APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1976, p. 808-818
Vol. 32, No. 6

Printed in U.S.A.

Nitrate Removal in Closed-System Aquaculture by Columnar Denitrification

W. L. BALDERSTON[1]* AND J. McN. SIEBURTH

Graduate School of Oceanography and Department of Microbiology, University of Rhode Island, Kingston, Rhode Island 02881

Received for publication 19 May 1976

The columnar denitrification method of nitrate-nitrogen removal from high-density, closed system, salmonid aquaculture was investigated and found to be feasible. However, adequate chemical monitoring was found to be necessary for the optimization and quality control of this method. When methanol-carbon was not balanced with inlet nitrate-nitrogen, the column effluent became unsatisfactory for closed-system fish culture due to the presence of excess amounts of nitrite, ammonia, sulfide, and dissolved organic carbon. Sulfide production was also influenced by column maturity and residence time. Methane-carbon was found to be unsatisfactory as an exogenous carbon source. Endogenous carbon could not support high removal efficiencies. Freshwater columns adapted readily to an artificial seawater with a salinity of 18‰ without observable inhibition. Scanning electron microscopy revealed that the bacterial flora was mainly rod forms with the Peritricha (protozoa) dominating as the primary consumers. Denitrifying bacteria isolated from freshwater columns were tentatively identified as species of *Pseudomonas* and *Alcaligenes*. A pilot plant column was found to behave in a manner similar to the laboratory columns except that nitrite production was never observed.

Aquaculture is being used increasingly as a method of food production. A large majority of fish aquaculture operations employ a flow-through or open-system type of water use whereby metabolic products are discarded into a nearby body of water. However, evidence (19) indicates that inorganic nitrogen (a major metabolic product of open systems) is the growth-limiting nutrient of coastal and estuarine phytoplankton and thus the cause of eutrophication.

An alternative to the open-system is the closed-system, in which 95 to 100% of the culture water is recirculated past the organisms being cultured. This type of system has been used to a limited extent in salmonid hatcheries (15), but little experience has been gained with high-density, closed-system aquaculture. One of the major problems and requirements in this type of system is the removal of metabolic wastes and their breakdown products. For fish, ammonia and urea have a toxic effect if allowed to accumulate in the culture medium (12). Ammonia removal is usually accomplished by nitrifying bacteria, which are readily enriched on aerated surfaces in a "biological filter" (16). However, the use of nitrification results in a slow but gradual increase (depending on the density of the fish) in nitrate-nitrogen; nitrate-nitrogen is reported to be toxic to fish at levels of 181 mg of NO_3^1-N/liter (12).

Many methods of nitrate removal from various types of wastewater have been investigated: nitrate assimilation from agricultural drainage water in shallow ponds by algae (4) and bacterial assimilation in sewage sludge mixtures (8) are a few examples. Anaerobic filter systems, utilizing a denitrifying microbial population attached to an inert substrate under anaerobic conditions, have also been studied (5). However, the quality of the filter effluent, other than nitrate content, has not been thoroughly investigated.

The purpose of the current study was to test whether a columnar denitrification filter could be used in high-density, closed-system salmonid aquaculture to control the level of nitrate-nitrogen and to maintain overall water quality control as it applies to this type of aquaculture. Laboratory columnar filters and their attached microbial flora were studied in terms of their development and the quality of the effluent produced. Pilot plant columns were also studied for their similarities to the laboratory columns.

[1] Present address: Chemclene Corp., Box 26, R.D. #1, Malvern, PA 19355.

MATERIALS AND METHODS

Aquaculture system. Atlantic and chinook salmon (*Salmo salar* and *Oncorhynchus tschawytscha*, respectively) were raised from fingerlings to 20 to 25 cm in length (about 500 g each) in the aquaculture facility at the University of Rhode Island, Kingston, R.I., in a high-density, partially closed system during the period of this study (17). The inorganic nitrogen transformations that took place in this aquaculture system were as follows: ammonia was produced in the system either directly or indirectly (from urea) by the fish or by the decomposition of unused food, and nitrification took place constantly in highly aerated biological filters, resulting in a nitrate build-up. This system was used as the source of the nitrate-rich aquaculture water used throughout the study and was a freshwater system at all times. Nitrate-nitrogen in this system was measured daily and usually ranged between 50 to 100 mg/liter; in some instances this level was lower, and nitrate (as KNO_3) was added to adjust the level back to the 50 to 100 mg of NO_3^--N/liter range prior to the use of the water as the laboratory denitrification column influent.

Laboratory apparatus and experiments. The laboratory denitrification column and associated apparatus are illustrated in Fig. 1. The large or inlet end of the 1.5-liter column was fitted with a neoprene stopper through which the appropriate fittings and instruments were inserted. The entire column was covered with aluminum foil to exclude all light. The 13-liter reservoir was fitted so that its contents could be sparged with any kind of gas. Salmon culture water was deoxygenated in this reservoir and then pumped down through the denitrifying column. A methanol solution was injected into the culture water just before the column inlet. Gases produced by denitrification accumulated at the top of the column and were removed through the cotton-plugged gas vent with the gas-sampling syringe. This was done at regular intervals to avoid significantly altering the column void volume.

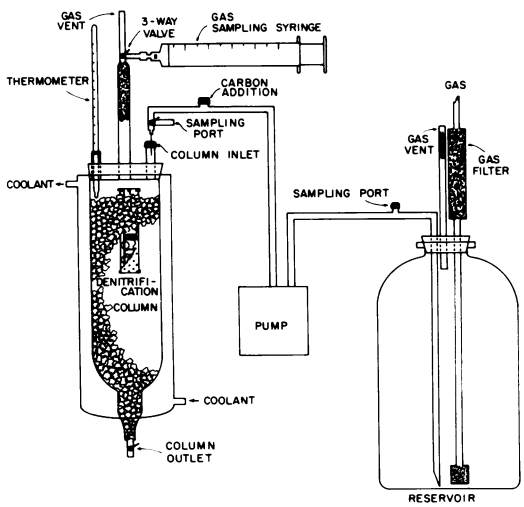

FIG. 1. *Laboratory denitrification column and supporting apparatus: detail in the column shows the substrate sampling apparatus used to obtain samples for the SEM.*

Laboratory columns were filled with 11-mm limestones. These stones were collected from the nitrifying prefilter of the aquaculture system (17), graded as to size, and inserted in the column within 1 h. Some columns were discarded after one experimental run, whereas others were operated for long periods of time (3 months) and up to three experimental runs. A continuous record of each column was kept so that its history was known prior to experimentation. All columns were run at 20°C (± 1°C) and 120-min residence times (the time required to pump one void volume through the column), unless otherwise noted.

During the selection and enrichment experiments and throughout most of the laboratory portion of the study, methanol was used as the exogenous carbon source. In all experiments of this type, a six-channel peristaltic pump (Extracorporeal Medical Specialities, Inc.) employing silicone-rubber pumping chambers and silicone-rubber connecting tubing was used to pump the culture water through the column and to add the methanol. Nitrogen was used as the reservoir sparging gas in these experiments. This procedure was checked for its ability to remove oxygen with a polarographic oxygen electrode (Yellow Springs Instrument Co.). The water in the reservoir was replaced daily with fresh culture water from the aquaculture system. Before fresh culture water was introduced into the reservoir it was analyzed for NO_3^--N. It was found that the NO_3^--N concentration in the reservoir did not change appreciably during the 24-h period. The initial NO_3^--N concentration thus measured was used to determine how much methanol to add to arrive at the desired C:N ratio.

In experiments using methane as the carbon source, methane (technical grade; Matheson Gas Products Co.) was dissolved in the culture water (as close to saturation as possible) through the gas sparging apparatus in the reservoir. A Brewer automatic pipetter (Baltimore Biological Laboratory) with glass connecting tubing was used to pump the methane-saturated water without significant loss of methane.

In experiments investigating the effect of changing the salinity on columnar dentrification, artificial seawater (ASW) salts (W. L. Balderston, Ph.D. thesis, University of Rhode Island, Kingston, 1974) were added to the reservoir in appropriate amounts. The salinity of the inlet water after methanol injection was checked with an optical salinometer (American Optical Instrument Co.) to confirm the correct salinity.

Pilot plant apparatus and experiments. A pilot plant column was studied for its similarities with the laboratory columns. It consisted of a fiberglass cylinder (height, 3.7 m; inner diameter, 0.61 m) closed at the bottom end. The column was designed so that samples could be withdrawn from various heights within the column through valved sampling

ports placed about 35 cm apart for the entire length of the column. The column was an upward flowing column (water was pumped from the bottom to the top) so that any gas produced was exhausted through the open, top end of the column. The packing material used was plastic Koch Flexirings (25 mm in diameter by 25 mm high; surface area:volume ratio = 0.311). Culture water from the aquaculture system was pumped directly through this column, which was located adjacent to the culture silos. Dilute solutions of methanol (prepared using a hydrometer) were metered into the column inlet water using a large, gear-driven chemical metering pump. The column was designed and operated by the aquaculture facility at the University of Rhode Island and normally operated at a residence time of approximately 200 min.

Analytical techniques. Nitrate-nitrogen and nitrite-nitrogen were analyzed using the method of Strickland and Parsons (22). Nitrate was reduced to nitrite, and nitrite was measured spectrophotometrically after the formation of an azo dye by the addition of sulphanilamide and *N*-(napthyl)ethylenediamine. Sulfide interference in nitrate samples was eliminated by the method of Wood et al. (24). The analyses were performed within 2 h of sampling. Ammonia (including ammonium measured as ammonia) was analyzed using an ammonia probe (Orion Research, Inc.) and the procedure of Barcia (1) within 5 to 30 min of sampling. Samples were analyzed for sulfide by the method of Cline (6).

Samples taken for dissolved organic carbon (DOC) analysis were filtered immediately after collection through a precombusted Gelman type A glass-fiber filter, acidified with 3.0% phosphoric acid (0.2 ml/5-ml sample), and stored at 5°C until analyzed within 2 h. In some cases, DOC samples (filtered and acidified) were stored at −50°C for various periods of time until the analysis could be performed. DOC was analyzed using a modified carbonaceous analyzer (Beckman Instrument Co.) connected to a recorder and a digital integrator (model CRS-208; Infotronics Corp.).

Samples taken for methane analysis were collected with a glass syringe and analyzed within 5 min using a gas chromatograph (model 1700; Varian Associates) equipped with a stainless steel column (6 feet by 1/8 inch; ca. 183 by 0.32 cm) packed with 80- to 100-mesh Porapak Q (Waters Associates, Inc.) and a flame ionization detector (145°C). Helium was used as the carrier gas (60 ml/min). The gas chromatograph was connected to an Infotronics model CRS-208 digital integrator.

Non-pH-corrected oxidation-reduction potential was measured with a combination platinum electrode (Orion Research, Inc.). When measuring the redox potential in an experimental column, the electrodes were inserted for the duration of the experiment. No growth or macroscopic changes of the platinum-sensing element were observed. An inlet electrode was positioned just above the limestone bed, and an outlet electrode was positioned directly in an unexposed (to air) portion of the column outlet.

SEM. Some of the methanol-supplemented laboratory columns were studied in terms of succession of the microorganisms attaching to the glass and limestone substrates using a scanning electron microscope (SEM). For these studies, a special substrate-sampling apparatus was designed so that portions of a substrate could be withdrawn from an active column at any time with minimal disturbance of the column (Fig. 1, detail). This apparatus consisted of a polypropylene drying tube (150 mm by 14 mm [inner diameter]) that was capped at one end and perforated with holes about 3 mm in diameter. This tube was inserted in the limestone bed of the column when the column was first made, with the open end level with the top of the bed and with a loose-fitting plastic cap. Inserted inside the perforated tube (and removable) was a small-diameter glass rod or cane to which were attached seven small plastic shelves (10 mm in diameter). Round glass cover slips (10 mm in diameter) were attached to the cane between the shelves with double-adhesive cellulose tape (3M Co.), and individual limestones were placed on each shelf.

Specimens were prepared for SEM by fixing in 3.0% gluteraldehyde in 0.1 M sodium cacodylate (pH 7.2) for 1 to 5 days at 5°C, fixing in 1.0% osmium tetroxide for 1 h at 22 to 24°C, dehydrating in a graded ethanol series to absolute ethanol, transfering to 100% amyl acetate, and critical-point drying with a critical-point drying system (Sorvall, Inc.). Specimens were observed using a Cambridge S-4 Steroscan SEM (Cambridge Instruments). Photographs were made on Polaroid 55 P/N film.

Microbiological studies. In anticipation of the possibility that the freshwater flora would be lost when the salinity of the culture water was increased, brackish water (15‰ salinity) denitrifying bacteria were isolated from the lower portion of the Narrow River (R.I.), where the salinity averaged 20‰. Samples of sediment, decaying algae, and water were enriched in anoxic medium A (0.01% Trypticase [BBL], 0.01% yeast extract [BBL], an 1.0% KNO_3 in 15‰ ASW). When good growth and/or gas production was evident in these anaerobic enrichments, serial dilutions were plated on medium B agar (0.1% Trypticase, 0.1% yeast extract, and 1.0% KNO_3 in 15‰ ASW) using the spread plate technique. All morphologically different colonies were picked and tested for their ability to denitrify (21) using medium B. Cultures from positive tests were purified by repeated streaking and kept as stock cultures on medium B agar butts at 5°C.

A sample of scraping from some of the stones on the top or inlet end of a freshwater column that used methanol as the exogenous carbon source was taken aseptically, homogenized with glass beads, and placed on medium B agar made with distilled water. All morphologically different colonies were picked and treated in the same way as the brackish water isolates.

Both freshwater and brackish water denitrifying isolates were tested for their Gram reaction and their ability to oxidize or ferment glucose (14). The isolates were observed using phase-contrast light microscopy for living morphology and using transmission electron microscopy (Zeiss EM9S-2) and negative staining (0.3% phosphotungstic acid or

1.0% ammonium molybdate and 2.0% ammonium acetate at pH 5.4) for type of flagellation. In addition, ability to denitrify was confirmed using media with and without nitrate and checked in media of both 0 and 15% salinity.

RESULTS

Column development. Columns filled with prenitrifying filter limestones, perfused with fish culture water containing nitrate and supplemented with methanol, were capable of developing a denitrifying flora that would remove 95 to 100% of the influent nitrate in 20 to 22 days (Fig. 2). The time needed to develop a 100% nitrate-removal capability is dependent to some extent on the amount of methanol added to the influent water, expressed as the methanol-carbon added:nitrate-nitrogen ratio (C:N ratio). When a C:N ratio of 1.0 and above was provided earlier, 100% nitrate removal was achieved as much as 6 days sooner. However, 95 to 100% nitrate removal does not necessarily mean that the column effluent is of a high quality (i.e., quality sufficient for the culture of fish); nitrite was produced in the developing columns and is correlated with C:N ratios of <1.0 as well as time from the start of carbon supplementation. Freshly started columns are capable of removing 5 to 25% of the influent nitrate without an exogenous carbon and energy source (Fig. 2), but this percentage decreases with time.

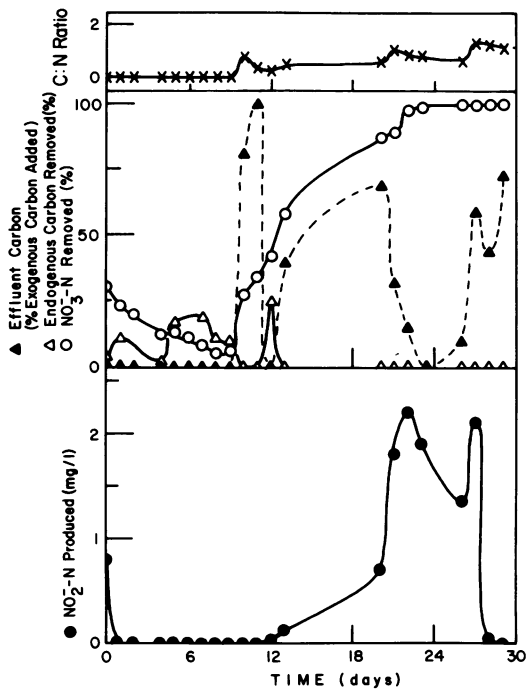

FIG. 2. *Column development: the effect of varying the C:N ratio on NO_3^--N removal, endogenous carbon removal, NO_2^--N production, and effluent carbon in a methanol-supplemented laboratory denitrification column. The average nitrate concentration in the column influent was 68 ± 10 mg of NO_3^--N/liter.*

Effluent carbon (measured as the DOC exclusive of endogenous carbon), expressed as a percentage of the exogenous carbon added, was used in this study as a measure of exogenous carbon utilization. In a developing column, effluent carbon is rarely zero and fluctuates widely (Fig. 2). There can be instances of no exogenous carbon utilization as well as fairly long periods of 25 to 50% utilization. There is some indication in developing columns that effluent carbon is related to the C:N ratio; when the C:N ratio is increased, effluent carbon increases (Fig. 2). The percentage of endogenous carbon removed was calculated by subtracting the effluent DOC from the influent DOC (before methanol addition); negative or zero values were interpreted as zero percent endogenous carbon removal.

SEM of glass and stone substrates in a developing column revealed an extensive, mainly bacterial flora developed in 17 days. Glass substrates seemed to reveal a more varied flora in the early stages of development (8 days), but this could be due to the fact that stones could not be scanned as extensively as glass surfaces due to the extremely uneven surfaces of the former. Flagellates, loricate ciliates, Testacea, and Peritricha were common on young glass surfaces, but most protozoans were absent from older glass surfaces (Fig. 3). The Peritricha were an exception to this rule. The same type of flora was seen on the stones.

At all stages of development the bacterial flora consisted mostly of rods. Occasionally spirochaetes and caulobacter-like organisms were observed, especially in mature, deep samples (e.g., 17 days). The early stages of bacterial flora development consisted of both sparse, even distribution (Fig. 3A) and microcolonies (Fig. 3B). Mature samples (17 days) exhibited a dense bacterial flora, usually embedded in a stringy matrix (Fig. 3F).

Carbon:nitrogen ratio. To investigate the relationship of the C:N ratio to the efficiency of columnar denitrification as well as the quality of the column effluent, several columns were observed during their developmental (0 to 95% nitrate removal) and mature stages using three C:N ratios: 1.0, >1.0, and <1.0. The C:N ratio of 1.0 was selected as the adequate C:N ratio for this type of system on the basis of the experience gained with the previous developing columns (i.e., this ratio allowed 100% nitrate re-

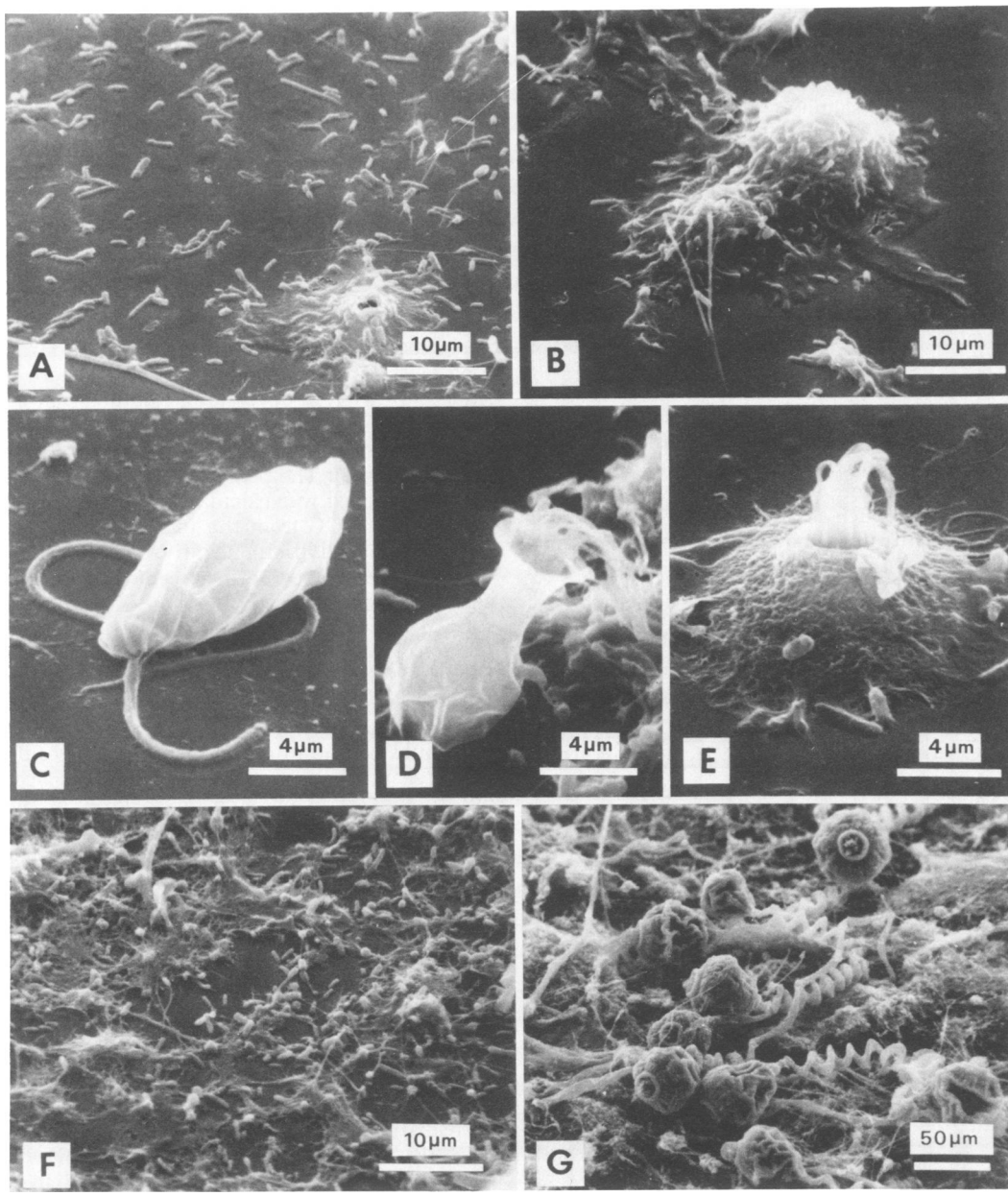

FIG. 3. *SEMs of the colonization sequence on glass substrates in a laboratory denitrification column. (A) Colonization after 8 days: bacteria and Testacea. (B) Colonization after 8 days: bacterial microcolony. (C) Colonization after 8 days: flagellate. (D) Colonization after 8 days: loricate ciliate. (E) Colonization after 8 days: Testacea with extended pseudopodia. (F) Colonization after 17 days. (G) Colonization after 17 days: Peritricha colony.*

moval and a column effluent low in nitrite and effluent carbon). The other C:N ratios were considered insufficient (<1.0) and excess (>1.0).

During the developmental stages of columns operated on a C:N ratio of 1.0 over a long period of time, effluent carbon varied considerably (similar to that seen in Fig. 2). However, these columns showed that effluent carbon decreased to a constant 20% after this initial variability.

For this particular C:N ratio, this amounted to about 4 mg of C/liter. This would seem to indicate that effluent carbon is related to column age.

Columns were studied for the effect of long-term, insufficient C:N ratios on the removal of nitrate and the quality of the effluent. These experiments showed that time and the resultant increase in column biomass did not allow for the development of complete denitrification (i.e., nitrite was produced). Nitrite production continued for a period when the columns would have been mature if the C:N ratio had been higher. In the developmental period of these columns (as with this period in all columns), nitrite production was only a part of the late stages of development (between 75 and 100% nitrate removal). The effluent carbon (the DOC content of the effluent exclusive of the endogenous carbon) of columns with low C:N ratios was lower (but not zero) than the effluent carbon of columns having adequate C:N ratios.

Columns that had a consistantly high C:N ratio had a much higher effluent carbon than columns with an adequate C:N ratio. Effluent carbon ranged between 50 to 75% for the former. For the C:N ratios used in these experiments this amounted to 14 to 27 mg of C/liter. However, nitrite production was rare, even though there were many times when nitrate removal was less than 100%.

Effect of salinity. The effect of increasing the salinity of the culture water passing through the laboratory columns in 2‰ increments from 0 to 18‰ is shown in Fig. 4. Salinity did not affect the efficiency of nitrate removal. Effluent carbon is not plotted, as it followed the same pattern seen in other columns with a C:N ratio of 1.0. It can be seen that nitrite was not produced, and ammonia production was at its normal low level. However, sulfide was produced beginning between days 15 and 25 or between 12 and 15‰ salinity. It appeared that a doubling of the residence time almost doubles sulfide production; this effect was reversible.

Sulfide production. The results of oxidation-reduction potential measurements in a mature, laboratory freshwater column are presented in Fig. 5. The outlet Eh remained fairly constant but was highly reducing. However, the inlet Eh began at about +200 mV and varied considerably with time. When the inlet electrode was installed, the inlet rubber stopper was cleaned of the growth attached to it. This seems to have created, to a small extent, a developing column situation as is reflected in nitrate removal efficiency. An Eh value of +200 mV indicates that very little, if any, oxygen is present and that alternate electron acceptors are being utilized (23). The decrease of the inlet Eh to the range of +200 to 0 mV probably indicates the reduction of nitrate, whereas the reduction of sulfate most likely is reflected by an Eh of 0 to −150 mV (9). Figure 5 also illustrates that sulfide production is directly related to residence time.

While the column used in the sulfide production experiments still had the redox potential electrodes installed, the effect of deoxygenating the influent water by sparging with nitrogen was studied. By measuring oxygen di-

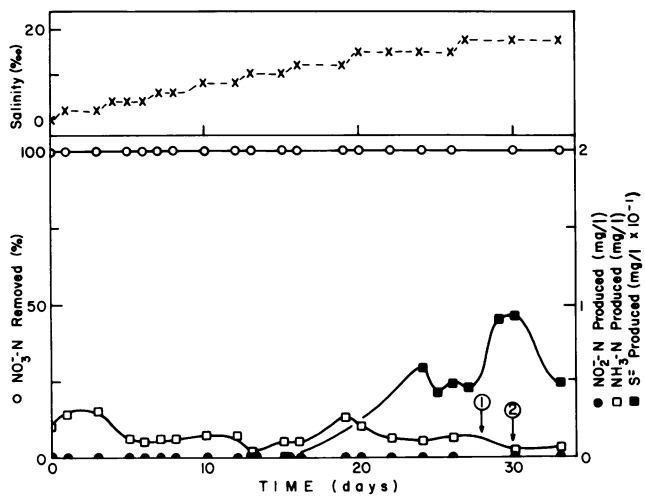

FIG. 4. *Effect of salinity and residence time on NO_3^--N removal, NH_3-N production; NO_2^--N production, and S^{2-} production in a methanol-supplemented laboratory denitrification column. (Arrow 1 indicates a change in residence time from 2 h to 4 h; arrow 2 indicates a return to the 2-h residence time.)*

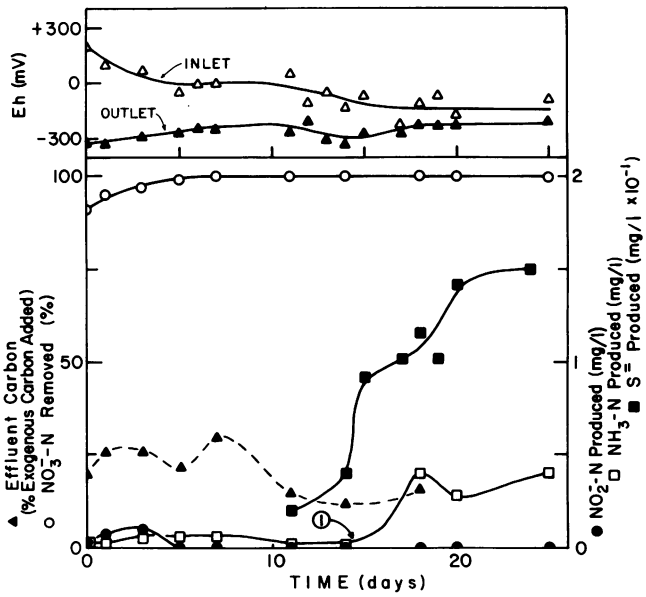

FIG. 5. *Variation of oxidation-reduction potential, S^{2-} production, and NH_3-N production with time (column maturity) and residence time in a methanol-supplemented laboratory (freshwater) denitrification column. (Arrow 1 indicates a change in residence time from 2 h to 5.7 h.)*

rectly, it was found that the dissolved oxygen in the reservoir decreased from 8.8 mg of O_2/liter to 1.2 mg O_2/liter after 20 min of sparging. However, the inlet Eh was relatively constant before and after sparging. It appears that when methanol is available as a carbon and energy source the flora in the inlet tubing (between the point where methanol is added and the point where the culture water is introduced into the column) and in the space above the limestone bed is capable of removing enough molecular oxygen so that an alternate electron acceptor must be used for microbial respiration. Therefore, deoxygenating the inlet water is probably not necessary.

Methane as exogenous carbon. Preliminary experiments utilized fresh, aerobically developed columns that were perfused with methane-saturated culture water. Even after more than 1 month under these conditions, a flora capable of significant nitrate removal did not develop. Residence times of 3 to 4 h did not change the results. Previously methanol-matured columns were then tried as a method of obtaining a flora that would be more likely to utilize methane. The results derived from these preconditioned columns are similar to the results obtained with the preliminary methane columns and are presented in Fig. 6. Although nitrate removal was low, nitrite and ammonia production were extremely high. Ammonia production was two to three times higher than in normal methanol columns. Both ammonia and nitrite production were positively correlated with nitrate removal (e.g., when the residence time was increased). The C:N ratio was similar to that used in methanol columns, but very little methane was actually utilized. An increase in residence time caused more nitrate to be removed, but this effect was temporary.

Pilot plant columns. Based on several short runs with the pilot plant column, the adequate C:N ratio of 1.0 and the effluent carbon were found to be similar for both the laboratory and pilot plant columns. However, in the pilot plant columns there was never any nitrite detected in the effluent, even at low C:N ratios or during periods when less than 100% of the nitrate was removed. Ammonia production was low, with some exceptions. As in the laboratory columns, sulfide production was related to column age and residence time.

Figure 7 depicts an 8-day run with the pilot plant column and presents daily data by column height to give some insight into the functioning of the various portions of the 3.7-m column. This was a mature column. The C:N ratio was high at the start, but leveled off close to 1.0 for the remainder of the run. No carbon was added on day 0. However, upon the addition of methanol there was an increase of dissolved carbon close to the inlet. Extrabasal car-

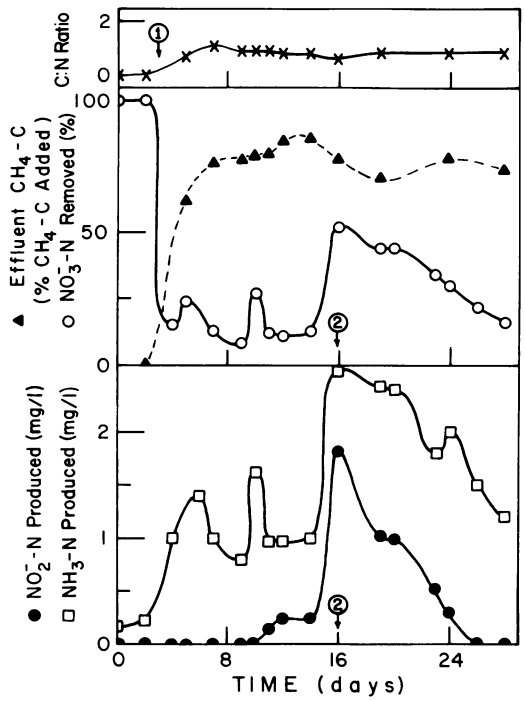

FIG. 6. *Effect of methane and residence time on NO_3^--N removal, NO_2^--N production, and effluent methane in a methane-supplemented laboratory denitrification column. (Arrow 1 indicates a change from methanol to methane; arrow 2 indicates a change in residence time from 2.2 h to 9.4 h.) The C:N ratio refers to methane-carbon only.*

bon is that amount of DOC above the endogenous amount and is expressed as a percentage of the exogenous carbon (methanol) added. With time, the extra-basal carbon level decreased closer to the inlet, and 100% nitrate removal occurred closer to the inlet. As the DOC content of the water was decreased closer to the inlet, sulfide was produced close to the outlet. The area of sulfide production was also the area of no nitrate; the top or outlet portion of the column was not being used for nitrate reduction at this time (days 6 and 7). Since there was a carbon and energy source available, sulfate was reduced. Again, an increased production of ammonia was seen in a sulfate-reducing condition.

For technical reasons concerning the aquaculture system (not the column), the column was operated on a somewhat intermittent basis. This is why data for only 8 days are presented. However, since the completion of this study, the column has been run for longer periods of time (1 month) with the same general results (T. L. Meade, personal communication).

Microbiological studies. As a result of enrichment of and isolation from samples of the brackish water environment, five possibly different denitrifying bacteria were isolated. Two of the five were determined to be the most active denitrifiers and were selected for subsequent use and study; they were designated isolate 11-6 and 11-9B. Three attempts were made to isolate denitrifying bacteria from a mature,

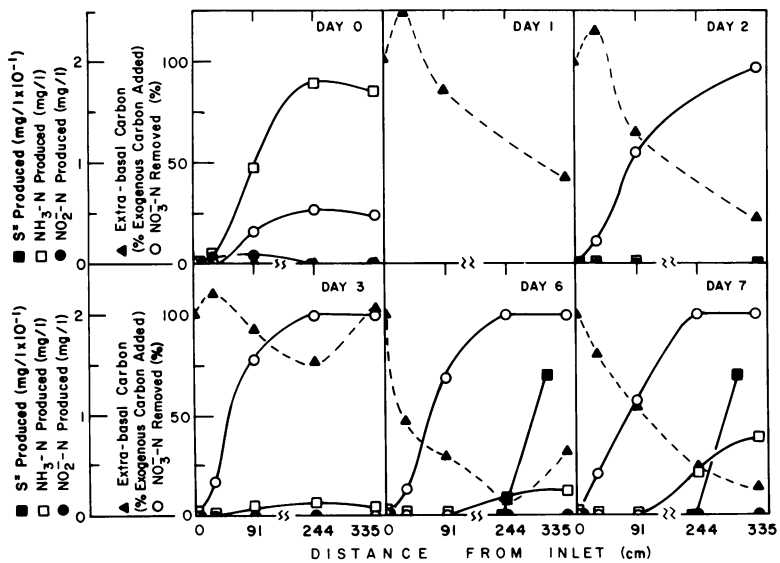

FIG. 7. *Daily nitrogen and carbon concentrations and S^{2-} production as a function of column height in a methanol-supplemented pilot plant denitrification column.*

methanol-supplemented, freshwater column. In the first two attempts the column effluent was used as the source of the oranisms; none could be isolated. Denitrifying bacteria were finally isolated from the slime growth at the column inlet, but only from plates that would normally be considered overgrown and uncountable. Two possibly different denitrifying bacteria were kept and designated isolates III-1 and III-2.

All isolates that were retained were gram-negative rods. All were highly motile except 11-9B. Broth cultures of this isolate contained few motile cells, but most cells attached to the cover glass, usually in rosette fashion. Negatively stained cells of each isolate observed with the transmission electron microscope showed that III-1 possesses two polar flagella and that 11-6 and 11-9B each possesses a single polar flagellum. However, the majority of 11-9B cells observed were not flagellated, nor were fragments of flagella found.

When each isolate was grown in media of 0 and 15‰ salinity, isolates III-1 and III-2 grew and denitrified well at both salinities, whereas isolates 11-6 and 11-9B could only grow and denitrify at 15‰ salinity.

Analysis of carbohydrate metabolism showed that III-1, III-2, and 11-6 could only oxidize glucose, whereas 11-9B could neither oxidize or ferment glucose. There was some indication that 11-6 could oxidize glucose at very low concentrations of oxygen. On the basis of these and the other diagnostic results only, isolates III-1, III-2, and 11-6 were tentatively identified as either *Pseudomonas* sp. or *Alteromonas* sp.; isolate 11-9B possibly belongs to the genus *Alcaligenes* (2).

DISCUSSION

During column development, effluent quality varies considerably. Although an exogenous carbon and energy source is needed to produce a high-quality effluent (effluent with low levels of nitrate, nitrite, and ammonia), freshly started, nonsupplemented columns can remove some nitrate. It is possible that the bacteria are using cellular carbon stored during aerobic conditions, the nitrifying prefilter in this case. But it also appears that the flora is utilizing endogenous carbon during this period. The removal of nitrate under these conditions is probably not due to denitrification but to nitrate assimilation.

From the long-term experiments with a C:N ratio of 1.0, it is apparent that biomass alone will not reduce the effluent carbon to 0%. However, if exogenous carbon is reduced (C:N ratio <1.0) to eliminate effluent carbon, denitrification is incomplete and nitrite is produced. Nitrite is reported to be extremely toxic to fish, with amounts as low as 0.15 mg of NO_2^--N/liter causing large salmonid mortalities (16). Therefore, it appears that constant effluent carbon will be a normal characteristic of this treatment process.

A large amount of effluent carbon could create an undesirable condition in closed-system aquaculture relying on nitrification for ammonia removal, as methanol can completely inhibit bacterial oxidation of ammonia at concentrations as low as 48 mg of CH_3OH-C/liter (10). Also, an increased level of DOC in a closed system might favor the selection of heterotrophs over chemolithotrophs, thus decreasing the ammonia-oxidizing capacity of the system. However, the small amounts of effluent carbon seen in properly managed columns should not present a problem.

With both low and adequate C:N ratios there is always a constant, low-level production of ammonia, whereas columns having a high C:N ratio (1.5 to 2.5) do not produce ammonia. Although some investigations of columnar denitrification of sewage and agricultural drainage water did not measure effluent ammonia (e.g., reference 13) or reported periodic ammonia analysis showing 0.5 mg of NH_3-N/liter in the effluent (20) or no ammonia production, none have reported constant, low-level production (0.1 to 0.5 mg of NH_3-N/liter). Low levels of ammonia should not be a problem in the aquaculture system due to the presence of the nitrifying filter; it only means that some of the inorganic nitrogen remains in the system.

Laboratory column oxidation-reduction potential measurements suggest that most of the nitrate reduction occurs in the top portion (inlet) of the column. This indicates a shorter residence time could be used. Preliminary experiments using a mature column in which the residence times were constantly decreased indicated that residence times shorter than 60 min could probably be used without difficulty.

The positive correlation between sulfide production and increased residence time in freshwater columns indicates that salinity or increased sulfate concentration is not the cause of sulfide production. Portions of a mature column are probably devoid of nitrate; in fact, this was seen in pilot plant columns. Sulfate would be the next electron acceptor to be reduced due to the free energy of its reaction. Longer residence times would probably tend to produce larger areas in the column that would contain no nitrate. This hypothesis assumes that there is a

carbon and energy source for the sulfate reducers. The effluent carbon of most columns studied assures this requirement is met.

Sulfide production has not been reported in sewage and agricultural drainage columnar denitrification. Hydrogen sulfide is extremely toxic to most fish in amounts less than 1 mg of S^{2-}/liter (12). It has also been reported that hydrogen sulfide (3) and sodium sulfide (10) are inhibitory to nitrifying bacteria and therefore the presence of these compounds in closed-system aquaculture might decrease the efficiency of the nitrifying filters. However, sulfide is easily oxidized by small amounts of air and might not be a problem (in small amounts) in the highly oxygenated water of the aquaculture system. The fact that methane could not be used as a carbon source by anaerobic, denitrifying microbial populations does not agree with the findings of Davies (7), who reported achieving 50% nitrate removal under conditions similar to those used in this study. It seems likely that methane-oxidizing bacteria require molecular oxygen to complete the first step of methane oxidation (the formation of methanol), even though the amount of oxygen actually preferred in the environment by methanotrophs is probably less than that found in air (e.g., see reference 18). It is also possible that anaerobic methane-oxidizing bacteria do exist and that they use an alternate electron acceptor as the source of oxidizing power; sources of inoculum other than the culture water (e.g., sludge from an anaerobic sewage digester) might prove fruitful in future investigations.

The activity of the column isolates at various salinities confirms the performance of the freshwater columns that were gradually exposed to increasing salinities and indicates that freshwater columns could be exposed to 15‰ salinity initially. This would mean the application of this type of filter system might be extended to the mariculture of many organisms. It should again be stressed that the identification of the column isolates (and the isolates from the brackish water environment) is very tentative; more definitive analysis of the biochemical activity of each isolate is needed. The main purpose of the isolation portion of this study was to acquire isolates that could be studied in terms of their activity (nitrate reduction and response to salinity changes) and its relation to the activity of whole columns.

In general, the results of this study indicate that columnar, microbial denitrification, with methanol as the carbon and energy source, can be used to remove nitrate-nitrogen from high-density, closed-system salmonid aquaculture water. However, this type of biological filtration does not appear to be as self-regulating or consistent as a nitrifying biological filter due to the necessity of an exogenous carbon source for the heterotrophic, denitrifying microorganisms. The maintenance of the adequate C:N ratio of 1.0 should prove to be particularly difficult in a system where nitrate levels will undoubtedly fluctuate. Constant monitoring will be essential. An automated constant water quality monitoring system coupled to an automatic exogenous carbon addition system could be invaluable in the management of a large-scale denitrification column.

ACKNOWLEDGMENTS

This investigation was supported in part by the Sea Grant Program of the National Oceanic and Atmospheric Administration (grant no. R/A-7) and the Biological Oceanography Program of the National Science Foundation (grant no. GA-32446).

The technical assistance of Paul W. Johnson and Donald Scales and the use of the salmonid culture facilities of T. L. Meade is gratefully acknowledged.

LITERATURE CITED

1. **Barcia, J.** 1973. Reliability of an ammonia probe for electrometric determination of total ammonia nitrogen in fish tanks. J. Fish. Res. Board Can. 30:1389–1392.
2. **Baumann, L., P. Baumann, M. Mandel, and R. D. Allen.** 1972. Taxonomy of the aerobic marine eubacteria. J. Bacteriol. 110:402–429.
3. **Bremner, J. M., and L. G. Bundy.** 1974. Inhibition of nitrification in soils by volatile sulfer compounds. Soil Biol. Biochem. 6:161–165.
4. **Brown, R. L.** 1971. Removal of nitrate by an algal system. Water Pollut. Control Res. Ser., report no. 13030ELY04/71-7. California Department of Water Resources, Sacramento.
5. **Christensen, M. H., and P. Harremöes.** 1972. Biological denitrification in water treatment, report no. 2-72. Technical University of Denmark, Lyngby.
6. **Cline, J. D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14:454–458.
7. **Davies, T. R.** 1973. Isolation of bacteria capable of utilizing methane as a hydrogen donor in the process of denitrification. Water Res. 7:575–579.
8. **Finstein, M. S.** 1966. Nitrogen and phosphorous removal from combined sewage components by microbial activity. Appl. Microbiol. 14:679–684.
9. **Graetz, D. A., D. R. Keeney, and R. B. Aspiras.** 1973. Eh status of lake-sediment water systems in relation to nitrogen transformations. Limnol. Oceanogr. 18:908–917.
10. **Hooper, A. B., and K. R. Terry.** 1973. Specific inhibitors of ammonia oxidation in *Nitrosomonas*. J. Bacteriol. 115:480–485.
11. **Johnson, P. W., and J. McN. Sieburth.** 1974. Ammonia removal by selective ion exchange, a back-up system for microbiological filters in closed-system aquaculture. Aquaculture 4:61–68.
12. **Jones, J. R. E.** 1964. Fish and river pollution. Butterworth, London.
13. **Jones, J. R.** 1971. Denitrification by anaerobic filters and ponds. Water Pollut. Control Res. Ser., report no.

13030ELY06/71-14. Environmental Protection Agency, Washington, D.C.
14. **Leifson, E.** 1963. Determination of carbohydrate metabolism of marine bacteria. J. Bacteriol. 85:1183–1184.
15. **Liao, P. B., and R. D. Mayo.** 1972. Salmonid hatchery water reuse systems. Aquaculture 1:317–335.
16. **Liao, P. B., and R. D. Mayo.** 1974. Intensified fish culture combining water reconditioning with pollution abatement. Aquaculture 3:61–85.
17. **Meade, T. L.** 1974. The technology of closed system culture of salmonids. Marine Tech. Rep. no. 30. Marine Advisory Service, University of Rhode Island, Kingston.
18. **Rudd, J. W. M., R. D. Hamilton, and N. E. R. Campbell.** 1974. Measurement of microbial oxidation of methane in lake water. Limnol. Oceanogr. 19:519–524.
19. **Ryther, J. H., and W. M. Dunstan.** 1971. Nitrogen, phosphorous and eutrophication in the coastal marine environment. Science 171:1008–1013.
20. **Smith, J. M., A. N. Masse, W. A. Feige, and L. J. Kamphake.** 1972. Nitrogen removal from municipal waste water by columnar denitrification. Environ. Sci. Technol. 6:260–267.
21. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43:159–271.
22. **Strickland, J. D. H., and T. R. Parsons.** 1972. A practical handbook of seawater analysis. Fish. Res. Board Can., Bull. 167, 2nd ed., Ottawa.
23. **Turner, F. T., and W. H. Patrick, Jr.** 1968. Chemical changes in water-logged soil as a result of oxygen depletion. Trans. Int. Congr. Soil Sci. 4:53–65.
24. **Wood, E. D., F. A. J. Armstrong, and F. A. Richards.** 1967. Determination of nitrate in seawater by cadmium-copper reduction to nitrite. J. Mar. Biol. Assoc. U. K. 47:23–31.