INVESTIGATION OF SULPHONAMIDES EFFECT ON ANAMMOX PROCESS

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Abstract The influence of selected sulphonamides such as sulphacetamide (SCM) and p-toluenesulphonamide (p-TSA) on the Anammox process was investigated. The short-term (14-hour) and long-term (90-day) exposure of mixed Anammox culture to SCM and p-TSA was applied. The aim of long-term exposure study (conducted in continuous-flow bioreactors) was to investigate a long-term influence of sulphonamides on Anammox process and on adaptation of Anammox culture to sulphonamides. The batch test performances resulted in the N-removal rate for non-acclimated and acclimated Anammox culture. The experiments were carried out at sulphonamides concentration of: 10, 20, 40, 80, 100 and 1000 mg/dm^3. The results confirmed that both sulphonamides inhibit the Anammox process, however, the effect is more evident in the case of SCM. The higher the sulphonamides concentration, the stronger inhibition of microorganisms' activity was observed.

Keywords: anammox, nitrogen removal, sulphonamide

INTRODUCTION

Efficient nitrogen removal from wastewater is a challenge since the stringent legal requirements were introduced in order to avoid the eutrophication problem and pollution of drinking water with nitrogenous compounds. Biological treatment comprises different processes, like ammonification, nitrification, denitrification or deammonification and they take place due to activity of different types of microorganisms.

Since late nineties, when the Anammox bacteria were discovered, new technologies for sustainable nitrogen removal from wastewater with high ammonium nitrogen concentration have been developed and applied.

The Anammox process (Eq. 1) consists of simultaneous nitrite and ammonium conversion under anaerobic conditions directly to gaseous nitrogen (Strous *et al.,* 1998).

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15} \qquad (1)$$

The partial nitritation coupled with the Anammox process can be applied for treatment of high-ammonium concentrated streams generated during dewatering of digested sludge or leachates from landfills of the waste (Egli *et al.,* 2001; Gut *et al.,* 2003). It also could be used for treatment of many industrial side streams e.g. from food sector (Keller *et al.*, 1997; Banas *et al*., 1999; Hippen *et al*., 2001; Carrera *et al*., 2003), animal waste (Waki *et al.,* 2007).

Previous researchers of the Anammox process were focused on an investigation of the influence of physical and chemical parameters (dissolved oxygen concentration, pH, temperature and alkalinity) and substrates as well as substrate – product concentration (Helmer *et al*., 2000; Van Dongen *et al*., 2001; Wyffels *et al*., 2003; Szatkowska *et al.,* 2004).

However, wastewater may contain many different chemical substances, which can inhibit microorganisms' growth and cause various troubles during biological treatment. These harmful substances, depending on their physical and chemical properties, can be not readily biodegradable and remain toxic during the entire wastewater treatment process (Schalk *et al.*, 1998; Halling-Sørensen *et al.,* 2000). Examples of such compounds are different pharmaceuticals such as sulphonamides, which are used in human therapy and animal husbandry. Sulphonamides are entering into the environment as effluents from pharmaceutical industry, municipal wastewater treatment plant as well as fish- and stock-farming. Sulphonamides were found in wastewater treatment plant effluents and surface water in the amount of the range from 10 to 2000 ng/dm^3 of the different sulphonamides such as sulphamethazine, sulphamethoxazole and sulphadiazine (Hartig *et al.,* 1999; Hirsch *et al.,* 1999). These compounds are not readily biodegraded and they reveal weak adsorption both, to soil and activated sludge (Ingerslev and Halling–Sørensen, 2000; Huang *et al*., 2001).

Due to sulphonamides properties, their migration in the environment may result in contamination of drinking water and food products. The inhibition effect of the sulphonamides on microorganisms growth of activated sludge or biofilm has been reported by Jjemba (2002). Besides, in the literature there are a few reports on sulphonamides' toxicity to nitrifying bacteria (Ingerslev and Halling-Sørensen, 2000; Al- Ahmad *et al*., 1999). Therefore, these compounds can induce serious trouble in self-purification of water as well as in biological wastewater treatment. Their removal during wastewater treatment, even in modern treatment plants is not sufficient and purified streams still contain these compounds.

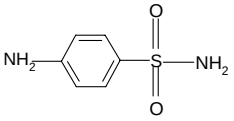
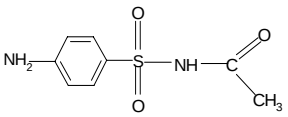
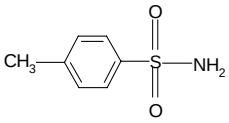
The Anammox bacteria are very sensitive to any type of toxic pollutants, however, there were no reports on investigation describing how sulphonamides contained in wastewaters could influence the Anammox process. Thus, the main aim of this study was to investigate how various sulphonamides influence the Anammox process during long-term (90 days) and short-term (14 hours) exposure to different their concentrations. It seemed to be very interesting to investigate the adaptive response of Anammox bacteria to sulphonamides present in wastewater of high ammonium ions concentration.

METHODOLOGY

In the current work sulphanilamide (SA), sulphacetamide (SCM) and p-toluenesulphonamide (p-TSA) were used. SA and SCM are antimicrobial substances, applied in medicine and veterinary treatment, but p-TSA is a metabolite of Chloramine T, used as a disinfectant against bacteria, viruses and fungi. Some properties of the tested sulphonamides are presented in Table 1. All sulphonamides were obtained from Sigma-Aldrich Co.

The results of our earlier investigations (Mędrzycka *et al.,* 2004) have confirmed the observations of very low biodegradability of investigated sulphonamides, reported earlier by other researchers (Halling-Sørensen *et al.,* 1998; Al.-Ahmed *et al.,* 1999). After 28 days (according to OECD procedure) p-TSA was degraded in 35% while SA and SCM only in 8% and 10.6 %, respectively (Table 1).

Table 1. Characteristics of sulphonamides used in the study

Sulphonamide	**Sulphanilamide (SA)**	**Sulphacetamide (SCM)**	**p-toluene-sulphonamide (p-TSA)**
Nomenclature	4-amino-benzene sulphonamide	N-[p- aminobenzene sulphonyl] – acetamide	4-metylbenzene sulphonamide
Molecular weight [g/mol]	172.21	214.20	171.22
Structural formula			
Solubility [g/dm^3]	7.5	6.7	3.16
Biodegradability * after 28 days (49 days) [%]	10.6 (17.4)	8.0 (15.7)	35.0 (47.0)

*(Mędrzycka et al., 2004)

Anammox anaerobic culture

Kaldnes rings with well-established Anammox bacterial culture were taken from the scale pilot plant of deammonification process (partial nitritation/Anammox system) at Himmerfjärden WWTP in Grödinge (Sweden) and were cultivated at the optimal conditions in a laboratory bioreactor (Gut *et al*., 2003; Szatkowska *et al*., 2004). Kaldnes rings ensured support for growth of the Anammox bacteria as well as hold them up in the system preventing the bacteria washing out.

Treated wastewater

The wastewater used in the batch tests was prepared from the supernatant taken from cultivation bioreactor and was supplemented with the additives of NH_4Cl and $NaNO_2$.

In the long-term experiments the high-strength ammonium supernatant from dewatering of the sludge in Gdansk WWTP was used. It usually contained about 700-800 mg NH_4-N/dm^3, thus it have to be diluted with tap water and additionally enriched with $NaNO_2$ in order to achieve the required concentrations of both forms of nitrogen.

EXPERIMENTAL PROCEDURE

Batch tests

In order to investigate the influence of sulphonamides on the Anammox process efficiency the series of batch tests were run in seven bottles (1 l working volume) in the closed reaction system. The test bottles equipped with magnetic stirrers were filled in 50 % of their volume with the Kaldnes rings. The initial concentrations of NH_4^+-N and NO_2^--N were set for each test. The test conditions have to assure that there is no inhibition of the process due to deficit or due to excessive amount of substrates. Thus, as it has been suggested by Van Dongen (Van Dongen *et al*., 2001) the NO_2^--N concentration shouldn't exceed 70 mg/dm^3, while the NH_4^+-N concentration have to be higher than stoichiometric value (to be sure that it is not a limiting substrate), (see eq.1). Required

initial nitrogen forms concentration (about 150 mg NH_4^+-N/dm^3 and about 65 mg NO_2^--N/dm^3) were obtained by addition of NH_4Cl and $NaNO_2$ solutions to the liquor taken from the Anammox cultivation reactor. The tests were run for 14 hours and the effect of different sulphonamides concentrations: 0, 10, 20, 40, 80, 100 and 1000 mg/dm^3 was investigated. The parameters: DO, pH, temperature were kept stable at the optimum level (DO was about 0.15 mg/ dm^3, pH was 7.8 – 8.3 and temperature was 29-32 ^{0}C).

Batch tests were carried out for non-acclimated and for acclimated Anammox cultures (after long-term exposure experiments).

Long-term exposure study

The long-term exposure study was carried out within 90 days and its aim was to investigate a long-term influence of sulphonamides on Anammox process and on adaptation of Anammox culture to sulphonamides. The experimental set-up was consisted of five reactors of one litre working volume. Each reactor was equipped with mixers to keep homogeneity of reactor volume. A water bath kept temperature in the range of 30 – 33 °C. Each reactor was filled in 50 % of working volume with Kaldnes rings carrying Anammox bacterial culture and then filled up to 1 dm^3 volume with wastewater. The reactors were operated simultaneously and were continuously fed with sulphonamides – enriched feeding wastewater. The feeding wastewater was prepared using the supernatant as a source of NH_4^+-N and $NaNO_2$ as a source of NO_2^--N. The required nitrite to ammonium nitrogen ratio (NAR) for Anammox bacteria is 1.3 (eq. 1).

Based on earlier investigations by Bąkowska (2006) it were decided the initial concentrations of ammonium and nitrite at levels of 150 mg NH_4^+-N/dm^3 and 195 mg NO_2^--N/dm^3. To obtain such concentrations the supernatant was diluted with tap water and further, the required volume of $NaNO_2$ solution was added.

The effect of different concentrations of each sulphonamide (20, 40, 80 100 and 1000 mg/dm^3) was investigated during the whole operating period. Additionally, the blank reactor was fed with sulphonamide-free feeding wastewater.

Analytical procedures

Samples were collected twice a week both, from inlet and outlet of each reactor during the long-exposure study and once per every two hours during batch test performance. The samples were analysed for ammonium nitrogen, nitrite and nitrate nitrogen with usage of TECATOR – AQUATEC 5400 ANALYZER or Hach DR 2000 spectrophotometers and typical analytical procedures. The process parameters such as pH, DO and conductivity were measured once per every day in long-term experiments and once per one hour during batch tests.

RESULTS AND DISCUSSION

Long-term exposure processes

In tables 2 - 5 the values of DO and pH measured during long-term exposure Anammox processes at different concentrations of both sulphonamides are presented.

Earlier studies showed that the DO is crucial factor in the Anammox process performance and in the oxygen-limiting conditions (below 0.2 mg O_2/dm^3) the microorganisms can use nitrite instead of oxygen as an electron acceptor. During the long-term processes the stable value of DO (0.19-0.22 mg O_2/dm^3) can be observed (Table 2 and 3).

The optimum pH range for Anammox is around 8 (Egli *et al.*, 2001). In the performed processes the pH was not corrected and in the influent it was slightly lower than 8 (7.25-7.35 in case of SCM and 7.63-7.80 in case of p-TSA), while in the effluent it was around 8 (7.83-8.14 in case of SCM and 7.74-8.03 in case of p-TSA, Table 2 and 3).

Table 2. Dissolved oxygen (DO) and pH of wastewater in the long-term Anammox processes at the presence of p-TSA

DO [mg O_2/dm^3]	**p-TSA concentration [mg p-TSA /dm^3]**				
	0	**20**	**40**	**80**	**1000**
Average	0.21	0.21	0.22	0.20	0.21
Standard deviation	0.02	0.05	0.08	0.03	0.04
Inlet pH	**p-TSA concentration [mg p-TSA /dm^3]**				
	0	**20**	**40**	**80**	**1000**
Average	7.63	7.64	7.71	7.80	7.76
Standard deviation	0.06	0.07	0.09	0.17	0.18
Outlet pH	**p-TSA concentration [p-TSA/dm^3]**				
	0	**20**	**40**	**80**	**1000**
Average	7.93	8.02	8.00	8.03	7.74
Standard deviation	0.08	0.15	0.15	0.32	0.11

Table 3. Dissolved oxygen (DO) and pH of wastewater in the long-term Anammox processes at the presence of SCM

DO [mg O_2/dm^3]	**SCM concentration [mg SCM/dm^3]**				
	0	**20**	**40**	**80**	**1000**
Average	0.19	0.20	0.19	0.21	0.19
Standard deviation	0.06	0.07	0.06	0.07	0.06
Inlet pH	**SCM concentration [mg SCM/dm^3]**				
	0	**20**	**40**	**80**	**1000**
Average	7.25	7.35	7.32	7.35	7.33
Standard deviation	0.20	0.22	0.21	0.25	0.28
Outlet pH	**SCM concentration [mg SCM/dm^3]**				
	0	**20**	**40**	**80**	**1000**
Average	8.12	8.09	8.13	8.14	7.83
Standard deviation	0.14	0.14	0.16	0.14	0.33

Table 4. Dissolved oxygen (DO) and pH of wastewater in the long-term Anammox processes at the presence of SA (65 days)

DO [mg O_2/dm^3]	**SA concentration [mg SA/dm^3]**			
	0	**20**	**40**	**1000**
Average	0.29	0.33	0.45	0.33
Standard deviation	0.09	0.09	0.54	0.06
Inlet pH	**SA concentration [mg SA/dm^3]**			
	0	**20**	**40**	**1000**
Average	7.95	7.99	7.90	8.02
Standard deviation	0.24	0.15	0.24	0.17
Outlet pH	**SA concentration [mg SA/dm^3]**			
	0	**20**	**40**	**1000**
Average	8.1	7.87	7.72	7.68
Standard deviation	0.24	0.22	0.24	0.31

Table 5. Dissolved oxygen (DO) and pH of wastewater in the long-term Anammox processes at the presence of SA (25 days)

DO [mg O_2/dm^3]	**SA concentration [mg SA/dm^3]**			
	0	**20**	**40**	**1000**
Average	0.31	0.33	0.46	0.35
Standard deviation	0.04	0.03	0.18	0.05
Inlet pH	**SA concentration [mg SA/dm^3]**			
	0	**20**	**40**	**1000**
Average	7.76	7.75	7.90	7.86
Standard deviation	0.19	0.08	0.24	0.14
Outlet pH	**SA concentration [mg SA/dm^3]**			
	0	**20**	**40**	**1000**
Average	8.29	7.63	7.72	7.33
Standard deviation	0.21	0.30	0.24	0.20

One of the factors that confirm proper Anammox process performance is the increase of pH during process run (Gut *et al.*, 2005). As it can be seen from the Tables 2 and 3 it takes place only in the reactors with sulphonamides concentration from 0 up to 80 mg/dm^3, while in the case when p-TSA or SCM concentration was 1000 mg/dm^3, the pH value decreased. This may suggest that Anammox process was disturbed by both sulphonamides at their high concentrations.

In Figs. 1 - 3 the N-removal efficiency (percent of total inorganic N-removal) during long-term exposure studies is presented. The results relate to processes with the addition of sulphonamides and without it (blank).

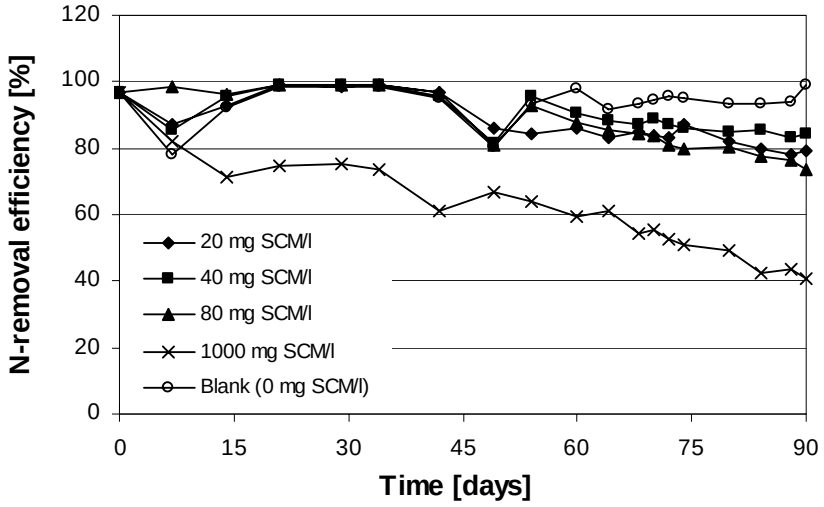


Figure 1. N-removal efficiency during long-term exposure study at different concentrations of SCM

During the initial stage of the process the N-removal efficiency in reactors with SCM was about 80-90 % and after 20 days of the run it has been stabilized at the value of 90-95 %. Only in case of 1000 mg/dm^3 of SCM concentration the removal efficiency was much lower (about 70 %) and after 45 days it decreased up to 40 % at the end of the run (after 90 days). In case of lower SCM concentrations the decrease of N-removal efficiency was also observed, however, after 90 days it has reached values of about 75-83 % (Fig. 1). Only in the blank reactor the process efficiency was high till the end of the investigations (90-95 %).

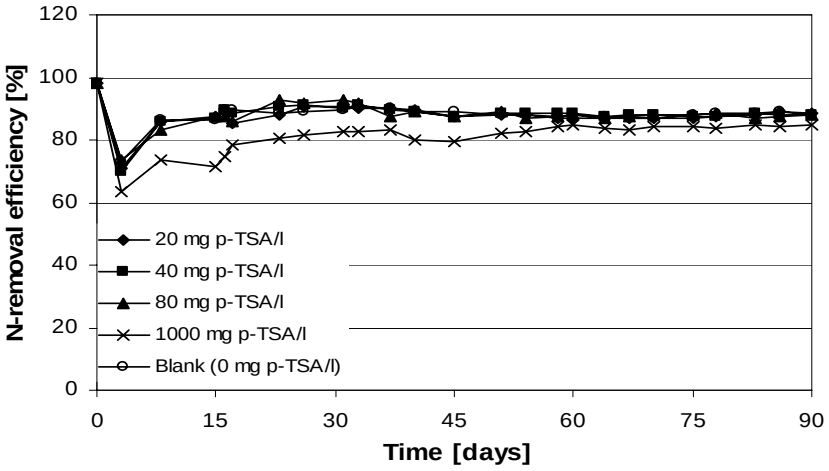


Figure 2. N-removal efficiency during long-term exposure study at different concentrations of p-TSA

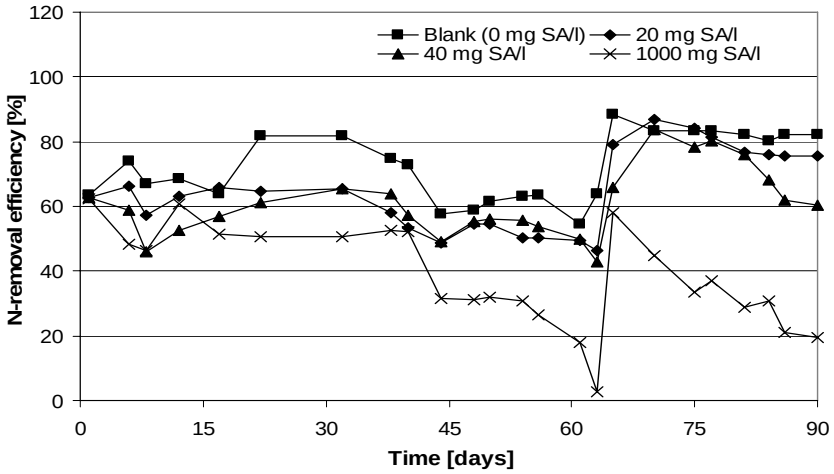


Figure 3. N-removal efficiency during long-term exposure study at different concentrations of SA

In case when p-TSA was added to the wastewater the changes in removal efficiency were not so great and from initial removal efficiency amounted to 65-70% it continuously has increased up to 85-90% at the end of the run (Fig. 2). However, also in the case of this sulphonamide, the removal efficiency at its highest concentration (1000 p-TSA mg/dm^3) is worse (by about 5-15%) than at its lower concentrations.

The long-term exposure study with using SA was divided into two periods: first - 65 days period and second - 25 day period. Initial concentration of 100 mg NH_4^+-N/dm^3 and 130 mg/dm^3 NO_2^--N were used in the first period. After first 65 days of the process run the initial concentrations of ammonium and nitrite forms were increased up to 150 mg NH_4^+-N/dm^3 and 195 mg NO_2^--N/dm^3. The change was made in order to investigate the process performance at higher ammonium and nitrite concentrations, usually observed in supernatant.

During the first period the N-removal efficiency in blank reactor and in reactors with 20 and 40 mg SA/dm^3 were fluctuated (Fig 3). Only in the reactor with 1000 mg SA/dm^3 the process efficiency has rapidly dropped after 40th day of run (up to 3 % removal). The average N-removal efficiency was about 67%, 56% and 38%, respectively (Fig. 3).

After the increase of NH_4^+-N and NO_2^--N concentrations (during the second period the N in all reactors was about 360 mg/dm^3) the N-removal efficiency increased rapidly in all reactors. Within this period the effluents' composition from blank reactor and reactors with 20 and 40 mg SA/dm^3 was similar). The effluent contained about 13-16 mg NH_4^+-N/dm^3 and 19 - 26 mg NO_2^--N/dm^3, respectively. At the highest SA concentration the effluents concentrations were about 100 mg NH_4^+-N/dm^3 and about 96 mg NO_2^--N/dm^3. The NO_3^--N production exceeded 11.2% only in reactor with 1000 mg SA/dm^3 and it was about 22%. In other reactors the nitrate production consumed about 6% of the total nitrogen.

During the second period ammonium and nitrite forms were removed with efficiency higher than during first period. The Anammox process remained stable in blank reactor and in reactor with 20 mg SA/dm^3 during the whole 25-day period (Fig. 3). It can be said that during long-term exposure to 20 mg SA/dm^3 the Anammox process was slightly disturbed. The obtained N-removal efficiency

was lower by about 14% than in blank reactor. However, at the presence of 1000 mg SA/dm^3 the Anammox process was accompanied by nitrification process.

Summarizing, one can state that the Anammox process remained stable in reactors with p-TSA during the whole 90-day period (Fig. 2), while in the reactors with SCM the Anammox process was slightly disturbed, especially at its concentration 1000 mg/dm^3. Probably, in the presence of 1000 mg SCM/dm^3 the Anammox process was accompanied by nitrification process because slight NO_3^--N concentration increase (from 2.5 mg NO_3^--N/dm^3 to 24.4 mg NO_3^--N/dm^3) and significant pH drop (from pH level about 8.2 to about 7.3) were observed within the experiment duration. Similar conclusions can be drawn when analyses the results of the Anammox process at the presence of SA, the nitrification proceeds at highest SA concentration.

Batch tests

In the batch test study the nitrogen removal rate during short-exposure of the mixed Anammox culture to sulphonamides was evaluated. The nitrogen removal rates in the Anammox process were obtained for non-acclimated and acclimated bacterial cultures and are presented in Figs 4 and 5, respectively.

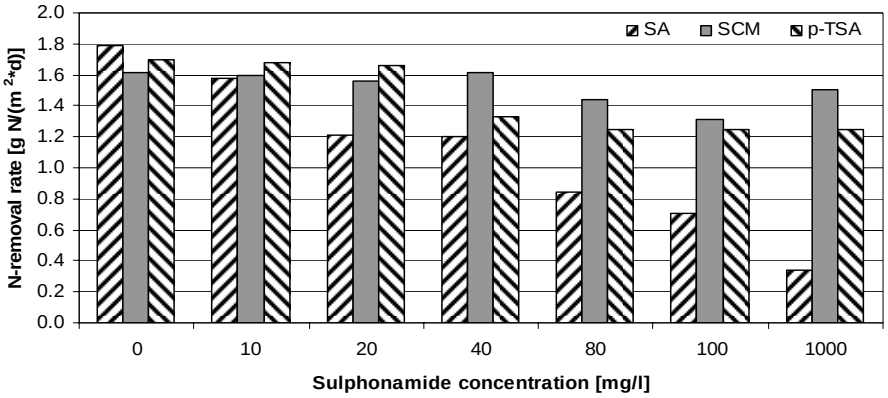


Figure 4. N- removal rates obtained during batch tests with non-acclimated Anammox culture

As it results from Fig. 4 (for non-acclimated Anammox bacterial culture) the nitrogen removal rate is equal to about 1.6 g N/m^2·d at the SCM content from 0 up to 40 mg/dm^3 and slightly higher - about 1.7 g N/m^2·d at the p-TSA content from 0 up to 20 mg/dm^3. The increase of sulphonamides concentration decreases microorganisms' activity and the rate of nitrogen removal drops to about 1.2 or 1.3 g N/m^2·d in case of 100 mg/dm^3 of SCM and p-TSA, respectively (Fig. 4). Further increase of sulphonamides content does not change substantially the rate of nitrogen removal.

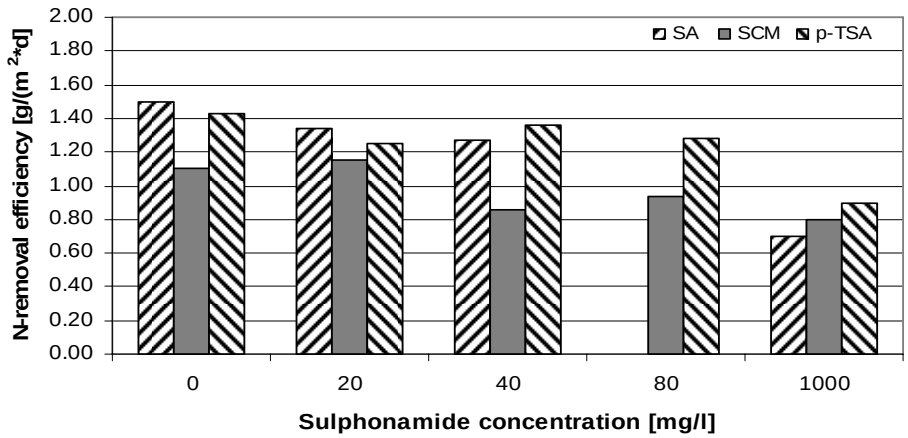


Figure 5. N - removal rates obtained during batch tests with acclimated Anammox culture

In the case of acclimated Anammox bacteria (taken from the long-term exposure experiments), the rate of nitrogen removal is lower than that, obtained for non-acclimated culture. When SCM was added to the wastewater the process rate has decreased up to 1.1–0.8 g $N/m^2 \cdot d$, and in the case of p-TSA use, the rate decreased only to 1.4-0.9 g $N/m^2 \cdot d$ (Fig. 5).

Thus one can conclude that the higher was the sulphonamides concentration, the lower N-removal efficiency was observed. Besides, the long-term exposure did not result in the evident acclimation of the bacteria. Moreover, a comparison of the N-removal changes between the non- and acclimated Anammox culture was made. Only the acclimated Anammox cultures to the presence 1000 mg/dm^3 SCM and 1000 mg/dm^3 p-TSA were more sensitive than non-acclimated cultures, as the rates of N-removal was lower about 24 % and 13.5 % for SCM and p-TSA, respectively.

It has been showed that p-TSA reveals less negative effect to Anammox bacteria than SCM does. The difference in the effect of both sulphonamides probably results from the fact that p-TSA is already a metabolite of toxic Chloramine T and its toxicity is lower than toxicity of the non-treated biologically SCM.

CONCLUSION

1) The results obtained from long-term exposure experiments and from batch tests confirm hypothesis that sulphanilamides can inhibit the Anammox process. It has been stated for SA and SCM. The higher the SA and SCM concentration, the greater inhibition effect was observed. The negative effect observed in case of p-TSA is much smaller.

2) The acclimation possibility of the Anammox bacteria to sulphonamides is doubtful. However, some of the results showed that microorganisms may acclimate to p-TSA presence in the process's environment and along with time the inhibition effect can be reduced. Hence, further research on the Anammox process in the presence of sulphonamides should be continued.

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