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Role of cyclic diguanylate in affecting microbial community shifts at different pH during the operation of simultaneous partial nitrification, anammox and denitrification process

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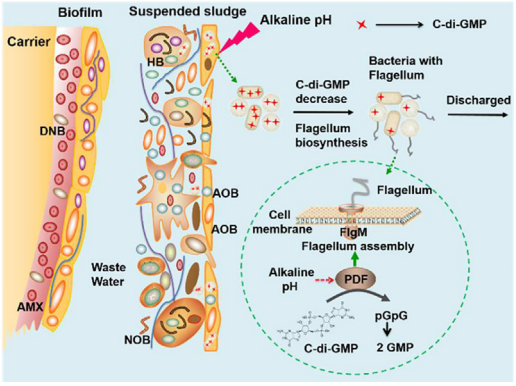
HIGHLIGHTS

- More comprehensive understanding of microbial community shifts induced by *c*-di-GMP was proposed.
- The increased alkaline pH reduced intracellular *c*-di-GMP content in flocculant.
- A decrease in *c*-di-GMP content increased bacterial motility causing microbial community shifts.

GRAPHICAL ABSTRACT



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ABSTRACT

The intracellular cyclic diguanylate acid (*c*-di-GMP) has emerged as a prominent second signal molecule that coordinates sessile-motile transition and biofilm formation in many bacteria. Herein, we study the role of *c*-di-GMP in affecting microbial community shifts at different pH levels during simultaneous partial nitrification, anammox and denitrification process (SNAD) in integrated fixed film activated sludge (IFAS) reactor. The results demonstrated that the contents of *c*-di-GMP notably decreased in suspended sludge, whereas the contents of *c*-di-GMP in biofilm had no significant change as pH gradually increased from 7.5 to 8.5. Most of the bacteria (*Blastocatella*, *Brevundimonas*) with flagella that have been reported to be regulated by *c*-di-GMP were present in suspended sludge, and the microbial community structure of suspended sludge had obvious change than biofilm. The increased alkaline pH reduced intracellular *c*-di-GMP content for increasing the motility of bacteria to be washed out from the reactor, causing the microbial community shifts in suspended sludge. This change would lead to the increase of nitrite-oxidizing bacteria which would inhibit anammox activity. Overall, this study provided more comprehensive information regarding the shifts of microbial community induced by *c*-di-GMP in SNAD-IFAS reactor.

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1. Introduction

The microbial communities are essential to the performance of wastewater treatment facilities and the shifts of microbial communities may lead to the changes of reactor performance. Many factors can account for the variation in microbial community, such as temperature, pH, substrate quality and the form of activity sludge (Lin et al., 2016; Yuan et al., 2015; Chen et al., 2009; Despland et al., 2012; Abzazou et al., 2016). The pH is a major factor influencing microbial composition and diversity in the bioreactor. In waste activated sludge fermentation bioreactor, the ratios of archaea to bacteria would change at different pH and the abundance of methanogenic archaea decreased while hydrolytic bacteria increased at pH 10 (Yuan et al., 2015). Two mainly general explanations may explain, singly or in combination, the mechanisms for pH affecting microbial composition and diversity. First, pH may not directly alter microbial community structure but may affect the characteristics of microbial growth environment, such as nutrient availability, cationic metal solubility, and organics characteristics (Lauber et al., 2009). A second mechanism is that pH directly imposes a significant stress on bacteria. The pH would affect microbial growth and respiration, metabolic function, transcriptional activity, and pH also exerted effects on nucleotide and protein metabolism (Belnap et al., 2011; Nicol et al., 2008). Although the mechanisms for the response of microbial community to pH were studied in soil (de Boer et al., 2012), there are few studies regarding the mechanisms for the response of microbial community to pH in the sustainable nitrogen elimination biotechnologies, and one reason has been omitted for long time, that is the regulation function of cyclic diguanylate (*c*-di-GMP).

In recent years, some investigations focus on the role of signal molecules on activated sludge (Feng et al., 2014; Wan et al., 2014). Among the signal molecules, the intracellular cyclic diguanylate (*c*-di-GMP) is regarded as the second signal messenger in bacteria that coordinates the cellular function associated with bacteria biofilm and the transition from a motile lifestyle to a sessile state. The molecule *c*-di-GMP was first time discovered as an allosteric activator of the cellulose synthase in *Gluconacetobacter xylinus* (Ross et al., 1987). Subsequently, it has been showed that *c*-di-GMP could regulate biofilm formation in *Escherichia coli*, *Klebsiella pneumonia*, and *Bacillus cereus* group (Lacanna et al., 2016; Schumacher and Zeng, 2016; Fagerlund et al., 2016), twitching motility and swarming in *Pseudomonas aeruginosa* (Baker et al., 2016). It was also noted that the *c*-di-GMP could control flagellum-based motility (Wolfe and Visick, 2008). Moreover, the relationship between *c*-di-GMP and aerobic granules was investigated and found that the contents of polysaccharides and proteins in granules were decreased in conjunction with the decline in *c*-di-GMP concentration, and the formation of filamentous aerobic granules was associated with the *c*-di-GMP content (Wan et al., 2014). Although the relationship between *c*-di-GMP and extracellular polymeric substances in anaerobic ammonium oxidation (anammox, AMX) reactor and completely autotrophic nitrogen removal over nitrite reactor had been studied by our previous researches (Guo et al., 2017; Wang et al., 2017), another regulation function of *c*-di-GMP about bacteria motility was ignored in more complex autotrophic nitrogen bioreactors.

In this study, we hypothesized that the important function of *c*-di-GMP regulation bacteria motility would affect the microbiological community shift at different pH levels during simultaneous partial nitrification, anammox and denitrification process (SNAD) in integrated fixed film activated sludge (IFAS) reactor. Three different pH conditions of 7.5, 8.0 and 8.5 were selected according to the study of Langone et al. (2014). The microbial community structure in the biofilm and suspended sludge was analyzed via Illumina MiSeq sequencing of the V4-V5 region of 16S rRNA gene. Furthermore, the content of intracellular *c*-di-GMP was detected in the biofilm and suspended sludge, and the swarming motility assay for detecting the correlation between intracellular *c*-di-GMP content and bacteria motile lifestyle are also addressed in this research.

2. Material and methods

2.1. SNAD-IFAS reactor establishment and operation

The laboratory-scale SNAD-IFAS reactor used in this experiment had a working volume of 8.0 L (Fig. 1). A 40% volume fraction of non-woven ring carriers (Wang et al., 2017) with average density of 1.1 g/cm^3 was employed in the SNAD-IFAS reactor. The raw material for manufacturing non-woven is polyester and the carrier-frame is made of polypropylene. A mechanical mixer installed in the reactor, and air was supplied from the bottom of the reactor, thus it can be considered as a type of homogenous reactor. A secondary clarifier was used for collecting activated sludge, and then the activated sludge returned to the system by pump. Sludge reflux ratio was set at 3.0.

The operating condition of the SNAD-IFAS reactor is displayed in Table 1. The reactor was operated at an ambient temperature of 25 ± 2 °C. During all stages, the hydraulic retention time (HRT) was 0.75 days. The reactor was operated for a total of 120 days and reactor operation was divided into four stages (I, II, III, and IV).

The first stage (I) was start-up of the reactor from day 0 to day 20. Non-woven carriers were used in the reactor to form biofilm. During this phase, the aeration rate was 160 mL/min. The low aeration rate in this phase was used to prevent washing away of the preliminary formation of biofilm. The seed sludge was anammox sludge prepared from a laboratory-scale up-flow anaerobic sludge bed reactor (UASB, MLSS = 2.3 g/L, Dalian University of Technology), which had been used to enrich anammox bacteria for 2 years under ambient temperature (25 ± 2 °C). The partial nitrification sludge taken from a laboratory-scale continuous-flow stirred tank reactor (CSTR, MLSS = 2.7 g/L, Dalian University of Technology) for over 5 months under ambient temperature (25 ± 2 °C). Whereas the denitrifying bacteria (DNB) were domesticated from anammox and partial nitrification sludge by adding organic compounds in the loading wastewater. This method of SNAD process start-up has been a common method in previous studies (Chen et al., 2009; Liang et al., 2014; Wang et al., 2018). The reactor was fed with synthetic medium contained (g/L): 0.019 CH_3COONa, 0.47 $(NH_4)_2SO_4$, 1.25 $KHCO_3$, 0.025 KH_2PO_4, 0.3 $CaCl_2 \cdot 2H_2O$, 0.2 $MgSO_4 \cdot 7H_2O$, 0.00625 $FeSO_4$, 0.00625 EDTA. The next three stages (II - IV, from day 20 to day 120, each phase for 30 days), the pH of the reactor was maintained at 7.5, 8.0 and 8.5 for phase II, phase III and phase IV, respectively, which was realized by adjusting sodium carbonate concentration in influent.

The concentrations of ammonium, nitrite, nitrate and total organic carbon (TOC) were analyzed every two days. For bacteria community and the content of *c*-di-GMP analysis, biofilm and suspended sludge were collected in triplicate from the reactor on day 50, 80, and 120, which was the final day of each operational phase. Before the long running test for the research on the change of pH in the reactor, which would affect the shifts of bacteria community (on day 20), batch experiments, swarming motility test were applied to examine the impact of pH change on the *c*-di-GMP content and bacterial motility.

2.2. Chemical and statistical analysis

According to the standard methods (APHA, 2005), the concentrations of ammonium and nitrite were analyzed using a visible colorimetric technique, whereas nitrate concentrations were determined using ultraviolet spectrophotometry. The concentrations of TOC were measured by a total organic carbon analyzer (TOC-V CPN, SHIMADZU) using the combustion-infrared method. The levels of dissolved oxygen (DO) and pH were detected with a digital portable DO and pH meters (WTW, Multi 3430, Germany), respectively. An analysis of statistical significant difference was carried out using Microsoft Excel® software (Student's *t*-test) at the 95% confidence interval ($p < 0.05$).