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# Wastewater denitrification process—the influence of methanol and kinetic analysis

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#### Abstract

The aim of this work was to achieve rapid nitrate removal from synthetic wastewater (SW) without nitrite accumulation and to improve the economic effectiveness of the process. For that purpose, experiments were carried out to determine the influence of methanol on denitrification rate in batch assays and in the continuous-flow stirred cultures. Nitrate–N (200 mgNO<sub>3</sub><sup>-</sup> –N/l) was reduced under anoxic conditions during approximately 4–6 h for the MeOH/NO<sub>3</sub><sup>-</sup> –N ratio above 2.5. Nitrite concentration was elevated to the maximum of 1.2 mgNO<sub>2</sub><sup>-</sup> –N/l and at the end of the tests nitrite concentrations were 0.06–0.1 mgNO<sub>2</sub><sup>-</sup> –N/l. At lower MeOH/NO<sub>3</sub><sup>-</sup> –N ratios the denitrification process stopped after exhaustion of methanol. The analysis of experimental results showed that denitrification was a zero-order reaction with respect to nitrate and a first-order reaction with respect to the biomass concentration (the first-order overall reaction). In the continuous denitrification process during 45 days the hydraulic retention time (HRT) was decreased from 62 to 28 h. Dissolved oxygen concentration fell from 5.50 to 0.40 mgO<sub>2</sub>/l during the first 4 h, but over 3 days of continuous flow it increased to 2.5 mgO<sub>2</sub>/l and remained at that level. Accumulation of nitrite ions in SW was similar to that in batch tests, but at an HRT of 28 h the nitrite concentration increased to 6 mgNO<sub>2</sub><sup>-</sup> –N/l. Complete denitrification at 25 °C was achieved at nitrate and methanol loading rates of 4.35 mgNO<sub>3</sub><sup>-</sup> –N/l h and 23 mgO<sub>2</sub>/l h, respectively.

Keywords: Batch denitrifying reactor; Continuous-flow stirred reactor; Denitrification; Mixed bacterial culture; Nitrite accumulation; Kinetic analysis

#### 1. Introduction

Excessive application of fertilizers and other nitrogen compounds in various industries, e.g. agricultural, pharmaceutical, dairy or food, contribute to nitrogen pollution [1,2]. A common method of treating Npollution is nitrification, followed by denitrification. Biological denitrification enables transformation of oxidized nitrogen compounds by a wide spectrum of heterotrophic bacteria into harmless nitrogen gas with accompanying carbon removal. This process has been well studied, but biological denitrification of wastewater is usually slow and lasts several days. According to Ref. [3], 10–50 mgNO<sub>3</sub><sup>-</sup> –N/l was removed with *Pseudomonas denitrificans* during 4 h and denitrification of 100 mgNO<sub>3</sub><sup>-</sup> –N/l with the suspended bacterial cells lasted 18 h [4]. Furthermore, different industrial wastewaters contained more than 200 mgNO<sub>3</sub><sup>-</sup> –N/l and biological denitrification of such wastewaters usually lasted a few days [2,5–7]. Thus, Zayed and Winter [2] investigated nitrate removal from dairy wastewater: with activated sludge removal of 250 mgNO<sub>3</sub><sup>-</sup> –N/l lasted 3 days, with an immobilized mixed culture the conversion of nitrate was faster, and after 2 days no nitrate or nitrite remained, and finally in the tests with the suspended pure culture 8 days were required to convert 100% of nitrate–N to nitrogen gas and to reduce the chemical oxygen demand (COD) from 1500 to 159 mgO<sub>2</sub>/l.

The denitrification process depends on a number of factors, such as change in environmental conditions (temperature and pH), dissolved oxygen (DO), presence or accumulation of nitrite during the process and availability of organic carbon. The nature and the amount of the organic substrate used as exogenous carbon source plays an important role in the success and

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price of denitrification. Various substrates have been used in many studies and a whole variety of compounds, mostly methanol [3,8,9], acetate [4,6,7], and ethanol [10]. Some studies used nutrient broth [11] and tryptic soy broth [12]. Relevant to the price and availability, methanol is most commonly used for bacterial denitrification [8]. The methanol to nitrate-nitrogen ratio is a measure of electron donor to electron acceptor ratio in biological denitrification. During the process it influences denitrification rate and accumulation of nitrite ions. As a consequence, an optimal methanol to nitratenitrogen ratio seems to be an advantageous parameter for achieving complete and cost-effective denitrification process, under minimal accumulation of nitrite ions.

In this study denitrification of synthetic wastewater (SW) was investigated in batch assays and in continuous-flow stirred cultures. Tests with different original biomass concentrations applied for denitrification and optimisation of methanol to nitrate–N ratio were carried out in the batch assays. The selected MeOH/  $NO_3^-$ –N value was applied to investigate denitrification in the continuous-flow stirred reactor. A simplified kinetic analysis was performed for quantitative comparison of nitrate consumption and biomass production rates.

#### 2. Materials and methods

#### 2.1. Experimental apparatus

Batch experiments were performed in 0.5 l closed, sterile serum bottles. Each bottle contained 0.3 l SW and 0.1 l biomass suspension. The stoppers were punctured with a thermometer and two disposable syringes with needles, one for measuring the gas produced and the other for sampling.



Fig. 1. Schematic of continuous-flow stirred reactor.

Continuous tests were carried out in the bioreactor (Fig. 1) of 0.3 l working volume. Every test began as a batch test. When nitrate was completely reduced, continuous flow of feed started. For continuous cultivation, SW was added to the reactor with a peristaltic pump at different flow rates to give different hydraulic retention times (HRT). Incubation was conducted at  $25\pm2$  °C, pH 6.8 and an agitation speed of 400 rpm under anoxic conditions.

#### 2.2. Synthetic wastewater

The original mineral medium comprised  $K_2$ HPO<sub>4</sub> 2.5 g, KH<sub>2</sub>PO<sub>4</sub> 1 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.17 g and NaCl 5 g/l deionised water. The solution was autoclaved and allowed to cool to room temperature prior to the addition of 0.1 ml trace metal mixture (the solution containing 0.5% w/v of each metal salt: MnSO<sub>4</sub>, CuSO<sub>4</sub>, FeCl<sub>3</sub>, and Na<sub>2</sub>MoO<sub>4</sub>). Nitrate ions, 200  $mgNO_3^- - N/l$  (the stock solution was an aqueous solution of NaNO<sub>3</sub> containing 10  $gNO_3^- - N/l$ ) and methanol were added to the original mineral medium to prepare SW. The methanol concentration during the investigation of the biomass concentrations effect on nitrate removal was 900 mg/l (MeOH/NO3-N weight ratio was 4.5). During optimisation of methanol to nitrate-nitrogen ratios, methanol concentrations were adjusted to give different MeOH/NO<sub>3</sub><sup>-</sup>-N weight ratios, from 1.5 to 4.5. The MeOH/NO $_3^-$ -N ratio in SW used as feed solution was 3.0. Phosphate salts in the mineral medium were used as buffer. Thus, the pH in SW remained unchanged throughout the experiments.

#### 2.3. Microorganisms

Microorganisms originated from the mixed liquid from aerobic municipal sewage treatment plant (Velika Gorica, Croatia) and activated sludge from an anaerobic–aerobic wastewater treatment plant (Anamet, Pliva, Savski Marof, Croatia). These two sludges (0.05 l fractions) were mixed and centrifuged at  $12557 \times g$ and 5 °C for 10 min. The biomass obtained was washed twice, diluted with mineral medium, refrigerated at 4 °C and kept for further use. Mixed bacterial culture was acclimated to nitrate ions up to 500 mgNO<sub>3</sub><sup>-</sup> –N/l at pH 6.8 and 35 °C under anoxic conditions. After each experiment the biomass suspension was prepared as described and used as inoculum in the next experiment.

#### 2.4. Analytical methods

During nitrate removal from SW, microbial growth was monitored and the kinetics of microbial growth was established. To study the kinetics of nitrate removal from the medium, samples were taken from the bottle or the reactor at the preset time and processed immedi-

ately. DO concentration, volatile suspended solids (VSS) and cell numbers, i.e. colony forming units (CFU), were monitored in the effluent and for control in the reactor at the midpoint of its height (Fig. 1). Liquid samples were then centrifuged at 5 °C and  $12557 \times g$  (Sigma 3K15, Osterode, Germany). The supernatant was used for nitrate and nitrite analysis.

Nitrate concentration in SW during denitrification was monitored spectrophotometrically on a Varian LS 80 (Varian, Mulgrave, Australia) by a chromotropic acid method at  $\lambda = 400$  nm [13]. Nitrite detection was based on a colorimetric reaction with  $\alpha$ -naphthylamine after diazotisation with sulphanilic acid [14] and photometric measurement at 500 nm (MA 9510-Iskra, Kranj, Slovenia). The concentration of DO and pH of SW were monitored with an by oxygen-meter MA 5485 and pHmeter MA 5750 (Metrel, Horjul, Slovenia). VSS and COD were determined according to Standard Methods [13] for expressing biomass and methanol concentrations. CFU were determined by plate count on standard nutrient broth and on the feed solution hardened by the addition of agar–agar, after repeated dilution with 0.9% NaCl. Different denitrifiers in the mixed bacterial culture were distinguished according to their colony forms and by optical microscopy after Gram staining. Bacterial species isolated as pure cultures were identified by API 20 E and API 20 NE systems and according to Bergey's Manual of Determinative Bacteriology [15].

#### 2.5. Calculations

The rate at which nitrate is converted to nitrite is calculated as the first-order overall function of nitrate (zero-order) and biomass concentration (first-order):

$$\frac{d[NO_3^- - N]}{dt} = -k_1[NO_3^- - N]^0[X]^1$$
(1)

and its integration form,

$$[NO_{3}^{-} - N] - [NO_{3}^{-} - N]_{0} = -k_{1}[X]t$$
<sup>(2)</sup>

Biomass growth rate is

$$\frac{\mathrm{d}[X]}{\mathrm{d}t} = \mu[X] \tag{3}$$

The relation between the rates of the substrate consumption and cell production, known as growth yield is

$$\frac{\mathrm{d}[X]}{\mathrm{d}t} = -Y \frac{\mathrm{d}[\mathrm{NO}_3^- - \mathrm{N}]}{\mathrm{d}t}$$
(4)

where  $[NO_3^- - N]$  is nitrate-N concentration  $(mgNO_3^- - N/l)$ , [X] the biomass concentration (gVSS/l) and  $k_1$  the reaction rate constant, specific denitrification rate  $(mgNO_3^- - N/gVSS h)$ ,  $\mu$  the specific growth rate constant (1/h) and Y growth yield  $(mgVSS/mgNO_3^- - N)$ .

Specific growth rates in this work were determined at different original VSS concentrations in the batch reactors at 25 °C by measuring VSS concentrations at regular time intervals and by plotting  $\ln([X]_t/[X]_0)$  vs. time. From Eq. (4) over any finite time period during exponential growth phase, growth yield is expressed as weight of bacteria formed/weight of substrate consumed.

The data from batch experiments were used to calculate the kinetic constants as described by the Monod equation. The Monod equation is written as follows [4,16,17]:

$$r_{\rm D} = \frac{d[{\rm NO}_3^- - {\rm N}]}{dt} = \frac{\mu_{\rm max}[{\rm NO}_3^- - {\rm N}]}{(K_{\rm N} + [{\rm NO}_3^- - {\rm N}])} \frac{[X]}{Y}$$
$$= \frac{k_{\rm D}[{\rm NO}_3^- - {\rm N}]}{(K_{\rm N} + [{\rm NO}_3^- - {\rm N}])}$$
(5)

where  $r_{\rm D}$  is the rate of nitrate utilization (mgNO<sub>3</sub><sup>-</sup> -N/l h),  $k_{\rm D}$  is maximum rate of nitrate utilization (mgNO<sub>3</sub><sup>-</sup> -N/l h),  $\mu_{\rm max}$  is the maximum specific growth rate (1/h), and  $K_{\rm N}$  is the half velocity constant (mg/l). If  $K_{\rm N} \ll [{\rm NO}_3^- - {\rm N}]$  in Eq. (5),  $K_{\rm N}$  is insignificant in comparison to  $[{\rm NO}_3^- - {\rm N}]$  and the Monod equation turns to zero-order reaction model [16].

In the presence of nitrite ions and DO in wastewater, addition of methanol may be calculated as proposed by McCarthy et al. [18]:

$$[CH_{3}OH] = 2.47[NO_{3} - N] + 1.53[NO_{2} - N] + 0.87[DO]$$
(6)

Volumetric loading rates and denitrification rates during denitrification in the continuous-flow stirred reactor were calculated as follows:

Organic load = 
$$\frac{\text{COD}_{\text{in}} \cdot R}{V} (\text{mgO}_2/\text{l h})$$

Nitrate - nitrogen load

$$=\frac{[\mathrm{NO}_{3}^{-}-\mathrm{N}]_{\mathrm{in}}\cdot R}{V} (\mathrm{mg}\mathrm{NO}_{3}^{-}-\mathrm{N/l}\,\mathrm{h})$$

Volumetric denitrification rate

$$=\frac{\{[NO_{3}^{-} - N]_{in} - [NO_{3}^{-} - N]_{effl}\} \cdot R}{V} (mgNO_{3}^{-} - N/l h)$$

Specific denitrification rate

$$=\frac{\text{volumetric rate}}{[X]} (\text{mgNO}_3^- - \text{N/gVSS h})$$

where  $[NO_3^- - N]_{in}$ ,  $[NO_3^- - N]_{effl}$  and  $[COD]_{in}$  represented the influent, effluent nitrate-nitrogen concentrations (mgNO<sub>3</sub><sup>-</sup> -N/l) and influent COD (mgO<sub>2</sub>/l). [X] is the biomass concentration (gVSS/l), R the influent wastewater flow rate (ml/h) and V the reactor volume

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(l). Dilution rate, D (1/h) and hydraulic retention time, HRT (h) are calculated from the flow rate values and the reactor volume.

#### 3. Results and discussion

# 3.1. Nitrate removal and kinetic of the batch denitrification process

Mixed microbial culture used for the removal of nitrate ions from SW, was first acclimated to nitrate ions up to 500 mgNO<sub>3</sub><sup>-</sup> - N/l at pH 6.8 and 35 °C under anoxic conditions (data are not shown). Complete nitrate removal was achieved from 2 to 6 h. In the course of the measurement of cell numbers, morphology of bacterial colonies was examined. After Gram staining of isolated pure cultures and biochemical tests six different bacterial species were identified in mixed culture. The isolated bacteria were Paracoccus sp., Pseudomonas stutzeri, Xanthomonas maltophilia, Ochrobactrum antrophi, Aeromonas salmonicida achromogenes and Staphylococcus sp. Among them, Paracoccus sp. and Pseudomonas stutzeri were the only true denitrifiers [19,20] and dominated making 80% mixed bacterial population. Bacteria belonging to the genera Pseudomonas, Xanthomonas, Ochrobactrum and Aeromonas were also present in some mixed cultures during similar investigations [3,19]. It was reported [18] that Staphylococcus sp. caused significant accumulation of nitrite and inhibited the denitrification process. However, in the present study there was no significant increase of Staphylococcus sp. cells, so it can be assumed that the presence of this bacterium did not affect accumulation of nitrite ions. As mentioned before, Paracoccus sp. and Pseudomonas stutzeri were reported as true denitrifiers, so their dominant presence in mixed culture obviously accomplished complete and rapid nitrate removal with minimum nitrite accumulation.

In order to determine the kinetic of denitrification process the influence of biomass concentration on nitrate removal were examined and a set of experiments were carried out. The original biomass concentrations were 1.2, 1.6, 1.8, 2.3, and 3.1 gVSS/l. The time required for complete denitrification was from 2.5 to 9 h (Fig. 2a). Nitrate removal was modelled according to Eq. (5) and results are presented in Fig. 2a and Table 1. A high degree linear relationship ( $R^2 > 0.99$ ) between nitrate concentrations vs. time and the Monod equation model confirmed that the denitrification process was well described with the Monod equation. Obtained model parameters  $\mu_{\rm max}$  and  $K_{\rm N}$  were 0.01095 1/h and 0.001 mgNO<sub>3</sub><sup>-</sup> –N/l, respectively. Value of  $\mu_{max}$  was similar to value  $\mu_{\text{max}} = 0.0125$  1/h, obtained during similar tests [4]. Since  $K_N$  was 0.001 mgNO<sub>3</sub><sup>-</sup> -N/l and the original nitrate concentration was 200 mgNO<sub>3</sub><sup>-</sup>-N/l, it was



Fig. 2. Nitrate removal (a) during the batch tests in comparison of the Monod model, and correlation (b) between nitrates removed and original biomass concentrations (Eq. (7)).

obvious that  $[NO_3^- - N]$  was much greater than  $K_N$  and nitrate removal approaches a zero-order reaction [3,8,16,21]. Partial reaction order with respect to biomass concentration was experimentally determined from Eq. (1). Eq. (1) was rearranged to equation:

$$([NO_3^- - N]_0 - [NO_3^- - N])/t = k_1[X]^m$$
(7)

The left side of this equation was measured experimentally and plotted against biomass concentration as shown in Fig. 2b. Linearity ( $R^2 > 0.99$ ) between  $k_1[X]^m$  and [X] indicated that m equalled one. Consequently, experimental results confirmed that denitrification was a first-order overall reaction (zero-order reaction with respect to nitrate and first-order reaction with respect to biomass concentration). By applying linear fit to nitrate–N concentrations vs. time points, in Fig. 2a, from the slope of the linear line and Eq. (2) specific denitrification rates were calculated. Rates  $k_1$  were in range from 17.9 to 25.4 mgNO<sub>3</sub><sup>-</sup> –N/gVSS h at 25 °C (Table 1). Those results were similar to the data obtained by Sozen and Orhon [22], who reported that

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Table 1		
Denitrification	kinetic	parameter

$X_0$ (mgVSS/l)	$[NO_{3}^{-}-N]_{0}$ (mgNO <sub>3</sub> -N/l)	$\mu_{\rm max}$ (1/h)	$K_{\rm N} ({\rm mgNO_3^-} - {\rm N/l})$	$R^2$
		,		
1.2	204.12	$0.01095 \pm 9 \times 10^{-5}$	0.001	0.9974
1.6	204.11	$0.01095 \pm 9 \times 10^{-5}$	0.001	0.9980
1.8	197.47	$0.01095 \pm 9 \times 10^{-5}$	0.001	0.9990
2.3	196.81	$0.01095 \pm 9 \times 10^{-5}$	0.001	0.9971
3.1	192.82	$0.01095 \pm 9 \times 10^{-5}$	0.001	0.9936

denitrification rates for different wastewaters were in the range from 17.0 to 32.0 mgNO<sub>3</sub><sup>-</sup> –N/gVSS h. Biomass yields were measured to estimate the amount of biomass that would be produced during nitrate removal. Specific growth rates,  $\mu$  calculated from batch experiments with different original biomass concentrations are small values (Table 2), but were within the range of the typical model parameter values of anaerobic digestion process [21]. Although the growth yield, Y is usually considered a constant, it can show a wide range of variations, even under meticulously controlled conditions. The results obtained compare well with Refs. [9,23].

In biological denitrification nitrite production is known to be one of the main problems [6], since nitrite ions inhibit bacterial growth. Therefore, nitrite levels must be regularly checked. Original nitrite concentrations were  $0.5-0.8 \text{ mgNO}_2^-$  -N/l, and during the tests increased to 1.2 mgNO $_2^-$  –N/l, but final nitrite concentrations were usually as low as  $0.06-0.1 \text{ mgNO}_2^- - \text{N/l}$ . Contrary to that, as pointed out in Ref. [11], during denitrification of a similar original concentration of nitrate (250 mgNO<sub>3</sub><sup>-</sup>-N/l) with a pure culture of Paracoccus denitrificans, the level of accumulated nitrite was 181 mgNO $_{2}^{-}$  –N/l and than entirely diminished. In this study, accumulated nitrite was lower or close to the proposed targeted highest contaminant level of 1.0 mgNO<sub>2</sub><sup>-</sup>-N/l. The lower nitrite accumulation during present investigation may be attributed to the symbiotic activity of bacterium in mixed bacterial culture.

Table 2	
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Values of specific denitrification rates,	, $k_1$ , specific growth rates, $\mu$ and	d
growth yields, Y for the batch tests		

[X] (g/l)	$k_1 \text{ (mgNO_3-N/gVSS h)}$	$\mu$ (1/h)	$Y (mgVSS/mgNO_3-N)$
1.2	17.88	0.0085	0.61
1.6	19.26	0.0090	0.57
1.8	18.87	0.0120	0.58
2.3	22.66	0.0112	0.52
3.1	25.38	0.0011	0.48

# 3.2. The influence of methanol to nitrate-nitrogen ratio on the denitrification process

Due to the importance of carbon source for denitrification, the next run was performed in order to quantify the influence of methanol to nitrate-nitrogen ratio on denitrification. Methanol was selected as the most suitable external carbon source because it is the least expensive and very efficient in denitrification [9]. Seven different methanol to nitrate-nitrogen ratios were tested separately. The control was methanol-free. From the results obtained the original biomass concentration selected for further tests was 1.8 gVSS/l, i.e. similar to that of Lie and Welander [24] who, in their denitrification experiments, applied 2.3–2.8 gVSS/l. It can be seen from Fig. 3 that nitrate concentration continuously decreased for all methanol to nitrate-nitrogen ratios exceeding 2.5 mgCH<sub>3</sub>OH/mgNO<sub>3</sub><sup>-</sup>-N. At lower methanol to nitrate-nitrogen ratios (2.0 and 1.5 mgCH<sub>3</sub>OH/  $mgNO_3^- - N$ ) nitrate concentration decreased during the first 4 h to half of the original values and maintained that level throughout the tests. Comparing these results with the control test and taking into account that SW contained only methanol as carbon source for microbial growth, it was clear that complete denitrification required a methanol to nitrate-nitrogen ratio of 2.5 mgCH<sub>3</sub>OH/mgNO<sub>3</sub><sup>-</sup>-N. That result was in good correlation with literature values relating to complete denitrification [8]. Complete nitrate removal at MeOH/ NO<sub>3</sub>-N ratios of 3.5, 4.0, and 4.5 mgCH<sub>3</sub>OH/  $mgNO_3^-$  – N lasted for 4.5 h (Fig. 3a). As can be seen from these results, at MeOH/NO<sub>3</sub>–N ratios over 3.5, the time for complete nitrate removal did not shorten further, but remained constant. Therefore, it seemed that a MeOH/NO<sub>3</sub><sup>-</sup>-N ratio of 3.5 mgCH<sub>3</sub>OH/  $mgNO_3^- - N$  was more than sufficient for complete denitrification. Comparison of the time required for complete nitrate removal at MeOH/NO<sub>3</sub><sup>-</sup>-N ratios of 3.0 and 2.5 suggested that the stoichiometric value was 2.5 under the experimental conditions. This MeOH/  $NO_3^- - N$  ratio was in accordance with the theoretical value calculated from Eq. (6):

$$[CH_{3}OH] = 2.47 \cdot 200 + 1.53 \cdot 0.7 + 0.87 \cdot 5.70$$
  
= 500.03 mgCH\_{3}OH/1 (8)

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Fig. 3. Time course of nitrate removal (a) and accumulation of nitrite ions (b) during the batch tests at different methanol/nitrogen ratios.

For MeOH/NO<sub>3</sub><sup>-</sup> –N ratios higher than 2.5 values of  $k_1$  were between 18.59 and 21 mgNO<sub>3</sub><sup>-</sup> –N/gVSS h,  $\mu$  were in range of 0.013–0.0176 1/h and Y were around 0.6 gVSS/gNO<sub>3</sub><sup>-</sup> –N, which are in accordance with Refs. [3,8]. Similar value Y = 0.53 g VSS/gNO<sub>3</sub><sup>-</sup> –N was determined during one of fundamental denitrification studies [18]. McCarthy et al. [18] proposed stoichiometry of denitrification reaction in which the MeOH/NO<sub>3</sub><sup>-</sup> –N ratio was 2.47:

$$NO_{3}^{-} + 1.08CH_{3}OH + H^{+}$$
  

$$\rightarrow 0.065C_{5}H_{7}O_{2}N + 0.478N_{2} + 0.76CO_{2}$$
  

$$+ 2.44H_{2}O$$
(9)

Throughout denitrification tests (MeOH/NO<sub>3</sub><sup>-</sup>-N ratio in SW above 2.5) with the original biomass concentration of 1.8 gVSS/l, approximately  $61 \pm 1.5$  ml gaseous N<sub>2</sub> were produced. A stoichiometric coefficient of 0.463 was calculated from the obtained value of produced N<sub>2</sub> gas. This stoichiometric value of generated N<sub>2</sub>, corresponded to the value obtained from Eq. (9). With an assumption that stoichiometric correlation H<sup>+</sup>/

 $NO_3^- = 1$ , as given in fundamental denitrification equations [8,18], and based on investigation results that the optimum values of MeOH/NO<sub>3</sub><sup>-</sup>-N ratio and *Y* were 2.5 and 0.60 gVSS/gNO<sub>3</sub><sup>-</sup>-N, respectively, a similar stoichiometric equation of investigated denitrification process at pH 6.8 and 25 °C was determined:

$$NO_{3}^{-} + 1.09CH_{3}OH + H^{+}$$
  

$$\rightarrow 0.074C_{5}H_{7}O_{2}N + 0.463N_{2} + 0.72CO_{2}$$
  

$$+ 2.421H_{2}O$$
(10)

Nitrite and biomass concentrations in SW were monitored. Nitrite dropped gradually (Fig. 3b) during the first 2 h, then increased up to  $1.2 \text{ mgNO}_2^-$  -N/l and rapidly decreased to  $0.11-0.44 \text{ mgNO}_2^--N/l$ . At MeOH/NO<sub>3</sub><sup>-</sup> -N ratios over 2.5 mgCH<sub>3</sub>OH/mgNO<sub>3</sub><sup>-</sup> -N, nitrite removal was monitored over 2 h additionally, after which nitrite was completely diminished. Monitoring of nitrite concentrations (Fig. 3b) showed that the curves followed the typical pattern of biological denitrification: transient increase in nitrite concentrations (nitrite was produced by nitrate reduction) was subsequently followed by nitrite reduction. Nitrite accumulation and reduction were similar as in previous tests, but with MeOH/NO $_3^-$  –N ratios above 2.5 there were fewer nitrite ions at the end of the experiment. In the similar study of Martienssen and Schöps [12] nitrite accumulation was as high as 100 mgNO<sub>2</sub><sup>-</sup> -N/l, and nitrate removal (200 mgNO $_3^-$  –N/l), even with cells in logarithmic phase, lasted for 8 h. The fast nitrate removal, achieved in the present study might have been partially due to low nitrite accumulation during denitrification.

# 3.3. The denitrification process in the continuous-flow stirred reactor

In the view of the results obtained and literature data [8,9], further investigation of the denitrification process in the continuous-flow stirred reactor was performed at a MeOH/NO<sub>3</sub><sup>-</sup>-N ratio of 3.0, in order to avoid carbon-limited conditions. Denitrification in the continuous-flow stirred reactor was monitored in the first set of experiments at different dilution rates (0.0162, 0.0194, 0.0217, 0.0271, and 0.0355 1/h) in order to achieve the optimum dilution rate. Each test started as a batch and when nitrate was completely reduced (in  $\approx 4$ h), continuous flow of feed solution was started. SW used as feed solution was prepared daily and checked for nitrate-N and nitrite-N concentrations, COD and DO were also controlled. Nitrite-N was usually 0.02  $mgNO_2^- - N/l$  and DO concentration was between 5.40 and  $6.20 \text{ mgO}_2/l$ . In the first test set only at dilution rate of 0.0162 1/h nitrate ions were completely reduced in the reactor. A nitrate concentration of 30 mgNO<sub>3</sub><sup>-</sup> -N/l was recorded in the effluent at steady state, at D = 0.0217 1/h (Fig. 4a). Nitrite concentration during that test reached

Fig. 4. Denitrification in the continuous-flow stirred reactor at different dilution rates.

a maximum  $(0.7 \text{ mgNO}_2^- - \text{N/l})$  on the second day, and then nitrite ions in the effluent fell to as low as 0.1-0.3mgNO<sub>2</sub><sup>-</sup> -N/l. Biomass concentration (gVSS/l) was measured in effluent solution and in the reactor (at the midpoint of its height). It decreased from 1.88 to 0.26 g VSS/l, and after the fifth day reached steady state. A similar trend was observed at all tested dilution rates.

Generally, denitrification is an anaerobic or anoxic process; however a complete nitrate removal was reported even at a DO concentration of 5 mgO<sub>2</sub>/l [7,12]. In this study, DO diminished from 5.50 to 0.40 mgO<sub>2</sub>/l during the first 4 h. During 3 days of continuous flow, the DO increased to 2.5 mgO<sub>2</sub>/l and remained at that level (Fig. 4b). The original COD value of 850 mgO<sub>2</sub>/l at D = 0.0217 1/h fell to 180 mg O<sub>2</sub>/l. Generally, at all examined dilution rates, the COD values at steady state were in the range 80 to 300 mgO<sub>2</sub>/l. During 7 days of the first set of experiments at different dilution rates specific denitrification rates were usually increased from 2 to 50 mgNO<sub>3</sub><sup>-</sup> -N/gVSS h (48–1200 mgNO<sub>3</sub><sup>-</sup> -N/gVSS day).

In the second test set denitrification started at the lowest dilution rate (D = 0.0162 1/h) because in previous tests it was observed that at higher dilution rates the effluent contained nitrate. During 15 days of continuous

Fig. 5. Time course of denitrification in the continuous-flow stirred reactor.

flow there was no trace of nitrate (Fig. 5a). Specific denitrification rate was  $2-58 \text{ mgNO}_3^- - \text{N/gVSS}$  h. The biomass concentration in SW determined during denitrification in the continuous-flow stirred reactor decreased, as in previous tests, from the original value of 1.88–0.5 gVSS/l, but after the fourth day a steady state was established. After 7 days of denitrification in SW, biomass flocks were formed. In similar denitrification tests, Yoo et al. [25] showed a relatively rapid fall of the original MLSS concentration in the intermittently aerated reactor at HRT of 25 h. Nevertheless, their results and the results obtained in the present study show that such a fall did not affect COD and nitrate removal. The numbers of bacterial colonies (CFU), grown on the standard nutrient agar and on the hardened feed solution, were compared in order to determine the presence of true denitrifiers. The same CFU values were found by each method. With respect to this, it was concluded that the mixed bacterial culture contained true denitrifying bacteria. The bacteria count during these tests decreased from 10<sup>9</sup> to 10<sup>8</sup> CFU/ml. The recorded plate count in SW corresponded to the values reported from similar denitrification tests [2,12]. Since the biomass concentration significantly decreased compared to the CFU/ml, it was assumed that the formation of stable biomass flocks was responsible for the increase of specific denitrification rates. The DO

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concentration fell from 5.50 to 0.40 mgO<sub>2</sub>/l, as mentioned before, and after some fluctuations during the first 7 days increased to 2.5 mgO<sub>2</sub>/l and remained at that level (Fig. 5b). That unexpected decrease and increase of DO during the first 7 days may be explained with fluctuations in biomass concentrations, formation of biomass flocks and consumption of O<sub>2</sub> by bacterial cells. The results obtained confirmed that complete and rapid denitrification could be achieved even at that DO concentration.

A higher dilution rate was then applied to investigate the biomass flock activity. From day 15 to 25, the dilution rate was increased to 0.0194 1/h and nitrate ions present in feed solution were reduced completely even at the midpoint of reactor height. Subsequently, the dilution rate was elevated to 0.0217 1/h for a further 10 days. But even at this time nitrate ions were not found in the effluent, as had been the case in the previous test performed with the same dilution rate, where nitrate concentration was 30 mgNO<sub>3</sub><sup>-</sup> -N/l. Apparently, a slow increase of the feed influent, accomplished with flock formation, influenced the bacterial biomass increasing its activity. Consequently, specific denitrification rates increased again, from 58 to 150 mgNO<sub>3</sub><sup>-</sup> –N/gVSS h. Finally, the dilution rate on day 35 was elevated to 0.0355 1/h. Nitrate concentrations in the effluent were  $1-3 \text{ mgNO}_3^- - \text{N/l}$  (Fig. 5A). Nitrite ions unexpectedly increased to 6 mgNO $_2^-$  –N/l, then fell and accumulated until steady state establishment at 6 mgNO $_2^-$  –N/l. COD value (Fig. 5B) and nitrate concentrations in the influent gave an organic carbon loading rate of 17-22 mgO<sub>2</sub>/l h and a nitratenitrogen loading rate of  $3.11-7.10 \text{ mgNO}_3^--\text{N/l}\text{ h}$ . Specific denitrification rates calculated from the experimental results at different dilution rates were in the high range, from 24 to 300 mgNO<sub>3</sub><sup>-</sup>-N/gVSS h. That unexpectedly high range is partially due to decrease of biomass concentration and a high stability of flocks. The results were comparable with the values reported in Ref. [19,22] and higher than those reported by Bernet et al. [1] or Lie and Welander [24]. Finally, in this study complete denitrification with low nitrite accumulation and 90% removal of organic load was achieved.

#### 4. Conclusions

This work investigates nitrate removal from SW in a batch denitrifying reactor and in a continuous-flow stirred reactor by the mixed bacterial culture. In the culture with the dominant *Paracoccus* sp. and *Pseudomonas stutzeri*, a high denitrification rate was achieved. Complete denitrification (200 mgNO<sub>3</sub><sup>-</sup> –N/l) with low accumulation of nitrite (up to 1.2 mgNO<sub>2</sub><sup>-</sup> –N/l) was found during approximately 6 h for the MeOH/NO<sub>3</sub><sup>-</sup> – N ratio above 2.5 in SW. At the end of the tests the

nitrite concentrations were 0.1 mgNO<sub>2</sub><sup>-</sup> –N/l. A kinetic analysis was developed to determine dependence of denitrification rate on biomass (first-order) and nitrate (zero-order) levels. The specific denitrification rates  $k_1$  were from 17.9 to 25.4 mgNO<sub>3</sub><sup>-</sup> –N/gVSS h at 25 °C.

In the continuous denitrification process during 45 days specific denitrification rates increased to 250 mgNO<sub>3</sub><sup>-</sup> –N/gVSS h, while HRT decreased from 62 to 28 h. The DO concentration fell from 5.50 to 0.40 mg O<sub>2</sub>/l during the first 4 h, but over 3 days of continuous flow it increased to 2.5 mgO<sub>2</sub>/l and remained at that level. Accumulation of nitrite ions in SW was similar as in the batch tests, but at HRT 28 h the nitrite concentration increased to 6 mgNO<sub>2</sub><sup>-</sup> –N/l. Complete denitrification at 25 °C was achieved at nitrate and methanol loading rates of 4.35 mgNO<sub>3</sub><sup>-</sup> –N/l h and 23 mgO<sub>2</sub>/l h, respectively, with specific denitrification rates mostly in the range from 100 to 200 mgNO<sub>3</sub><sup>-</sup> –N/ gVSS h.

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