



The use of crab-shell chitin for biological denitrification: Batch and column tests

Mary Ann Robinson-Lora, Rachel A. Brennan*

Department of Civil and Environmental Engineering, The Pennsylvania State University, 212 Sackett Building, University Park 16802-1408, USA

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ABSTRACT

Crab-shell chitin (SC-20) was evaluated for its ability to enhance biological denitrification in bench-scale tests. In the presence of SC-20, highly reducing conditions were generated, supporting both denitrification and sulfate reduction of aerated water. Rapid degradation of protein in SC-20 was observed to cause an initial high release of ammonium and carbon, while a slower, continuous release of calcium carbonate from the crab shell maintained the pH near 9 throughout the tests. In batch tests, denitrification rates of 2.4 ± 0.2 mg N/L-d were obtained. Columns receiving a continuous nitrate load of 24.5 mg N/L-d sustained complete denitrification for an average of 149 d (250 pore volumes). The denitrification rates and longevity of SC-20 chitin are comparable to, or better than, those previously reported for other polymeric substrates. This, in addition to its particle size, non-swelling nature, and ease of delivery in slurry form make SC-20 an attractive electron donor source for groundwater bio-denitrification.

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1. Introduction

Nitrate (NO_3^-) removal from water sources is a topic of intense research due to its implications on human health and water quality. Increased levels of nitrate and ammonia in groundwater result from intensive use of nitrogenous fertilizers, livestock manure, nutrient rich wastewater for irrigation, and improper sewage treatment and disposal. Some industrial activities, like the combustion of fossil fuels, and the production of fertilizers, explosives, glass, plastics, and cured meat can also contribute to nitrate pollution (ITRC, 2002). Natural levels of nitrate in groundwater are usually less than 3 mg/L as N. However, in contaminated areas, nitrate concentrations can exceed 200 mg/L as N (ITRC, 2002). The National Water Quality Assessment (NAWQA) program of the US Geological Survey reports that nitrate is the pollutant that most frequently exceeds its standard limits (Squillace et al., 2002).

The main health effect related to the consumption of high-nitrate water is the occurrence of methaemoglobinemia, especially in infants, or “blue-baby syndrome”. Some recent studies have tried to correlate high nitrate concentrations with cancer incidence, but evidence to date has been insufficient to prove that hypothesis (WHO, 2004). The maximum contaminant levels (MCLs) for nitrate and nitrite mandated by the USEPA (10 mg N/L and 1 mg N/L, respectively) and the World Health Organization and the European Union (50 mg/L as NO_3^- and 3 mg/L as NO_2^-), were established to prevent negative health effects.

Different technologies have been proposed to remove nitrate from drinking water. They include: ion-exchange, reverse osmosis,

electrodialysis, chemical nitrate reduction, catalytic denitrification, and biological denitrification (Kapoor and Viraraghavan, 1997). Of these, biological denitrification has proven to be cost-effective for both in-situ groundwater clean up and above ground treatment. Biological denitrification is a natural process that is part of the nitrogen cycle, and is commonly exploited in wastewater treatment plants for the removal of excess nitrogen. It has also been proven to be cost-effective for both in-situ and ex-situ groundwater treatment, using both inorganic and organic electron donors (Soares, 2000). Alternative solid electron donor sources like newspaper and cotton have been tested with positive results (Volokita et al., 1996a,b). These alternative materials have been successfully placed in trenches for in-situ remediation; however, their delivery and replacement can be difficult at large depths. Therefore, a particulate substrate is needed to overcome limitations on substrate delivery.

Chitin is the second most abundant biopolymer in nature, and is easily processed into particulate form. It is estimated that several gigatons of chitin are produced on earth annually (Howard et al., 2003), primarily by arthropods (insects and crustaceans), mollusks, and fungi. The main commercial source of chitin is crustacean shells (especially crab and shrimp), which are waste products of the seafood industry and are available at relatively low cost. Recent studies have shown that the degradation of chitin creates reducing conditions that can promote and sustain anaerobic-reductive processes similar to denitrification (Brennan et al., 2006a,b; Vera et al., 2001). Chitin fermentation has been shown to produce volatile fatty acids (VFAs), some alcohols, and ammonia. VFAs and alcohols can be used by denitrifying bacteria as electron donors, while ammonia is the preferred nitrogen source for bacterial growth (Rittmann and McCarty,

* Corresponding author. Tel.: +1 814 865 9428; fax: +1 814 863 7304.
E-mail address: rbrennan@engr.psu.edu (R.A. Brennan).

2001). Under aerobic conditions, the production of ammonia during in-situ remediation of nitrate could be undesirable due to potential nitrification. However, groundwater treated with chitin rapidly becomes anoxic due to fermentation processes, effectively eliminating the possibility of ammonia oxidation. Nonetheless, care should be taken when applying chitin in-situ to ensure that ammonia is consumed in the anoxic treatment zone and does not negatively impact downgradient aerobic waters.

It was hypothesized that chitinous material from crab shells can serve as an electron donor source to support biological denitrification at rates competitive with other organic substrates previously tested by others. Since the shell of crabs (and other crustaceans) is a waste-product of the seafood industry, its use provides a two-fold benefit: (1) it reduces the concern related to waste material disposal and degradation, and (2) it can reduce the overall cost of bioremediation technologies. The solid, particulate nature of chitin makes it easy to be delivered in-situ as part of a slurry to create a permeable reactive barrier or reactive zone, and once installed requires little maintenance compared with liquid electron donors that need to be continuously pumped into contaminated aquifers. It may also offer an additional support for biofilm development that at the same time acts as a food source. In addition, its particle size and non-swelling nature help to maintain porosity after injection and prevent clogging (Brennan, 2003). The objectives that guided this study were: (1) to evaluate the denitrification rates supported by chitinous material; (2) to determine the effect of operating conditions (i.e. chitin load, flow rate, and influent dissolved oxygen) on denitrification under continuous-flow conditions; and (3) to estimate the longevity of chitinous material as an electron donor source for denitrification.

2. Methods

2.1. Chemicals

All chemicals used in this study were reagent grade or better. Ultra high purity nitrogen gas (UHPNG, MG Industries) was used to purge solutions and samples. Stabilized chitinous material derived from Dungeness crab (ChitoRem[®] SC-20, JRW Bioremediation, LLC, Lenexa, KS), was used as the chitin source. Demineralization and deproteinization of SC-20 was conducted to determine the actual composition (carbon and nitrogen) of the material based on protocols described in previous studies (Beaney et al., 2005; Cira et al., 2002; Percot et al., 2002). Elemental analysis of the SC-20 chitin indicated that the total carbon and nitrogen contents are 23.55% and 6.97%, respectively (Agricultural Analytical Services Laboratory at The Pennsylvania State University). Limestone chips (1–2 mm) used in the batch test were obtained from Prairie Central (Champaign, IL). Washed and ignited, standard Ottawa sand (30–40 mesh, EMD Chemical Inc.) was used as an inert packing material in the column tests.

2.2. Groundwater sources

Natural groundwater used in the batch experiment was obtained from the Bald Eagle formation (Seven Mountains, Centre County, PA). Before use, the groundwater was amended with 0.137 g NaNO₃ or 0.163 g KNO₃ per liter to give a final concentration of 22.7 mg NO₃⁻-N/L, and deaerated with UHPNG for 2 h (final DO ≤ 0.5 mg/L, final pH = 6.5).

Synthetic groundwater was prepared for use in the enrichment cultures and the column study using reagent grade chemicals and milli-Q water (Table 1). Freshly prepared synthetic groundwater

had a pH of 6.5. The dissolved oxygen (DO) content of the synthetic groundwater was modified to the desired value by either bubbling it with laboratory air, or purging it with UHPNG for 2 h for anoxic water (≤0.5 mg O₂/L).

2.3. Inoculum preparation

Approximately 2 weeks before each column experiment was started, enrichment cultures were prepared to obtain an actively denitrifying inoculum. To prepare the enrichments, fresh garden soil (150 mg) and SC-20 chitin (75 mg) were placed in a 160-ml glass serum bottle. The bottle was purged with UHPNG for 15 min before adding 100 ml anoxic synthetic groundwater and then purged for 20 min more. The bottle was then sealed with a butyl rubber stopper and incubated in the dark with continuous agitation on an orbital shaker. After 6–7 d of incubation, when IC analysis confirmed that nitrate had been consumed, 5 ml of the actively denitrifying enrichment suspension was transferred to a new sealed bottle. The transfer bottle (160-ml serum bottle) was prepared with 75 mg of SC-20 chitin and 100 ml of synthetic groundwater as described above.

2.4. Batch experiment setup

A batch microcosm experiment was conducted as a preliminary evaluation of the capacity of chitin to enhance biological denitrification. In addition, the buffering capacity of limestone and the effect of different nitrate salts (i.e. sodium nitrate or potassium nitrate) on denitrification were evaluated by means of a 2^k full factorial experimental design (Table 2). Each set of test conditions containing chitin was conducted in duplicate, and singlet control bottles (without chitin) were also prepared. For each set of conditions, 150 mg garden soil and appropriate amounts of SC-20 chitin and limestone (Table 2) were added. The bottles were then purged for 15 min with UHPNG before adding 150 ml anoxic nitrate-amended natural groundwater and then purged for 20 min more. The bottles were sealed with butyl rubber stoppers and aluminum crimp tops, and incubated in dark at room temperature with continuous agitation on an orbital shaker. Samples (15 ml) were taken periodically and the extracted volume was replaced with new anoxic nitrate-amended groundwater. After the DO and pH were measured, samples were centrifuged (20,000 rpm for 15 min) and filtered (0.45 μm) before being stored for future analyses (4 °C for less than a week for nitrate, nitrite, chloride, phosphate, sulfate, and ammonium, and –10 °C for VFAs).

Table 1

Composition of synthetic groundwater used for growing enrichment cultures and for the column study

Ion	Conc. (mg/L)
HCO ₃ ⁻	36.3
NO ₃ ⁻ -N	22.7
Cl ⁻	14.3
SO ₄ ²⁻	6.9
PO ₄ ³⁻	4.15
BO ₃ ³⁻	0.02
MoO ₄ ²⁻	0.0033
Na ⁺	54
Ca ₂ ⁺	4.09
Mg ₂ ⁺	2.39
K ⁺	2.27
Fe ₂ ⁺	0.10
Mn ₂ ⁺	0.07
Zn ₂ ⁺	0.01
Co ₂ ⁺	0.01
Ni ₂ ⁺	0.01
Cu ₂ ⁺	0.01

Table 2
Full factorial (2²) design matrix for the batch experiment

Treatment No.	Bottle No.	Chitin	Limestone	Na ⁺	Chitin added (g)	Limestone added (g)	Nitrate salt added	
							(g)	formula
Active 1	1,2	+	+	+	0.075	0.375	0.137	NaNO ₃
Active 2	3,4	+	+	–	0.075	0.375	0.163	KNO ₃
Active 3	5,6	+	–	+	0.075	0	0.137	NaNO ₃
Active 4	7,8	+	–	–	0.075	0	0.163	KNO ₃
Control 1	9	–	+	+	0	0.375	0.137	NaNO ₃
Control 2	10	–	+	–	0	0.375	0.163	KNO ₃

2.5. Column test setup

Duplicate columns were constructed using 5.08 cm diameter by 30.48 cm long PVC threaded pipe nipples with end caps (United States Plastic Corp.). The columns were equipped with five lateral sampling ports spaced evenly along the length of the columns. Nylon bags filled with 3 mm diameter glass bead were installed into the end caps of the columns as support for the packing material. A polypropylene mesh disc (55 mm diameter, 149 µm pore size, Spectra/Mesh®) was placed above each glass bead bag, near the packing material at both ends of the columns, to prevent the loss of small particles contained in the SC-20. The packing material, consisting of a mixture of 100 g of SC-20 and approximately 600 g of sand, was placed in each column using a wet packing procedure. To ensure that the total length of each column was filled with packing material, additional sand was added at the top when necessary. The influent reservoir was connected to the columns using Tygon® laboratory tubing. The flow rate was controlled by means of a peristaltic pump (Masterflex L/S, Cole Parmer). After the packing procedure, the columns were flushed with the influent water in the upward direction for five pore volumes, until the sea salt from the surface of the SC-20 particles was flushed out and the effluent chloride concentration returned to background values. The columns were then dosed in line with 5 ml of inoculum and operated in a closed-loop mode for 1.2 d (inoculation period). Following the inoculation period, effluent lines were opened and synthetic groundwater was continuously pumped through the columns at a flow rate of 0.45 ml/min (Darcy velocity = 0.32 m/d). The columns were operated for 212 d. Samples (~15 ml) were taken periodically from the effluent line and analyzed as previously described. To examine the effect of chitin treatment on water quality from a biological perspective, separate effluent samples for total bacterial counts (1 ml) were periodically taken using aseptic techniques, and were immediately diluted, plated, and incubated. Additional samples (2 ml) were also taken from the side ports using plastic syringes. The extracted volume was filtered (0.45 µm) before being stored at –10 °C for future analyses (nitrate, nitrite, chloride, phosphate, sulfate, VFAs, and dissolved carbon). Tracer (NaCl) tests were conducted in both columns before they were taken out of operation. One of the columns was subsequently sacrificed to obtain samples to visualize the biofilm attached to both sand grains and crab shell particles by means of Scanning Electron Microscopy (Column 1), and the other column was sacrificed to measure the remaining chitinous material (Column 2).

2.6. Analytical methods

Electrodes were used to measure pH (Accumet® BASIC, AB15 connected to a Thermo-ORION pH probe), DO (Accumet® RESEARCH, AR40 equipped with a YSI BOD probe), and ammonium concentrations (ISE ORION 9512). Nitrate, nitrite, chloride, phosphate, and sulfate ions were measured by ion chromatography (IC, Dionex DX-100), the analytical procedure for which is described elsewhere (APHA, 2005). VFAs were determined by high performance liquid

chromatography (HPLC, Waters 2695 separation module) using a Photodiode Array Detector (Waters 2996). Filtered samples (1.5 ml) were acidified with 0.15 ml of 2N sulfuric acid for the analysis. Separation and elution of the VFAs was carried out on an analytical column (HPX-87H, Biorad) using a 0.005 M sulfuric acid eluent at a flow rate of 0.6 ml/min. Dissolved carbon (DC) was measured using a TOC analyzer (Shimadzu TOC-V CSN). Samples were neither acidified nor sparged during analysis. Total bacterial counts were made following the heterotrophic plate count method (HPC, APHA, 2005) with R2A agar (Difco Laboratories). Colony forming units (CFU) were counted after 7 d of incubation at 28 °C. Total carbon and nitrogen analyses were conducted by combustion, using Fisons NA 1500 Elemental Analyzer, at the Agricultural Analytical Services Laboratory at The Pennsylvania State University.

2.7. Sample extraction for SEM

Scanning electron microscopy (SEM) was used to visualize the biofilm attached to the sand and chitin grains throughout Column 1 at the end of the experiment. Glutaraldehyde (2.5%) in phosphate buffer saline solution (10 mM Na₂HPO₄/NaH₂PO₄ and 150mM NaCl, pH 7.4) was used as a fixation agent. Biofilm was fixed inside the column by passing the fixation agent through the column and letting it stand overnight. The column was then opened to obtain samples of packing material from between each of the lateral sampling ports. Extracted samples were preserved in the fixation agent for 4 d at 4 °C before final preparation, following the protocols of the Scanning Electron Facility at The Pennsylvania State University, prior microscopic visualization. Images were taken using a JEOL JSM5400 (Peabody, MA) scanning electron microscope. Digital images were collected using IMIX-PC v.10 software (PGT, Princeton, NJ).

2.8. Analysis of remaining chitinous material

The chitinous material remaining in Column 2 at the end of the experiment was quantified as follows. Samples of the packing material from between each of the side sampling ports were taken and preserved at 4 °C for 30 d. Biofilm was detached from the particles by sonication and washed out with milli-Q water. Washed packing material was dried at 105 °C for 24 h, then weighed and ashed at 550 °C for 3.5 h. Remaining ashes were dissolved and removed from sand using a 1+1 hydrochloric acid solution. The sand was then washed with demineralized water, dried at 105 °C for 24 h, and weighed. The remaining chitinous material was then calculated by weight difference.

3. Results

3.1. Batch tests

Statistical analysis of the measured parameters revealed that there were no significant differences between the four treatments

evaluated in the batch test ($\alpha = 0.05$). Therefore, all eight active bottles were treated as replicates. Nitrate concentration decreased below the MCL after 7 d (Fig. 1). Nitrate was completely consumed after 13 d. Some nitrite accumulation was observed; however, less than 15% of the nitrate-N initially added was converted to nitrite and accumulated nitrite was reduced to below the detection limit (0.05 mg/L) after 13 d in all but one of the active bottles. Ammonium was gradually released, reaching a maximum of 5–6 mg N/L after 13 d (Fig. 1). In contrast, no appreciable changes in any nitrogen species concentrations were observed in the control bottles. The overall denitrification rate in the active bottles was 2.40 ± 0.20 mg N/L-d (p -value = 0.00). This value was calculated assuming zero-order kinetics and using the concentrations of total soluble oxidized nitrogen species (nitrate + nitrite) measured during the first 7 d. Negligible accumulation of gaseous by-products of the reaction (i.e. NO, N₂O) was also assumed.

Volatile fatty acids were not detected to any appreciable extent during the batch experiments. Acetate, formate, and caproate were detected on the 13th day in some of the treatment bottles, but most concentrations were below the working range of the instrument calibration curve (<0.1 mM, data not shown). The pH in the active bottles rapidly increased, from pH 6.5 to a maximum pH 8.5 in 4 d, and remained fairly constant throughout the rest of the experiment (Fig. 1). Lesser pH changes in the control bottles were observed, reaching a maximum of 7.0–7.5 after 4 d (data not shown).

3.2. Column test results

3.2.1. Initial flushing and inoculation periods

Soluble components of SC-20 were rapidly released in the column experiment, causing high concentrations of anions (chloride and sulfate), ammonium, and some VFAs. An initial flushing period of 3 d was necessary to allow the effluent salt concentrations to decrease to influent values (data not shown). Some nitrate reduction with simultaneous, but low, nitrite accumulation was observed during the flushing period. The inoculation period was extended for only 1.2 d, due to a high gas (presumably N₂) accumulation in the lines. After this period, complete nitrate reduction was observed, but some nitrite also accumulated (0.5–1.8 mg N/L) during the first 12 d of continuous operation.

3.2.2. Nitrogenous species

Complete denitrification was achieved in both columns on the 13th day after inoculation (Figs. 2A and B). The complete denitrifi-

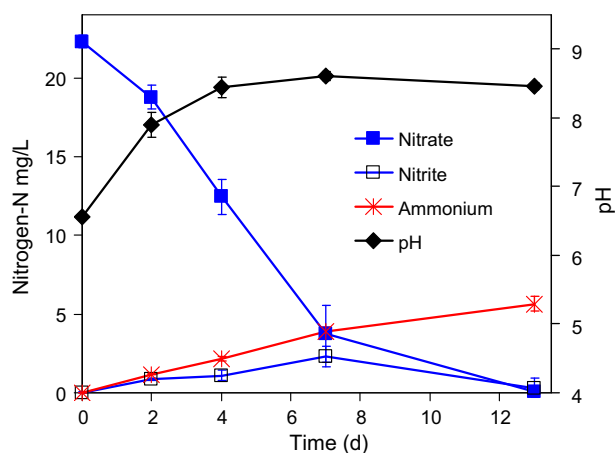


Fig. 1. Change in pH, nitrate, nitrite, and ammonium concentrations over time for the batch test. Data points represent averages from eight replicates; error bars represent 1 standard deviation.

cation period lasted for 130–167 d in Columns 1 and 2, respectively.

The analysis of aqueous port samples showed that complete nitrate reduction was achieved in the front end of both columns shortly after inoculation (Fig. 3 A and B). As the chitin was exhausted, nitrate breakthrough along the lateral ports always occurred sooner in Column 1 than in Column 2. Simultaneous with nitrate reduction, some nitrite accumulation was observed. Nitrite accumulation tended to reach its maximum (between 5 and 8 mg N/L) when nitrate reduction started to be incomplete (Fig. 3 C and D). Nitrite accumulation tended to be higher in Column 2 than in Column 1.

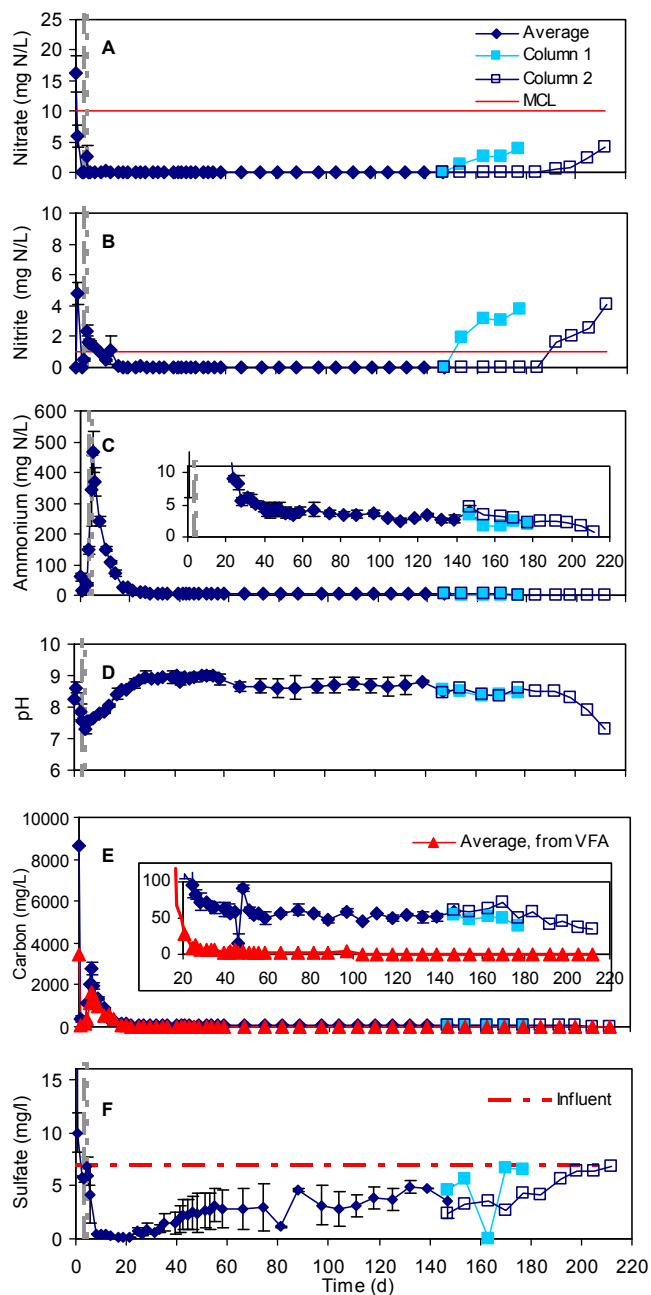


Fig. 2. Change in effluent nitrate (A), nitrite (B), ammonium (C), pH (D), measured dissolved carbon (E), and sulfate (F) over time in the column test (note the different y-axis scales). Solid triangles in panel E represent total carbon concentration from measured VFAs. Vertical dashed and dotted lines indicate the end of flushing and inoculation periods, respectively. Data points represent duplicate averages; error bars represent 1 standard deviation.

Significant amounts of ammonium were released during the inoculation period. Maximum ammonium concentrations of approximately 470 mg N/L were observed two days after inoculation period. After this, ammonium concentrations rapidly decreased to values near 6 mg N/L in 23 d. Concentrations continued to decrease thereafter, but at a much slower rate (Fig. 2C).

3.2.3. Volatile fatty acids and dissolved carbon

VFA and DC concentrations in the effluent of the columns followed a very similar pattern to ammonium, indicating the hydrolysis and fermentation of protein and chitin from the crab shell. A quick release of formate occurred during the flushing period, reaching a maximum concentration of 117 mM. After inoculation, acetate and butyrate were the most dominant VFAs, reaching maximum values (30 and 12.6 mM, respectively) after 5 d. Concentrations rapidly decreased to less than 1 mM after 28 d. No VFAs were detected in the effluent after 100 d of operation (data not shown). During VFA analysis by HPLC, an unknown component was observed to elute at approximately the elution time of caproate throughout the experiment. However, DC results and mass balances calculations indicated that this component could not be caproate; therefore, it was not taken into account in the final analysis. This “mystery” component may have been an artifact of chitin fermentation, and has been observed by our research team previously (Brennan, 2003).

Very high DC concentrations were observed at the beginning of the test (day 0). Carbon decreased during the flushing period, and started increasing again during and after inoculation. At any time, the measured DC concentration was higher than the total carbon released from VFAs (Fig. 2E). While no VFAs were detected after 100 d, a DC concentration of about 50 mg/L was relatively stable until near the end of the test. A slight decrease in DC concentrations was observed to be coincident with nitrate/nitrite breakthrough.

3.2.4. Other observations

Coincident with the high release of VFAs, pH values at the beginning of the test rapidly decreased from pH 8.6 down to pH 7.3 after inoculation, and then rapidly increased and remained rel-

atively stable at pH 8.5–9.0 (Fig. 2D). It was also observed that for Column 2, pH values started to decrease considerably when the nitrate/nitrite breakthrough occurred.

No sulfate was detected in the effluent of either column during the first 37 d after inoculation (Fig. 2F). After this period some effluent sulfate was observed but the concentrations were always lower than those in the influent. The gradual increase in sulfate over the course of the experiment may be indicative of a loss of reducing power throughout the test due to the consumption of substrate.

All heterotrophic plate counts from effluent samples were of the order of 10^6 CFU/ml (data not shown). Gram staining and microscope visualization indicated that most colonies were formed by gram negative, rod-shaped bacteria. Some gram negative cocci and gram positive rod-shaped bacteria were also observed. SEM Micrographs from biofilm samples collected after column exhaustion (Fig. 4) show at least two different morphologies (rods and cocci) colonizing the packing material, with a higher diversity on SC-20 particles than on sand grains. It was also noticed that not all particles were colonized and that most of the observed cells were localized in clusters.

The chitinous material remaining in exhausted Column 2 consisted mainly of coarse particles (> 1 mm). The physical appearance of these particles resembled that of chitinous materials obtained after chemical deproteinization (obtained from JRW Bioremediation, LLC). After calcination and acid wash, the remaining SC-20 corresponded to approximately 38% of initial load by mass.

3.2.5. Nitrogen and carbon balances

Nitrogen and carbon balances were made based on the initial mass of chitin that was used to pack the columns (100 g) and its original nitrogen and carbon content. For the nitrogen balance, effluent nitrate, nitrite, and ammonium concentrations were integrated over time to obtain output nitrogen mass, and it was assumed that ammonium was the nitrogen source utilized for bacterial growth (Table 3). For the carbon balance, the total carbon content of the SC-20 chitin was used as the input and the effluent DC concentrations were used to calculate the output (Table 3). Since DC comprises all carbon species (organic and inorganic), it

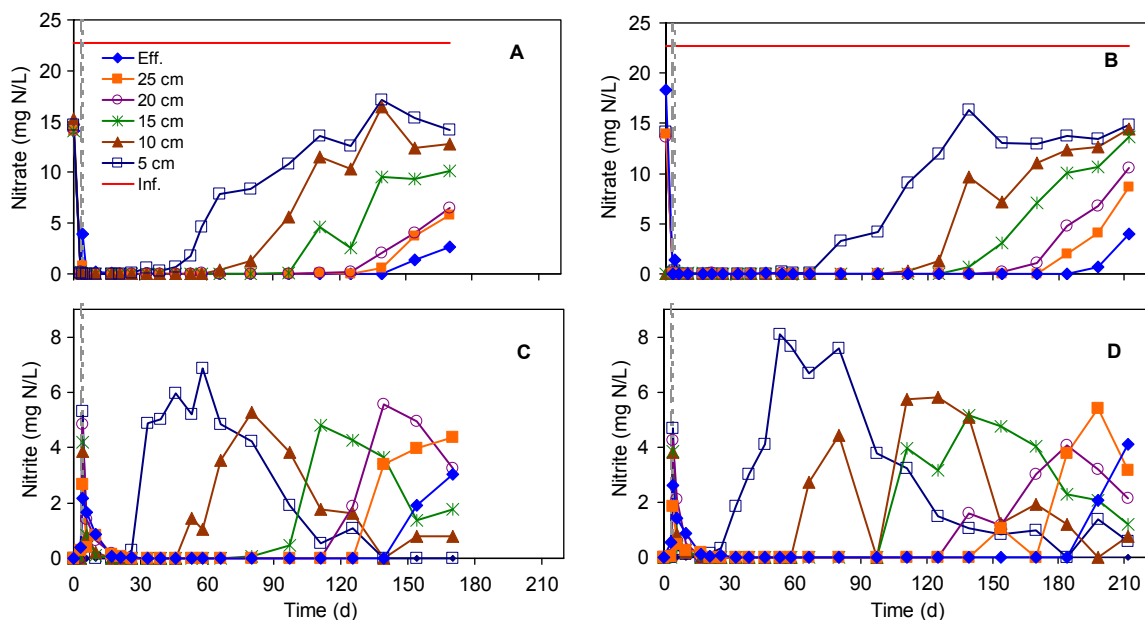


Fig. 3. Change nitrate and nitrite concentrations in side ports over time in the column test. (A): nitrate in Column 1; (B): nitrate in Column 2; (C): nitrite in Column 1; (D): nitrite in Column 2 (note the different y-axis scales). Numbers in the legend indicate distance from the influent. Vertical dashed and dotted lines indicate the end of flushing and inoculation periods, respectively.

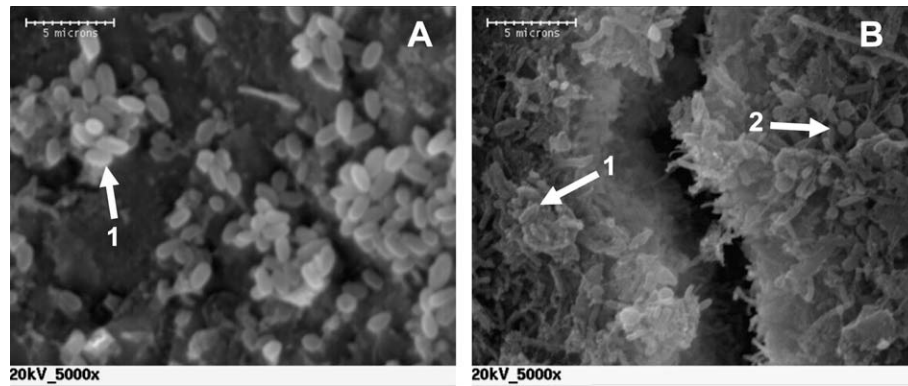


Fig. 4. SEM micrographs of the biofilm developed onto (A) a sand grain, colonized by clusters of rods (1), and (B) a cracked SC-20 particle, colonized by a more diverse assemblage of rods (1) and cocci (2). Samples were taken after column exhaustion. Bar represents 5 μm .

Table 3
Nitrogen and carbon mass balances for the column test

Mass (mg) of		Column 1	Column 2
Nitrogen			
IN	N from SC-20	3970	3970
	N from influent	2608	3124
OUT	N from effluent nitrite	68	57
	N from effluent nitrate	60	41
	N from effluent ammonium	2479	2443
RXN	N consumed for nitrate and oxygen reduction	946	1149
TOTAL	total N input	6578	7094
	total N output	2607	2541
Carbon			
IN	C from SC-20	23550	23550
OUT	C measured from effluent DC	18489	19795

was not necessary to take microbially mediated conversion of carbon into account.

4. Discussion

4.1. Batch tests

4.1.1. The effect of chitin addition on denitrification rates

The calculated denitrification rate in this study (2.4 mg NO_3^- -N/L-d) is comparable to or higher than those which have been previously reported for other substrates. Reported denitrification rates with mixed VFAs as the primary electron donor source for denitrification in wastewater range from 0.054 to 0.754 g NO_3^- -N/gVSS-d (reviewed in Elefsiniotis et al., 2004; VSS: volatile suspended solids). A direct comparison with our rates is not possible since the rates in Elefsiniotis et al. (2004) are expressed in terms of the biomass present in the system and the initial biomass was not reported. However, it is likely that the biomass concentration in our groundwater-amended microcosms was significantly lower than that typically found in wastewater, indicating that we may have achieved a higher overall denitrification rate. A more direct comparison of denitrification rates can be obtained from Devlin et al. (2000), who reported an initial denitrification rate of 0.55 mg NO_3^- -N/L-d for indigenous microorganisms from a nitrate-contaminated site using acetate as the electron donor – four times lower than our observed rate with crab-shell chitin.

4.1.2. The effect of limestone and chitin on pH

Limestone is commonly used as a source of alkalinity to maintain an appropriate pH in water treatment processes. However,

limestone did not appear to significantly affect pH in the batch test. An additional test was conducted to differentiate the pH effect of chitin and limestone addition separately. Results indicated that the effect of chitin addition on pH is greater than the effect of limestone addition and that SC-20 alone supplies enough buffering capacity to maintain the pH within appropriate values for denitrification. The increase in pH observed in this study could be due to one or more of the following processes: (1) the generation of alkalinity from nitrate reduction; (2) the dissolution of calcium carbonate from the crab shells (demineralization results indicate that SC-20 contains approximately 78% mineral matter by mass); and (3) the release of the weak base ammonium from chitin.

4.2. Column tests

4.2.1. Initial phase

During the initial 3 d (5 pores volumes) of operation, approximately 14–20 mg of chloride, 0.4–1.0 mg of sulfate, 0.4–0.9 mg of ammonium (as N), and 2–10 mg of formate (as C) were released per gram of SC-20. This observation is not surprising given that SC-20 is derived from marine organisms, in which chitin is closely associated with proteins, lipids, pigments, and marine salts (reviewed in Beaney et al., 2005, and Percot et al., 2002). In future applications, it may be beneficial to wash the material prior to use, to eliminate analytical interferences due to high salt content released from the crab shell.

4.2.2. Nitrate and nitrite reduction

Previous column studies using alternative cellulose-rich materials, like newspaper, cotton, and wheat straw to support denitrification have been reported (Soares and Abeliovich, 1998; Volokita et al., 1996a,b). Under similar residence times, chitin was able to support denitrification with about the same column life time as newspaper, even though the chitin mass was one-fourth that of newspaper (Table 4). Results reported using cotton as a substrate show higher denitrification rates than the ones obtained in this study (Volokita et al., 1996a, Table 4). The cause of this difference is likely due to differences in the density and diversity of the bacterial community obtained from different natural environments (i.e. garden soil from different sites). The use of a known denitrifying bacterium or inoculum taken from a nitrate-rich environment could make the denitrification process more efficient. Despite the high initial release of carbon and ammonium, chitin may offer two major advantages over the other substrates: longevity and ease of delivery.

The total nitrate that was removed varied from 26–31 mg N per g of SC-20. Nitrate and nitrite profiles from the port samples indi-

Table 4
Comparison of results from the column test with previous studies

Electron donor	Chitin	Newspaper	Cotton
Mass (g)	100	400	380
Applied nitrate concentration (mgN/L)	22.7	22.5	Up to 68 45
Retention time (h)	16.2	16.5	20.6 11.8
Maximum denitrification rate (mg N/L _{col} -d)	24.5	32.7	79.1 91.6
Column life time (d)	130–167	–160	–210
Source	This study	(Volokita et al., 1996b)	(Volokita et al., 1996a)

cate different kinetics. In Column 1, both oxidized species were efficiently consumed, while in Column 2 more nitrate was reduced at the expense of nitrite accumulation. A number of factors could have caused the observed differences. The heterogeneous nature of SC-20, as well as differences in the concentration and diversity of the used inoculum are likely to be responsible for the different nitrate removal rates.

4.2.3. Ammonium and carbon species

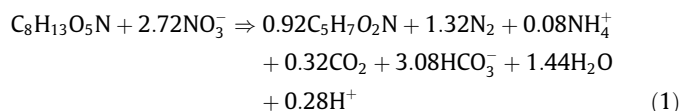
Unlike what was observed during the batch test, high concentrations of ammonium and VFAs were observed to be released during the column test. One of the main differences between the two tests is the liquid-to-solid ratio: 2000 and 3.9 ml per gram of chitin in the batch and column tests, respectively. This could be the cause of such different concentration ranges. The VFAs (acetate, butyrate and propionate) detected after inoculation are in agreement with what has been reported in previous studies using chitin (Brennan et al., 2006b; Vera et al., 2001). The coincidence between ammonium and VFA profiles indicates the occurrence of chitin/protein breakdown and fermentation. Breakdown and fermentation of chitin-associated proteins in crustacean shells have been previously reported, and have shown that up to 70% of the initial protein content is removed during the first 7 d of fermentation (Cira et al., 2002; Oh et al., 2000; Yang et al., 2000). These observations indicate that the initial high VFA and ammonium release in this study is due more to the rapid degradation of protein in the crab shells, rather than to actual chitin degradation. Analysis of hydrolysates of chitin-associated proteins have shown that the most common amino acids consist of short carbon chains (4 or less carbons, Iijima et al., 2005; Shahidi and Synowiecki, 1991). This could explain the prevalence of short chain fatty acids in the effluent of the columns in this study.

Effluent DC concentrations reveal that other organic compounds, different from VFAs, were also released. During the beginning of the test, it is likely that some non-hydrolyzed proteins, lipids, and carotenoids associated with SC-20 were released in the effluent, and played a major role in increasing the DC levels. Once those compounds were flushed out and protein degradation ceased, it is more likely that the soluble products of microbial activity (Rittmann and McCarty, 2001) started to become more important. The relatively stable DC concentrations after 50 d of operation may indicate that the columns reached a pseudo-steady state until the available carbon from the chitin supply neared exhaustion. Then DC levels started to decrease.

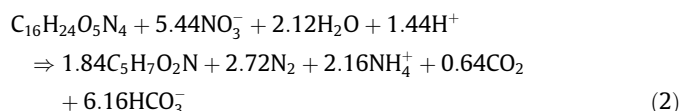
The maximum nitrogen removal using chitinous material as an electron donor source was estimated for this study by assuming that the only products of chitin and protein degradation are acetate and ammonium, and that energetics follow standard denitrification metabolism (Rittmann and McCarty, 2001). Based on the over-

all stoichiometry (Eqs. (1) and (2)), a maximum of 187 mg of nitrate-N can be reduced per gram of pure chitin, and 216 mg of nitrate-N reduced per gram of pure protein. However, based on the composition of SC-20, which is only 10% chitin and 12% protein, the maximum mass of nitrate that could have been reduced is approximately 45 mg nitrate-N per gram of SC-20. Due to the high nitrogen content in the protein, the theoretical ammonium that would be released from chitin + protein is about 70% higher than what would be assimilated by denitrifiers, and hence, our observation of excess ammonium in the effluent is not unexpected.

Denitrification with chitin:



Denitrification with protein:



The high ammonium and organic matter levels in the effluent, due to rapid protein degradation, may indicate a potential drawback in the use of chitinous material for denitrification in highly aerobic aquifers. In other applications (such as in-situ bioremediation of chlorinated solvents, for example), it could be beneficial to have enhanced carbon and nutrient delivery at the beginning of the process to stimulate bacterial activity and decrease the duration of a lag period. Once the protein content is almost exhausted, chitin starts to play a major role as the electron donor source. The electron donor release from chitin is slow enough to maintain reducing conditions with minimum carbon accumulation. This may represent a major advantage of this solid substrate over other alternatives.

Additional post-chitin treatment (for example, aeration or air stripping) may be necessary to improve effluent water quality if the nitrogen demand of the microbial community surrounding the zone of treatment is insufficient to consume the excess fermentation products. Fortunately, since chitin addition increases pH, it will facilitate the removal of ammonium by air stripping. An alternative solution would be a pretreatment of the material before use, which includes washing and/or deproteinization. Such material is already commercially available, although it retails for higher cost (JRW Bio-remediation, LLC). High bacterial counts were observed in effluent samples; therefore, additional filtration and/or disinfection of chitin-treated water would also be necessary if potable water quality is desired.

4.2.4. Carbon and nitrogen balances

Although the two columns behaved somewhat differently, mass balances were very similar. Output nitrogen mass was only 36 to 40% the total input, and it was mainly due to ammonium release. In this test, the total ammonium that was released was only approximately 62% of what was expected from the degradation of SC-20. However, if the theoretical ammonium needed for the reduction of nitrate and oxygen is taken into account, the percentages increase to about 88%. Similarly, the carbon output is about 81% of what was expected from the complete degradation of the SC-20.

Carbon and nitrogen balances show a high percentage of recovery of these elements. This indicates that the packed SC-20 was almost exhausted by the time nitrate/nitrite breakthrough occurred. Considering that both columns had the same initial organic load, and that their mass balances were very similar, differences in their longevity are likely due to divergences in

hydraulics and microbial activity. During this study, no gaseous nitrogen species (i.e. NO, N₂O, and N₂) were measured, so it was expected that the input nitrogen mass would be higher than the sum of the effluent plus the consumed nitrogen. The lack of mass balance for carbon may be due to the loss of some carbon (especially CO₂) during sample preparation for DC analysis. Finally, it is possible that residual carbon and nitrogen on the particles consist of somewhat recalcitrant compounds that are difficult for microorganisms to metabolize. Therefore, these compounds would lead to little carbon release into the effluent.

It is also important to clarify that the SC-20 particles also contain high amounts of minerals (up to 78% by mass). Crabs incorporate these minerals into their shells for rigidity and strength (Percot et al., 2002). Therefore, the exhaustion of the electron sources did not lead to the disappearance of the material. In addition, since the packing material consisted of a mixture of SC-20 particles and sand, the columns only experienced a very small decrease in their packed length after exhaustion (<2 cm).

4.2.5. Engineering significance

The findings of this study increase the understanding of how chitinous materials can be used as alternative electron donor sources to support bioremediation processes. Given the potential of chitin as electron donor source, it would be interesting to evaluate its performance for denitrification under different operating conditions. Similar chitin loads could be used to treat water contaminated with higher nitrate concentrations. For example, wastewaters from the metal finishing and nuclear weapons production industries contain extremely high nitrate concentrations (greater than 1,000 mg N/L), and would benefit from nitrate reduction despite a relatively minor increase in ammonium. Chitin could also be used to treat the rejection effluent from reverse osmosis or ion exchange technologies, in which the nitrate concentration is higher than the one used in this study. Alternatively, raw chitin could also be diluted by mixing it with other solid electron donor sources so that the proportion of carbon to nitrogen is reduced to a level that is appropriate for enhancing denitrification, without leaving an undesirable ammonium residual in the treated water.

5. Conclusions

In summary, this study demonstrated that SC-20 chitinous material provides electron donors that can be used to enhance biological denitrification. In the column tests, SC-20 was able to support complete denitrification for several months at rates that were comparable with previous studies with other solid-phase electron donors. The longevity of this material is also competitive with what has been reported for different cellulosic substrates. SC-20 also provided enough buffering capacity to maintain an alkaline environment and reducing conditions, suitable for the activity of denitrifiers. On average, 28.5 mg of nitrate-N can be removed per gram of SC-20 under continuous operation. Degradation of SC-20 appears to involve rapid degradation of protein, followed by a slower chitin breakdown. Removal of excess ammonium and organic matter during the initial application of SC-20 may be needed, depending on the demand of the surrounding microbial community near the zone of treatment.

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