Effects of media length on biofilms and nitrification in moving bed biofilm reactors

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ABSTRACT

Biofilms grown on free-floating plastic media are increasingly being used to cultivate biofilms in integrated fixed film activated sludge (IFAS) and moving bed bio reactor (MBBR) systems for wastewater treatment with the common goal of increasing nitrogen removal. Fundamental principles of fluid dynamics dictate that the length of internal media channels affects fluid velocities and shear forces across biofilm surfaces, which in turn should affect rates of mass transfer and biofilm growth and activity, but little is known about media length effects on water quality and biofilm characteristics. It was hypothesized that length affects biofilm thickness, microbial populations and their activities, and system performance. Nitrification rates and biofilm characteristics were monitored in parallel continuous flow, bench-scale MBBR systems with media length as a controlled variable. Longer media produced biofilms with approximately twice the thickness and twice the mass per unit area than did media with one-third their length. Based on calculated head losses, the combined effects of length and constriction of internal channels led to an estimated 77% reduction in fluid velocity through the longer media relative to the shorter media. Longer media demonstrated more rapid development of nitrite oxidizing bacteria (NOB) activity than the shorter media over much of the study, as indicated by measurements of nitrate and nitrate, but AOB activity was similar in the two media. Both biomass and NOB activity were concentrated toward media ends, while ammonia oxidizing bacteria (AOB) activity was uniformly distributed across the media, based on testing of sectioned media. 16s rRNA amplicon sequencing indicated the presence of several putative heterotrophic nitrifying families, particularly Xanthomonadaceae, Comamonadaceae and Microbacteriaceae, as well as the autotrophic Bradyrhizobiaceae (which includes the NOB Nitrobacter) were common on both media throughout the study. The short media enriched for Nitrosomonadaceae, which includes the AOB genus Nitrosomonas, while minimal autotrophic AOBs were found in the long media biofilm. These results provide insights to the design of media for improved performance, particularly with respect to nitrite versus nitrate production, which may be useful to improve nitrification and for energy saving processes for nitrogen removal such as deammonification. The research also provides fundamental insights regarding the effects of media geometry on biofilm structure and function, which advance our understanding of environmental factors affecting biofilm development.

1. Introduction

Biofilms are commonly utilized in a variety of biotechnological processes, including for the removal of pollutants in wastewater treatment systems to protect public health and environmental resources. Biofilms used for wastewater treatment can be fixed-in-place, as in trickling filters, or they can be grown on mobile, free-floating media, as in moving bed bioreactors (MBBRs) and integrated fixed film activated sludge (IFAS) systems (Ødegaard et al., 2006). MBBR and IFAS systems typically contain plastic media designed to provide large surface areas to encourage microorganism attachment and growth (Rusten et al., 1995). Nitrogen removal from wastewater is critical for the protection of downstream ecosystems from ammonia toxicity and eutrophication. A central process for nitrogen removal is nitrification, which consists of two steps: ammonia oxidation to nitrite by ammonia oxidizing bacteria (AOBs) and nitrite oxidation to nitrate by nitrite oxidizing bacteria (NOBs). IFAS and MBBR systems are commonly used to enhance nitrification, in part because the biofilms’ long residence times are conducive.
to enrichment of slow growing nitriﬁcation bacteria.

IFAS and MBBR media are commercially available in a variety of shapes and sizes [1,2], including chip-type media, which consist of flat or curved plastic disks (e.g., Z-carriers by AnoxKaldnes, Lund, Sweden), mesh-type media, which are similar but they allow ﬂow through mesh openings (e.g. BioﬁlmChip by AnoxKaldnes), porous or sponge-type media, and media with internal tube-like channels (e.g. K3 media by AnoxKaldnes). The last of these types is most commonly used in practice (and is therefore often referred to as “conventional media”), with many different sizes and geometries available from a variety of manufacturers [3] #1592 [1]; #1598). In this paper such media will be referred to as tubular media.

[1] noted that there is a general need to better understand how bioﬁlm media geometry affects bioﬁlm structure and function, as it fundamentally affects the local ﬂow velocities and cross-ﬂow shear. These ﬂow conditions in turn strongly inﬂuence bioﬁlm growth and morphology, including diversity, bioﬁlm structure and quantity [4]. They also affect mass transfer of substrates, including diffusion from the bulk solution through a laminar ﬂuid boundary layer and through the bioﬁlm, as well as advective transport through channels in the bioﬁlm structures [5,6].

The length of the internal channels in tubular media is of interest as it is a fundamental but apparently unstudied design parameter that directly affects cross-ﬂow velocity and shear. Based on the Darcy-Weisbach equation for ﬂow through a cylindrical channel, length is inversely correlated with the velocity of a liquid passing through a pipe for a given pressure differential (Eq. (1)) due to the effect of length on frictional resistance [7]. Consequently, length is also inversely correlated with the shear stress on the interior channel surface (Equation (2)); [8].

\[
v = \sqrt{\frac{\Delta h}{L} \cdot \frac{2gD}{f_0}} \quad \text{(Eq. 1)}
\]

\[
\tau = \frac{\Delta h}{4D} \cdot \rho \cdot g \cdot D \quad \text{(Eq. 2)}
\]

where: \( v \) = ﬂow velocity (m/s), \( \frac{\Delta h}{L} \) = pressure differential as head loss (\( \Delta h \)) per length (L) of pipe (m/m), \( g \) = gravitational constant (m/s²), \( D \) = pipe diameter (m), \( \tau \) = shear stress (Pa).

Eq. (1) indicates that bioﬁlms inside media with longer internal channels experience lower cross-ﬂow velocity than bioﬁlms in shorter internal channels (Eq. (1)), although the hydrodynamics associated with free ﬂoating media in aerated bioi reactors are inherently complex [9]. Cross-ﬂow velocities are important because they fundamentally affect bioﬁlm thickness and structure, mass transfer, production of exopolysaccharides, and metabolic and genetic bioﬁlm behaviors [10]. The tendency for higher shear forces to produce thinner, more dense, more shear-resistant bioﬁlms is well documented [11–14], suggesting that media length may be correlated with thickness and negatively correlated with density.

Furthermore, rates of mass transfer could be greater in shorter media than in longer media, as increasing cross-ﬂow velocity also increases rates of mass transfer, due in part to its effects on the diffusion-limited boundary layer thickness [1,10,15,16], which effects the supply rate of oxygen and nutrients to the bioﬁlm and the export of products from the bioﬁlm. On the other hand, bioﬁlms grown under low cross-ﬂow velocity were reported to exhibit higher diffusivities [12], and so the net effect of cross-ﬂow velocity on mass transfer is not clear.

There are only limited studies of how media geometries can affect bioﬁlm development and performance, particularly with respect to nitrification, and there appear to be no studies that speciﬁcally evaluate the effect of media length [17]. reported that biomass thickness was greater near tubular media edges (near the bulk solution) than it was in the channel middles, based on visual inspection of media cross-sections. The authors concluded that most of the bioﬁlm activity may have occurred near the media edges, which suggests that shorter media, with their relatively small interior regions, may produce more biomass and more activity per unit area than longer media. This hypothesis has not been tested, and it is somewhat contradictory to the discussion above regarding length effects on cross-ﬂow velocities.

[18] evaluated chemical oxygen demand (COD) removal rates by bioiﬂms grown on various commercially available tubular media, but they did not evaluate length as an independent variable, and they did not evaluate nitrogen removal or bioi thickness [19]. reported sponge-type media performed less COD removal than tubular media [3]. reported that under high loading conditions mesh-type media (AnoxKaldnes BioﬁlmChip M and P) became clogged and performed less ammonia oxidation than tubular media (AnoxKaldnes K3 media), although nitrite and nitrate were not measured, and so NOB activity was not evaluated.

Several studies of media geometry effects have focused on chip-type media with ridges of varying heights (Z-carriers; AnoxKaldnes, Lund, Sweden) to produce bioﬁlms with varying thicknesses. Multiple studies reported that while bioﬁlm thickness (ridge height) had no effect on NOB activity (nitrite consumption), thicker bioiﬂms provided greater NOB activity (nitrite consumption and nitrate production) [20–22]. While these studies provided information about bioﬁlm thickness on chip-type media, there remains a knowledge gap regarding tubular media length effects on bioﬁlm thickness and the resulting effects on AOB and NOB activities.

The central hypothesis tested in this study is that tubular MBBR media with shorter internal channels produce thinner bioiﬂms with lower NOB activities than longer media. The objective was to test this hypothesis by comparing bioiﬂms grown on tubular media in laboratory scale, continuous ﬂow reactors with length as a controlled experimental variable. An additional objective was to assess differences in bioiﬂms grown near media edges with those grown in media interiors. Measurements of nitrogen species, biomass, thickness, microbial populations, and AOB and NOB activities were conducted in the continuous systems and in batch tests.

2. Materials and methods
2.1. Reactors and media

Two custom sets of tubular media that differed in length by a factor of 3 were made by carefully cutting 4.8 mm inner diameter high-density polyethylene tubing into 7.5 mm (R-Short) and 22.5 mm (R-Long) sections (Fig. 1) using a pipe cutter, with no losses biomass by visual observation. These lengths were selected to span a range relevant to commercial media; for example the 7 mm length AnoxKaldnes K1 media and 15 mm Sinta FRP PED9 media. Two laboratory scale MBBR systems were operated (Fig. 2), one of which contained the R-Short media and one of which had R-Long media. Reactor and media parameters are listed in Table 1. The reactors were operated identically except for the media each contained. Each reactor contained 100 linear feet of media, resulting in a ﬁll volume of 27% (volume of media including voids/total reactor volume). The biologically active surface area (the internal area) in each reactor was 43.4 m²/m³ reactor volume in each reactor. (It is common practice to report the internal surface area as the biologically active area in IFAS and MBBR systems, as external surfaces tend to be scour by collisions with other media; [23].

2.2. Synthetic feed

Synthetic feed was added as a concentrated stream (2.2 mL/min) diluted with dechlorinated tapwater (4.8 mL/min) to reduce the volume of chemical feed requiring preparation. The net (diluted) feed concentrations are listed in Table 2. Inﬂuent ammonia concentrations were gradually increased from 20.5 to 200 mg NH₃-N/L during the startup phase, with the goal of maintaining effluent concentrations in the range 10–30 mg NH₃-N/L to balance the objectives of avoiding both ammonia
limitation of nitrification activity and inhibition of AOBs and NOBs due to ammonia toxicity (Anthonisen et al., 1976; Kim et al., 2005; Rusten et al., 1994; Ødegaard et al., 2006). These ammonia concentrations were high relative to domestic wastewater, and were selected to produce highly nitrifying biofilms, with no organic carbon to reduce competition for space from heterotrophs.

2.3. Reactor inoculation and operation

Before startup, plastic media was inoculated by immersion in 10 L of fresh activated sludge from an aeration basin at the Albuquerque, New Mexico, Southside Wastewater Reclamation Facility (SWRF) for 3 days at room temperature with coarse-bubble mixing. The SWRF utilizes a Modified Ludzack-Ettinger activated sludge configuration to perform nitrification and denitrification. After inoculation, the media solution in each MBBR was decanted to remove suspended solids and replaced with 5 L supernatant from settled activated sludge and 5 L of fresh primary effluent. The continuous synthetic feed was then introduced to each reactor to begin the experiment.

The pH of each reactor was kept between 7.15 and 7.50 using a pH controller with automatic 0.5 M HCl and 0.7 M Na₂CO₃ addition (Fig. 2). The reactors were kept in a water bath at 21 °C. The reactor walls and probes were cleaned daily to prevent biofilm growth. Mixing was by coarse bubble aeration (air flow 1.2 m³/h), which was selected to provide continuous circulation of the media.

2.4. Batch tests

Batch tests were conducted to compare activities on media end and middle sections. On Day 123, thirteen R-Long media pieces with mature biofilms were cut into 3 equal sections with a pipe cutter, each with a length equal to the R-short media (7.5 mm). Thirteen end sections and 13 middle sections were then tested separately in 100 mL batch tests, providing approximately the same specific surface values (m² internal...
area/m² reactor volume) as in the continuous systems. Thirteen media from the R-Short reactor were also tested in a 100 mL batch reactor for comparison. After cutting, media pieces were gently conditioned with batch test solution (see below). Two sets of tests were performed on these media. In the first, the batch test solution was the same as the feed solution (Table 2), except it contained 40 mg NO₂-N/L and 0 mg NH₃-N/L added to test nitrite oxidation (NOB activity) only. The second batch test included 40 mg NH₃-N/L and 40 mg NO₂-N/L to test both AOB and NOB activity. The latter tests were conducted twice for each media. pH was 7.35 at the beginning of each test, and was measured but was not controlled during the tests. The media were added to batch reactors (150 mL Erlenmeyer flasks) containing 100 mL synthetic feed, and mixing was provided via stir bar/plate at 100 rpm. Nitrogen species were measured as described below periodically through the 3 h batch tests. DO was measured to be nearly constant throughout the test and was not controlled.

2.5. Analytical methods

Nitrogen species were measured on 0.45 μm-filtered samples using Hach (Loveland, CO) kits for ammonia (AmVer TNT High Range Set, Hach, Loveland CO), nitrite (NitrIver 3 TNT Low Range Set), and nitrate (NitraVer X High Range Reagent Set) with a Hach DR-2700 spectrophotometer. Samples were diluted as recommended by the manufacturer.

Biofilm biomass was measured by harvesting biomass from randomly selected carriers (12 for R-Short and 4 for R-Long, providing the same total surface area of each type). Biomass was removed from the media by brushing using Proxabrush dental cleaning tools (Sunstar Americas Inc., USA) and rinsing with deionized water. The number of media removed from each reactor was accounted for in calculations of nitrogen fluxes. The removed biomass was measured using Standard Methods 2505B and 2540E (American Public Health Association et al., 2012) for total and volatile solids, respectively. The reactor total biofilm solids (TBS) and volatile biofilm solids (VBS) were calculated based on the total number of media in the reactors.

Select biomass samples were analyzed by 16s ribosomal RNA (rRNA) gene amplicon sequencing using the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) three times throughout the study for each reactor (Days 28, 92–95, and 171–178). DNA extraction, amplification, and sequencing were performed by MRDNA (Shallowater, TX, USA) using the universal prokaryote primers 357F and 785R, which target the V3 and V4 regions of the 16s RNA gene [24], with a barcode placed on the forward primer. A 30 cycle polymerase chain reaction (PCR) was used (5 cycles for PCR products) with the HotStarTaq Plus Master Mix Kit (Qiagen, Germany) under the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. PCR products were checked by electrophoresis. Pooled samples were purified using calibrated Ampure XP beads. The pooled and purified PCR products were used to prepare DNA sequence libraries following the Illumina MiSeq protocol. Sequences were joined, depleted of barcodes, sequences less than 150bp were removed, and sequences with ambiguous base calls were removed. Sequences were denoised, operational taxonomic units (OTUs) were generated, and chimeras were removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII, and NCBI (DeSantis et al., 2006).

2.6. Statistical analyses

Statistical significance of differences between sets of measurements was determined using 2-tailed t-tests with unequal variances. Diversity and evenness of microbial populations were calculated based on fractions of total OTUs using the Shannon Diversity and Evenness Indices [25].

3. Results and discussion

3.1. Biomass

The two continuous flow bioreactors were operated for 229 days. From the first measurement on day 8 through the end of the study, the R-Long media consistently yielded greater attached biomass (both as concentration per reactor volume and mass per surface area) than the R-Short media (Fig. 3). For all measurements after day 76, when the biofilm mass in each reactor was at approximately steady state, the R-Long media had 1.8 times more attached biomass (0.41 ± 0.01 mg VBS/cm²) than the R-short media (0.22 ± 0.02 mg VBS/cm²), and this difference that was statistically significant (p < 0.01). These results are consistent with images of the media cross sections (Fig. 4), which indicated a thicker biofilm inside the R-Long media (approximately 1100 μm) than the R-short media (approximately 580 μm) (Fig. 4). The R-Long biofilm also reached its maximum value more rapidly than the short media biofilm (Fig. 5). Taken together, these results confirmed the hypothesis that longer media media leads to greater biomass growth per unit area, likely because of the cross-flow velocities expected in the longer media channels (Eq. (2)), as cross-flow velocity has been shown to be inversely related to biofilm thickness in other systems [11,13,14].

It is also notable that the greater accumulation of biofilm in the longer media led to a narrower channel (diameter approximately 2.5 mm, area approximately 4.9 mm²) than in the shorter media (diameter approximately 3.6 mm, area approximately 10 mm²) (Fig. 4). This constriction of the cross sectional area would tend to further decrease fluid velocity and shear forces across the biofilm (Eqs. (1) and (2)). The combined effects of length and cross sectional area on fluid velocity and shear stress can be calculated using Eqs. (1) and (2), based on the media lengths (Lₚ and Lₛ) and diameters (Dₚ and Dₛ) in the long and short media, respectively. Assuming the same pressure differential and friction factor values, the ratio of the average fluid velocity in the short media (vₛ) to that in the long media (vₚ) is the square root of (Dₚ/Dₛ) * (Lₛ/ Lₚ), which yields a predicted 52% reduction in fluid velocity and a 77% reduction in shear stress in the long media relative to the short media (although this calculation ignores the effects of entrance and exit pressure losses, which would likely be similar for the two media).

3.2. Nitrification

There was little difference in AOB activities in the R-Short and R-Long reactors throughout the study, as indicated by their similar quantities of ammonia uptake (calculated as influent – effluent concentrations) (Fig. 5a). As ammonia uptake increased over the first 64 days, the influent ammonia concentration was increased from 20.5 to 200 mg NH₃-N/L to reduce the potential for inhibitory ammonia

![Fig. 3. Attached biomass on R-Short and R-Long media as volatile biofilm solids (VBS) expressed as concentration (mass per reactor volume) and mass per media interior area.](image-url)
concentrations in the reactors, as described in Materials and Methods. Beginning on approximately day 50 the R-Long media yielded higher NOB activity rates than the R-Short media, as indicated by its relatively high effluent NO$_3^-$ and low NO$_2^-$ concentrations (Fig. 5b). For example, from days 58–92 the effluent nitrite concentrations was 124 ± 9 in the R-Short reactor, but it was only 98 ± 7 mg N/L in the R-Long reactor, and the difference between these values was statistically significant (p < 0.00001). These results supported the study’s hypothesis that not only...
did shorter media lead to thinner biofilms, but also that the thinner biofilms were associated with lower NOB activity during this phase of the study.

The finding of less NOB activity in the thinner biofilm was consistent with [20]; who reported lower NOB activity in a thinner biofilm (<200 μm) compared to 3 thicker biofilms grown on chip-type media with ridges ranging from 200 to 500 μm. Piculell et al. suggested this result was due to NOB being space limited in the thinner biofilms. It has been reported that AOB tend to be located nearer to biofilm surfaces and some systems, while NOB tend to occur in deeper biofilm regions in biofilms grown in trickling filters [26] and rotating disk reactors [5]. However, others did not find such stratification in nitrifying biofilms grown on clay beads [27]. [5] suggested NOB may be found in deeper biofilm regions because the greater turnover rate near the surface (due to grazing and sloughing) would select against slower growing NOBs, and/or because the higher oxygen concentrations in outer biofilm layers inhibit NOBs such as Nitrospira spp. Either of these phenomena could help explain why thinner biofilms may disfavor NOB activity, as the deeper regions favoring NOBs would be limited. Another explanation as to why NOBs may be favored in thicker biofilms is that thicker biofilms provide greater resistance to loss of the nitrite produced by AOBs to the bulk solution by diffusion, thereby increasing nitrite availability to NOB. Further research is needed to clarify the mechanisms by which thinner biofilms appear to disfavor NOB, but not AOB, growth.

The finding that selection of media length may be a useful design strategy to manipulate nitrite production is particularly relevant to development of deammonification processes [20], which can provide substantial energy savings relative to conventional nitrification/denitrification for nitrogen removal from wastewater [28]. Deammonification consists of two steps: ammonia oxidation to nitrite by AOBs and anaerobic oxidation of ammonia (anammox), whereby nitrite is reduced and ammonia is oxidized to produce nitrogen gas by anammox bacteria. One of the key challenges to stable operation of deammonification processes is avoiding nitrite oxidation to nitrate through the repression of NOB activity [28].

The differences in NOB activity in the two biofilms decreased over time, however, and after 105 days, the two reactors performed similarly. Ammonia uptake was in the range 180–190 mg NH₃-N/L (3.71–3.92 g N/(m²·d)) through the end of the study and they exhibited similar nitrite and nitrate production rates as well, indicating that the NOB activity in the R-Short biofilm eventually caught up to the R-Long media biofilm. Further research is needed to determine whether NOB activity can be repressed for longer periods of time.

3.3. Biomass and activity variations along media length

Differences in biomass mass and activity along the media length were investigated by cutting R-Long media into thirds, which were divided into “middle” and “end” sections for further analyses (Day 123). The average biomass on the R-long end sections was 0.41 ± 0.02 mg VBS/cm² compared to 3 thicker biofilms grown on chip-type media with ridges ranging from 200 to 500 μm. Piculell et al. suggested this result was due to NOB being space limited in the thinner biofilms. It has been reported that AOB tend to be located nearer to biofilm surfaces and some systems, while NOB tend to occur in deeper biofilm regions in biofilms grown in trickling filters [26] and rotating disk reactors [5]. However, others did not find such stratification in nitrifying biofilms grown on clay beads [27]. [5] suggested NOB may be found in deeper biofilm regions because the greater turnover rate near the surface (due to grazing and sloughing) would select against slower growing NOBs, and/or because the higher oxygen concentrations in outer biofilm layers inhibit NOBs such as Nitrospira spp. Either of these phenomena could help explain why thinner biofilms may disfavor NOB activity, as the deeper regions favoring NOBs would be limited. Another explanation as to why NOBs may be favored in thicker biofilms is that thicker biofilms provide greater resistance to loss of the nitrite produced by AOBs to the bulk solution by diffusion, thereby increasing nitrite availability to NOB. Further research is needed to clarify the mechanisms by which thinner biofilms appear to disfavor NOB, but not AOB, growth.

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The greater biomass quantity found near the media ends relative to middle sections was in agreement with visual observations of media sections reported by Ref. [17]. Possible reasons for this result include substrate consumption by the biofilms as the fluid flowed through the media channels, resulting in lower substrate concentrations in the media interiors, and/or local mixing effects that increased rates of mass transfer to the biofilm near the media. Further research is needed to evaluate these hypotheses.

Based on these results, batch testing of the R-Long media sections was performed to assess whether AOB and/or NOB also activity varied along the R-Long media length. Nitrite was added in all batch tests to insure NOB activity was not be limited by nitrite production from AOBs, with two replicate tests containing both ammonia and nitrite, and one additional test with nitrite only. The results are shown in Fig. 7, along with the R-Short media for comparison. The AOB activities were similar across all sections (Fig. 7), with average values of 3.29, 3.10, and 3.05 g N/(m²·d) for the R-Short media, R-Long middle sections, and R-Long end sections, respectively. These results suggest that the AOB activity was distributed evenly along the R-Long media interior. This is consistent with the similar ammonia uptake in the R-Long and R-Short reactors (Fig. 5).

However, NOB activity (measured as nitrate flux) varied greatly across the three biofilms, with the lowest flux by the R-Short media and the largest flux by the R-Long end sections (Fig. 7). The differences between each of the three average nitrate flux values were all statistically significant (p < 0.05, based on averages of 3 batch tests each). These results indicate NOB activity was most concentrated towards the ends of the R-Long media, where the biofilm was thickest. NOB activity was linearly correlated with biofilm quantity for measurements across all three biofilm types (Fig. 7). To our knowledge these results provide the
first measurement of activity variations across MBBR media length. The fluxes shown in Fig. 7 can be considered an indication of maximum rates of AOB and NOB activity, as excess ammonia and nitrite were added in the respective tests. It was somewhat surprising that the measured NOB activity was higher than the AOB activity, as in the continuous system NOB activity was less than AOB activity, based on the observation that only about half of the ammonia taken up by AOBs was oxidized to nitrate by NOBs (effluent nitrite and nitrate concentrations were about the same) (Fig. 5). Based on these results, the biofilms with different thicknesses (short media, long media middles, and long media ends) had similar absolute quantities of AOBs, but the thicker biofilms contained greater quantities of NOBs than the thinner biofilms (but possibly similar fractional compositions), and the NOBs for some reason were less active in the continuous systems than in the batch tests. It is likely that local nitrite concentrations differed in the batch and continuous systems (nitrite was produced by AOBs in the continuous systems, and it diffused into the biofilms from the bulk solution in the batch tests), which could explain the apparent greater relative activities of the NOBs in the batch tests. These results suggest the existence of an excess NOB activity capacity that was not utilized in continuous operation, particularly in the thicker biofilms. Further research is needed to determine the specific reasons for this phenomenon, which could provide insights to the development of systems for nitrite production for use in nitritation/denitrification and anammox systems.

3.4. Microbial populations

The changes in bacterial populations, as percentages of OTUs, in the two biofilms over the course of the study are shown in Fig. 8. The fractional changes shown in Fig. 8 indicate changes in composition but not necessarily changes in absolute quantities, as the total attached biomass increased over time (e.g., from approximately 0.06 to 0.4 mg/cm² from the early (before approximately day 50) to late stages (after approximately day 140) for R-Long media; Fig. 3). The eight most common families across all samples (numbers 1 through 8 in Fig. 8) included both known and suspected nitrifiers, which correspond to the high nitrification rates shown in Fig. 5. The family *Nitrosomonadaceae* (Group 2 in Fig. 8), which includes the common wastewater treatment AOB genus *Nitrosomonas* [29], was detected in all samples. However, this family comprised less than 0.5% of the OTUs in all samples except the Late Phase (Day 171) short media biofilm sample, where it comprised 18% of all OTUs (Fig. 8). The only known autotrophic NOB detected was the family *Bradyrhizobiaceae*, which includes the genus *Nitrobroctubes*, a commonly reported autotrophic NOB in wastewater treatment systems [30]. *Bradyrhizobiaceae* comprised 4–28% of all OTUs across all samples (Group 6 in Fig. 8). Its presence as a fraction of total OTUs tended to decrease throughout the study in both the long and short media biofilms (Fig. 8), although this trend was somewhat balanced by the overall biomass increase during the study (Fig. 3).

The six other most common families (the heterotrophic *Microbacteriaceae*, *Comamonadaceae*, *Pseudomonadaceae*, *Nocardiaceae*, *Sphingomonadaceae*, and *Xanthomonadaceae*) have all been suggested to include nitrifiers in previous studies. Janka et al. [31] suggested the families *Comamonadaceae* and *Microbacteriaceae* may have performed heterotrophic nitrification and/or denitrification in MBBRs operated for simultaneous nitrification/denitrification [32], also suggested *Comamonadaceae* may be a heterotrophic nitrifier and/or denitrifier [33], and reported that *Microbacteriaceae* increased with increasing nitrification rates in cement tank sediments, and a strain of this family demonstrated both autotrophic and heterotrophic nitrification [34].

[35] suggested *Pseudomonadaceae*, *Nocardiaceae*, *Sphingomonadaceae*, and *Xanthomonadaceae* have all been suggested to include important producers of extracellular polysaccharides in these systems [41], and so it is possible it may have played a similar role in biofilm formation in this study, in addition to potential ammonia oxidation. It is notable that while several of these families have been suggested to denitrify in wastewater treatment systems, including *Xanthomonadaceae* [40,41] and *Comamonadaceae* [32,42], they did apparently not perform this function in the current study, as denitrification was negligible (the average ratios of ammonia nitrogen removed to nitrite plus nitrate nitrogen produced was 0.98 in the R-Short and R-Long reactors from day 40 to the end of the experiment).

During the early phase of the study (Day 28) when both reactors exhibited increasing ammonia uptake (Fig. 5) and both biofilms were still in the early stages of growth (Fig. 3), the short and long media exhibited similar population compositions, and the families previously

**Fig. 8.** Population changes at the family level in the short and long media throughout the early, middle, and late stages of the study, as percentages of OTUs based on 16S rRNA gene amplicon sequencing. Averages of two separate samples and sequencing analyses are shown for each column. The 12 most common families are shown. Families 1 through 8 include known or suspected AOBs and/or NOBs (blue box in legend; see text). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
suggested as nitrifiers (groups 1–8 in Fig. 8) comprised 61–62% of the OTUs. By the middle stage samples (Days 92–95), when the attached biomass had greatly increased (Fig. 3), Groups 1–8 increased to 81–88% of the total OTUs in the long and short media biofilms. The long media biofilm contained a greater fraction of Bradyrhizobiaceae (16%), which includes the NOB Nitrobacter, than was found in the short media biofilm (7%). This result was consistent with the greater NOB activity (greater NO_3_ and lower NO_2_ production) by the long media biofilm than the short media biofilm at this time (Fig. 5). The long media biofilm also contained a greater fraction of Xanthomonadaceae (35%) but less Microbacteriaceae (15%) than the shorter media biofilm (25% and 26%, respectively).

By the late stage of the study (Days 171–178), when the biofilms performed similarly, the short media contained a greatly increased fraction of the AOB Nitrosomonadaceae (from 0.4% on day 95 to 18% on day 171). The Xanthomonadaceae and Microbacteriaceae fractions decreased over this time frame (from 25 to 13% and 26 to 20%, respectively), and the ammonia consumption was little changed, suggesting that the autotrophic Nitrosomonadaceae may have taken the place of Xanthomonadaceae and Microbacteriaceae with respect to heterotrophic ammonia oxidation in the short media biofilm. Similarly, the increase in the family Pseudomonadaceae (from <0.1% on day 95 to 5% on day 171) corresponded with a decrease in the NOB Bradyrhizobiaceae, which could indicate Pseudomonadaceae contributed to heterotrophic nitrite oxidation in the short media biofilm.

The population compositions of the long media ends and middles, measured at the end of the study, were very similar, suggesting that the higher NOB activity measured on the media ends relative to the middles (Fig. 7) was due to differences in the attached biomass (Fig. 6) rather than differences in the microbial populations.

Calculation of the Shannon Diversity and Evenness Indices indicated that while the shorter media biofilm population was initially less diverse and less even than the long media biofilm population (Day 28), in all later samples this relationship was reversed (Fig. 9). This result differed from that reported on biofilms grown in chip media, where thicker biofilms were associated with greater diversity and evenness [22]. The reason for this difference is not known, but the result demonstrates that greater thickness does not necessarily produce greater diversity in nitrifying biofilms, with environmental conditions related to each specific study apparently playing important roles.

4. Conclusions

There has been little research on how specific aspects of media geometry may affect biofilm structure and function in systems designed for nitrogen removal from wastewater. This study provides the first evaluation of tubular media length effects on nitrification in MBBR systems. Longer media yielded a thicker biofilm with nearly twice the biomass per unit area compared to media with 1/3 length, probably because of reduced cross-flow velocities and shear through the media. While both media had similar AOB activities, the thinner biofilm on the shorter media exhibited lower NOB activity (nitrate production) through much of the study, although this difference disappeared by the end of the experiment. Greater biofilm quantities and NOB activities existed near media ends compared to middle sections, but AOB activity was uniform throughout the media.

These findings suggest that media length may be a useful parameter in the design of nitrifying systems, including for low energy deammonification processes, which require the suppression of nitrite oxidation to nitrate. Further research is needed to determine the effects of length in systems containing organic carbon, and whether the apparent inhibition of NOBs in the thinner biofilms produced on shorter media can be maintained over longer terms, possibly by further reducing media length, increasing diameter, or increasing mixing to further reduce media thickness. The media used in this study were comprised of single tubular channels (Fig. 4), which differ somewhat from the extruded plastic media comprised of multiple interior channels commonly used in practice (e.g. ADD EXAMPLE). The results presented in this study have generally been normalized to interior surface area to facilitate comparisons with other studies. It is notable that local hydraulic conditions, caused by differences in spinning behaviors in response to aeration mixing, for example, could differ between the media used in this study and those in practice. Although these differences may well be minor, further research is needed to confirm the results reported herein in extruded plastic media. This research also provides fundamental insights to how engineering design of biofilm attachment surfaces can be used to manipulate biofilm thickness, microbial populations, and activity, which may be of importance to the broader biofilm research community.

CRediT authorship contribution statement

Kody A. Garcia: Investigation, Writing – original draft, Formal analysis.
Patrick McLec: Investigation. Andrew J. Schuler: Conceptualization, Resources, Writing – review & editing, Formal analysis, Funding acquisition.

Declaration of competing interest

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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