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Dynamics of nitrate and nitrite in saturated sand filters with enhanced substrate conditions for denitrifying bacteria

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ABSTRACT

Substrate conditions for denitrifying bacteria were enhanced by adding carbon sources to a laboratory-scale sand filter system. Temperature, oxidation–reduction potential, and hydrogen ion concentration were measured through the recirculation of nitrogen-dosed wastewater and carbon sources that were mixed to encourage microbial growth, with denitrifying bacteria identified by standard plate counts. Two different external carbon sources (sucrose and ethanol) were added, with and without activated sludge amendments. Nitrate, nitrite, and chemical oxygen demand (COD) concentrations were monitored relative to an untreated control and a treatment with activated sludge under an initial hydraulic loading rate of 0.508 m^3/m^2 d and a hydraulic retention time of 2.5 h. Nitrate decay rates were only significantly enhanced for the ethanol treatment without addition of activated sludge. Nitrite initially accumulated when carbon sources were added, but no accumulation was evident by the end of the experiment after 150 min. COD declined when carbon sources were added, but activated sludge had no effect on the rate at which the COD declined. The increased rate of nitrate removal with the addition of ethanol is of technical interest, as the volume of wastewater treated in a unit volume of filter medium for denitrification doubled with ethanol compared with sucrose at the same concentration.

Keywords: Nitrate; Nitrite; Chemical oxygen demand; Saturated sand filters; Denitrification

1. Introduction

High nitrate concentrations in drinking water have been linked to both methemoglobinemia and bladder cancer. Furthermore, treated municipal wastewater that contains high amounts of nitrate can enter water bodies and potentially stimulate algal blooms, degrade water quality, and reduce biological diversity. This eutrophic phenomenon is the major cause of the hypoxic zone "dead zone" in water [1]. Nitrite accumulation (an intermediate product of nitrate reduction during the denitrification process) is not beneficial in wastewater treatment as extra oxygen for N

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removal is required. Furthermore, there can be problems associated with discharging of wastewater into natural water bodies without treating nitrite [2]. Glass and Silverstein [3] reported that nitrite accumulation could inhibit denitrification because it hinders bacterial development.

Denitrification is a progressive reaction converting nitrate to nitrogen gas by enhancing indigenous denitrifying microorganisms [4]. Cui et al. [5] reported no clear difference in nitrate removal between planted and unplanted sand filters. Thus, we highlighted in this experiment the role of adding different C sources such as ethanol and sucrose (external C sources) and activated sludge (internal C sources) in unplanted sand filters. The addition of external C sources and internal C sources to the filter material has been considered a stable source of carbon for denitrifying bacteria, potentially enhancing nitrogen removal and reducing the hydraulic retention time and the treatment area required per unit volume of wastewater and thus reducing treatment costs [6–9]. Ethanol and sucrose have often been the choice in practice as they are cheap and convenient [6,10].

Constantin and Fick [11], Dold et al. [12], Gomez et al. [13], Hallin and Pell [14], Rodriguez-Caballero et al. [15] have worked with ethanol on denitrification. Few authors have worked with sucrose as a carbon source. Gomez et al. [7], Dold et al. [12], Gomez et al. [13], Hallin and Pell [14], Shah and Coulman [16], Stensel et al. [17] have used activated sludge to stimulate denitrifiers, in addition to the carbon sources and nutrients. A few studies have not used activated sludge as an inoculum, relying only on the contribution of the carbon sources along with the main nutrient substrates [18,19]. However, there is a paucity of information on whether adding activated sludge to the main substrate media can further enhance microbial activity to accelerate mass nitrogen removal. Furthermore, no investigation has compared sucrose and ethanol saturated with N compounds with and without activated sludge.

A review by Mohseni-Bandpi et al. [20] covered the applications of C sources on N removal in bioreactors, but not in sand filters. A sand filter system also provides a simplified analogue for saturated flow in groundwater and near-stream riparian zones as well as wetland sediments [21]. Al-Saedi et al. [22] showed that downflow saturated conditions were preferable to unsaturated conditions for mass nitrogen removal, as the final concentrations are similar between the saturated and unsaturated zone. It was, therefore, advantageous to save space and operate this filter bed as an entirely saturated system, as the measured saturated water content was seven times greater than the measured water content of the unsaturated zone. Furthermore, operating a sand filter in an upflow mode can give greater displacement of entrapped air than is possible in the downflow mode, which could provide a preferable condition for denitrification [15]. Therefore, we now seek to establish if removal rates can be accelerated by addition of organic sources to upflow saturated sand filters.

The aims of the study were to: (1) examine how biodegradation of the external C sources (ethanol and sucrose) is influenced by the internal C source (AS) and how this interaction affects the denitrification rate; (2) assess the behaviour of denitrifiers in sand filters treating nitrate enriched wastewater.

2. Materials and methods

2.1. Experimental setting, sampling and design

This study used six parallel perspex vertical upflow columns. The column bases had plastic filters to support the sand layer and evenly percolate the wastewater through the columns. The characteristics of the sand columns used in the experiments are given in Table 1.

To enhance nitrate removal under fully saturated conditions in the vertical flow filters, we applied the following steps in the laboratory: (1) initial upflow of wastewater through the core bases to uniformly distribute treated water through the filters; and (2) addition of different liquid organic carbon sources (ethanol and sucrose) with and without activated sludge.

Prior to commencing the experiment, indigenous microorganism activity was stimulated by feeding the system with the nitrate-enriched synthetic wastewater once a week for 3 weeks [23] (the flow was not continuous) as an adaptation period at an influent flow rate and a hydraulic loading rate of 0.003 m^3/d and 0.508 m^3/m^2 d, respectively. Synthetic wastewater was prepared using potassium nitrate as the only source of nitrate. Two columns were used as controls (CONT) with only KNO_3 added, two columns had liquid sucrose added (SUC), and two columns had ethanol added (ETH) at C:N ratios of 5 for SUC and ETH.

Nitrate reduction using ethanol and sucrose as carbon sources is represented in the following stoichiometric reactions [11,13]:

$$C_{12}H_{22}O_{11} + 9.6NO_3^- + 9.6H^+ \rightarrow 12CO_2 + 4.8N_2 + 15.8H_2O \quad (1)$$

$$5C_2H_5OH + 12NO_3^- \rightarrow 6N_2 + 10HCO_3^- + 2OH^- + 9H_2O \quad (2)$$

At the end of the adaptation period, the recirculated wastewaters were completely flushed from the columns. This was followed by an investigation period with a controlled upward flow from a header tank at an influent flow rate and a hydraulic loading rate of 0.003 m^3/d and 0.508 m^3/m^2 d, respectively. The investigation period involved two operating periods. In the first operating period, the columns were saturated from below with a new synthetic wastewater (the same as flushed). Once saturated, the flow was switched off and denitrification allowed to progress under static, saturated conditions. During the adaptation and investigation periods, a positive head was kept to drive the filter solution out for sampling. Sampling was undertaken by re-establishing the flow briefly at 10, 20, 30, 50, 70, 90, 120 and 150 min (until nitrate concentrations reached close to zero) to force about 10 mL of wastewater to accumulate at the soil surface for sampling by syringe (Fig. 1).

In the second operating period, the flushed columns were fed with the same synthetic wastewater described previously (CONT, SUC and ETH), but with 4 g/L of mixed liquor activated sludge/L wastewater added. Thus, the second period involved two columns with KNO_3 (CONT) and activated sludge (CONT + AS), two columns with liquid sucrose added with activated sludge (SUC + AS), and two columns with ethanol added with activated sludge (ETH + AS) at the same C/N ratio.

Table 1
Column characteristics

Parameters	Values and units
Column height	34 cm
Internal diameter	6 cm
Surface area	28.26 cm^2
Height of coarse sand bed	16 cm
Volume of coarse sand bed	452 cm^3
Average coarse sand diameter	0.7 mm
Dry bulk density	1.33 g/cm^3
Average sand bed porosity	50%
Average column pore volume	225 mL
Organic matter content	0.3%

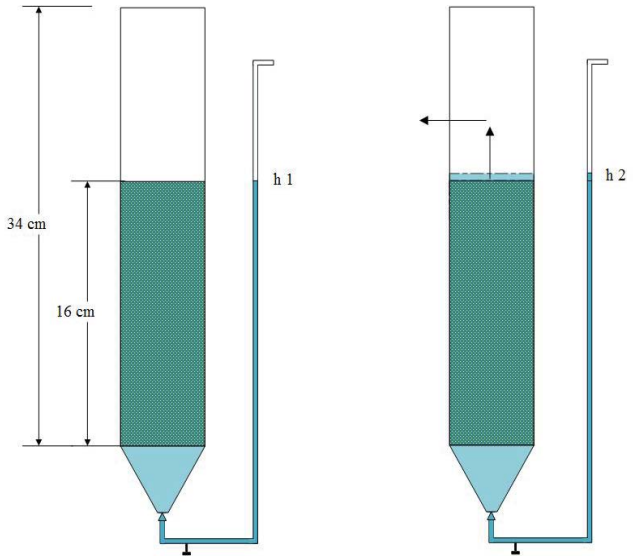

Fig. 1. Schematic diagram of the sampling procedure for one laboratory-scale vertical flow sand filter. h1: hydraulic head before sampling, h2: hydraulic head through sampling, so $h1 - h2 = \Delta h$.

The treatment procedure was repeated twice for each column. Before repeating the treatment, the sand beds were sterilised and repacked, applying the same procedure of recirculating and feeding for each column. The activated sludge was supplied from a wastewater treatment plant with total inorganic nitrogen compounds of 7 mg/L and total nitrogen of 56.3 mg/L to give an organic nitrogen concentration of 49.3 mg/L.

As described above, all measurements were taken during the operating period, giving four replicates. At each stage, three treatments were randomly allocated to two columns and sample was taken from each column. In order to account for potential laboratory measurement error, samples were taken in triplicate. After each stage, the columns were repacked, therefore the replicates (columns) can be considered independent and the experimental design can be described as a randomised complete block design.

2.2. Analytical measurements

The temperature was measured using an OxyGuard Handy Polaris 2 meter. Oxidation–reduction potential (redox) and hydrogen ion concentration (pH) were measured using a water quality probe WP/81. For NO_3 and NO_2 concentrations, the drawn wastewater samples were filtered through 0.45 μm membrane filters (Millipore HVLP04700) and then analysed with a HACH DR/6000 spectrophotometer (USA) using HR Ferrous sulphate and HR Cadmium reduction methods for nitrite and nitrate tests respectively.

Organic matter content of the sand was determined by loss on ignition, which is the difference in the dry weight of the ignited sample and the heated sample divided by the heated sample. Chemical oxygen demand (COD) tests were performed in triplicate for each sample using the digestion method with a HACH DR/6000 spectrophotometer (USA).

2.3. Standard quantification of denitrifying bacteria

Denitrifying bacteria were quantified by a standard plate count, which is often used in estimating viable bacterial cells in water [24]. This method used nitrate sucrose agar medium as described by Rodina, 1972 [25]. The pH of the agar medium (Table 2) was adjusted each time to 7.0 by adding small spikes of sodium bicarbonate. During the adaptation period (21 d), 1 g of soil was transferred—using a sterilised spoon—from the anoxic zone to a sterile glass bottle containing 100 mL of 0.9% NaCl. The soil samples underwent sonication for 5 min to separate the biofilm from the inert substrate. A dilution series (1:10) with 10 mL Pyrex tubes was made using a sterile saline solution (0.9% NaCl). 0.1 mL was extracted from each dilution and spread on the prepared medium in Petri dishes, with three replicates for each dilution. The dishes were incubated anaerobically using AnaeroGen system OXOID (28°C–30°C) for 2 weeks. The number of bacteria (CFU) per gram of sample was calculated on plates of the series displaying ~10 to 100 CFU.

2.4. Statistical analysis

The nitrate decay data were analysed using treatment as a grouping factor in the fitted exponential nonlinear regression to investigate the consistency of a nonlinear relationship across the six treatments. We specifically sought to determine whether treatments had a significant effect on the response, assuming that nitrate concentration declines exponentially with time. The fitted model tested whether the rates and curvature of the fitted curves differ in response to the treatments.

An exponential curve, as formulated below, demonstrated the best fit

$$y = y_0 + be^{kt} \tag{3}$$

where y_0, b and k ($k < 0$ for exponential decay and $k > 0$ for exponential growth) are the parameters and the explanatory variable t is the time when the measurements of nitrate concentration were taken. The statistical significance of the differences between the treatments for peak NO_2 concentration and production rate was tested.

Some additional statistical analyses were conducted using ANOVA techniques for factorial treatment structures.

All statistical analyses were conducted using Genstat 20th Edition (VSN International Ltd., Hemel Hempstead, UK).

2.5. Kinetic coefficients of denitrification

Estimating values of kinetic parameters is beneficial for predicting effluent concentrations under any operating conditions [16].

The change of NO_3 stock in the water column of the wetland during the investigation (dNO_3/dt) equals the difference between NO_3 sources (NO_3 input [in] and nitrification) and NO_3 sinks (NO_3 output [out], denitrification, and uptake of NO_3 [up] [mg/m^2/d]) [26].

$$\frac{dNO_3}{dt} = \text{in} + \text{nit} - \text{out} - \text{denit} - \text{up} \tag{4}$$

where in = NO_3^- input (mg/m^2/d), nit = nitrification (mg/m^2/d), out = NO_3^- output (mg/m^2/d), denit. = denitrification (mg/m^2/d) and up = uptake of NO_3^- by plant (mg/m^2/d).

The effect of the nitrification process and uptake of NO_3^- by plants on the denitrification rate has been neglected by many researchers [27], reducing Eq. (4) to:

$$\text{denit.} = \text{in} - \text{out} - \frac{dNO_3}{dt} \tag{5}$$

The loss of nitrate in a vertical flow wetland treatment system follows the first order kinetic model (Eq. (6)), particularly when the influent nitrate concentration in the wastewater is about 100 mg/L [26,27].

$$\frac{C_e}{C_0} = e^{-k_T t} \tag{6}$$

$$k_T = k_{20}\left(1.06\right)^{T-20} \tag{7}$$

where C_e = effluent N concentration (mg/L), C_0 = influent N concentration (mg/L), k_T = removal rate coefficient at temperature T°C (1/d), and k_{20} = removal rate coefficient at 20°C (1/d).

3. Results

3.1. Filter bed operating conditions

Average influent temperature was 19.2°C and average effluent temperature was slightly lower at 18.1°C, indicating slight ambient cooling over the course of the experiment.

Average influent pH levels were 7.5 (±0.2), and average effluent values were 7.8 (±0.4), indicating a slight rise in pH throughout the course of the experiments. Over most of the experimental period, pH values were optimal (7.0–7.5) for promoting denitrification [15].

Influent redox values in the feed wastewater averaged 194 ± 1 mV. Effluent redox values were 30, 14, 24, –105, 22 and –122 mV for CONT, CONT + AS, SUC, SUC + AS, ETH and ETH + AS, respectively. Redox values >100 mV promote aerobic conditions and <100 mV promote anaerobic conditions [28]. The oxidation–reduction conditions for all columns were identical in the first week of the adaptation period and became completely anaerobic (highlighted values) at day 10 for SUC + AS and ETH + AS, and at days 15 and 17 for ETH and SUC, respectively, and at days 19 and 15 for CONT and CONT + AS, respectively (Table 3).

Table 2
Nitrate sucrose agar medium composition used in this study [40]

Component	Mass or volume
Tap water	1,000 mL
$NaNO_3$	2 g
K_2HPO_4	1 g
$MgSO_4{\cdot}7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4{\cdot}7H_2O$	Trace
Sucrose	30 g
Agar	15 g

Table 3
Redox values over the adaptation period

Time (d)	CONT	CONT + AS	SUC	SUC + AS	ETH	ETH + AS
0	194	193	195	194	195	195
1	174	160	166	149	164	144
3	166	142	157	144	147	143
5	160	142	145	142	146	143
7	154	127	144	121	126	118
9	147	113	133	109	123	104
11	136	107	128	78	115	77
13	122	101	116	10	113	8
15	115	98	101	–56	98	–65
17	107	91	88	–90	86	–95
19	95	87	83	–104	75	–116
21	30	14	24	–105	22	–122

3.2. Nitrate nitrogen removal

Nitrate decay curves for all treatments are presented in Fig. 2 with an influent concentration of 100 mg/L. No significant statistical differences were observed between CONT and other treatments where organic C sources were used.

A sequence of four statistical models was compared. The first fitted all three parameters in Eq. (3), y_0, b and k, the same for all six treatments, the second model allowed only different y_0 values for each treatment, the third allowed different y_0 and b values and in the final fourth model all three parameters differ for each treatment, all fitted parameters are given in Table 4. While fitting the models, the percentage variance was monitored and as expected it grew from 88% for the first model (reflecting a good curve selection and fit) to 93% for the final model. Most importantly, the significance of the change of the fit while increasing the different number of parameters for the treatment curves, as described above, was followed. In particular, there was a highly significant difference ($p < 0.001$) between the last two models where the parameter k was fitted the same for all treatments (model 3) and different (model 4), explaining the pattern observed in Table 4, more precisely that the decay for treatment ETH is much higher than for the rest of the treatments.

The results suggest that different asymptote parameters (y_0), different rate (linear) parameters (b), and different nonlinear shape parameters (k) are required to obtain the best model fit to the data. Additional analysis revealed that time and treatment are highly significant ($p < 0.001$) and their interaction is borderline significant, ($p = 0.052$). The latter can be attributed to the pattern observed in Fig. 2, where the dynamics of ETH treatment in time is different from the rest of the treatments. Moreover, five of the six treatments have similar rates of decay (–0.01), with the ethanol treatment (ETH) having much higher rate of decay (–0.03; Table 4).

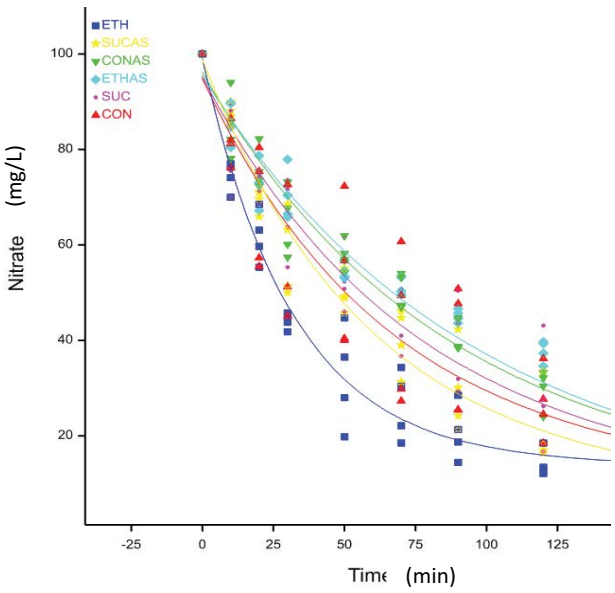

Fig. 2. Nitrate concentration decay over time.

3.3. Nitrite production and decay

Influent nitrite concentrations were 0, 0, 7, 6, 1 and 0 mg/L for CONT, CONT + AS, SUC, SUC + AS, ETH, ETH + AS, respectively. Nitrite production and decay trends are shown in Fig. 3. Three comparative metrics can be obtained to describe the general dynamics: time to peak, peak concentration and reaction time. The latter were analysed statistically across treatments and the results are summarised in Table 5.

It can be seen that for the concentration peaks treatments SUC, SUC + AS, ETH and ETH + AS are significantly higher than the controls (with no significant difference between the CONT and CONT + AS peaks). At the same time, all SUC and ETH treatments with and without AS did not significantly differ from each other. Similarly, for the reaction time there was no significant difference between the CONT and CONT + AS but all SUC and ETH treatments had significantly longer reaction times. This reflects the increased production of nitrite compared with the controls and hence an increased time required for decay. There was no significant difference between ETH and SUC, but the addition of AS significantly reduced the reaction time for ETH and significantly increased the reaction time for SUC.

3.4. COD removal

The COD had an average influent concentration of 11, 62, 365, 433, 441 and 584 mg/L for CONT, CONT + AS, SUC, SUC + AS, ETH and ETH + AS, respectively. CONT and CONT + AS differed from the columns supplemented with external carbon sources (Fig. 4). The COD values for CONT and CONT + AS increased in the first 50 min of the experiment. At the end of the experiment, the average effluent

Table 4
Coefficient values

Parameter	Estimate	Standard error (s.e.)
k Treatment CON	–0.015	0.002
b Treatment CON	83.79	5.82
y_0 Treatment CON	11.01	6.43
k Treatment CON + AS	–0.012	0.002
b Treatment CON + AS	87.55	8.82
y_0 Treatment CON + AS	8.540	9.79
k Treatment ETH	–0.031	0.003
b Treatment ETH	85.04	3.68
y_0 Treatment ETH	13.94	2.75
k Treatment ETH + AS	–0.011	0.002
b Treatment ETH + AS	89.50	10.2
y_0 Treatment ETH + AS	5.90	11.3
k Treatment SUC	–0.013	0.002
b Treatment SUC	86.04	7.18
y_0 Treatment SUC	9.130	8.01
k Treatment SUC + AS	–0.017	0.002
b Treatment SUC + AS	89.42	5.01
y_0 Treatment SUC + AS	9.380	5.41

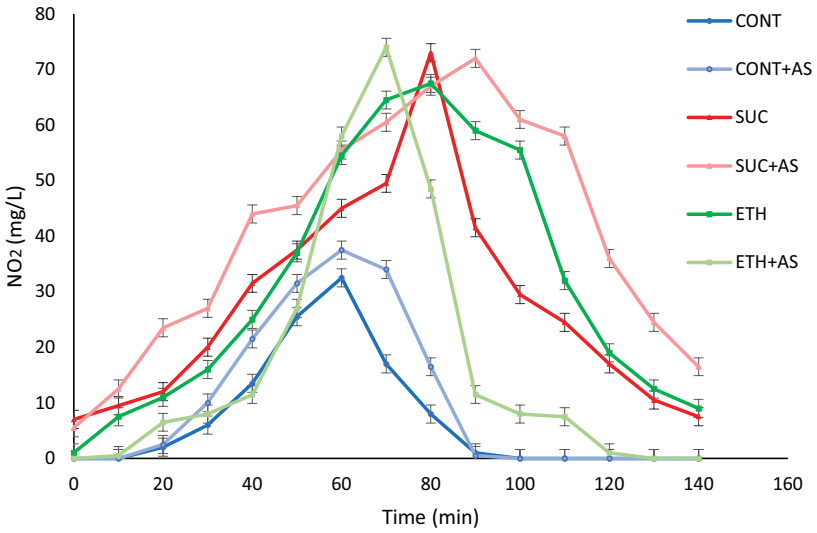

Fig. 3. Nitrite concentration over time.

Table 5
Metrics for nitrite accumulation across filter treatments: predicted means and LSD values

Columns	Mean of peak concentration (mg/L)	Mean of reaction time (min)
CONT	32.5	80.0
CONT + AS	37.5	85.0
SUC	73.0	130.0
SUC + AS	72.0	145.0
ETH	67.5	135.0
ETH + AS	74.0	95.0
LSD (0.05)	10.3	7.1

COD values had declined to 1 and 11 mg/L for CONT and CONT + AS treatments. Average effluent concentrations of COD for SUC, SUC + AS, ETH and ETH + AS were 48, 59, 48 and 87 mg/L, respectively. The biodegradation of organic matter mainly occurred in the first 50 min of the total time (2.5 h).

3.5. Behaviour of denitrifying bacteria during the adaptation period

Table 6 shows an increase in the bacterial population during the adaptation period, indicating successful growth of denitrifying bacteria before sampling commenced. The inoculated activated sludge was a mixture of autotrophic/heterotrophic bacteria from a wastewater

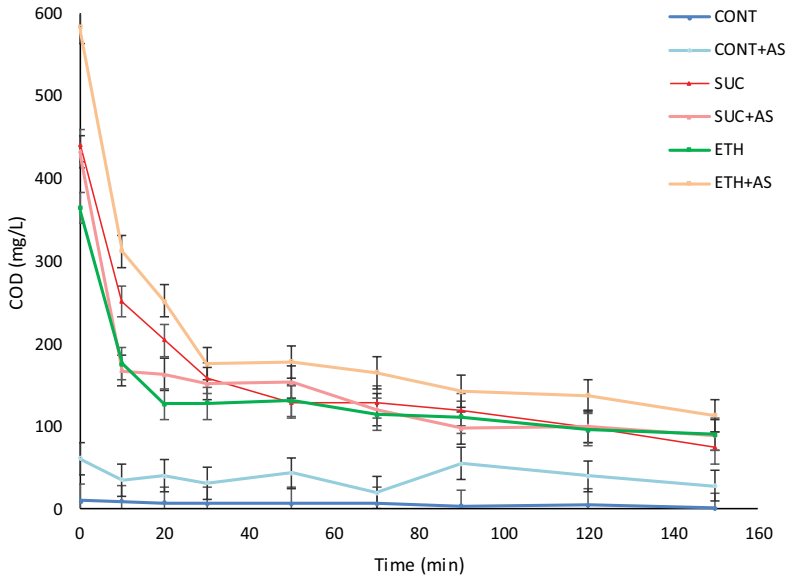

Fig. 4. COD concentrations over time.

treatment plant. Bacterial counts on day 1 indicate that the addition of AS increased bacterial numbers for all treatments. By day 7, the count numbers of all treatments were an order of magnitude higher than the control treatments. However, by day 21, count numbers had increased substantially in the controls, so that only the ETH + AS treatment had higher numbers. At the start of the experiment (after 21 d of recirculating the flow), the bacterial counts in the control equalled the sucrose treatments, and the CONT + AS treatment exceeded all but the ETH + AS treatment. All treatments, therefore, had active microbial populations of similar magnitude to the start of the experiment.

4. Discussion

4.1. Operating conditions

Eqs. (1) and (2) show that 575 mg/L of sucrose and 309 mg/L of ethanol are required to denitrify 1,000 mg/L of nitrate. Consequently, a lesser concentration of nitrate removal would be expected in the sucrose columns than in ethanol using the same quantity (concentration) of organic carbon (Fig. 2). Therefore, if ethanol is used instead of sucrose in the denitrification process, the amount added/unit volume can be about half that of sucrose, using the same C/N ratio.

The stoichiometric reactions (Eqs. (1) and (2)) demonstrate that the theoretical C/N ratios should be 1.07 and 0.72 for sucrose and ethanol, respectively, for complete denitrification. However, Shah and Coulman [16] recommended a C/N ratio of about 3 to maintain an adequate carbon source for complete elimination of inorganic nitrogen. In addition, in the case of a heterogeneous culture, the carbon source could be consumed by other microorganisms or by assimilation of the carbon source in the biofilm which is usually used for cellular growth [3]. Therefore, a C/N ratio of 5 was chosen for this study to safely exceed the advised value.

Microbial growth and metabolic rates are strongly related to temperature [29], which can affect the performance of denitrifying bacteria [30]. Denitrification generally functions well at water temperatures from 15°C to 30°C [4,30] so we operated our system in this range.

In a filter matrix, the chemistry and biology of water are influenced by pH because healthy aquatic microorganisms can only function within a restricted pH range [31]. The results indicated a slight increase in pH level during the recirculation period as denitrification is an alkaline-producing process, consuming hydrogen ions and producing carbon dioxide. In theory, every 1,000 mg/L of NO_3^- can consume 309 mg/L of ethanol and produce 820 mg/L of alkalinity as HCO_3 (stoichiometric Eq. (2)).

Numerous researchers have reported that the dominant respiration activities and the related terminal electron acceptors, and hence elimination of nitrogen compounds are actively influenced by the prevailing oxidation–reduction potential (redox) in the filter bed matrix [9,24,32,33]. Therefore, redox can be a powerful tool for predicting the biological state of filters (e.g., aerobic, anoxic or anaerobic). Reddy and DeLaune [28] reported that the oxidation state of N compounds (from nitrate to nitrogen gas) in denitrification drops from +5 to 0, as it is mediated by microbial activity, whereby facultative microbes acquire the enzymes that permit them to utilise nitrate, nitrite and nitrous oxide as the terminal electron acceptor during the oxidation of organic carbon under anoxic conditions. Although aerobic microbial growth is usually dominant [1], the presence of anoxic/anaerobic conditions (low redox values) in this study was observed during the adaptation period, which was due to the saturated conditions and selection of suitable substrate medium to promote microbial growth and denitrification [9,16].

4.2. Nitrate nitrogen removal

Fig. 2 shows that the removal of NO_3 was achieved efficiently at the top of the saturated zone as long as the anoxic conditions and microorganisms were maintained.

Under an initial nitrate concentration of 100 mg/L, the average decay rate of nitrate for the CONT columns was 0.0118/min, which is higher than values reported elsewhere [34,35]. Zaman et al. [35] studied nitrate removal in a wetland treating nitrate-rich wastewater without any addition of C sources. Their decay rates were 0.00174/min and 0.00255/min in surface and groundwater samples under initial nitrate concentrations of 1.5 and 8 mg/L, respectively. Burchell et al. [34] studied enhanced nitrate removal by adding a dredged material as an organic C source into subsurface filters; their nitrate decay values for controls were 0.0000111, 0.00004167, 0.0000382 and 0.0000354/min across four investigation periods under initial nitrate concentrations of 30, 40, 60 and 120 mg/L, respectively, with a significant improvement in the performance of columns treated with organic matter relative to the control.

The initial recirculation in our study resulted in sufficient bacteria in the control for reasonably efficient decay, as there were no differences between most of the treatments. While many studies have investigated the effect of adding organic carbon on nitrate removal rate [6,12], we are not aware of any study that has compared the effect of adding commercial organic carbon sources sucrose and ethanol with the control on the performance of denitrification in filters.

As the growth of denitrifying bacteria is affected by the organic carbon contribution, and considering ethanol as the most appropriate carbon source [7], adding ethanol increased the rate of growth (Table 6) and the apparent decay rate (Fig. 2). The initial decline in nitrate with added ethanol was related to the reduced state of the organic source in the wastewater [36]. However, in the current study, the observed nitrate reduction with ETH + AS did not differ significantly from the controls or sucrose treatments, and only ETH produced a significantly higher decay rate. Makinia, 2010 [37] reported that only readily biodegradable compounds (including sucrose and ethanol) are considered substrates in heterotrophic growth under anoxic conditions. Thus, adding activated sludge may have hindered denitrification because the non-biodegradable compounds (particularly the soluble part) in wastewater cannot be easily metabolised by microorganisms [37]. Furthermore, the inoculated activated sludge was a mixture of autotrophic/eterotrophic microorganisms from a wastewater treatment

Table 6
Denitrifying bacteria growth over the recirculation period

	Denitrifying bacteria number (CFU × 10^4)					
Time (d)	CONT	CONT + AS	SUC	SUC + AS	ETH	ETH + AS
1	0.01	0.002	0.06	0.3	0.023	0.04
3	0.024	0.02	0.2	0.32	0.042	0.08
5	0.2	0.06	0.8	0.98	0.54	0.2
7	0.8	0.13	1.62	1.07	2.03	1.9
14	2	3.43	2.2	2.1	8.208	10.02
21	25.87	40.233	30.12	34	12.011	64

plant. Hallin and Pell [14] confirmed that adding ethanol to the substrate media might affect the ability of denitrification to use other substances in the substrate rather than the external carbon sources. Andersson et al. [27] reported that adding an external C source alters the microbiology of the activated sludge due to the new biomass production, which changes the overall biocoenosis (an association of different organisms forming a closely integrated community) of the sludge. However, the selective effect of ethanol for very special groups of bacteria, which require ethanol for their growth, has not been well documented [27].

Rodriguez-Caballero et al. [15] studied the treatment efficiency of biological sand filters fed tap water (control), tap water + ethanol, and tap water + nutrients + ethanol. They reported that up to day 8, the tap water + nutrients + ethanol treatment had similar enzyme activity as tap water + ethanol treatment; after which, it started to decline. To date, no investigation has compared sucrose and ethanol saturated with N compounds with and without activated sludge.

4.3. Nitrite production and decay

Nitrite accumulation in the denitrification process has been attributed to several factors including environmental conditions (abundance or lack of oxygen) [2], increased competition between nitrate and nitrite reductases for electrons [3,38], and changes in the metabolism of competing bacteria [2]. In this study, the control columns had the lowest accumulation concentration peak relative to other columns (Fig. 3 and Table 5). Ge et al. [2] reported that the accumulation range increased when C sources were added due to a shift in the microbial population (a temporary increase in nitrate respiratory bacteria that only reduce nitrate to nitrite compared with true denitrification bacteria that reduce nitrate to nitrite and then to nitrogen gas). The ETH + AS treatment had a faster decay rate of nitrite than the other treatments, which could be attributed to the competition of true denitrifiers over nitrate respirators [2]; this was also supported by the bacterial count data, with the highest denitrifying bacteria count in the ETH + AS treatment.

4.4. COD removal

COD is an indicator of the total organic fractionation of wastewater (readily + slowly + inert) [37]. The COD levels of CONT + AS initially accumulated and then declined. Barnard and Meiring [36] affirmed that an increase in the COD removal rate is an indicator of the reduced state of organic compounds in wastewater due to a bacterial performance. Therefore, some accumulation of COD in the CONT + AS columns could be attributed to the bacterial limitation to oxidise the organic matter in the activated sludge. Christensson and Welander [39] attributed low COD removal to endogenous respiration in the column. An enhanced COD removal rate in the columns fed with the external carbon sources was observed (Fig. 4), which may be due to the presence of an electron acceptor (nitrate) and an electron donor (carbon source), that stimulates the simultaneous removal of COD and nitrate [15].

4.5. Denitrifying bacteria behaviour

Hallin et al. [23] used an activated sludge inoculum as the control and ethanol with activated sludge in a predenitrification activated sludge system with an average mixed liquor of 2,560 and 2,790 mg/L for control and ethanol treatments, respectively. The nitrogen removal efficiency with ethanol was approximately double that of the control after 12 d of adaptation. The authors attributed the rapid increase in denitrification rate with activated sludge fed ethanol to increased enzyme activity rather than altered bacterial species composition. Furthermore, Gomez et al. [13] concluded that the presence of various denitrifying bacteria might affect denitrification due to differences in the ability of these bacteria to reduce nitrate. In our study, denitrifying bacteria in the sludge might not be fully adapted to ethanol due to the incomplete availability of the particular microbial population that is necessary for metabolising ethanol [27,40]. The decline in the denitrification capacity of ethanol with added activated sludge may be due to a change in bacterial species composition, but this will not be evident in bacterial counts. Therefore, evaluation of the functional microbial community along with a taxonomic identification of the 16S rDNA fragments to identify species of denitrifying bacteria is required.

5. Conclusions

Only the ethanol treatment significantly increased the rate of nitrate decay. This effect was suppressed when activated sludge was added with ethanol. For the control columns, the microbial population must have been

sufficient to initiate nitrate decay. Therefore, there is a need to expand the research undertaken here to better define the role of ethanol (it might be carried with macrophytes) in enhancing nitrate loss from saturated sand filters and why activated sludge depresses this effect. In addition, using different C/N ratios (lower and higher than the investigated ratio) for ethanol with different concentrations of activated sludge is recommended.

Overall, no nitrite was released from this system after 2.5 h. At the end of the recirculation period, the ethanol columns had the lowest denitrifying bacteria count, but the ethanol with activated sludge columns had the highest denitrifying bacterial count. The enhanced nitrate decay with ethanol and suppression by addition of activated sludge suggests a possible shift in bacterial species rather than enzyme activity.

In the non-treated sand, the heterogeneous composition of activated sludge initially hindered COD removal. However, the addition of C sources resulted in an efficient COD removal, and no effect of activated sludge on COD removal was observed.

From a practical perspective, the increased nitrate decay rate observed with the addition of ethanol is of interest, as the volume of wastewater treated in a unit volume of filter material for nitrate decay doubled with ethanol compared with sucrose at the same concentration.

For denitrifying bacteria, analysis of the functional microbial communities involved in N removal and taxonomic identification of the 16S rDNA fragments to identify species of denitrifying bacteria is recommended.

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