Biological Treatment of Anaerobically Digested Swine Wastewater and Subsequent Nutrients Recovery by *Spirulina platensis* Cultivation

July 2017

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A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences

University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Environmental Studies

(Doctoral Program in Sustainable Environmental Studies)

Abstract

Anaerobic digestion is widely applied in large-scale swine farms to reduce the discharge of solid wastes and wastewater to the environment. The effluent from the digester, i.e. anaerobically digested swine wastewater (ADSW) is attracting more attention due to its huge production amount and complex pollutants (especially antibiotics), which contributes a lot to the pollution status of water environment in China. In order to control the environmental pollution caused by the discharge of ADSW, a new and strict national discharge standard for livestock and poultry breeding will be implemented in China. However, it is still a big challenge to use single biological process or single nutrients recovery process for ADSW treatment to achieve the discharge requirements for total nitrogen (TN) and total phosphorous (TP) in an effective and economic way.

In this study, an integrated process using novel biological nitrogen removal reactor followed by nutrients recovery technology was used to deal with ADSW. The intermittently aerated sequencing batch reactor (IASBR) was expected to remove most of ammonium nitrogen (NH_4^+-N), TN and antibiotics from ADSW. Then the effluent from IASBR, which still can't satisfy the discharge requirements for TN and TP, was used to cultivate *Spirulina platensis* to further remove and recover nutrients (nitrogen and phosphorus).

The IASBR achieved significantly high removal rates of 97.5±1.4% and 93.8±12.8% for NH_4^+-N and TN under a chemical organic demand (COD) to TN (COD/TN) ratio of 2.4, hydraulic retention time (HRT) of 3 days and temperature above 20°C. As a result of the inhibition to nitrite oxidizing bacteria (NOB) during the whole operation, partial nitrification-denitrification was achieved in IASBR with nitrite accumulation rate greater than 80%. The influent COD/TN ratio, nitrogen loading, and temperature greatly affected the removal of NH_4^+-N and TN. The extremely low influent COD/TN ratio and lack of enough carbon source exerted unexpected negative effect on denitrification process, resulting in large accumulation of nitrite and deterioration of TN removal. The increase in free ammonia (FA) concentration at lower COD/TN ratio inhibited the growth of ammonium oxidizing bacteria (AOB), followed by worsen NH_4^+-N removal. Both NH_4^+-N and TN loading increased when IASBR was run at a shorter HRT, mainly leading to the increase in FA concentration and restrained the partial nitrification. Denitrifying bacteria were much more sensitive than nitrifying bacteria to lower temperature, resulting in dramatic increase in FA concentration,

thus inhibiting both nitrification and denitrification processes. The IASBR effluent should be treated further not only for TN removal but also for TP removal.

The removal characteristics of eleven veterinary antibiotics (including tetracyclines, sulfonamides, quinolones and macrolides) were also studied by using the IASBR system. Results showed that the removal of antibiotics was greatly influenced by COD volumetric loading, which could achieve up to 85.1%±1.4% at 0.17±0.041 kg COD/(m^3·day), while dropped to 75.9%±1.3% and 49.3%±12.1% when COD volumetric loading increased to 0.65±0.032 and 1.07±0.073 kg COD/(m^3·day), respectively. Tetracyclines, the dominant antibiotics in ADSW, were removed by 87.9% in total at the lowest COD loading, of which 30.4% were contributed by sludge sorption. In contrast, sulfonamides were removed about 96.2%, almost by biodegradation. Long solid retention time (SRT) seemed to have little obvious impact on antibiotics removal, while a shorter SRT of 30-40 days could reduce the accumulated amount of antibiotics in sludge. The ratio of COD/TN in the influent was regarded as an unimportant impact factor for the removal of antibiotics. Both sludge sorption and biodegradation were found to be the major contributors to the removal of antibiotics. Mass balance analysis revealed that greater than 60% of antibiotics in the influent were biodegraded in the IASBR, whereas averagely 24% were adsorbed by sludge under the condition that sludge sorption gradually reached its equilibrium.

A local *Spirulina platensis* strain ZJWST-S1 was found to be able to proliferate quickly in the pretreated ADSW. Single factor experiments showed that the strain grew fast in a Zarrouk medium as the dosage of sodium bicarbonate ($NaHCO_3$), nitrate nitrogen (NO_3^--N) and phosphate phosphorus (PO_4^{3-}-P) were not less than 4, 40 and 3 mg/L, respectively. No growth inhibition was observed when the culturing medium contained nitrite nitrogen (NO_2^--N) of 0-120 mg/L. However, NH_4^+-N beyond 60 mg/L would inhibit the growth of *Spirulina*. In the two runs of raceway pond cultivation, the average area biomass productivity achieved 4.5 g/(m^2·d) in IASBR effluent lower than that in Zarrouk medium, most probably due to the influence of high chrominance in the IASBR effluent. The *Spirulina platensis* ZJWST-S1 removed almost all NH_4^+-N, 68.7% of TN and 79.1% of TP from the IASBR effluent, among which 91.5±3.4% of TN and 92.4±4.8% of TP reduced from ADSW were converted to *Spirulina platensis* biomass. The concentrations of TN and TP at the end of cultivation could meet the new discharge standard.

The innovative integrated process of IASBR coupling with *Spirulina platensis* cultivation can not only help to enhance the removal of TN and TP to satisfy the new national discharge

standards, but also help harvest high-profit animal feed grade proteins to attain additional incomes. Single use of IASBR or *Spirulina platensis* cultivation couldn't meet discharge standards directly. Although IASBR could enhance the removal of NH_4^+-N and TN, the effluent TN and TP still couldn't reach to the discharge requirements. *Spirulina platensis* cultivation could further remove and recover nitrogen and phosphorous. But without the pretreatment of IASBR, *Spirulina platensis* need to be cultivated in diluted ADSW and suffered negative effects due to the existence of inhibitory and toxic substances such as NH_4^+-N and antibiotics. The research set up a promising novel integrated process to be possibly applied in ADSW treatment and will contribute to the sustainable development of large-scale swine farms to a great extent in China.

Key words: Anaerobically digested swine wastewater (ADSW); Intermittently aerated sequencing batch reactor (IASBR); *Spirulina platensis*; Nitrogen; Antibiotics; Nutrients recovery

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Acronyms and abbreviations

Anaerobically digested swine wastewater	ADSW
Ammonium oxidizing bacteria	AOB
Biochemical oxygen demand of 5 days	BOD_5
Chemical oxygen demand	COD
Ratio of chemical oxygen demand to total nitrogen	COD/TN
Free ammonia	FA
Free nitrous acid	FNA
Hydraulic retention time	HRT
Intermittently aerated sequencing batch reactor	IASBR
Liquid chromatography-tandem mass spectrometry	LC-MS/MS
Mixed liquor suspended solids	MLSS
Nitrite accumulation rate	NAR
Ammonium nitrogen	NH_4^+-N
Nitrite oxidizing bacteria	NOB
Nitrate nitrogen	NO_3^--N
Nitrite nitrogen	NO_2^--N
Oxidation-reduction potential	ORP
Phosphate phosphorus	PO_4^--P
Solid phase extraction	SPE
Solid retention time	SRT
Suspended solids	SS
Temperature	Temp.
Total nitrogen	TN
Total phosphorous	TP

Chapter 1 Introduction

1.1. Anaerobically digested swine wastewater and related environmental issues

In most regions of the world, pig numbers are increasing all the way as reported by the Food and Agriculture Organization (FAO). Table 1-1 shows the pig number during 2010-2014 worldwide (FAOSTAT, 2016). Nearly 60% of the world's pig population is in Asia.

Especially in China, the consumption habit of residents is given priority to pork, as a result, the output of pork accounted for 65.1% of all meat production in 2014. In fact, the production of pork increased from 4.56 million tons to 5.67 million tons during the year 2005 to 2014. In order to satisfy the increasing demand for pork consumption, the traditional scattered swine farms were gradually replaced by large-scale breeding farms. Therefore, in recent ten years, large-scale breeding industry has developed rapidly in China. This change improves the efficiency of management and decreased the cost of production, but also brings a strong pressure on the ecological environment. In fact, the inventories of pigs are 480 million heads in 2014 in China, producing about more than 4 billion tons of swine wastewater annually. In the meantime, about 2.89 million tons of chemical oxygen demand (COD), 0.29 million tons of ammonium nitrogen (NH_4^+-N), 1.39 million tons of total nitrogen (TN) and 0.23 million tons of total phosphorus (TP) were discharged into the environment in 2014 (CSYE, 2015). Thus swine wastewater is becoming the major pollution source in rural and suburb area.

Swine wastewater is the mixed wastewater of pig urine and piggery washing water with high COD of 5,000-20,000 mg/L, TN of 800-2,000 mg/L and TP of 25-65 mg/L (Shin et al., 2005). Anaerobic digestion process, with high organic pollutants decomposition, low energy consumption and low sludge production rate, has become an important technology for large-scale swine farm wastewater treatment (Sakar et al., 2009). Figure 1-1 shows the typical treatment process involving anaerobic digestion in large-scale swine farms. Swine wastewater is usually first collected in a tank, then digested in anaerobic digesters. Finally the anaerobic digested residues could be used as farmland fertilizer through composting, and the biogas can be utilized for electricity generation or heat supply. The effluent from the digester, i.e. anaerobically digested swine wastewater (ADSW) containing high concentrations of organic pollutants, nitrogen, phosphorus and antibiotics still need further polishing before final discharge.

1.2. Characteristics of ADSW

Table 1-2 showed the typical wastewater quality of ADSW (Rajagopal et al., 2011). After anaerobic digestion, COD concentration of ADSW often decreases drastically from 10,000 to 1,500 mg/L in average. However, anaerobic digestion is generally not good at removing nitrogen and phosphorous.

In fact, the primary characteristics of ADSW could be summarized as follows:

(1) ADSW contains high concentrations of organics, especially a great deal of refractory organic pollutants. Anaerobic digestion process can easily remove plenty of biodegradable organics, and the unbiodegradable pollutants will take an important proportion in the digested wastewater. Based on Table 1-2, the ratio of COD to biochemical oxygen demand of 5 days (BOD_5) or COD/ BOD_5 in ADSW is about 0.25 averagely much lower than that of domestic wastewater (usually beyond 0.4), which makes it more difficult to treat ADSW by using traditional biological processes.

(2) TN and TP levels are quite high in ADSW. Anaerobic microorganisms need only a few nutritional elements for growth in anaerobic digestion, resulting in still quite high concentrations of TN and TP in ADSW. It should be noticed that NH_4^--N is the main species of nitrogen in ADSW, and TP almost existed as inorganic phosphorous.

(3) The ratios of chemical oxygen demand to total nitrogen or COD/TN in ADSW often maintain at an extremely low level, even below 1.0 sometimes. Normally, a COD/TN ratio of 4-10 is required for a complete TN removal in conventional biological treatment processes (Wu et al., 2003). The imbalanced COD/TN ratio is a big obstacle for TN removal, resulting in increased treatment cost caused by adding huge amount of external carbon source.

(4) Large variations in water quality occur at different times. In fact, the characteristics and wastewater qualities of ADSW fluctuate greatly with the change of seasons, which is most probably attributable to the variation in feeds for pigs and the efficiency of the anaerobic digestion facilities. Besides, lack of professional and precision management of swine farms also contributes a lot to the above changes.

(5) Moreover, the pollution problem of veterinary antibiotics and heavy metals in ADSW also has been concerned. In fact, only a small fraction of the ingested antibiotics may be absorbed by the organisms, and greater than 85% of them run off through animal excretion and are finally discharged into water, sediment, soil, and other related environments (Bailey et al., 2016; Li et al., 2012; Wegst-Uhrich et al., 2014). Although the concentrations of residual antibiotics in livestock wastewater are generally around μg/L levels, they can still exert negative effects on drinking water safety and public health (Richardson and Ternes, 2011; Zhou et al., 2007). And heavy metals such as Cu, Zn, As, Pb, Cd, Ni and Cr were all detectable in ADSW, which are mainly attributable to the additives in the feed (Cortes-Esquivel et al., 2012; Ma et al., 2013).

Therefore, ADSW without effective treatment will bring about serious environmental problems. High concentrations of nitrogen and phosphorous will increase the risk of eutrophication in rivers and lakes, and it is also difficult for ecological remediation because of the exotic and harmful substances leached into the groundwater. Meanwhile, antibiotics and heavy metals in ADSW can be a big threat for the environmental safety and public health.

1.3. Treatment technologies for ADSW and their challenges

In the past, ADSW is traditionally spread on farmlands. However, the high concentrations of TN and TP in ADSW usually exceed the growth demands of plants, and also exceed or are approaching the limits of land bearing capacity. For example, in Europe, according to the European Communities (Good Agricultural Practice for the Protection of Waters) Regulations (S. I. No. 610, 2010), the maximum amount applied to land every year is equivalent to 170 kg N/ha. But many lands are no longer suitable for land spreading because organic nitrogen loading from livestock is approaching or already at the limit of 170 kg N/ha. Besides, the available farmlands have become less and less to afford the huge amounts of ADSW from large scale swine farms, especially in developed areas, like Yangtze River delta in East China. Therefore, a great deal of ADSW has to be discharged into the environment leading to serious environmental pollution. In order to control the pollution status brought about by ADSW, a new set of standards entitled as discharge standard of pollutants for livestock and poultry breeding will be implemented in China right now. As show in Table 1-3, the new standard has much higher requirements for swine wastewater discharge, especially for COD, TN and TP.

In view of the simultaneous removals of organic pollutants, nutrients (TN and TP) and other pollutants, natural ecological process and biological treatment process have been principally used for ADSW treatment.

1.3.1. Ecological treatment

Ecological treatment technologies usually utilize natural water and soil or artificial ecological system to treat wastewater. Constructed wetland (CW) and stabilization ponds (SP) are the two main ecological processes for ADSW treatment in last decade (Forbes et al., 2010; Poach et al., 2007; Westerman and Bicudo, 2002). Costa and Medri (2002) reported that the SP systems could remove approximately 97% of organic pollutants, 90% of TN, and 93% of TP in swine wastewater treatment. Hunt et al. (2002) pointed out that CWs could be very effective in the removal of TN from anaerobic lagoon-treated swine wastewater. Liu et al. (2014) compared the difference between CW and SP for the treatment of ADSW. They found that SP system performed much better than CW for NH_4^+-N removal and no obvious difference in COD and TP removal existed between SP and CW. Generally, both CW and SP are easily affected by temperature and exhibit unstable removals of COD, TN and TP due to the change of seasons. Furthermore, these two ecological processes can only suffer a quite lower loading rate or treat diluted ADSW, which will result in a great land demand for ADSW treatment. Therefore, the applications of ecological treatment technologies have been unavailable in land shortage areas.

1.3.2. Biological treatment

Biological processes are mainly used to treat ADSW due to their effective treatment abilities and reasonable treatment cost. Aerobic biological processes including conventional activated sludge process (Osada et al., 1991), anoxic/oxic (A/O) process (Anup et al., 2011), sequencing batch reactor (SBR) (Deng et al., 2008; Kim et al., 2004; Zhan et al., 2009), membrane bioreactor (MBR) (Shin et al., 2005; Sui et al., 2015) are commonly studied for ADSW treatment.

Basically, these biological treatment technologies exhibit high potential for COD removal, but relatively lower TN removal during ADSW treatment. Therefore, in order to enhance TN removal for ADSW, researchers tried to make some improvements on these conventional aerobic processes. Kim et al. (2004) designed an integrated real-time control system based on SBR, which was operated with automatic addition control of an external carbon source and utilized oxidation-reduction potential (ORP) and the pH as parameters to control the anoxic phase and oxic phase, respectively. Sui et al. (2015) investigated the effectiveness of combining ammonia stripping (AS) and MBR processes to enhance TN removal from ADSW. Nevertheless, due to the imbalanced COD/TN ratio in ADSW, plenty of external carbon sources and alkalinity must be added into the reactor to achieve an excellent performance of denitrification process for most of these biological processes, resulting in large increase in treatment cost.

Intermittently aerated sequencing batch reactor (IASBR) is an innovative bio-technology based on SBR, which has been developed to treat ammonium-rich wastewater for nitrogen removal through creating multiple aerobic-anoxic alternating environments during one SBR operation cycle (Li et al., 2011a). Nitrogen can be removed efficiently by using IASBR process. Zhang et al. (2011) reported that the IASBR achieved high removal rates of 89.8% and 76.5% for COD and TN, respectively, with COD/TN ratio being 3.0 and TN loading rate being 0.38 kg N/(m^3·d) in the influent. Guo et al. (2008) compared a continuously-aerated reactor with an IASBR in the treatment of synthetic wastewater. Results showed the efficiency of NH_4^+-N and TN removal was 80% and 70% in the continuously-aerated reactor, respectively; while, the concentration of effluent NH_4^+-N from IASBR was always below the detection limit with TN removal around 86%.

Most importantly, efficient partial nitrification has been detected in IASBRs (Li et al., 2008; Zeng et al., 2008). Partial nitrification process can decrease the oxygen demand because it's unnecessary to convert nitrite (NO_2^--N) to nitrate (NO_3^--N) for denitrification. Theoretically, 2.86 kg COD/kg N is required for traditional TN removal through NO_3^--N reduction, while 1.72 kg COD/kg N is required via NO_2^--N (Akunna, et al., 1992). Therefore, the existence of partial nitrification-denitrification process in a bioreactor can save 25% oxygen demand in the nitrification stage and reduce 40% of organic carbon needs in the denitrification stage. So compared with conventional biological technologies, IASBR process can remove TN more effectively and decrease energy consumption and external carbon source supplement in the treatment of low COD/TN ratio wastewater. Therefore, IASBR is a suitable biological process for swine wastewater treatment. Pan et al. (2014) achieved excellent COD, TN, and NH_4^+-N removal rates about 89.8%, 76.5%, and 99.1%, respectively when using IASBR to treat swine wastewater with COD, TN, and NH_4^+-N concentrations of 11,540 ± 860 mg/L, 4041 ± 59 mg/L, and 3808 ± 98 mg/L, respectively.

Although IASBR can remove most of NH_4^+-N and TN from ADSW and take much lower treatment cost compared with traditional biological processes, the effluent TN from IASBR still doesn't meet the new national discharge standard. The high concentration of TN in ADSW requires higher TN removal rates than 95% for a biological process to satisfy the new national discharge standard, which is quite difficult to achieve. Meanwhile, traditional biological process including IASBR usually can't decrease the concentration of TP from 40 mg/L to 5 mg/L directly because of the intrinsic disadvantages of traditional biological phosphorus removal process, and physiochemical methods such as chemicals dosing will be often considered for advanced removal of TP, which will tremendously increase the treatment cost for ADSW.

1.4. Nutrients recovery from ADSW and its bottlenecks

As mentioned above, the effluent from biological treatment processes should be treated for further TN and TP removal from treated ADSW. Compared with some physiochemical methods, nutrients recovery processes are paid more attention because they not only help to reduce the concentrations of TN and TP but also help to attain high-value products.

1.4.1. Struvite crystallization technology

Struvite crystallization technology is often considered as a feasible choice for simultaneous recovery of nitrogen and phosphorous from swine wastewater (Song et al., 2011; Suzuki et al., 2007). Vanotti et al. (2017) used struvite crystallization process combining with gas-permeable membrane technology to recover nitrogen and phosphorous. And phosphorous removal efficiency was beyond 90% and phosphorous recovery efficiency was about 100%. However, these nutrients recovery methods are usually costly because of the addition of chemicals for pH adjustment and supplement of external magnesium compounds.

1.4.2. Cultivation of *Spirulina* for nutrients recovery

Another nutrients recovery process, cultivation of *Spirulina*, has been concerned worldwide. *Spirulina*, an ancient microalga rich in protein, amino acid, vitamin and bioactive components, has been acknowledged as an effective immune-enhancer and feed additive that can prevent disease, promote animal growth and improve the appearance of the animal products and nutrition values (Ajayan et al., 2012; Rodrigues et al., 2010). *Spirulina* cultivation based wastewater has been increasingly researched since 1957 (Kim et al., 2007;

Markou and Georgakakis, 2011; Oswald and Gotaas, 1957). The Advantage of this process is that high valued algal biomass can be produced during the removal of inorganic nutrients from wastewaters.

A few researchers had attempted the cultivation of *Spirulina* with swine wastewater to attain algae powder and recover nutrients. Canizares and Dominguez (1993) cultured *Spirulina*in with 2 L of aerobically biodegraded swine wastewater, and obtained algae powder containing 36% of protein. Chaiklahan et al. (2010) cultured *Spirulina* in a 100 L of outdoor raceway pool with five times diluted ADSW supplemented with 4.5 g/L of sodium bicarbonate and achieved a production rate of 12 g/(m^2·day). Li et al. (2011b) cultured *Spirulina* in 50 milliliters of swine wastewater added with 10% Zarrouk medium, and obtained powder with a production rate of 7.5 g/(m^2·day). The cultivation of *Spirulina* using swine wastewater could not only remove and recover nitrogen and phosphorus from swine wastewater, but also help reduce the cultivation cost of *Spirulina*. The harvested algae powder can be applied as animal feed, given that the quality can reach the national standard of product grade.

However, ADSW contains some inhibitory and toxic substances for the growth of *Spirulina*. NH_4^+-N and NO_2^--N are common inorganic nitrogen forms in ADSW. The high concentrations of NH_4^+-N and NO_2^--N in ADSW are considered to be toxic to algae growth, although trace of them may benefit algae growth (Markou and Georgakakis, 2011). More importantly, the high concentration of veterinary antibiotics in ADSW is one of biggest challenges for *Spirulina*. Cao et al. (1999) tested the sensitivities of 6 antibiotics for *Spirulina platensis* and the results showed *Spirulina platensis* was highly sensitive to hygromycin and chloramphenicol. Wang et al. (2001) reported that both *Spirulina platensis* and *Spirulina maxium* were sensitive to ampicillin, streptomycin, and chloramphenicol. The inhibitory concentrations of ampicillin, streptomycin, and chloramphenicol were 5.0-50.0 μg/mL, 5.0 μg/mL and 0.1 μg/mL in liquid culture, respectively.

In order to resolve these negative effects on the growth of *Spirulina*, some researchers attempted to dilute ADSW. But it is not an economical way due to the waste of huge amount of fresh water. Nevertheless, biological treatment process can help to remove most inhibitory and toxic substances such as NH_4^+-N, NO_2^--N and antibiotics. Therefore, it will be a better choice to use biological treatment process to achieve its bio-safety before culturing *Spirulina* with ADSW.

1.5. Research objectives and thesis structure

As described above, it is still a big challenge to treat ADSW to satisfy the new discharge standard of pollutants for livestock and poultry breeding in China. Due to the special characteristics such as high concentrations of TN and TP, imbalanced COD/TN ratio, it's quite tough to use single biological treatment process or single nutrients recovery process to achieve the discharge limit values for TN and TP in an effective and economic way.

Therefore, the main objective of this study is to establish an innovatively integrated process combining biological treatment with nutrients recovery, achieving enhanced TN and TP removal from ADSW. A novel biological treatment process IASBR was expected to remove most of NH_4^+-N, TN and antibiotics from ADSW. Then the effluent from IASBR, which still can't reach the discharge requirements for TN and TP, was used to cultivate *Spirulina platensis* to further remove and recover nitrogen and phosphorus. This combination is expected to not only achieve high-value feed additives, but also qualify final effluent with TN and TP concentrations satisfying the discharge standards. It should be noted that IASBR is a necessary pretreatment process for the cultivation of *Spirulina platensis*, because the inhibitory and toxic substances such as NH_4^+-N and antibiotics could be removed by IASBR, that guaranteeing the bio-safety for *Spirulina*.

The main contents of this thesis are composed of three parts. In chapter 2, a lab-scale IASBR was applied to treat ADSW to explore the removal characteristics of nitrogen. The removals of NH_4^+-N and TN were investigated under different influent COD/TN ratios, nitrogen loadings and temperature. The nitrite accumulation rate (NAR), concentrations of free ammonia (FA) and free nitrous acid (FNA) were studied to understand the partial nitrification-denitrification process in IASBR. In chapter 3, the removal rates of eleven veterinary antibiotics (including tetracyclines, sulfonamides, quinolones and macrolides) in the reactor were investigated under different COD volumetric loadings, solid retention times (SRTs) and COD/TN ratios. In chapter 4, a *Spirulina platensis* strain ZJWST-S1, which was strongly adaptable and resist to high concentrations of ADSW, was selected for cultivation to further remove and recover nitrogen and phosphorus from the IASBR effluent. Flask tests in illuminating incubator were carried out to study the growth behavior of the local strain ZJWST-S1, and its favorable requirement for carbon, nitrogen and phosphorus and the tolerance limits to NH_4^+-N and NO_2^--N were also explored. An indoor raceway pond was then carried out to cultivate *Spirulina platensis* by using the effluent from IASBR, in which algae yield and wastewater quality were investigated. The thesis structure is displayed in Figure 1-2.

Table 1-1. Global population of pigs.

Regions	2010	2011	2012	2013	2014
World	974.97	968.88	971.69	978.64	986.66
Africa	31.51	31.39	32.98	33.81	34.54
American	163.85	166.92	166.66	165.54	170.36
Asia	585.10	577.65	583.53	591.16	590.88
Europe	189.16	187.53	183.23	182.95	185.53
Oceania	5.35	5.39	5.29	5.18	5.35

Unit: million heads.

Source: FAOSTAT, 2016.

Table 1-2. Typical wastewater quality of anaerobically digested swine wastewater (ADSW).

COD	BOD_5	TN	NH_4^+-N	NO_2^--N	NO_3^--N	TP	pH
1275±384	312±96	1372±346	975±313	≤10	≤50	42±12	6~9

Unit: mg/L, except pH.

Table 1-3. New discharge standards for wastewater from livestock and poultry breeding in China.

Item	Discharge limit (Old)	Discharge limit (New)
Suspended solids (SS)	200	150
BOD_5	150	40
COD	400	150
NH_4^+-N	80	40
TN	/	70
TP	8	5.0
Cu	/	1.0
Zn	/	2.0

Unit: mg/L.

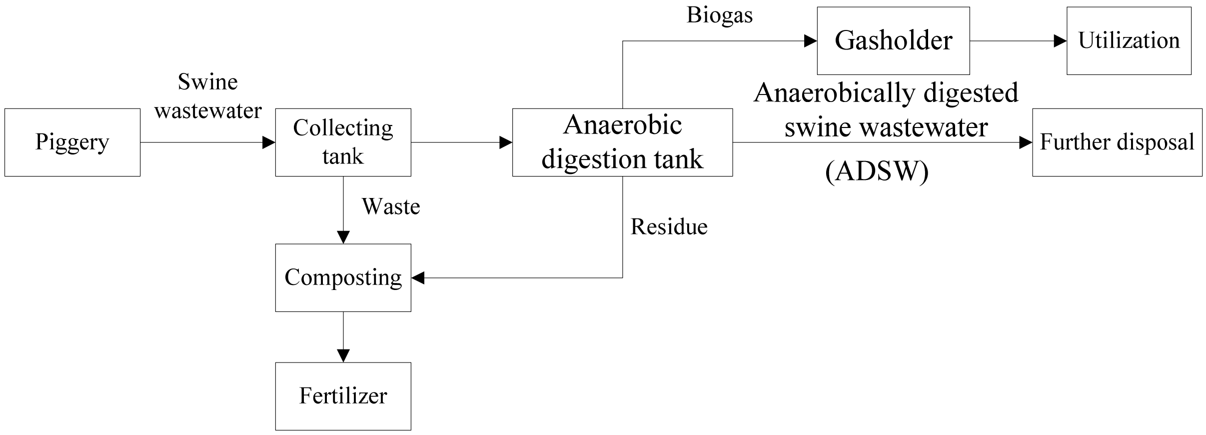

Figure 1-1. Typical treatment processes for swine wastewater in large-scale swine farms.

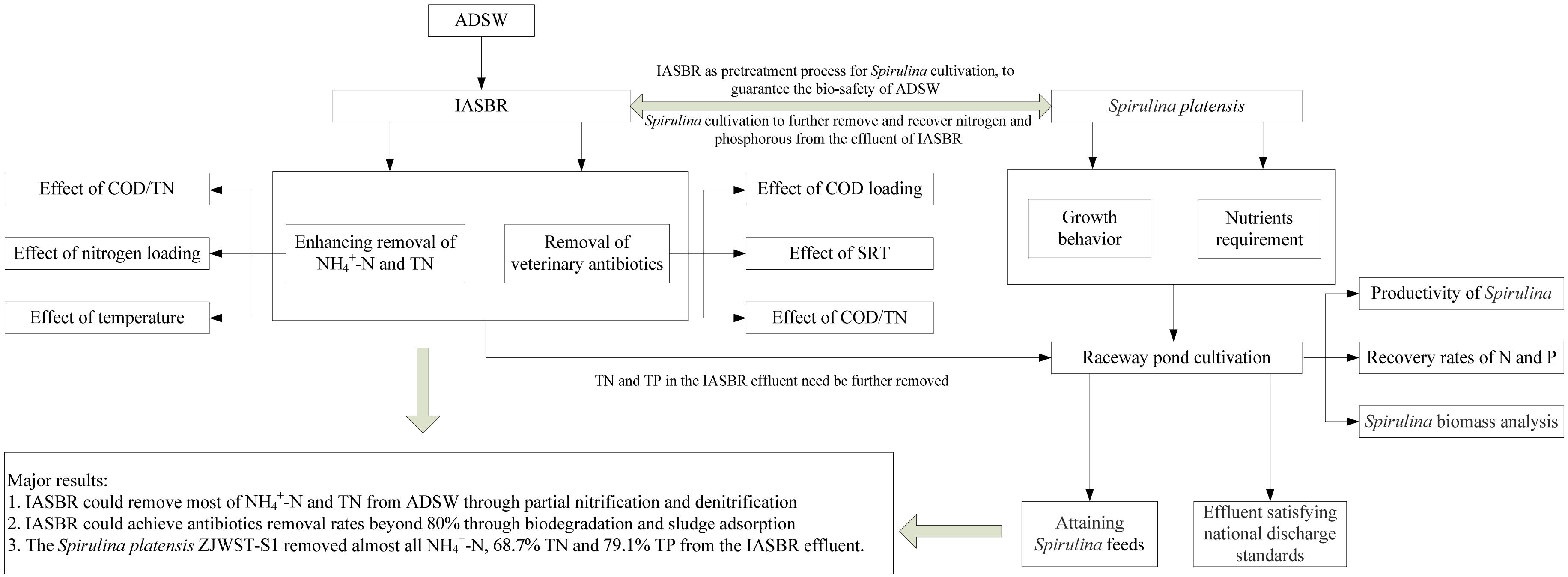

Figure 1-2. Structure of this thesis.

Chapter 2 Removal of nitrogen from ADSW using intermittently aerated sequencing batch reactor

2.1. Introduction

In China, all intensive swine farms are required to equip with a biogas digester for anaerobic digestion pretreatment. However, the digested effluent, i.e. anaerobically digested swine wastewater (ADSW), which is usually characterized as high concentrations of NH_4^+-N and TN with low COD/TN ratio, should be treated further. The conventional biological treatment processes usually have some problems like inefficient removal of NH_4^+-N and TN from ADSW due to their easy acidification trend during nitrification process (Sombatsompop et al., 2011; Yang et al., 2016). The poor nitrogen removal may be attributed to lack of enough biodegradable organic substrate serving as an electron donor, and shortage of alkalinity (Deng et al., 2008; Yang et al., 2015). A biological wastewater treatment process, intermittently aerated sequencing batch reactors (IASBR), is considered to be an efficient technology for enhanced removal of NH_4^+-N and TN through a partial nitrification-denitrification process for low COD/TN ratio wastewater treatment. Using IASBR to achieve partial nitrification has one major advantage, i.e. no need for precise control of dissolved oxygen (DO), pH and temperature in the reactor (Ciudad et al., 2007; Pambrun et al., 2008). However, it should be noticed that partial nitrification by this process is sensitive to operational conditions, and the stable partial nitrification in IASBR system during a long-term operation is likely unreliable when treating wastewater with fluctuating influent (Norton et al., 2009).

So in this chapter, a lab-scale IASBR was set up to study the removal characteristics of nitrogen from ADSW. The removal of NH_4^+-N and TN were investigated under different influent COD/TN ratios, nitrogen loadings and temperature. The nitrite accumulation rate (NAR), concentrations of free ammonia (FA) and free nitrous acid (FNA) were also determined to understand the partial nitrification-denitrification process in IASBR.

2.2. Materials and methods

2.2.1. Wastewater characteristics

The wastewater, i.e. ADSW used in study was collected from the effluent pipe of an anaerobic digester in a large-scale swine farm in Jiaxing City, China. The wastewater was pretreated by coagulation and flocculation to remove suspended solids (SS), and stored at 4°C prior to use. The characteristics of this wastewater were as follows: COD of 1,151±321 mg/L, NH_4^+-N of 987±218 mg/L, TN of 1,423±211 mg/L, pH of 7.8-8.3, and alkalinity of 4,000-6,000 mg/L (in terms of $CaCO_3$), with NO_3^--N below 20 mg/L and NO_2^--N below 1 mg/L.

2.2.2. Experimental setup and operation conditions

The IASBR was composed of a stainless-steel cylinder of Φ20 cm × H 45 cm with an effective volume of 10 L. The operation of the IASBR was controlled by a programmable logic controller following the sequence of 10 min filling, 4 cycles of 40 min stirring (anoxic) and 60 min aeration (aerobic) alternatively, 60 min settling, and 10 min drainage as shown in Figure 2-1. DO was around 0.5-2.0 mg/L during aeration.

Seed sludge was collected from a municipal wastewater treatment plant near Jiaxing City, China. The initial mixed liquor suspended solids (MLSS) concentration in the IASBR was 6.3 g/L. The IASBR was operated for seven runs under different operation conditions (Table 2-1). From day 1 to day 20, the IASBR system started with the diluted ADSW. Its hydraulic retention time (HRT) gradually decreased from 10 days to 7 days to achieve the acclimation of sludge. In Run 1 (day 21 to day 62), the IASBR system was fed with the raw ADSW at a HRT of 7 days. There's no sludge discharge during these days with temperature maintained between 25-30°C. In Run 2 (day 63 to day 117) and Run 3 (day 118 to day 138), sodium acetate was added into the influent to increase the COD/TN ratio from 0.8 to 2.4 in average, during which HRT was decreased from 5 days to 2 days, respectively. Excess sludge was discharged and the corresponding sludge retention time (SRT) was 10-15 days. The operation temperature was still maintained between 25-30°C. And in Run 4 (day 139 to day 188), HRT returned to 3 days. In Run 5 (day 189 to 233), Run 6 (day 234 to 261) and Run 7 (day 262 to 284), as the focus was on temperature effect on nitrogen removal, the reactor temperature was decreased from 25-30°C in Run 4 to 20-25°C in Run 5, 15-20°C in Run 6, and 10-15°C in Run 7. During these periods, HRT was remained at 3 days and the influent COD/TN ratio was around 2.4 averagely. SRT was also kept at 10-15 days.

2.2.3. Analytical methods

COD, NH_4^+-N, TN, NO_2^--N, NO_3^--N, MLSS were analyzed according to standard methods (APHA, 1998). DO, pH and temperature were determined with portable instruments (DKK-TOA CORPORATION, DO-31P, HM-30P), respectively. All water used was Milli-Q water.

The nitrite accumulation rate (NAR) used to evaluate the partial nitrification was calculated according to equation (2-1) (Li et al., 2014). The concentration of free ammonia (FA), the substrate for ammonia oxidizing bacteria (AOB) and also the inhibitor for both AOB and nitrite oxidizing bacteria (NOB), was calculated according to equation (2-2). The concentration of free nitrous acid (FNA), the substrate for NOB and also the inhibitor for both AOB and NOB, was calculated according to equation (2-3) (Anthonisen et al., 1976).

$$NAR = \frac{C_{NO_2^- - N}}{C_{NO_2^- - N} + C_{NO_3^- - N}} \times 100\% \tag{2-1}$$

$$C_{FA} = \frac{17}{14} \times \frac{10^{pH} \bullet C_{NH_4^+ - N}}{e^{6344/(273+T)} + 10^{pH}} \tag{2-2}$$

$$C_{FNA} = \frac{46}{14} \times \frac{10^{pH} \bullet C_{NO_2^- - N}}{e^{-2300/(273+T)} + 10^{pH}} \tag{2-3}$$

in which, $C_{NH_4^+ - N}$, $C_{NO_2^- - N}$, $C_{NO_3^- - N}$ were ammonium nitrogen, nitrite nitrogen and nitrate nitrogen concentrations, respectively, mg/L; C_{FA} is FA concentration, mg/L; C_{FNA} is FNA concentration, mg/L; T is temperature, °C; and pH is negatively logarithm of the hydrogen ion activity.

2.3. Results and discussion

2.3.1. Performance of ammonium nitrogen removal

The removal of NH_4^+-N is usually achieved through nitrification in a biological wastewater treatment process. As shown in Figure 2-2, the effluent NH_4^+-N concentration and NH_4^+-N removal rate fluctuated during the operation from Run 1 to Run 4. In Run 1, the NH_4^+-N removal rate gradually increased from 88.2% to 96.1% at first stage, and then decreased to only 65.8% at the end of Run 1. The effluent NH_4^+-N changed correspondingly from 125.1 mg/L to 32.5 mg/L, and then eventually to 299.2 mg/L. In Run 2, due to the addition of external carbon source, IASBR recovered good performance for NH_4^+-N removal, reaching to 93.2±6.1%. However, in Run 3, the NH_4^+-N removal in IASBR deteriorated again, possibly due to the increase of NH_4^+-N loading rates. The NH_4^+-N removal rate declined to the lowest value of 49.1% and the effluent NH_4^+-N concentration increased to the highest value of 489.5 mg/L. In Run 4, under suitable operation conditions, the IASBR system maintained excellent and stable NH_4^+-N removal at COD/TN ratio of 2.4 and HRT of 3 days. Except on day 142, the effluent NH_4^+-N concentration was always below 10 mg/L, with NH_4^+-N removal rate at a high level of 97.5±1.4%.

The change of NO_2^--N, NO_3^--N and NAR in IASBR were shown in Figure 2-3. The NARs in IASBR system reflected a high level of 85.5±6.9%, at least beyond 60% during Run 1 to Run 4. NO_2^--N took the dominant role over NO_3^--N during these periods, which indicated that partial nitrification was achieved in IASBR system. In Run 1, an obvious accumulation of NO_2^--N was observed at a concentration of 751.9±64.4 mg/L. In Run 2, with the addition of external carbon source, the effluent NO_2^--N concentration decreased drastically from 731 mg/L (day 66) to 4.8 mg/L (day 117), and the effluent NO_3^--N concentration declined from 135.2±38.1 (Run 1) to 22.1±3.1 (Run 2). Nevertheless, the effluent NO_2^--N concentration from IASBR fluctuated in Run 3 under the shorter HRT of 2 days. In addition, HRT returned to 3 days, the effluent NO_2^--N decreased from 176.2 mg/L to 25.4 mg/L in Run 4 finally. The effluent NO_3^--N concentration always maintained at a lower level of 11.1±5.25 mg/L during Run 3 and Run 4.

2.3.2. Effects of major factors on ammonium nitrogen removal

The changes of effluent NH_4^+-N, NO_2^--N, and NO_3^--N from the IASBR system during Run 1 to Run 4 were mainly attributable to the effect of influent COD/TN ratio and NH_4^+-N loading. In fact, different influent COD/TN ratio and NH_4^+-N loading can directly influence the concentrations of FA and FNA, which is closely associated with the growth of nitrification bacteria including AOB and NOB. Generally, FA is considered as the substrate for AOB and the real inhibitor for NOB. Moreover, much high concentration of FA could negatively influence both NOB and AOB. Anthonisen et al. (1976) pointed out the inhibitory concentrations of FA were 10-150 mg/L and 0.1-1 mg/L for AOB and NOB, respectively. So NOB is much more sensitive to FA than AOB. FNA, which is the product of AOB, is considered as the substrate for NOB and the inhibitor for both AOB and NOB. Researchers claimed different inhibitory concentrations of FNA for AOB and NOB. Vadivelu et al. (2007) reported that the inhibitory concentration of FNA was 0.4 mg/L and 0.02 mg/L for AOB and NOB, respectively, and NOB was more sensitive to FNA than AOB. But Anthonisen et al.

(1976) found that FNA's inhibition on AOB and NOB was started from 0.06 mg/L and 2.8 mg/L, respectively, and AOB was more sensitive to FNA than NOB. These results can be attributed to the different wastewater treatment and different biological processes. The concentrations of FA and FNA, NH_4^+-N loading, and NAR from Run 1 to Run 4 were shown in Table 2-2.

Firstly, the effect of COD/TN ratio was discussed. In Run 1, the concentrations of FA and FNA were 3.8±5.1 mg/L and 0.45±0.26 mg/L during this period. FA showed a decline trend at first from 8.6mg/L to 0.3 mg/L, and then increased to 9.2 mg/L again. FNA increased fast from 0.12 mg/L to 0.78 mg/L and then maintained at relatively stable value at about 0.62 mg/L. Therefore, NOB was inhibited apparently at first with a quick increase of FNA, resulting in the accumulation of huge amount of NO_2^--N. It should be noted that the high concentration of FA during initial stage of Run 1 may also contribute to the inhibition of NOB. Moreover, AOB was inhibited at the latter period of Run 1 as the concentration of FA returned to a high level of 9.2 mg/L, leading to the increase of NH_4^+-N in the effluent. By the way, when FNA increased rapidly during the initial period of Run 1, the NH_4^+-N removal was not affected, so FNA of 0.45±0.26 mg/L was not the primary factor for AOB inhibition. In Run 2, sodium acetate was added into the influent to increase the COD/TN ratio from 0.8 to 2.4 averagely. FNA decreased from 0.45±0.26 mg/L in Run 1 to 0.15±0.39 mg/L in Run 2 (even declined to 0.02 mg/L at the end of this period). The inhibition of FNA to NOB was relieved greatly. Similarly, FA increased slightly from 3.8±5.1 mg/L in Run 1 to 4.6±4.3 mg/L in Run 2, while AOB was not inhibited by FA any more due to the actual decrease of FA from initial 9.4 mg/L to 4.1 mg/L in Run 2, improving the NH_4^+-N removal effectively. The extremely low concentration of NO_3^--N and quite high NAR indicated that NOB was still inhibited by FA in IASBR. The drastic decrease of NO_2^--N should be ascribed to the enhancement of NO_2^--N denitrification due to the increase of influent COD/TN ratio. Therefore, the appropriate COD/TN ratio alleviated the inhibition of FA to AOB and achieved better NH_4^+-N removal, simultaneously improving denitrification process and reducing nitrite accumulation.

Secondly, NH_4^+-N loading is another influencing factor on NH_4^+-N removal. As shown in Table 2-2, Run 3 reflected much higher NH_4^+-N loading of 0.055±0.012 kg N/(kg MLSS·day) than Run 4. The influence of FNA on AOB and NOB could be neglected because of their extremely low concentrations. In fact, the higher NH_4^+-N loading led to larger increase of FA (79.2±35.4 mg/L) in IASBR, strongly inhibiting the growth of AOB. After HRT returned to 3 days in Run 4, NH_4^+-N loading decreased to 0.022±0.006 kg N/(kg MLSS·day) with the corresponding decline of FA (3.8±3.2 mg/L), which only restrained the growth of NOB. As a result, IASBR recovered to stable and excellent NH_4^+-N removal due to the relief of FA inhibition.

Besides, the effect of temperature on NH_4^+-N removal was shown in Figure 2-4. When the temperature changed between 15-30°C from Run 4 to Run 6, IASBR always maintained its high and stable NH_4^+-N removal rate greater than 90%. As the temperature declined to 10°C, NH_4^+-N removal deteriorated sharply with a much lower removal rate of 36.4±4.2%. In fact, nitrifying bacteria is sensitive to the change of environmental conditions. Low temperature will reduce the rate of reproduction of nitrifying bacteria, and the decrease in temperature can also reduce the metabolism rate of bacteria, thus reducing the rate of nitrification. The optimum temperature of nitrification is reported to be 20-30°C, and nitrification process comes to a halt with temperature below 5°C (Groeneweg et al., 1994; Salvetti et al., 2006). The concentrations of FA and FNA, NH_4^+-N loading, and NAR from Run 4 to Run 7 were showed as Table 2-3. Although temperature was decreased from 30°C to 10°C, NAR was always greater than 80%, indicating that IASBR still maintained steady partial nitrification regardless of the variation of temperature. NOB was still primarily inhibited by FA from Run 4 to Run 6. In Run 7, huge amount of NO_2^--N was accumulated in the IASBR system, possibly not only due to the strong inhibition of NOB under quite high concentrations FA and FNA, but also due to the breakdown of denitrification under extremely low temperature. The increasing FA concentrations of 118.2±48.4 mg/L intensely repressed the activity of AOB, that resulting in poor NH_4^+-N removal finally in Run 7. Therefore, the critical temperature for NH_4^+-N removal was above 15°C in this study.

2.3.3. Performance of total nitrogen removal and its impact factors

When NH_4^+-N is converted to NO_2^--N or NO_3^--N by nitrifying bacteria, TN will be removed through denitrification in a biological wastewater treatment process. As shown in Figure 2-5, the imbalanced COD/TN ratio greatly affected TN removal. In Run 1, at influent COD/TN ratio of 0.8±0.2, IASBR showed a very low TN removal rate of 18.3±12.2% most probably due to lack of enough carbon source. With the addition of sodium acetate, TN removal rate increased to 92.4±13.3% at influent COD/TN ratio of 2.4±0.5 in Run 2. Enough carbon source could supply abundant electron donors for NO_2^--N to accomplish denitrification process, thus effluent TN concentration declined quickly with the corresponding decrease of

NO_2^--N. According to studies of Bortone and Libelli (1999) and Matějů et al. (1992), the lowest influent COD/TN ratio for denitrification should be 4.0, which is much higher than the required COD/TN ratio of 2.4 in this study. The results may be attributed to the denitrification process through NO_2^--N, which can reduce 40% carbon demand compared to NO_3^--N denitrification.

Influent TN loading also influenced TN removal in IASBR. In Run 3, TN removal rate decreased to 64.6±21.7% under TN loading of 0.098± 0.026 kg N/(kg MLSS·day). Because AOB was inhibited under higher FA concentration in Run 3, NH_4^+-N couldn't be converted to NO_2^--N effectively, thereby TN removal declined as a result of lack of enough electron donor of NO_2^--N for denitrificaiton. When TN loading decreased to 0.042± 0.012 kg N/(kg MLSS·day), IASBR achieved high TN removal of 93.8±12.8% again. More than 90% of NH_4^+-N was oxidized to NO_2^--N by AOB, and then NO_2^--N was removed through denitrification, achieving the excellent TN removal.

Meanwhile, the effect of temperature on TN removal was also investigated from Run 4 to Run 7. As shown in Figure 2-6, when the temperature maintained above 25°C, IASBR exhibited high TN removal performance greater than 90% in Run 4. After that, TN removal rate decreased to 81.7±6.4% when temperature varied between 20-25°C in Run 5. As temperature declined continuously, TN removal deteriorated remarkably. In Run 6, TN removal rate dropped to 54.3±8.8%, and NO_2^--N accumulated to 95.8±53.4 mg/L, much higher than that in Run 5 (43.6±25.8 mg/L) (Table 2-3). In contrast, IASBR still performed satisfactorily with NH_4^+-N removal rate greater than 90%. Salvetti et al. (2006) also indicated that biological treatment system could maintain a good performance of nitrification, but denitrification rate might decrease obviously at 15°C. Therefore, it could be concluded that denitrifying bacteria was much more sensitive to temperature than nitrifying bacteria. In Run 7, the activities of nitrifying bacteria and denitrifying bacteria were restrained at the same time with temperature below 15°C, and IASBR showed an extremely low TN removal rate of 30.7±13.7. As it is known, denitrification rate varies greatly with the change of temperature, and different operation conditions exert different influences in biological treatment processes. Cao et al. (2013) reported the effects of temperature on denitrification rate for activated sludge. Their results showed that denitrification rate decreased about 29.2% and 42.2% at 15°C and 10°C, respectively, in comparison to that at 21°C. Welander and Mattiasson (2003) studied low temperature impact on the suspended carrier biofilm denitrifying process, and found that temperature didn't have significant impact on denitrification rate when varied between 10-30°C. In this study, denitrification process would be inhibited when temperature below 20°C, which is different from the inhibition temperature for nitrification (15°C).

2.4. Summary

Partial nitrification-denitrification was achieved in IASBR system due to the inhibition of NOB during the whole operation. Influent COD/TN ratio, nitrogen loading, and temperature greatly affected the removal of NH_4^+-N and TN simultaneously. A quite low COD/TN ratio and lack of enough carbon source exerted unexpectedly negative influence on denitrification process, resulting in the great accumulation of nitrite. At the same time, low influent COD/TN ratio might cause the increase of FA and inhibited the growth of AOB, thus followed by a worse NH_4^+-N removal. Both NH_4^+-N and TN loadings increased under a shorter HRT, which mainly led to the increase of FA and restrained the partial nitrification. As a result, both NH_4^+-N and TN removal deteriorated under a higher nitrogen loading. Denitrifying bacteria were found to be much more sensitive than nitrifying bacteria. A lower temperature resulted in the drastic increase of FA, leading to both nitrification and denitrification processes worsened. In conclusion, The IASBR could achieve excellent removal rates of NH_4^+-N and TN greater than 90% at influent COD ratio of 2.4, HRT of 3 days and temperature above 20°C. However, the TN concentration in the IASBR effluent maintained between 90-150 mg/L, which still exceeded the discharge requirement of 70 mg/L. Moreover, IASBR was not good at removing TP with removal rate of about 30% and effluent TP concentration of 20-30 mg/L from IASBR, so TP removal was not discussed in this study. The IASBR effluent should be further treated not only for TN removal but also for TP removal.

Table 2-1. Operation conditions of intermittently aerated sequencing batch reactor (IASBR) for nitrogen removal.

Period	HRT (d)	COD/TN	SRT (d)	Temp. (°C)
Start (1-20d)	7	Diluted ADSW	No sludge discharge	25-30
Run 1 (21-62d)	7	0.8±0.2 (Raw ADSW)	No sludge discharge	25-30
Run 2 (63-117d)	5-3	2.4± 0.5	10-15	25-30
Run 3 (118-138d)	2	2.4± 0.8	10-15	25-30
Run 4 (139-188d)	3	2.4± 0.9	10-15	25-30
Run 5 (189-233d)	3	2.5± 0.8	10-15	20-25
Run 6 (234-261d)	3	2.4± 0.6	10-15	15-20
Run 7 (262-294d)	3	2.3± 0.3	10-15	10-15

HRT -Hydraulic retention time; COD/TN -Ratio of chemical oxygen demand to total nitrogen; SRT -Solid retention time; Temp. -Temperature; ADSW -Anaerobically digested swine wastewater

Table 2-2. Ranges of FA, FNA and NO_2^--N concentrations, NH_4^+-N loading, and NAR from intermittently aerated sequencing batch reactor (IASBR) during Run 1 to Run 4.

Item	Run 1	Run 2	Run 3	Run 4
FA (mg/L)	3.8±5.1	4.6±4.3	79.2±35.4	3.8±3.2
FNA (mg/L)	0.45±0.26	0.15±0.39	0.0015±0.0021	0.0019±0.0015
NO_2^--N (mg/L)	751.9±64.4	145.8±213.4	64.2±95.1	44.3±35.3
NAR (%)	88.8±7.2	84.3±9.8	84.2±5.5	85.6±2.8
NH_4^+-N loading (kg N/(kg MLSS·day))	0.028 ± 0.005	0.021 ± 0.008	0.055 ± 0.012	0.022 ± 0.006

FA -Free ammonia; FNA - Free nitrous acid; NO_2^--N -Nitrite nitrogen; NH_4^+-N -Ammonium nitrogen; NAR -Nitrite accumulation rate.

Table 2-3. Ranges of FA, FNA and NO_2^--N concentrations, NH_4^+-N loading, and NAR from intermittently aerated sequencing batch reactor (IASBR) during Run 4 to Run 7.

Item	Run 4	Run 5	Run 6	Run 7
FA (mg/L)	3.8±3.2	4.7±4.8	2.5±4.1	118.2±48.4
FNA (mg/L)	0.0019±0.0015	0.0025±0.0018	0.0038±0.0016	0.13±0.11
NO_2^--N (mg/L)	44.3±35.3	43.6±25.8	95.8±53.4	364.5±92.1
NAR (%)	85.6±2.8	92.2±8.8	81.9±2.7	84.2±10.5
NH_4^+-N loading (kg N/(kg MLSS·day))	0.022 ± 0.006	0.021 ± 0.009	0.023 ± 0.012	0.025 ± 0.013

FA -Free ammonia; FNA - Free nitrous acid; NO_2^--N -Nitrite nitrogen; NH_4^+-N -Ammonium nitrogen; NAR -Nitrite accumulation rate.

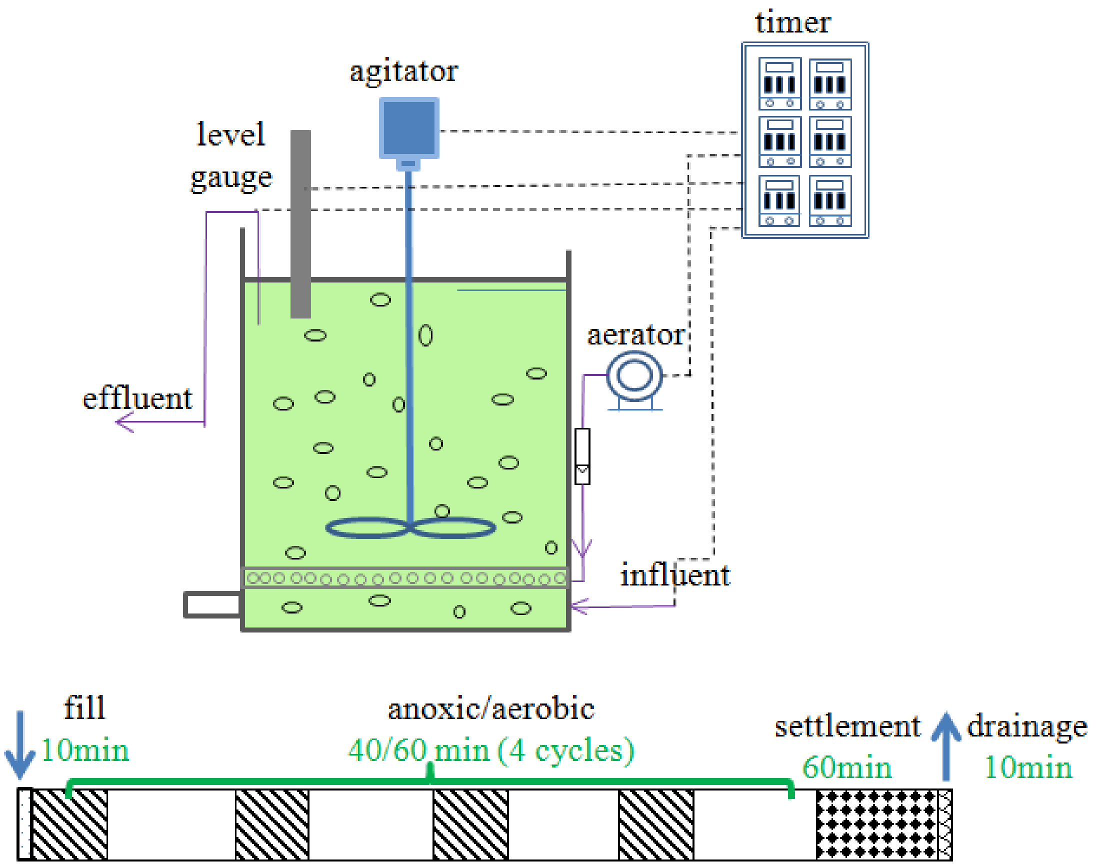

Figure 2-1. Schematic of lab-scale intermittently aerated sequencing batch reactor (IASBR) and its operation cycle.

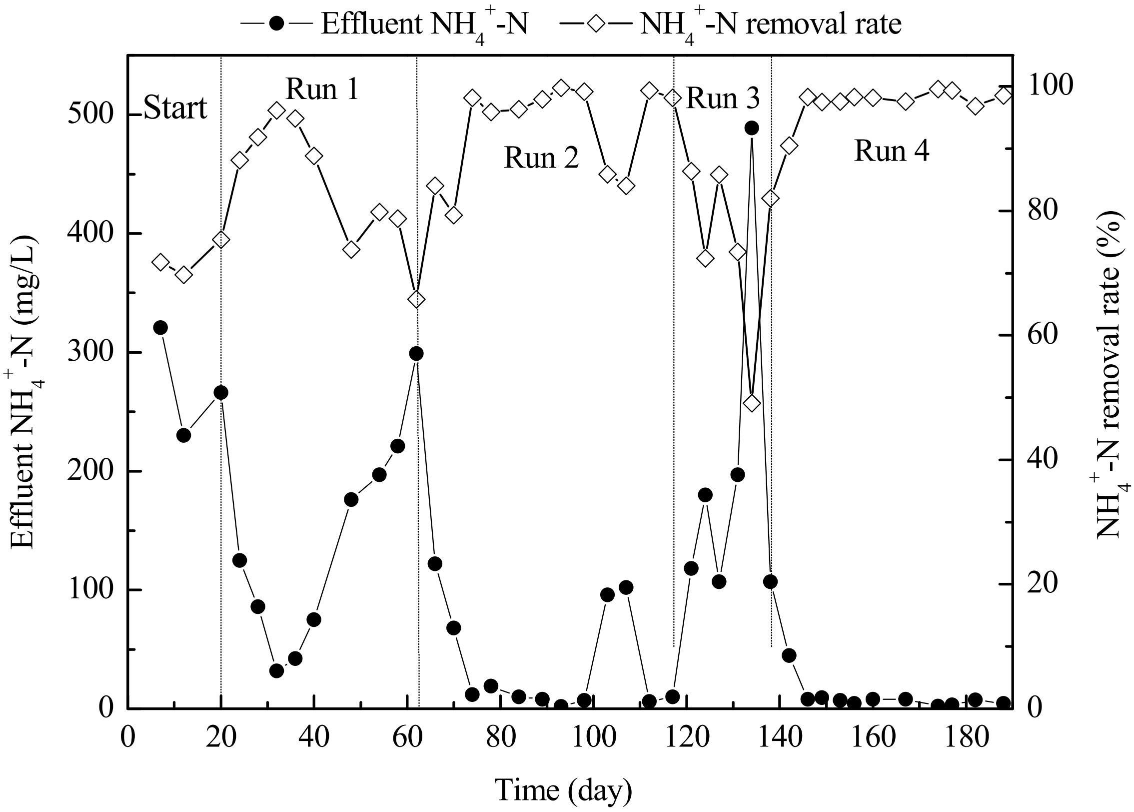

Figure 2-2. NH_4^+-N removal in intermittently aerated sequencing batch reactor (IASBR) during Run 1 to Run 4.

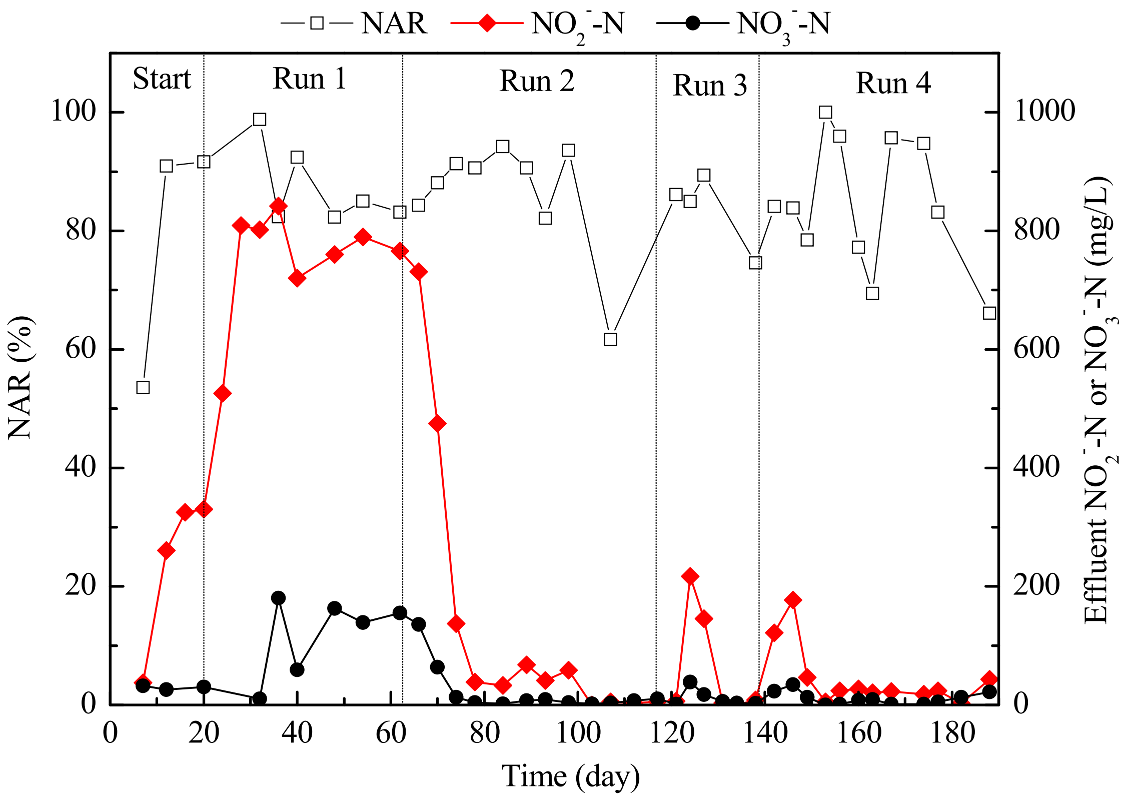

Figure 2-3. Changes of NO_2^--N, NO_3^--N and NAR in intermittently aerated sequencing batch reactor (IASBR) during Run 1 to Run 4.

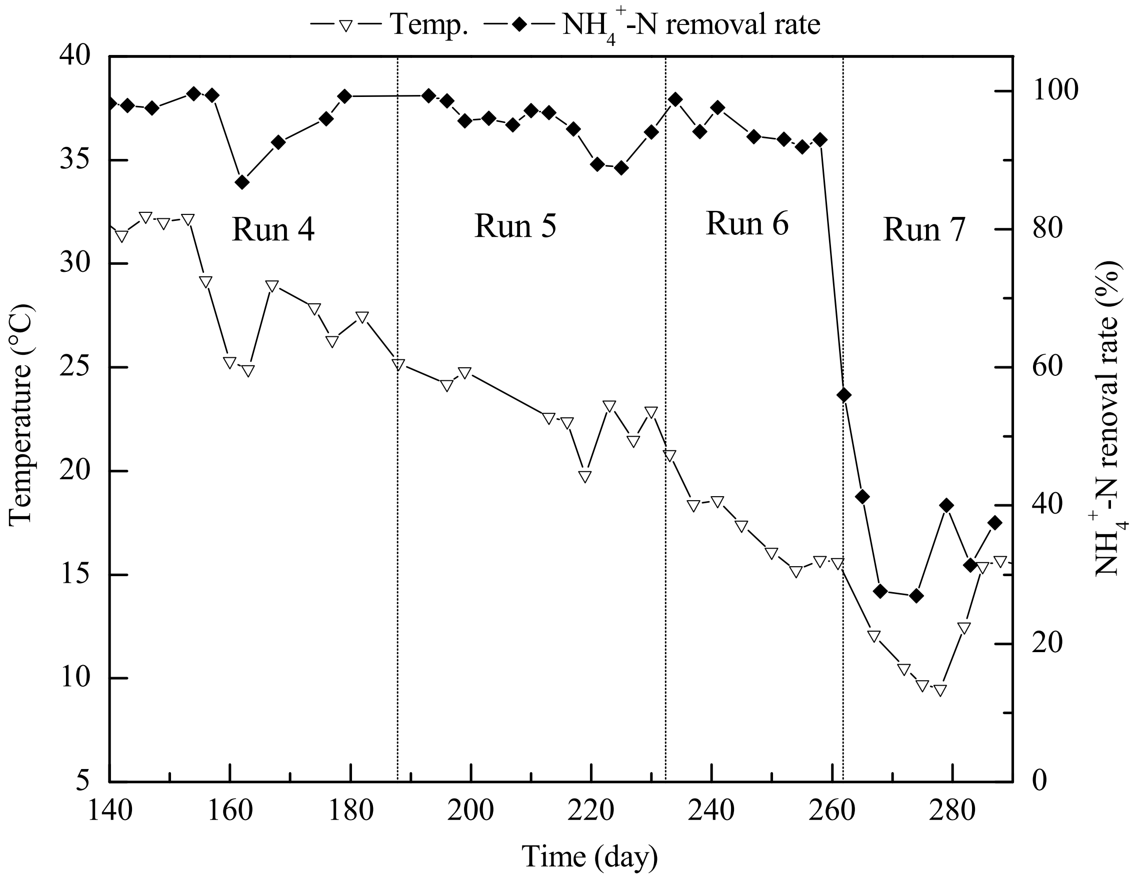

Figure 2-4. Effect of temperature on NH_4^+-N removal in intermittently aerated sequencing batch reactor (IASBR) during Run 4 to Run 7.

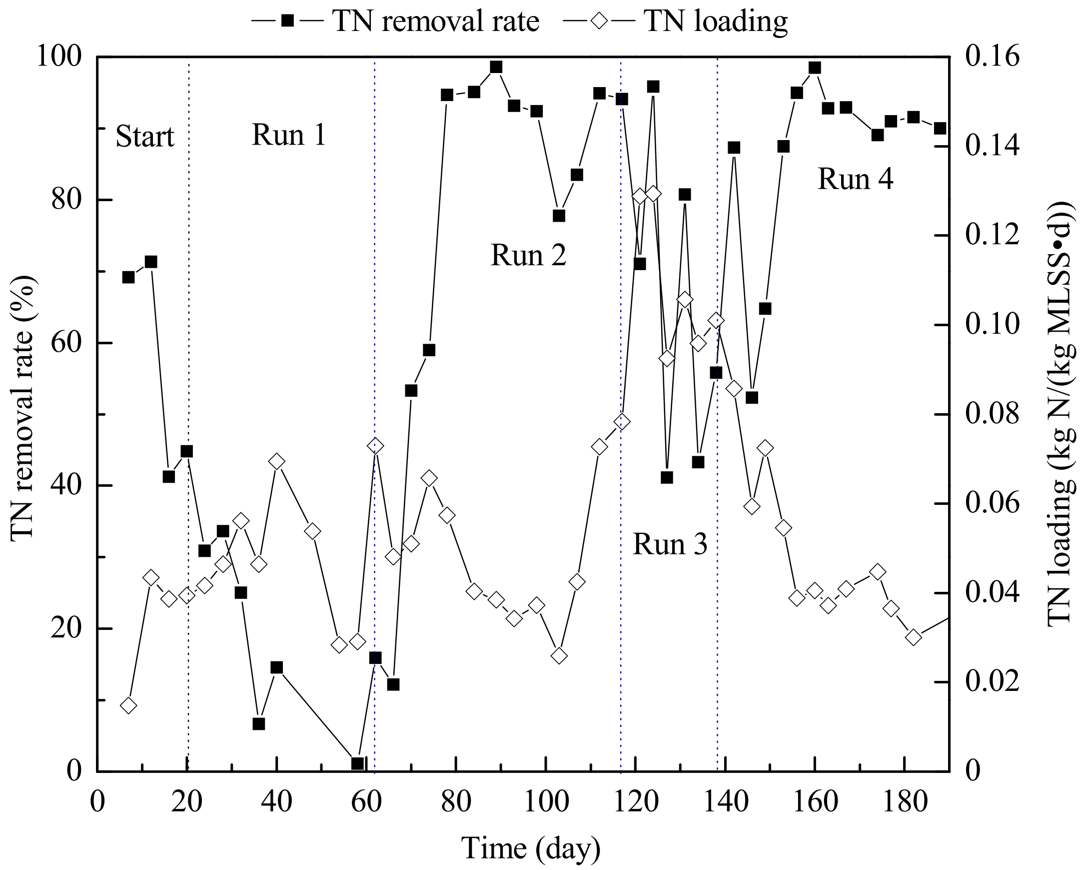

Figure 2-5. TN removal in intermittently aerated sequencing batch reactor (IASBR) during Run 1 to Run 4.

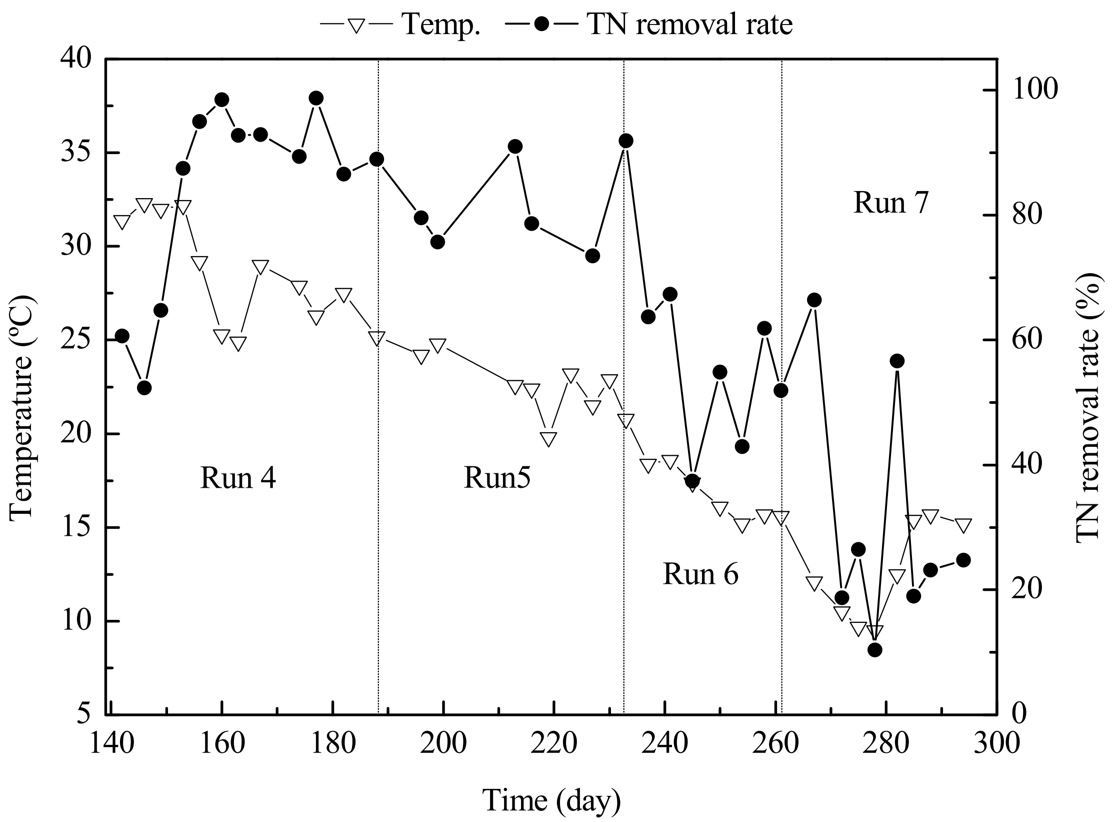

Figure 2-6. Effect of temperature on TN removal in intermittently aerated sequencing batch reactor (IASBR) during Run 4 to Run 7.

Chapter 3 Removal of veterinary antibiotics from ADSW using intermittently aerated sequencing batch reactor

3.1. Introduction

Veterinary antibiotics have been widely used in intensive livestock farming to reduce bacterial infection and promote livestock growth. However, their continuous misuse and resultant pollutions have triggered increasing concerns and serious environmental issues recently (Srinivasan et al., 2014; Luo et al., 2011). Due to the dietary habits of Chinese people, pig farming is developing rapidly and pig farms have been reported to be the major contributors to residual antibiotics in the aquatic environment (Li et al., 2016).

Some researchers reported veterinary antibiotics removals from swine wastewater by using other treatment processes. For instance, Ben et al. (2009) found that an effective degradation of the six selected antibiotics (five sulfonamides and one microcline) could be achieved under the optimum condition by using Fenton's reagent to treat swine wastewater pretreated with sequencing batch reactor (SBR), independent on the tested COD and suspended solids (SS) levels. Huang et al. (2015) tested the performance of vertical up-flow constructed wetlands (VUF-CWs) on swine wastewater containing tetracycline compounds (TCs), achieving 69.0%-99.9% of removal efficiencies. However, little information is available on the differences and characteristics of sludge sorption and biodegradation regarding to antibiotics removal in biological processes, especially IASBR for swine wastewater treatment. More importantly, the existence of antibiotics in ADSW has been demonstrated a negative influence on the growth of *Spirulina*. Therefore, it’s necessary to study the removal performance and characteristics of antibiotics in IASBRs for swine wastewater treatment.

In this chapter, a lab-scale IASBR was applied to remove veterinary antibiotics from ADSW in order to avoid the inhibitory effect on subsequent *Spirulina* cultivation. The removal rates of eleven antibiotics by the IASBR were studied under different COD volumetric loadings, SRTs and COD/TN ratios. And the relationships between antibiotics biodegradation and sludge sorption in IASBR were also explored.

3.2. Materials and methods

3.2.1. Wastewater and chemicals

The ADSW was also collected from the effluent pipe of an anaerobic digester in a large-scale swine farm in Jiaxing City, China. The wastewater was pretreated by coagulation and flocculation to remove suspended solids, and stored at 4°C prior to use. Two batches of ADSW were sampled for this study: (1) Batch 1 ADSW was used for the experiments from day 1 to day 78, which had a COD of 1,210±291 mg/L, NH_4^+-N of 798±188 mg/L, TN of 1,439±195 mg/L, pH of 7.8-8.1, antibiotics of 44.4±1.1 μg/L, and alkalinity of 4,800-6,200 mg/L (in terms of $CaCO_3$); and (2) Batch 2 was used for the experiments from day 79 to day 193, having a COD of 1,060±278 mg/L, NH_4^+-N of 852±131 mg/L, TN of 1,387±187 mg/L, pH of 7.8-8.3, antibiotics of 30.6±0.3 μg/L, and alkalinity of 5,200-6,800 mg/L (in terms of $CaCO_3$). All the above indices were determined with standard methods (APHA, 1998).

Eleven veterinary antibiotics including tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), doxycycline (DC), sulfamethoxazole (SMX), sulfadimidine (SMD), ciprofloxacin (CIP), norfloxacin (NOR), enrofloxacin (ENR), tylosin (TYL) and roxithromycin (RTM) were detectable in the ADSW and selected for this study. All standard samples of these antibiotics were purchased from Dr. Ehrenstorfer GmbH (Germany). Simatone ordered from AccuStandard (USA) was used as the internal standard substance (Ben et al., 2008; Yang et al., 2010). The surrogate standard substances including thiabendazole-d4 (TBH-D_4), sulfamethoxazole-d_4 (SAX-D_4), ciprofloxacin-d_8 (CFX-D_8), and erythromycin-^{13}c-d_3 (ETM-^{13}C-D_3) were bought from Toronto Research Chemicals (Canada). 5.0 mg of each antibiotic standard was dissolved into 100 mL volumetric flask with methanol as solvent to prepare the standard mixture solution of antibiotics. The simatone-methanol solution of 10 mg/L was used as the internal standard solution. 0.1 mg of each surrogate standard was dissolved into 100 mL volumetric flask with methanol as solvent to prepare the surrogate standard mixture. All the above solutions were stored at 4°C, and all chemicals used were of analytically pure grade.

3.2.2. Experimental setup and operation conditions

The IASBR was composed of a stainless-steel cylinder of Φ25 cm × H 40 cm with an effective volume of 15 L. The operation of the IASBR was described as section 2.2.2. DO was around 0.5-2.0 mg/L during aeration, and the water temperature was kept at 30±1°C during the whole operation.

Seed sludge was collected from a municipal wastewater treatment plant near Jiaxing City, China. The initial MLSS concentration in the IASBR was 6.8 g/L. The IASBR was operated for three runs under different operation conditions (Table 3-1). In Run 1 (day 1 to day 64), the IASBR system was fed with raw ADSW at a long HRT of 7 days. In Run 2 (day 65 to day 98) and Run 3 (day 99 to day 193), sodium acetate was added into the influent to increase the COD/TN ratio from 0.8 to 2.4 on average, and at shorter HRT of 5 days and 3 days, respectively. Excess sludge was discharged only in Run 3 and the corresponding sludge retention time (SRT) was 30-40 days. The influent, effluent, and activated sludge in the IASBR were sampled on day 34, 54, 62, 70, 93, 136, and 184, respectively for detection of antibiotics.

3.2.3. Solid phase extraction (SPE) of antibiotics

For wastewater samples, after filtration through a 0.7 μm glass fiber membrane (Whatman, UK), 20 mL of the filtrated wastewater was diluted 10-fold by Milli-Q water prior to antibiotics extraction. EDTA-2Na (0.2 g) was then added to the diluted wastewater, with pH adjusted to 4.0 using 10% HCl. Later, 200 mL of diluted wastewater was loaded into a pre-conditioned Oasis HLB column (6 cc/200 mg, Waters, USA) at a flow rate of 2-3 mL/min, followed by 10 min of vacuum drying. The extracted antibiotics in the Oasis HLB column were eluted with 5 mL of methanol, and then concentrated to approximately 0.5 mL under a stream of nitrogen gas. Finally, the solution was diluted to 2 mL with methanol after the addition of 20 μL of simatone standard solution and filtered through a 0.22 μm PTFE filter (Anpel, China) before antibiotics detection.

Briefly, for sludge samples, 200 μL of the surrogate standard mixture containing TBH-D_4, SAX-D_4, CFX-D_8, and ETM-^{13}C-D_3 at 1 mg/L each was added to 0.2 g of 24 hr freeze-dried activated sludge. The sludge sample was subsequently immersed in 5 mL of extraction solution which was composed of methanol, 0.1 mol/L of EDTA-2Na and citrate buffer (pH 4) at a volumetric ratio of 3:1:2. The sludge sample and extraction solution mixture was vortexed for 1 min, ultrasonicated for 15 min, and centrifuged at 3,500 r/min for 5 min. After the first extracted supernatant liquid was collected, the extraction process was repeated twice. The three extracts were pooled, diluted with Milli-Q water to 200 mL, combined with 0.2 g of EDTA-2Na, and adjusted to a pH of 4.0 with 10% HCl. The extract was finally loaded into two pre-conditioned, serially connected SAX-HLB columns (Waters, USA) to remove humus particles and concentrate the antibiotics. The elution of antibiotics from the HLB column and further treatments were the same as those for the wastewater samples described as above.

3.2.4. Analytical method for antibiotics with liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Antibiotics were determined using a liquid chromatography (LC) (Waters e2695, Waters, USA) coupled with a triple quadrupole-linear mass spectrometer (MS) (Waters TQ Detector, Waters, USA). Aglient Eclipse XDB-C18 column (4.6 mm×150 mm, 5 μm pore size) was used for separation of antibiotics. The injection volume was 10 μL with column temperature at 30°C. A combination of three mobile phases was used at a constant flow rate of 0.3 mL/min. Mobile phase A was composed of 99.9% water and 0.1% formic acid (*V/V*). Mobile phases B and C were methanol and acetonitrile, respectively. The separation of antibiotics was achieved with a gradient program described as follows: the mobile phase ratio of A:B:C was 90:4:6 at 0 min and maintained for 10 min, 90:0:10 at 11 min, 87:0:13 at 13 min, 78:0:22 at 15 min, 55:0:45 at 25 min, 0:0:100 at 26 min and maintained for 5 min, 90:4:6 at 33 min and maintained for 12 min for column equilibration. The MS system equipped with an electrospray ionization (ESI) source and operated in the positive ion mode. The optimal conditions for the MS system were determined as capillary temperature 120°C, desolvation temperature 350°C, capillary voltage 4.0 kV, and desolvation gas flow 550 L/hr. The limits of detection (LOD) for the antibiotics in the wastewater and activated sludge were 4-71 ng/L and 0.4-7.1 μg/kg, respectively, with signal to noise ratios (S/N) of 3. The fortified recovery rates of the antibiotics in the wastewater ranged from 73% to 105.2% with standard deviation (SD) of 3.1 to 10.2% (n=3), slightly higher than those in the activated sludge (57.4%-104.6%, SD = 1.9%-10.9%, n=3). The recovery rates and LODs in this study were comparable to previous research works (Yang et al., 2010; Ben et al., 2008).

3.2.5. Analysis of mass balance for antibiotics

Samples from the IASBR during stable operation stage between day 34 to day 62 were used for mass balance analysis in terms of the 11 antibiotics. The influent was from batch 1 ADSW with total concentration of antibiotics of 44.88±1.15 μg/L, and total concentration of antibiotics 6.69±0.58 μg/L in the effluent. The mass balance between the liquid phase, sludge phase, and biodegradable portion of the antibiotics was calculated based on equations (3-1) and (3-2):

$$M_{inf} - M_{eff} = M_S + M_d \qquad (3\text{-}1)$$

$$Q{\cdot}C_{inf} - Q{\cdot}C_{eff} - Q{\cdot}C_S{\cdot}\mathrm{SS}\cdot 10^{-3} = \mathrm{MLSS}{\cdot}C_S{\cdot}V{\cdot}10^{-3} + M_d \qquad (3\text{-}2)$$

where M_{inf} (μg) is the total amount of antibiotics in the influent, M_{eff} (μg) is the total amount of antibiotics in the effluent, M_S (μg) is the total amount of antibiotics adsorbed in the sludge, M_d (μg) is the total amount of antibiotics biodegraded by microorganisms, C_{inf} is the concentration of antibiotics in the influent (μg/L), C_{eff} is the concentration of antibiotics in the effluent (μg/L), SS (g/L) is the concentration of suspended solids in the effluent, C_S (μg/kg) is the concentration of antibiotics in the sludge, Q (L) is the total amount of treated wastewater, V (L) is the effective volume of the reactor, and MLSS (g/L) is the concentration of sludge in the reactor. Since the suspended solids concentration in the effluent was below 5 mg/L which was considered to be negligible when compared to the influent, equation (3-2) could be simplified as equation (3-3):

$$Q{\cdot}C_{inf} - Q{\cdot}C_{eff} = \mathrm{MLSS}{\cdot}C_S{\cdot}V{\cdot}10^{-3} + M_d \qquad (3\text{-}3)$$

And C_{inf} and C_{eff} were calculated based on the average antibiotic concentrations on day 34, 54, and 62, and the total volume of treated wastewater (60 L) on day 34 to 62, respectively. M_d was calculated by subtracting M_{eff} and M_{S} from M_{inf}. In consideration of the change of MLSS and sorption concentration of antibiotics on the sludge, the mass balance was calculated as equation (3-4):

$$Q{\cdot}C_{inf} - Q{\cdot}C_{eff} = (\mathrm{MLSS}_{62}{\cdot}C_{S\text{-}62} - \mathrm{MLSS}_{34}{\cdot}C_{S\text{-}34})\cdot V{\cdot}10^{-3} + M_d \qquad (3\text{-}4)$$

3.3. Results and discussion

3.3.1. Performance of veterinary antibiotics removal from ADSW

The removals of eleven veterinary antibiotics by the IASBR are shown in Table 3-2. In Run 1, samples were collected three times, respectively on day 34, 54, and 62 for antibiotics removal determination. In Run 2 and Run 3, two times of determination (day 70 and 93 in Run 2, and day 136 and 184 in Run 3, respectively) were conducted. The concentrations of total detected veterinary antibiotics in Batch 1 ADSW on day 34, 54, 62, 70 ranged from 42.93 μg/L to 46.54 μg/L, much higher than those in Batch 2 ADSW (day 93, 136 and 184, 30.16-31.06 μg/L). The components of antibiotics in the two batches ADSW were also quite different. The tetracyclines including TC, CTC, OTC, and DC averagely accounted for 69.7±3.7% of total antibiotics in Batch 1 ADSW, which were the second most abundant antibiotics in Batch 2 (36.8±1.3%). In Batch 1 ADSW, DC and TC were the two dominant tetracyclines, accounting for >90% of the total tetracyclines. In Batch 2 ADSW, the concentrations of DC and TC decreased substantially, whereas OTC and CTC remained stable resulting in their dominance among the tetracyclines. The sulfonamides, especially SMD, were the second most abundant classes of antibiotics in Batch 1 ADSW (19.3±1.1% of total detected antibiotics), which became the most abundant antibiotics in Batch 2 ADSW amounting to 40.3±2.4% of the total antibiotics. The quinolones including NOR, CIP, and ENR exhibited similar low concentrations of below 4.5 μg/L. Among the macrolides, TYL was detected at very low concentrations in the ADSW (0.04-0.98 μg/L), whereas RTM was not detectable in all the ADSW samples used in this study (< LOD). In fact, the characteristics and water quality of ADSW fluctuated greatly with the change of seasons, most probably attributable to the variation in feeds to pigs, characteristics of swine wastewater and efficiency of the anaerobic digestion facilities. Besides, lack of professional and precision management of swine farms also contributed a lot to the above changes, especially the characteristics of ADSW, thus the change of antibiotics concentrations in ADSW.

In Run 1 and Run 2, the IASBR achieved high total veterinary antibiotics removals of averagely 85.1±1.4% and 75.9±1.3%, respectively. The concentrations of total detected antibiotics in the effluents of Run 1 and Run 2 ranged from 5.94 μg/L to 10.88 μg/L. The tetracyclines and sulfonamides were significantly removed from the influent by the IASBR in Run 1 and Run 2, with removal rates of 81.1%-91.3% and 92.1%-98.5%, respectively. The remaining sulfonamides were only detectable at 0.1-1.0 μg/L. In Run 3, however, the removal of antibiotics decreased dramatically, only about 37.3% on day 184.

Figure 3-1 shows the relationship between the influent concentrations of antibiotics and their removal rates. The removal rates of tetracyclines and sulfonamides almost remained >80% when their influent concentrations were higher than 5.0 μg/L; however, their removal rates greatly fluctuated and were difficult to achieve stable levels greater than 80% when their influent concentrations were lower than 5.0 μg/L. The removal rates of quinolones and macrolides changed irregularly and seemed to have no direct relationship with their influent concentrations, among which three of quinolones and one of macrolides were all below 5 μg/L. Therefore, the antibiotics removal could maintain much more stable at relatively higher efficiency when influent ADSW contained high concentrations of antibiotics, which is to some extent in agreement with McAdam et al. (2011) who stated that the removal rates of trace organic pollutants were higher at higher influent concentrations than those at lower ones. The above results may also attribute to the refractory characteristics of antibiotics and their unfavorable competition against other abundant organics in ADSW.

3.3.2. Sorption of veterinary antibiotics onto sludge

Antibiotics adsorbed on the sludge were detected, as shown in Table 3-3. No sludge was intentionally discharged during Run 1 and Run 2. The concentrations of eleven veterinary antibiotics in the sludge increased with the operation of IASBR in Run 1, from 5.02 mg/kg on day 34 to 13.78 mg/kg on day 62. The total amount of antibiotics in the sludge increased from 413.3 μg on day 34 (MLSS of 5.5 g/L) to 1,055.7 μg on day 62 (MLSS of 5.1 g/L), resulting in an increase in the sludge antibiotics content from day 34 to day 62. The sludge concentration in IASBR increased from 5.1 g/L on day 62 to 6.9 g/L on day 93 in the presence of sufficient external carbon source. However, the increase of sludge concentration didn't help to increase antibiotics sorption amount onto sludge particles, resulting in almost similar total antibiotics contents of 13-14 mg/kg in sludge in Run 2 as that on day 62 in Run 1. The total amount of antibiotics accumulated in sludge slightly increased from 1,306.2 μg on day 70 to 1,386.9 μg on day 93, and most of the antibiotics in the influent ADSW were steadily removed through biodegradation instead of sludge sorption. Therefore, it is reasonable to suppose that the sorption of veterinary antibiotics on sludge reached their equilibrium state in Run 2. In addition, biodegradation may play a dominant role in antibiotics removal when the sorption of veterinary antibiotics onto sludge reaches the sorption capacity of sludge before being discharged. Excess sludge was intermittently discharged in Run 3, and the concentration of total antibiotics in the sludge declined remarkably to 10.42 mg/kg on day 136, and again reached a similar concentration of 10.24 mg/kg on day 184. Meanwhile, the total amount of antibiotics in the sludge slightly decreased from 1,279.2 μg on day 136 to 1,193.4 μg on day 184. These results imply that sludge sorption was an important pathway for the removal of veterinary antibiotics in ADSW and a balanced sorption capacity of sludge could be achieved at a long SRT during stable operation, while a shorter SRT could decrease the balanced sorption capacity of the sludge. The existence of a balanced sorption capacity could be attributable to the establishment of antibiotics adsorption-desorption equilibrium in the sludge (Morissette et al., 2015; Yang et al., 2011). More specifically, the tetracyclines were the most abundant in the sludge in accordance with the influent ADSW composition, amounting to 94±2.1% of the total adsorbed antibiotics. The macrolides were detected at the lowest concentrations, which is in consistence with their low concentrations in ADSW. In contrast, the sulfonamides, the second abundant antibiotics in ADSW, were detected at low concentrations in the sludge (4.2 to 0.2 mg/kg).

3.3.3. Effect of COD volumetric loading on antibiotics removal

Organic loading usually has a profound influence on organics removal in bioreactors, including trace organic pollutants such as antibiotics, pharmaceuticals and endocrine disrupting chemicals (EDCs) (Carranza-Diaz et al., 2014; McAdam et al., 2011). In Run 1, the COD volumetric loading was 0.17±0.041 kg COD/(m^3·day) with MLSS of 5.1-6.8 g/L. The insufficient carbon source may limit the growth of microorganisms, leading to decreased sludge concentration thus relatively low and fluctuant COD removal rates (averagely 60.3±15.7%). When the COD volumetric loading was increased to 0.65±0.032 kg COD/(m^3·day) in Run 2 and 1.07±0.073 kg COD/(m^3·day) in Run 3, correspondingly, average COD removal rates were increased to 88.3±4.7% and 90.8±5.5%, respectively (Table 3-4). The effluent CODs during Run 1, Run 2 and Run 3 were averagely 454, 396 and 296 mg/L, respectively. This observation together with the increase in MLSS from Run 1 to Run 2 and Run 3 (Table 3-4) indicates that external carbon source or increasing organic loading favored the growth of microorganisms, thus enhanced the removal of organic pollutants.

However, antibiotics removal showed a different trend with the change of COD volumetric loading. The removal rates of eleven veterinary antibiotics slightly decreased as the COD volumetric loading increased from Run 1 to Run 2, and abruptly dropped to 49.3±12.1% when COD loading was further increased to 1.07±0.073 kg COD/(m^3·day) in Run 3. This phenomenon implies that COD volumetric loading could significantly impact the antibiotics removal, and higher organic loading may have strongly negative effect on the competition of antibiotics over easily biodegradable carbon source (sodium acetate in this study) during biological wastewater treatment. This observation to some extent agrees with Conkle et al. (2010). In Run 1, MLSS was determined to gradually decrease from initial 6.8 g/L to 5.1 g/L on day 62. The obvious decrease in MLSS in the reactor possibly reflected that a large proportion of the microorganisms might be in an endogenous respiration phase because of the lower organic loading applied and lack of readily biodegradable substances. Although veterinary antibiotics were determined at trace levels in terms of μg/L in the influent, the microorganisms might utilize them as much as possible for survival, resulting in somewhat amelioration of competition between antibiotics and other organics. In Run 2, the increase in sludge concentration was most probably resulted from the sufficient supply of readily biodegradable carbon source (sodium acetate in this study) and less need for antibiotics functioned as carbon source, thus decreased antibiotics removal rates noticeably. Moreover, when the reactor was operated at a much higher COD volumetric loading (Run 3), the microorganisms seemed to prefer to utilize the abundant readily biodegradable organics in the influent for their survival and metabolisms. Therefore, other organics than antibiotics achieved an overwhelming removal in terms of biodegradation, resulting in the remarkable decline of antibiotics removal in Run 3. As shown in Figure 3-2, all the four antibiotics classes exhibited decreased removal rates at higher COD volumetric loadings. Results also showed that the sulfonamides exhibited higher removal rates than tetracyclines in Run 1 and Run 2, but lower in Run 3. These observations suggest that the removal of sulfonamides is more likely to be affected by organic loadings than that of tetracyclines, attributable to the higher adsorption capacity of tetracyclines onto sludge. Namely, regarding to veterinary antibiotic removal COD volumetric loading may pose even greater influence on biodegradation than sludge.

3.3.4. Effect of SRT on antibiotics removal

SRT might also impact the antibiotic removal by IASBR systems as in other bioreactors. A too short SRT will lead to greatly declined sludge concentrations and thereby a significant decrease in sorption and degradation of antibiotics and other organic pollutants (Xia et al., 2012). No sludge was intentionally discharged in Run 1 and Run 2 due to the lower organic loadings applied, and their MLSS was maintained at 5.1-7.1 g/L. In Run 3, the organic loading was increased and SRT was shortened to 30-40 days with MLSS ranged between 7.4-8.5 g/L. As shown in Table 3-4, sludge discharge did not significantly affect the removals of generally organic pollutants indicated by COD using the IASBR system. On the other hand, the accumulated antibiotics were found to slightly decrease from 13.4 mg/kg on day 93 (Run 2) to 10.43 mg/kg on day 136 (Run 3), and maintained at a stable level of 10.24 mg/kg on day 184 (Table 3-3). Therefore, the applied shorter SRT might not be the reason for the poor antibiotics removal in Run 3. As known, SRT could definitely influence the balanced sorption capacity of sludge, and a shorter SRT could reduce the accumulation of antibiotics in sludge. In this study, due to the fact that both HRT and SRT were changed in Run 2 and Run 3, it is difficult to interpret the real impact of SRT on antibiotics removal, which needs further investigation in the followed-up research.

3.3.5. Effect of COD/TN ratio on antibiotics removal

The COD/TN ratio in the influent was found to significantly influence nitrogen removal. As shown in Table 3-4, TN removal was poor and fluctuated (35.8±18.2%) under operation at a low COD/TN ratio of 0.8±0.2 in Run 1, and NO_2^--N was detected to accumulate at 751.9±98.3 mg/L due to lack of sufficient carbon source for denitrification. This high concentration of NO_2^--N might also exert a negative effect on the growth of nitrifying and denitrifying bacteria with resultant low TN removal. In Run 2, along with the addition of sodium acetate, the COD/TN ratio in the influent increased to 2.4±0.5 averagely. As a result, the TN removal increased to 85.9±7.4%, even though the reactor was operated under a shorter HRT and higher nitrogen volumetric loading conditions (compared to Run 1). However, due to a much shorter HRT applied in Run 3, the further increased nitrogen loading might impact nitrogen removal negatively. On the contrary, antibiotics removal appears not to be significantly influenced by COD/TN ratio, as Run 1 and Run 2 achieved high removal rates for total antibiotics (Table 3-4).

3.3.6. Removal mechanisms of veterinary antibiotics

Generally, both sludge sorption and biodegradation are the main removal pathways for antibiotics in biological processes rather than other possible pathways like volatilization or hydrolysis (Dorival-García et al., 2013; Li and Zhang, 2010). The IASBR system achieved excellent antibiotics removal when treating the raw ADSW without any external carbon source from day 34 to day 64. The role of sludge sorption for antibiotics removal can be seen more clearly before the bioreactor reached the balanced sorption capacity of sludge. Therefore, mass balance analysis based on data from days 34 to 62 was conducted to explore the removal routes of veterinary antibiotics in this IASBR system. As shown in Table 3-5, the results reveal that 15.1% of the antibiotics were remained in the effluent, with 60.8% being biodegraded and 24.1% adsorbed by sludge. This observation indicates that during a long and stable operation of the IASBR system biodegradation plays a more important role in antibiotics removal than sludge sorption. Specifically, 95% of the influent sulfonamides were biodegraded, indicating their easy biodegradability. Li and Zhang (2010) reported that two sulfonamides (SMX and sulfadiazine) were predominantly removed through biodegradation in an activated sludge process. Both Wu et al. (2009) and Yu et al. (2011) noticed the weak sorption of SMX and SMD in a biological system, and results from the latter work indicate that SMX possesses much stronger biodegradability than SMD. In this study, 96.8% of influent SMD was removed by biodegradation, much higher than SMX (71.9%). This observation is probably attributable to the huge difference in their initial concentrations in the influent ADSW. The poor sorption of sulfonamides can be explained by the acid-base equilibrium processes involved. The amphoteric sulfonamides with functional groups are in anionic form at neutral and basic pH, resulting in a low adsorption to the activated sludge (Ben et al., 2014; Yang et al., 2011). On the other hand, only 57.5% of influent tetracyclines could be biodegraded with 30.4% left in the sludge, suggesting that unlike sulfonamides this class of antibiotics was more difficult to biodegrade, thus sludge sorption would be a more important removal route. Both Li and Zhang (2010) and Prado et al. (2009) claimed that TC exhibited good adsorbability and low biodegradability, which could be mainly removed by adsorption in biological processes. However, in this study, greater than 60% of influent TC, OTC and DC were removed through biodegradation. CTC was an exception, nearly all of which was adsorbed on the sludge. These results may be brought about by the starvation conditions under lower organic loading in Run 1, which promoted microorganisms to utilize antibiotics at trace levels and lower biodegradability. As observed by Shi et al. (2011), the removal of tetracyclines can be described as a quick sorption and then a slow biodegradation. In this study, neither biodegradation nor sorption could achieve excellent removal of quinolones, leaving 41.6% in the effluent. In addition, 25.9% of influent quinolones were removed by biodegradation, slightly higher than that by sorption, which is quite different from the findings of Dorival-García et al. (2013) who claimed that sorption by sludge played a dominant role in the elimination of six commonly found quinolones (ciprofloxacin, moxifloxacin, norfloxacin, ofloxacin, pipemidic acid, and piromidic acid) from wastewaters. Additionally, no comment could be made on macrolides due to the fact that their influent concentrations were too low in this study.

3.4. Summary

The IASBR was an efficient biological treatment system for the removal of veterinary antibiotics from ADSW and would greatly decrease negative influence on the *Spirulina* growths for subsequent nutrients recovery process. The removal of veterinary antibiotics was significantly decreased under higher organic volumetric loading or shorter HRT. A shorter SRT could reduce the accumulation of antibiotics and the balanced antibiotics sorption capacity of sludge. The COD/TN ratio in influent was a key factor for nitrogen removal, but not for veterinary antibiotics in ADSW treatment by using IASBR. The influent sulfonamides underwent obvious biodegradation in the bioreactor. The removal of both tetracyclines and quinolones was contributed by biodegradation and sludge sorption. Although the IASBR could achieve excellent removal rates (>80%) for all studied veterinary antibiotics from ADSW under lower COD volumetric loading, nearly 24% of eleven antibiotics were found to adsorb onto the activated sludge and were not completely decomposed. IASBR could removed most of antibiotics in ADSW and created a bio-safety condition for *Spirulina*.

Table 3-1. Operation conditions of intermittently aerated sequencing batch reactor (IASBR) for antibiotic removal.

Parameters	Run 1	Run 2	Run 3
Stage	day 1-day 64	day 65-day 98	day 99-day 193
HRT (day)	7	5	3
SRT (day)	62*	98*	30-40
Influent COD/TN	0.8±0.2	2.4±0.5	2.4±0.4
Influent COD (mg/L)	1,210±291	3,252±159	3,218±219
Influent NH_4-N (mg/L)	798±188	805±169	832 ±151
Influent TN (mg/L)	1,439±195	1,392±138	1,321 ±198
Sludge discharge	No	No	Intermittently

*Estimated according to MLSS in the reactor and suspended solids concentration in the effluent.

HRT - Hydraulic retention time; SRT - Solid retention time; COD - Chemical oxygen demand; TN -Total nitrogen; NH_4^+-N -Ammonium nitrogen.

Table 3-2. Variations in antibiotics concentrations in the influent and effluent of intermittently aerated sequencing batch reactor (IASBR).

ADSW batch	Run	Operation		DC[a]	TC	OTC	CTC	SMD	SMX	ENR	CIP	NOR	TYL	RTM	Total tetracyclines	Total sulfonamides	Total quinolones	Total macrolides	Total antibiotics
1	1	Day 34	Inf.	19.43	11.36	1.59	1.08	6.38	1.34	1.02	1.04	1.07	0.13	—[b]	33.46±3.85	7.72±1.88	3.13±2.18	0.13±0.06	44.44±3.48
			Eff.	2.94	1.71	0.29	0.21	0.12	0.03	0.19	0.36	1.24	0.04	—	5.15±2.56	0.15±0.08	1.79±0.56	0.04±0.02	7.13±2.34
		Day 54	Inf.	17.62	9.22	1.05	1.93	8.60	0.25	1.39	2.84	3.53	0.11	—	29.82±2.98	8.85±2.02	7.76±2.25	0.11±0.07	46.54±2.14
			Eff.	1.04	0.62	0.36	0.58	0.23	0.20	0.38	0.55	1.88	0.10	—	2.60±1.27	0.43±0.12	2.81±1.12	0.10±0.05	5.94±1.17
		Day 62	Inf.	15.44	11.19	1.71	2.92	8.88	0.22	0.56	0.77	1.86	0.11	—	31.26±3.68	9.1±2.56	3.19±1.88	0.11±0.08	43.66±3.19
			Eff.	1.14	1.06	0.37	1.04	0.23	0.21	0.39	0.33	1.61	0.07	—	3.61±1.22	0.44±0.28	1.33±0.64	0.07±0.04	7.01±1.05
	2	Day 70	Inf.	16.5	9.51	0.84	2.31	8.29	0.21	1.54	1.25	2.01	0.47	—	29.16±2.33	8.5±1.44	4.8±1.67	0.47±0.22	42.93±1.98
			Eff.	3.21	1.77	0.02	0.39	0.25	—	0.51	0.46	0.66	0.34	—	5.39±1.45	0.25±0.12	1.62±1.15	0.34±0.12	10.88±1.21
2		Day 93	Inf.	3.78	1.28	3.01	3.35	12.16	0.41	2.26	2.11	1.14	0.98	—	11.42±1.98	12.57±2.12	5.51±2.15	0.98±0.11	30.48±1.87
			Eff.	0.79	0.73	0.31	0.33	0.81	0.18	0.35	1.65	0.48	0.31	—	2.16±1.65	0.99±0.43	2.48±1.92	0.31±0.08	6.94±1.15
	3	Day 136	Inf.	3.56	1.01	2.82	3.45	11.21	0.19	1.01	2.91	4.25	0.65	—	10.84±1.22	11.4±2.87	8.17±3.76	0.65±0.28	31.06±1.67
			Eff.	0.47	0.46	0.94	1.34	4.36	—	0.67	1.33	2.37	0.07	—	3.21±1.31	4.36±1.22	4.37±1.88	0.07±0.11	12.01±1.43
		Day 184	Inf.	3.87	1.51	2.97	3.13	12.72	0.23	1.23	1.91	2.36	0.23	—	11.48±3.45	12.95±2.12	5.5±2.97	0.23±0.08	30.16±2.51
			Eff.	0.9	0.67	1.6	2.91	7.44	0.21	1.2	3.9	—	0.07	—	6.08±2.28	7.65±1.27	5.1±1.21	0.07±0.12	18.9±2.11

[a]Average value of triplicate tests for each antibiotic class. [b] '-': means not detected. Unit: μg/L.

TC -Tetracycline; CTC -Chlortetracycline; OTC -Oxytetracycline; DC -Doxycycline; SMX -Sulfamethoxazole; SMD -Sulfadimidine; CIP -Ciprofloxacin; NOR -Norfloxacin; ENR -Enrofloxacin; TYL -Tylosin; RTM -Roxithromycin.

Table 3-3. Variations in antibiotics concentrations in the activated sludge of intermittently aerated sequencing batch reactor (IASBR).

ADSW batch	Run	Operation	DC[a]	TC	OTC	CTC	SMD	SMX	ENR	CIP	NOR	TYL	RTM	Total tetracyclines	Total sulfonamides	Total quinolones	Total macrolides	Total antibiotics
1	1	Day 34	2.541	1.517	0.150	0.673	0.002	0.002	0.015	0.093	0.021	0.004	—[b]	4.881±1.882	0.004±0.002	0.128±0.066	0.004±0.001	5.017±1.632
		Day 54	4.396	3.054	0.154	1.656	0.001	0.007	0.113	0.155	0.115	0.007	—	9.260±2.543	0.008±0.002	0.384±0.092	0.007±0.003	9.659±2.432
		Day 62	6.838	3.858	0.265	1.823	0.053	0.032	0.185	0.257	0.437	0.028	—	12.784±3.335	0.085±0.039	0.880±0.115	0.028±0.011	13.777±2.967
	2	Day 70	7.634	2.448	0.270	2.850	0.044	0.016	0.115	0.119	0.226	0.034	—	13.202±3.165	0.060±0.041	0.460±0.124	0.034±0.018	13.755±2.798
2		Day 93	7.808	1.047	0.406	3.294	0.031	0.019	0.276	0.285	0.193	0.039	—	12.554±2.023	0.049±0.034	0.754±0.135	0.039±0.021	13.397±1.961
	3	Day 136	1.506	0.259	1.240	6.466	0.172	0.019	0.219	0.280	0.251	0.007	—	9.471±1.729	0.190±0.068	0.750±0.087	0.007±0.002	10.418±1.534
		Day 184	1.423	0.215	1.350	6.337	0.168	0.018	0.207	0.270	0.242	0.007	—	9.325±1.432	0.187±0.065	0.718±0.103	0.007±0.005	10.237±1.454

[a]Average value of triplicate tests for each antibiotic class. [b] "-" means not detected. Unit: mg/kg.

TC -Tetracycline; CTC -Chlortetracycline; OTC -Oxytetracycline; DC -Doxycycline; SMX -Sulfamethoxazole; SMD -Sulfadimidine; CIP -Ciprofloxacin; NOR -Norfloxacin; ENR -Enrofloxacin; TYL -Tylosin; RTM -Roxithromycin.

Table 3-4. Average COD, antibiotics, NH_4^+-N and TN removal rates during Run 1, Run 2, and Run 3.

Item	Run 1	Run 2	Run 3
MLSS (g/L)	5.1-6.8	5.3-7.1	7.4~8.5
Influent COD volumetric loading (kg COD/(m^3·day))	0.17±0.041	0.65±0.032	1.07±0.073
COD removal rate (%)	60.3±15.7	88.3±4.7	90.8±5.5
Antibiotics removal rate (%)	85.1±1.4	75.9±1.3	49.3±12.1
NH_4^+-N removal rate (%)	62.5±14.8	89.1±8.8	51.2±28.6
TN removal rate (%)	35.8±18.2	85.9±7.4	54.1 ± 19.5

COD - Chemical oxygen demand; NH_4^+-N -Ammonium nitrogen; TN -Total nitrogen; MLSS -Mixed liquor suspended solid.

Table 3-5. Mass balance analysis on antibiotics during operation from day 34 to day 62.

Substances	M_{inf} (μg)	M_{eff} (μg)	M_s (μg)	M_d (μg)	M_{eff}/M_{inf} (%)	M_s/M_{inf} (%)	M_dM_{inf} (%)
Total antibiotics	2,694.0	402.0	642.4	1,619.6	15.1	24.1	60.8
Tetracyclines	1,890.0	228.1	575.0	1,086.9	12.1	30.4	57.5
Sulfonamides	516.0	19.8	6.2	490	3.8	1.2	95.0
Quinolones	282.0	150.0	58.9	73.1	53.2	20.9	25.9
Macrolides	6.6	4.1	1.8	0.7	62.1	27.3	10.6

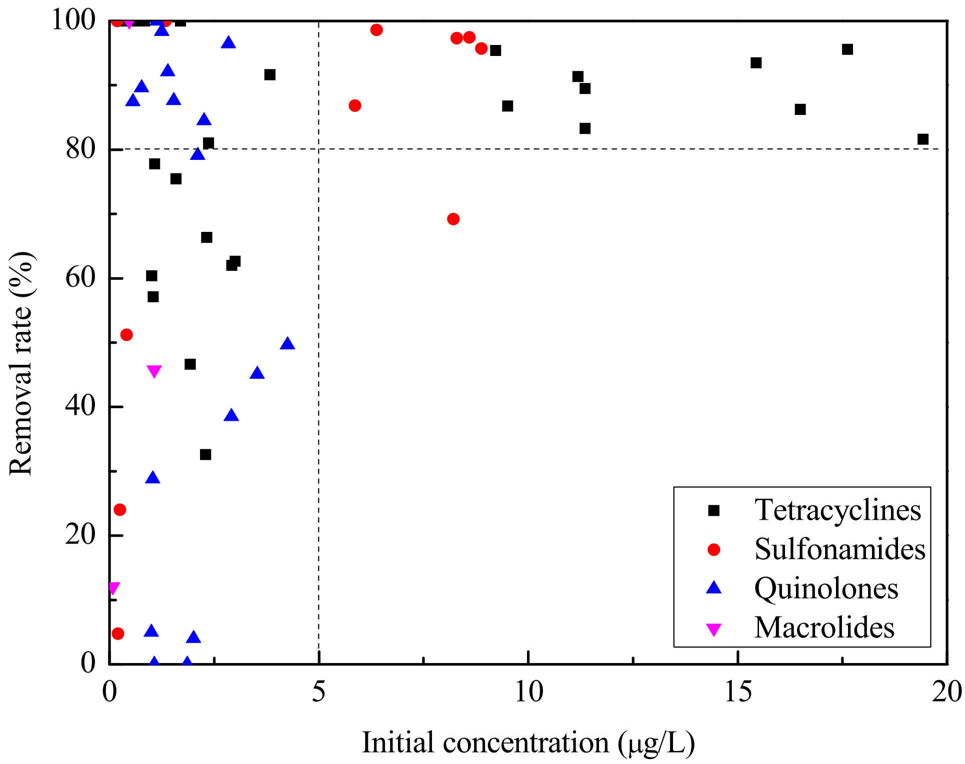

Figure 3-1. Variations in removal rates of antibiotics under different influent antibiotics.

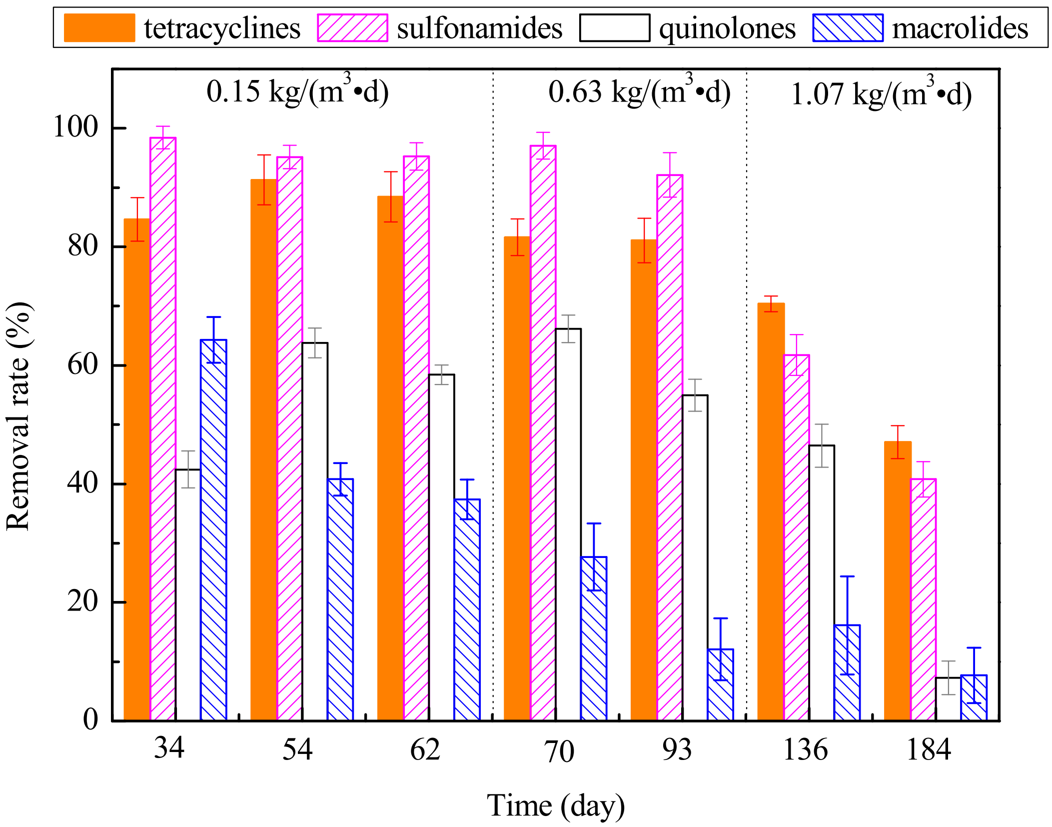

Figure 3-2. Variations in removal rates of antibiotics under different influent COD volumetric loadings.

Chapter 4 Cultivation of *Spirulina platensis* for nutrients recovery from ADSW

4.1. Introduction

Swine wastewater not only contains high concentrations of nutrients (nitrogen and phosphorus), but also contains abundant of trace elements such as zinc and magnesium, bioactive substances such as protein, amino acids, sugars and ribose, and growth regulators such as vitamins and growth hormone (Xia, 2011). It has been proven that ADSW can be used for *Spirulina* cultivation. However, most of previous studies reported that the cultivation of *Spirulina*the required dilution of ADSW and supplement of nitrogen and phosphorus compound fertilizers. The primary challenge is the poor resistance of common *Spirulina* strains to the complex contaminants in ADSW, especially the high concentrations of NH_4^+-N and antibiotics. The concentration of NH_4^+-N in ADSW is usually 500-1,500 mg/L in China. Few *Spirulina* can grow fast in NH_4^+-N concentration over 60 mg/L (Cheunbarn and Peerapornpisal, 2010; Geng et al., 2004). Besides, as described in Section 1.4.2, the high concentration of veterinary antibiotics will also inhibit the growth of *Spirulina* (Cao et al., 1999; Wang et al., 2001). Therefore, in order to create a bio-safety condition for *Spirulina*, IASBR was used to pre-treat ADSW. IASBR system showed excellent removal rates of NH_4^+-N and antibiotics finally, but the concentrations of TN and TP in IASBR effluent still didn't meet the new discharge standard.

So in this chapter, in order to further remove and recover nitrogen and phosphorous, the feasibility of culturing *Spirulina* with the IASBR effluent was investigated. A *Spirulina platensis* strain ZJWST-S1, which was strongly adaptable and resist to high concentrations of ADSW, was selected for cultivation in undiluted ADSW. Its growth behaviors and nutrient requirements were first confirmed by single factor analysis. Then the IASBR effluent was used to cultivate *Spirulina platensis* for satisfying the discharge limits of TN and TP. An indoor raceway pond experiment was then carried out for two runs, in which the algae yield and wastewater quality were studied.

4.2. Materials and methods

4.2.1. Source of *Spirulina platensis*

The *Spirulina* strain used in this study was named ZJWST-S1, isolated from an ADSW storage pool in Jiaxing City, China. The pool received ADSW from a large scale swine farm 0.15 km away where ADSW was sampled for this study. The inoculum enlargement of strain ZJWST-S1 was carried out in a Zarrouk (Zarrouk, 1966). In order to compare the growth behavior with local strain ZJWST-S1, three other *Spirulina* strains were also used in this study. *Spirulina* Ns-90020 was provided by Wuhan Botanical Garden, Chinese Academy of Sciences. *Spirulina* DLMM S6 and DLMM S2 were provided by School of Life Sciences, Xiamen University, China. *Spirulina* Ns-90020, DLMM S6, DLMM S2 were the strains which were commonly used in China for commercial cultivation. The strain ZJWST-S1 and other three commercial strains have been identified as *Spirulina platensis* by morphological features including width of trichome, cell size, shapes of the helix, gas vesicles and calyptra on the end cells with a microscope (400×magnification; Olympus, Japan) shown as in Table 4-1.

4.2.2. Flask tests on growth behaviors and nutrient requirements

Flask tests in illuminating incubator were carried out in order to compare the growth behavior of the local strain ZJWST-S1 with the commercialized strains, and investigated the nutrients requirement of ZJWST-S1. The *Spirulina platensis* strains were filtered through a 400 mesh filter, washed by a Zarrouk medium without nitrogen and phosphorus, and then were inoculated into culture medium or ADSW in 250 mL Erlenmeyer flasks. The flasks were then allocated in an illuminating incubator of 25±2°C for nine days. The photoperiod was 12 hr light (6,000±2,000 lux) and 12 hr dark. The solution was manually mixed once per day. All experiments were carried out in triplicate. The influence of inorganic carbon, nitrogen and phosphorous on the growth of ZJWST-S1 was studied by adjusting the dosage of $NaHCO_3$, $NaNO_3$ or K_2HPO_4 in the Zarrouk medium. The inhibition of NH_4^+-N or NO_2^--N on *Spirulina* growth was investigated by dosing different concentrations of $(NH_4)_2SO_4$ or $NaNO_2$ into the Zarrouk medium. The components of Zarrouk medium are shown in Table 4-2 and Table 4-3. The ADSW was taken from a large scale swine farm in Jiaxing City, China. The wastewater was pretreated with an iron-carbon internal electrolysis followed by activated sludge process. The pretreated effluent was characterized as 133±28 mg/L of COD, 1,031±49 mg/L of TN, 6.1±0.8 mg/L of NH_4^+-N, 39±11 mg/L of TP, and 7.8±1.1 mg/L of antibiotics. This pretreated effluent was used for *Spirulina* cultivation after pH was adjusted to 8.0 or higher with 0.1g/L of sodium bicarbonate.

4.2.3. *Spirulina platensis* cultivation in a raceway pond

Spirulina platensis cultivation tests were carried out in two indoor raceway ponds of 240 L (2.0 m in length×0.6 m in width×0.3 m in depth) which were prefilled with 140 L of IASBR effluent and Zarrouk medium, respectively. *Spirulina* ZJWST-S1 at the log phase with an initial absorbance of approximately 0.25 at 560 nm (OD_{560}) (initial biomass concentration approximately 0.25 g/L) was inoculated into the IASBR effluent. The IASBR effluent was characterized as 126.4±9.2 mg/L of COD, 145.2±6.8 mg/L of TN, 15.1±1.8 mg/L of NH_4^+-N, 21.5±2.4 mg/L of TP, and 5.5±2.2 mg/L of antibiotics. Water temperature was maintained at 20-25°C. Light intensity was 5000 lux and the light-dark ratio was 14 hr: 10 hr. The system was continuously stirred with a paddle agitator at 30 rpm/min. Two runs, totally 20 days, were carried out in order to investigate biomass production. In Run 2, the cultivation was operated in the corresponding original medium after harvest of Run 1. Sodium bicarbonate with a final concentration of approximately 13.5 g/L was added into the IASBR effluent in order to guarantee adequate inorganic carbon source for *Spirulina platensis* growth.

4.2.4. Analytical methods

The cell concentration in the mixed liquor was indicated from the chlorophyll *a* concentration (OD_{560}) with a spectrophotometer (Precisoin Instruments, China). Biomass was evaluated using the method of dry cell weight by filtering the mixed liquor samples through a 400 mesh filter, washing the solids with acidified water (pH 4, carboxylic acid solution) to eliminate salt precipitates and alkalinity, and finally drying the washed solids in an oven at 60°C for 4 hr and weighing. The dry cell weight showed a linear relationship with OD_{560} as follows: $y=0.7178x+0.0025$ (R^2 >0.997), in which Y (g/L) is the dry cell weight; x is the absorbance at 560 nm. The crude protein content of dry algae biomass (%) was determined with a Coomassie brilliant blue method (Sedmak and Grossberg, 1977). The moisture content in the algal powder were determined after washing algae biomass with acidified water, and then drying at 105°C; The ash content in the algal powder was measured after burning the dry biomass at 600°C for 4 hr. Heavy metals (Pb, As, Cd, and Hg) in the algae powder were determined with an atomic absorption spectro-photometer (Agilent Technologies, USA) after microwave digestion (Sineo, China). Bacteria, coliforms, molds and pathogens were determined with plate counting methods.

Nitrogen and phosphorus in *Spirulina* cell was estimated according to Stumm and Morgan (1996), who proposed *Spirulina* cell component as a formula of $C_{106}H_{263}O_{110}N_{16}P$. Conversion efficiency of TN or TP from ADSW to *Spirulina platensis* was calculated as equation (4-1):

$$\text{Conversion efficiency of TN or TP}\,(\%) = \frac{\text{Net increase in dry weight of } \textit{Spirulina}}{\text{Net reduction of TN or TP from ADSW}} \times \text{Percentage of TN or TP in the cell of } \textit{Spirulina} \quad (4\text{-}1)$$

4.3. Results and discussion

4.3.1. Comparison in growth behaviors of *Spirulina platensis* in ADSW and Zarrouk medium

The nine days' productivity of *Spirulina platensis* ZJWST-S1, Ns-90020, DLMMS6 and DLMMS2 was compared in Table 4-4 and Table 4-5. In Zarrouk medium, the productivity of ZJWST-S1 was similar to those of DLMM S6 and DLMM S2, much higher than that of Ns-90020.The crude protein in dry biomass was 62.8 ±6.1% for ZJWST-S1, slightly higher than but not significantly different from that of DLMMS2 (59.4±6.2%) and DLMMS6 (61.7±10.5%), and lower than that of Ns-90020 (70.3±5.8%). But ZJWST-S1 showed much higher biomass productivity of 29.1±13.2 $g/m^2 \cdot d$ than other three *Spirulina platensis* strains. As the medium was changed to ADSW, only ZJWST-S1 remained fast growth. DLMM S6 grew a little, while Ns-90020 and DLMM S2 did not grew at all. The growth rate and productivity of ZJWST-S1 in ADSW medium showed little different from those in a Zarrouk medium. The crude protein contained in dry biomass of ZJWST-S1 slightly decreased to 59.4±2.5%, much higher than that of DLMMS6 (45.1±5.3%). Although ADSW has been pre-treated by an iron-carbon internal electrolysis process followed by activated sludge process, Ns-90020 and DLMM S2 still can't adapt to the condition, mainly because they are often cultured with clean freshwater for food in China. DLMMS6 was once used to culture with wastewater, but it showed a relatively lower biomass productivity. Therefore, comparing with other three *Spirulina platensis* strains, the local *Spirulina platensis* strain of ZJWST-S1 was the best choice for cultivation in ADSW.

4.3.2. Effect of carbon, nitrogen and phosphorus on growth of *Spirulina platensis*

According to Stumm and Morgan (1996), the photosynthesis process of algae is interpreted as equation (4-2). Therefore, inorganic carbon, nitrogen and phosphorus are principal nutrients required by algae growth. Single factor experiments were carried out by flask tests and the growth curves of ZJWST-S1 were studied in Zarrouk medium that was adjusted by the dosage of $NaHCO_3$, $NaNO_3$ and K_2HPO_4, as shown in Figure 4-1, Figure 4-2 and Figure 4-3.

$$106CO_2+16NO_3^-+HPO_4^{2-}+122H_2O+18H^+ \xrightarrow{\text{Light}} C_{106}H_{263}O_{110}N_{16}P+138O_2 \qquad (4\text{-}2)$$

Spirulina can use CO_2, HCO_3^-, and CO_3^{2-} as carbon source. HCO_3^- and CO_3^{2-} can be converted to CO_2 by carbonic anhydride (CA), and then utilized by *Spirulina* (Badger and Price, 2003; Jaiswal et al., 2005). Growth curves in Figure 4-1 showed an initial lagging phase of three days in the group without $NaHCO_3$, while two days in other groups. ZJWST-S1 cultured with $NaHCO_3$ of 2.0 g/L or less showed yellowish green and a significant slower growth rate. The nine days' productivity greatly increased as the dosage of $NaHCO_3$ increased from 2.0 g/L to 4.0 g/L, and the productivity increased little when further increased $NaHCO_3$ dosage. The result was in consistent with Hu et al. (2012) who also reported that *Spirulina* could gradually adapt to the low concentration of inorganic carbon and be able to achieve as high productivity as that in a Zarrouk medium even if the dosage of $NaHCO_3$ was reduced to 1/4. In ADSW, HCO_3^- and CO_3^{2-} were also considered as the indicator of alkalinity. And the raw ADSW usually contains alkalinity of approximately 4000-6000 mg/L. The partial nitrification and denitrification processes in IASBR will consume partial alkalinity, which might result in the decrease of HCO_3^- and CO_3^{2-} and the shortage of inorganic carbon source for *Spirulina*. As a result, the external inorganic carbon should be added into the IASBR effluent for *Spirulina* cultivation.

Growth curves in Figure 4-2 and Figure 4-3 did not show a significantly different growth rate between the test groups containing nitrate (NO_3^--N) of 40-825 mg/L and PO_4^{3-}-P of 10-89 mg/L. But it should be pointed out that the higher biomass productivity could be achieved under higher concentrations of NO_3^--N than the lower ones, mainly because the gradually decreasing NO_3^--N couldn't satisfy the optimal nitrogen requirement when reaching to a lower concentration of NO_3^--N. The effect of phosphorous for ZJWST-S1 was observed through the change of *Spirulina* color. ZJWST-S1 in medium containing 0-3 mg/L of PO_4^{3-}-P turned yellowish and settled down. This suggests that phosphorus of at least 3-10 mg/L was required for high productivity, although too much phosphorus was unnecessary. The same high grow rate of *Spirulina* was also achieved by Shi and Chen (1989) in a medium with PO_4^{3-}-P of 5.6 mg/L as in a Zarrouk medium. However, they found that the growth rate of *Spirulina* began to slow down after five days with the exhaustion of phosphorus.

4.3.3. Effect of ammonium and nitrite nitrogen on growth of ZJWST-S1

NH_4^+-N and NO_2^--N, which were usually considered to be toxic to algae growth, were common inorganic nitrogen forms in ADSW, especially in IASBR effluent. Figure 4-4 showed that growth inhibition effect became stronger with the increase in NH_4^+-N concentration. NH_4^+-N at 60 mg/L or above led to a significant decrease in the algal productivity. Commonly, the existence form of NH_4^+-N in the liquid is associated with pH and temperature. Under the higher temperature and alkaline pH, free ammonia (FA) toxic for photosynthetic organisms will dominate in the liquid. Unfortunately, *Spirulina* strains are mainly cultured in slightly alkaline environment, so *Spirulina* can't survive under high NH_4^+-N concentration. However, NO_2^--N did not inhibit the growth of ZJWST-S1 in all groups as shown in Figure 4-5. On the contrary, it seemed to slightly promote the growth, especially under the concentrations of 30-60 mg/L. The stimulation of NO_2^--N on the growth of *Spirulina platensis* was also reported by Markou and Georgakakis (2011). The algae can convert NO_3^--N and NO_2^--N to NH_4^+-N, and then assimilated by algae, and the light is an important for this conversion process (Converti et al., 2006).

4.3.4. Raceway pond cultivation on productivity of ZJWST-S1

Raceway pond cultivation of *Spirulina platensis* ZJWST-S1 was carried out for two runs in IASBR effluent and Zarrouk medium, as shown in Figure 4-6. In Run 1, the biomass productivity and the biomass concentration after 10 days' cultivation were 9.7 g/(m^2·d) and 1.07 g/L in Zarrouk medium, respectively, and 5.1 g/(m^2·d) and 0.68 g/L in IASBR effluent, respectively. In Run 2, the cultivation was operated in the original medium of Run 1 without any other treatment after biomass harvest. Growth of ZJWST-S1 in Run 2 performed similar to Run 1. ZJWST-S1 still grew faster in Zarrouk medium, with biomass productivity of 7.5 g/(m^2·d). The growth was still lower in the IASBR effluent, with biomass productivity of 4.5 g/(m^2·d). Comparing with the growth in Zarrouk medium, ZJWST-S1 grew slower in the IASBR effluent no matter in Run 1 or Run 2. The main reason may be attributed to low quality of IASBR effluent, especially its high chrominance. Depraetere et al. (2013) evaluated the potential of color removal methods for enhancing the growth rate and biomass yield of

Spirulina using swine wastewater as a nutrient source. Chrominance removal using biopolymers such as chitosan resulted in a doubled initial growth rate and a 50% increase in the final biomass yield. Liu et al. (2017) found the decolorization of biological effluent by ozone oxidation could significantly increase the growth rate of *Spirulina*. The high value of chrominance in the culturing medium primarily influences the utilization of light for *Spirulina*, thereby causing negative effect on the photosynthesis process.

4.3.5. Nutrients removal and recovery from ADSW

The concentrations of NH_4^+-N, TN and TP in raceway pond were analyzed during the cultivation of *Spirulina platensis* ZJWST-S1 in the IASBR effluent as shown in Figure 4-7. After 20 days' cultivation (two runs), the concentrations of NH_4^+-N, TN and TP in the raceway pond were decreased averagely from 15.1±1.8 mg/L, 145.2±6.8 mg/L and 21.5±2.4 mg/L to 0.8±0.5, 45.5±3.1 mg/L and 4.5±0.8 mg/L, respectively, achieving removal rates of 94.7 %, 68.7% and 79.1%. The removal of NH_4^+-N in the IASBR effluent took precedence over NO_2^--N and NO_3^--N because NH_4^+-N was utilized preferentially by *Spirulina platensis* (Malerba et al., 2015). When NH_4^+-N was exhausted in raceway pond, NO_2^--N and NO_3^--N would be transformed to NH_4^+-N for the utilization of *Spirulina platensis* (Converti et al., 2006). The concentration of TN remained 45.5 mg/L at the end of Run 2, satisfying the new discharge standard for TN finally. Meanwhile, the concentration of TP was only 4.5 mg/L at the end of Run 2, also satisfying the discharge requirement for TP. The *Spirulina* generally converts inorganic phosphorus into organic substances through photosynthesis phosphorylation combined with substrate level phosphorylation and oxidative phosphorylation. According to the mass balance analysis, results revealed that 91.5±3.4% of TN and 92.4±4.8% of TP reduced from ADSW were converted to *Spirulina platensis* biomass. Namely, most of the removed TN and TP from IASBR effluent had been utilized by microalgae.

The concentrations of Pb, As, Cd, and Hg in the harvested *Spirulina platensis* ZJWST-S1 biomass were 0.098±0.024, 0.76±0.07, 0.07±0.01 and 0.06±0.01 mg/kg dry biomass, respectively, which were lower than the Chinese *Arthrospira* Standard for Animal Feed Grade (GB/T 17243-1998). All the other parameters such as moisture and crude protein etc. could meet the standards as shown in Table 4-6. The use of IASBR effluent as the culturing medium to produce animal feed protein with the locally isolated *Spirulina platensis* ZJWST-S1 strain would reduce the *Spirulina* biomass manufacturing cost and increase the income of the farmers, finally providing a sustainable alternative for ADSW management.

4.4. Summary

The *Spirulina platensis* strain ZJWST-S1 was able to proliferate quickly in undiluted ADSW. Single factor experiments showed that the pre-treated ADSW can be used as a natural culturing medium for producing ZJWST-S1. In the two runs of raceway pond cultivation, the average area biomass productivity achieved 4.5 g/(m^2·d) in IASBR effluent lower than that in Zarrouk medium due to the influence of high chrominance in IASBR effluent. The *Spirulina platensis* ZJWST-S1 removed almost all NH_4^+-N, 68.7% of TN and 79.1% of TP from the IASBR effluent. And 91.5±3.4% of TN and 92.4±4.8% of TP reduced from ADSW were converted to *Spirulina platensis* biomass. And the concentrations of TN and TP at the end of cultivation could meet the new discharge standard. Using ADSW to culture ZJWST-S1 can not only help harvest high-profit animal feed grade proteins, but also help remove and recover nitrogen and phosphorus nutrients from ADSW. Compared to industrial wastewater, ADSW contains little toxicants and is relatively eco-safe. However, high concentrations of NH_4^+-N and antibiotics in ADSW were observed to have negative effect on *Spirulina platensis* cultivation. Moreover, suspended solids, bacteria and parasitic ovum in ADSW should also be removed because these pollutants would contaminate algae and thereby decrease the quality of the algal products. Therefore, IASBR process, which showed excellent removal for NH_4^+-N and antibiotics, is strongly proposed.

Table 4-1. Morphological characteristics of ZJWST-S1 and other three strains used in this study.

Strains	Width of trichome (μm)	Shapes of helix end and end cell	Calyptra on end cells	Gas vesicles	Cell length (μm)
ZJWST-S1	5.5-6.0	Both ends rounded	+	+	4-5
Ns-90020	5.3-5.7	Both ends rounded	+	+	5
DLMM S6	5.5-5.8	Both ends rounded	+	+	4
DLMM S2	5.8-6.0	Both ends rounded	+	+	4
S.platensis characteristics*	5.0-6.0	Both ends rounded	+	+	2-6
S.maxima characteristics*	6.0-8.0	Diminished at one end	-	-	8-12
*S.subsalsa*e characteristics*	1.5-2.5	Diminished at both ends	-	-	2-5

*Source: Hu and Wei, 2006.

'+': present; '-': absent.

Table 4-2. Composition of ingredients in Zarrouk medium.

No.	Components	Contents	Unit
1	NaCl	1.0	g/L
2	$CaCl_2$	0.04	g/L
3	$NaNO_3$	2.5	g/L
4	$FeSO_4 \cdot 7H_2O$	0.01	g/L
5	EDTA-Na	0.08	g/L
6	K_2SO4	1.0	g/L
7	$NaHCO_3$	16.8	g/L
8	K_2HPO_4	0.5	g/L
9	$MgSO_4 \cdot 7H_2O$	0.25	g/L
10	A_5	1.0	mL
11	B_6	1.0	mL

Table 4-3. Composition of microelements in Zarrouk medium.

	Components	Contents	Unit
A_5	H_3BO_3	2.86	g/L
	$MnCl_2 \cdot 4H_2O$	1.8	g/L
	MoO_3	0.01	g/L
B_5	NH_4VO_3	22.9	mg/L
	$NiSO_3 \cdot 7H_2O$	47.8	mg/L
	$NaWO_4$	17.9	mg/L

Table 4-4. Productivity of different *Spirulina* strains in Zarrouk medium after 9 days' cultivation.

Spirulina platensis	Max. specific growth rate (d^{-1})	Growth of generation (hr)	Floatation ratio (%)	Productivity ($g/(m^2 \cdot d)$)	Crude protein (%)
ZJWST-S1	1.64	13.9	91.6	29.1±13.2	62.8±6.1
Ns-90020	1.02	23.2	91.8	15.5±8.1	70.3±5.8
DLMM S6	1.57	11.7	92.6	15.8±9.3	61.7±10.5
DLMM S2	1.78	10.5	98.2	18.2±6.4	59.4±6.2

Table 4-5. Productivity of different *Spirulina* strains in ADSW medium after 9 days' cultivation.

Spirulina platensis	Max. specific growth rate (d^{-1})	Growth of generation (hr)	Floatation ratio (%)	Productivity ($g/(m^2 \cdot d)$)	Crude protein (%)
ZJWST-S1	1.55	14.2	92.4	24.2±9.6	59.4±2.5
Ns-90020	no growth	no growth	—	—	—
DLMM S6	0.33	49.6	51.4	8.1±3.8	45.1±5.3
DLMM S2	dead	dead	—	—	—

Table 4-6. Quality of *Spirulina* ZJWST-S1 powder produced using IASBR effluent.

Properties	*Spirulina* powder cultivated in ADSW	Feed grade standard*
Moisture (%)	4.15±0.35	≤7
Crude protein (%)	61.5±4.2	≥50
Ash (%)	6.3±0.6	≤10
Pb (mg/kg)	0.098±0.024	6.0
As (mg/kg)	0.76±0.07	1.0
Cd (mg/kg)	0.07±0.01	0.5
Hg (mg/kg)	0.06±0.01	0.1
Total plate count (cells/g)	$1.7\times10^3\pm0.2\times10^3$	5×10^4

*The Chinese *Arthrospira* Standard for Feed Grade (GB/T 17243-1998).

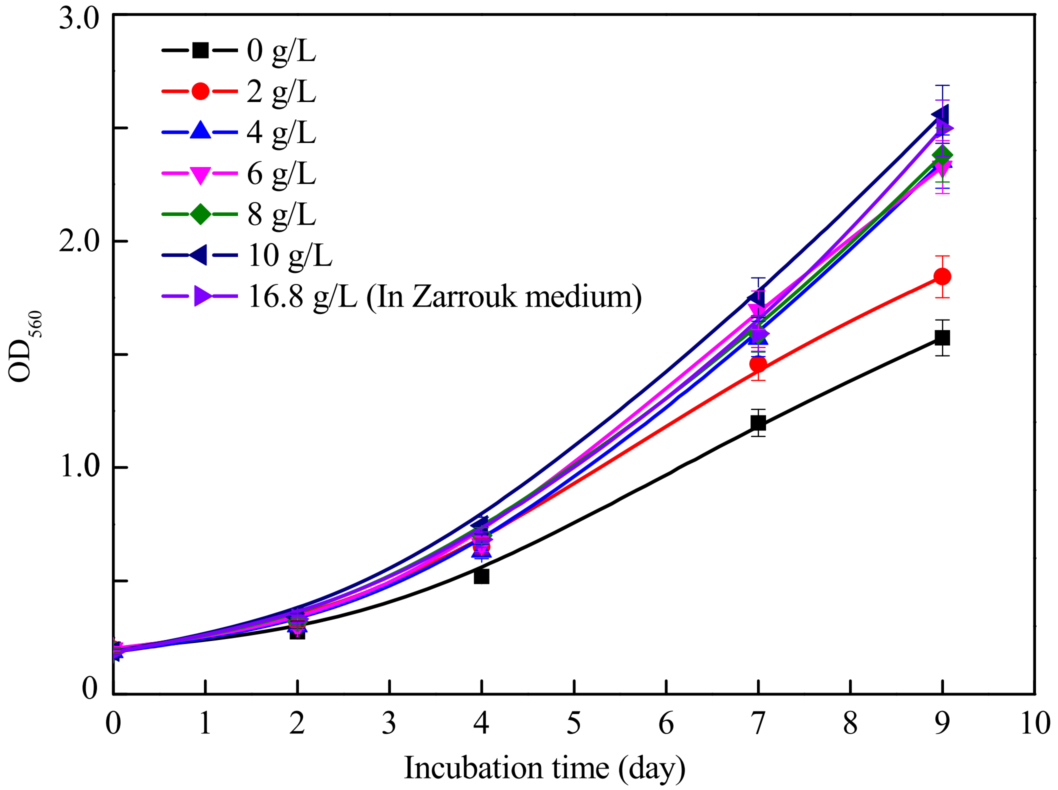

Figure 4-1. Growth curves of *Spirulina* ZJWST-S1 in Zarrouk medium with different dosage of $NaHCO_3$.

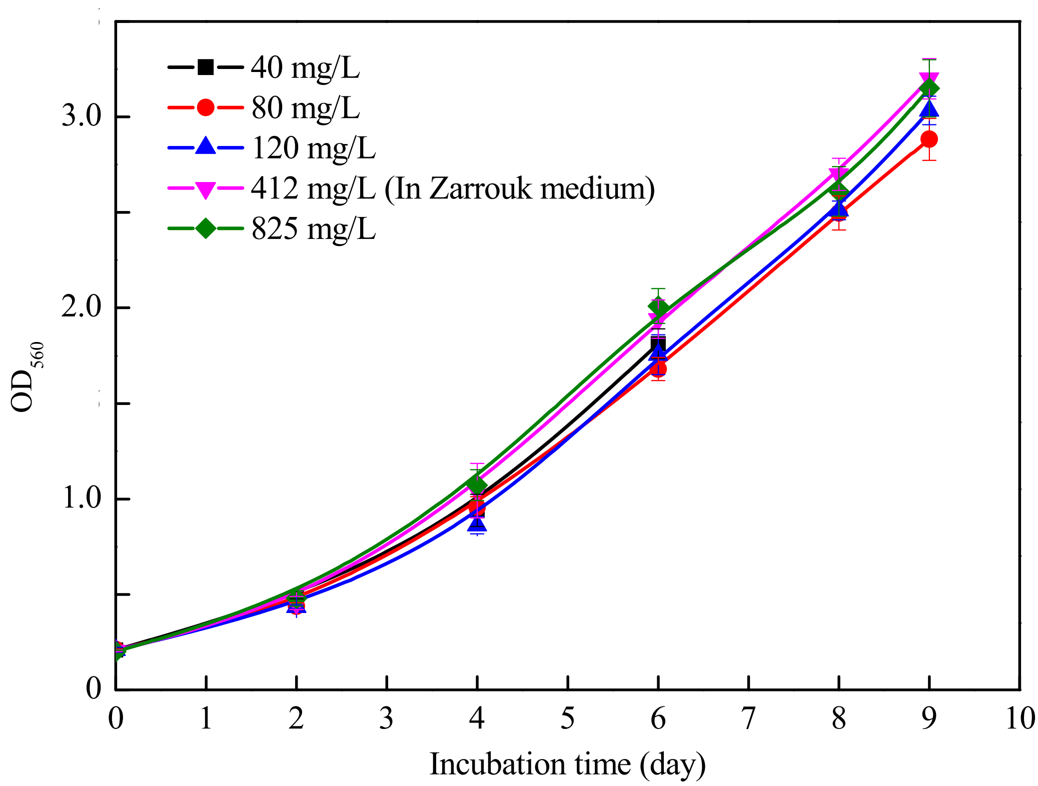

Figure 4-2. Growth curves of *Spirulina* ZJWST-S1 in Zarrouk medium with different dosage of NO_3^--N.

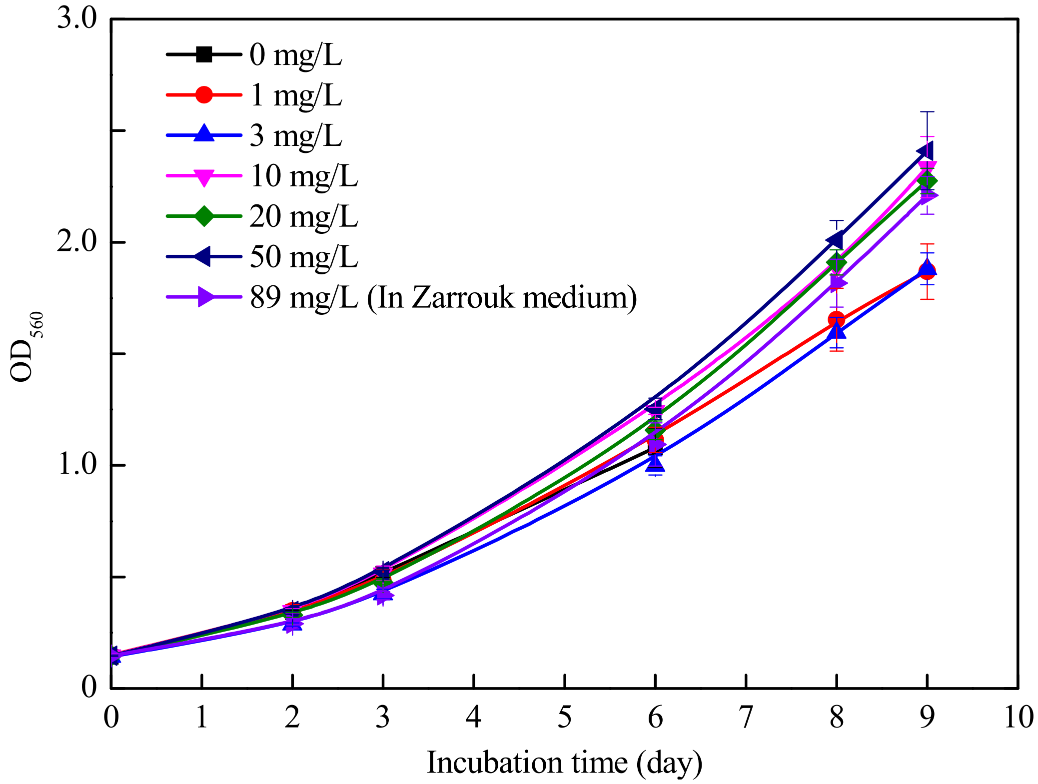

Figure 4-3. Growth curves of *Spirulina* ZJWST-S1 in Zarrouk medium with different dosage of PO_4^{3-}-P.

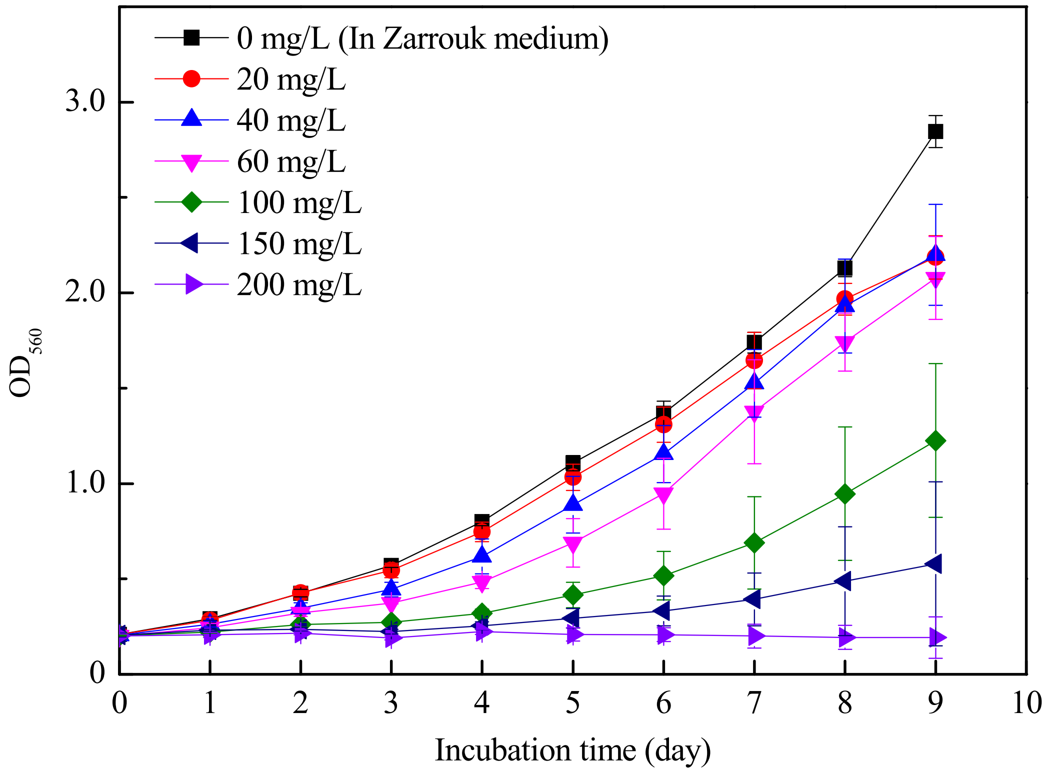

Figure 4-4. Growth curves of *Spirulina* ZJWST-S1 in Zarrouk medium with different dosage of NH_4^+-N.

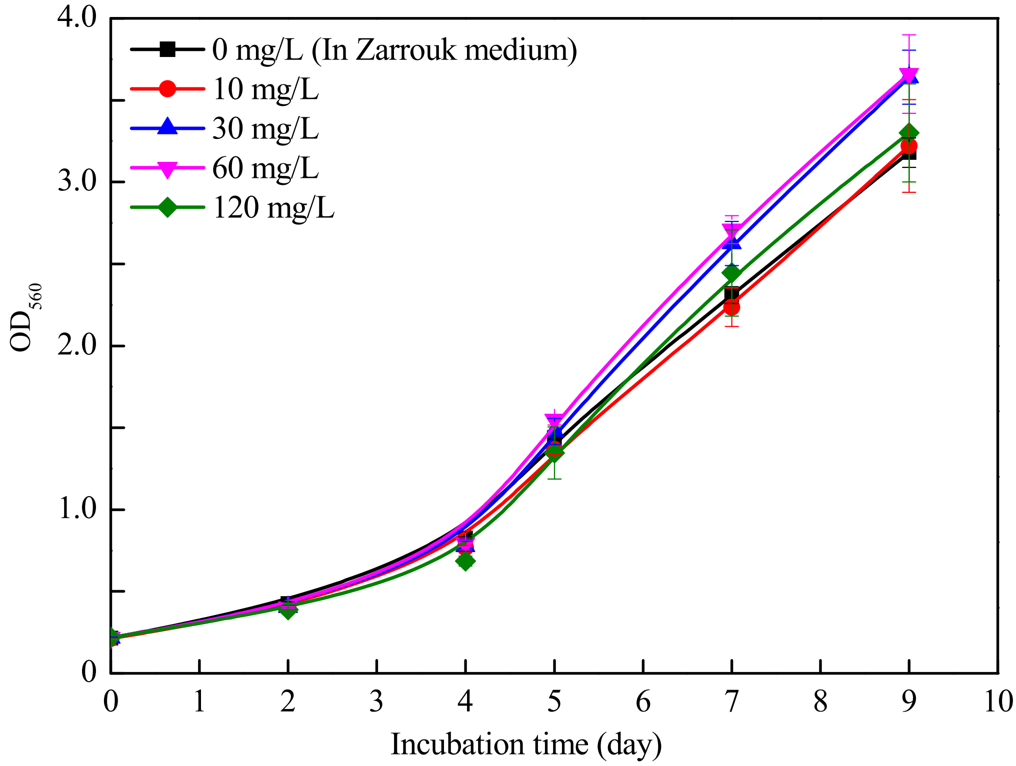

Figure 4-5. Growth curves of *Spirulina* ZJWST-S1 in Zarrouk medium with different dosage of NO_2^--N.

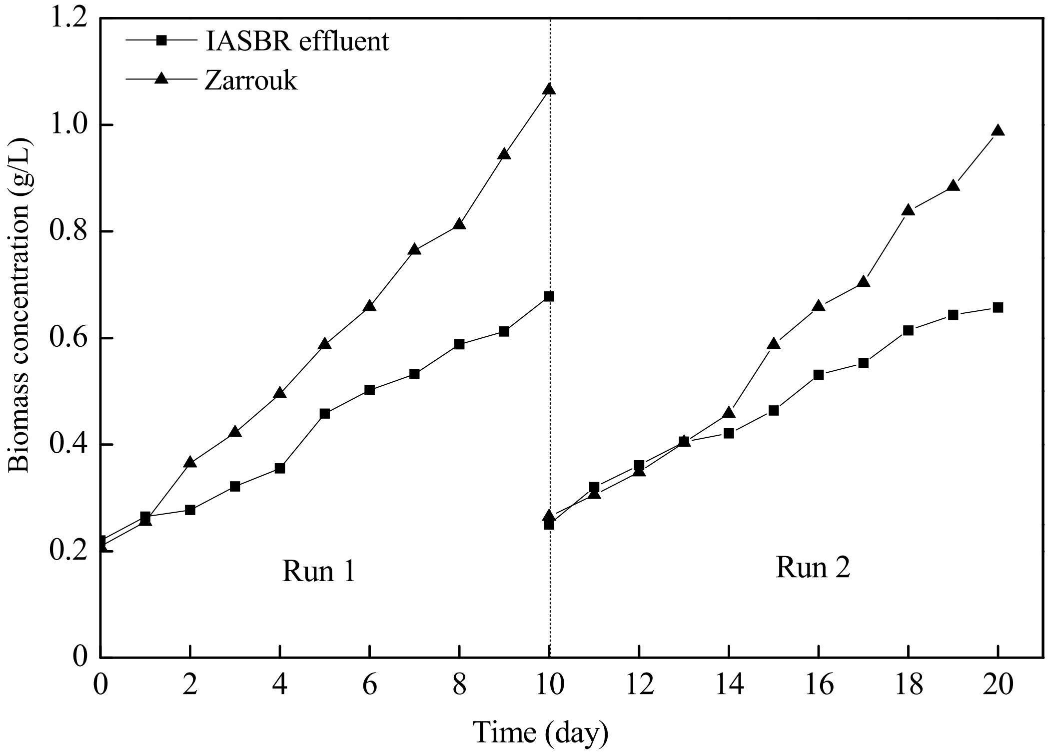

Figure 4-6. Growth curves of *Spirulina platensis* ZJWST-S1 in Zarrouk medium and IASBR effluent in raceway pond cultivation.

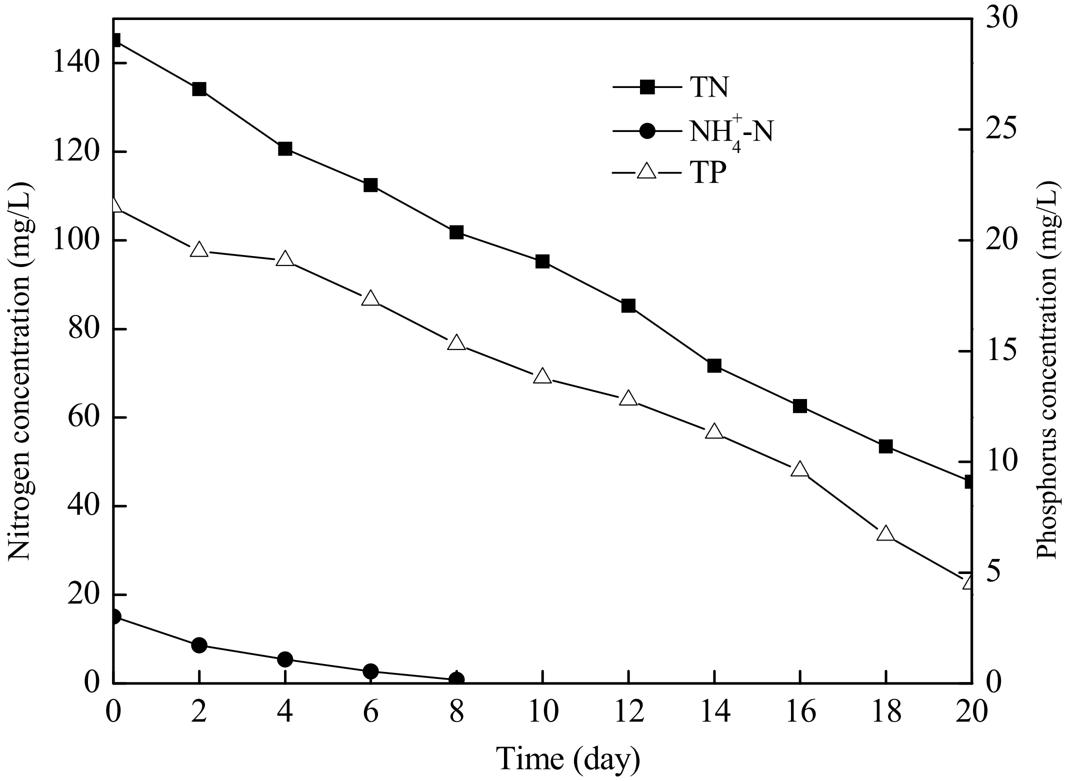

Figure 4-7. Changes in concentrations of NH_4^+-N, TN and TP in IASBR effluent during cultivation of *Spirulina platensis* ZJWST-S1.

Chapter 5 Conclusions and future research

5.1. Conclusions

In this study, a biological treatment process of intermittently aerated sequencing batch reactor (IASBR) followed by nutrients recovery of *Spirulina platensis* cultivation were applied in an attempt to enhance the treatment of anaerobically digested swine wastewater (ADSW) and nutrients recovery. Conclusions can be drawn as follows:

(1) The IASBR achieved greatly high removal rates of 97.5±1.4% and 93.8±12.8% for NH_4^+-N and TN under a COD/TN ratio of 2.4, HRT of 3 days and temperature above 20°C. Partial nitrification-denitrification was achieved in IASBR due to the inhibition of NOB during the whole operation. The increase of FA under lower COD/TN ratio inhibited the growth of AOB thus followed by deteriorated NH_4^+-N removal. Lack of enough carbon source resulted in the great accumulation of NO_2^--N and aggravated TN removal in IASBR. The increase of nitrogen loading mainly led to the increase of FA and restrained the partial nitrification. Denitrifying bacteria were much more sensitive than nitrifying bacteria. A lower temperature resulted in the drastic increase of FA, inhibiting both nitrification and denitrification processes.

(2) Both sludge sorption and biodegradation were found to be the major contributors to the removal of antibiotics, and greater than 60% of antibiotics in the influent were biodegraded in the IASBR, whereas averagely 24% were adsorbed by sludge. The removal of eleven veterinary antibiotics was greatly influenced by COD volumetric loading, which could achieve up to 85.1%±1.4% at 0.17±0.041 kg COD/(m^3·day), while dropped to 49.3%±12.1% when COD volumetric loading increased to 1.07±0.073 kg COD/(m^3·day). Tetracyclines, the dominant antibiotics in ADSW, were removed by 87.9% in total at the lowest COD loading, of which 30.4% were contributed by sludge sorption. Above 90% of Sulfonamides were removed by biodegradation. A shorter SRT could reduce the accumulation of antibiotics and the balanced antibiotics sorption capacity of sludge. The COD/TN ratio in influent was a key factor for nitrogen removal, but not for veterinary antibiotics in ADSW treatment by using IASBR.

(3) A local *Spirulina platensis* strain ZJWST-S1 was selected for culturing in IASBR effluent. No growth inhibition was observed when the culturing medium contained NO_2^--N of

0-120 mg/L. However, NH_4^+-N beyond 60 mg/L would inhibit the growth of *Spirulina*. In the two runs of raceway pond cultivation, the *Spirulina platensis* ZJWST-S1 removed almost all NH_4^+-N, 68.7% of TN and 79.1% of TP from the IASBR effluent. And 91.5±3.4% of TN and 92.4±4.8% of TP reduced from ADSW were converted to *Spirulina platensis* biomass. The concentrations of TN and TP at the end of cultivation could meet the new discharge standard.

(4) In this study, the integrated process of IASBR coupling with *Spirulina platensis* cultivation can remove TN and TP successfully to satisfy the new national discharge standard. Single use of IASBR or *Spirulina platensis* cultivation couldn't meet discharge standards directly. Although IASBR could enhance the removal of NH_4^+-N and TN, the effluent TN and TP still couldn't reach to the discharge requirements. *Spirulina platensis* cultivation could further remove and recover nitrogen and phosphorous. But without the pretreatment of IASBR, *Spirulina platensis* need to be cultivated in diluted ADSW and suffered negative effects due to the existence of inhibitory and toxic substances such as NH_4^+-N and antibiotics. Therefore, the use of IASBR is necessary before the cultivation of *Spirulina platensis* to guarantee the bio-safety for *Spirulina*.

5.2. Future research

The biological treatment process combined with *Spirulina* cultivation was a new attempt for ADSW treatment. Due to the limitation of research time, some improvements and further studies should be done in the future.

(1) The mechanism for partial nitrification and denitrificaiton in the IASBR system should be clarified more clearly. The achievement of partial nitrification and denitrificaiton is actually determined by microbial communities in a biological system. So it's necessary to figure out the relationship between the variations of microbial communities and the change of operation conditions such as pH, DO, temperature, COD or nitrogen loading, and so on. And finally, in a real application of IASBR for ADSW treatment, the operation conditions can be controlled much more precisely to maintain more stable partial nitrification and denitrificaiton in the IASBR system.

(2) The effect of antibiotics in ADSW should be studied in more detail for the growth of *Spirulina*. The inhibition of antibiotics on *Spirulina* has been demonstrated by many other researchers. However, the influence of antibiotics contained in the raw ADSW is not very clear for *Spirulina* cultivation in this study. And the inhibition concentrations for different kinds of antibiotics should be indicated accurately.

(3) The effluent from IASBR still reflects high color level, which would decrease the productivity of *Spirulina platensis.* The next step is to enhance the removal of color and improve its transparency to increase the productivity of *Spirulina*.

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Acknowledgements

First and foremost, I would like to sincerely appreciate my supervisor, Professor Zhenya Zhang, for providing me the opportunity to study for doctorate in University of Tsukuba. My research and thesis benefited a lot from his instructive advice and professional guidance. I'm so lucky to have a supervisor like him who is conscientious, benevolent, and generous.

Second, I would like to express my heartfelt gratitude to Professor Zhongfang Lei, who instructed and help me a lot in my research. I could not complete my papers and thesis without her patient and carefully instructions. And many thanks should give to Professor Lujun Chen and Professor Rui Liu, from Yangtze Delta Region Institute of Tsinghua in China, where I undertook my most of research work.

Special gratitude should give to my thesis committee members, Professor Zhenya Zhang, Professor Zhongfang Lei, Professor Motoo Utsumi, and Professor Kazuya Shimizu, for their valuable and helpful suggestions and comments on my thesis.

Finally, I would like to thank my wife and beloved family for their supporting to me all through these years.

Publications

1. **Wei Zheng**, Zhenya Zhang, Rui Liu, Zhongfang Lei. Removal of veterinary antibiotics from anaerobically digested swine wastewater using an intermittently aerated sequencing batch reactor. *Journal of Environmental Sciences*, available online 21 April 2017, in press. https://doi.org/10.1016/j.jes.2017.04.011 (IF 2.208)
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