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High nitrate removal from synthetic wastewater with the mixed bacterial culture

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Abstract

The applicability of the mixed bacterial culture, originated from two-stage anaerobic–aerobic industrial yeasts production wastewater treatment plant for high rate denitrification processes was investigated. After acclimation to nitrate, the dominant strains were *Pseudomonas* and *Paracoccus* sp. Complete denitrification with low accumulation of nitrite-N (0.1 mg/l) was found in synthetic wastewater, obeying a zero-order reaction with respect to nitrate and a first-order reaction with respect to biomass concentration. Denitrification was then monitored in the continuous-flow stirred reactor at different hydraulic retention time, HRT (62–28 h) in order to achieve the optimal HRT. Nitrate was completely removed during following 45 days, at 25 °C with HRT, which we reduced from 62 to 28 h. Yet still, at 28 h HRT, high average specific denitrification rate of 142 mg NO_3^--N/g VSS h was obtained.

Keywords: Continuous flow denitrifying reactor; Denitrification; Kinetic model; Mixed bacterial culture; Nitrite accumulation

1. Introduction

Nitrogen-containing compounds released into environment can create serious problems, such as eutrophication of rivers, deterioration of water quality and potential hazard to human health, because nitrate in the gastrointestinal tract can be reduced to nitrite ions. In addition, nitrate and nitrite have the potential to form N-nitrous compounds, which are potent carcinogens (Forman, 1991). Biological removal of nitrate is widely used in the treatment of domestic and complex industrial wastewaters (Delanghe et al., 1994; Lemmer et al., 1997; Sozen and Orhon, 1999; Kesserü et al., 2003; Dong and Tollner, 2003). Biological denitrification enables transformation of oxidized nitrogen compounds by a wide spectrum of heterotrophic bacteria into harmless nitrogen gas with the accompanying carbon removal. Based on its price and availability, methanol is most commonly used as additional carbon source for bacterial denitrification (Wang et al., 1995). This process has been well studied, but biological denitrification of wastewater is usually slow and lasts several days. Thus, a high rate denitrification process is needed. Great efforts were made recently to slightly increase nitrate removal rate. In that respect, the adapted mixed bacterial cultures from various industrial wastewater treatment plants proved to be very useful. According to literature,

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(Almeida et al., 1995a; Zayed and Winter, 1998; Peyton et al., 2001) different industrial wastewaters contain more than 200 mg NO_3^--N/l, and their biological denitrification usually takes a few days. Thus, Zayed and Winter (1998) studied nitrate removal from dairy wastewater: with the use of activated sludge, 250 mg NO_3^--N/l were removed within three days. The immobilized mixed culture facilitated nitrate conversion, so that after two days there was no nitrate i.e. nitrite left. However, in the tests with the suspended pure culture eight days were required to convert complete nitrate-N to nitrogen gas. This example illustrates that adaptation of mixed bacterial cultures from an industrial wastewater treatment plant, optimisation of pH and temperature and selection of carbon source may enable rapid denitrification (Glass and Silverstein, 1998).

In general, nitrite production is known to be one of the main problems in biological denitrification, because nitrite ions are inhibitors of bacterial growth (Almeida et al., 1995b). At the same time, complete nitrate removal may be prolonged by nitrites present or accumulated during the biological denitrification process. As pointed out in literature, (Blasczyk, 1993) the denitrification with methanol (up to 250 mg NO_3^--N/l) by *Paracoccus denitrificans* lasted nine days, the level of accumulated nitrite was 180 mg NO_2^--N/l and then it was completely reduced. In the same study, biological denitrification in the presence of nutrient broth lasted 12 h, and no nitrite accumulation was observed. Furthermore, Martienssen and Schöps (1999) reported that *Staphylococcus* sp. present in mixed culture caused significant accumulation of nitrite and inhibited the denitrification process. On the contrary, the presence of *Pseudomonas stutzeri* in mixed culture seems to enable complete and fast denitrification with no nitrite accumulation (Lazarova et al., 1994).

The aim of the present paper was to investigate the applicability of the mixed microbial cultures of an industrial yeast production wastewater treatment plant, for high rate denitrification processes. The active sludge of this plant comprises microorganisms acclimated to nitrates, and variety of other substances. Therefore, biomass prepared from the active sludges of this wastewater treatment plant was used for investigations of the kinetics of the biological denitrification process. Attempts were made to optimise the temperature, pH values and methanol to nitrate ratio to achieve as rapid nitrate removal as possible, without nitrite accumulation, and to improve economical effectiveness of the process. The denitrification of synthetic wastewater was investigated in a batch and in the continuous-flow stirred reactor. To investigate denitrification in the continuous-flow stirred reactor the selected MeOH/NO_3^--N value was applied and a simplified kinetic analysis was performed for quantitative comparison of nitrate consumption and biomass production rates.

2. Methods

2.1. Organisms and culture media

The microorganisms originated from the active sludge of the two-stage anaerobic–aerobic wastewater treatment plant of the Pharmaceutical industry "Pliva" in Savski Marof, Croatia, treating high-loaded wastewaters of the yeasts production (total influent nitrogen was usually 1 g N/l). Sludge (from 50 ml samples) from both stages of this plant were mixed and centrifuged at 12,557*g* and 5 °C for 10 min. The obtained biomass was washed twice, diluted with mineral medium, refrigerated at 4 °C and kept for further use. The mixed bacterial culture was acclimated to nitrate ions (100–500 mg NO_3^--N/l) at pH = 6.8 and 35 °C under anoxic conditions. After every experiment the biomass suspension was prepared as described and used as inoculum in the next experiment.

Composition of the medium was slightly different from the originally proposed by Wang et al. (1995). Mineral medium contained (g/l): K_2HPO_4 2.5; KH_2PO_4 1; $MgSO_4 \cdot 7H_2O$ 0.1; $CaCl_2 \cdot 2H_2O$ 0.17; NaCl 5 and deionised water to 1 l. In the batch tests performed at different pH, mineral medium was prepared with $NaH_2PO_4 \cdot 2H_2O$ rather than with KH_2PO_4. Different ratios of two phosphate salts were used to achieve the desired pH of mineral medium. Phosphate solutions were then autoclaved and allowed to cool to room temperature before the addition of 0.1 ml of trace metal mixture (the aqueous solution containing 5 g of each metal salt/l: $MnSO_4$, $CuSO_4$, $FeCl_3$ and Na_2MoO_4). That mineral medium was used to prepare synthetic wastewater (SW). For each experiment nitrate from 100 to 500 mg NO_3^--N/l (stock solution was aqueous solution of $NaNO_3$ containing nitrate 10 g NO_3^--N/l), 2 moles of methanol per mole of nitrate and yeast extract 0.1 g/l were added separately. Yeast extract was necessary because vitamin and co-factor requirements of the microbial culture were unknown. Phosphate salts in the mineral medium were used as buffer. Thus, pH in SW remained unchanged throughout the experiments.

2.2. Experimental set-up

The experiments were performed in 0.5 l closed serum bottles. Each clean sterile serum bottle was filled with 0.3 l of SW and 0.1 l of biomass suspension. The stopper was punctured with a thermometer and two disposable syringes with needles, one to measure the produced gas and the other one for sampling. During denitrification tests, the inoculated bottles entirely immersed in a water bath at constant temperature were placed on the magnetic stirrer. All experiments were conducted under anoxic conditions (the headspace gas contained O_2 initially, but this would rapidly have been consumed by

bacteria) at 400 rpm. In the first part, the effect of various nitrate concentrations (100–500 mg NO_3^--N/l) at 35 °C and pH = 6.8 was examined. In the second run, the influence of temperature and pH on biological denitrification was studied. For that purpose, the bottles with SW (200 mg NO_3^--N/l and pH = 6.8) were incubated at 15 °C, 20 °C, 25 °C, 30 °C and 35 °C. To determine the influence of pH on denitrification (200 mg NO_3^--N/l), batch tests were performed at different pH (5.9, 6.8, 7.4, 7.9 and 8.4) and at 25 ± 0.5 °C under anoxic conditions. SW was adjusted to desired pH by changing the ratio of phosphate salts (K_2HPO_4 and $NaH_2PO_4 \cdot 2H_2O$) in mineral medium.

Continuous tests were carried out in the bioreactor 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, synthetic wastewater was pumped with a peristaltic pump at different flow rates into the reactor to give different hydraulic retention times (HRT). Incubation was conducted at 25 ± 2 °C, pH 6.8 and an agitation speed of 400 rpm under anoxic conditions. Effluent wastewater and produced gas were collected in the fluid and gas collector. In order to determine nitrogen gaseous compounds the gas collector contained saturated KOH solution for CO_2 absorption. 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, the samples were taken from the bottle at the predetermined time and processed immediately. All the experiments were repeated (performed twofold) and data reported here represent the average values. Volatile suspended solids, nitrate and nitrite concentrations measured during duplicate measurements differed by 0.2–5 mg/l. Cell numbers determined by plate count differed by less than 5%.

2.3. Analytical methods

Liquid samples were centrifuged at 5 °C and 12,557*g* (Sigma 3K15, Osterode, Germany). Thus, obtained supernatant was used for nitrate and nitrite analysis. Nitrate concentration in SW during the course of the experiment was monitored spectrophotometrically on Varian DMS 80 (Varian, Mulgrave, Australia) by chromotropic acid method at $\lambda = 400$ nm (American Public Health Association, 1989). Nitrite detection (Höll, 1979) was determined by absorbance measurements at $\lambda = 500$ nm on photometer (MA 9510—Iskra, Kranj, Slovenia). Concentration of dissolved oxygen and pH of SW were regularly monitored by oxygenmeter MA 5485 and pH-meter MA 5750 (Metrel, Horjul, Slovenia).

Cell numbers were determined by plate count on the standard nutrient broth after repeated dilution with NaCl (m/V ratio = 9 g/l). Different denitrifiers in the mixed bacterial culture were distinguished according to their colony forms and by optical microscopy (Olympus BX 50 F4, Olympus optical Co., Japan) 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 (Holt et al., 1994). Volatile suspended solid (VSS) and sludge volume index (SVI) was determined according to Standard Methods (American Public Health Association, 1989).

2.4. Denitrification kinetic analysis

A general kinetic model of nitrate removal is given:

$$dC_N/dt = -kC_N^n C_X^m \quad (1)$$

where t is time in hours, C_X and m are biomass concentration (mg VSS/l) and its partial reaction order, C_N and n are nitrate concentration (mg NO_3^--N/l) and its partial reaction order, and k is the specific denitrification rate with a unit depending on the values of m and n ($mg^{(1-n)}l^{(m+n-1)}$/mg VSS^mh).

Nitrate concentration depending on the time of partial reaction order (n) is given by

$$\left(\frac{C_N}{C_{N0}}\right)^{(1-n)} = 1 - \frac{(1-n)kC_X}{C_{N0}^{(1-n)}}t \quad (n \neq 1) \quad (2)$$

and

$$\frac{C_N}{C_{N0}} = \exp(-kC_X t) \quad (n = 1) \quad (3)$$

Rate constant is also determined by the widely applied Monod equation (Casey, 1997; Li et al., 2001; Sarioglu and Horan, 2001), which we employed for calculating kinetic constants using data from batch experiments:

$$r_D = \frac{dC_N}{dt} = \frac{\mu_{max} \cdot C_N}{(K_N + C_N)} \cdot \frac{C_X}{Y} = \frac{k_D \cdot C_N}{(K_N + C_N)} \quad (4)$$

where r_D is the rate of nitrate utilization (mg NO_3^--N/l h), C_N is the nitrate concentration (mg NO_3^--N/l), C_X is the biomass concentration (mg VSS/l), μ_{max} is the maximum specific growth rate (1/h), K_N is the half velocity constant (mg NO_3^--N/l), Y is the growth yield (mg VSS/mg NO_3^--N), and K_D is the maximum rate of nitrate utilization (mg NO_3^--N/l h), which include the influence of microbial concentration, maximum specific growth rate and growth yield. The growth yield, Y is usually considered as a constant during modelling the process but it can vary, according to reaction conditions.

If $K_N \ll C_N$ in Eq. (4), K_N is insignificant compared to C_N, and the Monod equation turns to a zero-order reaction model (Timmermans and Van Haute, 1983; Beaubien et al., 1995; Li et al., 2001). The kinetic

parameters of the Monod equation were determined using the Nelder–Mead simplex method of non-linear parameter search incorporated in Micromath Scientist program. The initial guess of the kinetic parameter is entered into the program. Using this set of parameters the response curves are generated by the Runge–Kutta numerical integration method. Once the optimal kinetic parameters were established, the final optimal theoretical curve was compared with the experimental data plot. In order to determine the Monod equation constants, μ_{max} and k_N, the specific growth rate constant, μ (1/h) and growth yield, $Y_{VSS/nitrate\text{-}N}$ (mg VSS/mg NO_3^--N) were previously calculated. Specific growth rates, μ in this work were determined at different original biomass concentrations in the batch reactors at 25 °C by measuring biomass concentrations at regular time intervals and by plotting $\ln(C_{Xt}/C_{X0})$ versus time.

Finally, the rate of nitrate conversion was calculated as the first-order overall function of nitrate (zero-order, $n = 0$) and biomass concentration (first-order, $m = 1$):

$$\frac{dC_N}{dt} = -k_{den} C_N^0 C_X \quad (5)$$

and its integration form,

$$C_N - C_{N0} = -k_{den} C_X t \quad (6)$$

where k_{den} is the specific denitrification rate, mg NO_3^--N/g VSS h.

Denitrification rates increase with temperature, depending on activation energy of the reaction, as given by Arrhenius equation:

$$k_{den} = k_0 \exp\left(-\frac{E}{RT}\right) \quad (7)$$

where k_0 is frequency factor and has the same unit as k_{den}, E is activation energy (J/mol), R is gas constant (8.314 J/mol K), and T is temperature (K). The overall relationship between nitrate concentration, temperature and biomass concentration can be expressed as

$$\frac{(C_{N0} - C_N)}{t} = C_X k_0 \exp\left(-\frac{E}{RT}\right) \quad (8)$$

or,

$$\ln\left\{\frac{(C_{N0} - C_N)}{t}\right\} = \ln(C_X k_0) - \frac{E}{RT} \quad (9)$$

where C_{N0} and C_N are the initial and instant nitrate concentrations (mg NO_3^--N/l) at time t.

Generally, the obtained activation energy, E for biological denitrification was in activation energy range of enzyme-catalysed reactions, which were usually 16–84 kJ/mol and most commonly 46 kJ/mol (Shuler and Kargi, 1992).

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

$$\text{Organic load} = \frac{[\text{COD}]_{in} \cdot R}{V} \quad (\text{mg } O_2/\text{l h})$$

Nitrate-nitrogen load

$$= \frac{\lfloor C_{NO_3^-\text{-N}} \rfloor_{in} \cdot R}{V} \quad (\text{mg } NO_3^-\text{-N/l h})$$

Volumetric denitrification rate

$$= \frac{\{[C_{NO_3^-\text{-N}}]_{in} - [C_{NO_3^-\text{-N}}]_{effl}\} \cdot R}{V} \quad (\text{mg } NO_3^-\text{-N/l h})$$

Specific denitrification rate

$$= \frac{\text{volumetric rate}}{C_X} \quad (\text{mg } NO_3^-\text{-N/g VSS h})$$

where $[C_{NO_3^-\text{-N}}]_{in}$, $[C_{NO_3^-\text{-N}}]_{effl}$ and $[\text{COD}]_{in}$ represented the influent, effluent nitrate nitrogen concentrations (mg NO_3^--N/l) and influent COD (mg O_2/l). C_X is the biomass concentration (g VSS/l), R the influent wastewater flow rate (ml/h) and V the reactor volume (l). Dilution rate, D (l/h) and hydraulic retention time, HRT (h) are calculated from the flow rate values and the reactor volume.

3. Results and discussion

3.1. Effect of nitrate concentration on biological denitrification

The nitrate concentration as a function of time was shown in Fig. 1. The initial concentration of NO_3^--N in synthetic wastewater was increased stepwise from

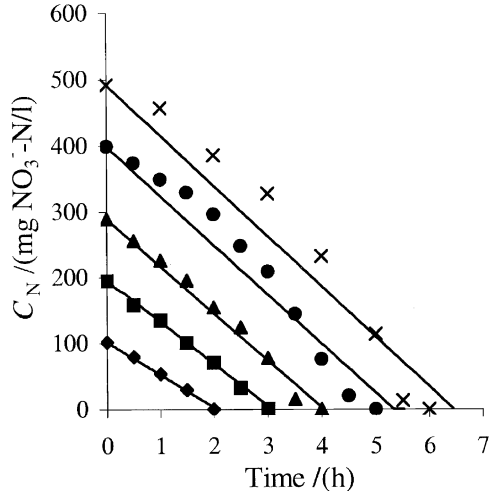

Fig. 1. Nitrate-N removal during investigation of nitrate removal at pH = 6.8 and 35 °C under anoxic conditions at different initial NO_3^--N concentrations: 100 mg/l (♦), 200 mg/l (■), 300 mg/l (▲), 400 mg/l, (●), 500 mg/l (×); nitrate concentrations was simulated according to Monod model, Eq. (4) (—).

100 mg NO_3^--N/l to 500 mg NO_3^--N/l. The reductions of nitrate ions were modelled according to the Monod Eq. (4) and the model acceptably predicted the nitrate concentration decrease. As shown in Fig. 1, for the initial nitrate concentrations of 400 and 500 mg NO_3^--N/l, the predicted nitrate removal based on the Monod model is a curve rather than a straight line. This indicates that the employed model is incomplete for nitrate concentrations above 400 mg NO_3^--N/l. Furthermore, it is clear that denitrification is a complex process, and that validity and accuracy of simplified assumptions leads to some disagreement with the kinetic model. Monod model constants, μ_{max} and K_N were 0.02 l/h and 0.003 mg NO_3^--N/l. Since K_N was very small in comparison to C_N in Eq. (4), the Monod model equation turns to zero-order reaction model. The initial biomass concentration was 1.8 g VSS/l and it was observed that nitrate removal was followed by slow increase of bacterial biomass. By counting cell number per millilitre, it was established that the initial biomass concentration of 1.84 g VSS/l was related to 1.8×10^9 CFU/ml. Cell number usually increased to 2.1×10^9 CFU/ml after 3 h of SW denitrification.

Furthermore, the mixed bacterial culture was capable of complete removal of 100–500 mg NO_3^--N/l from synthetic wastewater during 2–6 h. The produced nitrite-N during the experiments was between 0.30 and 1.70 mg NO_2^--N/l, but terminal nitrite ions were as low as to 0.11 mg NO_2^--N/l. Monitoring of nitrite concentrations showed that the nitrite concentration points followed the typical pattern of biological denitrification: transient increase in nitrite concentrations (nitrite was produced by nitrate reduction) was subsequently followed by nitrite reduction. The results suggest that nitrite production was insignificant and that denitrification was not inhibited by nitrite ions. In order to achieve a fast denitrification without nitrite accumulation, selected mixed culture originating from an industrial wastewater treatment plant seemed to be more advantageous over pure culture (Blasczyk, 1993) or other referred mixed cultures (Glass and Silverstein, 1998; Martienssen and Schöps, 1999).

The mixed culture used for investigation of nitrate removal, comprised two dominant bacterial strains identified as *Pseudomonas stutzeri* and *Paracoccus* sp.; other identified species were *Xanthomonas malthophilia*, *Ochrobactrum antrophi*, *Aeromonas salmonicida achromogenes* and *Staphylococcus* sp. Among isolated bacteria *Paracoccus* sp. and *Pseudomonas stutzeri* were reported as true denitrifiers, so their dominant presence in mixed culture obviously accomplished a complete and rapid nitrate removal with the minimum nitrite accumulation. Furthermore, *Pseudomonas* strain was also present in mixed cultures used for denitrification of wastewaters (Almeida et al., 1995b; Wang et al., 1995). 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 high accumulation of nitrite ions as previously mentioned (Martienssen and Schöps, 1999).

3.2. Temperature and pH effect on the denitrification process

Nitrate removal from SW (200 mg NO_3^--N/l) at 15 °C, was very slow and lasted for 18 h (Fig. 2a). At the same time, during the first 3 h 32.5% and 50.8% of nitrate were removed from SW at 20 and 25 °C, respectively. Complete denitrification was achieved in 9 and 5.5 h, respectively. At 25 and 30 °C there was no significant difference. As shown in Fig. 2a, at 35 °C complete denitrification was achieved in 3 h, which was very fast compared to other tested temperatures. Although denitrification at 25 °C was slower than at 35 °C, for economical reasons the former temperature can be proposed as the operating temperature. The simulated curves in

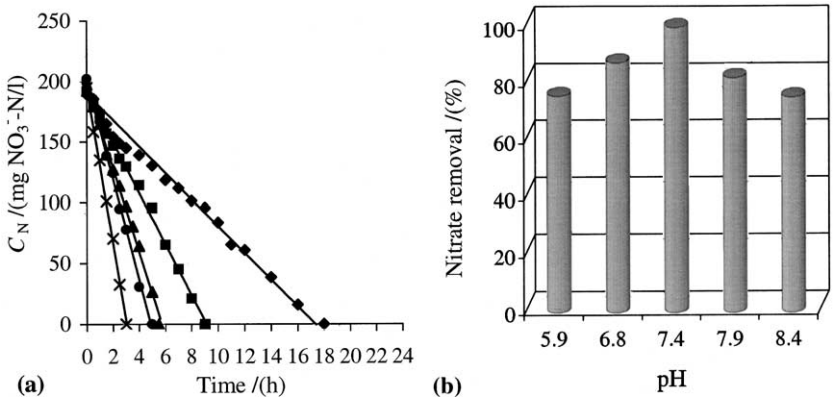

Fig. 2. Removal of 200 mg/l NO_3^--N during the denitrification process at pH = 6.8 at different incubation temperatures: 15 °C (♦), 20 °C (■), 25 °C (▲), 30 °C (●), 35 °C (×); (a) nitrate concentrations simulated according to Monod model, Eq. (4) (—) and (b) nitrate-N removal (200 mg/l) at 25 °C under anoxic conditions at different pH values.

Fig. 2a show that the Monod model acceptably predicts the nitrate reduction.

Optimal pH for denitrification is usually within neutral range (Wang et al., 1995; Casey, 1997). Nitrate removal at investigated pH levels during 5.5 h was in the range of 75–100%. As presented in Fig. 2b, SW denitrification was the fastest at pH = 7.4. The decrease of the nitrate removal rate to 75% was observed at the lowest and at the highest examined pH value of SW. The specific denitrification rate (k_{den}) was found to be a function of pH-value (Timmermans and Van Haute, 1983). In comparison to experimental points, a good agreement between calculated and the experimental values were observed. However, change of pH in the investigated range did not cause significant change of k_{den}, as cited in literature (Timmermans and Van Haute, 1983; Lemmer et al., 1997). It was revealed that acclimation of mixed culture originated from an industrial wastewater treatment plant was responsible for achieving rapid denitrification even at different pH values in range from 5.9 to 8.4, as reported in similar observation (Beaubien et al., 1995).

During these investigations, the nitrite concentration in the SW was monitored and results were similar to previous results. Nitrite accumulation was lower or close to the proposed targeted highest contaminant level of 1.0 mg NO_2^--N/l. Nitrite accumulation of up to 1.7 mg NO_2^--N/l during the first 3 h was observed, but by the end of the experiments it was almost entirely diminished. Comparison of results shows insignificant effect of nitrite ions on nitrate reduction. Nevertheless, nitrite production was lower at pH = 6.8 (up to 0.6 mg NO_2^--N/l), which was chosen as optimal parameter for further investigation.

3.3. Denitrification kinetic analysis

Kinetic analysis of obtained data was conducted and a high degree linear relationship ($R^2 > 0.99$) between nitrate concentrations versus time as shown in Fig. 3, was observed, confirming a zero-order reaction ($n = 0$) with respect to nitrate concentration (Timmermans and Van Haute, 1983; Wang et al., 1995; Casey, 1997). From the slope of the linear line in Fig. 3 with the initial biomass concentration (C_{X0} = 1.84 g VSS/l), Eq. (6) gave the specific denitrification rate, k_{den} = 18.7 mg NO_3^--N/g VSS h at 25 °C. The obtained value was in the range from 17.0 to 32.0 mg NO_3^--N/g VSS h, as was reported for different wastewaters (Timmermans and Van Haute, 1983; Sozen and Orhon, 1999).

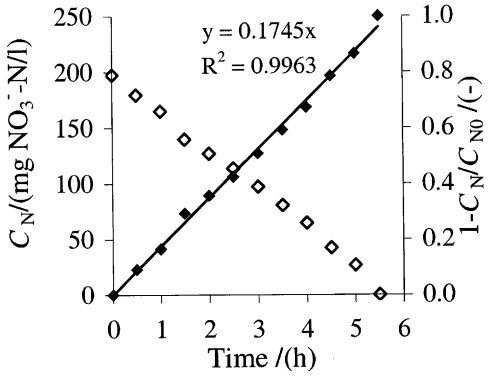

Fig. 3. Nitrate concentrations (◊) and ($1 - C_N/C_{N0}$) versus time (♦); data obtained from tests conducted at 25 °C and pH = 6.8 under anoxic conditions and linear regression (—).

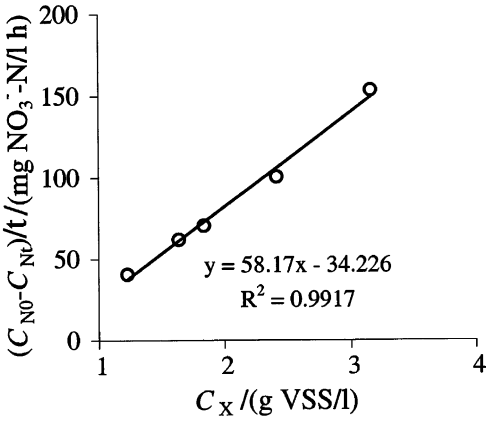

Fig. 4. Nitrate removal from SW at different biomass concentration, 25 °C and, at pH = 6.8 under anoxic conditions: Experimental results (○) and linear regression (—).

The determination of the reaction order (m) with respect to biomass concentration was performed by conducting denitrification experiments with different initial biomass concentration (C_{X0}) and by rearranging Eq. (3) in the following form:

$$(C_{N0} - C_N)/t = k_{den} C_X^m \quad (10)$$

The left side of Eq. (10) can be measured experimentally and plotted against biomass concentration as shown in Fig. 4. Linearity ($R^2 > 0.99$) between $k_{den} \cdot C_X^m$ and C_X indicated that m equalled one. Therefore, the kinetic model of SW denitrification was a first-order overall reaction.

The left-hand side of Eq. (9) was measured and plotted against the reciprocal of temperature as shown in Fig. 5a. High degree linearity ($R^2 > 0.99$) is known to provide a reliable estimate of the activation energy (E) and frequency factor (k_0). Activation energy for nitrate reduction was 50.1 kJ/mol and k_0 was 1.1×10^{10} mg NO_3^--N/g VSS h. Accordingly, this E value is in very good agreement with the findings of Wang et al. (1995), who reported activation energy value of 58.2 kJ/mol obtained at pH = 7.1. Specific denitrification rate was calculated using Monod equation (4), and was found to be a temperature function, as shown in Fig. 5b. Furthermore, temperature coefficient $Q_{10} = k_{den(T+10)}/k_{denT}$ was 2.3, indicating that specific denitrification rate

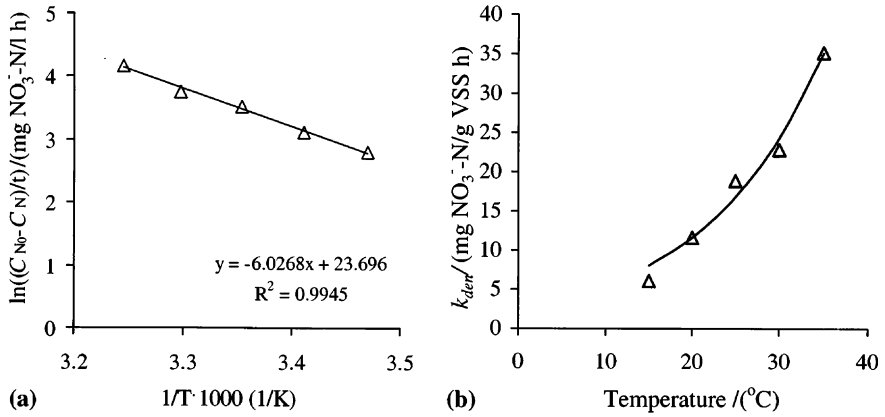

Fig. 5. The estimation of activation energy (a) and (b) effect of temperature on the specific denitrification rate: experimental results (△) and linear regression (—).

went down with temperature decrease of 10 °C by the factor 2.3 (Delanghe et al., 1994). Conversely, higher values (Q_{10} = 3.3) for denitrification with methanol were obtained at pH = 9 (Timmermans and Van Haute, 1983). Comparison of results obtained during present study and above mentioned indicated that the present process was less sensitive to temperature changes, which may be due to the use of acclimated mixed culture from an industrial wastewater treatment plant.

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

Methanol was selected as the most suitable external carbon source because it is the least expensive and very efficient in denitrification (Purtschert and Gujer, 1999). In order to quantify the influence of methanol to nitrate-nitrogen ratio on denitrification seven different methanol to nitrate-nitrogen ratios were tested separately. The control was methanol-free. It can be seen from Fig. 6 that nitrate removal continuously increased for all methanol to nitrate-nitrogen ratios (calculated on a mass basis) exceeding 2.5 mg CH_3OH/mg NO_3^--N. At lower methanol to nitrate-nitrogen ratios (2.0 and 1.5 mg CH_3OH/mg NO_3^--N) nitrate removal was 42% and 38%, respectively. At 2.5 mg CH_3OH/mg NO_3^--N complete denitrification was achieved during 6 h. Total nitrate removal at MeOH/NO_3-N ratios of 3.5, 4.0 and 4.5 mg CH_3OH/mg NO_3^--N lasted for 4.5 h. Results obtained at MeOH/NO_3-N ratios over 3.5 indicated that the time for complete nitrate removal remained constant. Therefore, it seemed that MeOH/NO_3^--N ratio of 3.5 mg CH_3OH/mg NO_3^--N was more than sufficient for complete denitrification. Comparison of the time required for complete nitrate removal at MeOH/NO_3^--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 equation proposed by McCarthy et al. (1969):

$$[C_{CH_3OH}] = 2.47 \times [C_{NO_3^-\text{-N}}] + 1.53 \times [C_{NO_2^-\text{-N}}] + 0.87 \times [C_{DO}]$$
$$[C_{CH_3OH}] = 2.47 \times 200 + 1.53 \times 0.7 + 0.87 \times 5.70 = 500.03 \text{ mg } CH_3OH/l \quad (11)$$

For MeOH/NO_3^--N ratio of 3.5, the maximum value of k_{den} = 21 mg NO_3^--N/g VSS h was found (Fig. 6). Specific denitrification rates varied with different methanol concentrations. The values of k_{den} increased with MeOH/NO_3^--N ratios below 3.5 and decreased at the ratios above 3.5. The obtained data suggest that the model is somewhat incomplete and that the kinetics is much more complex.

Specific growth rate, μ was within the range of 0.0130–0.0176 l/h and Y was around 0.60 g VSS/g NO_3^--N, which was in accordance with literature (Timmermans and Van Haute, 1983; Wang et al., 1995). A similar value, Y = 0.53 g VSS/g NO_3^--N was determined during one of the fundamental denitrification studies (McCarthy et al., 1969).

Throughout denitrification tests with the original biomass concentration of 1.8 g VSS/l, approximately

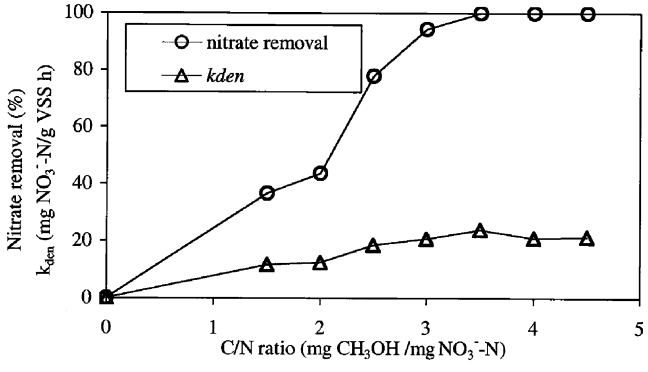

Fig. 6. Nitrate removal (○) from SW and the specific denitrification rates, k_{den} (△) at different methanol/nitrogen ratios at 25 °C and, at pH = 6.8 under anoxic conditions.

$61 \pm 1.5\,cm^3$ gaseous N_2 were produced. Stoichiometric coefficient of 0.463 was calculated from the obtained value of produced N_2 gas. Stoichiometric ratios were calculated on a mass basis. The optimum values of $MeOH/NO_3^-$-N ratio and Y were 2.5 and 0.60 g VSS/g NO_3^--N, respectively. Based on that results and with an assumption that stoichiometric correlation $H^+/NO_3^- = 1$, as was given in fundamental denitrification equations (McCarthy et al., 1969; Timmermans and Van Haute, 1983), a stoichiometric equation of investigated denitrification process at pH 6.8 and 25 °C was determined:

$$NO_3^- + 1.09CH_3OH + H^+ \rightarrow 0.074C_5H_7O_2N + 0.463N_2 + 0.72CO_2 + 2.421H_2O \quad (12)$$

3.5. The denitrification process in the continuous-flow stirred reactor

In the view of the obtained results and literature data (Timmermans and Van Haute, 1983; Purtschert and Gujer, 1999), further investigation of denitrification process in the continuous-flow stirred reactor was performed at $MeOH/NO_3^-$-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 HRT (61.7, 51.6, 46.1, 36.9 and 28.2 h) in order to achieve the optimum HRT. Each test started as a batch and when nitrate was completely reduced (in approximately 4 h), continuous flow of feed solution was started. Synthetic wastewater used as feed solution was prepared daily and checked for nitrate-N, nitrite-N concentrations, COD and dissolved oxygen concentration (DO). Nitrite-N was usually 0.02 mg NO_2^--N/l and DO was between 5.40 and 6.20 mg O_2/l. In the first test set only at HRT of 61.7 h nitrate ions were completely reduced in the reactor. Nitrate concentration of 30 mg NO_3^--N/l was recorded in the effluent at steady state, at HRT = 46.1 h. Biomass concentration (g VSS/l) was measured in effluent solution and in the reactor (at the midpoint of its height-for control). It decreased from 1.88 g VSS/l to 0.26 g VSS/l, and after the fifth day reached steady state. Similar trend was observed at all tested HRT. During seven days of the first set of experiments at different HRT, specific denitrification rates were usually increased from 2 to 50 mg NO_3^--N/g VSS h. After seven days of denitrification in SW, biomass flocs were formed (Fig. 7). These bio-flocs were different sizes: large ones were average $170 \times 23\,\mu m$ and small were $14 \times 9\,\mu m$. In similar denitrification tests, Yoo et al. (1999) showed a relatively quick drop 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 drop did not affect COD and nitrate removal.

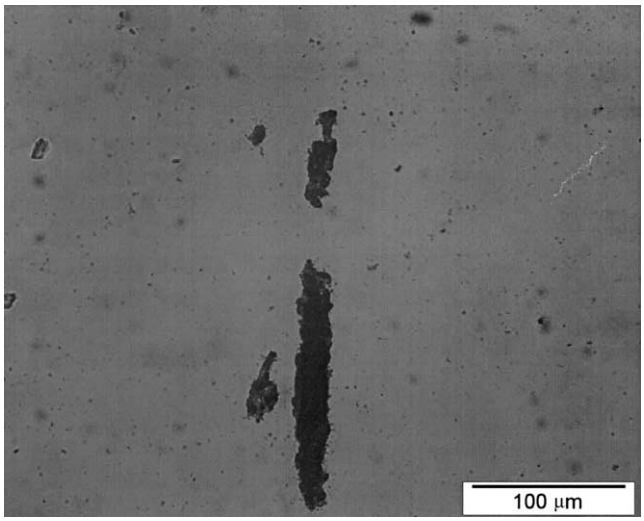

Fig. 7. Biomass flocs formed during continuous nitrate removal from SW.

The numbers of bacterial colonies (CFU), grown on the standard nutrient agar and on the hardened feed solution, were mutually 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. Bacteria count during these tests decreased from 10^9 CFU/ml to 10^8 CFU/ml. The recorded plate count in SW corresponded to the values reported from similar denitrification tests (Zayed and Winter, 1998; Martienssen and Schöps, 1999). Since the biomass concentration significantly decreased compared to the CFU/ml, it was assumed that the formation of stable biomass flocs was responsible for the increase of specific denitrification rates.

Generally, denitrification is an anaerobic or anoxic process; however, a complete nitrate removal was reported even at dissolved oxygen concentration of 5 mg O_2/l (Patureau et al., 1997; Martienssen and Schöps, 1999; Huang and Tseng, 2001). In this study, dissolved oxygen (DO) was diminished from 5.50 mg O_2/l to 0.40 mg O_2/l during the first 4 h and after some fluctuations during the first three days raised to 2.5 mg O_2/l and remained at that level. That decrease and increase of DO during the first three days may probably be explained with fluctuations in biomass concentrations, formation of biomass flocs and consumption of O_2 by bacterial cells. The obtained results confirmed that complete and fast denitrification could be achieved even at that DO concentration.

In the second test set denitrification started at the highest HRT (61.7 h) because in previous tests it was observed that at lower HRT the effluent contained nitrate. During 15 days of continuous flow nitrate was completely reduced (Fig. 8a). Biomass concentration in

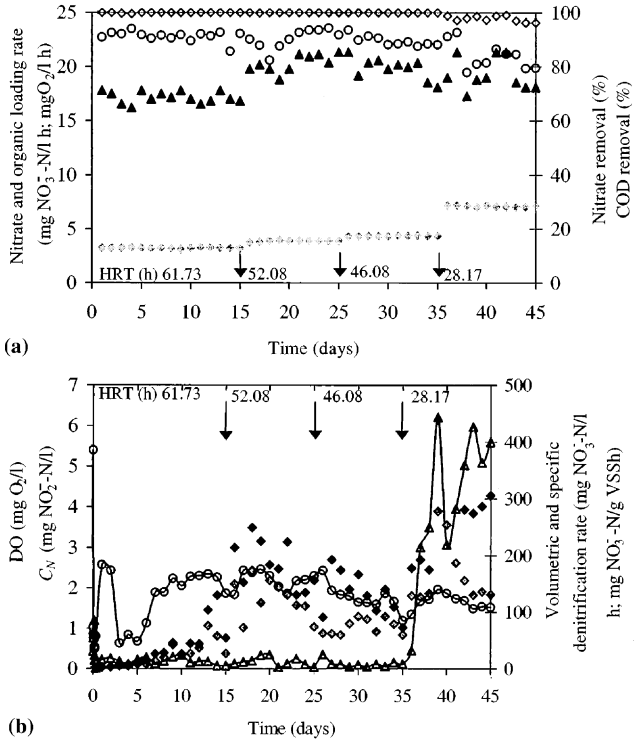

Fig. 8. Denitrification in the continuous-flow stirred reactor. (a) Nitrate (♦) and organic loading rate (▲), nitrate (◊) and COD removal (○) from SW and (b) nitrite effluent concentration (△), DO effluent concentration (○), volumetric denitrification rates (◊) and specific denitrification rates (♦).

Table 1
Literature values of specific denitrification rates obtained during studying the denitrification process

The specific denitrification rate, k_{den}	Temperature (°C)	References
56.3 mg NO_3^--N/g MLVSS h	25	Timmermans and Van Haute (1983)
10–20 mg NO_3^--N/g VSS h	20	Henze et al. (1994)
19.16–31.25 mg NO_3^--N/g VSS h	20	Beaubien et al. (1995)
32.6–35.25 mg NO_3^--N/g VSS h	35	Bernet et al. (1996)
10–33 mg NO_3^--N/g VSS h	15	Hallin et al. (1996)
23–54 mg NO_3^--N/g MLSS h	25	Glass and Silverstein (1998)
32–111 mg NO_3^--N/g VSS h	25	Sozen and Orhon (1999)
0.54–1.16 mg NO_3^--N/g MLSS h	25	Yoo et al. (1999)
12–27.6 mg NO_3^--N/g biomass h	30	Peyton et al. (2001)
1.88–2.88 mg NO_3^--N/g MLSS h	25	Sarioglu and Horan (2001)
2.16–3.29 mg NO_3^--N/g MLSS h	30	Sarioglu and Horan (2001)
21.0 mg NO_3^--N/g VSS h	25	This paper—batch test
142 mg NO_3^--N/g VSS h	25	This paper—continuous-flow test

SW determined during denitrification in the continuous-flow stirred reactor decreased, as in previous tests, from the original value of 1.88 g VSS/l to 0.5 g VSS/l, but after the seventh day, steady state was established and biomass flocs were formed.

Lower HRT was then applied to investigate the biomass flocs activity. From day 15 to 25, HRT was decreased to 51.6 h. Nitrate ions present in feed solution were reduced completely and an average specific denitrification rate of 142 mg NO_3^--N/g VSS h was obtained. Subsequently, HRT was lowered to 46.1 h for further 10 days. However, even at this time, nitrate was completely reduced, whereas in the previous tests performed with the same HRT nitrate was present in effluent (30 mg NO_3^--N/l). Apparently, it seems that the shortage of the nitrate in the influent and the increase of its feed rate, accomplished with flocs formation, slightly decreased the apparent bacterial biomass efficiency. Consequently, average specific denitrification rate of 102 mg NO_3^--N/g VSS h was obtained. Finally, HRT on day 35 was decreased to 28.2 h to continue for the last 10 days of the experiment. The COD value and nitrate concentrations in the influent gave an organic carbon loading rate of 17–22 mg O_2/l h and nitrate-nitrogen loading rate of 3.11–7.10 mg NO_3^--N/l h (Fig. 8a). The nitrates were under these conditions in excess, as indicated by the presence of 1–3 mg NO_3^--N/l in the effluent. Unfortunately, nitrites (6 mg NO_2^--N/l) appear in the effluent, too (Fig. 8b). However, nitrate removal efficiency higher than 96%, and an average specific denitrification rate of 173 mg NO_3^--N/g VSS h was obtained under these conditions. Obviously, to avoid significant nitrite and nitrate breakthrough into the effluent, specific denitrification rates somewhat lower than this maximum one have to be applied. The value of 142 mg NO_3^--N/g VSS h, reported before, seems to be more acceptable. Furthermore, biomass with volume index of 23.21 ml/g VSS was obtained, indicating good settling characteristic of denitrifying bio-flocs (Yoo et al., 1999).

Some representative specific denitrification rates, reported during the last 10 years in literature, are presented in Table 1 together with results of this research. The denitrification rates of the batch tests obtained in the present work are comparable with the upper range of the published data. However, the value of k_{den} = 142 mg NO_3^--N/g VSS h of the continuous flow measurements, was higher than the published specific denitrification rates. The obtained results indicate to the superiority of the acclimated mixed bacterial culture originated from the anaerobic–aerobic industrial wastewater treatment plant for high rate denitrification process.

4. Conclusions

Mixed bacterial culture originating from an industrial wastewater treatment plant after acclimation, reduced completely in the batch tests 200 mg/l nitrate at 25 °C during 5.5 h, producing only 0.11 mg NO_2^--N/l. The denitrification proceeded under anoxic conditions with the mixed bacterial culture containing mostly *Pseudomonas* and *Paracoccus* sp. strains, obeying a zero-order reaction with respect to nitrate concentration and a first-order reaction with respect to biomass concentration. The specific denitrification rate of 21 mg NO_3^--N/g VSS h was obtained in the batch process under optimal conditions. In the continuous denitrification process complete denitrification was achieved at 25 °C with the nitrate and methanol loading rate of 4.35 mg NO_3^--N/l h and 23 mg O_2/l h, respectively. Explicitly high average specific denitrification rate of 142 mg NO_3^--N/g VSS h, could be obtained even at HRT of 28 h.

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