Methods for increasing the rate of anammox attachment in a sidestream deammonification MBBR
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ABSTRACT
Deammonification (partial nitritation-anammox) is a proven process for the treatment of high-nitrogen waste streams, but long startup time is a known drawback of this technology. In a deammonification moving bed biofilm reactor (MBBR), startup time could potentially be decreased by increasing the attachment rate of anammox bacteria (AMX) on virgin plastic media. Previous studies have shown that bacterial adhesion rates can be increased by surface modification or by the development of a preliminary biofilm. This is the first study on increasing AMX attachment rates in a deammonification MBBR using these methods. Experimental media consisted of three different wet-chemical surface treatments, and also media transferred from a full-scale mainstream fully nitrifying integrated fixed-film activated sludge (IFAS) reactor. Following startup of a full-scale deammonification reactor, the experimental media were placed in the full-scale reactor and removed for activity rate measurements and biomass testing after 1 and 2 months. The media transferred from the IFAS process exhibited a rapid increase in AMX activity rates (1.1 g/m²/day NH₄⁺ removal and 1.4 g/m²/day NO₂⁻/C₀ removal) as compared to the control (0.2 g/m²/day NH₄⁺ removal and 0.1 g/m²/day NO₂⁻/C₀ removal) after 1 month. Two out of three of the surface modifications resulted in significantly higher AMX activity than the control at 1 and 2 months. No nitrite oxidizing bacteria activity was detected in either the surface modified media or IFAS media batch tests. The results indicate that startup time of a deammonification MBBR could potentially be decreased through surface modification of the plastic media or through the transfer of media from a mature IFAS process.

Key words | anammox, biofilm adhesion, deammonification, MBBR, sidestream treatment

INTRODUCTION
The combination of partial nitritation and anaerobic ammonia oxidation (anammox), commonly known as deammonification, is an economical option for sidestream treatment because of decreased aeration energy requirements, no required external carbon or alkalinity, and decreased sludge production over traditional nitrification/denitrification. The ANITA™ Mox process consists of a single-stage deammonification moving bed biofilm reactor (MBBR) for treating high nitrogen waste streams. In this process ammonia oxidizing bacteria (AOB) and anammox bacteria (AMX) exist simultaneously within a biofilm attached to plastic carriers. A key component of the process is controlling dissolved oxygen to limit the growth of nitrite oxidizing bacteria (NOB) which compete with AMX for substrate and to limit oxygen penetration into the biofilm thereby maintaining an anoxic environment for AMX to grow.

Deammonification systems must be closely monitored and controlled during startup to prevent growth of NOB and irreversible nitrite inhibition of AMX. Reducing startup time would be extremely beneficial as it would require less attention from operators and reduce the risk of irreversibly inhibiting the process. Seeding a reactor with 2–10% pre-colonized media has been shown to reduce startup times from 1 year down to 2–4 months (Lemaire et al. 2011; Christensson et al. 2015). Contrary to reports that seeding decreases startup time, some argue that seeding is not necessary (Ling 2009; Kanders et al. 2014). The debate is whether the origin of the AMX biomass on the virgin
media is from the seed media or the wastewater itself. Regardless of the origin of AMX biomass, several months of operation are required for AMX to establish on virgin media, and this could be a barrier to widespread adoption of deammonification technology.

Media in MBBR and integrated fixed-film activated sludge (IFAS) processes is typically made from high density polyethylene (HDPE) due to low cost. However, HDPE has a low surface energy and is hydrophobic making it unfavorable for bacterial growth (Bergbreiter 1994; Goddard & Hotchkiss 2007). Although interactions between surfaces and bacteria are complex and not completely understood, it is well known that bacterial adhesion is affected by surface energy, hydrophobicity, and roughness (An & Friedman 1998; Goddard & Hotchkiss 2007). Bacterial adhesion to a surface occurs in two phases: the initial attachment of a bacterial cell to the substratum, which is governed by physicochemical interactions, followed by biological cell growth and extra cellular polymer production (An & Friedman 1998; Hermansson 1999). The study of bacterial adhesion is broad and typically the goal is to prevent biofilms on surfaces such as ship hulls, food processing equipment, and biomedical devices. However, the promotion of biofilms is desirable for biodegradation of plastics (Roy et al. 2011) and biological wastewater treatment (Hadjiev et al. 2007).

Plastic surfaces can be modified using chemical, plasma, or thermal processes to have increased hydrophilicity, thereby increasing the rate of bacterial attachment and growth (Bergbreiter 1994). Studies on adhesion of nitrifying bacteria have typically demonstrated that surfaces with higher energy and hydrophilicity correlate with higher nitrification rates, increased biomass accumulation, and increased shear resistance (Kim et al. 1997; Terada et al. 2004; Lackner et al. 2009; Khan et al. 2011; Khan et al. 2013), although one report did conclude that the most hydrophobic surface had the most nitrifying biomass formation (Sousa et al. 1997). Although Chen et al. (2012) examined AMX attachment to surface modified carbon fibers in a deammonification reactor, no studies have been published on increasing AMX attachment to surface modified plastic.

Another potential method of increasing rates of AMX attachment is through the development of a preliminary biofilm composed of nitrifiers and/or heterotrophs. The hypothesis is that the existing biofilm creates a preferential environment for AMX by providing protection from oxygen and NO₃ in the bulk liquid. While it is known that development of a preliminary biofilm may encourage the attachment of AMX, the transfer of media from a mature, mainstream, fully nitrifying IFAS process to a sidestream deammonification reactor with the intent of decreasing startup time is a novel approach. Zekker et al. (2012) showed that AMX developed faster in an anoxic reactor containing media pre-colonized with a nitrifying biomass than in an anoxic reactor containing virgin media. The concern with using media pre-colonized with nitrifying biomass in an aerobic deammonification process versus an anoxic anammox process is the potential proliferation of NOB.

The current study represents the first work on increasing the rates of AMX attachment through surface modification of plastic biofilm carriers. The objectives of this study were to test whether rates of AMX biofilm growth and ammonia/nitrite removal on HDPE media could be increased through wet chemical surface treatment and through the transfer of media with a mature biofilm from a full-scale mainstream fully nitrifying IFAS process.

**MATERIALS AND METHODS**

**Full-scale deammonification reactor startup**

An existing 390 m³ tank at the 76 mL/day James River Wastewater Treatment Plant (JRWWTP) in Newport News, VA was modified to install the ANITA™ Mox process. This was the first full-scale installation of a sidestream deammonification MBBR process in North America. The mainstream process at JRWWTP utilizes IFAS operated in an MLE configuration. Anaerobically digested waste activated and primary sludge is dewatered using centrifuges and the centrate is sent to an equalization basin which is then treated in the deammonification MBBR. The average centrate characteristics throughout the study period (Day 115–Day 197) were 950 mg/L NH₄-N, 1,020 mg/L total Kjeldahl nitrogen (TKN), 3,450 mg/L as CaCO₃ Alkalinity, 3.63 Alkalinity/NH₄-N ratio, and 500 mg/L chemical oxygen demand (COD).

The reactor was seeded with 10% pre-colonized Anox™ K5 carriers (AnoxKaldnes, Sweden) from an established sidestream deammonification MBBR process (Sjölunda WWTP Malmö, Sweden). The total media fill in the reactor is 32% to meet a design NH₄-N removal rate of 2 g/m²/day. By Day 120 the sidestream deammonification MBBR was achieving greater than 85% NH₄ removal at the design loading rate, signaling the end of startup (Figure 1). Variations in removal percentages during startup up are due to frequent changes in loading rate (Figure 1). After startup was complete the removal loading rate and removal percentages were more...
consistent. Nitrate production ratio was calculated as NO$_3^-$ produced over NH$_4^+$ removed. A nitrate production ratio of less than 12% indicates that NO$_3^-$ produced is solely from AMX while a ratio greater than 12% indicated the presence of NOB. Figure 2 shows the time course of influent and effluent NH$_4^+$-N, NO$_3^-$-N, NO$_2^-$-N, and sCOD. The temperature was maintained at 30°C using supplemental heating as needed. AMX activity on the virgin carriers was first detected in bench scale activity tests after 3 months of operation. After 2 months, it was determined, by calculating the theoretical biomass production based on AMX yield, that production of AMX biomass was not the limiting factor in biofilm development leading to the hypothesis that attachment of the bacteria to the new virgin media must have been limiting.

**Experimental media**

Wet chemical (as opposed to plasma or thermal) methods of surface modification were explored for this study due to considerations over what would be feasible in a full-scale process. Three 1 L batches of K5 carriers were treated with potassium permanganate, Fenton’s reagent (FeSO$_4$ plus H$_2$O$_2$), and ozone with the intent of oxidizing the surface functional groups thereby increasing the hydrophilicity. Media with biofilm from the existing deammonification MBBR served as a positive control while virgin K5 carriers served as a negative control. All of the media that was chemically oxidized (and the virgin control) was wetted in an aerated container of tap water for 1 week prior to treatment. A small piece of an HPDE sheet was treated along with each batch of media in order to measure contact angle. The experimental media, along with the positive and negative control, were placed in a perforated aluminum box with individual compartments for each batch of media and placed in the existing sidestream deammonification MBBR on Day 115. Dimensions of the box were approximately 12 inches high × 18 inches wide × 6 inches deep. The box allowed for bulk liquid to flow through the media while allowing the media to be removed from the tank for testing. The box had six individual compartments each with a volume of 3 L. The media fill in each compartment was 30% to reflect conditions in the overall tank. The experimental media were removed once after 30 days and again after 60 days to measure AMX, AOB and NOB activity as well as biomass concentration.

**Fenton’s reagent treatment**

The media were placed in deionized water and the pH was adjusted to 3 with H$_2$SO$_4$. Next 50% H$_2$O$_2$ was added to reach a concentration of 5,000 mg/L H$_2$O$_2$. Then 6.95 g of FeSO$_4$ was added to reach a H$_2$O$_2$/Fe$^{2+}$ molar ratio of 4. The reaction took place for 24 h. A sample was periodically tested for peroxide to ensure that a residual was being maintained. The final residual was approximately 15 ppm H$_2$O$_2$.

**Ozone treatment**

An ozone generator (Ozonology, Inc., Model L-100) was connected to a reactor containing the media in deionized water. Ozone was produced using ambient air at room temperature and was bubbled through the water at the maximum concentration that the generator could provide for 24 h.
A sample was periodically tested to ensure a residual of greater than 0.5 ppm was maintained.

**Potassium permanganate treatment**

Two grams of KMnO₄ was added to 3 L of deionized water with the media in a glass beaker and continuously stirred for 48 h at room temperature. A residual was assumed to be maintained as indicated by the purple color of the solution.

**IFAS media**

One litre of media (Anox™ K3, AnoxKaldnes, Sweden) with fully nitrifying biofilm was removed from the existing full-scale mainstream IFAS process at JRWWTP on Day 115. The IFAS process has been in operation for approximately 2.5 years.

A batch test was performed to evaluate the effect of short term exposure of the IFAS media to sidestream conditions on NOB activity. Two 1 L samples of media were collected from the IFAS process and drained. One sample was placed in bulk from the IFAS process while the other was placed in bulk from the sidestream process. Bench scale activity tests were performed after 4 h at 20 °C to measure AOB and NOB rates. Nearly all NOB activity was assumed to be on the media as demonstrated by Regmi et al. (2011) at JRWWTP.

**Contact angle measurements**

The pieces of HDPE sheet were analyzed for each surface treatment. The Fenton’s reagent and KMnO₄ pieces were cleaned with a 1 M H₂SO₄ solution prior to measurement to remove residual metal oxide. The contact angles were determined by goniometry for the three probe liquids ultrapure water, diiodomethane (99%, Sigma-Aldrich, USA), and formamide (>99.5%, Sigma-Aldrich, USA). The sessile drop technique was used (Ramé-Hart Instrument Co., Goniometer Model# 400-22-300 with DROPimage Standard, NJ, USA) as previously described in Khan et al. (2011). At least five 5 µL droplets were measured with each liquid.

**Surface energy calculations**

Surface energies were calculated from the contact angle measurements as described in Liu et al. (2008) and Khan et al. (2013) according to Van Oss et al. (1986). In summary the total surface energy ($\gamma_{total}$) is the sum of the Lifshitz-van der Waals (LW) and Lewis acid-base (AB) components (Equation (1)).

$$\gamma_{total} = \gamma_{LW} + \gamma_{AB}$$  \hspace{1cm} (1)

The acid-base component ($\gamma_{AB}$) is related to the electron-acceptor ($\gamma^+$) and electron-donor ($\gamma^−$) parameters for the given liquid or substrata by Equation (2).

$$\gamma_{AB} = 2\sqrt{\gamma^+ \cdot \gamma^-}$$  \hspace{1cm} (2)

The Young-Dupré equation (Equation (3)) describes the relationship between $\gamma_{LW}^S$, $\gamma^+$, $\gamma^−$, and contact angle ($\theta$) for a surface (S) and a drop of liquid (L).

$$\gamma_L(\cos \theta_L + 1) = 2\sqrt{\gamma_{LW}^S \cdot \gamma_L^S} + 2\sqrt{\gamma^+ \cdot \gamma_L^+} + 2\sqrt{\gamma^- \cdot \gamma_L^-}$$  \hspace{1cm} (3)

If $\gamma_{LW}^S$, $\gamma_L^S$, and $\gamma_L^S$ are known then $\gamma_L^+$, $\gamma_L^−$, and $\gamma_L^-\gamma$ can be calculated from contact angles using three different liquids. Reference values for $\gamma_{LW}^S$, $\gamma_L^S$, and $\gamma_L^S$ were from Good & van Oss (1992) and were confirmed with the supplier.

**Bench scale maximum activity testing**

Bench scale maximum activity tests were performed once at 30 days and once at 60 days. AOB and NOB activity was measured under aerobic conditions while AMX activity was measured under anoxic conditions. For all of the samples, the biofilm was thin enough that AMX activity was inhibited in the aerobic test. The bulk liquid test was only performed under aerobic conditions since the amount of AMX activity in the bulk was negligible. The bulk liquid AOB activity rates were subtracted from the rates that included media and bulk liquid in order to obtain AOB rates on the media alone. In order to mimic conditions in the full scale reactor, the bench scale reactors were filled with 30% media (1 L) by volume and bulk liquid from the full-scale reactor to reach a total volume of 3 L. All reactors were fully mixed and dissolved oxygen was monitored and manually controlled to above 4 mg/L in the aerobic test. For the anoxic test, the reactor was covered and sparged with a blend of nitrogen gas with 380 ppm of CO₂, pH was monitored and manually controlled to stay within the range of 6.5−7.5. Temperature was controlled using a water bath to match the temperature in the full-scale reactor.
(30 °C). Samples were taken at regular intervals, immediately filtered through 0.45 micron filter membranes, and analyzed for NH₄⁺, NO₃⁻, and NO₂⁻. The maximum activity rates were evaluated by linear regression of the change in nitrogen species over the experimental period. AOB rates were determined from NOx production, NOB from NO₃⁻ production, and AMX from both NH₄⁺ and NO₂⁻ consumption.

Performance monitoring of full-scale reactor

Samples for on-site monitoring of the full-scale reactor were immediately filtered through 0.45 micron filter membranes following collection, and analyzed using HACH TNT kits and a HACH DR2800 spectrophotometer. The influent sample was analyzed for NH₄⁺ and the process (effluent) sample was analyzed for NH₄⁺, NO₃⁻, and NO₂⁻. sCOD was measured using standard methods.

Biomass concentration measurements

The weight of the biomass per square meter of surface area was measured at 30 and 60 days. For this measurement, nine pieces of media were removed from each compartment. Measurements were made according to Regmi et al. (2011) with the exception that a 25 mg/L disodium EDTA solution was used to remove biofilm instead of H₂SO₄.

Statistics

Statistical analysis to test if maximum activity on the experimental media was significantly different from the control was generated using SAS software. The slope of the linear regression from each experimental batch test was tested against the control to determine p values.

RESULTS AND DISCUSSION

Contact angle and surface energy measurements

The ozone treated HDPE had the lowest water contact angle indicating that it was the most hydrophilic with the new media control being the most hydrophobic (Table 1). As expected, all three of the surface treatments produced a more hydrophilic surface than the new media control. The surface energy results did not correlate with water contact angle as both the ozone and Fenton’s reagent treatment produced a similar surface energy higher than the control, while the potassium permanganate treatment produce a lower surface energy than the control (Table 1).

Short-term sidestream IFAS media experiment

When media from the IFAS tank were placed in bulk liquid from the sidestream deammonification MBBR, NOB activity was reduced by 55% within 4 h (1.6 g/m²/day to 0.9 g/m²/day). These results suggested that NOB activity would be quickly inhibited, most likely via free ammonia, in sidestream conditions eliminating or reducing the competition with AMX for substrate and space within the biofilm.

Activity and biomass results for experimental media

There was measureable AMX activity on the new media control after 1 month (compared to the 3 months it took for original new media during startup) implying that shear conditions inside the perforated box did not match the overall tank conditions, or the larger background AMX population in the tank accelerated attachment. After 1 month, the positive control seed media had slightly higher AMX activity (7.6 g/m²/day NH₄⁺ removal and 6.9 g/m²/day NO₂⁻ removal) and higher biomass (47.9 g/m²) than the original

<table>
<thead>
<tr>
<th>Contact angle (degrees)</th>
<th>Surface energy parameters (mJ/m²)</th>
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<tbody>
<tr>
<td>θ_w θ_D θ_F</td>
<td>γ_LW γ_γ γ_AB γ_total</td>
</tr>
<tr>
<td>New media control</td>
<td>89.5 ± 1.1 51.4 ± 2.0 77.8 ± 3.0</td>
</tr>
<tr>
<td>Fenton’s reagent</td>
<td>85.4 ± 3.2 42.3 ± 1.0 71.2 ± 1.4</td>
</tr>
<tr>
<td>KMnO₄</td>
<td>83.4 ± 2.2 53.0 ± 2.5 73.4 ± 3.7</td>
</tr>
<tr>
<td>Ozone</td>
<td>76.0 ± 3.1 36.2 ± 1.1 55.2 ± 5.3</td>
</tr>
</tbody>
</table>
seed media in the full scale reactor (5.5 g/m²/day NH₄⁺ removal, 6.9 g/m²/day NO₂⁻ removal, and 42.0 g/m² biomass). This also supports that the experimental media were exposed to lower shear conditions than the overall reactor. After 2 months, the control seed media had slightly less AMX activity (4.5 g/m²/day NH₄⁺ removal and 4.9 g/m²/day NO₂⁻ removal) and about the same biomass (43.6 g/m²) as the original seed media in the full scale reactor (5.5 g/m²/day NH₄⁺ removal, 6.9 g/m²/day NO₂⁻ removal, and 41.7 g/m² biomass) indicating that perhaps the experimental conditions were closer to that of the overall reactor. Regardless of the difference between the experimental conditions and the conditions in the overall tank, the experimental media can still be compared to the new media control.

After 1 month, there was significantly more AMX activity on the IFAS media (1.1 g/m²/day NH₄⁺ removal and 1.4 g/m²/day NO₂⁻ removal) as compared to the control (0.2 g/m²/day NH₄⁺ removal and 0.1 g/m²/day NO₂⁻ removal) (Figure 3). Initial biomass on the IFAS media prior to placement in the deammonification reactor was 18.7 g/m². Biomass increased after 1 month and was much higher than on the other experimental media due to the preliminary biofilm (Figure 4). At the same time, in the full-scale reactor, the maximum AMX rates (from bench scale tests) on the original new media were 1.4 g/m²/day NH₄⁺ removal and 2.4 g/m²/day NO₂⁻ removal (after 5 months of operation). AMX activity and biomass on the IFAS media after 2 months was again much higher than the control and the surface modified media (Figures 3 and 4). IFAS AMX activity was 2.0 g/m²/day NH₄⁺ removal and 3.2 g/m²/day NO₂⁻ removal while the new media control was 0.3 g/m²/day NH₄⁺ removal and 1.1 g/m²/day NO₂⁻ removal. After 2 months, AMX activity on the IFAS media was comparable to that on the original new media in the overall reactor (3.2 g/m²/day NH₄⁺ removal and 3.6 g/m²/day NO₂⁻ removal) which had been in the reactor for 6 months. The IFAS media biomass decreased from 1 month to 2 months (Figure 4) but was higher than the initial biomass from the IFAS reactor (18.7 g/m²). One explanation for decreased biomass while AMX activity increased could be that more AMX biomass was colonizing the biofilm while inactive biofilm was sloughing off of the media. AOB activity on the IFAS media was slightly higher than the control at both 1 and 2 months (approximately 1.5 g/m²/day compared to 1.3 g/m²/day). No NOB activity was detected at either 1 or 2 months as indicated by nitrate production ratio less than 12%. These results suggest that media from a mature IFAS process could be used to achieve immediate AMX growth in a sidestream process without risk of NOB proliferation. The ammonia removal rate for the new media control was unusually low for the test at 2 months (Figure 3). It is frequently seen in the activity tests that the NO₂⁻ removal results are more consistent than the NH₄⁺ results. Another common observation is that nitrite removal develops first before NH₄⁺ removal as AMX is establishing on the new media.

All three batches of surface-treated media had higher attached biomass than the new media control (Figure 4) at both 1 and 2 months. Statistical analysis was performed to determine if the AMX activity (based on NO₂⁻ removal) for the surface modified media was significantly higher
(p ≤ 0.05) than the new media control. Results after 1 month showed that the AMX activity for both Fenton’s reagent treated media and KMnO₄ treated media was significantly higher than the control (p = 0.0037 and p < 0.0001, respectively) while the ozone treated media was not significantly higher (p = 0.14) (Figure 3). After 2 months, the AMX activity for Fenton’s reagent treated media and KMnO₄ treated media was again significantly higher than the control (p < 0.0001) while the ozone treated media was not. Although ozone treated media had the lowest water contact angle (Table 1), it did not have the highest biomass density or AMX activity. In fact, the ozone treatment seemed to make a direct comparison to the original new media startup time.

The surface modified media had higher biomass density and hydrophilicity than the control new media. AMX activity rates were significantly higher for the Fenton’s reagent and potassium permanganate treated media than the new media control after 1 month and 2 months, while ozone treatment did not result in increased AMX activity. Media from the IFAS tank provided a preliminary biofilm that led to a rapid increase in AMX activity and NOB were inhibited. The large amount of AMX activity in the tank (plus reduced mixing) makes it difficult to make a direct comparison to the original new media during startup. While the results clearly demonstrate that the surface modification and preliminary biofilm led to higher rates of AMX biofilm development compared to the control, further study such as a pilot operation is warranted in order to provide conclusive evidence of reduced startup time.

CONCLUSIONS

The authors wish to thank the HRSD James River Wastewater Treatment Plant staff for their role in monitoring the full-scale process, as well as for construction of the box to house the experimental media. We also thank Virginia Tech’s Laboratory for Interdisciplinary Statistical Analysis (LISA), specifically Jon Atwood for performing the statistical analysis. Patrick McLee was supported by the National Science Foundation, Grant 1337077.

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First received 3 February 2016; accepted in revised form 31 March 2016. Available online 15 April 2016