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Advancements in biofilm carriers and gas-permeable membranes: assessment of zeolite technologies for shortcut nitrogen removal applications in wastewater

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Abstract

The partial nitrification-anammox (PNA) process and other shortcut nitrogen removal processes have been widely studied because of their potential to offer cost savings during wastewater treatment; nevertheless, sustainable examples of full-scale mainstream shortcut nitrogen removal are lacking. The recent development of novel biofilm supports, specifically, zeolite-coated hollow fiber membranes and zeolite-coated biofilm carriers, that locally concentrate ammonium are promising for enhancing mainstream PNA. The ideal application of these technologies is yet to be determined, however. In this study, zeolite-coated carriers were tested in flow-through reactors under both anaerobic and aerobic conditions and zeolite-coated hollow fiber membranes were tested in a membrane-aerated flow-through configuration with varying operating times, lumen oxygen concentrations, and with the presence and absence of amended nitrite. Under anaerobic conditions, reactors containing zeolite-coated carriers had significantly greater ammonium and total nitrogen removal (84.0 ± 16.2% and 89.4 ± 17.1%, respectively) compared to reactors containing control carriers ($P = 0.005$). Anammox-specific 16S rRNA (Amx) genes and two genes associated with denitrifiers (*nirS* and *nosZ*) were preferentially retained in the bulk liquid and in the carrier biofilms in zeolite-coated carrier reactors at a statistically significant level. Genes specific to aerobic ammonium oxidizers (*amoA* genes) were preferentially retained in the bulk liquid of the zeolite-coated carrier reactors. The aerated zeolite-coated carrier reactors also had higher ammonium removal rates (83.8 ± 10.9%) and higher TN removal rates (69.1 ± 16.1%) compared to the aerated control reactors (30.8 ± 23.4%, $P = 0.002$ and 37.4 ±27.4%, $P = 0.05$ for ammonia and TN, respectively). Again, despite aeration, *amoA* genes were only preferentially retained in the liquid of the reactors containing zeolite-coated carriers. In experiments with zeolite-coated membranes, Amx genes were

preferentially retained at significantly higher quantities under only two of the experimental conditions: two-week operation with 100% oxygen delivered in the membrane lumen and two-week operation with nitrite supplemented in the influent. Overall, the zeolite-coated carriers present promising potential for deployment in both anaerobic and aerated environments to enhance nitrogen removal and in particular, the retention of anammox bacteria. The zeolite-coated membranes require more study before their optimal deployment strategy is clear.

Introduction

Nitrogen removal is an important part of wastewater treatment and protects the environment from excess nutrients. Wastewater technologies have been designed to remove both ammonium and nitrite/nitrate, forming harmless nitrogen gas, typically through the application of combined nitrification and denitrification processes. Although effective, these processes are energy and resource intensive, resulting in an industry shift towards implementing lower cost "shortcut" nitrogen removal processes.[1,2]

Shortcut nitrogen removal can make use of a variety of microbial metabolic processes, with the ultimate goal of streamlining microbiological oxidation and reduction for lower oxygen, carbon, and/or alkalinity requirements.[3–5] In general, shortcut nitrogen removal combines nitrification, anammox, and denitrification processes. Partial nitrification is the process of converting half of the influent ammonium to nitrite via the activity of aerobic ammonia oxidizing bacteria (AOB) (Eq. 1).[6] Subsequently, ammonium and nitrite are converted to nitrogen gas by anaerobic ammonium oxidizing (anammox) bacteria (Eq. 2).[7] Combined, this process is referred to as partial nitrification-anammox (PNA). Alternatively, the nitrite produced by AOB can be converted to nitrogen gas by nitrite-consuming denitrifiers (Eq. 3).[6]

(Eq. 1) $$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$$

(Eq. 2) $$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$$

(Eq. 3) $$0.33NO_2^- + 1.33H^+ + e^- \rightarrow 0.17N_2 + 0.67H_2O$$

Implementing shortcut nitrogen removal processes substantially reduces operating costs compared to conventional nitrification and denitrification. The anammox process reduces oxygen demand by approximately 60% with overall operating cost savings estimated at 60 to 90% if implemented for mainstream treatment.[3,5,8] Nitrite shunt, or nitritation/denitritation, can reduce oxygen demand by 25% and reduce overall energy costs by 60%.[4] Additional benefits associated with shortcut nitrogen removal are the elimination or reduction of carbon or alkalinity addition, a reduction in sludge production, and the potential decrease in reactor footprint.[1,4,8] Nevertheless, the implementation of such processes for mainstream treatment have presented challenges because typical mainstream conditions do not allow for the retention of certain microorganisms, such as anammox.[1]

One way to improve shortcut nitrogen removal for mainstream wastewater treatment is the use of biofilm. Biofilm growth decouples the solids retention time and the hydraulic retention time (HRT) to retain slow growing, autotrophic microorganisms.[9] In some cases, specific biofilm carriers are used to further improve performance.[10] As biofilms develop and aerobic and anaerobic zones are created, complete nitrogen removal can occur in a single biofilm.[10–12] Technologies that take advantage of these benefits include integrated fixed film activated sludge (IFAS) and moving bed biofilm reactors (MBBR).[13] Membrane aerated biofilm reactors

(MABR) supply oxygen at the membrane base of the biofilm, creating a unique substrate profile that may enhance total nitrogen removal within a single biofilm.[14] Many studies have shown that biofilm technologies work well for shortcut nitrogen removal processes, but that there is still room for improvement, particularly with respect to enhancing the colonization and retention of anammox biomass.[10,13,15–17]

Because of their ability to sorb and therefore concentrate ammonium, zeolite-coated membranes and biofilm carriers could improve current biofilm technologies for shortcut nitrogen removal by improving the rate at which anammox bacteria colonize solid supports as well as their retention, and potentially retaining AOB as well.[16,18,19] In previous work, we showed that zeolite technologies can attract and retain anammox bacteria under mainstream conditions.[18] With enough zeolite in the system, anammox bacteria were retained and outperformed systems without zeolite amendment with respect to both ammonium and total nitrogen (TN) removal. The wastewater conditions under which zeolite-coated carriers or membrane technologies can be applied for enhanced performance has yet to be explored, however. Aerated zeolite-coated membranes with anaerobic bulk conditions might encourage simultaneous AOB and anammox growth if biofilm growth enables anaerobic zones on the outer biofilm layers. Under aerated conditions, it is possible that AOB and anammox could coexist on carriers colonized with thick biofilms. Regardless of the bulk conditions, both zeolite-coated carrier and membrane technologies should concentrate ammonium at the base of the biofilm and therefore retain and enrich anammox bacteria, if oxygen concentrations are not too high. The overall AOB and anammox bacteria retention will likely depend on bulk conditions, the mass of zeolite incorporated into these biofilm supports, and the oxygen permeance of the zeolite-coated membranes[9,18,20–23].

In this study, we explored how operating conditions impact the performance of novel zeolite-coated biofilm support technologies with respect to nitrogen removal and microbial enrichment/retention. We hypothesized that anammox bacteria will be preferentially retained in the presence of zeolite coatings and low oxygen concentrations, whereas AOB will be preferentially retained on zeolite-coated membranes that are able to supply oxygen and sorb ammonium. This research clarifies how one can implement these novel zeolite-coated biofilm supports for enhanced wastewater nitrogen removal and suggests other applications for these supports.

Experimental

Biofilm attachment materials. Several methods of attaching zeolite onto a support were tested in this research to create a material that maximized ammonium removal per support surface area. Detailed methods describing these processes are provided in the SI or in previous publications.[18,19,24,25] Briefly, for development of zeolite-coated membranes, attachment onto a polymer surface was tested with four different methods of surface functionalization,[24–29] one method of embedding the zeolite into the polymer membrane, and one method of growing zeolite on alumina hollow fibers, which we have tested previously and is described in Huff Chester et al.[18] For development of porous zeolite-coated carriers, deposition into a porous polyethylene (PE) matrix was tested, also described previously (Feinberg et al.).[19] Control materials without zeolite were generated for some of these zeolite-coated supports as previously described.[18,19]

Scanning electron microscope (SEM) images were taken using methods described in Huff Chester et al.[18]

Synthetic wastewater and seed. Three types of synthetic wastewater with ammonium concentrations of 35 mg-N/L were used, depending on the experimental objectives. One wastewater contained carbon at 200 mg/L and nitrite at 21 mg-N/L, modified from Huff Chester et al.,[18] and was intended to mimic mainstream wastewater in which partial nitrification (PN) was active. The second synthetic wastewater was identical to this but did not contain nitrite, mimicking mainstream wastewater influent.[18] A third synthetic wastewater was prepared without carbon and was modified from Peterson et al.[30] to limit the potential for heterotrophic activity. Tables S1 and S2 detail the synthetic wastewater contents. Wastewater was autoclaved and sealed until used. The activated sludge and anammox sludge inoculum and their storage conditions were described previously.[18]

Experimental set-up and operation

Sorption measurements and isotherm tests. Sorption tests were carried out on membranes and carriers as described by Huff Chester et al.[18] Areas of membranes were measured using a calipers to determine ammonium removal per area of membrane. Carrier surface area was calculated as the apparent surface area, and not the surface area of the internal porous network. Sorption tests were also conducted for isotherm fitting, also described in Huff Chester et al.[18] Briefly, carriers were added to 10 mL of autoclaved synthetic wastewater amended with varying ammonium concentrations and then mixed on a rotator for 48 hours. Ammonium measurements were taken with an ammonium probe (Orion, Thermo Scientific). Ammonium sorbed per unit mass of membrane or per carrier (q_e) and liquid equilibrium ammonium concentration were fit to Langmuir (Eq. 4), Freundlich (Eq. 5), and linear isotherms (Eq. 6) using the R nonlinear least squares function (nls). Constants are described in Table S3.

$$(Eq. 4) \quad q_e = \frac{q_{max} \cdot K \cdot C_f}{1 + K \cdot C_f} = \frac{41.56 \cdot 0.000946 \cdot C_f}{1 + 0.000946 \cdot C_f}$$

$$(Eq. 5) \quad q_e = k_f \cdot C_f^{\frac{1}{n}} = 0.0465 C_f^{\frac{1}{1.0588}}$$

$$(Eq. 6) \quad q_e = k_f \cdot C_f$$

Testing of carriers for ammonium sorption through a developed biofilm. In addition to testing fresh membranes and carriers for ammonium sorption, carriers upon which a biofilm layer had developed were also tested for ammonium sorption to ensure that the zeolite deposited into the PE remained intact and ammonium could still exchange into the zeolite with biofilm present. Two sets of zeolite-coated and control carriers were tested for ammonium sorption post-biofilm growth: one set that was harvested at the end of an aerated flow-through experiment (described below) and a second set that was harvested after being submerged in a DEMON reactor (described previously by Peterson et al.[30]) for 14 days. Once harvested, carriers were subjected to 925 Gy of gamma irradiation (GI) on a Cs-137 irradiator (JL Shepherd & Associates),[31] after which they were tested for abiotic ammonium sorption in an ammonium chloride solution (29.5 ± 0.4 mg-N/L), as previously described.[19]

Carrier bioavailability test. Carriers were formed such that the zeolite particles were entrapped in a porous structure of polyethylene. Carriers were tested to ensure that the ammonium sorbed to the zeolite particles within the porous network was accessible for microbial use, specifically for anaerobic ammonium oxidation. First, ammonium was exchanged into the zeolite in the carriers by adding 387 carriers to 2 L of 100 mg-N/L ammonium chloride solution for 72 hours, after which the carriers were transferred to batch reactors. Control carriers were not pre-sorbed with ammonium. Carriers were then divided into three different reactor types: 1) zeolite-coated carriers to which an anammox enrichment culture was added, 2) zeolite-coated carriers amended with sodium azide (10 mM), as an abiotic control, and 3) control carriers to which an anammox enrichment culture was added. Serum bottles were used as reactors and contained 50 mL of headspace (97% N_2:3% H_2). Each reactor contained 43 carriers and 50 mL of synthetic carbon-free wastewater containing nitrite and was seeded with 2 mL of settled anammox biomass. This experiment was operated on a shake table (60 rpm) in a glove bag under anaerobic conditions at room temperature (21 ± 2°C). The only ammonium added to the systems was the ammonium sorbed to the carriers. Ammonium and nitrite were sampled every 4 hours to monitor ammonium and nitrite degradation.

<u>*Oxygen transfer characteristics of hollow fiber membranes.*</u> Permeance testing of membranes was conducted for both zeolite-coated and uncoated alumina hollow fiber membranes using the methods described by Ahmed and Semmens.[32] To summarize, 10 membranes were potted in a long reactor, sealed on one end (dead-end configuration) (Figure 1).

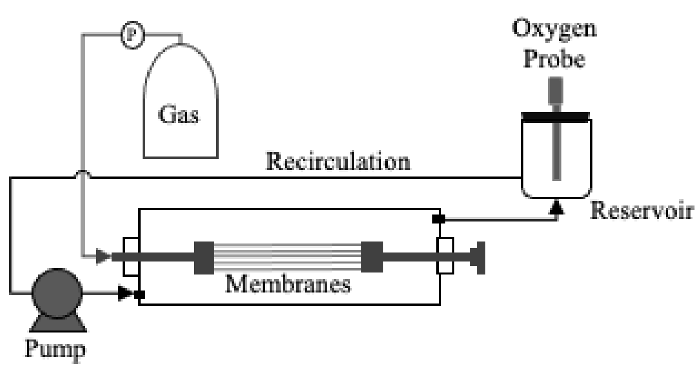

Figure 1. Schematic of membrane permeance test step-up

Oxygen was fed to the membranes (lumen side) at 5 PSI and oxygen transfer occurred from the membrane into bulk reactor water as the water flowed over the membrane surface at 0.01 cm/min. Prior to testing, the water was deoxygenated via an N_2 gas purge and recirculated in the reactor system until the oxygen probe (Unisense, Opto-3000) inserted in a reservoir at the end of the reactor measured <0.5 mg DO/L. Leakage tests with no oxygen fed through the membranes were also performed to enable the subtraction of oxygen leaking into the system from the oxygen permeating through the membranes. Once the oxygen mass transfer coefficient (k) was determined, the mass flux of oxygen (J) into the zeolite and control membrane reactors was calculated using Eq. 6. Here A is the area of the membrane, C^* is average equilibrium dissolved oxygen concentration in the membrane, and C_L is the oxygen concentration in the bulk liquid.

$$(Eq. 6) \qquad J = kA(C^* - C_L)$$

Carrier flow-through reactors - anaerobic and aerobic. Flow-through reactors were designed to test the nitrogen-removal performance of zeolite-coated and control carriers under mainstream wastewater treatment conditions, as well as their ability to attract and retain anammox bacteria and AOB. Triplicate carrier flow through reactors (CFTR) were packed with 100 zeolite-coated carriers to obtain a "high ammonium zone" of 10 mL. Triplicate control CFTRs were set up similarly but contained control carriers. The reactor set-up is shown in Figure 2. Synthetic wastewater containing carbon flowed through the reactors continuously, with a hydraulic residence time (HRT) of 17 hours. The experiment was operated twice, once without aeration and with synthetic wastewater amended with nitrite (CFTR), and once with aeration provided by a stone diffuser and synthetic wastewater with no nitrite added. Oxygen measurements were taken at the reactor exit periodically throughout the aerated carrier flow through reactor (ACFTR) experiment with an oxygen probe (Unisense, Opto-3000). Carrier and liquid biomass samples were harvested from a side port in the reactor for microbial analysis throughout the experiment. Effluent was collected in a vial to which phosphoric acid was added so that the pH of the effluent was adjusted to less than four to ensure that no biological reactions occurred after collection but prior to analysis. Samples were immediately filtered (0.45 μm) and stored sealed at 4°C until analyzed for COD, ammonium, total nitrogen, nitrate, and nitrite as described below.

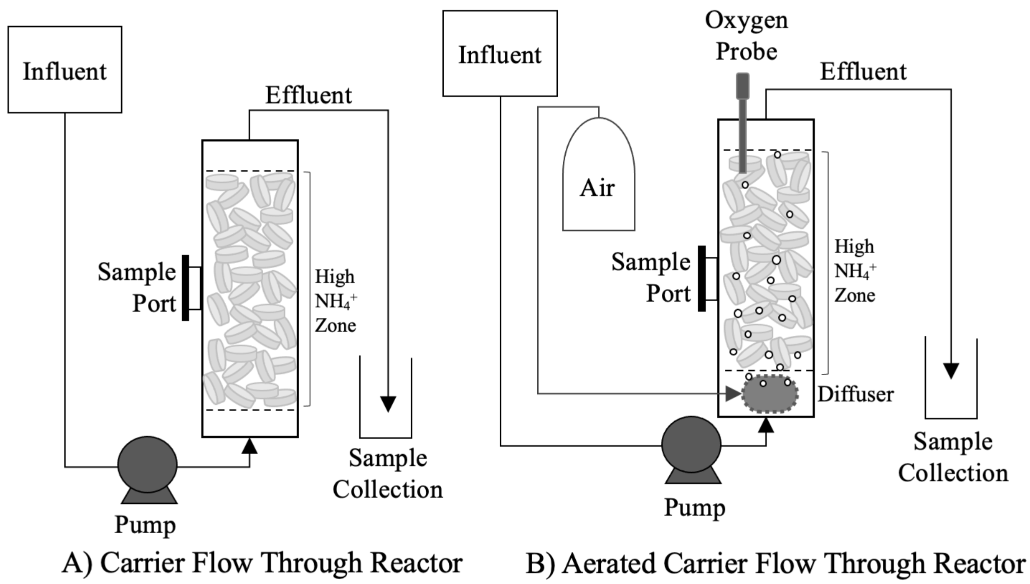

Figure 2. Schematic of CFTR and ACFTR reactor set-up

<u>*Membrane aerated flow-through reactor.*</u> Zeolite-coated and alumina control membranes were also tested in a flow-through configuration to assess the growth and retention of anammox bacteria and AOB on the membranes. Carbon- and nitrite-free synthetic wastewater was fed continuously into the reactors. Triplicate reactors were set up for both the zeolite-coated membranes and the alumina control membranes. Reactors were 70 mL in volume and contained a single potted membrane, as shown in Figure 3. Oxygen was introduced into the membrane lumen from a compressed tank at 5 PSI in dead-end mode. Experiments were performed for 1, 7,

14, and 24 days to monitor colonization of the membranes; at the end of the experiment the membrane was harvested and biomass was extracted from the membrane surface (see below). Additional experiments were performed for 14 days in which N_2 or air were fed through the membrane. A final experiment was performed for 14 days in which nitrite was added to the synthetic wastewater fed to the system. For all experiments, 0.5 mL of activated sludge and 0.2 mL of settled anammox sludge were added to the reactors at the start of the experiment. Reactor liquid was recirculated for 24 hours at 1.0 mL/min to encourage biofilm attachment. After 24 hours, settled sludge was drained and the flow-through experiment was started with a wastewater HRT of 23 hours. At the end of each experiment membranes were carefully harvested and cut into equal sample lengths of 52 ± 3.4 mm for DNA extraction. Oxygen was monitored with an oxygen probe throughout the experiment; the bulk liquid of the reactors quickly became and stayed anaerobic during all experiments.

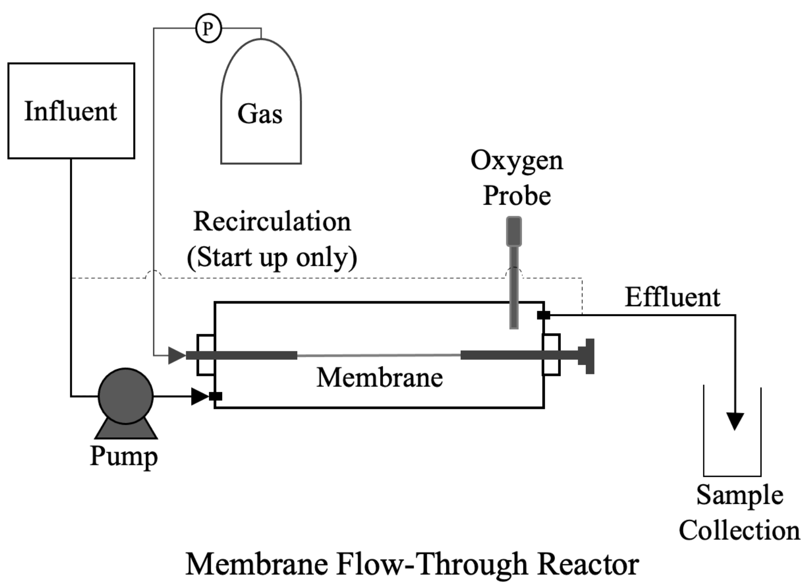

Figure 3. Schematic of MFTR reactor set-up

Analytical methods. Analytical methods, specifically nitrogen measurement methods, were described previously in Huff Chester et al.[18] Briefly, ammonium concentrations for the sorption experiments were measured with an ammonium probe (Orion, Thermo Scientific). Ammonium and TN concentrations in reactor effluent samples were measured colorimetrically (Hach). Nitrite and nitrate concentrations in reactor effluent samples were measured with ion chromatography (930 Compact IC Flex, Metrohm). Dissolved organic carbon (DOC) measurements in reactor samples were analyzed using a TOC-L total organic carbon analyzer (Shimadzu) after first filtering samples through a 0.22 µm syringe filter. A 5-point calibration curve was generated from 500 mg/L stock solution ranging from 10 to 500 mg/L. Typical limits of detection were 2 mg/L.

Molecular methods. DNA extractions and qPCR for Bacterial 16S rRNA, Anammox 16S rRNA, *amoA*, *nirK*, *nirS*, *nosZ*, and *nxrA* genes were performed as previously described in Huff Chester et al.[18]

Data and statistical analysis. Ammonium removal was calculated for the ammonium sorption experiments using Eq. 7.

$$(Eq. 7) \qquad Removal = \frac{C_{initial} - C_{final}}{C_{initial}}$$

Non-parametric Wilcoxon rank sum tests were used to compare data from the CFTR and ACFTR experiments, namely the nitrogen concentrations, DOC concentrations, and the qPCR data for the zeolite-coated and control carrier reactors. Comparisons of the ammonium concentrations from the carrier-biofilm sorption tests were also performed using the non-parametric Wilcoxon rank sum test. Parametric paired Student t tests were used to compare the qPCR data in the reactors containing zeolite-coated versus control membranes in the MFTR experiments after first checking for normality using the Shapiro-Wilk test. Typically, p-values less than 0.05 were considered significant, but p-values less than 0.1 were also reported and statistical significance with 90% confidence was clearly indicated. Statistical tests were performed with R software.

Results and Discussion

Characterization of membranes and carriers for bioreactor deployment. Materials prepared using different methods of zeolite attachment (described in the SI) were tested for ammonium sorption. SEM images of the developed materials are show in Figure 4.

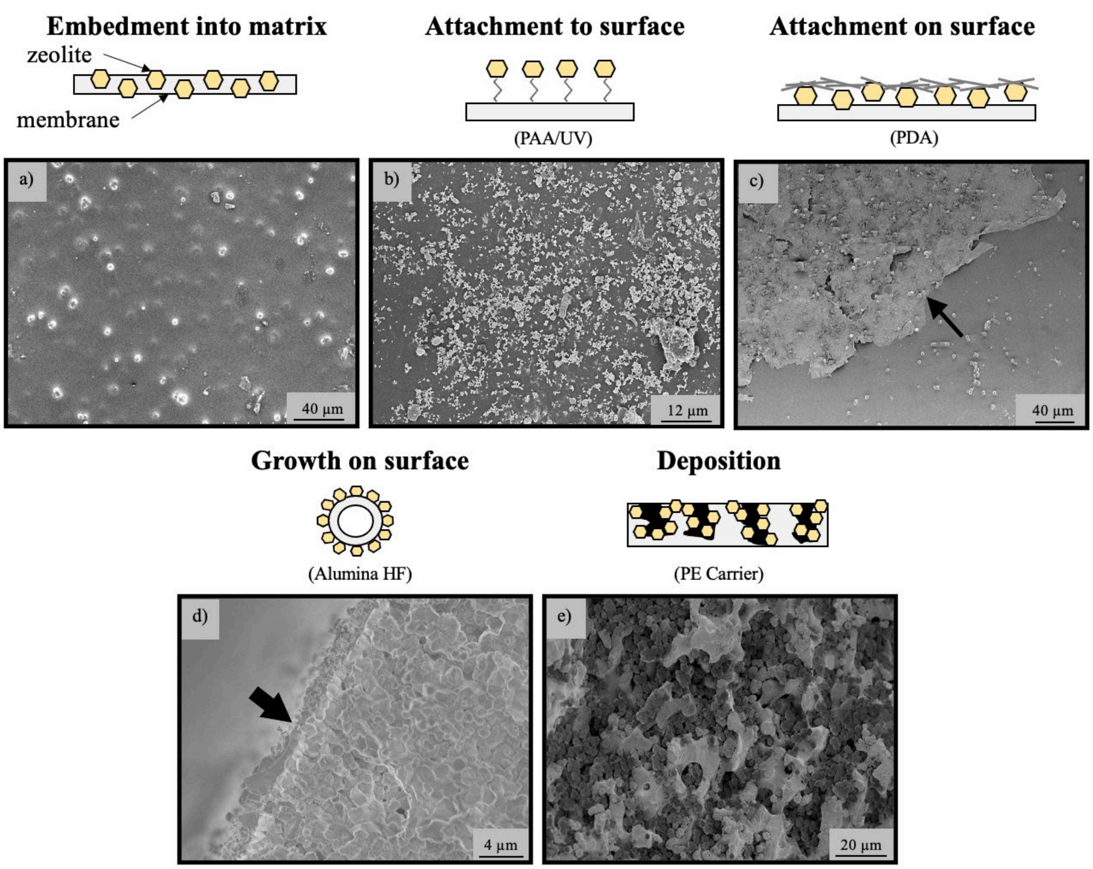

Figure 4. SEM images of membrane and carrier surfaces. a) Embedment into polymer membrane matrix, b) attachment to membrane surface using UV/PAA method, c) attachment to membrane using PDA method, d) growth of zeolite on alumina hollow fiber membrane, and e) deposition into porous PE carrier. Panel e is adapted with permission from Feinberg et al. Porous Polyethylene-Supported Zeolite Carriers for Improved Wastewater Deammonification. *ACS EST Eng.* 2021, *1* (7), 1104–1112. Copyright 2021 American Chemical Society.

Ammonium sorption from synthetic wastewater as a function of carrier surface area is shown for each membrane and carrier type in Figure 5. Most of the zeolite coating methods were successful in attaching zeolite and facilitating at least some ammonium sorption from synthetic wastewater, except for the embedment method, which did not result in substantial ammonium removal ($1.9\times10^{-5} \pm 4.1\times10^{-6}$ mg-N/L/mm^2) (Figure 5). Membranes developed from the attachment methods all obtained some level of ammonium sorption (0.013 ± 0.002, 0.012 ± 0.003, 0.0019 ± 0.0006, and 0.00029 ± 0.0004 mg-N/L/mm^2 for functionalization enabled by ultraviolet (UV), poly(acrylic acid)/UV, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide, and polydopamine, respectively). The zeolite-coated alumina hollow fiber membranes had the highest ammonium sorption per mm^2 of membrane, removing 0.029 ± 0.004 mg-N/L/mm^2; these membranes were therefore selected for further testing, both with respect to their sorption capacity and their potential to transfer oxygen. Likewise, the zeolite-coated porous biofilm carriers also showed excellent ammonium sorption capability (0.065 ± 0.03 mg-N/L/mm^2) (Figure 5) and were also studied further.

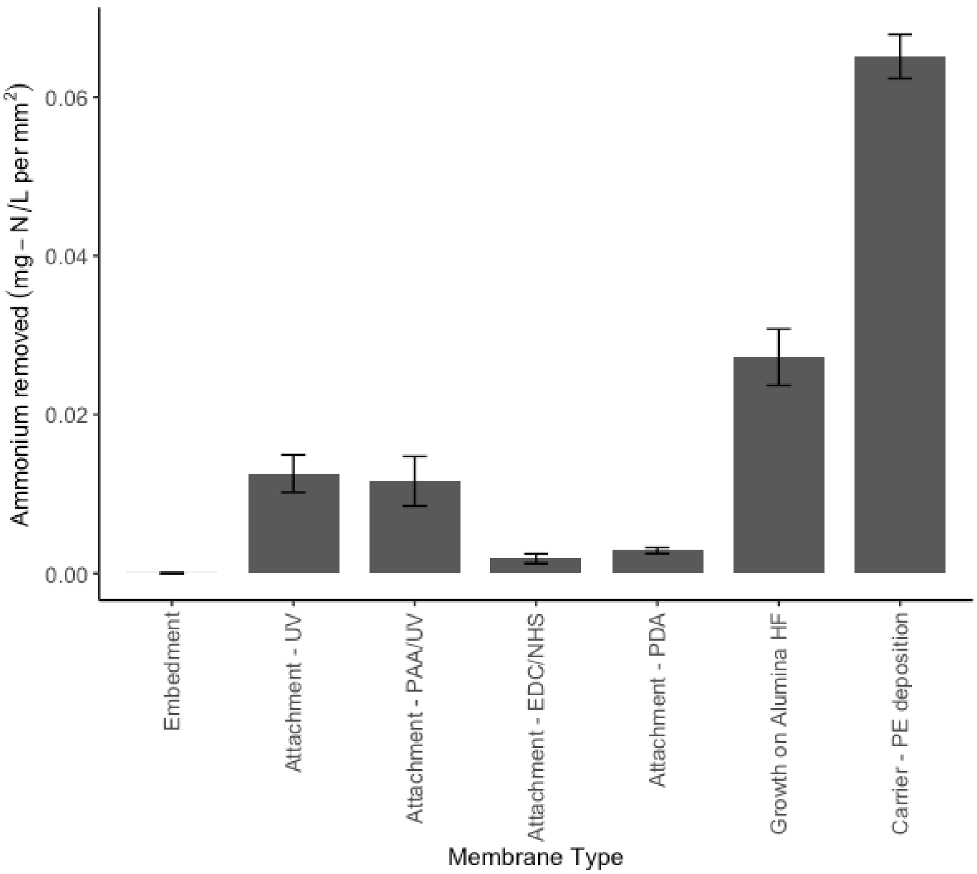

Figure 5. Ammonium removal from synthetic wastewater via sorption to zeolite-functionalized materials using zeolite attachment or coating methods. Error bars indicate the standard deviation of triplicate experiments. Note: For the porous carrier within which the zeolite was deposited, the apparent surface area, excluding the internal pore structure, was used for sorption calculations.

Isotherms for ammonium sorption of the zeolite-coated membranes and carriers were also developed and were fit to linear, Langmuir, and Freundlich isotherm models. All isotherms fit the data for the zeolite-coated membranes well.[18] Zeolite carriers were tested beyond the linear region. As a result, the Langmuir isotherm model provided the best fit, with an

R^2 of 0.992; the Freundlich isotherm model also provided a good fit to the data, with an R^2 of 0.981. The linear isotherm fit was poor, with an R^2 of 0.85.

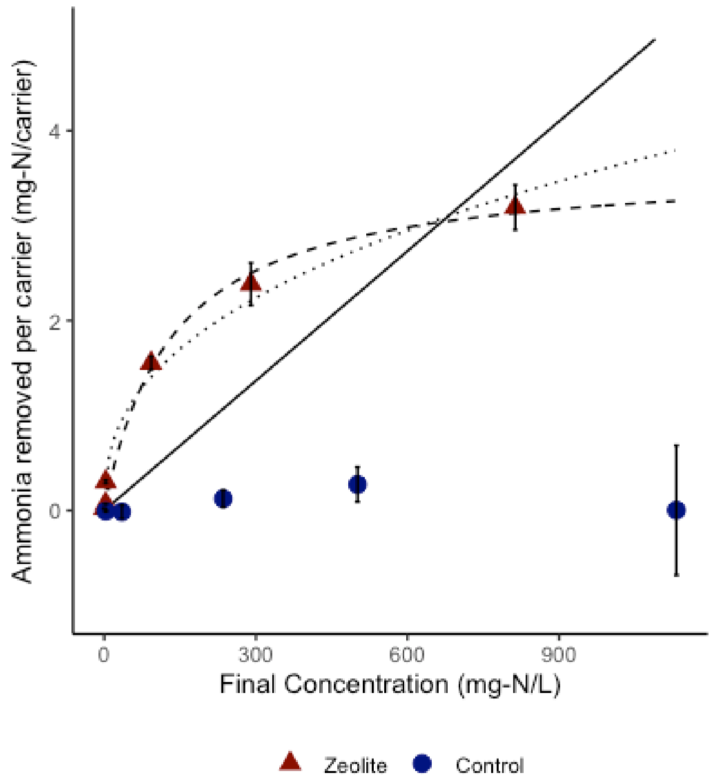

Figure 6. Ammonium sorption isotherms for zeolite-coated and control carriers in synthetic wastewater. Lines indicate model fits, with the solid line showing the linear isotherm fit, the dashed line showing the Langmuir isotherm fit, and the dotted line showing the Freundlich isotherm fit. Control membranes and carriers were not fit to isotherm curves, as they failed to sorb ammonium. Error bars indicate the standard deviation of triplicate experiments.

Carriers coated with biofilm were also tested for ammonium sorption to determine if biofilm growth created substantial blockage of ion exchange sites on the zeolite, as has been reported in other studies.[29] Zeolite-coated carriers, first gamma irradiated to inactivate the biofilm, showed considerable ammonium removal (Figure 7) indicating that biofilm growth did not prevent ammonium sorption on zeolite-coated carriers. In fact, all zeolite-coated carriers showed ammonium sorption, removing significantly more ammonium compared to the control carriers ($P = 0.0035$). The GI-ACFTR zeolite-coated carriers removed more ammonium compared to the GI-Amx zeolite-coated carriers ($84.6 \pm 2\%$ vs. $49.8 \pm 1\%$, respectively, $P<0.0001$), which is likely an indication that the GI-Amx biofilm was thicker than the GI-ACFTR biofilm and some blockage of the exchange sites did occur upon growth of a thick enough biofilm.

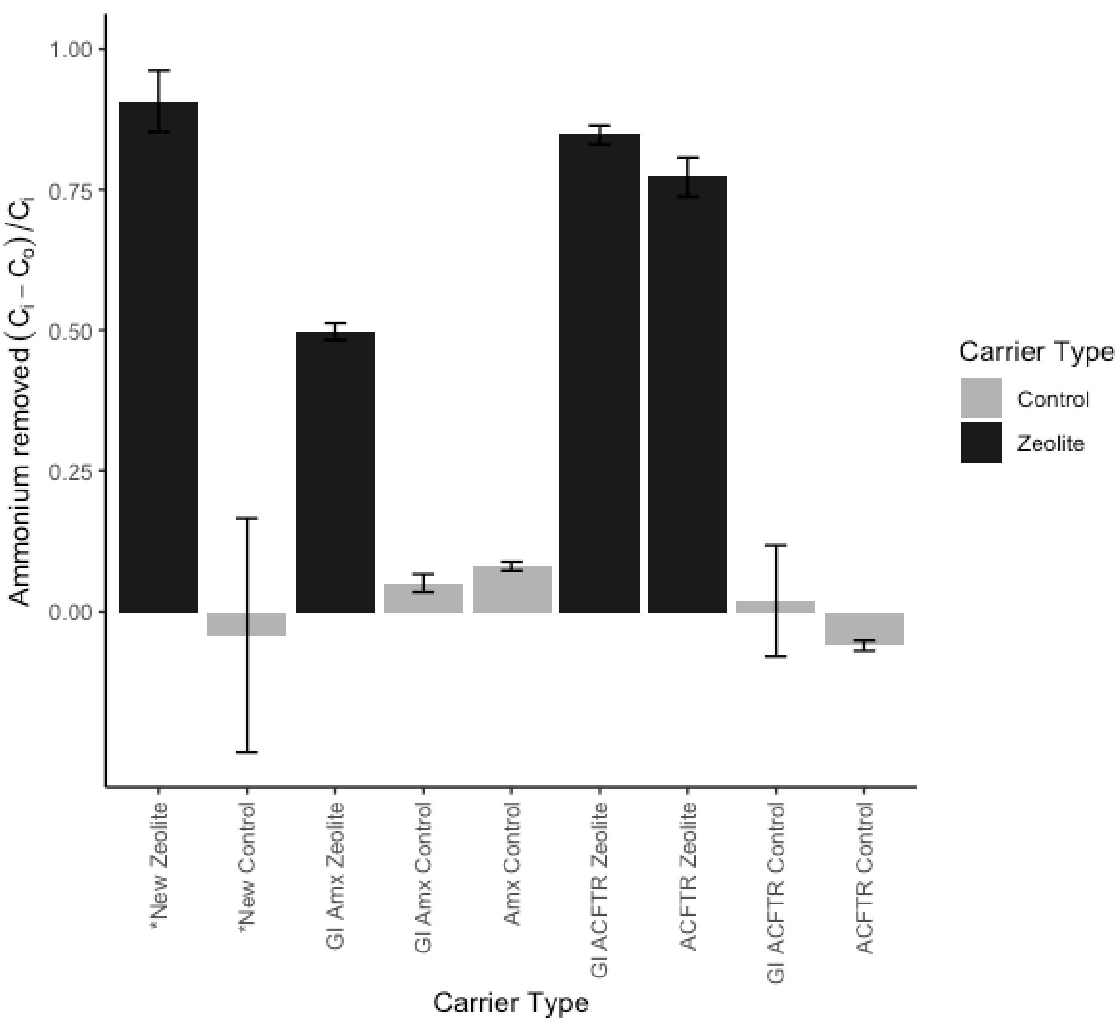

Figure 7. Ammonium removal [(C_i-C_f)/C_i] via sorption to biofilm-covered zeolite-coated and control carriers subjected to gamma irradiation; these were compared to pristine (no biofilm present) zeolite-coated and control carriers. Amx indicates carriers submerged in an anammox enrichment reactor for biofilm growth. ACFTR indicates carriers sampled from the aerated CFTR experiment and also covered in biofilm growth. GI indicates gamma-irradiated carrier samples. Error bars indicate standard deviation of triplicate experiments.

Permeance tests with zeolite-coated alumina hollow fibers showed that the fibers were capable of transferring oxygen to water, with mass transfer coefficients calculated to be 6.3×10^{-6} cm/s for the zeolite-coated membranes and 2.8×10^{-6} cm/s for the control membranes. It is unclear why the zeolite-coated membranes were capable of greater oxygen transfer, but it could have been a result of their different surface chemistry. Measurements of the zeta potential and hydrophobicity of the zeolite-coated and control membranes were attempted, the curvature of the hollow fiber surfaces, however, made these parameters impossible to measure. The reasons for the greater oxygen transfer rates with the zeolite-coated membranes, therefore, could not be confirmed. The mass flux of oxygen into the system was calculated from the mass transfer coefficients to be 2.4×10^{-7} mg/s for pure oxygen and 5.04×10^{-8} mg/s for air fed to the lumen of the zeolite-coated membranes. For the control membranes, the mass flux was 1.0×10^{-7} mg/s for pure oxygen and 2.2×10^{-8} mg/s for air.

Our previous work on the zeolite-coated alumina hollow fibers (Huff Chester et al),[18] as well as work by others,[30,31] has suggested that ammonium sorbed to zeolite is accessible to microorganisms; nevertheless, given the sponge-like structure of the zeolite-coated carriers and the fact that porous materials of this kind have not been previously tested, the bioavailability of the sorbed ammonium with the zeolite-coated carriers was verified. As described in the methods, if the ammonium that was sorbed to the carriers was bioavailable, the nitrite in the bottles containing zeolite-coated carriers and anammox bacteria should degrade. Data presented in Figure 8 supports this view, with the nitrite concentrations in the zeolite-coated carrier treatments decreasing from 19.16 ± 0.5 to 0 ± 0 mg-N/L within 25 hours and no appreciable nitrite decrease in the control carrier treatments. Indeed, the rate of nitrite degradation in the zeolite-coated carrier treatments was significantly greater (0.97 ± 0.04 mg/L per hour) than that in the control carrier treatments (0.052 ± 0.005 mg/L per hour) ($P = 0.027$). Additionally, negative control reactors containing zeolite-coated carriers amended with sodium azide showed an increase in ammonium to an average of 56.6 ± 3.7 mg-N/L, as the ammonium desorbed into solution from the carriers and was not consumed biologically. These results clearly demonstrated the ability of the anammox culture to access sorbed ammonium from the zeolite-coated carriers.

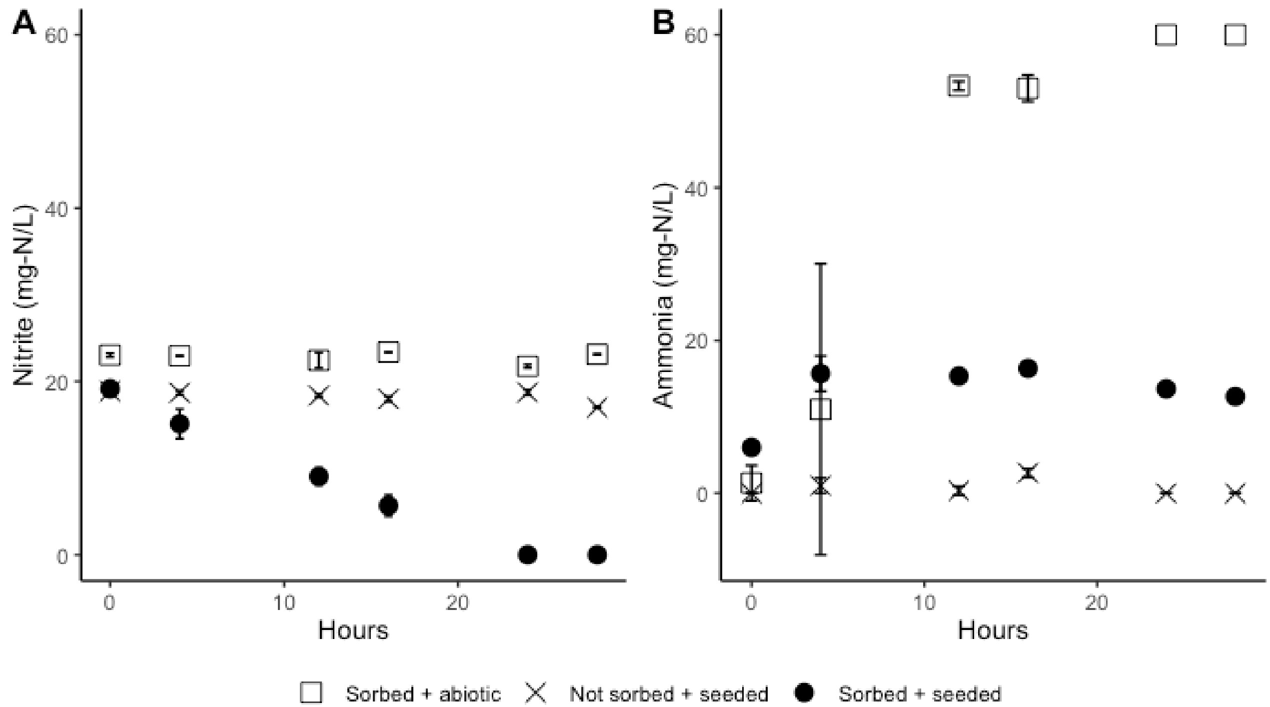

Figure 8. Measured ammonium and nitrite concentrations over time from the bioavailability tests, indicating that the ammonium sorbed to the carriers was bioavailable. Error bars show standard deviations of triplicate experimental replicates.

Overall, material testing was able to show that a variety of methods could be used to attach zeolite to supports and additional experiments with two of the most promising materials showed that ammonium sorption followed both a Langmuir and Freundlich sorption model. It was further shown that ammonium sorption occured through a layer of biofilm, and ammonium exchanged into the zeolite-coated carriers was biologically accessible.

Zeolite coated substrates demonstrate shortcut nitrogen removal in wastewater system. The results in the previous section suggested that both the zeolite-coated alumina hollow fiber membranes and PE carriers should be excellent candidates for deployment into mainstream wastewater systems for enhanced shortcut nitrogen removal. We investigated these prospects in carrier flow-through bioreactors, aerated carrier flow-through bioreactors, and membrane flow-through bioreactors. Zeolite-coated and control carriers were tested in a flow-through system (Figure 1) fed with synthetic wastewater containing ammonium and nitrite at concentrations of 15.7 ± 2.33 mg-N/L and 17.4 ± 4.0 mg-N/L, respectively, for a TN concentration of 37.2 ± 6.9 mg-N/L. The influent also contained DOC at a concentration of 167.7 ± 66.2 mg/L. Influent and effluent concentrations of ammonium, TN, nitrite, and nitrate are shown in Figure 9. DOC influent and effluent concentrations are shown in Figure 10. As a result of an error, no nitrite was added to the influent on Day 25. This is evident in the results shown in Figure 9, where the influent concentration on Day 25 was 0.5 mg-N/L, compared to the average concentration on all other days of 17.4 ± 4.0 mg-N/L.

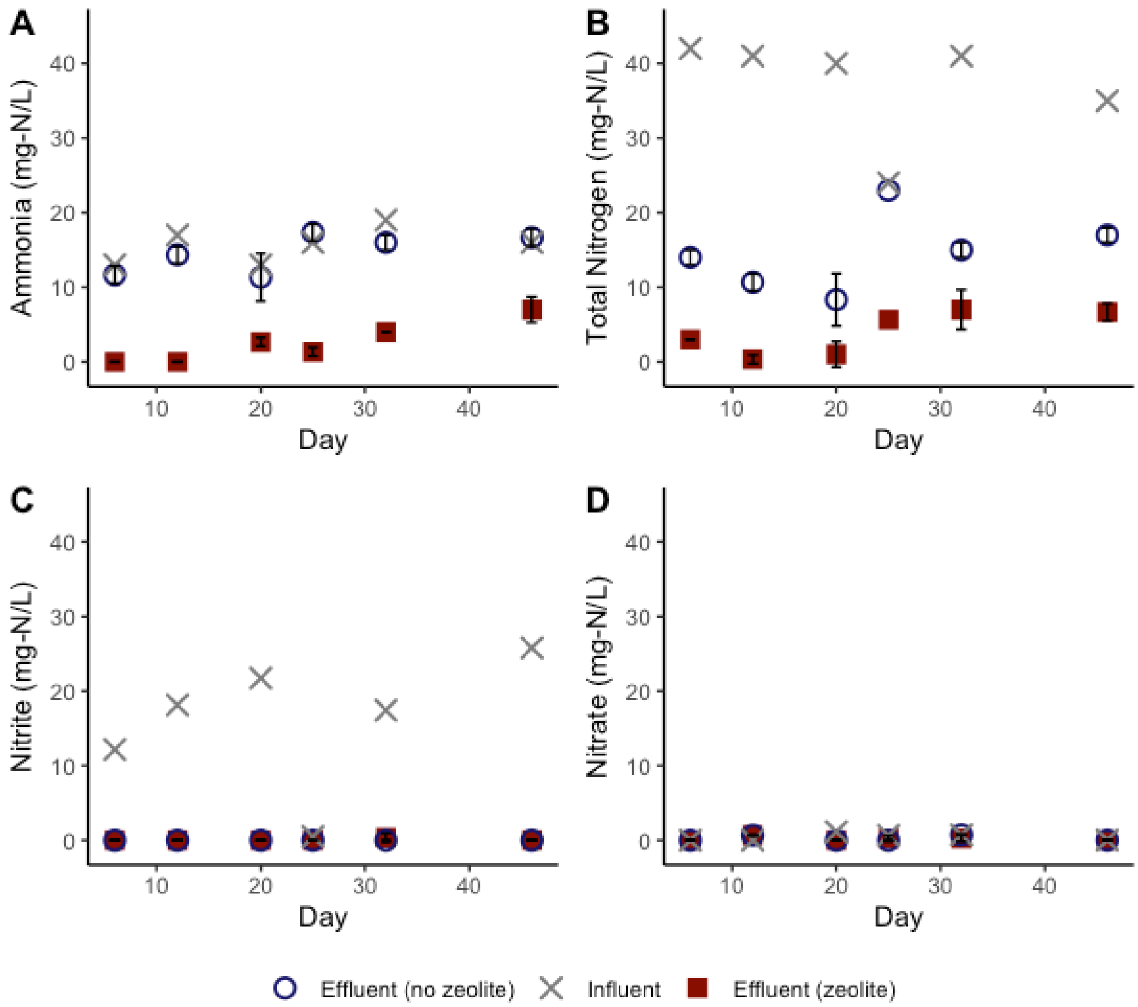

Figure 9. Influent and effluent concentrations of A) Ammonium, B) TN, C) Nitrite, and D) Nitrate in the CFTR experiment. Error bars indicate the standard deviation of triplicate reactors.

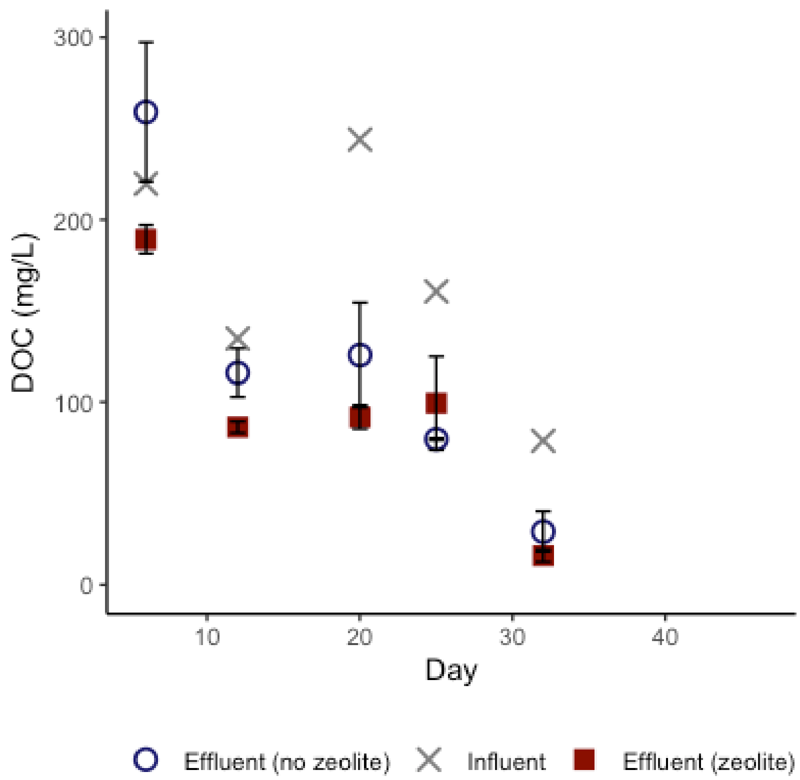

Figure 10. Influent and effluent DOC concentrations in the CFTR experiment. Error bars indicate standard deviation of triplicate reactors.

Over the course of the 46-day experiment, the reactors containing zeolite-coated carriers removed significantly more ammonium (84.0 ± 16.2%) and TN (89.4 ± 17.1%) on average, and therefore had much lower average effluent ammonium (2.50 ± 2.62 mg-N/L) and TN (3.94 ± 2.98 mg-N/L) concentrations compared to control reactors (14.56 ± 2.79 and 14.7 ± 5.37 mg-N/L for ammonium and TN, respectively) ($P < 0.0001$ for both ammonium and TN) (Figure 9). It cannot be determined from the chemical data alone whether this was a result of the retention/enrichment of anammox bacteria on the zeolite-coated carriers and enhanced anammox activity, or a result of abiotic ammonium sorption coupled with the denitrification of amended nitrite. Examination of the microbial data, Figure 11, however, shows that retention of anammox bacteria on the zeolite-coated carriers is at least partially responsible for the enhanced nitrogen removal in the reactors containing zeolite-coated carriers.

The log of the anammox gene copies per carrier or per mL was statistically greater in both the carrier biofilm samples and in the bulk liquid samples from the reactors containing zeolite-coated carriers, compared to the same samples taken from the reactors containing control carriers ($P = 0.002$ for the carrier biofilm samples and $P = 0.01$ for the bulk liquid samples). The average log 16S rRNA copies per mL or per carrier for anammox (Amx) in the zeolite-coated carrier reactor liquid and carrier biofilm samples were 6.85 ± 0.38/mL and 5.53 ± 0.38 per carrier, respectively, compared to 6.13 ± 0.69/mL and 4.84 ± 0.54 per carrier for the control reactor liquid and carrier biofilm samples, respectively. This equated to a statistically greater percent of Amx genes in the liquid of the zeolite-coated ($10.02 \pm 9.91\%$) versus control carrier ($4.64 \pm 5.90\%$) reactors ($P = 0.01557$). There was also a greater percent of Amx genes in the carrier biofilm in the zeolite-coated ($1.6 \pm 0.49\%$) versus control carrier ($0.33 \pm 0.31\%$) reactors, but only at the 90% confidence level ($P = 0.07359$). When coupled with the chemical data, these results suggest that the zeolite-coated carriers were able to enhance the retention, and likely the activity, of the anammox bacteria in the system. Amx gene copies per mL decreased on Day 25 in the liquid of the zeolite-coated reactors, but not in the carrier biofilm samples. This was thought to be a result of the lack of nitrite in the feed on Day 25. The control reactors appeared to be less affected by the lack of nitrite, with no clear decline of Amx gene copies per mL occurring with the lack of nitrite addition.

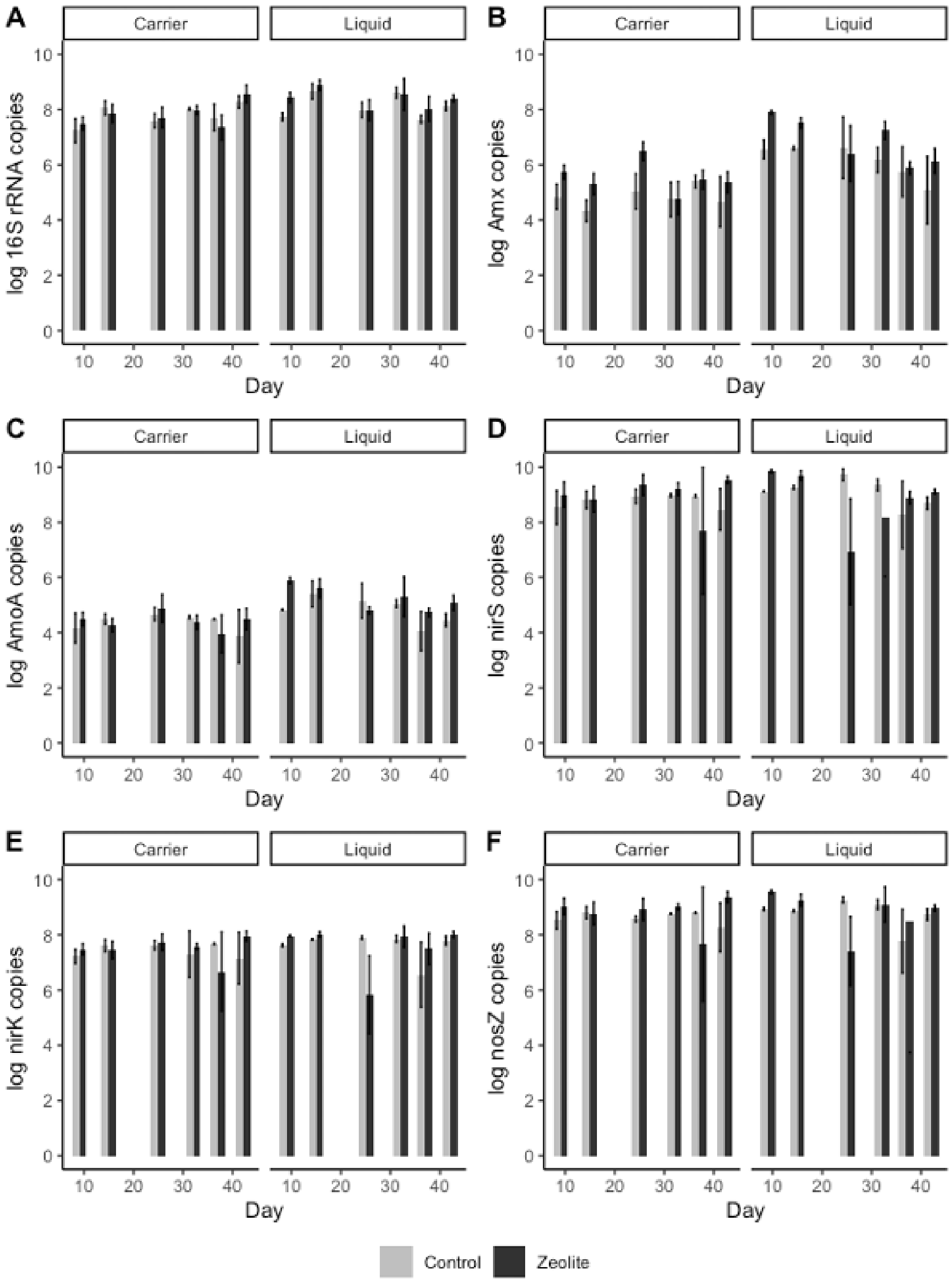

Figure 11. qPCR results from the CFTR experiment for the A) 16S rRNA, B) Amx, C) *amoA*, D) *nirS,* E) *nirK,* and F) *nosZ* genes, showing the log number copies per carrier for the carrier biofilm samples and the log number copies per mL for the reactor liquid from CFTR. Light grey indicates samples from control carrier rectors and

dark grey indicates samples collected from zeolite-coated carrier reactors. Error bars show the standard deviation of the reactors run in triplicate.

Both reactors also showed excellent nitrite removal and little nitrate production, with effluent nitrite concentrations of 0.069 ± 0.27 mg-N/L and 0 mg-N/L for the zeolite-coated and control carrier reactors, respectively, and effluent nitrate concentrations of 0.23 ± 0.33 mg-N/L and 0.28 ± 0.35 mg-N/L for the zeolite-coated and control carrier reactors, respectively (Figure 10). This suggests that denitrification was occurring in both treatments. Not surprisingly, denitrifiers, quantified by the number of *nirK*, *nirS* and *nosZ* gene copies per mL or per carrier present, were detected in the bulk liquid and carrier biofilm samples in both reactors. The *nirS* ($P = 0.037$) and *nosZ* ($P = 0.0071$) genes were significantly enriched in the reactors containing zeolite-coated carriers (Figure 11), with the carrier biofilm samples containing average log *nirS* and log *nosZ* gene copies of 8.93 ± 0.66 and 8.79 ± 0.58 per zeolite-coated carrier, respectively, compared to 8.78 ± 0.34 and 8.62 ± 0.26 per control carrier, respectively (Figure 11). On Day 25, *nirK, nirS,* and *nosZ* copies per mL in the liquid decreased in the zeolite-coated carrier reactors, likely from the lack of nitrite in the influent. A similar decrease was not observed in the carrier biofilm samples, suggesting, as with the anammox bacteria, that the denitrifying communities on the carriers were more stable. Again, as observed with the Amx genes, a similar decrease was not observed in the control carrier reactors.

Another group of nitrogen-cycling bacteria that was analyzed in these reactors was the ammonium oxidizing bacteria, specifically, the *amoA* gene. Interestingly, even with no oxygen supplied to the reactors, the log *amoA* gene copies per mL in the bulk liquid of the zeolite-coated carrier reactors (5.24 ± 0.29/mL) was significantly higher than that in the control reactors (4.83 ± 0.37/mL) (P = 0.009) (Figure 11). The carrier biofilm samples themselves did not have significantly different numbers of *amoA* gene copies per carrier (P = 0.50), perhaps because of the lack of oxygen supply within the reactors. There was no significant difference in log 16S rRNA gene copies per mL or per carrier in either the bulk liquid or carrier biofilm samples between the zeolite-coated carrier and control carrier reactors (P = 0.9244 for the carrier biofilm samples and P = 0.1810 for the bulk liquid samples).

Overall, Amx genes were in higher abundance in the carrier biofilm samples and bulk liquid samples in the zeolite-coated carrier reactors compared to the control reactors, with some denitrifying genes, *nirS* and *nosZ*, also in greater abundance in these samples (Figure 11). The bulk liquid in the reactors containing zeolite-coated carriers also had higher numbers of *amoA* genes compared to the liquid in the control reactors. This indicates that the presence of the zeolite coating on these novel carriers did attract a unique microbial community, not only on the carrier surface, but also within the reactor bulk liquid. This provides evidence that the combination of zeolite-facilitated abiotic ammonium sorption with the apparent zeolite-enhanced retention/enrichment of N-cycling bacteria improves total nitrogen removal under mainstream-like operating conditions (Figure 9).

Aerated carrier flow-through reactors. To determine whether operating the system with active aeration would encourage more substantial colonization of AOB in the reactors containing zeolite-coated carriers, the experiment was repeated with no added nitrite in the influent and with active aeration within each reactor. DO levels were high in the bulk reactor liquid throughout the experiment, with 95.1 ± 6.5% and 89.9 ± 20.0% of DO saturation measured in the zeolite-coated carrier and control carrier reactors, respectively. Over the course of the 30-day experiment, the reactors containing zeolite-coated carriers again removed significantly more ammonium and TN, and therefore had much lower effluent ammonium (2.1 ± 1.9 mg-N/L) and TN (6.5 ± 3.6 mg-N/L) concentrations compared to control reactors (9.1 ± 3.7 mg-N/L and 12.7 ± 3.7 mg-N/L for ammonium and TN, respectively) ($P<0.0001$ for ammonium and $P = 0.0012$ for TN) (Figure 11). As with the previous experiment, effluent nitrite and nitrate concentrations were low and very similar in the two types of reactors ($P = 0.85$ and $P = 0.83$ for nitrite and nitrate respectively), which suggests that either ammonium sorption or ammonium sorption, oxidation, and rapid denitrification on the carrier surface was occurring in the zeolite-coated carrier reactors (Figure 12). Log *amoA* copies were 5.55 ± 0.24/mL and 5.41 ± 0.15 per carrier in the reactor samples containing zeolite-coated carriers and 5.20 ± 0.40/mL and 4.79 ± 0.67 per carrier in the control reactor samples (Figure 13). As with the CFTR experiments, the log *amoA* copies per mL or per carrier were only higher in the bulk liquid of the zeolite-coated carrier reactors, and at only a 90% confidence interval, and not within the biofilm on the carriers ($P = 0.065$ and $P = 0.132$ for the liquid and carrier samples, respectively) (Figure 13), which suggests that ammonium oxidation was occurring in the bulk liquid, but was not significantly enhanced on the surface of the zeolite-coated carriers.

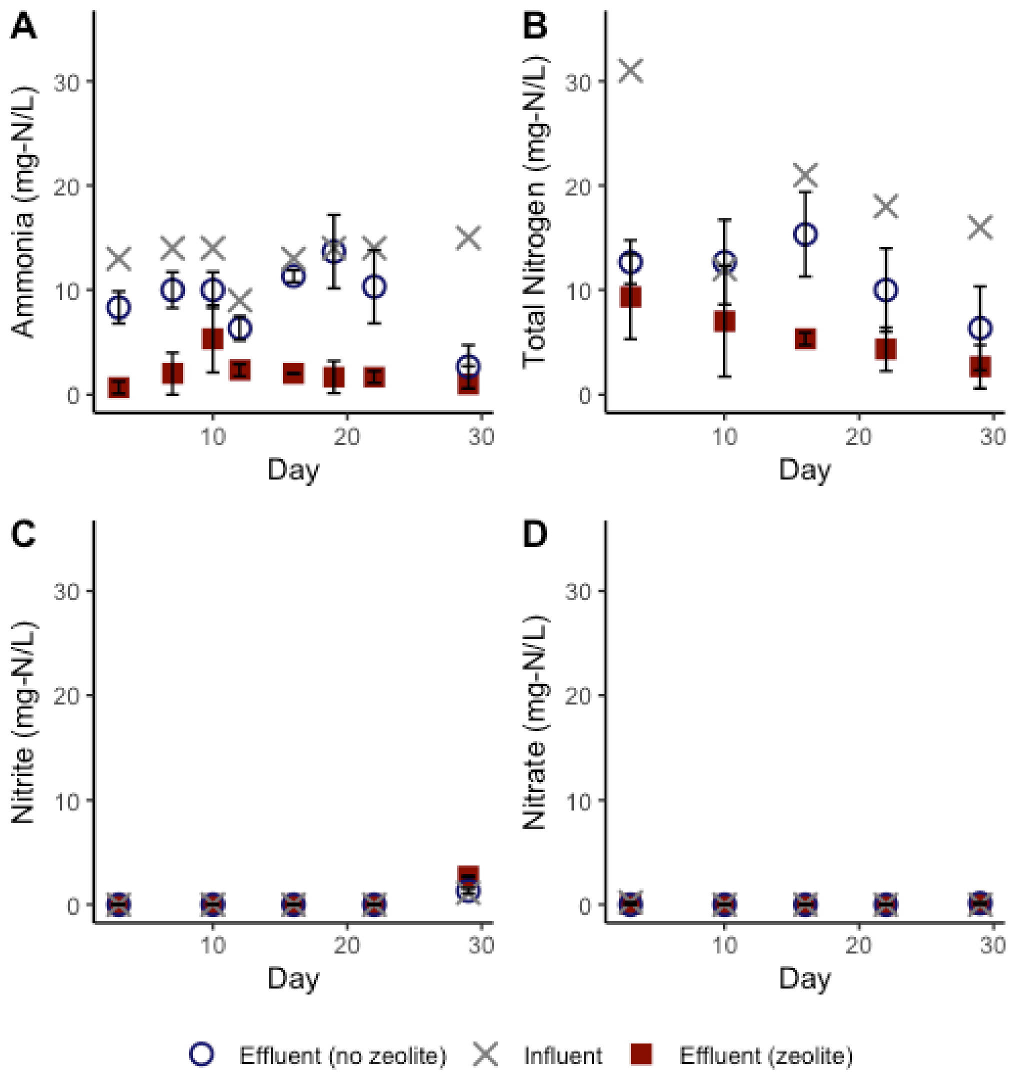

Figure 12. Influent and effluent concentrations of A) Ammonium, B) TN, C) Nitrite, and D) Nitrate in the ACFTR experiment. Error bars indicate the standard deviation of triplicate reactors.

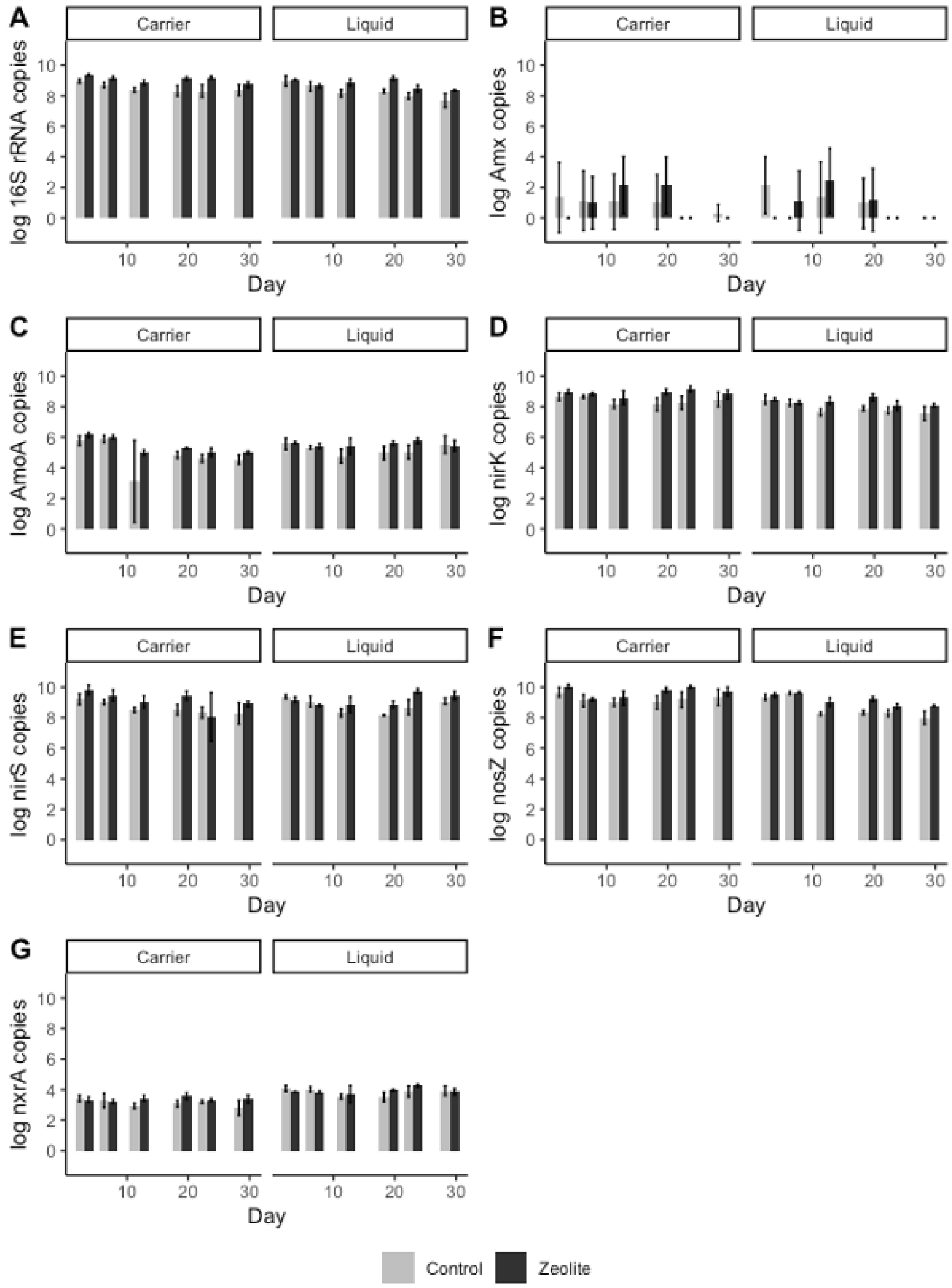

Figure 13. qPCR results from the ACFTR experiment for the A) 16S rRNA, B) Amx, C) *amoA*, D) *nirK,* E) *nirS,* F) *nosZ*, and G) *nxrA* genes, showing the log number copies per carrier for the carrier biofilm samples and the log number copies per mL for the reactor liquid from ACFTR. Light grey indicates samples from control

carrier rectors and dark grey indicates samples collected from zeolite-coated carrier reactors. Error bars show the standard deviation of the reactors run in triplicate.

Unexpectedly, 16S rRNA gene copies per carrier were significantly higher in the carrier biofilm and were also higher at the 90% confidence interval in the reactor bulk liquid in samples taken from the reactors containing zeolite-coated carriers compared to samples taken from control reactors ($P = 0.0087$ and $P = 0.065$ and for carrier biofilm and reactor liquid samples, respectively) (Figure 13). In addition, the zeolite-coated carriers had higher quantities of *nirK* ($P = 0.009$), *nosZ* ($P = 0.026$), and *nxrA* ($P = 0.0411$) per carrier than the control carriers (Figure 13). In the liquid samples, only *nirK* was in higher quantities per mL, and only at a 90% confidence interval, in the zeolite-coated carrier reactors compared to the control reactors ($P = 0.065$). Consistent with these higher biomass numbers, effluent DOC was significantly lower ($P<0.0001$) in the zeolite-coated carrier reactors (18.1 ± 13.0 mg/L) compared to that in the control carrier reactors (40.6 ± 14.3 mg/L) (Figure 14).

The higher DOC and HRT of 17 hours might not have facilitated substantial autotrophic ammonium oxidation (Figure 12) in this experiment but did appear to lead to abundant heterotrophic growth. Indeed, the zeolite-coated carriers did accumulate greater quantities of bacteria when compared to the control carriers, and this community included denitrifiers and nitrite oxidizing bacteria. More research is needed to understand exactly how best to use the zeolite-coated carriers under aerated conditions to enhance the enrichment, retention, and activity of AOB, nitrite oxidizing bacteria, and denitrifiers to enhance shortcut nitrogen removal under highly aerobic conditions that are less amenable to anammox activity. Nevertheless, these zeolite-coated carriers appear to be promising for deployment in both anaerobic and aerated environments.

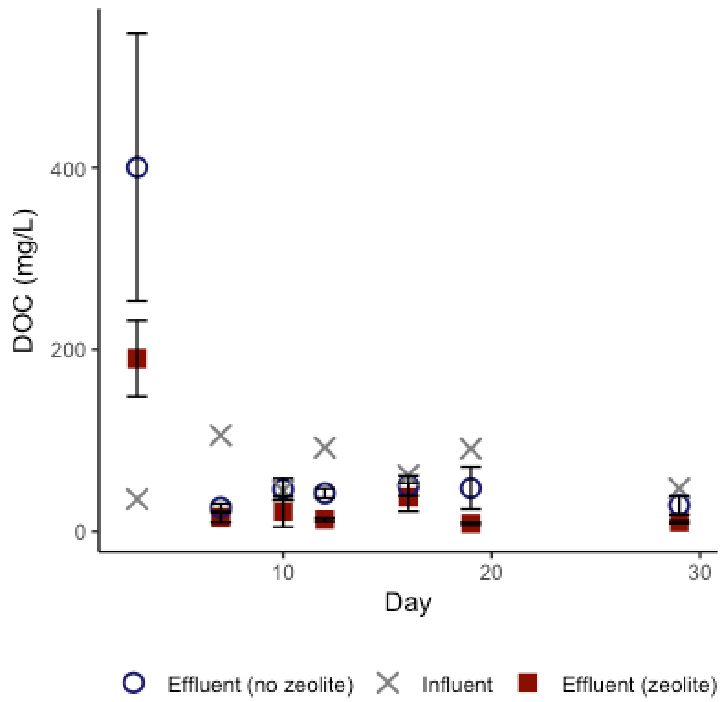

Figure 14. Influent and effluent DOC concentrations in the ACFTR experiment. Error bars indicate standard deviation of triplicate experiments.

Membrane flow-through bioreactors. The zeolite-coated hollow fiber membranes offer another material that shows promise with respect to its ability to sorb ammonium and retain anammox bacteria.[18] The ability of these materials to enrich and retain anammox bacteria as well as AOB under a variety of operating conditions, however, is important for understanding how best to deploy and utilize these fibers for enhancing shortcut nitrogen removal in mainstream wastewater treatment. These experiments were not designed to achieve substantial nitrogen removal, with an HRT=bulk SRT of 23 hr and a single membrane serving as the only mechanism for aerating the reactor, but rather, to determine whether differential microbial growth, particularly of AOB, could occur on the membrane surface because of the membrane's ability to both sorb ammonium and transfer oxygen. Nitrogen removal results from the final day of each experiment (Figure 15) suggest that there is perhaps some nitrification occurring, although neither the nitrite nor the nitrate effluent concentrations correlate with experiment duration or membrane lumen oxygen concentration. Additionally, there was no difference in performance between the reactors containing zeolite-coated versus plain alumina membranes. In a full-scale application, a much larger quantity of membrane surface area would need to be added to stimulate substantial ammonium oxidation.

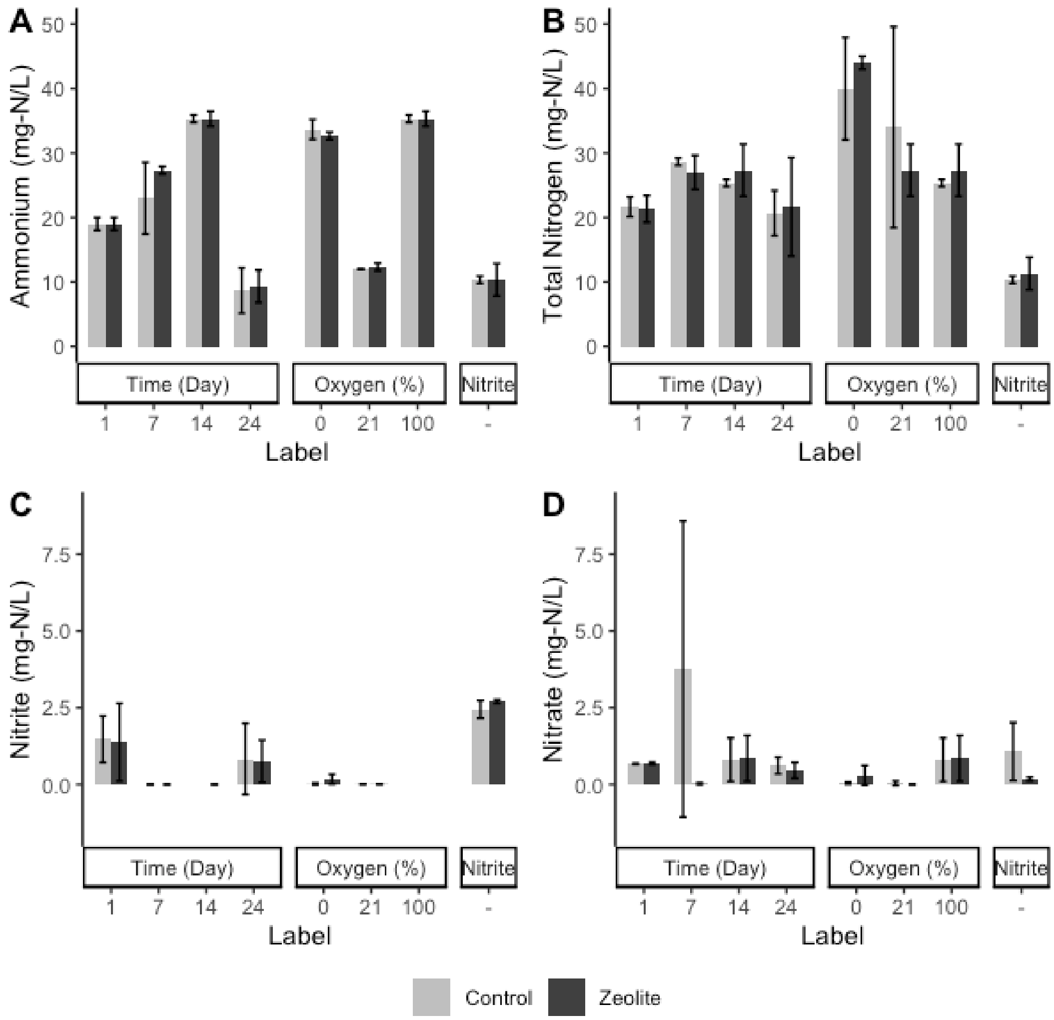

Figure 15. Effluent concentrations of A) Ammonium, B) TN, C) Nitrite, and D) Nitrate at the end of each of the MFTR experiments. Labels at the bottom of each panel indicate the experiment performed. “Time (days)” indicates experiments operated for different durations of time, “Oxygen (%)” indicates the oxygen concentration supplied to the membrane lumen, and “Nitrite” indicates the experiment in which nitrite was added to the influent. The data from the 14 day and 100% oxygen experiments are the same. Light grey indicates samples from control membranes and dark grey indicates samples collected from zeolite-coated membranes. Error bars show the standard deviation of the reactors run in triplicate.

With respect to biomass growth on the membrane surface, the results are varied, with the zeolite-coated membranes only having higher quantities of total bacteria (16S rRNA gene copies per membrane) in one experiment, the 1-week experiment ($P = 0.044$), and only having higher quantities of *amoA* copies at the 90% confidence interval ($P = 0.056$) in the two-week, 100% oxygen experiment (Figure 16). The quantities of anammox bacteria generally increased in both the plain alumina membrane reactors and in the zeolite-coated membrane reactors with operation time (Figure 16). As observed previously,[18] anammox bacteria were generally retained/enriched on the zeolite-coated membranes, with higher quantities measured in the 1-day experiment ($P = 0.09195$) and significantly higher quantities measured in the two-week experiment ($P = 0.02704$), as well as in the two-week experiment to which nitrite was added ($P = 0.04128$). As observed with the carrier experiments, the zeolite coating only retained anammox bacteria and did not preferentially retain AOB.

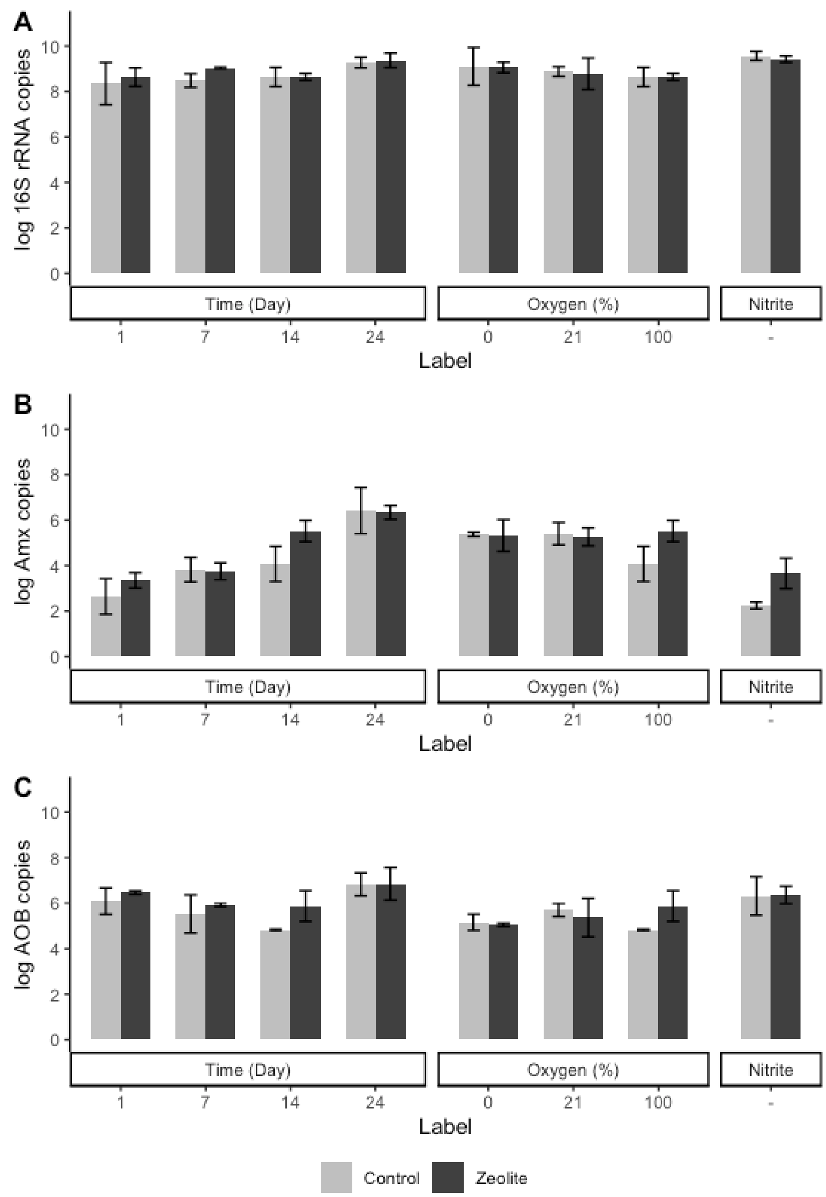

Figure 16. qPCR results from the MFTR experiments for the A) 16S rRNA, B) Amx, and C) *amoA* genes, showing the log number copies per membrane. Experiments included varied operation time, varied membrane lumen oxygen concentrations, and an experiment to which nitrite was added in the influent. Light grey indicates samples from control membranes (plain alumina hollow fibers) and dark grey indicates samples from zeolite-coated membranes (alumina hollow fiber core with zeolite coating). The data from the 14-day experiment and the 100% oxygen experiment are the

same but are shown twice for comparison to other experiments. Error bars show the standard deviation of triplicate experimental replicates.

Implications for the use of biofilm supports incorporating zeolite coatings. Under most of the conditions tested, anammox bacteria were retained/enriched at higher quantities on zeolite-coated surfaces compared to control surfaces. This was likely a result of the zeolite-coatings concentrating ammonium at their surface as the ammonium exchanged into the zeolite cages. Anammox bacteria were also retained to a greater degree and under more experimental conditions than AOB. Even though the half saturation constant (K_s) for anammox bacteria is low (0.07 g N/m^3)[7] compared to that for AOB (2.4 g N/m^3),[32] anammox bacteria seem to grow much more effectively on surfaces with high ammonium concentrations. A possible explanation for the different behavior of anammox bacteria and AOB is the strong tendency for anammox to grow in biofilms or granules, which may have predisposed these organisms to form biofilm on the zeolite-coated carriers and membranes.[33] Additionally, while the half saturation constant for AOB is slightly higher than that for anammox bacteria, it is still low, indicating AOB can function well in low ammonium environments. An unexpected finding from this work was the apparent preferential retention/enrichment of denitrifiers on zeolite-coated carriers under both aerated and anaerobic conditions. This indicates that these zeolite-coated carriers should facilitate a range of shortcut nitrogen processes, including the bypass of nitrate production and denitrification via the nitrite shunt, as long as PN can be encouraged. More work is needed to better understand why denitrifying communities are retained and under what conditions AOB can be enriched and retained on zeolite-coated surfaces.

Others have also investigated zeolite particles[16,34-36] and carriers containing zeolite particles[37] as a way to retain anammox bacteria in target environments and have reported results similar to those of our study. When zeolite particles were added as media for a continuous-flow fixed bed biofilter, not only was an increase in the retention of anammox bacteria observed, but consistent, high rates of nitrogen removal (95%) were achieved and maintained over the course of 570 days.[36] Another study added zeolite particles to sequencing batch reactors, and again, not only was anammox biomass retention improved, but the specific anammox activity in the system also increased.[16] A study using small spherical cages as biofilm carriers with zeolite particles inside also found increased retention of anammox bacteria compared to control carriers when operating a PNA system.[37] AOB were also successfully retained in this system, making up 19% of the total biomass in the attached biofilm. In our work we were specifically trying to create surfaces that could be economically mass produced (*e.g*., PE carriers) (Feinberg et al.)[19] and also could be easily retained within a system that might otherwise allow zeolite particles to wash out. Nevertheless, the results of other researchers[16,34–37] are consistent with our findings and offer additional exciting possibilities for the application of zeolite-modified surfaces for enhanced nitrogen removal.

Potential applications for these biofilm support technologies are systems with low ammonium concentrations that would benefit from the localized concentration of ammonium and increased retention of anammox bacteria, such as mainstream wastewater treatment operated at low DO concentrations. The carbon amended in these experiments did not appear to negatively affect the selection and retention of anammox on zeolite surfaces, in fact, it appeared to facilitate greater growth of the overall carrier-supported biomass and the retention of denitrifiers. IFAS systems may be of particular interest for this type of zeolite-coated carrier, providing the recycling of solids, and therefore AOB, along with the carrier biofilm-based retention of anammox bacteria.[13] Such systems could be retrofitted into existing activated sludge processes, reducing the need to continuously bioaugment anammox sludge for full-scale mainstream anammox nitrogen removal, as well as improve start up times for anammox activity, as biofilm carriers would be expected to be retained in the system with screens. Of course, it will be important to further test this promising novel technology with real wastewater, as contaminants in wastewater and variations in the C:N ratio could have an impact on nitrogen removal (Li et al., 2020; Madeira and de Araújo, 2021). It is possible that other applications where ammonium concentrations are low, such as in some treatment wetlands or stormwater systems, could also benefit from the use of zeolite-coated biofilm supports and the retention of anammox and denitrifying bacteria.[1] These systems typically are anoxic,[38] indicating they would also be an ideal setting for application of this technology. Nevertheless, work is needed to verify this, as the conditions for stormwater treatment (highly variable flows, periods of drying, very low ammonium concentrations) may not be consistent with biological nitrogen removal in this manner.

Conclusions

Zeolite-coated biofilm carriers were able to increase the removal of ammonium and total nitrogen compared to reactors containing control carriers under both aerobic and anaerobic operating conditions. In addition, under anaerobic conditions anammox bacteria and denitrifiers were preferentially retained in the bulk liquid and in the carrier biofilms in zeolite-coated carrier reactors. Aerobic ammonium oxidizers were preferentially retained in the bulk liquid of the zeolite-coated carrier reactors under both aerobic and anaerobic conditions. The zeolite-coated carriers retained their ability to adsorb ammonium even when coated with biofilm, though thick biofilm did appear to block some sorption sites. The adsorbed ammonium was bioavailable. Results with zeolite-coated hollow fiber membranes were less definitive. The membranes were able to transfer oxygen and in some cases were able to preferentially retain anammox bacteria, but this was not always the case such as when 0% and 21% oxygen were fed to the membrane lumen. This suggests that additional research is needed to be able to apply such zeolite-coated hollow fiber membranes reliably to support mainstream anammox. Overall, the application of zeolite-coated carriers for promoting anammox activity and short-cut nitrogen removal in mainstream wastewater appears promising, with potential applications in other areas, such as stormwater treatment, as well.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

We would like to thank Justin Garrison and Karen Cook for their assistance in the laboratory and Michael J. Semmens for helpful conversations regarding permeance measurements. The work was supported by the Environment and Natural Resources Trust Fund as recommended by the Legislative Citizen Commission on Minnesota Resources and by the University of Minnesota via the Joseph T. and Rose S. Ling Chair in Environmental Engineering.

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