45-50 mg N/L. However, unlike growth, PHA utilization and glycogen production were not completely stopped at higher initial FNA concentrations. PHA utilization decreased more slowly, reaching approximately 60% of the initial rate at the highest concentration range applied (0.020-0.082 mg HNO_2-N/L), while in the case of glycogen replenishment, a sharp decrease took place when FNA was increased from 0.00005-0.02 mg HNO_2-N/L, and followed by a slower decrease, to approximately 40% at

274 the highest FNA concentration range studied (0.040-0.082 mg HNO_2-N/L). These findings thus
275 demonstrated that the stronger FNA adverse effects on the anabolic activities rather than the
276 catabolic activities are not only obtained from the direct exposure to FNA as have been reported
277 in several previous studies [14, 32], but also from the accumulated inhibition in the subsequent
278 FNA-free phase(s). Indeed, it has been reported that FNA depletes the intracellular energy pool
279 of bacteria [20, 32], which would lead to lower anabolic (energy-consuming) activities. The
280 reduced energy reserve is possibly due to the following mechanisms: (1) extra energy
281 requirement to carry out detoxification process by expelling protons out of the cells; and (2)
282 increase in energy requirement for cellular maintenance. A hypothesis could be made that after
283 FNA had been removed, expulsion of protons out of the cells (detoxification) would still
284 continue to gradually restore the transmembrane proton gradient which was previously disrupted
285 by FNA diffusion into the cells. This would then enable adenosine triphosphate (ATP) synthase
286 to synthesize ATP through oxidative phosphorylation, and therefore allowing bioactivities to
287 progressively recover.
288
289 **3.3.2 Reversibility of inhibition**
290 Recovery tests in the subsequent cycle(s) were carried out after the exposure to aerobic condition
291 explained in the above section. As can be seen from Figure 3a, the rates of acetate uptake were
292 negatively correlated with the previously-applied FNA concentrations, demonstrating that
293 inhibition still continued in the following FNA-free cycle. During this recovery period, a
294 detoxification process was also suggested by the increasing energy expenditure as reflected by
295 the increasing glycogen consumption per C-mole of acetate uptake (Gly/C) (see Figure 3b). The
296 increasing Gly/C ratio could be also linked to the shift in PHV/PHB content where the

297 percentage of PHV content was observed to increase with FNA concentration, while the
298 percentage of PHB content, on the other hand, decreased as FNA concentration increased. This
299 arises from the dual role of glycogen in the anaerobic metabolism of GAOs, i.e. to generate ATP
300 and to maintain the intracellular redox balance via $NADH_2$ production. The additional ATP
301 requirement which prompted glycogen degradation also resulted in additional $NADH_2$
302 production. The produced $NADH_2$, however, could be in excess of that required for PHA
303 synthesis. Consumption of the excess $NADH_2$ to maintain the redox balance was therefore
304 performed through the propionate-succinate pathway where pyruvate was converted to
305 propionyl-CoA which was subsequently condensed and reduced to the PHV polymer [31]. As a
306 result, increase in the ratio of PHV/PHB took place as Gly/C ratio increased. A graphical
307 illustration for this series of biotransformation is shown in Figure 4.
308
309 Figure 5a shows as the number of cycles increased, lower increases in Gly/C as well as
310 PHV/PHB ratios were obtained. Furthermore, both Gly/C and PHV/PHB ratios had reached the
311 initial state at the third anaerobic recovery cycle of test 6 where an FNA concentration of 0.0013
312 mg HNO_2-N/L was applied. During tests 10, 16, and 17, the ratios of PHV/PHB had notably
313 shifted from approximately170% at the first recovery cycle to 115-130% at the third cycle. This
314 suggested that the higher energy demand due to prior FNA exposure had gradually been restored
315 to the non-inhibited levels, demonstrating the phenomenon of reversible FNA inhibition. In
316 addition, the reversibility phenomenon was also confirmed by the increasing acetate uptake rates
317 until the third recovery cycle (see Figures 5b and 6). The recoverability of GAOs' carbon uptake
318 and metabolisms from FNA inhibition obtained in this study thus supported its application in the
319 nitrite-shortcut AB process for an energy-efficient wastewater treatment system.

4. Conclusions

This article discusses the application of GAOs in the AB process in an effort to recover energy and material resources from wastewater in the form of microbial storage products. However, the recycle stream from B-stage, where partial nitrification for nitrogen removal occurs, could pose risk of incidental nitrite leakage to the contact tank in A-stage. This leakage may inhibit the carbon uptake of GAOs and also their growth rates in the A-stage tank, subsequently affecting the carbon capture performance of A-stage. The inhibition continued for a few cycles of the alternating phases in the FNA-free environment, and eventually was found to be reversible. The recovery capacity of GAOs thus confirms their potential role in carbon entrapment of the AB process. Regardless, a proper control measure, for the level of nitrite/FNA in particular, needs to be deployed in conjunction with partial nitrification scheme of the AB process.

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References

1. De Clippeleir, H., S.E. Vlaeminck, F. De Wilde, K. Daeninck, M. Mosquera, P. Boeckx, W. Verstraete, and N. Boon, *One-stage partial nitritation/anammox at 15 °C on pretreated sewage: feasibility demonstration at lab-scale.* Applied Microbiology and Biotechnology, 2013. **97**(23): p. 10199–10210.

2. Dana Kathleen, F., *Optimization of intermittent aeration for increased nitrogen removal efficiency and improved settling*, in *Environmental Engineering*. 2014, Virginia Polytechnic Institute and State University: Blacksburg, Virginia.
3. Xiao, L., W. Liu, S. Pradhan, K. Boopathy, R. Wikramanayake, E. Jordan, and J.Y. Hu. *Biosorption of organics by live and inactivated aerobic bacteria.* in *IWA Leading Edge Technology Conference*. 2011. Singapore.
4. Lim, C.P., S. Zhang, Y. Zhou, and W.J. Ng, *Enhanced Carbon Capture Biosorption through Process Manipulation.* Biochemical Engineering Journal, 2015. **93**: p. 128-136.
5. Haider, S., K. Svardal, P.A. Vanrolleghem, and H. Kroiss, *The effect of low sludge age on wastewater fractionation (SS, SI).* Water Science and Technology, 2003. **47**(11): p. 203-209.
6. Lim, C.P., S. Zhang, E. Mar'atusalihat, L. Ganda, Y. Zhou, and W.J. Ng, *Energy Recovery from Municipal Wastewater through Carbon Storage - Potential Application of Novel Chromatiales Bacteria.* International Journal of Environmental Science and Technology, 2015: p. Submitted.
7. Böhnke, B., *Das Absorptions-Belebungs-Verfahren.* Korrespondenz Abwasser, 1977. **24**(77): p. 33-42.
8. Pujol, R. and J.P. Canler, *Biosorption and dynamics of bacterial populations in activated sludge.* Water Research, 1992. **26**(2): p. 209-212.
9. Huda, S.M.S., H. Satoh, and T. Mino, *Anaerobic digestion of polyhydroxybutyrate accumulated in excess activated sludge.* Journal of Water and Environment Technology, 2013. **11**: p. 429-438.

10. Sakai, K., S. Miyake, K. Iwama, D. Inoue, S. Soda, and M. Ike, *Polyhydroxyalkanoate (PHA) accumulation potential and PHA-accumulating microbial communities in various activated sludge processes of municipal wastewater treatment plants.* Journal of Applied Microbiology, 2015. **118**(1): p. 255-266.
11. Yu, J., X. Lan, S. Sreerama, R. Wikramanayake, R. Knauf, W. Liu, E. Jordan, and J. Hu. *An energy saving solution for water reclamation – a hybrid process combining bio-sorption and anaerobic digestion. Process concept and bio-sorption using anaerobic/aerobic sludge mixtures*. in *IWA Leading Edge Technology Conference*. 2009. Singapore.
12. Wett, B. and J. Alex, *Impacts of separate rejection water treatment on the overall plant performance.* Water Science and Technology, 2003. **48**(4): p. 139-146.
13. Anthonisen, A.C., R.C. Loehr, T.B.S. Prakasam, and E.G. Srinath, *Inhibition of nitrification by ammonia and nitrous acid.* Journal of Water Pollution Control Federation 1976. **48**(5): p. 835-852.
14. Vadivelu, V.M., J. Keller, and Z. Yuan, *Effect of free ammonia and free nitrous acid concentration on the anabolic and catabolic processes of an enriched Nitrosomonas culture.* Biotechnology and Bioengineering, 2006. **95**(5): p. 830-839.
15. Almeida, J.S., S.M. Júlio, M.A.M. Reis, and M.J.T. Carrondo, *Nitrite inhibition of denitrification by Pseudomonas fluorescens.* Biotechnology and Bioengineering, 1995. **46**(3): p. 194-201.
16. Zhou, Y., M. Pijuan, and Z. Yuan, *Free nitrous acid inhibition on anoxic phosphorus uptake and denitrification by poly-phosphate accumulating organisms.* Biotechnology and Bioengineering, 2007. **98**(4): p. 903-912.

17. Pijuan, M., L. Ye, and Z. Yuan, *Free nitrous acid inhibition on the aerobic metabolism of poly-phosphate accumulating organisms.* Water Research, 2010. **44**(20): p. 6063-6072.
18. Ye, L., M. Pijuan, and Z. Yuan, *The effect of free nitrous acid on the anabolic and catabolic processes of glycogen accumulating organisms.* Water Research, 2010. **44**(9): p. 2901-2909.
19. Vadivelu, V.M., Z. Yuan, C. Fux, and J. Keller, *The Inhibitory Effects of Free Nitrous Acid on the Energy Generation and Growth Processes of an Enriched Nitrobacter Culture.* Environmental Science & Technology, 2006. **40**(14): p. 4442-4448.
20. Ye, L., M. Pijuan, and Z. Yuan, *The effect of free nitrous acid on key anaerobic processes in enhanced biological phosphorus removal systems.* Bioresource Technology, 2013. **130**: p. 382-389.
21. Smolders, G.J.F., J. van der Meij, M.C.M. van Loosdrecht, and J.J. Heijnen, *Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence.* Biotechnology and Bioengineering, 1994. **43**(6): p. 461-470.
22. Oehmen, A., R.J. Zeng, A.M. Saunders, L.L. Blackall, J. Keller, and Z. Yuan, *Anaerobic and aerobic metabolism of glycogen accumulatingcorganisms selected with propionate as the sole carbon source.* Microbiology, 2006. **152**(9): p. 2767-2778.
23. APHA, *Standard Methods for the Examination of Water and Wastewater*. 19th ed. ed. 1995, Washington, DC: American Public Health Association.
24. Oehmen, A., B. Keller-Lehmann, R.J. Zeng, Z. Yuan, and J. Keller, *Optimisation of poly-[beta]-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems.* Journal of Chromatography A, 2005. **1070**(1-2): p. 131-136.

25. Bond, P.L., R. Erhart, M. Wagner, J.r. Keller, and L.L. Blackall, *Identification of some of the major groups of bacteria in efficient and nonefficient biological phosphorus removal activated sludge systems.* Applied and Environmental Microbiology, 1999. **65**(9): p. 4077-4084.

26. Amann, R., *Fluorescently labeled, ribosomal-RNA-targeted oligonucleotide probes in the study of microbial ecology.* Molecular Ecology, 1995. **4**: p. 543-553.

27. Crocetti, G.R., J.F. Banfield, J. Keller, P.L. Bond, and L.L. Blackall, *Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge processes.* Microbiology, 2002. **148**(11): p. 3353-3364.

28. Wong, M.T., F.M. Tan, W.J. Ng, and W.T. Liu, *Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic-aerobic activated sludge process.* Microbiology, 2004. **150**: p. 3741-3748.

29. Meyer, R.L., A.M. Saunders, and L.L. Blackall, *Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing.* Microbiology, 2006. **152**(2): p. 419-429.

30. Wett, B., K. Buchauer, and C. Fimml. *Energy self-sufficiency as a feasible concept for wastewater treatment systems*. in *IWA Leading Edge Technology Conference*. 2007. Singapore.

31. Filipe, C.D.M., G.T. Daigger, and C.P.L. Grady, *A metabolic model for acetate uptake under anaerobic conditions by glycogen accumulating organisms: stoichiometry, kinetics, and the effect of pH.* Biotechnology and Bioengineering, 2001. **76**(1): p. 17-31.

32. Zhou, Y., L. Ganda, M. Lim, Z. Yuan, S. Kjelleberg, and W.J. Ng, *Free nitrous acid (FNA) inhibition on denitrifying poly-phosphate accumulating organisms (DPAOs).* Applied Microbiology and Biotechnology, 2010. **88**(1): p. 359-369.

Table

Table 1. Experimental conditions applied in batch tests

No.	pH	NO_2^- (mg NO_2^--N/L)	FNA × 10^3 (mg HNO_2-N/L)
1	8	2	0.05
2	8	13	0.34
3	8	37	1.00
4	8	67	1.77
5	7	0	Control
6	7	2.3	0.53
7	7	5.6	1.30
8	7	10.6	2.44
9	7	15.8	3.65
10	7	22.0	5.08
11	7	33.3	7.69
12	7	41.7	9.63
13	7	53.8	12.41
14	7	63.9	14.75
15	7	71.6	16.53
16	7	91.5	21.11
17	7	105.5	24.35
18	7.5	2.2	0.16
19	7.5	26.8	1.95
20	7.5	58.0	4.24
21	7.5	86.9	6.34
22	6.5	31.8	23.23
23	6.5	62.4	45.54
24	6.5	89.6	65.39
25	6.5	113.0	82.49

Figure Captions

Figure 1. Effects of FNA on anaerobic carbon uptake rate of GAOs in the direct inhibition tests

Figure 2. Accumulated inhibitory effects of FNA on aerobic metabolisms of GAOs: a. bacterial growth, b. PHA degradation, and c. glycogen production

Figure 3. Effects of FNA on a. anaerobic carbon uptake recovery of GAOs, and b. PHV/PHB and Gly/C ratios under FNA-free condition

Figure 4. Illustration on the effects of FNA exposure on biological carbon transformation and ATP flow of GAO

Figure 5. a. PHV/PHB and Gly/C ratios and b. carbon uptake rate in multiple cycles of FNA-free anaerobic period: ● FNA = 1.30×10^{-3} mg N/L; ○ FNA = 5.08×10^{-3} mg N/L; ▼ FNA = 21.11×10^{-3} mg N/L; Δ FNA = 24.35×10^{-3} mg N/L

Figure 6. COD and nitrite profiles in the initial, inhibition and recovery periods (Test 10)

Figure 1
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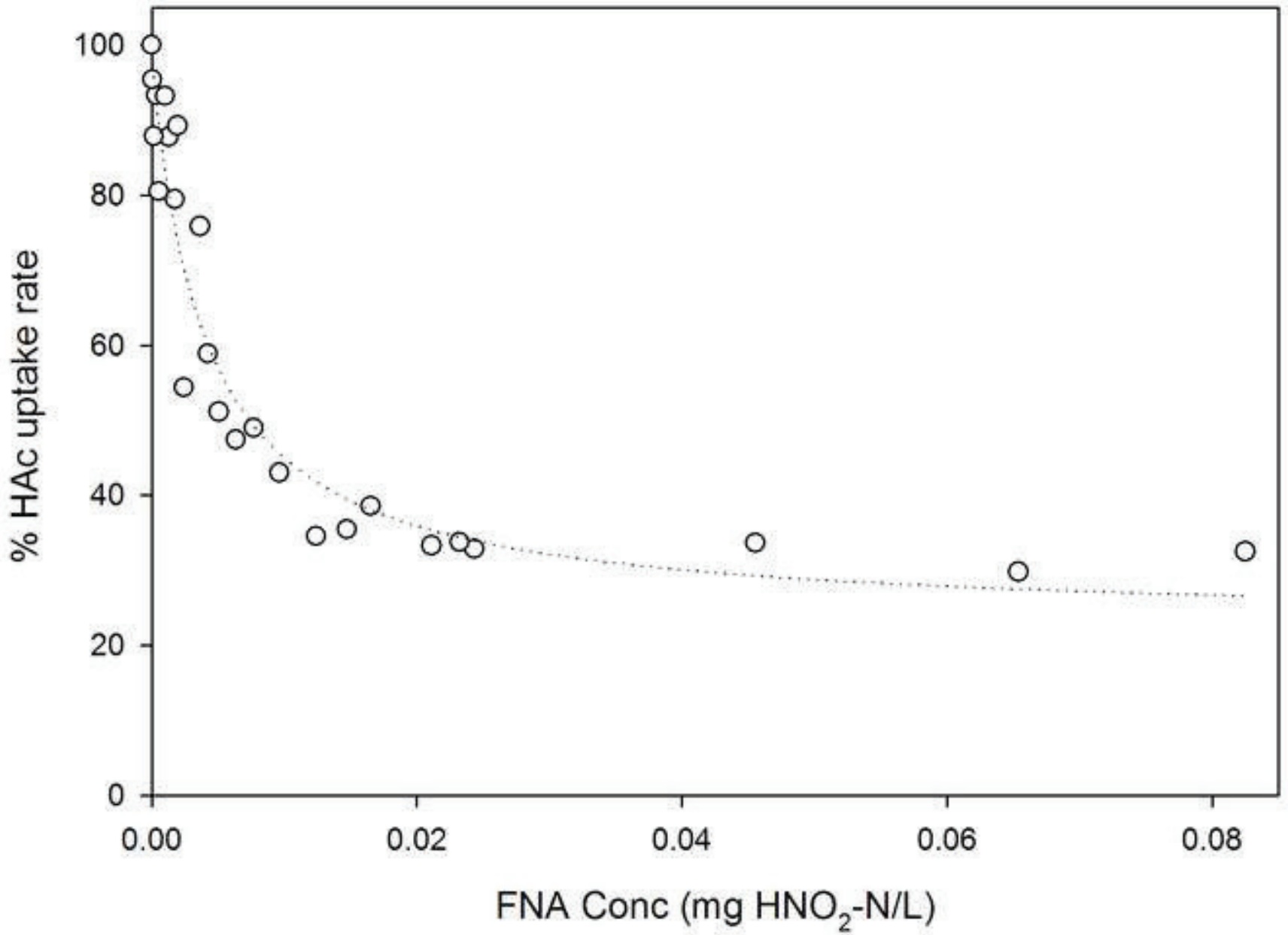

Figure 2
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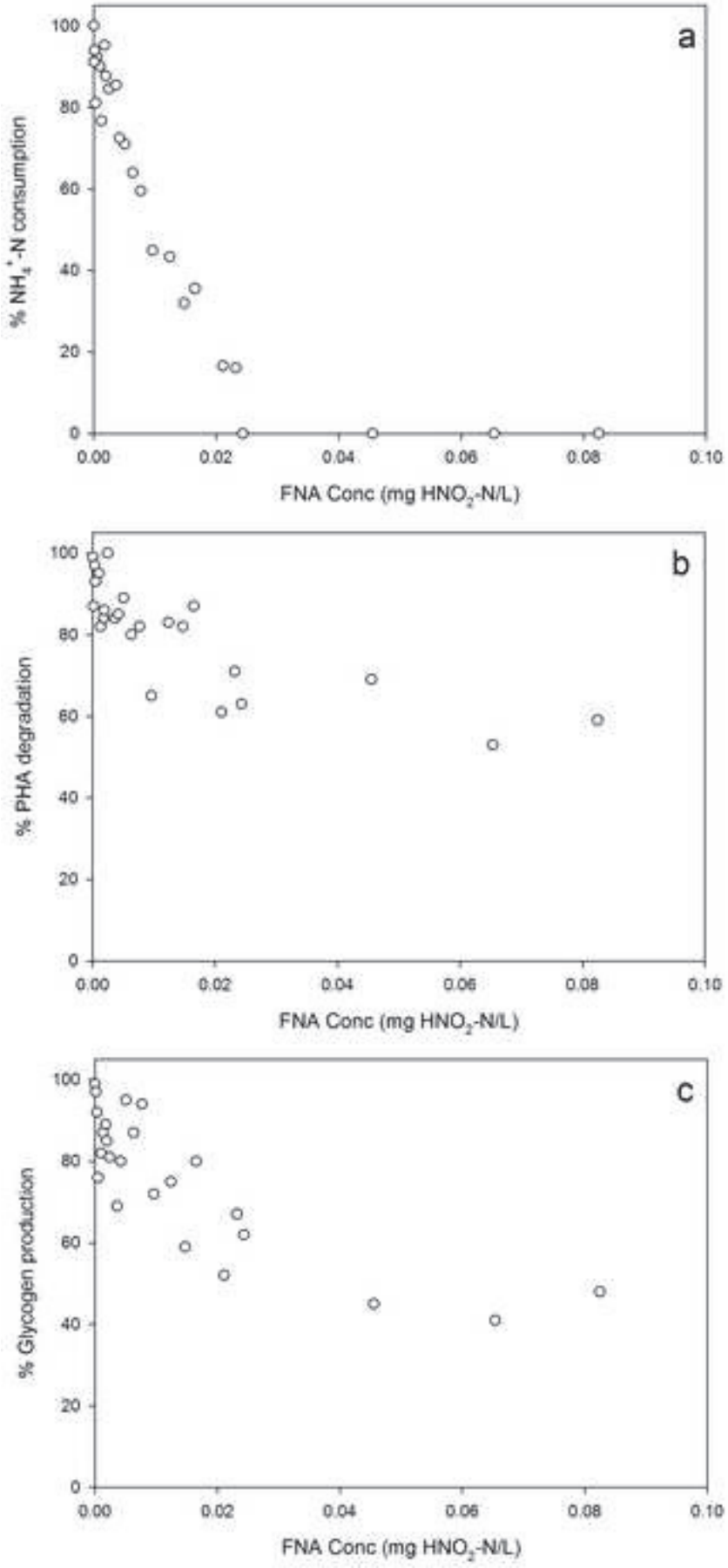

Figure 3

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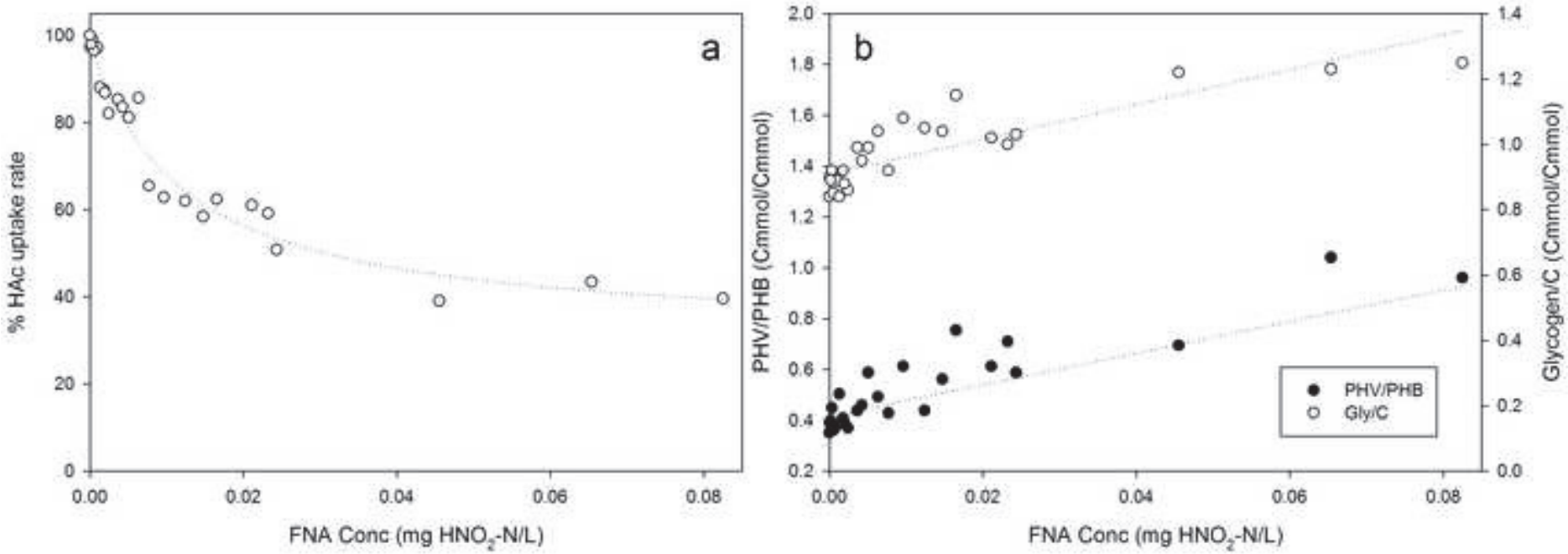

Figure 4

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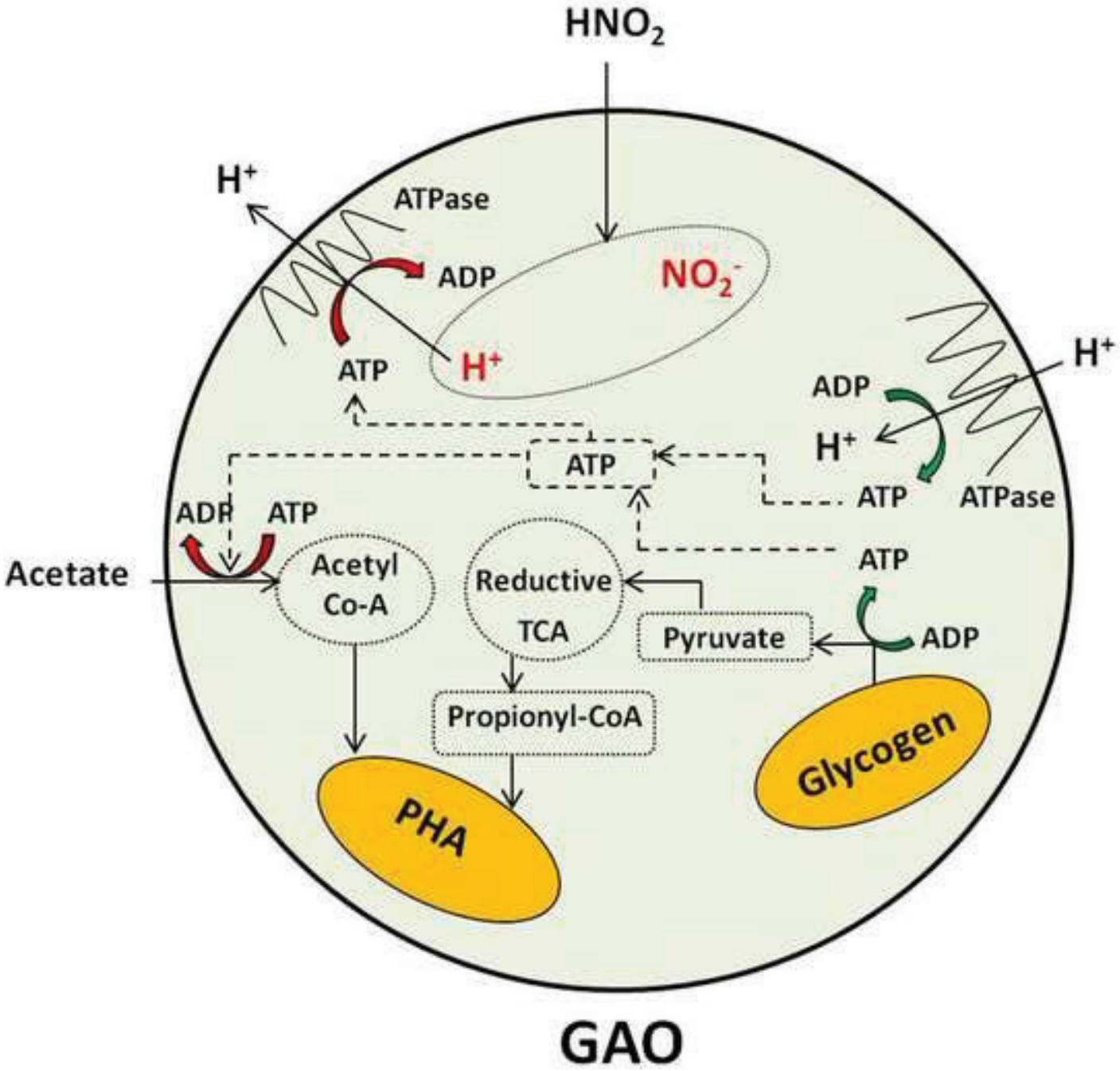

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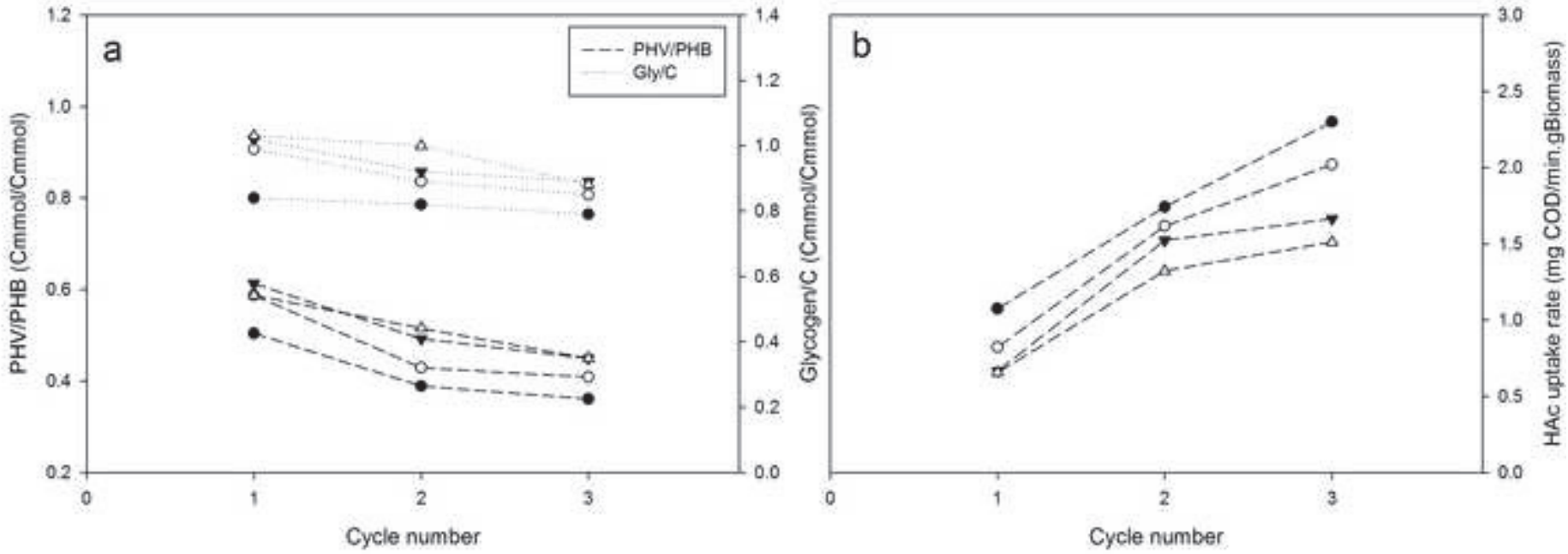

Figure 6
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