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Tetrad-forming-organism-dependent deterioration of enhanced biological phosphorus removal in a full-scale wastewater treatment plant

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Proliferation of tetrad-forming organisms (TFOs) has been suggested to cause deterioration of enhanced biological phosphorus removal (EBPR) in wastewater treatment plants. TFOs are known store glycogen as an energy source, thus they are called glycogen-accumulating organisms (GAOs) and can compete with polyphosphate-accumulating organisms (PAOs) for volatile fatty acids. Wastewater temperature and sludge age have proven to be important factors in determining EBPR stability. The efficiency of BPR decreased with an increase of wastewater temperature. An increased population of TFOs and a higher level of glycogen were found in the activated sludge when the efficiency of EBPR was low. After decreasing the age of sludge to 5 days, EBPR stabilized and started functioning effectively. Glycogen concentration in the activated sludge decreased with decreasing wastewater temperature.

Key words: biological phosphorus removal, enhanced biological phosphorus removal, polyphosphate-accumulating organisms, tetrad-forming organisms, glycogen-accumulating organisms

INTRODUCTION

The enhanced biological phosphorus removal (EBPR) is a microbial process widely used for removing phosphorus from wastewater to avoid eutrophication of water bodies. EBPR presents an environmentally friendly alternative to phosphorus removal by chemical precipitation. High levels of phosphorus removal occur in EBPR systems because they select polyphosphate-accumulating organisms (PAOs) (Mino et al., 1998). The PAO metabolism is induced by cycling PAOs through anaerobic/aerobic conditions with volatile fatty acid supplied to the anaerobic phase (Mino et al., 1998). In some laboratory-scale EBPR reactors, deterioration of EBPR has been reported to be due to proliferation of the other group of microorganisms that can compete with PAOs for carbon sources without phosphate release under anaerobic conditions, indicating no involvement of poly-P in their metabolism (Cech, Hartman, 1990, 1993; Bond et al., 1999). Cech and Hartman (1990, 1993) found that cocci-shaped cells arranged in distinctive tetrads were abundant in systems without phosphate release under anaerobic conditions. The organisms were initially called "G bacteria" (Cech, Hartman, 1993; Seviour et al., 2000) and later became known as tetrad-forming organisms (Tsai, Liu, 2002; Wong et al., 2004). Cech, Hartman (1993) have shown that G-bacteria accumulate carbohydrates intracellularly. The term "glycogen–accumulating organisms" (GAOs) was proposed by Mino et al. (1995) and is defined as the phenotype of organisms that store glycogen aerobically and consume it anaerobically as their primary source of energy for taking up organic substrates. By consuming VFAs, PAOs accumulate polyphosphate in their cells, whereas GAOs do not. The current definition of GAOs is based on their phenotype, but it can be suggested from above presented material that tetrad-forming organisms have a GAO phenotype. According to Liu et al. (1996), GAOs are either cocci (0.8–2.5 μm in diameter) occurring in pairs, tetrads or aggregates, or large oval rods.

The growth of GAOs in the EBPR process is affected by some factors.

1. The GAOs are mezophylic microorganisms with optimal growth temperature from 20 to 38 °C, while the PAOs are psychrophylic microorganisms (Vazquez et al., 2007; Griffiths et al., 2002). Thus, when the temperature of wastewater decreases seasonally, the amount of GAOs decreases and the efficiency of phosphorus removal increases (Brdjanovic et al., 1997).

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2. A long age of sludge in a warm season. At the wastewater temperature of 20 °C, phosphorus removal is efficient when sludge age does not exceed 10 days (Whang, Park, 2006; Erdal et al., 2003).

3. An enhanced content of glucose in the influent stimulates the growth of GAOs (Erdal et al., 2003; Cech, Hartman, 1990).

4. A low P/C ratio in the influent also stimulates the growth of GAOs (Liu et al., 1997; Oehmen et al., 2006).

5. A low pH value (pH ≤ 7.25) is favourable for the growth of GAOs (Cokgor et al., 2004; Filipe, Daigger, 2001).

6. Nitrite inhibits phosphate uptake by PAOs, thus the presence of nitrite might enhance the presence of GAOs – competitors to PAOs in full-scale wastewater treatment plants (Saito et al., 2004).

7. GAOs, unlike PAOs, are not able to readily switch between acetate and propionate uptake (Oehmen et al., 2005). Thus, regularly alternating the carbon source between acetate and propionate provides PAOs with a competitive advantage over GAOs in EBPR systems (Lu et al., 2006).

The effect of temperature and sludge age on EBPR has been studied in laboratory conditions (Whang et al., 2006; Erdal et al., 2003; Cokgor et al., 2004). Very little is known on the effects of these parameters in full-scale systems.

The objectives of this study were as follows: to detect tetrad-forming organisms (TFOs) in activated sludge systems at both low and high BPR efficiency; to evaluate the effect of temperature and sludge age on EBPR during the summer–autumn period in a full-scale wastewater treatment plant.

MATERIALS AND METHODS

The research was carried out in a full-scale wastewater treatment plant of Utena (Lithuania) with biological phosphorus and nitrogen removal in the summer–autumn period of 2007. The research was repeated in the same period of 2008. Two aeration basins were used for the biological wastewater treatment. The inflowing part in each aeration basin is used as an anaerobic zone for biological phosphorus removal. Nitrogen removal according to the intermittent denitrification technology was carried out in a simultaneous way of nitrification and denitrification. Oxygen supply was controlled by a NADH sensor according to sludge activity. A NADH sensor is a fluoremeter installed in a stainless steel case and emitting 340 nm shafts through the quartz glass. The light from the instrument penetrates to the microorganisms and detects the NADH compounds which, when illuminated, emit 460 nm shafts, and the NADH sensor registers them. NADH is being constantly converted from the oxidator NAD into the reducing agent NADH in active sludge. The sensor fixes only NADH which gives information on how much energy the microorganisms possess.

Sampling. Samples were taken on average three times per week during the time, when the temperature of mixed liquid in the aeration basins reached 20 °C and decreased to 16 °C. Wastewater samples were taken from the wastewater treatment plant after mechanical treatment and from the effluent. The following data were analyzed: temperature (τ), pH, BOD_7, COD, total suspended solids (TSS), total phosphorus (TP), total nitrogen (TN). The following parameters were estimated: phosphate concentration in the anaerobic zone $(PO_4)_{an}$, phosphate concentration in the aeration zone $(PO_4)_{aer}$, nitrate concentration in the anaerobic zone $(NO_3)_{an}$, nitrate concentration in the aeration zone $(NO_3)_{aer}$, and nitrogen concentration ammonium in the aeration zone $(NH_4)_{aer}$. The activated sludge concentration in the aeration basin (a), the concentration of returned sludge (a_c) and volatile suspended solids in the aeration basin were also measured. The average flows (Q_d) in the aeration basin were fixed. All analyses were carried out by standard methods confirmed by the Ministry of Environment of the Republic of Lithuania (Unifikuoti..., 1994, 2000, 2005, 2005).

The load of activated sludge (A), sludge age (Θ), and the increase of activated sludge were estimated from the obtained data. A statistical processing of the obtained results was performed. The values that did not fit into the reliability interval of 95% were eliminated following the statistical equations (Martinėnas, 2004). The average wastewater flow was about 10 000 m^3/d and the load of activated sludge 60 $mgBOD_7$ / gMLVSS · d; wastewater pH in the aeration basin fluctuated within a small interval (7.4–7.5). The average wastewater composition during the experiment: BOD_7 268 mg/l, COD 562 mg/l, TSS 115 mg/l, TN 58 mg/l, TP 12 mg/l.

Samples for microscopic observation of mixed liquid were taken from the end of the aeration zone. These samples were immediately prepared as smears (20 μl) on 24 × 4 mm slide glasses and air-dried. The air-dried smears were stained with methylene blue by the method described in Lindrea et al. (1999). The samples were divided into two groups: A – when the efficiency of phosphorus removal was low and B when it was high.

Microscopy. Bright field microscopy was performed with an Olympus AX70 microscope with 1000 × magnification. The smears were analyzed for presence of TFOs. Cell size, cell shape and the distribution of TFOs were examined. The amount of TFOs was assessed using the subjective 0–6 rating developed by Griffiths (2002), zero being the lowest score and 6 denoting excessive numbers present; 64 photographs were taken for image analysis quantification.

Glycogen measurements were performed according to the anthrone method (Severina, Solovyova, 1989): 1 ml of mixed liquid was mixed up with 3 ml 30% KOH in a 10 ml tube. The tube was closed and heated at 100 °C for 1 hour to break down the cells and to solubilize the glycogen homopolymer. The resulting solution was diluted with 4 ml distilled water and centrifuged at 1 600 g for 15 min. The concentration of glycogen in the supernatant was measured using the anthrone reagent. An aliquot of 3 ml of anthrone reagent (100 mg of anthrone in 50 ml of 95% sulfuric acid) was added to 1.5 ml of a sample in the test tube kept in a water bath at 5 °C. The tube was closed and placed in a hot water bath (90 °C) for 10 min. Upon cooling the samples to room temperature, the absorbance A_{670} was measured with a spectrophotometer.

RESULTS AND DISCUSSION

The period of unstable biological phosphorus removal started from 20 July 2007 and continued till 21 August, i. e. 32 days. A significant fluctuation of phosphorus removal efficiency with a trend to decrease was observed during this period (Fig. 1). The effect of biological treatment addressing the total phosphorus in the aeration basin fluctuated from 37 to 91%, the concentration of phosphate in the aeration zone varied within 0.58–4.5 mg/l, the concentration of ammonium nitrogen in the aeration zone was 0.64–1.6 mg/l and concentration of nitrate in the aeration zone 0.36–4.9 mg/l. The concentration of nitrate and nitrite in returned sludge was low (less than 1 and 0.1 mg/l, respectively). Thus, nitrite is not likely to interfere with the metabolism of phosphate-accumulating organisms in the anaerobic phase.

The temperature of wastewater ranged from 21.6 to 24.5 °C during this period. The deterioration of BPR might be caused by a more intensive proliferation of GAOs in comparison with PAOs, as a temperature above 20 °C is more favourable to the first than to the other.

From 20 July to 17 August, sludge age fluctuated from 22 to 6 days. A higher sludge age means a higher residence time for PAOs, which can improve P removal. However, when the growth of GAOs is stimulated by an increased wastewater temperature, a longer age of sludge is favourable for the proliferation of GAOs in EBPR systems. This might be the explanation for the data in Fig. 1; e. g. from August 1 to 6, the sludge age increased from 11 to 18 days, while the efficiency of BPR decreased significantly. When sludge age was reduced to 5 days (20th of August), EBPR stabilized and started to function effectively. After that, the age of sludge was increased to 20 days and the efficiency of EBPR remained very high – about 97%. It might be suggested that new sludge had grown, in which PAOs became dominant because their anaerobic-acetate uptake rate and aerobic-biomass yield were higher in comparison with GAOs (Whang, Park, 2006).

The microscopic images of activated sludge samples are presented in Fig. 2. An appreciable difference in the size of TFO population was observed while comparing sludge with a low (samples A) and a high (samples B) BPR efficiency: ac-

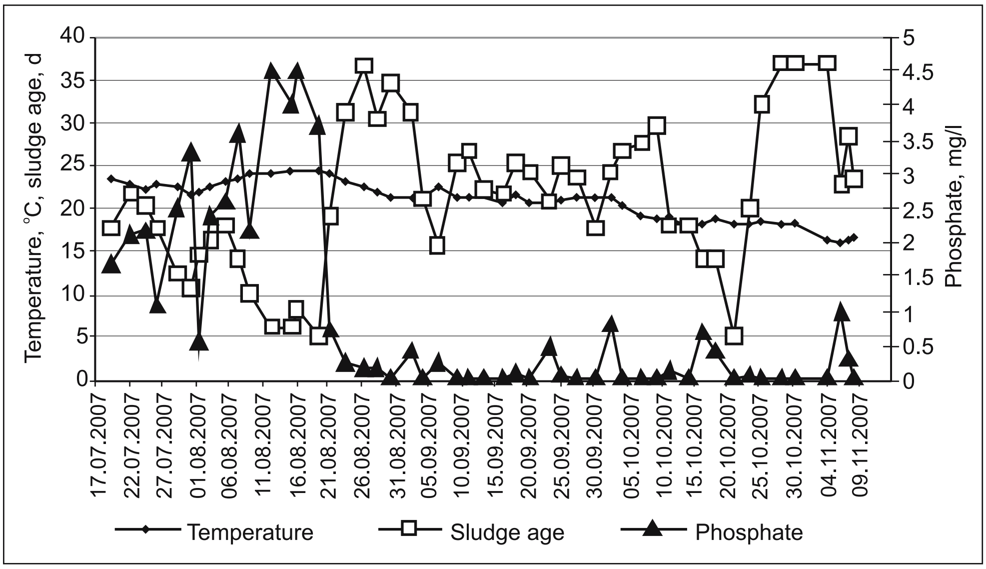

Fig. 1. Dynamics of phosphate in the aeration zone, sludge age and temperature

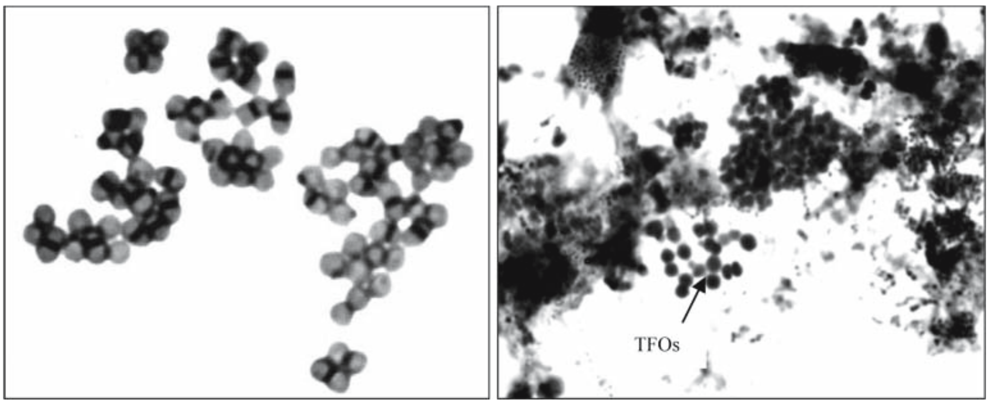

Fig. 2. Microscopic images of TFOs

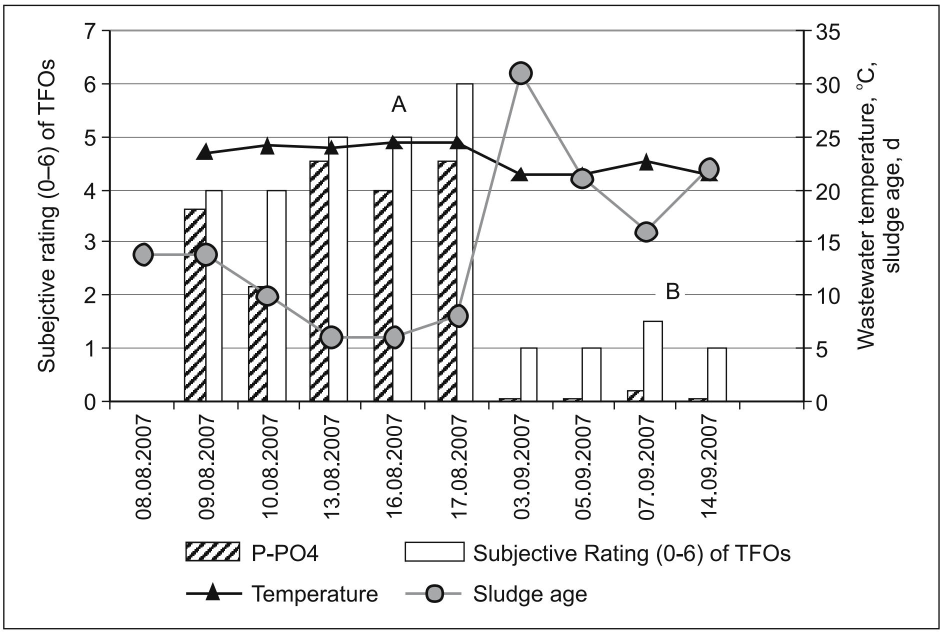

Fig. 3. Comparison of TFO populations in samples from A and B

cording to the subjective rating of Griffiths et al. (2002), the size of TFO population within 4 to 6 points in samples A and from 1 to 1.5 in samples B (Fig. 3). The bacteria, arranged in distinctive tetrads, are very similar in morphology to the GAOs reported by Liu et al. (1996), Maszenan et al. (1998), Seviour et al. (2000), Wong et al. (2004).

It is likely that GAO-dominated activated sludge will have a higher glycogen content in comparison with PAO-dominated one. For this reason, the concentration of glycogen was measured from 8 October 2007 to 28 November 2007. Changes of glycogen concentration, wastewater temperature and sludge age are shown in Fig. 4. At the beginning of the measurements, the concentration of glycogen in activated sludge from the end of the aeration zone was 72.7 mg gly/gMLSS and the concentration of phosphates in the aeration zone was low (0.13 mg/l). After five days, glycogen concentration increased to 131.2 mg gly/gMLSS. The concentration of phosphates in the aeration zone increased, too, showing a low efficiency of BPR. After that, sludge age was decreased to 5 days, and glycogen concentration in activated sludge decreased 2.5 times. After two days when sludge age reached 20 days, glycogen concentration in activated sludge increased and was about 88 mg gly/gMLSS during the next five days. The temperature of wastewater ranged from 18.9 to 18.2 °C, while the concentration of phosphates in the aeration zone was very low (0.024–0.054 mg/l). When the temperature of wastewater decreased to 18°C, glycogen concentration in activated sludge decreased to 60.5 mg gly/gMLSS. A further decrease of glycogen concentration (to 43 mg gly/gVDSM) was observed with a decrease of wastewater temparature to 16.3 °C.

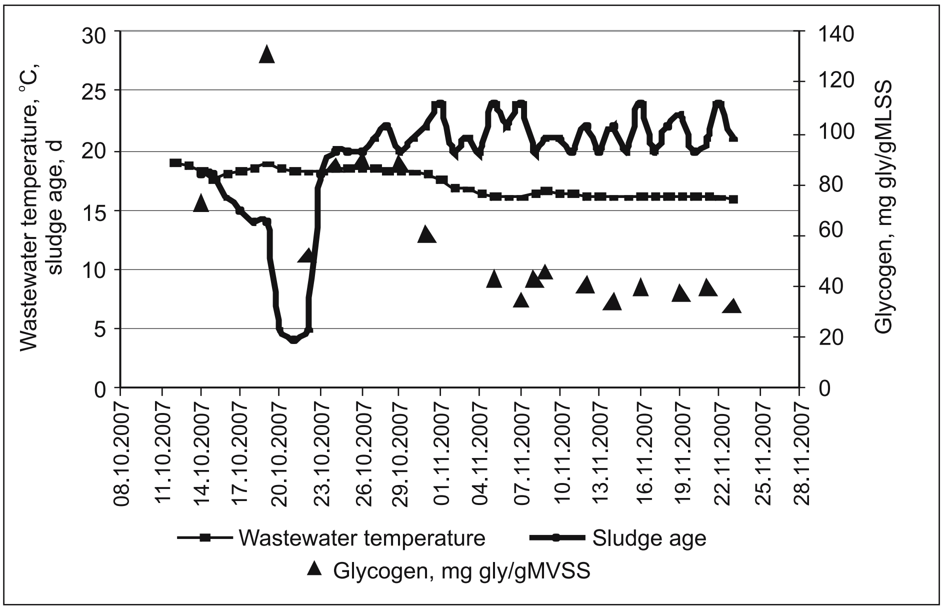

Fig. 4. Changes of glycogen concentration and wastewater temperature

CONCLUSIONS

The results of our research have shown that the deterioration of EBPR process in a full-scale wastewater treatment plant could be expected during the warm season of the year when wastewater temperature exceeds 20 °C. The increasing content of tetrad-forming cocci and the rising level of glycogen in activated sludge might signal the oncoming failure of EBPR in a wastewater treatment plant.

The reduction of sludge age to 5 days, followed by an evident increase, is shown to be an effective means of EBPR recovery and stabilization. It is likely that PAOs become dominant in the newly growing sludge as a result of their faster growth in comparison with GAOs.

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References

1. Bond P. L., Erhart R., Wagner M., Keller J., Blackall L. L. 1999. Identification of some of the major groups of bacteria in efficient and nonefficient biological phosphorus removal activated sludge systems. *Applied & Environmental Microbiology*. Vol. 65. N 9. P. 4077–4084.
2. Brdjanovic D., van Loodsdrecht M. C. M., Hooijmans C. M., Alaerts G. J., Heijnen J. J. 1997. Temperature effects on physiology of biological phosphorus removal. *Journal of Environmental Engineering.* Vol. 123. N 2. P. 144–153.
3. Cech J. S., Hartman P. 1990. Glucose-induced breakdown of enhanced biological phosphate removal. *Environmental Technology.* Vol. 11. P. 651–656.
4. Cech J. S., Hartman O. 1993. Competition between polyphosphate and polysacharide accumulating bacteria in enriched biological phosphate removal systems. *Water Research.* Vol. 27. N 7. P. 651–656.
5. Cokgor E. U., Yagci N. O., Randall C. W., Artan N., Orhon D. 2004. Effects of pH and substrate on the competition between glycogen and phosphorus accumulating organisms. *Journal of Environmental Science & Health, Part A*. Vol. 39. N 7. P. 1695–1704.
6. Erdal U. G., Erdal Z. K., Randall C. W. 2003. The competition between PAOs (phosphorus accumulating organisms) and GAOs (glycogen accumulating organisms) in EBPR (enhanced biological phosphorus removal) systems at different temperatures and the effects on systems performance. *Water Science & Technology*. Vol. 47. N 11. P. 1–8.
7. Filipe C. D. M., Daigger G. T., Grady C. P. L. 2001. pH as a key factor in the competition between glycogen-accumulating organisms and polyphosphate-accumulating organisms. *Water Environmental Research*. Vol. 73. P. 223–232.
8. Griffiths P. C., Stratton H. M., Seviour R. J. 2002. Environmental factors contributing to the “G bacteria” population in full-scale EBPR plants. *Water Science & Technology.* Vol. 46. N 4–5. P. 185–192.
9. Lindrea K., Seviour E. M., Seviour R. J., Blackall L. L., Soddell J. A. 1999. Practical methods for the examination and characterization of activated sludge. In: Seviour, R. J., Blackall, L. L. (eds.) *The Microbiology of Activated Sludge.* Dordrecht, The Netherlands: Kluwer Academic Publishers. 300 p.
10. Liu W. T., Mino T., Nakamura K., Matsuo T. 1996. Glycogen accumulating population and its anaerobic substrate uptake in anaerobic-aerobic activated sludge without biological phosphorus removal. *Water Research.* Vol. 30. P. 75–82.
11. Liu W., Nakamura K., Matsuo T., Mino T. 1997. Internal energy-based competition between polyphosphate and glycogen-accumulating bacteria in biological phosphorus removal reactors-effect of P / C feeding ratio. *Water Research*. Vol. 31. N 6. P. 1430–1438.
12. Lu H., Oehmen A., Virdis B., Keller J., Yuan Z. G. 2006. Obtaining highly enriched cultures of Candidatus *Accumulibacter phosphatis* through alternating carbon sources. *Water Research*. Vol. 40. N 20. P. 3838–3848.
13. Martinenas B. 2004. *Statistical analysis of experimental data*. Vilnius: Technika. 101 p.
14. Maszenan A. M., Seviour R. J., Patel B. K. C., Rees G. N., McDougall B. M. 1998. The hunt for the G-bacterial in activated sludge biomass. *Water Science & Technology*. Vol. 37. N 4–5. P. 65–69.
15. Ministry of Environment of the Republic of Lithuania. 1994. *Unified Methods of the Wastewater and Surface Water Quality Researches. Chemical Analysis Methods.* Part I. 224 p.
16. Ministry of Environment of the Republic of Lithuania. 2000. *LAND 32–2000. Water Quality. Estimation of Ammonium Nitrogen*. 10 p.
17. Ministry of Environment of the Republic of Lithuania. 2005. *LAND 66–2005. Water Quality. Estimation of Nitrate*. 7 p.
18. Ministry of Environment of the Republic of Lithuania. 2005. *LAND 58: 2003 Water Quality. Estimation of Phosphate*. 24 p.
19. Mino T., Liu W T., Kurisu F., Matsuo T. 1995. Modeling of glycogen-storage and identification capability of microorganisms in enhanced biological phosphate removal processes. *Water Science & Technology.* Vol. 31. N 2. P. 25–34.
20. Mino T., van Loosdrecht M. C. M., Heijnen J. J. 1998. Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Research.* Vol. 32. P. 3193–3207.
21. Oehmen A., Yuan Z. G., Blackall L. L., Keller J. 2005. Comparison of acetate and propionate uptake by polyphosphate accumulating organisms and glycogen accumulating organisms. *Biotechnology & Bioengineering.* Vol. 91. P. 162–168.
22. Oehmen A., Zeng R. J., Saunders A. M., Blackall L. L., Keller J., Yuan Z. 2006. Anaerobic and aerobic metabolism of glycogen accumulating organisms selected with propionate as the sole carbon source. *Microbiology.* Vol. 152. P. 2767–2778.
23. Saito T., Brdjanovic D., van Loosdrecht M. C. M. 2004. Effect of nitrite on phosphate uptake by phosphate accumulating organisms. *Water Research.* Vol. 38. P. 3760–3768.
24. Severina S. E., Solovyova G. A. 1989. *Uchebnik biokhimii*. Moskva. 201 p.

25. Seviour R. J., Maszenan A. M., Soddell J. A., Tandoi V., Patel B. K. C., Kong Y., Schumann P. 2000. Microbiology of the "G-bacteria" in activated sludge. *Environmental Microbiology.* Vol. 2. N 6. P. 581–593.
26. Tsai C. S., Liu W. T. 2002. Phylogenetic and physiological diversity of tetrad-forming organisms in deteriorated biological phosphorus removal systems. *Water Science & Technology***.** Vol. 47. N 1–2. P. 179–184.
27. Vazquez M. C. L., Song Y., Hooijmans C. M., Brdjanovic D., Moussa M. S., Gijzen H. J., van Loosdrect M. M. C. 2007. Short-term temperature effects on the anaerobic metabolism of glycogen accumulating organisms. *Biotechnology & Bioengineering.* Vol. 97. N 3. P. 483–495.
28. Whang L. M., Park J. K. 2006. Competition between polyphosphate and glycogen-accumulating organisms in enhanced-biological-phosphorus-removal systems: Effect of temperature and sludge age. *Water Environmental Research.* Vol. 78. P. 1–4.
29. Wong M. T., Tan F. M., Ng W. J., Liu W. T. 2004. Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic-aerobic activated sludge processes. *Microbiology*. Vol. 150. P. 3741–3748.

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TETRADAS FORMUOJANČIŲ ORGANIZMŲ ĮTAKA FOSFORO BIOLOGINIO ŠALINIMO PROCESO PABLOGĖJIMUI NUOTEKŲ VALYKLOJE

Santrauka

Fosforo biologinio šalinimo iš nuotekų procesas gali sutrikti dėl tetradas formuojančių organizmų (TFO) intensyvaus dauginimosi veikliajame dumble. Yra žinoma, kad šie organizmai nekaupia savo ląstelėse polifosfatų, o energijos atsargas kaupia glikogeno pavidalu, todėl jie pavadinti glikogeną akumuliuojančiais organizmais (GAO) ir gali konkuruoti dėl lakiųjų riebalų rūgščių su polifosfatus akumuliuojančiais organizmais (PAO). Tyrimų, atliktų veikiančioje nuotekų valykloje, rezultatai parodė, kad fosforo biologinio šalinimo efektyvumas priklauso nuo nuotekų temperatūros ir dumblo amžiaus. Pakilus nuotekų temperatūrai, fosforo biologinis šalinimas pablogėja. Esant mažam fosforo šalinimo efektyvumui, nuotekų valyklos veikliajame dumble rasti padidėję TFO kiekiai ir padidėjusi glikogeno koncentracija (palyginus su tais atvejais, kai fosforo biologinio šalinimo efektyvumas buvo didelis). Sutrumpinus dumblo amžių iki 5 parų, fosforo biologinio šalinimo procesas atsistatė, ir sistema ėmė dirbti efektyviai. Sezoniškai mažėjant nuotekų temperatūrai, glikogeno koncentracija veikliajame dumble mažėjo.

Raktažodžiai: biologinis fosforo šalinimas, efektyvesnis biologinis fosforo šalinimas, polifosfatus kaupiantys organizmai, tetradas formuojantys organizmai, glikogeną akumuliuojantys organizmai