



Enhanced nutrients removal resulting from energy metabolism improvement in the anoxic-anaerobic-oxic process (reversed AAO)

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ABSTRACT

Comparison of energy metabolism between the conventional anaerobic-anoxic-oxic (AAO) process and the anoxic-anaerobic-oxic process (reversed AAO) was carried out. Although in terms of biological nutrient removal, both processes offered excellent removal efficiencies for organic matter and total phosphorus, there was a significant difference in regards to nitrogen removal. It was found that the ammonia nitrogen in the effluent in the AAO and the reversed AAO processes was 3.45 ± 0.64 and 1.53 ± 0.48 mg L⁻¹, respectively, with an influent ammonia nitrogen of 23.22 ± 1.77 and 23.07 ± 1.57 mg L⁻¹, respectively (Mixed liquor suspended solids (MLSS): $3,000 \pm 500$ mg L⁻¹ and HRT: 12 h). This study investigated whether reversing the anaerobic and anoxic compartments could change the microbial activity and eventually promote efficient microorganism removal ability. Several key enzymatic indicators such as, 2,3,5-triphenyl tetrazolium chloride dehydrogenase activity (TTC-DHA), 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride electron transport system (INT-ETS), and adenosine triphosphate were measured and confirmed the improvement in energy metabolism in the reversed AAO process. Owing to the increased microbial activity, both processes showed identical phosphorus removal efficiency while less carbon source was consumed in reversed AAO process. Therefore, more carbon sources can be used for denitrification because of the highly efficient phosphorus removal mechanism. These findings highlight the improved removal capacity of reversed AAO process, demonstrating its real applicability.

Keywords: Reversed AAO; Microbial activity; Energy metabolism; Dehydrogenase activity; Electron transport system; Adenosine triphosphate

1. Introduction

In recent decades, booming industrialization and urbanization have caused water resources to deteriorate [1]. Freshwater lakes are suffering from eutrophication due to a massive input of nutrient elements such as nitrogen and phosphorus [2,3]. As such, the wastewater treatment plant (WWTPs) processes (e.g., oxidation ditch, sequencing batch reactor, and anaerobic-anoxic-oxic or AAO) were developed to improve the removal of nitrogen and phosphorus.

For example, the AAO process, which was developed for the simultaneous biological removal of nitrogen and phosphorus from wastewater, was shown to have high efficiency and stability, low energy consumption, and relatively low operational costs compared with old technologies [4–6]. In this process, phosphate-accumulating organisms (PAOs) dominate the phosphorus removal efficiency, it stores carbon source (mainly volatile fatty acids) from wastewater in the form of polyhydroxyalkanoates (PHAs) with the release

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of phosphorus [7]. In the subsequent oxic compartment, PHAs can be oxidized as energy for adsorbing phosphorus in excess. For nitrogen removal, first, ammonia is oxidized to nitrate by ammonia-oxidizing bacteria and nitrite-oxidizing bacteria in a two-step process. Second, after recycled to the anoxic compartment, nitrate is eliminated by transforming into gas and exits the atmosphere in the anoxic compartment. However, in the face of stricter wastewater treatment standards, the AAO process shows some deficiencies. For example, the limited carbon source in the wastewater is one influencing factor because both phosphorus and nitrogen removal depend on the autotrophic bacteria, which consume carbon source. To solve this problem, Zhang and Gao [8] developed a new AAO process to improve wastewater treatment without adding more carbon sources by placing the anoxic compartment before the anaerobic compartment, namely reversed AAO process (from anaerobic-anoxic-oxic to anoxic-anaerobic-oxic) [9,10]. The reversed AAO process costs 19.4% less energy consumption in WWTPs than AAO process while attains a comparable performance in the removal of organic matter and nitrogen and better efficiency in phosphorus removal [8,10,11]. As a result, currently more than 100 WWTPs in China use the reversed AAO process for enhanced nutrient removal [12,13].

Although widely adopted in WWTPs, few studies have investigated the metabolic microorganism changes using the reversed AAO process, which is crucial for explaining the enhanced nutrient-removal mechanisms. In this study, we hypothesized that shifting the sequence of the anaerobic and anoxic compartments can exert significant influence on microbial metabolism and activity. Correspondingly, several key enzymatic indicators such as 2,3,5-triphenyl tetrazolium chloride dehydrogenase activity (TTC-DHA), 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride electron transport system (INT-ETS), and adenosine triphosphate (ATP) were assessed for the changes of microbial activity [14–16]. TTC-DHA is widely used as an indicator of bacterial respiration and can quantify the bioactivity of activated sludge [17–19]. Dehydrogenases are oxidation–reduction enzymes that participate in the transport of electrons from the organic substrate to final electron acceptors [20]. Thus, dehydrogenase activity can reflect the strength of microbial respiration [21]. ETS is an electron transport system that reflects microorganism activity. The electron transfer rate in the microbial respiration chain indirectly indicates the activity of microbial respiration and then quantifies the biological activity of activated sludge. In microbial respiration, electrons are transferred to a natural terminal electron acceptor through the ETS. Tetrazolium salts such as 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride are used as ETS indicators [22]. Microorganisms present in activated sludge, mainly bacteria, use ATP as their energy currency, which is instrumental in driving all cellular activities [23]. The purpose of this study, therefore, is to test the hypothesis that the shifting in the reversed AAO process can improve microbial activity and eventually benefit the process more efficiently in utilizing carbon sources. Furthermore, although fewer carbon sources can be used for phosphorus release in anaerobic compartment, directly entering oxic compartment can maintain an identical phosphorus removal efficiency with AAO process.

2. Materials and methods

2.1. Bioreactor

Two lab-scale AAO and reversed AAO systems were established in parallel as shown in Fig. 1. The working volume for each system was 64 L, with 16 L of anaerobic compartment, 16 L anoxic compartment, and 32 L oxic compartment. The AAO system consists of anaerobic-anoxic-oxic compartments in sequence (Fig. 1a). The reversed AAO process adjusted the anoxic and anaerobic compartment, with the sequence of anoxic-anaerobic-oxic compartment. Meanwhile, the reversed AAO process removed the mixed liquor recirculation, but it did show a different rate of returned sludge (Fig. 1b).

A stirrer was used to mix the anaerobic/anoxic compartments, and an aerator was used to provide aeration in the oxic compartment. The hydraulic retention time was maintained for 12 h for both processes. The two processes were kept in operation for more than 150 d. Water samples and sludge samples were taken after both processes reached to a steady state (after day 120). Other operating conditions are listed in Table 1.

To figure out the levels of various parameters at different reaction times in one cycle, jar tests were carried out at the same time. The jar tests were conducted with the same system parameters as the lab-scale bioreactors, but reflected the differences between the two processes more precisely. The sludge in the jar test was inoculated with activated sludge from the two bioreactors on day 150. Nitrate was added in the sludge in the reversed AAO jar test under anoxic condition and stirred 3 h to stimulate the anoxic and anaerobic conditions. To make sure the jar of the AAO process was under anaerobic conditions, the sludge was elutriated in the first place to eliminate the nitrate. After 1.5 h of reacting, nitrate was added into the jar to stimulate the mixed liquor recirculation in the anoxic compartment. After 3 h of mixing, aeration by aerator was performed for another 3 h in both AAO and reversed AAO jar systems. Plastic wrap was used to isolate the jars from the outside.

2.2. Synthetic wastewater

The synthetic wastewater was made as follows (per 120 L): full cream milk powder, 10.85 g; NaAc·3H₂O, 13.36 g; NaPr, 18.85 g; NH₄Cl, 19.66 g; KH₂PO₄, 4.44 g and 12 mL trace element (see the composition in Table 2). The pH was adjusted to 7.0–8.0 by NaHCO₃ solution. The bioreactor was inoculated with activated sludge from the Tuandao Wastewater Treatment Plant (Qingdao, China). Table 3 presented the main characteristics of the influent.

2.3. Analytical methods

Water samples were collected regularly from the two bioreactors for measurement of the chemical oxygen demand (COD), total nitrogen (TN), ammonia nitrogen (NH₄⁺-N), phosphate (PO₄³⁻). Sludge samples were taken from the oxic compartment in the two jar systems for the measurement of TTC-DHA and INT-ETS. ATP and NADH in the sludge, ammonia nitrogen and phosphorus of the water at different

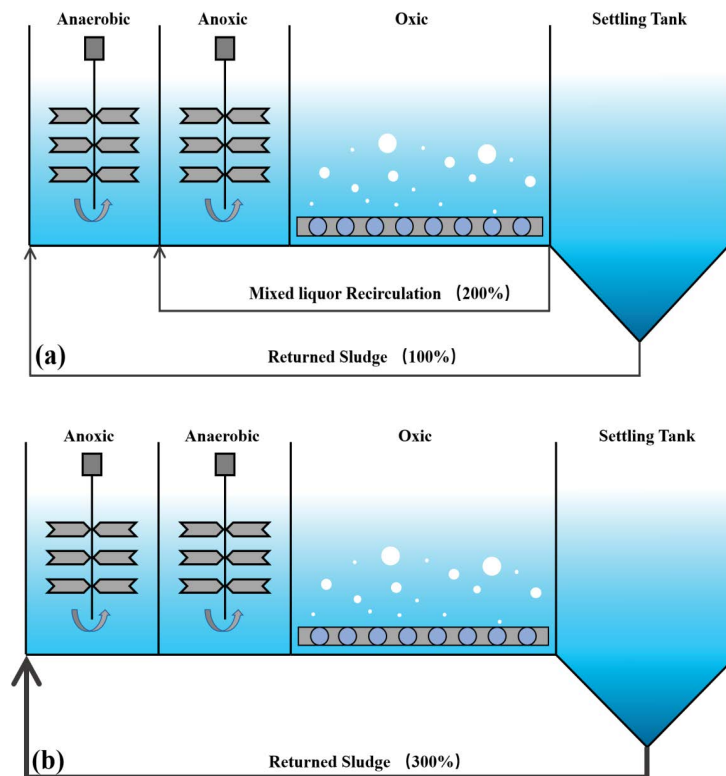


Fig. 1. Schematic diagrams of the anaerobic-anoxic-oxic (AAO) process (a) and reversed AAO process (b).

Table 1
Operating conditions of the systems

	AAO	Reversed AAO
Mixed liquor suspended solids (mg L^{-1})	2,500–3,500	2,500–3,500
Sludge retention time (d)	18–22	18–22
Dissolved oxygen (mg L^{-1})	1.0–3.0	1.0–3.0
Temperature ($^{\circ}\text{C}$)	20	20
Sludge returning ratio	100%	300%
Mixed liquor recirculation	200%	–

AAO: anaerobic-anoxic-oxic; Reversed AAO: anoxic-anaerobic-oxic process.

Table 2
Components of trace minerals

Trace minerals	Amount (g L^{-1})
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	1.440
H_3BO_3	0.140
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.024
KI	0.160
MnCl_2	0.110
$\text{Na}_2\text{MoSO}_4 \cdot 2\text{H}_2\text{O}$	0.050
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.110
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.090

Table 3
Main characteristics of the synthetic wastewater

Parameters	Range (mg L^{-1})
COD_{Cr}	320–400
TN	40–50
Phosphorus	8–10

time intervals in jar systems were also determined. The samples were replicated at least three times and the mean values were presented. The COD, TN, $\text{NH}_4^+\text{-N}$, PO_4^{3-} and ATP were measured according to the Chinese Standard Methods for Examination of Water and Wastewater Analysis (2002). The TTC-DHA test procedure was developed by Lenhard [24] and the optimal procedure of the test was later put forward by Han et al. [19], which was used in our study. The INT-ETS test was conducted following the procedures from Kenner and Ahmed [25]. The test for NADH used EnzyChrom™ NAD⁺/NADH test kits from Bioassay Systems (America).

3. Results and discussion

3.1. Biological nutrient-removal performance

Fig. 2 compares the biological removal performance of four different nutrients (COD, PO_4^{3-} , $\text{NH}_4^+\text{-N}$ and TN) in the two systems. Both processes showed similar removal efficiency for organic matters. The average

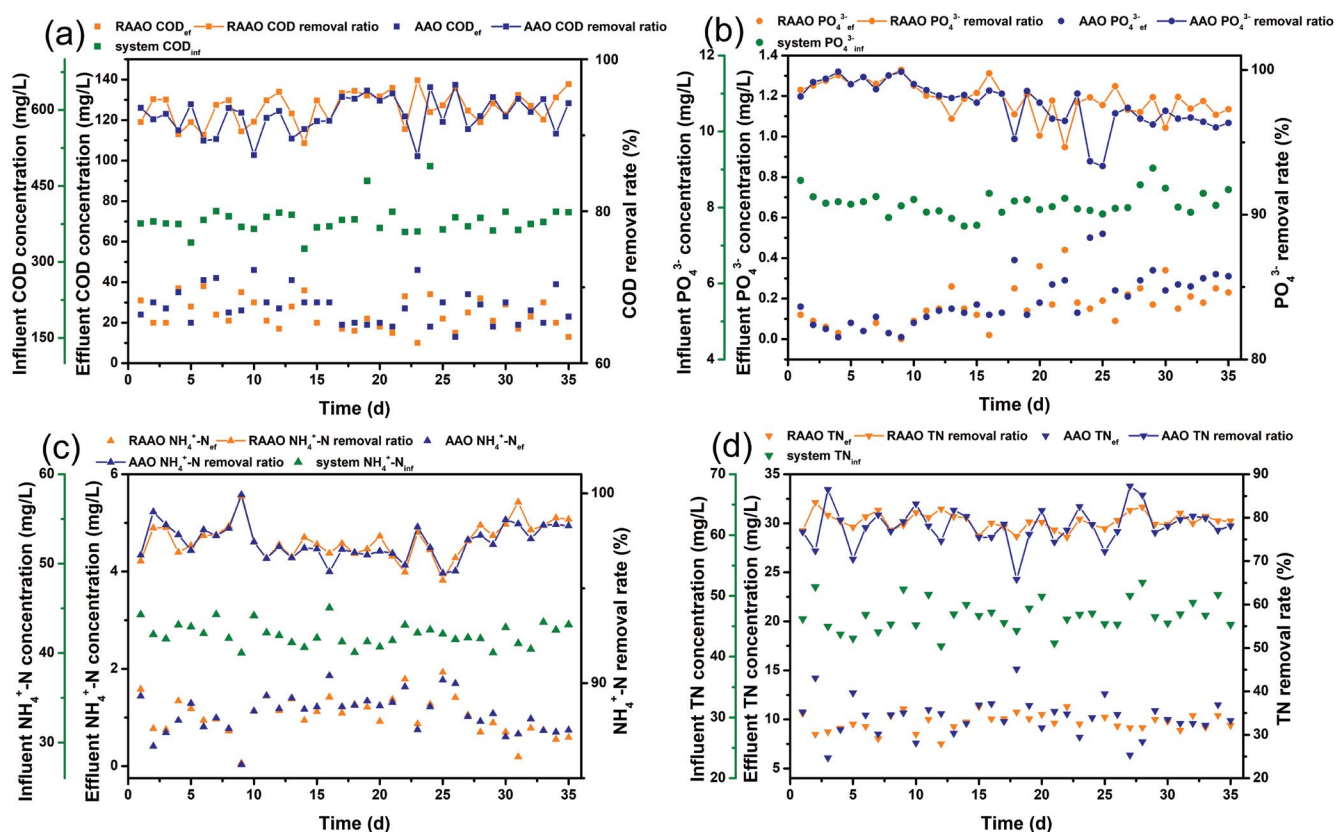


Fig. 2. Influent/effluent concentration and removal ratio of the anaerobic-anoxic-oxic (AAO) and reversed AAO processes in four main parameters: (a) COD, (b) PO₄³⁻, (c) NH₄⁺-N and (d) TN.

concentration of effluent COD concentrations was 27.71 ± 9 and 24.23 ± 8 mg L⁻¹ for the AAO and reversed AAO processes, with an average removal efficiency of 92.67% and 93.62%, respectively. The effluent phosphate concentrations in reversed AAO process were slightly lower than AAO process (Fig. 2). Fig. 2c depicted that the ammonia nitrogen concentrations in influents and effluents, with the average removal efficiency of 97.45% and 97.55% for AAO and reversed AAO process, respectively. Better and stable total nitrogen removal of reversed AAO process is shown in Fig. 2d. The effluent total nitrogen concentrations were 10.19 ± 1.89 and 9.70 ± 0.87 mg L⁻¹ for the AAO and reversed AAO process, respectively, with the average removal efficiency of 78.13% and 79.22%.

Unlike other studies on reversed AAO, both processes were stable in the removal of nutrients and showed no evident differences in the present study. This similarity may be due to the composition of the influent synthetic wastewater. Most of the COD materials used in this study was biodegradable; however, in an actual WWTP, more than 70% of the COD in the influent is not easy to biodegrade.

3.2. Microbial activity of the microorganisms

3.2.1. NADH and NADH/NAD⁺ ratio

NADH and NADH/NAD⁺ were determined along the experiment. Biological processes that utilize microorganisms

to consume substrate usually depend on the electron transport chain coupling to various organic matters [26]. NAD⁺ and NADH are mainly utilized by the enzymes that catalyze substrate oxidation and play an important role in electron transfer [27]. A high NADH level will reveal a shortage of electron acceptors whereas a large amount of substrate consumed will result in a low NADH level [28]. Fig. 3a shows the changes of the NADH level in the entire reaction. A great pronounced decrease was observed in the AAO process comparing with the reversed AAO process, which indicated that a large amount of carbon sources was utilized under anaerobic condition. While consuming a considerable percentage of the original carbon source, the AAO process failed to improve its phosphorus removal performance, which can be explained that the amount of the substrate consumed is not used for high-efficient phosphorus removal entirely. The long-term NADH/NAD⁺ ratio is shown in Fig. 3b. It is generally accepted that the NADH/NAD⁺ ratio, similar to the NADH, can also reflex the balance between electron donor and electron acceptor in living cells [29]. In comparison with the reversed AAO process, the NADH/NAD⁺ ratio was lower in the first 1.5 h but pronouncedly higher in the following 1.5 h in the AAO process. Both processes showed a low NADH/NAD⁺ ratio under their respective anaerobic condition indicating phosphorus release was sufficient in both processes under the circumstance that carbon sources in anaerobic zone of reversed AAO process were limited due to the previous complete

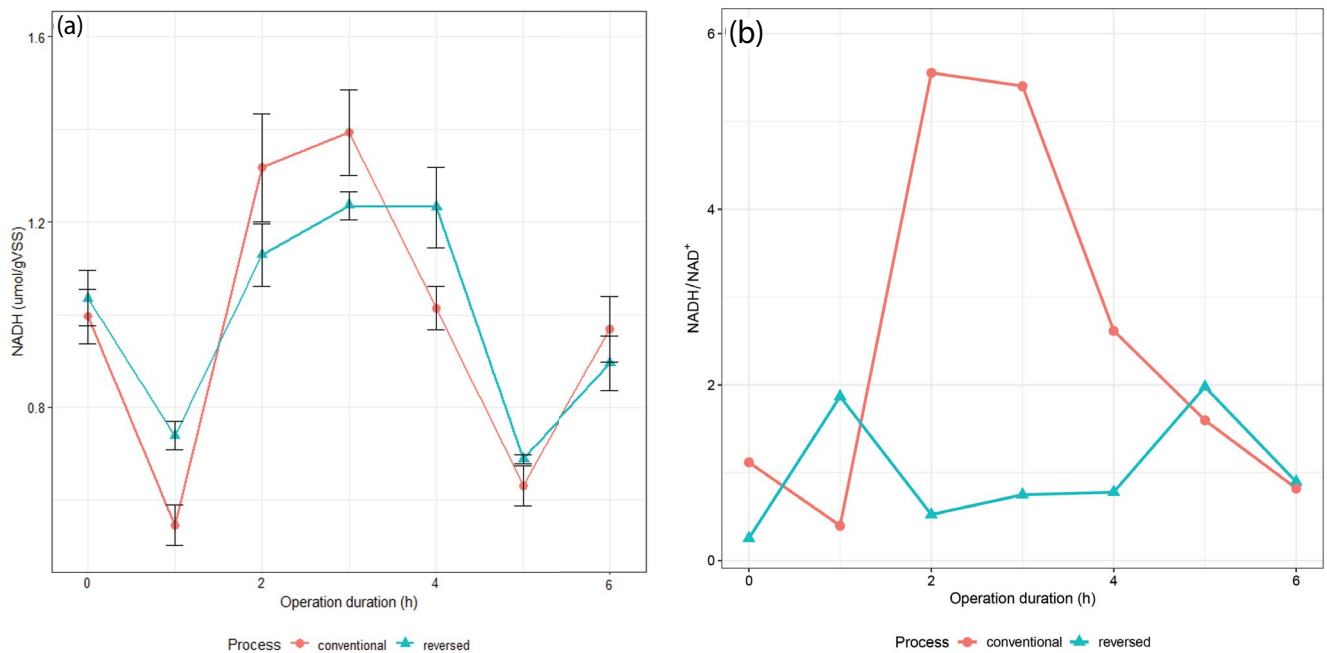


Fig. 3. Concentration of NADH (a) and the ratio of NADH/NAD⁺ (b) in the anaerobic-anoxic-oxic (AAO) and reversed AAO process.

denitrification. It was assumed that although the PAOs in reversed AAO process had fewer carbon sources to uptake, the pure complete anaerobic condition while less nitrate was present in the process raised the microbial activity of the PAOs, eventually increased the phosphorus removal efficiency [30]. An abrupt increase was also observed in the AAO process, which could be the response to a large number of electron acceptors (NO_3^-) present under anoxic condition. The recycled mixed liquor brought a lot of nitrate to this compartment to be denitrified; however, the denitrification was not sufficient because a large part of the carbon sources in influent was used for phosphorus releasing.

3.2.2. TTC-DHA

Dehydrogenase is an enzyme that oxidizes a substrate by reducing an electron acceptor. Thus, the TTC-DHA of microorganisms can reflect the efficiency of substrate degradation [31]. As shown in Fig. 4, the TTC-DHA activities in reversed AAO and AAO have a similar tendency with aeration hours. A peak was observed in the first hour in both processes followed by a steady decrease. The TTC-DHA in the reversed AAO process was significantly lower than that of AAO process because the wastewater in the reversed AAO process went through the anaerobic compartment that reduced microbial activity before entering the oxic compartment. When entering the oxic compartment, both systems had an increase in TTC-DHA, because of a relatively high substrate concentration in the preliminary stage of aeration. In addition, more dehydrogenase was needed to metabolize the substrate. With the decrease of the substrate, the TTC-DHA level also declined. Throughout the aeration, the TTC-DHA level in reversed AAO process was

higher than that of AAO process: the average TTC-DHA level in the oxic compartment was 9.66 and 9.02 mg TF/(g VSS h), in the reversed AAO and AAO process (c.a. 7.1% higher in the reversed AAO), respectively, which confirms that higher substrate degradation efficiency occurred.

3.2.3. 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride electron transport system

Fig. 5 shows the INT-ETS activity in the aeration stage of the reversed AAO and AAO process. The ETS activity was performed using the INT method to assess the different microbial activity in both processes. The ETS activity evaluates the biological activity by analyzing the activity of microorganism respiration [32,33]. As shown in Fig. 5, the ETS activity in the reversed AAO was higher than in the AAO over the whole operation period significantly, with the average ETS activities in the oxic compartment of the reversed AAO and AAO process were 180.21 and 129.66 mg TF/(g VSS h), respectively, which means the ETS activity in the oxic compartment of reversed AAO process was 38.99% higher than in the AAO process. It is also noteworthy that, when entering aerobic conditions, the ETS activity in reversed AAO process almost doubled than that of AAO process, which perfectly correlates the high respiration rate of microorganisms in the reversed AAO process.

Furthermore, the DHA and ETS levels in oxic compartments were in agreement, indicating that the reversing of anaerobic-anoxic compartments caused an enhanced microbial activity in the oxic compartment of reversed AAO process. The results of the DHA and ETS validated the hypothesis that with higher microbial activity, the reversed AAO process can remove identical or higher phosphorus than AAO process with fewer carbon sources.

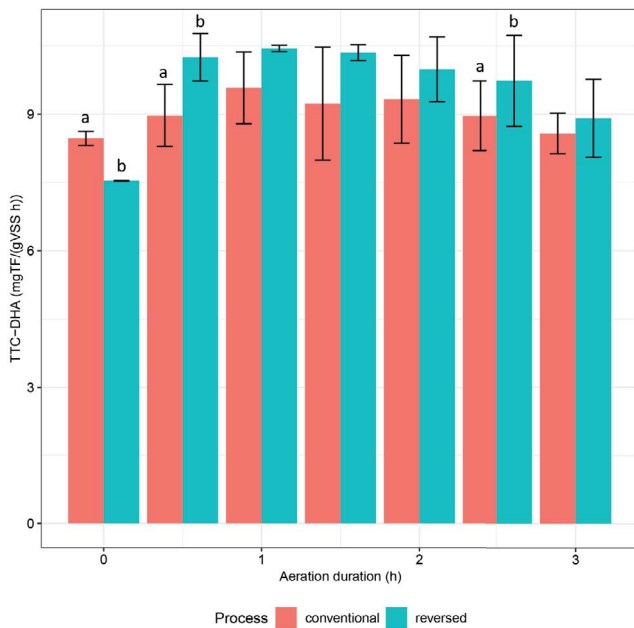


Fig. 4. TTC-DHA activity in the two systems (mean values \pm standard deviations followed by the different letter differ significantly in each fraction).

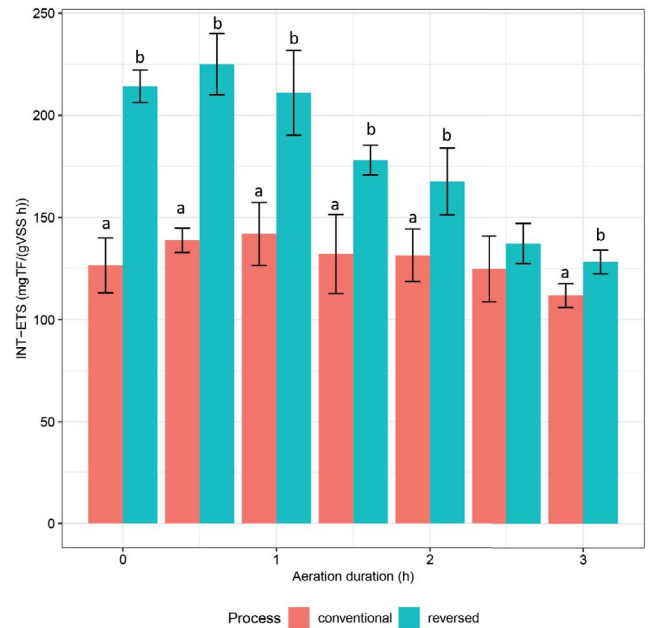


Fig. 5. 2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride electron transport system activity in the two systems (mean values \pm standard deviations followed by the different letter differ significantly in each fraction).

3.2.4. Adenosine triphosphate

Fig. 6 shows the level of the ATP amount and its relationship with time. ATP, a complex organic chemical found in all forms of life, provides energy to drive the metabolism of microorganisms. The more ATP in a microorganism, the more active it is. As shown in Fig. 6, under anaerobic and anoxic conditions (the first 3 h), ATP levels were low in both processes. In the aerobic condition, ATP had a significant increase due to the aeration. The ATP levels were significantly higher in reversed AAO process under the oxic condition, with 5.8% higher than that of the AAO process, which will accelerate the substrate oxidation progress.

3.3. Ammonia and phosphate levels in jar test in time sequence

Fig. 7 shows the concentrations of ammonia and phosphate present among the operation duration. In the first 3 h, the concentrations of ammonia in the two systems remained constant due to the absence of oxygen (Fig. 7a). In the oxic compartment, the concentrations of ammonia in the reversed AAO and AAO were reduced to 1.53 and 3.45 mg L⁻¹, respectively. Obviously, the bacteria in the reversed AAO had greater bioactivity toward nitrification.

A significant difference in phosphate removal was observed in the two processes (Fig. 7b). The phosphate in the AAO process increased to 47 mg L⁻¹ and remained at a high level compared with the reversed AAO process. It is confirmed that more carbon sources were used for phosphorus releasing in AAO process. For reversed AAO process, while using not enough substrate (the available carbon source in the reversed AAO was mostly used for denitrification in the anoxic compartment), the PAOs in the reversed AAO process released relatively less phosphorus (33 mg L⁻¹).

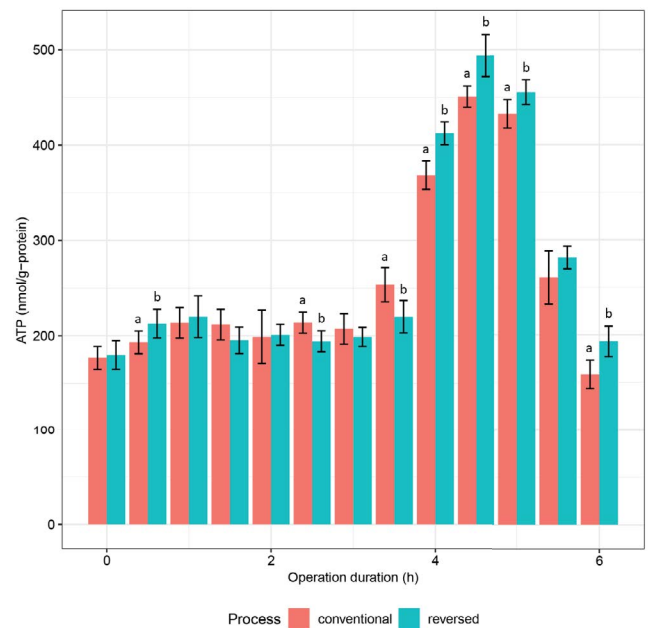


Fig. 6. ATP concentrations in the two systems (mean values \pm standard deviations followed by the different letter differ significantly in each fraction).

However, both processes had an identical concentration of phosphorus in the effluent. These results were in accordance with the previous enzymatic indicator levels, demonstrating that the reversing of the AAO process had significantly improved microbial activity in reversed AAO process.

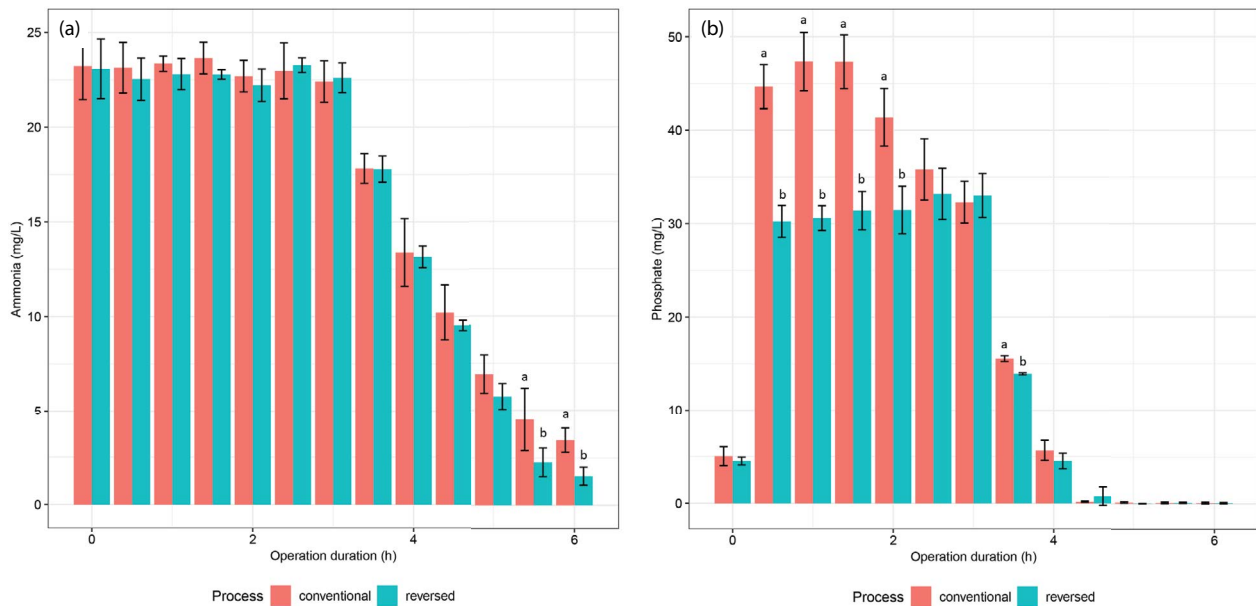


Fig. 7. Ammonia (a) and phosphate (b) concentrations in different reactors (mean values \pm standard deviations followed by the different letter differ significantly in each fraction).

3.4. Enhanced nutrients removal mechanism

For reversed AAO process, due to the shifting of the anaerobic and anoxic compartments, the wastewater was first mixed with the recycled nitrate for complete and fast denitrification, which consumes a big part of the carbon sources. The huge consumption of the carbon sources for denitrification resulted in enhanced nitrogen removal efficiency. Then, the nitrate-eliminated wastewater undergoes anaerobic conditions where PAOs uptake carbon sources for phosphorus releasing. It is well-stated that limited carbon sources might lead to compromised phosphorus removal efficiency due to insufficient phosphorus release; however, in reversed AAO process, the identical phosphorus removal efficiency was observed in this study. One potential explanation is that, in the reversed AAO process, the recycled nitrate was almost eliminated under the anoxic condition because of the adequate carbon sources. In such a case, the impact of nitrate on the PAOs for phosphorus release is insignificant [10,34], as little nitrate present in the anaerobic condition and phosphorus release is more complete with a high bioactivity level. Furthermore, the PAOs entered oxic compartment directly after releasing phosphorus while a significant delay in the anoxic compartment of AAO process made the PAOs less efficient in phosphorus uptake than reversed AAO process [35,36]. Hence, although the shifting will cause fewer available carbon sources for PAOs, the improved microbial activity due to the shift will cover the problem of insufficient carbon sources and eventually enhance the nutrients removal efficiency in reversed AAO process.

4. Conclusion

The present study targeted the metabolism mechanism in AAO and reversed AAO process. Both the AAO

process and the reversed AAO process showed excellent organic matter removal and phosphorus removal performance while the reversed AAO process performed better in nitrogen removal due to adequate carbon sources. The TTC-DHA, INT-ETS and ATP analyses showed higher levels in reversed AAO process, which confirmed that the microorganisms involved in more metabolic activities, suggesting that shifting the sequence of the anaerobic and anoxic compartments improves microbial activity. Also, the shift made the PAOs in reversed process enter oxic compartment directly after releasing phosphorus, avoided the delay in AAO process. This sequence enhanced the phosphorus removal efficiency of PAOs, which resulted in an identical phosphorus concentration in the effluent compared with AAO process using fewer available carbon sources.

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Declaration of interest statement

The authors would like to declare that there is no any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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