Protein to polysaccharide ratio in EPS as an indicator of non-optimized operation of tertiary nitrifying MBBR

Baisha Ren, Bradley Young, Fabio Variola and Robert Delatolla

ABSTRACT

The protein (PN), polysaccharide (PS), and extracellular DNA (eDNA) percent concentrations of extracellular polymeric substances (EPS) of biofilm samples harvested from a pilot-scale nitrifying moving bed biofilm reactor (MBBR) were investigated at various operating temperatures and hydraulic retention times (HRTs). Chemically measured EPS PN/PS ratios were shown to correlate to Raman intensity ratios of amide III to carbohydrate at 362 rel. cm⁻¹. The study also demonstrates that tertiary nitrifying MBBR systems may be optimized to operate at HRTs as low as 0.75 to 1.0 h as opposed to conventional HRTs of 2.0 to 6.0 h. The EPS of the nitrifying MBBR biofilm exhibited the lowest percent PN content and the highest percent PSs and eDNA content. In particular, PN/PS ratios lower than 3 were indicative of non-optimal operation of the nitrifying MBBR systems, whereas PN/PS ratios with values significantly below 3 were observed for ammonia underloaded systems at high operating temperatures and hydraulically overloaded systems at low HRTs. This study demonstrates that the PN/PS ratio in EPS is a potential metric to identify non-optimal operation of nitrifying MBBR systems.

Key words | extracellular polymeric substances (EPS), hydraulic retention time (HRT), nitrifying MBBR biofilm, protein to polysaccharide ratio, Raman spectroscopy, temperature

INTRODUCTION

Wastewater treatment solutions incorporating biofilm systems are becoming more popular because of increasingly stringent regulations pertaining to wastewater effluent discharges (Ward et al. 2011; Gardner et al. 2012). Biofilm treatment systems provide attractive alternatives to conventional suspended growth systems due to their small land footprints and low intensive operational requirements (Chen et al. 2012; Hoang et al. 2014). In this context, the moving bed biofilm reactor (MBBR) system is a flexible and effective biofilm wastewater treatment technology, with a strong adaptability to shock and influent loading, along with a distinctive capability to achieve high removal efficiencies of nitrogenous and carbonaceous pollutants (Odegaard 2006; WEF 2009). Further, the MBBR technology has been shown to require simpler maintenance, lower operational intensity, and lower operating cost than other biofilm systems with respect to ammonia removal (Rusten et al. 1998; WEF 2009).

The biocarriers housed in MBBR systems provide protected surfaces for bacterial attachment and biofilm growth (WEF 2009). A major component of biofilm is the extracellular polymeric substances (EPS) that house the sessile cells. The EPS composition has been shown to influence the physicochemical properties of the biofilm and hence the performance of the treatment technology (Flemming & Wingender 2010). In particular, EPS has been shown to kinetically control the biofilm surface characteristics, the adsorption ability of the biofilm and the substrate mass transfer dynamics, which govern the global kinetics of the biofilm technology (WEF 2009; Sheng et al. 2010). Therefore,
achieving a deeper understanding of the EPS characteristics by identifying the relationship between their composition and the functional performance of the system is a fundamental prerequisite for the rational design of the next generation of biofilm technologies. In particular, the protein to polysaccharide (PN/PS) ratio in EPS is currently conventional for the measurement of EPS in biological wastewater treatment (Li & Yang 2007; Adav & Lee 2008; Liang et al. 2010; Bassin et al. 2012; Wang et al. 2014). This work investigates the effects of temperature and hydraulic retention time (HRT) on the EPS composition (especially the PN/PS ratio) and ammonia removal efficiency of nitrifying MBBR biofilms used for wastewater treatment with two methods, chemical assays and confocal Raman spectroscopy, being used to characterize the EPS of the nitrifying biofilm. Further, the study investigates the biofilm morphology and thickness as well as the viability of embedded cells at different HRTs.

**METHODS**

**Nitrifying MBBR pilot plant configuration and operation**

Wastewater effluent of the Masson-Angers aerated lagoon treatment plant (Gatineau, Quebec, Canada) was diverted into a tertiary nitrifying MBBR pilot plant, installed as an upgraded nitrifying treatment unit. The exposure to low temperatures during winter operation restricts nitrification year-round in the Masson-Angers aerated lagoon, hence creating the need for a tertiary nitrifying upgrade technology. This pilot plant comprises two 223 L cylindrical aerated reactors, which were filled with AnoxKaldnes™ K5 carriers. The carriers have a bulk specific surface area of 800 m²/m³, a diameter of 10 mm, and a depth of 7 mm. The pilot reactors had been in operation for approximately a year prior to the experimental work presented in this paper. Therefore, the harvested carriers used in this study were assumed to house mature biofilm.

Reactor 1 was operated at a constant HRT of 2.2 h, a constant normalized ammonia loading rate (relative to a 60% carrier fill ratio) of 864 ± 7 g N/m³d, and at varying HRTs of 0.5, 0.75, 1.0, and 3.0 h (between the months of June and July). Hence, the actual filling ratios of reactor 2 ranged from 11 to 60% to maintain a constant loading rate at various HRTs. During the operation of reactor 2 at various HRTs, the biofilm morphology and thickness along with the embedded cell viability was added to the investigative methods used in the study to further gain information on the biofilm and biomass during various operational conditions. The operating conditions are summarized in Table 1.

The airflow rate of the MBBR reactors was adjusted to ensure adequate mixing of the carriers in the reactors while achieving conventional dissolved oxygen (DO) concentrations in the nitrifying MBBR reactors (WEF 2009). This airflow rate corresponded to stable DO concentrations between 4 and 6 mg/L at all operational conditions investigated. Additionally, there was sufficient alkalinity in the MBBR influent, 152.3 ± 3.0 mg CaCO₃/L, to allow for complete nitrification with limited effect on pH. In order to allow the biofilm to acclimatize to all operational conditions, the two reactors were operated at steady state for at least 2 weeks at each operational condition before removal efficiency, biofilm EPS, biofilm thickness, and embedded cell viability were tested. Steady state conditions are defined by effluent constituent concentrations that vary by less than ±10%.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Reactor 1</th>
<th>Reactor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD loading rate (g/m³d)</td>
<td>494 ± 23</td>
<td>965 ± 45</td>
</tr>
<tr>
<td>Ammonia loading rate (gN/m³d)</td>
<td>442 ± 12</td>
<td>864 ± 7</td>
</tr>
<tr>
<td>Influent COD concentration (mg/L)</td>
<td>22 ± 1.1</td>
<td>22 ± 1.1</td>
</tr>
<tr>
<td>Influent ammonia concentration (mgN/L)</td>
<td>19.7 ± 0.8</td>
<td>19.7 ± 0.8</td>
</tr>
<tr>
<td>DO (mg/L)</td>
<td>4.5 ± 0.2</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>pH</td>
<td>7 ± 0.1</td>
<td>7 ± 0.1</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>22, 15, 10, 5</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>HRT (h)</td>
<td>2.2</td>
<td>0.5, 0.75, 1.0, 3.0</td>
</tr>
</tbody>
</table>

COD: chemical oxygen demand.
Constituent measurements

The influent and effluent ammonia (NH₄⁺/NH₃-N), nitrite (NO₂⁻), and nitrate (NO₃⁻) concentrations within the reactors were measured by using standard methods 4500C-NH₃, 4500B-NO₂⁻ and 4500B-NO₃⁻ (APHA 2012). DO concentration and temperature within the reactors were measured with a HACH LDO® probe (HACH, ON, Canada). The pH was measured with a CORNING® Pinnacle 530 pH meter (Corning, MA, USA).

EPS extraction and chemical analysis

To minimize the effects of transport and storage on the EPS composition of the biofilm, the extraction and chemical analysis of the carrier EPS for each set of conditions were initiated within 1 h of harvesting. The extraction and chemical analysis was completed within 8 h of the samples arriving in the laboratory.

Ten to 15 MBBR carriers were collected at every studied operation condition and biofilms were abraded from the interior surface of the harvested MBBR carriers and weighed; the volatile solids (VS) concentration (g VS/g biofilm) was quantified by standard method 2540E (APHA 2012). Three separate EPS solutions were prepared from the harvested carriers and extracted EPS using approximately 0.15 of biofilm from each of the harvested carriers. The EPS extraction procedure was carried out according to the cation exchange resin (CER) method (Frølund et al. 1999). Biofilms were first suspended in 40 mL of extraction buffer and then CER (Dowex Marathon C, 20–50 nm mesh, sodium form, Fluka 91973), which was previously prewashed for 1 h, was added at a dose of 75 ± 10 g CER/g VS. Cation exchange was carried out at 4 °C with a magnetic stirrer speed of 600 rpm and resin contact time of 1 h. The extracted EPS were harvested by centrifugation at 12,000 g for 0.5 h at 4 °C.

The EPS components were quantified in terms of PN, PS, and extracellular DNA (eDNA). All the tests were performed in triplicate to ensure statistical significance. The modified Lowry method (Frolund et al. 1995) was applied for PN determination, and bovine serum albumin (Sigma, Canada) was used as standard. The PS content was measured by the phenol-sulfuric acid method (DuBois et al. 1956) using D-glucose as a standard. The eDNA content was measured using the diphenylamine colorimetric method (Sun et al. 1999) and calf thymus DNA (Sigma, Canada) was used as a standard.

Raman analysis

Raman spectra were acquired on carriers harvested from both pilot reactors during the aforementioned operating conditions. All the measurements were carried out at room temperature with a WITec Alpha 300 microscope (WITec, GmbH, Ulm, Germany) equipped with a 65× water immersion objective (Carl Zeiss, Canada) and a He-Ne laser (532 nm). MBBR carriers were harvested from the reactors, then sliced longitudinally and fixed on a Petri dish (47 mm, Fisher, Canada). Three locations across the inner surface of the carriers were randomly selected on each carrier segment. Three Raman spectra were collected at randomly selected regions of the samples, with an integration time of 10 s and 10 accumulations (a total of nine spectra per sample). Spectral analysis was carried out using OriginPro software (OriginLab, USA).

CLSM image acquisition and analysis

Carriers were harvested from reactor 2 at four different HRTs. A Zeiss LSM 510/Axio imager M.1 confocal microscope (Carl Zeiss, Canada) was used for confocal laser scanning microscopy (CLSM) imaging at various wavelengths (488, 514, and 543 nm) with a 63× water immersion objective. For each measurement, five carrier segments were fixed on a Petri dish and stained with live and dead cell staining (SYTO 9 and propidium iodide). Five stacked 214 × 143 μm images with a depth interval of 5–6 μm were collected, totaling 25 images per sample. Live/dead cells’ numbers, as well as the biofilm area (cell surface coverage), were quantified by using the NI Vision Assistant 7.1 software (LabView 8.0-National Instruments Canada).

VPSEM image acquisition and analysis

Carriers were harvested from reactor 2 at four different HRTs. A Tescan Vega II-XMU variable pressure scanning electron microscope (VPSEM) (Tescan USA Inc., PA, USA)
USA), operated at a pressure of 40 Pa, was used to acquire images of the biofilm morphology and thickness directly on the harvested carriers. Four sampled carriers for each specific HRT investigated in this study were analyzed at a magnification of 60×. Images were acquired at five randomly selected regions on each carrier. The biofilm thickness was measured on the acquired images using Axio Vision LE software (Carl Zeiss, Canada).

**Statistical analysis**

The statistical significance of variations in constituent concentrations, composition of EPS, PN/PS ratio, biofilm thickness, and percent cell viability were determined by applying the t-test with a p-value less than 0.05 signifying significant difference. Correlation between the PN/PS ratio and the Raman band intensity ratios was determined with the Pearson’s correlation test. A p-value of 0.1 was used to indicate a significant correlation relationship.

**RESULTS AND DISCUSSION**

**Effect of temperature on nitrifying MBBR biofilm**

**Raman spectra of EPS and EPS percent composition**

Figure 1 illustrates the Raman spectra of nitrifying MBBR biofilm EPS. The Raman bands were identified according to previous work (Maquelin et al. 2002; Ivleva et al. 2009; Wagner et al. 2009). In particular, Raman bands in the 350–500 rel. cm⁻¹ region were attributed to carbohydrates. The peak at 862 rel. cm⁻¹ was assigned to C-C stretching (ν_{C-C}) and to the C-O-C glycosidic link of carbohydrates. The S-S bond of PNs was detected at 505 rel. cm⁻¹. The bands at 1,635 rel. cm⁻¹ and 1,278 rel. cm⁻¹ were assigned to amide I and amide III, respectively. These bands were used to identify the presence of PNs. These peaks could also be indicative of the asymmetric stretching of the COO⁻ (ν_{COO⁻}) group of carbohydrates and/or the CH₂ twisting vibration (ν_{CH₂}) as well as the =C-H bonds of lipids. The prominent C-H deformation (CH₂ def) band located at approximately 1,455 rel. cm⁻¹ mainly originates from the amino acid side chains of PNs and carbohydrates as well as the -CH₃, -CH₂, and C-H functional groups in lipids. In the 1,000–1,200 rel. cm⁻¹ region, the band at 1,030 rel. cm⁻¹ was assigned to the C-C and C-O vibrations of lipids and/or the C-H bond of PNs; the band at approximately 1,116 rel. cm⁻¹ was assigned to the stretching vibrations of PNs/lipids (ν_{C-C}/ν_{C-N}) and/or the C-C and C-O-C glycosidic link of carbohydrates; the band at approximately 1,161 rel. cm⁻¹ originated from the stretching vibrations of PNs (ν_{C-C}/ν_{C-N}) or of the C-C and C-O ring of carbohydrates. The band at 1,752 rel. cm⁻¹ may originate from stretching vibrations of carbohydrates (ν_{C=O}) or the C = O ester of lipids. In addition, several bands were assigned to the amino acid bands of phenylalanine and tyrosine, and the RNA/DNA nucleotide base-ring bands of guanine (G) and thymine (T) were also observed in the Raman spectra.

The Raman spectra of the EPS of the MBBR biofilm sampled at different temperatures did not demonstrate a difference in the Raman band positioning across the various temperatures. The chemically determined PN, PS, and eDNA content of the nitrifying MBBR biofilm EPS measured at different temperatures are shown in Figure 2. The PN percentage of the nitrifying MBBR EPS significantly increased from 39.3 ± 3.2% at the temperature of 22 °C to 58.3 ± 1.7% at a temperature of 15 °C. As expected, as the PN percentage increased, the PS and eDNA percentage exhibited the inverse trend. The content of PS significantly decreased from 33.63 ± 3.4% at 22 °C to 19.04 ± 0.8% at 15 °C, and the content of eDNA significantly decreased.
from 27.09 ± 0.2% at 22 °C to 22.67 ± 1.0% at 15 °C. As the temperature of the operating nitrifying MBBR system continually decreased from 15 to 5 °C, no statistical changes were observed in the PN and PS percentages. However, the eDNA percentage showed a significant decrease from 22.67 ± 0.2% to 17.58 ± 0.9%.

PN/PS ratio and ammonia removal efficiency

Figure 3(a) demonstrates the PN/PS ratio of nitrifying MBBR biofilm EPS and the corresponding ammonia removal efficiency at various operational temperatures. A constant loading rate (442 ± 12 g N/m³d) and HRT (2.2 h) were set in reactor 1 for operation at both warm and cold temperatures (with cold temperatures expectantly showing a significant decrease in ammonia removal efficiency).

Based on previous studies and conventional design, the loading rate of the nitrifying MBBR reactor at a temperature of 22 °C and at the low C/N ratio of the lagoon effluent is low for an MBBR nitrifying system operating at warm temperatures of 22 °C (Houweling et al. 2007; WEF 2009; Ferrai et al. 2010). The assumed low loading conditions at 22 °C are supported by the low effluent ammonia concentration at this temperature (Table 2). The low nitrite concentrations and elevated nitrate concentrations are indicative of stable complete nitrification in the system. At 22 °C, the intrinsic ammonia removal efficiency of the cells is highest relative to the other temperatures studied in this research, which likely results in strong competition among microorganisms embedded in the biofilm matrix and subsequently the cells approaching a state of famine. Hence, the underloaded conditions at 22 °C resulted in a lower availability of substrate that may cause a lower rate of extracellular enzymatic PN production, an enhanced production of PSs due to low substrate availability, and eDNA excretion due to cell lysis. It follows that these factors might have caused the observed low EPS PN/PS ratio of 1.21 ± 0.20. The low PN/PS ratio of 1.21 ± 0.20 is hence likely indicative of a nitrifying biofilm that is substrate underloaded.

A decrease in temperature from 22 to 15 °C did not yield a significant change in the ammonia removal efficiency of the system, thereby supporting the hypothesis that the system was underloaded at 22 °C. The transition from an underloaded condition at 22 °C to a more optimized operation of the MBBR system at 15 °C was marked by a higher EPS PN/PS ratio of 3.08 ± 0.21 (Figure 3(a)) and a low effluent ammonia concentration (Table 2). The measured PN/PS ratio of the nitrifying MBBR EPS
remained stable at a value of approximately 3 at temperatures lower than 15 °C. At lower temperatures, the cell kinetics decreased, resulting in significantly higher effluent ammonia concentrations at 10 and 5 °C (3.6 ± 0.0 mg N/L and 10.9 ± 0.1 mg N/L) as compared to 15 °C (0.5 ± 0.1 mg N/L). In turn, significantly lower ammonia removal efficiencies were observed at 10 and 5 °C (82.6 ± 3.2% and 46.2 ± 2.4%) as compared to 15 °C (97.9 ± 2.0%). Therefore, at temperatures of 15 °C and lower, the MBBR system appears to be adequately loaded. This state of operation corresponds to an EPS PN/PS ratio of approximately 3, which is supported by previous research on tertiary MBBR systems that demonstrate an EPS PN/PS ratio value of 3 during adequately loaded operation at a low operational temperature of 1 °C and HRT of 2.2 h (Young et al. 2016a).

Raman analysis was used to complement the chemical assays at varying temperatures and HRTs. In particular, the ratio between the amide III band (1,278 rel. cm⁻¹) and the carbohydrate bands at 362 and 862 rel. cm⁻¹ (νC-C) were calculated and compared to the chemically measured PN/PS ratios (Figure 3(b)). Although the amide III band may be associated with PN and lipid content, it is mostly associated with EPS PNs in the biofilm because of the characteristic relatively low lipid-to-PN content (Conrad et al. 2003; Almomani et al. 2014). The Pearson’s correlation test validated a correlation between the EPS PN/PS ratio and the intensity ratio of amide III to carbohydrate at 362 rel. cm⁻¹, with a correlation coefficient of $R = 0.98$ and $p = 0.02$. However, when compared to the intensity ratio of amide III to $\nu_{C-C}$ at 862 rel. cm⁻¹, the EPS PN/PS ratio did not show a significant correlation ($R = 0.886$ and $p = 0.11$).

### Effect of HRT on nitrifying MBBR biofilm

#### Raman spectra of EPS and EPS percent composition

Similar to the temperature experiments, the Raman spectra of nitrifying MBBR biofilm EPS at varying HRTs showed no qualitative differences. The EPS percentage composition determined by chemical analyses demonstrated that as the HRT value increases from 0.5 to 0.75 h, the PN percentage of EPS significantly increased from 33.1 ± 4.5% to 60.0 ± 3.4%, while the eDNA percentage decreased from 35.9 ± 2.2% to 13.4 ± 1.3% (Figure 4). No significant variations were observed in percentage PS composition between 0.5 and 0.75 h. However, a statistically relevant change in percentage PS was determined between HRTs of 0.5 and 3.0 h, exhibiting values of 31.05 ± 2.3% and 20.69 ± 0.2%, respectively. The percentages of PN, PS, and eDNA at HRTs of 1.0 and 3.0 h remained stable.

### PN/PS ratio and ammonia removal efficiency

Figure 5(a) shows the effects of HRT with a constant hydraulic loading rate of 864 ± 7 g N/m³d and temperature of 20 ±
At an HRT of 0.5 h, the dissolved ammonia residence time in the system appeared to be too short to allow for complete metabolic uptake of the bulk concentration ammonia by the nitrifying population embedded in the biofilm. As such, operation at an HRT of 0.5 h produced an effluent ammonia concentration that is significantly larger than the effluent at HRTs of 0.75, 1.0, and 3.0 h (Table 2). At all HRT values, the effluent nitrite and nitrate concentrations are indicative of complete nitrification. Therefore, an HRT of 0.5 h hydraulically overloads the nitrifying MBBR system and restricts the availability of substrate to the nitrifying population. At these conditions, the response of the percentage of EPS PN and PS along with the PN/PS ratio is similar to that observed during the experiments at 22 °C, where the system was underloaded. As conventional HRT values for ammonia removal MBBR systems are between 2 and 6 h (Ødegaard 2006), it is expected that as the HRT increases to 3.0 h, the ammonia removal efficiency will increase as well. The EPS and ammonia removal efficiency response at HRT values of 0.75 and 1.0 h indicate that nitrifying MBBR systems may be optimized to operate at HRTs as low as 0.75 to 1.0 h. Subsequently, these results show once again that as the nitrifying MBBR system approaches optimal operation, the EPS PN/PS ratio approaches a value of approximately 3. Hence, the EPS PN/PS ratio of nitrifying MBBR systems significantly decreases below a threshold value of 3 when the systems are underloaded or hydraulically overloaded. Using Pearson's correlation, the EPS PN/PS ratio to the ammonia removal efficiency shows a significant correlation ($R = 0.955$ and $p = 0.06$), while the EPS PN/PS ratio to the effluent ammonia concentration shows a significant negative correlation ($R = -0.955$ and $p = 0.07$). As in the case of the experiments at different temperatures, the Raman band intensity ratios of the amide III band to the carbohydrate bands were calculated. Significant correlations were observed between the chemically measured EPS PN/PS ratios and both Raman band ratios ($R = 0.922$ and $P = 0.08$ for carbohydrate at 362 cm$^{-1}$; $R = 0.932$ and $p = 0.07$ for $\nu_{C-C}$ at 862 cm$^{-1}$) (Figure 5(b)).

**Biofilm thickness and cell viability**

To further investigate the effects of varying HRTs, the biofilm morphology and thickness as well as the viability of embedded cells were investigated in this phase of the study. VPSEM images were collected to monitor changes in biofilm morphology at various HRTs while the system was operated at constant ammonia loading rate and temperature (Figure 6(a)–6(d)). The observed morphology of the MBBR nitrifying biofilm did not significantly differ across
HRTs. The biofilm remained porous with a limited filamentous morphology. VPSEM images were also used to investigate changes in biofilm thickness at different hydraulic loading rates (Figure 6(e)). The biofilm thickness was also observed to not significantly change at the different HRTs, showing a thickness that ranged from 80 to 110 μm, which is in agreement with previous research on tertiary nitrifying MBBR biofilm (Almomani et al. 2014; Hoang et al. 2014; Young et al. 2016b).

In the CLSM images shown in Figure 7(a) and 7(b), the dead cells fluoresce red (propidium iodide stained) and the live cells fluoresce green (SYTO 9 stained) (the full colour version of this figure is available in the online version of this paper, at http://dx.doi.org/10.2166/wqrjc.2016.040). The percentage of live cells per total cells was calculated and is shown in Figure 7(c). The results demonstrate that as the MBBR HRT increases, the percentage of viable bacteria also increases. In particular, HRTs of 1.0 and 3.0 h are characterized by a statistically greater percentage of live cell values (76.0 ± 3.7% and 89.2 ± 2.8%) when compared to the percentage of live cells measured at an HRT of 0.5 h (54.5 ± 2.2%). This finding supports the statistically greater ammonia removal rates and PN/PS ratios being observed at HRTs of 1.0 and 3.0 h (2.76 ± 0.80 and 2.95 ± 0.03) as compared to 0.5 h (1.10 ± 0.22); with higher percentage PN being likely due to greater extracellular enzymes during higher ammonia removal activity. Moreover, the highest live cell percentage was observed at the highest ammonia removal efficiency and as the PN/PS ratio approached the threshold value of 3.

The total live cell number per carrier was quantified from CLSM images by enumerating the live cells per biofilm area and combining these data with the biofilm thicknesses previously determined by VPSEM image analysis (Figure 7(c)). The live cell number per carrier shows no significant difference in the quantity of live cells per carrier across various
HRTs. Therefore, although the percentage of live cells in the bacterial communities increased with increasing HRTs, the total viable cell counts showed no statistical variations across HRT, thereby indicating that the quantity of viable bacterial cells remained stable.

CONCLUSION

The ammonia removal efficiency and EPS response to varying conditions demonstrate that tertiary nitrifying MBBR systems may be optimized to operate at HRTs as low as 0.75 to 1.0 h as opposed to conventional HRTs of 2.0 to 6.0 h. The Raman spectroscopic analysis of the EPS at all operating temperatures and HRTs showed a correlation between the Raman intensity ratio of amide III/carboxydrate at 362 rel. cm⁻¹ and the chemically measured PN/PS ratios. Further, the results presented in this study demonstrate a concomitant increase in PN content and a decrease in PSs and eDNA content of EPS in nitrifying MBBR biofilm at the highest operational temperature and lowest HRT. In particular, it was shown that the EPS PN/PS ratio of nitrifying MBBR systems is reduced to a value below 3 when the system was substrate underloaded (observed at the highest operational temperature) or hydraulically overloaded (observed at the lowest HRT). Conversely, when the system is no longer substrate underloaded (at lower operational temperatures) and no longer hydraulically overloaded (at longer HRTs), the EPS PN/PS ratio is approximately 3.

Hence, this work demonstrates that the PN/PS ratio of EPS is a potential indicator for non-optimized operation of MBBRs, paving the way for the use of PN/PS measurements to quantify the efficiency of an operating nitrifying MBBR system. In particular, the overdesign of a nitrifying MBBR unit that is in operation may be difficult for operators to identify as overdesigned, as overdesigned systems and optimized systems will produce similar effluent ammonia concentrations. The PN/PS ratio measurement may also be used to identify or rule out hydraulic overloading of the operating unit as the cause of poor performance.

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