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# Effect of operational strategies on activated sludge's acclimation to phenol, subsequent aerobic granulation, and accumulation of polyhydroxyalkanoates

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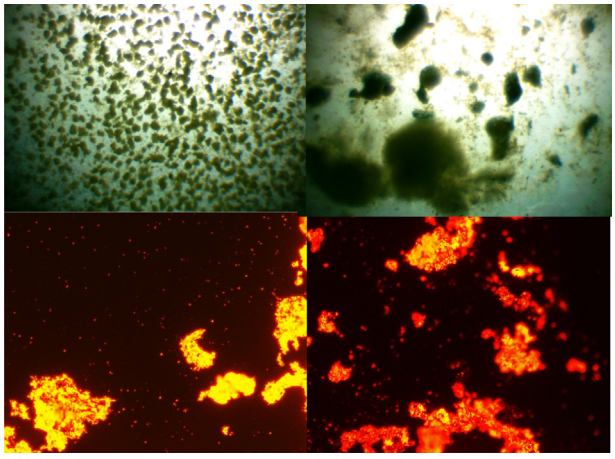
## HIGHLIGHTS

- Activated sludge was acclimated to phenol with 2 different strategies.
- Acclimated sludge later underwent aerobic granulation process.
- Sludge acclimated with phenol only degraded phenol and formed granules faster.
- Sludge acclimated with phenol + acetate formed more stable and robust granules.
- Both sludge exhibited significant PHA accumulation in early granulation stage.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Aerobic granules, a relative novel form of microbial aggregate, are capable of degrading many toxic organic pollutants. Appropriate strategy is needed to acclimate seed sludge to the toxic compounds for successful granulation. In this study, two distinct strategies, i.e. mixed or single carbon sources, were experimented to obtain phenol-acclimated sludge. Their effects on reactor performance, biomass characteristics, microbial population and the granulation process were analyzed. Sludge fed with phenol alone exhibited faster acclimation and earlier appearance of granules, but possibly lower microbial diversity and reactor stability. Using a mixture of acetate and phenol in the acclimation stage, on the other hand, led to a reactor with slower phenol degradation and granulation, but eventual formation of strong and stable aerobic

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granules. In addition, the content of intracellular polyhydoxyakanoates (PHA) was also monitored, and significant accumulation was observed during the pre-granulation stage, where PHA >50% of dry weight was observed in both reactors.



## 1. Introduction

Aerobic granular sludge (AGS) is a relatively new wastewater treatment technology that is receiving increasing research and application interests. AGS is usually formed in aerated column type sequencing batch reactors (SBRs) [1], and characterized by its round shape, compact structure, high density and settling speed [2]. AGS reactors have demonstrated remarkable ability to remove toxic and recalcitrant substances [3], which include phenol [4], *p*-nitro phenol [5], trichloroethylene [6] and halogenated phenols [7], and high concentrations and stressful loadings have been applied [8].

Some of these substances, e.g. phenol and halogenated phenols, though toxic are able to support microbial growth. Their toxicity is generally associated with concentration levels. For example, phenol, a pollutant found in many industrial effluents [9], can be biodegraded efficiently under low concentrations [10]. However it poses severe cytotoxicity at higher levels by affecting the mobility of cell membrane [11], showing typical substrate inhibition effect. Therefore removal of high strength phenol requires special measures. Some researchers adopted cell immobilization, i.e. formation of biofilm or granules, which improved cellular resistance to phenol toxicity [9,12,13]. In addition, whether as attached or free-moving cells, microorganisms can greatly benefit from a proper acclimation procedure to these substrates [10].

To adapt common activated sludge to a toxic but biodegradable substance, at least two strategies can be used. One is to provide it as the sole carbon and energy source, and stepwise increase the concentration. The other is to use a benign substance like glucose as the supplementary substrate, and gradually replace it with the toxic one [14]. These two strategies might have various impacts. Using the first strategy, it is possible that an over dramatic increase could lead to severe inhibition or even total reactor collapse [15]. On the other hand, including a benign substrate in the carbon sources can quickly increase the microbial population during early acclimation stages, providing better resistance against toxicity. However it might also hinder the acclimation process, as the sludge preferentially degrades the benign substrates, thus fails to develop the activity towards the toxic substances. Indeed both beneficial and adverse effects have been reported for the biogenic organic compounds [16].

Phenol degradation by aerobic granules has been studied before, using fully acclimated activated sludge as the seed [4,13]. However, in those studies the acclimation process was conducted rather tentatively, and little is known on its possible impact on the granulation process. Therefore in this study, it is intended to test the two strategies in parallel, to acclimate common activated sludge to phenol for aerobic granulation. The objective is to assess these two strategies, and obtain primary knowledge on their relative effects on the efficiency and extent of phenol degradation, the structure and physiological state of the microbial population, and the relative effects on subsequent granule formation. Successful application of aerobic granule in the removal of toxic substances can benefit from an efficient acclimation process and a stable resultant culture. Therefore this study also aims at providing guidance for further engineering application. In addition, accumulation of PHA has been briefly studied with aerobic granules [17,18]. This study also intends to investigate this phenomenon with a toxic compound as the growth substrate.

## 2. Materials and methods

### 2.1. Reactor setup and operational strategy

The study was carried out in two lab-scale SBRs ($R_1$ and $R_2$) with the height of 157 cm, inner diameter of 6 cm, and a working volume of 3 L. Aeration was provided by air stones placed at the bottom, at a rate of 5 L $min^{-1}$, resulting in a superficial upflow air velocity of approx. 3 cm $s^{-1}$. The whole operation was divided into two phases, i.e. acclimation and granulation phases. During acclimation, $R_1$ was fed with two carbon sources, sodium acetate (NaAC) and phenol, and $R_2$ with phenol only. The concentration of NaAC in $R_1$'s influent was gradually decreased and that of phenol increased, but their combined carbon loading (calculated as g TOC $d^{-1}$) was kept constant. Also kept constant were its cycle time (4 h), settling time (30 min per cycle) and volumetric exchange ratio (50%). In contrast, phenol of increasing concentration was applied in $R_2$, and the cycles were prolonged according to each increase of phenol concentration. Correspondingly, there were less cycles in a day, with longer settling time and higher exchange ratio per cycle. Throughout acclimation, the total settling time per day was kept constant at 180 min, and the hydraulic retention time was 8 h for both reactors. At the end of acclimation, the cycle length, settling time and exchange ratio of $R_2$ was adjusted to the same level as those of $R_1$, and the two reactors were operated identically. The detailed operational strategies and parameters are listed in Table 1.

After 36 days of acclimation, phenol concentration in the reactors when the cycles started rose from 50 mg $L^{-1}$ to 250 mg $L^{-1}$, thus achieving acclimation. Granulation was then initiated by gradually decreasing the settling time from 30 min to 2 min per cycle over a period of 7 weeks. During this phase, the reactors were operated identically, and phenol concentration at cycle beginning was further increased to 500 mg $L^{-1}$ in the last two weeks. The detailed conditions are listed in Table S1.

### 2.2. Seed and feeding medium

The reactors were seeded with activated sludge obtained from a domestic wastewater treatment plant in east Shanghai. Analytical grade chemicals and tap water were used to make the reactor influent, which contained the carbon sources, $NH_4Cl$, $KH_2PO_4$, macronutrients and trace elements, and buffer solution if necessary. Changes in the carbon sources can be seen in Table S2, while constant TOC/N ratio of 10:1 and TOC/P ratio of 15:1 (w/w) were applied throughout the cultivation, and macronutrients were supplied in the influent as (in mg $L^{-1}$): $CaCl_2$, 20; $MgSO_4$, 23.4; and $FeSO_4{\cdot}7H_2O$, 30. 1 mL stock solution of trace elements was added into per liter of reactor influent, whose detailed composition is also listed in Table S2. When pH of the reactor effluent dropped below 6.5, 10 mL 1 M $NaHCO_3$ was added into per liter influent as buffer.

**Table 1**
Operational strategies and parameters for $R_1$ and $R_2$ during the acclimatization phase.

| Strategy | $R_1$ | | | | $R_2$ | | | |
|---|---|---|---|---|---|---|---|---|
| | Two carbon sources, fixed cycle time | | | | Single carbon source, varied cycle time | | | |
| Operation time (d) | 1–7 | 8–18 | 19–27 | 28–36 | 1–7 | 8–18 | 19–27 | 28–36 |
| Cycle length (h) | 4 | 4 | 4 | 4 | 1 | 2 | 3 | 4 |
| Cycles per day | 6 | 6 | 6 | 6 | 24 | 12 | 8 | 6 |
| Influent volume per cycle (L) | 1.5 | 1.5 | 1.5 | 1.5 | 0.375 | 0.75 | 1.125 | 1.5 |
| Volumetric exchange ratio (%) | 50 | 50 | 50 | 50 | 12.5 | 25 | 37.5 | 50 |
| Influent time per cycle (min) | 10 | 10 | 10 | 10 | 7 | 7 | 7 | 10 |
| Aeration time per cycle (min) | 197 | 197 | 197 | 197 | 43 | 96 | 148 | 197 |
| Setting time per cycle (min) | 30 | 30 | 30 | 30 | 7.5 | 15 | 22.5 | 30 |
| Effluent time per cycle (min) | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 3 |
| Phenol concentration at cycle start ($mg\,L^{-1}$) | 50 | 100 | 180 | 250 | 50 | 100 | 180 | 250 |

### 2.3. Monitoring of reactor performance and biomass characteristics

Various parameters were monitored as indicators to the reactors' performance, including the influent and effluent pH, and phenol and chemical oxygen demand (COD) concentrations. Phenol was measured by a colorimetric method as described previously [12]. COD of centrifuged aqueous samples was determined colorimetrically after 2 h digestion at 150 °C with $K_2Cr_2O_7$. Sludge characteristics like sludge volume index (SVI) and specific oxygen utilization rate (SOUR) under various phenol concentrations were determined according to standard methods [19]. When measuring SOUR, a known amount of biomass was dispensed into a 200 mL dissolved oxygen (DO) bottle. Medium containing all nutrients except the carbon source was aerated until it was saturated with oxygen, and was then mixed with the biomass. The bottle was sealed with a DO meter, and the change of DO concentration therein was recorded at suitable time intervals as the endogenous respiration rate. Then various carbon sources, e.g. NaAc and phenol were added into the bottle, which was resealed and the rate was measured again. The measured $O_2$ consumption rate was divided by the SS content in the bottle to give SOUR.

For mixed liquor suspended solids (MLSS) in the reactor and effluent and PHA content in the biomass, 10 mL mixed liquor was taken near the end of a cycle and centrifuged (10000*g*, 10 min). The sediment was washed with 10 mL deionized water, and SS was measured after overnight drying at 105 °C. Subsequently the PHA content was determined by a successive digestion method [20]. The dried sludge was mixed with 10 mL sodium dodecyl sulfate (1% w/v pH 10), and shaken at 200 rpm, 37 °C until complete dispersion. The mixture was centrifuged again, and washed in turn by 20 mL sodium hypochlorite (active chlorine > 6%) and deionized water. Separation was achieved by centrifuge for 4 min at 7000*g*, and the residue dried for 24 h at 105 °C. The remaining mass was weighed and taken as the amount of PHA. In several occasions, the reactor effluent over 24–48 h was collected, and was used to measure the sludge retention time (SRT). The effluent was thoroughly mixed and 50 mL of it was taken to determine the MLSS content therein. The total MLSS in the effluent was then calculated, and SRT computed as the MLSS content in the reactor divided by MLSS in the effluent.

Biomass morphology was observed by digital camera imaging, and light microscopy after staining by PHA specific dye Nile Blue A [21]. Image processing software ERDAS and ARCGIS were used to analyze some of the images and computing the granules' sizes. To study microbial diversity, biomass samples were obtained regularly, and their genomic DNA extracted using a DNA Isolating Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), according to the manual provided by the supplier. The variable V3 region of the bacterial 16S rDNA was amplified by PCR with primers 8F 5′-GAGAGTTTGATCCTGGCTCAG-3′ with a GC clamp and 518 R (5′-ATTACCGCGGCTGCTGG-3′), on a BIO-RAD thermal cycler. 2–4 μL of the extracted DNA was used as the template in a 25 μL reaction mixture, which also contained 1.5 U of Lac Taq DNA polymerase, 2.5 μL of 10 × Lac PCR buffer, 2 μL of 10 mM dNTP mixture, 0.4 μL of the forward and reverse primer (10 μM each). PCR was started with 10 min activation at 95 °C. 35 cycles were then performed, with temperature program consisting of 1 min denaturing at 95 °C, 1 min annealing at 55 °C (−0.1 °C each cycle) and 1.5 min synthesis at 72 °C. Finally a 10 min extension step at 72 °C was set and the products were kept at 12 °C till collection.

The amplified products were separated by DGGE on 8% polyacrylamide gels with a linear gradient of 35–55% denaturant (100% denaturant contains 40% v/v formamide and 42% w/v urea) on a Dcode™ Universal Mutation Detection System (Bio-Rad, USA). Electrophoresis was run for 9 h at 120 V and 60 °C in 1 × TAE buffer. Selected DNA bands from the DGGE gels were excised, purified by Axygen DNA extraction kit (USA) and re-amplified by the same PCR protocol described above, but with 8 F primer (no GC clamp). Cloning and sequencing were performed on the DNA products according to previous work [22,23] (Sangon Biotech, China). The sequences were then analyzed by BLAST search in the GenBank for species identification.

## 3. Results and discussion

### 3.1. Overall reactor performance and biomass morphology change

The primary aim of this study was to acclimate common activated sludge to high concentration of phenol, therefore the ability of phenol removal is a good indicator to the efficiency of acclimation. In addition, several arbitrary criteria were also included as indications that the operation was successful and could proceed to the next stage. These criteria were: 1st, phenol provided to the reactors was totally degraded for at least 6–7 days; 2nd, organic substances in the effluent (in terms of COD) were constantly low; 3rd, biomass in the reactors was healthy and it SOUR on phenol was on a reasonable level; 4th, biomass settles normally and no significant washout was observed. Therefore the influent and effluent supernatant phenol and COD concentrations were regularly monitored. In day 1–5, residual phenol was detected in both $R_1$ and $R_2$′ effluents ($R_2$ higher than $R_1$), but was generally lower than $0.5\,mg\,L^{-1}$ ever since. Similarly effluent COD stabilized at around 30–40 $mg\,L^{-1}$ from the 2nd week on, with minor fluctuations. Small rise of effluent COD could sometimes be observed after increase of phenol loading or decrease of settling time, which usually dropped back to lower levels within 2–3 days. This might indicate a minor shock to the biomass and its immediate adaptation. The detailed data, including those of influent and effluent pH are shown in Fig. S1. The corresponding phenol and COD removal were >99% and around 95% for both reactors during most time of the operation, indicating the acclimation strategies

were successful in terms of the degradation of carbon sources and removal of phenol.

In terms of changes in biomass morphology, Fig. S2 shows several critical stages in the general process of shifts from flocs to small granule and further to bigger granules, in both $R_1$ and $R_2$ by digital imaging. No significant difference was observed in the first 40 days between the two reactors, where yellow pinpoint flocs dominated (see image on day 28 as an example). However on the images of day 46 (Fig. S2), 10 days after granulation phase started, small granules were observed in $R_2$, meanwhile $R_1$ harbored only flocs. Thus it seems that $R_2$ started granulation earlier than $R_1$, which might be correlated to the toxic substrate (phenol) used as its sole carbon source. When faced with environmental stresses like toxic substances, microorganisms often form aggregates (e.g. biofilm), as a barrier against various toxicities [24]. Such could be the case in this study, where repetitive applications of phenol stressed the microbial populations and promoted them to form granular structure. In a previous study, phenol degraders were isolated from mature aerobic granules, and their phenol degradation kinetics were determined and compared to those of the seed granules. It was found that the inhibition constant, $K_I$, of the whole granules was much higher than those of the individual isolates. Thus it was suggested that granular structure could protect biomass from phenol toxicity, especially at higher concentrations [25].

In the next 30 days, granules continued to develop in both reactors. Images on day 75 (Fig. S2) show that granules in $R_1$ were still in formation, but those in $R_2$ had become bigger and distinct. Some images from then on were used to measure the sizes of granular biomass, and the results are presented in Fig. S3(a). However, around that time the reactors went through some disturbance (power cut for 24 h), after which $R_2$ reactor rapidly deteriorated. In the last 10 days, small but robust granules eventually formed in $R_1$, while $R_2$ experienced severe fungi outgrowth, and the few remaining granules were white and fluffy (see images of day 84 and 85). Meantime $R_2$'s effluent quality also worsened, which became yellow and turbid, and significant amount of biomass was observed therein. The yellow color was most likely caused by phenol's degradation intermediate, 2-hydroxymuconic semialdehyde [26], and Fig. S1 also shows that at the end of operation phenol concentration in $R_2$'s effluent rose to over 50 mg $L^{-1}$. These phenomena suggest that the phenol degradation ability of $R_2$ was greatly reduced, and unconsumed substrate and intermediates accumulated in the reactor's bulk liquid. Changes in biomass morphology and loss of phenol degradation ability occurred simultaneously, and very possibly correlated. Though it was not clear which feature was the cause, they both suggested that $R_2$ had failed. The operation was therefore terminated at that stage. As these two reactors were operated in parallel all the time, it would seem that $R_1$ was more resistant to the reactor disturbance, e.g. absence of aeration and starvation from phenol. It was possible that $R_1$'s acclimation strategy generated biomass that was more stable and robust, which benefitted the later aerobic granulation process.

### 3.2. *Effect of acclimation strategy on general biomass characteristics*

The changes of reactors' MLSS concentrations are presented in Fig. 1, which showed a parallel trend in the first 3 weeks. From then to the end of acclimation, $R_2$ constantly exhibited a higher accumulation of biomass than $R_1$. As the onset of granulation in $R_2$ was earlier than $R_1$, it was possible that its improved sludge structure contributed to better retention of biomass. This hypothesis was collaborated by the data on SVI, which was measured as an indicator of the sludge's settling ability (results also shown in Fig. 1). SVI of the seed activated sludge was around 200 mL $g^{-1}$, which dropped dramatically to 50–100 mL $g^{-1}$ in the first 10 days. This was probably

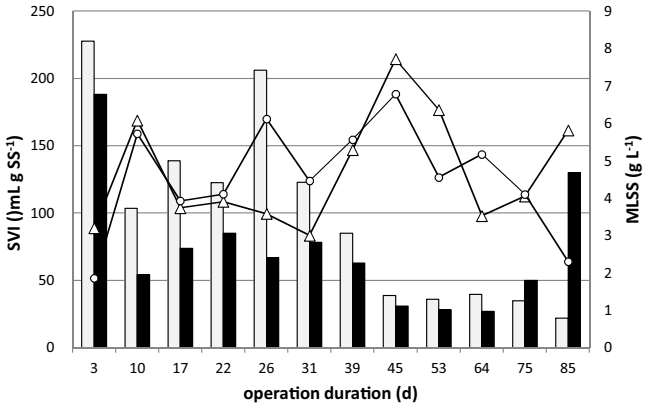


**Fig. 1.** Changes of reactors' MLSS and SVI during operation. (Δ) $R_1$ MLSS; (○) $R_2$ MLSS; (□) $R_1$ SVI; (■) $R_2$ SVI.

caused by the harsh operational conditions applied (high turbulence, short settling time) as compared to common wastewater treatment plant. As a result, disintegration of activated sludge flocs can occur and cause rapid biomass washout, and the remaining sludge had to settle fast enough to be retained in the reactors. However, as the sludge adapted, biomass slowly accumulated, and SVI rose again to the end of acclimation. During this period $R_1$'s SVI was always significantly higher than that of $R_2$, as its MLSS was lower. This indicates that $R_2$'s acclimation strategy (single toxic carbon source, shorter cycle, frequent feeding) was more effective to improve biomass' settling ability, which benefits MLSS accumulation and possibly acclimation.

From day 10 to the end of acclimation phase, $R_1$'s MLSS fluctuated between 4.5 and 6.5 g $L^{-1}$, while that of $R_2$ showed a continuous decline until day 31. Subsequently both increased dramatically and reached peak values on day 45. Day 45 was in the first stage of granulation (day 37–50), when a relative long settling time (15 min) was still employed. By this time the biomass had very likely fully acclimated to the toxic substrate and reactor environment, and its settling ability was moderately good (SVI 123 and 78 mL $g^{-1}$, respectively). Therefore it was possible that such a combination led to best retention of biomass in the reactors, which laid good foundation for the subsequent aerobic granulation. When settling time was further decreased, MLSS declined in the granulation phase in both reactors, probably caused by biomass washout. However after reactor disturbance, $R_1$ quickly recovered and its MLSS rose to >6 g $L^{-1}$ at the end. In contrast, $R_2$ continued to decline, and its final failure was accompanied by an MLSS only slightly over 2 g $L^{-1}$. In this phase, SVI in both reactors further dropped to around 20–40 mL $g^{-1}$. $R_2$ still performed better than $R_1$, but the difference was much smaller, suggesting rapid improvement of $R_1$'s biomass settling ability. Reactor disturbance did not significantly influence $R_1$, and its SVI continued to decline to 22 mL $g^{-1}$ on day 85. However biomass deterioration and fungi outgrowth in $R_2$ caused its SVI to rise to 130 mL $g^{-1}$ in the end, resulting in significant biomass washout and deterioration of effluent quality.

The sludge retention time (SRT) was measured on several occasions, and the results shown in Fig. S3(b) collaborated well with the observations above. On day 33 (near end of acclimation), SRTs in $R_1$ and $R_2$ were 1.1 and 4.3 days, respectively, and MLSS in $R_1$'s effluent was significantly higher than that of $R_2$. It seems that in the acclimation phase, $R_1$'s biomass adopted a strategy of "fast growth-high washout", while $R_2$ was characterized by better biomass accumulation and less discharge through effluent. However when granulation started, the trend was reversed and $R_1$'s SRT significantly increased. SRT in $R_2$ also rose to 7.2 days on day 59. After the disturbance, $R_1$ showed higher stability than $R_2$. Con-

**Table 2**
Identification of some unique populations in $R_1$ and $R_2$'s sludge at the end of operation.

| Band | Closest relative | Similarity | Closest relative accession no. | Reference |
|---|---|---|---|---|
| I | *Delftia acidovorans* strain SPH-1 | 100% | NR_074691.1 | [39] |
| II | Uncultured actinobacterium clone A-C2F-D09 | 99% | AY307871.1 | [40] |
| III | Uncultured bacterium clone A21 | 100% | HM007533.1 | [41] |
| IV | Uncultured bacterium clone HW_59 | 100% | KR080931.1 | [42] |
| V | Uncultured *Sphingomonas* sp. clone AGS_GMP7 | 99% | KM209211.1 | [43] |
| VI | Uncultured bacterium clone UE05 | 96% | AB456238.1 | [44] |
| VII | Uncultured beta proteobacterium clone GC0AA9ZH02PP1 | 100% | JQ919449.1 | [45] |
| VIII | Uncultured unclassified bacterium clone QEDR3CD12 | 97% | CU922343.1 | [46] |

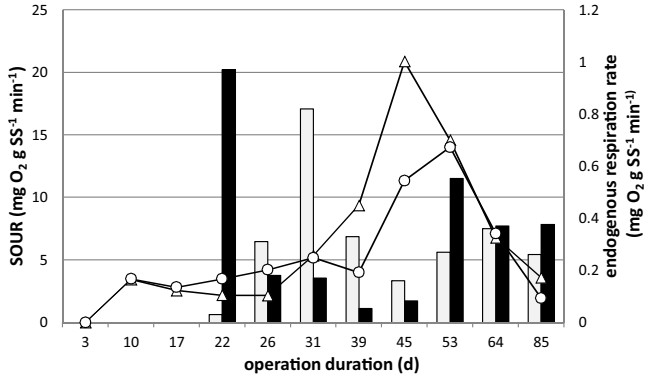


**Fig. 2.** Change of SOUR and endogenous respiration rate of the biomass. (△) $R_1$ SOUR; (○) $R_2$ SOUR; (□) $R_1$ endogenous respiration rate; (■) $R_2$ endogenous respiration rate.

tinued granulation in $R_1$ resulted in an SRT of 20.5 days at day 80, corresponding to the formation of fast settling granules, while that in $R_2$ dropped to 1.5 days, indicating collapse of the sludge.

### 3.3. Substrate degradation activities

SOUR indicates biomass' activity in taking up certain substrates, but is much easier and faster to measure, and has high sensitivity [27,28]. Therefore biomass' SOUR on phenol was measured regularly as the indicator of its activity in phenol degradation, and its changes are shown in Fig. 2 together with those of endogenous respiration rates (ERR). The phenol concentrations applied in SOUR measurement equaled to what the biomass encountered at cycle beginnings at that point in operation, i.e. 50 for day 3 and 10, 100 for day 22, 180 for day 26, 250 to day 64, and 500 mg $L^{-1}$ on day 85, therefore more closely simulating the real cycles in the reactors. SOUR on phenol first increased then decreased during the whole operation, with the peak values appearing around 45–55 days, first stage in the granulation phase. Noting that MLSS also peaked around the same time, it seemed that the pre-granulation stage was a critical point, which was characterized by dramatic and sudden changes. In a previous study [13] where phenol was used as the substrate for aerobic granulation, a same trend was also observed. The pre-granulation phase was characterized by rapid buildup of both SOUR and biomass, which would suggest that phenol was consumed very quickly in the reactors, resulting in long starvation time in a cycle. Rapid shifts of feast and farming regime, as well as significant starvation are proposed to be crucial factors in stimulating granule formation [29], which might be related to these observations. When granules were formed and matured, the SOURs in both cases dropped to a much lower but stable level. Researchers studying biofilm processes suggest that the biofilm formation is often accompanied by formation of inert biomass and occurrence of diffusion limitation of both oxygen and substrate [30]. Such phenomena would result in lower observed specific substrate degradation activity and oxygen uptake rate, which bears similarity to the present situation. In a previous study [31], the total biomass from a phenol degradation AGS reactor was sifted to various size fractions. It was discovered that the flocs and small granules fractions degraded phenol much faster than bigger granules, suggesting the occurrence of diffusion limitation in the granules. However it is not clear if the dramatic buildup of biomass and SOUR immediately before granulation were unique features to toxic substrates like phenol, or universal in aerobic granulation on other benign substrates like glucose and acetate.

In terms of the individual reactors, $R_2$'s SOUR was higher than $R_1$ during acclimation, which was understandable as phenol was the only carbon source in $R_2$. However when granulation started, $R_1$ rapidly improved and its SOUR surpassed that of $R_2$ on day 45. Therefore $R_1$ showed high adaptability, as although it was fed dual substrates during acclimation (one of them benign), its phenol uptake ability was rapidly enhanced once the acclimation was completed. In the later granulation phase, the SOURs of both reactors dropped in parallel, and at the end of operation had the values of 3.57 and 1.93 mg $O_2$ g $SS^{-1}$ $min^{-1}$ ($R_1$ and $R_2$ respectively, on 500 mg $L^{-1}$ phenol). Though $R_1$'s SOUR was moderate, combined with its high biomass content it was able to totally remove phenol from the reactor. In contrast, the biomass in $R_2$ not only experienced significant washout, but also lost much of its phenol degrading ability, which resulted in residue phenol in the effluent.

In some cases, SOUR was also measured with several different phenol concentrations (50 and 100 mg $L^{-1}$ on day 22; 50, 100, 180 mg $L^{-1}$ on day 26), and the results are shown in Fig. S4(a). On both days (late acclimation phase), $R_2$ exhibited higher phenol-dependent SOUR than $R_1$, under all phenol concentration levels tested. This agrees well with the previous inference, that employing single carbon source in $R_2$ enabled it to develop phenol degradation activity faster. Both reactors exhibited higher SOURs on day 26 than on day 22, on the corresponding phenol levels, indicating the progression of acclimation. However the degree of improvement was more significant for $R_2$ than $R_1$. In addition, for both reactors, higher phenol concentrations resulted in lower SOUR, indicating a Haldane type kinetics [25], where the substrate uptake rate is inhibited by high substrate concentrations above a threshold level. However $R_1$ showed lower level of inhibition than $R_2$. On day 22, $R_1$'s SOUR decreased from 2.66 to 2.17 mg $O_2$ g $SS^{-1}$ $min^{-1}$ when phenol concentration was increased from 50 to 100 mg $L^{-1}$. The percentage of decrease was therefore 18.4%, while the corresponding figure for $R_2$ was 37.5% (5.57–3.48 mg $O_2$ g $SS^{-1}$ $min^{-1}$). Similarly on day 26, on phenol levels 100 and 180 mg $L^{-1}$, $R_1$ exhibited SOUR drops of 19% and 28%, respectively, from 50 mg $L^{-1}$ phenol. Meanwhile the decreases were 25% and 47.3% for $R_2$. It would seem that $R_1$, though slower in developing phenol degradation activities, was more robust and resistance to high phenol concentrations.

As sodium acetate was used as the supplementary substrate in the acclimation phase, SOURs on acetate and/or phenol was measured on day 39 (shortly after acclimation), and the results are shown in Fig. S4(b). The measurements were done in two sets, in the first of which acetate was added to the biomass first, and phenol was added later after the first SOUR was measured. The second set

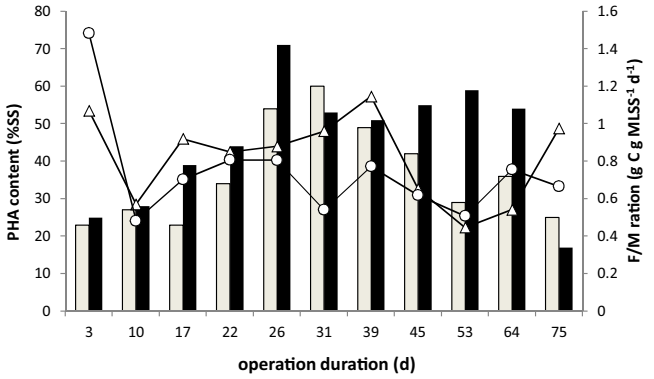


**Fig. 3.** Changes of biomass PHA content and F/M ratio in the reactors. (△) $R_1$ F/M ratio; (○) $R_2$ F/M ratio (□) $R_1$ PHA content; (■) $R_2$ PHA content.

employed phenol first, followed by acetate addition. The aim was to see which of these two substrates was taken up faster, and if they interfered with each other. It was observed that at this point $R_1$ had higher SOUR than $R_2$, and both reactors exhibited preferential intake of phenol than acetate. Indeed SOUR on 650 mg $L^{-1}$ sodium acetate was negligible compare to that on 250 mg $L^{-1}$ phenol, indicating the success of both acclimation strategies. Moreover, when phenol degradation is already underway, adding acetate to the culture did not significantly interfere with phenol SOURs (comparing the 2nd and 3rd columns). On the contrary when phenol was added after acetate, SOUR was dramatically enhanced to a level similar to that measured with phenol alone. This implies that acetate was not taken up and/or degraded by the biomass. However, acetate is a simple and non-toxic fatty acid, which should be an easy carbon source for aerobic microbes to assimilate and degrade. Why the phenol-grown biomass exhibited such low activity towards the benign substrate is not exactly clear, and could be an interesting point to explore. It was possible that phenol toxicity had something to do with it. As mentioned in the introduction, phenol poses toxicity to cells by affecting their membrane fluidity. In response to this toxicity, some microorganisms actively modify their membrane components to increase the rigidity of its structure [32]. Such changes might also affect the uptake efficiencies of some other substances, e.g. acetate, therefore hinder their degradation and the dependent oxygen utilization.

### 3.4. PHA accumulation and F/M ratio

Fig. 3 shows PHA contents in the biomass and the corresponding food/microbes (F/M) ratio at that point, while visual confirmation of intracellular PHA by fluorescent staining is presented in Fig. S5. After staining with Nile Blue and excited with green light, PHA showed as bright orange clusters under fluorescence microscope. PHA content was quantified by a gravimetric method, and was found to increase significantly in the acclimation phase, while granulation saw its general decline. PHA content in $R_2$ was generally higher than $R_1$, except at the end when $R_2$ sludge failed, indicating that phenol is an effective substrate for PHA synthesis. The highest value appeared around day 26 to day 31, when PHA contents in both reactors reached over 50% MLSS (w/w), and abundant oily brown colored residue was observed after biomass digestion (data not shown). In most stages of granulation $R_2$'s sludge still sustained high PHA levels, meanwhile $R_1$ showed a gradual decrease of PHA with granulation, and the granules finally formed had PHA content of around 25%. It is not clear if formation of aggregates affects microorganism's capacity for PHA accumulation, but a previous study [33] reported that in an acetate-fed nitrogen limiting culture of very low SRT, biofilm formation caused loss of most of its PHA storage capacity.

Many studied have focused on PHA production from various carbon-rich streams, usually using acetate as a model carbon source. However few reports are available on PHA accumulation in microbial aggregates, and even less using toxic compounds like phenol as the substrate for PHA accumulation. Recently a few researchers have studied PHA accumulation in aerobic granules both in reactors and batch conditions, with ethanol or sodium acetate as the carbon source [17,18,20]. The resultant PHA contents were in the range of 18–44% biomass (w/w), which agree well with the observation in this study. On the other hand, Maskow and Babel [34] operated a pure suspended culture reactor continuously fed with phenol, and obtained a PHA content of around 50% of cell dry mass. A more recent study tested 5 kinds of alkylphenols as possible carbon sources for PHB production by a pure culture *Bacillus* sp. CYR1 [35]. Phenol was identified as the substrate resulting in the highest PHB accumulation (51%).The maximum results obtained in this study was comparable, though mixed cultures were employed. Although pure cultures have the advantage of possibly higher maximum PHA content, mixed cultures are easier and cheaper to maintain, and more robust and versatile under changing operational conditions. This study exhibited, for the first time to the authors' knowledge, that mixed cultures can be used to produce PHA from toxic substances like phenol. High concentration was tolerated, degradation was complete and the high PHA level could be sustained for a long period during nearly 3 months of operation.

PHA can be accumulated by cultures experiencing shifting aerobic/anaerobic conditions, and also by aerobic cultures operated under SBR mode where the substrate concentration changes dynamically [36]. In the latter case the feast/famine regime is believed to be responsible, as microorganism adopt PHA storage as a strategy for faster substrate uptake when it is in abundance, to be used in the famine stage for sustained growth. In this case the ratio of carbon amount to biomass at the beginning of each cycle could be important, as PHA is accumulated when carbon is in excess (compared to growth need). F/M ratio is therefore calculated as the daily dosage of carbon divided by the biomass content in the reactor, and its value is shown in Fig. 3 too. F/M varied between 0.4 and 1.6 g C g $MLSS^{-1}$ $d^{-1}$ throughout the operation, and its trend was generally in parallel to the change in PHA content in the acclimation phase, except in the first week. This agrees well with the general principle that excessive carbon can be stored by cells for maintenance and growth during the famine period, therefore high F/M promotes accumulation of PHA. However in the granulation stage, $R_2$ showed an unusual correlation of PHA content and F/M, while that in $R_1$ was normal. One possible reason is that aerobic granules have different microbial ecology and PHA storage from suspended cells. Conversely some researchers also reported that under low substrate levels, microbes might enhance their PHA synthesis ability to gain extra competitive advantage in a consortium [33,37]. This could also be the mechanism for this observation, but the exact reason is not clear and more in-depth study is underway.

### 3.5. Effect of acclimation strategy on microbial diversity

The microbial diversity was preliminarily studied via molecular biology methods, and Fig. S6 shows the results of DGGE analysis. Phenol acclimation caused distinctive shifts of microbial population from the seed sludge in both reactors. At the end of acclimation phase, $R_1$ seemed to harbor more diverse microbial populations than $R_2$ (lanes 6 and 11, respectively), though much similarity was still observed. Though preliminary, this suggests that using dual substrate, one benign and the other toxic, during acclimation might help to preserve higher microbial diversity in the biomass. Such a culture, though slower in developing toxic substrate degradation

activities, is more versatile and stable, as various microbial populations can grow out and dominate when the operational conditions are changed. Using a single toxic carbon source and more frequent feeding, on the other hand, can obtain an acclimated culture more rapidly. However it might also pose too much stress in the early stages of operation, as initially only a small fraction of the seed activated sludge had innate phenol degradation ability. Non-phenol degraders face dual challenges of harsh conditions and toxic substrate, and were fast washed out. Loss of their genetic diversity might in turn compromise the robustness of the consortium.

Granulation further increased the difference between $R_1$ and $R_2$'s populations, and distinct bands appeared in individual reactors at the end of operation. Some of these bands were further identified and the results are listed in Table 2. Bands I–V were dominant in $R_1$'s sludge, while VI–VIII were observed with abundance only in $R_2$. Results indicate that bands II and IV were microbial populations found in natural river and sediment environments [39,41], while bands I and III were characterized by their degradation activities [38,40], especially that of aromatic substances [38]. In addition, band V was found in the aerobic granulation process [42]. On the other hand, bands VI to VIII were also associated with the degradation of phenolic and big molecule compounds [43–45], but usually under anaerobic conditions. It suggests that the formation of filamentous granules in $R_2$ might have led to diffusion limitation inside the granules and formation of anaerobic cores, a phenomenon noted by previous researchers [46].

## 4. Conclusion

Two distinct acclimation strategies were used to adapt activated sludge to high concentration of phenol, which was later subject to aerobic granulation process. Acclimation strategy was found to significantly affect biomass' general characteristics, phenol degradation activity, and granulation efficiency. Reactor fed with phenol only exhibited faster acclimation and granulation, but lower microbial diversity and stability. In contrast, reactor fed with a combination of acetate and phenol in the acclimation stage formed granules more slowly but was more robust and resistant to operational disturbance. PHA accumulation by phenol-grown biomass was found to peak at the point just before significant granule development, and a content of over 50% dry weight was detected for 40 days. This value was comparable to previously reported results by phenol and aromatics grown pure cultures, and demonstrated that mixed cultures can be employed to produce PHA from toxic substrates.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.05.074.

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