Effects of porous carrier size on biofilm development, microbial distribution and nitrogen removal in microaerobic bioreactors

Muhammad Ahmad^a, Sitong Liu^a, Nasir Mahmood^b, Asif Mahmood^c, Muhammad Ali^d,
Maosheng Zheng^e and Jinren Ni^{a,*}

^aDepartment of Environmental Engineering, Peking University; Key Laboratory of Water and Sediment Sciences, Ministry of Education, Beijing 100871, China.

^bKey Laboratory for Green Chemical Technology of the Ministry of Education, School of Chemical Engineering and Technology, Tianjin University; Collaborative Innovative Center of Chemical Science and Engineering (Tianjin), Tianjin 300072, China.

^cDepartment of Physics, South University of Sciences and Technology, Shenzhen, P.R. China

^dWater Desalination and Reuse Center (WDRC), Biological and Environmental Science and Engineering (BESE), King Abdullah University of Science and Technology (KAUST),
Thuwal 23955-6900, Saudi Arabia.

^eResources and Environmental Research Academy, North China Electric Power University, Beijing, China

*Corresponding author:

Jinren Ni;

Department of Environmental Engineering, Peking University, Beijing 100871, China;

Tel.: +86-10-62751185; fax: +86-10-62756526;

E-mail address: nijinren@iee.pku.edu.cn

Abstract

1 In this study, effects of porous carrier's size (polyurethane-based) on microbial characteristics
2 were systematically investigated in addition to nitrogen removal performance in six
3 microaerobic bioreactors. Among different sized carriers (50, 30, 20, 15, 10, 5 mm), 15 mm
4 carrier showed highest nitrogen removal (98%) due to optimal micro-environments created for
5 aerobic nitrifiers in outer layer (0~7 mm), nitrifiers and denitrifiers in middle layer (7~10 mm)
6 and anaerobic denitrifiers in inner layer (10~15 mm). *Candidatus brocadia*, a dominant
7 anammox bacteria, was solely concentrated close to centroid (0~70 μm) and strongly
8 co-aggregated with other bacterial communities in the middle layer of the carrier. Contrarily,
9 carriers with a smaller (<15 mm) or larger size (>15 mm) either destroy the effective zone for
10 anaerobic denitrifiers or damage the microaerobic environments due to poor mass transfer.
11 This study is of particular use for optimal design of carriers in enhancing simultaneous
12 nitrification-denitrification in microaerobic wastewater treatment processes.

13 **Keywords:** Carrier size; microbial distribution; simultaneous nitrification and denitrification;
14 *Candidatus brocadia*; co-aggregation; wastewater treatment.

1. Introduction

Removal of nitrogen from wastewater is a major concern because presence of nitrogen strongly affects aquatic life in waterbodies. Among many biological nitrogen removal methods, nitrification-anammox have received great attention (Cao et al., 2017; Gao et al., 2014; Lackner et al., 2014; Niu et al., 2016; Wang & Gao, 2016). During this process, two bacterial species (ammonia oxidizing bacteria (AOB) and anaerobic ammonium oxidation (anammox)) work in co-operation with each other to treat wastewater rich in nitrogenous species with low carbon contents (Cema et al., 2016). Under microaerobic conditions, the AOB partially converts ammonium (NH_4) to nitrite (NO_2) which is utilized by anammox bacteria (prior to the anammox reaction, part of the ammonium has to be oxidized into NO_2) to give high nitrogen removal rates (NRR) (Dai et al., 2015). This process can be operated in different principles either in separate reactors for nitrification and denitrification/anammox processes (Vazquez-Padin et al., 2010) or simultaneously in a single reactor (simultaneous nitrification and denitrification, SND) by developing biogranules whose outer part is comprised of AOB for nitrification and inner region is constituted of anammox (Huang et al., 2016). Although these methods exhibited promising results, however their long-term applications are hampered by (i) their inadequacy to control dissolved oxygen (DO), as DO value $> 0.5\%$ of air saturation inhibits the growth of anammox bacteria (ii) favorable environment for the growth of nitrite oxidizing bacteria (NOB) which compete with AOB for oxygen and anammox for NO_2 (iii) bacterial granule deterioration and (iv) clogging of outer zone which limits mass transfer towards inner zone *etc.* (Guillén et al., 2016; Liu et al., 2016; Ma et al., 2016).

Using highly porous 3-dimensional (3D) carriers is a good option to overcome these shortcomings as carriers could provide the desired microenvironment for microbial growth. Various carriers have been explored previously including polymeric gels (Ali et

al., 2015), porous non-woven (Ni et al., 2010) stripes, zeolites (Montalvo et al., 2012) and granular activated carbon (Chen et al., 2013) *etc.* However, application of such carriers is mainly limited by smaller surface area, porosity, poor mechanical properties as well as inaccessibility of core of the carrier by biomass. For example, the zeolites exhibit much higher surface area ($\sim 2000 \text{ m}^2/\text{g}$), however very small pore openings ($\sim 1 - 1.5 \text{ nm}$) drastically limit mass diffusion (Mehlhorn et al., 2012). Similarly, gels can provide better environment for biomass growth but are largely limited by poor mechanical properties (Prasomsri et al., 2015). Polymeric carriers with high mechanical strength present ideal candidates in this regards for the growth of biomass (Le et al., 2016). In addition to carrier type, the carrier size could be highly important for the accessibility and efficiency of the biomass in treating nitrogen-containing pollutants. For instance, the smaller carriers are prone to high oxygen penetration through porous medium which lead to dominant aerobic environment while larger carriers cause hurdles in mechanical mixing leading to low shear stress as well as starvation of anammox in the carrier's core (Kumarasamy & Maharaj, 2015; Arrojo et al., 2006). On the other hand, the optimization of carrier size is also critical in maintaining balance between aerobic and anaerobic zones. An ideal size carrier is expected to effectively shield the anammox bacteria inside the core layers of the carrier while AOB on the outer layers can decompose dissolved oxygen. Therefore, there is a need to find out suitable carrier size for the development of efficient biofilm structure with distinctive aerobic and anaerobic zone that could allow the growth of multiple species toward efficient nitrogen removal from wastewater. To the best of our knowledge, no reports are yet available regarding effect of carrier size using nitrification-anammox process.

Herein we utilized a porous carrier made of functional polyurethane foam (FPUF), which is highly porous, halogen-free, flame retardant, flexible and cube-shaped, to examine how carrier size affects the microbial distributions, biofilm compositions, and mutualism (among

anammox bacteria, AOB, and denitrifying bacterial communities) for efficient operation of nitrification-anammox process. This study will open a new gateway for designing an optimum size carrier that favors the efficient distribution of bacterial communities for the rapid enrichment and stable performance of micro-aerobic wastewater treatment process.

2. Materials and methods

2.1. Experiment set-up and operation

Six cylindrical shape bioreactors with working volume of 2.1 L were operated continuously (continuous mode) under limited aerobic conditions for a period of 180 days. Briefly, FPUF was selected as a carrier and prepared according to information contained in its patent (Ye et al., 2005) and cut into cubes of the six sizes: 5, 10, 15, 20, 30, and 50 mm in diameter, subsequently named R₅, R₁₀, R₁₅, R₂₀, R₃₀, and R₅₀, respectively (see Table S1 for characteristics of carrier). All reactors fed with synthetic wastewater (Table S2) and were operated under identical and specific environmental conditions; (i) hydraulic retention time (HRT) was changed from 10 to 4 h (ii) temperature (37 °C) (iii) pH 7–8 (iv) total nitrogen (TN) 110– 840 mg/L. The experimental methods are schematized in Fig. S1. Additionally, the detail description of the whole experiment is sequentially provided in electronic supplementary information (ESI).

2.2. Cryosectioning: Cutting strategy for carrier sample preparation

Three sets of carriers were collected from top, middle and bottom of the reactors on 50, 100 and 180th days, respectively and gently washed with 1×PBS (phosphate-buffered saline) to remove loosely adhered biomass. The carriers were frozen immediately (-70 °C) to avoid any sample destruction during microtome cutting. The carriers were cut in distinct layers of 10-mm thickness each. Systematically, R₅ and R₁₀ have only single layer while carrier R₁₅, R₂₀, R₃₀ and R₅₀ have subsequent 2, 2, 3 and 5 equal size layers. All carrier

layers (10-mm size) were cut using microtome. 10-mm layers of each carrier size was further cut into many equal slices of 150 μm . The strategy for carriers cryosectioning is picturized in Fig. S2 (reason of carrier cutting strategy is discussed in the ESI).

2.3. Characterizations of biofilm structure

Bacterial communities, thickness, roughness, adhesion, porosity, surface area and pore size distribution were characterized by using Scanning Electron Microscopy (SEM), Fluorescence in Situ Hybridization (FISH), Atomic Force Microscopy (AFM), Raman and Brunauer–Emmett–Teller (BET) (microscopic results are summarized and presented only for sampled collected on day 180, other description and protocols are provided in ESI). Furthermore, quantitative information contained in AFM images (3D rendering, roughness, adhesion) was extracted and quantified using a “Nanoscope” software package (Bruker, USA) as described by Dong et al. (2015).

2.4. Bacterial community analysis by CLSM and High-throughput sequencing

To extract bacterial quantitative information, two approaches were used; (i) 16S rRNA and (ii) Confocal laser scanning microscope (CLSM). Both techniques were performed to detect composition and quantify abundances of anammox, AOB and denitrifiers (see Table S3 for probes detail). More than 30 CLSM images were randomly collected for the purpose of microbial community analysis as described previously (Daims & Wagner, 2011). The details protocol of both techniques are provided in ESI. All microscopic analysis was carried out after 180 days of operation.

2.5. Biofilm image analysis

To understand the dynamic interplay between and within bacterial populations, quantitative measurements and statistical analysis of their localization patterns within the biofilms are necessary to address complex biofilm systems. Therefore, “daime” software (Daims et al., 2006) was used to extract quantitative data from CLSM images for three

purposes (i) to determine abundances of anammox (*Candidatus brocadia* and *Candidatus jettenia*), AOB and denitrifiers) (ii) to identify bacterial spatial arrangement pattern (iii) to explore bacterial distribution (evenness or unevenness). Brief description about software package is specified in ESI and Fig. S3 which is also documented in published studies (Almstrand et al., 2013; Daims et al., 2006).

2.6. Chemical analysis

To obtain quick, reliable clues about carrier size effects, NH₄, nitrate (NO₃) and NO₂ were monitored periodically. Samples (from influent and effluent tanks) were collected 3 times in a week and immediately analyzed using UV spectrophotometer. Dissolved oxygen (DO) and pH was determine using online industrial standard apparatus (Fig. S4) which were installed within each reactor and their detail is provided in ESI.

3. Results and discussion

3.1. Impact of carrier size on biofilm topography

Previous studies have described microbial adhesion to the surfaces of different carriers result in the formation of biofilm with varying topography (Huang et al., 2015). Such variation in biofilm topography (roughness, kurtosis and skewness *etc.*) affects growth of biofilm over the carrier surface (Dong et al., 2015). These topographical variations occur due to severe hydrodynamic conditions produced due to mixing speed, crossflow velocities and abrasion (interaction between biofilm and substrate particles) (Kaplan, 2010). We therefore hypothesized that a suitable carrier size could balance these hydrodynamic stresses and form biofilm with appropriate topographical features. To verify this idea, we used AFM to characterize biofilms of different sized carriers (3D visualization) collected on 180th day of reactors operation (Fig. 1 and Fig. S5). These results highlighted that roughness vary depending on the carrier size, increasing (0.47–1.97) with carrier size from R₅ to R₁₅ and then decrease (1.97–0.23) continuously as

carrier size increased from moderate to higher (R_{15} – R_{50}). Interestingly, biofilm formed on different carriers also presented different folds and ridges. Large number of ridges on R_{15} carrier (Fig. 1b) as compared to other carriers (Fig. 1a, 1c-f and Fig. S5) resulted in the formation of much smaller and complicated interspaces on the carrier surface. Therefore, it was easier for biofilm sheets to connect over R_{15} carrier which ultimately resulted in high surface area ($1200 \text{ m}^2/\text{m}^3$). That is the reason why thick biofilm ($2250 \text{ }\mu\text{m}$) was observed on R_{15} carrier as compared to the other carrier ($488 \text{ }\mu\text{m}$), (Table 1).

Although roughness ascertains initial adhesion of bacterial communities on the carrier surface however fail to describe their long-term association with biofilm. A combination of skewness and kurtosis with roughness data could explain progressive growth of biofilm by describing its symmetry (peaks and valleys). These observations prompted us to calculate skewness and kurtosis to differentiate biofilm growth over the surface of carriers. A positive value for skewness and a slightly larger value of kurtosis indicated low adhesion capabilities of the biofilm for R_5 , R_{10} , R_{30} , and R_{50} . The relative flat surfaces blocked interactions between bacteria and biofilm forcing bacterial communities to live as suspension (Fig. 1 and Fig. S5). In contrast, negative skewness and smaller kurtosis values (dominated by ridges and valleys where strong bacterial adhesion can occur) indicated ideal biofilm topography formed on R_{15} carrier, which eventually confirmed strong binding of bacterial communities inside biofilm (see ESI for more description). Moreover, the negative skewness values showed slight deviations towards the core of the R_{15} carrier (Fig. 1b) which confirmed distribution of holes and valleys resulting in micro-tunnels that ultimately facilitated better mass transfer (Chatterjee et al., 2014; Zaky et al., 2012). Taken together, these results confirmed our hypothesis and suggested that shear forces showed variable effect on carrier size which ultimately changed topography of all carrier's biofilm resulting in ideal topography on R_{15} carrier.

R₁₅ carrier biofilm was further examined by coupling AFM with Raman spectroscopy to confirm the role of topography. AFM coupled with Raman enabled simultaneous investigation of biofilm structure and composition with in R₁₅ carrier. Homogenous distributions of diverse microbial communities (anammox, AOB and denitrifiers) dispersed into extra polymeric substances (EPS) matrix were observed as shown in Fig. 1g. Larger sized aggregates indicated anammox bacterial communities, a portion of anammox was zoomed in and high resolution image was taken (Fig. 1h). Uniformly distributed bacterial communities could be clearly observed in encapsulated aggregates. Further magnification confirmed presence of ridges and crevices as well as high roughness in the anammox communities as shown in Fig. 1i. The presence of large number of ridges is suitable for further adhesion/growth of anammox and denitrifiers communities. In addition, biofilm chemistry was evaluated with the help of Raman spectra (Fig. 1j) which depicted that biofilm was mainly composed of proteins, carbohydrates, DNA/RNA and amide. Contrarily, presence of wide bands corresponded to ammonium, hydroxylamine and hydrazine which were clearly indicative of anammox activities in the biofilm (Patzold et al., 2006; Viridis et al., 2012). The AFM and AFM-RAMAN integrated investigation clearly showed that carrier size had profound effect in designing effective biofilm topography over R₁₅ carrier which played a vital role in nitrogen removal.

Fig. 1

3.2. Effect of carrier size on biofilm texture

Several papers have discussed the importance of biofilm texture which directly affect the bacterial colonization (Felföldi et al., 2015; Mehlhorn et al., 2012). In context to above discussion, we next examined what type of texture was formed on different carrier's biofilm and whether it influenced the entrapment of biomass within carrier. This idea was confirmed by SEM observation of all carriers. The bare FPUF carrier showed highly rough

1 and porous structure that was perfect for the growth of various bacterial species (Fig. S6a).
2 SEM images of different layers showed mature biofilm formation on both the external
3 surface and within internal pores, indicating that the carrier could serve as an ideal support
4 material for bacterial growth. For carrier in different sizes, variations in biofilm
5 compactness were observed. Smaller carriers (R_5 , R_{10}) exhibited loose biofilm layers on
6 the carrier surface (Fig. S6b, c). It is speculated that growth of bacterial communities on
7 relatively smaller carriers was hampered due to large shear forces which detached the
8 bacterial film from the surface. On the contrary, the biofilms produced in different layers
9 of R_{15} (Fig. S6d, e) showed strong networking of bacterial communities. The biofilm
10 formed in R_{15} carrier comprised of a network of spherical communities indicating strong
11 bonding between bacteria-carrier and the bacterial communities (Fig. S6f–i). Strong
12 aggregation resulted due to uniform pore size and EPS distribution which helped to form
13 very close woven meshes that have highly porous and compact nature (Fig. S6j–l). The
14 uniform distribution of bacteria was observed on R_{15} carrier due to appropriate cross-flow
15 velocities which directly stimulated the excretion of EPS whereas in larger and very small
16 size carriers, heterogeneous distribution of EPS resulted in continuous deterioration of
17 bacterial aggregates as shown in Fig. S7 (Hou et al., 2015; Ohashi & Harada, 1994; Trinet
18 et al., 1991).

19 Increasing carrier size (R_{20} , R_{30} , R_{50}) gradually deteriorated biofilm textures caused by
20 easy penetration or saturation of DO (Fig. S8). Less growth of bacterial community within
21 extra-large carriers (R_{30} , R_{50}) could be attributed to fewer nutrients penetration deep inside
22 the carrier, which ultimately caused catastrophic loss of bacterial communities (Fig.
23 S8a–g) with scattered arrangement (Fig. S8h–j) and adversely affecting their
24 water-treatment properties. From these results, it is obvious that among all carriers R_{15}
25 carrier shows suitable bacterial biofilm and these results are consistent with AFM results.

3.3. Impact of carrier size on EPS-Adhesion mediation

Our results demonstrated that the carrier size strongly affect biofilm structure and texture that is why dominant biomass was observed in R₁₅ carrier. The possible mechanism behind this observation might be strong correlation between bacterial adhesion and EPS (Flemming & Wingender, 2010). To test this possibility, the adhesion capabilities of different carrier's biofilms were assessed and presented in Fig. 2. After 30 days of reactor operation, dominant biofilm adhesion (40 nN) was observed on R₁₅ carrier as compared to R₅, R₂₀ and R₅₀ (15 nN, 32 nN and 20 nN). After 180 days of reactors operation, bacterial adhesion became stronger (150 nN) on R₁₅ carrier, much higher than R₅, R₂₀ and R₅₀ (41nN, 110 nN and 63 nN) (Fig. 2a). Significant variation in adhesion on R₁₅ carrier could be ascribed to dependency of adhesion on EPS production (Fig. 2b) which also helped R₁₅ carrier in yielding thickest biofilm with highly porous biofilm structure (Fig. 2c) and compact nature (Fig. 2d). Optimum retention of EPS (Fig. 2e) and strong adhesion suggest that dense bacterial population on R₁₅ is responsible for high biodegradation and nitrogen removal rates (Fig. 2f).

Lower EPS concentration in the core of large carriers (R₃₀-R₅₀) was due to excessive starvation (Fig. S7) resulting from limited substrate and nutrient diffusion into the core of carrier. For instance, the EPS production levels decreased gradually due to increased consumption by denitrifying bacteria in the core layer of the carrier. In essence, the EPS consumption limited the time for nutrient-bacterial interaction inside the carrier, causing poor retention of bacterial communities within the core of large carriers (R₃₀, R₅₀). Hence, the optimum EPS–adhesion linkage in R₁₅ carrier promoted bacterial attachment and shielded their surface features from turbulent hydrodynamic conditions which oppositely affected others carrier biofilm dynamics. These observations are consistent with SEM analysis which confirmed the formation of porous biofilm in R₁₅ carrier.

Fig. 2

3.4. Impact of carrier size on development of microenvironments

3.4.1. Microbial community distribution and activity dynamics

We further examined the influence of carrier size on the microbial abundance using FISH analysis. The FISH analysis of R₅ carrier (Fig. S9a) suggested existence of pure niche of AOB with few cells belonging to anammox and denitrifiers due to oxygen saturation. Much similar to R₅, biofilm on R₁₀ carrier also showed limited communities of anammox (Fig. S9b). However, in comparison to R₅, slightly higher concentration of anammox with limited denitrifiers were observed on R₁₀ carrier due to slightly improved anaerobic conditions for anammox growth. In contrast, the biofilm on R₁₅ revealed distinct aerobic (AOB rich) and anaerobic (anammox rich) zones. Fig. S9c represents the first layer of R₁₅ carrier (10 mm) where balanced habitats were observed with mutual habitats of AOB, anammox and denitrifiers. In comparison to first layer, dominant communities of anammox and denitrifiers were observed in the inner layer (5mm in thickness) of the R₁₅ carrier (Fig. S9d). Increasing carrier size exhibited detrimental effects on the anaerobic zone. For example, low cell densities of denitrifiers were observed in the inner layer of R₂₀ carrier as shown in Fig. S9e, f. Further increase in carrier size increased negative effects on bacterial growth towards the core of the carrier resulting in overall loss of anammox communities (Fig. S9g–n). The FISH results confirmed the suitability of R₁₅ carrier for efficient growth of bacterial communities in distinct aerobic and anaerobic zones of the carrier.

3.4.2. Distribution of aerobic and anaerobic bacteria in differentiated zones

To determine the localization of bacterial communities with greater accuracy, we performed high-throughput sequencing that allowed visualization of complex microenvironments inside carriers. We observed that the biofilms inside R₅, R₁₀, R₂₀, R₃₀ and

R₅₀ comprised of dominant population of AOB bacteria (Fig. S10). The absence of anammox in relatively smaller carriers (R₅ and R₁₀) could be ascribed to high oxygen penetration into thin biofilms which potentially destroyed anaerobic zones destroying anammox and denitrifiers niche. Similarly, anammox were deprived of nutrients towards the core of too large carriers (R₃₀, R₅₀) leading to deficiency of anammox communities. On the other hand, ideal biofilm chemistry with particular zones for aerobic and anaerobic bacteria was observed in R₁₅ (Fig. S10c) due to formation of more thickened and compact biofilm in outer layer which limited oxygen penetration into second layer (deeper layer). Highest abundances of AOB were found between 0–7 mm (aerobic zone) from surface while anammox and denitrifier's maximum activities were registered between 10–15 mm (anaerobic zone) which strongly depend on carrier size. Interestingly, a mutualistic zone was recorded between 7–10 mm, where all bacterial communities performed significant bioactivities under highly collaborative environment (Fig. 3d). More specifically, the anammox bacteria "*Candidatus (Ca.) brocadia*" abundance increased from 61% to 96% from the first layer to the center of the carrier where the anaerobic condition was suitable for anammox bacteria metabolism and growth. The abundance of another anammox bacteria "*Ca. jettenia*" was higher inside anoxic zone and anaerobic zones (37% in R₁₅) due to higher nitrogen concentrations and denser biofilm (Huang et al., 2014). A similar trend was found for denitrifiers which could be assigned to their dependency on anammox bacteria (consume EPS as a source of carbon produced by anammox bacteria).

To further explore the evenness or unevenness of bacterial communities in biofilms on the carriers, the CLSM images were analyzed using "daime" software. The variable curves of the bio-volume fractions for different carriers strongly deviated from each other (Fig. 3a-c and Fig. S11). In general, smooth curves show an evenness of species distributed on the entire biofilm, while the significant variation in curves indicate the degree of

unevenness of the bacterial communities. The variable behaviors demonstrated uneven formation of bacterial communities inside the larger carriers (R_5 , R_{30}) while in the medium carrier (R_{15}), the communities were formed in a smooth and regular pattern (Fig. 3b) which was also confirmed by AFM and Raman analysis. The bacterial evenness down to center of R_{15} carrier clearly highlighted the role of carrier size on biofilm formation and growth. This further verified successful formation of well-arranged bacterial communities which can exhibit higher nitrogen removal performance in comparison to uneven and scattered communities.

To observe the effect of carrier size over bacterial interactions, “daime” software was further applied on CLSM images to ascertain whether bacterial communities grow in aggregate or random states. Fig. 3d is an overview of the arrangement pattern and regions (distance ranges inside R_{15} carrier) where bacterial communities were distributed. In Fig. 3e-i, the horizontal dashed line separates mutual co-aggregation and mutual avoidance, while the dash line represents the random distribution of bacterial communities (Almstrand et al., 2013; Daims et al., 2006). Inside R_{15} , the AOB communities are strongly co-aggregated at $< 30 \mu\text{m}$ (Fig. 3e) while all bacterial communities were randomly distributed throughout all others carriers (Fig. 3f). The strong nutrients stratification and oxygen penetration that formed highly competitive environments resulted in very thin biofilms formation, which is consistent with previous studies (Du et al., 2016; Huang et al., 2014). On the other hand, within R_{15} carrier, all bacterial communities were arranged in a regular and uniform pattern which revealed highly cooperative and optimum environment (Fig. 3g). It was also found that anammox bacterial communities like to grow in aggregates when the distance between them is $< 200 \mu\text{m}$; co-aggregation becomes very stronger as the distance decreased to a few microns ($0\text{--}70 \mu\text{m}$).

Inside anoxic zone, anammox bacterial communities showed co-aggregation with denitrifiers when the distance between them exceeded 70 μm (Fig. 3h). On the other hand, AOB and denitrifiers avoid aggregation when the distance between them is $< 60 \mu\text{m}$ and like to grow in co-aggregation beyond this distance ($> 60 \mu\text{m}$). Fig. 3h demonstrates the mutual avoidance of "*Ca. brocadia*" from "*Ca. jettenia*", AOB, and the denitrifiers, at shorter distances ($< 70 \mu\text{m}$). "*Ca. jettenia*" showed a similar pattern to "*Ca. brocadia*", and showed relationships within its own niche; but distances between communities were likely shorter as compared to "*Ca. brocadia*" (Fig. 3i). This shorter distance proves that "*Ca. jettenia*" bacteria form smaller aggregates than "*Ca. brocadia*". Development of anoxic and oxic zones within carrier favors the growth of diverse microbial communities in carrier. These bacterial communities showed mutualistic behavior and rely on each other for nutrients resulting in formation of thickened biofilm. This distinct microbial distribution was only observed in R_{15} carrier confirming that an appropriate size of carrier is of great significance for efficient nitrogen removal.

Fig. 3

3.5. Effect of carrier size on nitrogen conversion rates

It is clear that variation in carrier size markedly affect the bacterial distributions and biofilm formation over the carrier. The developed carriers were further used to investigate the mutual effect of carrier size and bacterial distributions on the nitrogen removal efficiency. The carriers were exposed to nitrogen-rich wastewater for a total of 180 days, this operation period of 180 days was divided into three different phases (Phase 1; startup–50 days, Phase 2; 50–100 days and Phase 3; 100-180 days) as shown in Fig. 4a-b. In the initial 50 days, all carriers except R_{15} (96%) showed almost similar nitrogen removal efficiencies ($\sim 90\%$ @ $0.4 \text{ kg N/m}^3/\text{d}$ loading rate) in anaerobic conditions with anammox sludge (Fig. 4c, d). High nitrogen removal efficiency suggested that biofilms

were formed by transforming high nitrogen in wastewater. Similar nitrogen removal efficiencies for all carriers confirmed that anammox bacterial inoculation had been successfully completed within 50 days of stable reactor performance. The carriers were exposed to higher concentrations of nitrogen in limited aerobic conditions (0–0.8 mg/L) along with introduction of AOB sludge at the start of phase 2. All reactors were loaded with increasing concentrations of nitrogenous wastewater (while lowering HRT from 10 to 7 h). In contrast to phase 1 where all carriers showed similar nitrogen removal efficiency, variable nitrogen removal efficiencies were observed for different carriers after day 15 of phase 2. Among all carriers, the R₁₅ showed prominent nitrogen removal efficiency (NH₄-N = 236 mg/L, and NO₂-N = 300 mg/L). Decrease in nitrogen removal performance of several other carriers (R₅, R₁₀, R₂₀, R₃₀ and R₅₀) was attributed to poor biofilm formation.

To obtain stable reactor performance over longer testing time, the phase 2 was further extended to 180 days (phase 3). Among all carriers, the R₁₅ exhibited excellent nitrogen conversion efficiency (98% @ 1.93 kg/m³/d loading rate) towards the end of phase 3. Higher nitrogen removal efficiency values for R₁₅ confirmed that optimal carrier size was very important for efficient growth of bacterial communities along with zonal distribution of aerobic and anaerobic bacteria to achieve better operation of the biological wastewater process. In addition, while using R₁₅, the correlation between SMPs (soluble microbial products) and NO₃ concentrations was monitored over the period of reactor operation where a mutual decrease in SMPs and NO₃ concentrations was observed after phase 1 as shown in Fig. 4f. The AOB convert NH₄-N to NO₂-N while anammox utilize NO₂-N and release SMPs with fraction of NO₃. Denitrifiers further transformed NO₃ to nitrogen gas while maintaining their metabolism by using SMPs. Thus, this cyclic conversion of

nitrogen species ensures efficient conversion without degradation of microbial zones in R₁₅.

3.6. Effects of carrier size on shear stress

It is generally believed that stability of biofilms is very important for long term stable operation of biofilm coated carriers. Although the carriers show high mechanical stability but the bacterial layers grown on the FPUF carriers are prone to high shear forces created by mechanical mixing speed. Hence, in addition to carrier size, the effect of stirrer rotation speed (0–250 rpm) upon resultant bacterial biofilm was further investigated (Fig. 4e and Table 2). In R₁₅ reactor, no significant loss in nitrogen removal activity was witnessed as the mixing speed was increased gradually from 80 rpm to 250 rpm, even at 250 rpm 99 % nitrogen removal was observed. Such a high nitrogen removal rate at 250 rpm is attributed to balance between cohesive and shear forces. On the other hand, increasing mixing speeds resulted in gradual decrease in nitrogen removal efficiency ~20-30 % in larger and smaller carrier. This reduction in nitrogen removal efficiency was due to imbalance between cohesive and shears forces which completely faded anaerobic environments. This not only deteriorate anaerobic environments but also shifted denitrifiers habitats into reactors suspension which lead to severe sludge washout, ultimately resulted rapid decline in nitrogen removal efficiency (Fig. 4e). Hence, among all carriers, the R₁₅ carrier exhibited its suitability for growth of bacterial communities for better nitrogen removal efficiency. Conclusively, differential nitrogen removal efficiency depended on different sized carriers chracterized by high porosity, morphology and surface roughness. These features strongly affected biofilm thickness-oxygen penetration which played a vital role in fabricating aerobic, anoxic and anaerobic zones that ultimately helped to balance shear forces and resulted in optimized biofilm on R₁₅ carrier.

Fig. 4

4. Conclusions

The study revealed 15-mm carrier (R_{15}) exhibited highest nitrogen removal efficiency (98 %) due to development of optimum aerobic, anoxic and anaerobic zones for the growth of diverse bacterial communities. Higher surface roughness of biofilm favoured bacterial adhesion over carrier surface resulting in thicker biofilm formation. As compared to R_{15} , the bacterial zones were destroyed in other carriers (R_5 – R_{50}) due to high oxygen penetration, improper mixing of carriers, shear forces and poor mass transfer. This research would benefit towards integrating distinct aerobic, anoxic and anaerobic zones into single system for the advancement of the simultaneous nitrification-denitrification based treatment processes.

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Figures captions

Fig. 1. AFM images of different sized carrier's layers, representing different topography and roughness; (a) R_5 first (b) R_{15} first, (c) R_{20} first, (d) R_{30} third, (e & f) R_{50} fourth and fifth. Different colors differentiating bacterial community and most likely SMPs, (g–j) Coupled AFM and Raman spectroscopy results for R_{15} . S_{sk} , S_{ku} indicates skewness and kurtosis respectively.

Fig. 2. EPS-adhesion variation and their effect on nitrogen removal performance (a) EPS production between 1-180 d, (b) role of EPS in adhesion of bacterial communities, (c & d) porosity variations and pore size distributions of different sized carriers, (e) EPS variations during the experiment, (f) effects of biofilm thickness on nitrogen removal efficiency.

Fig. 3. Bio-volume fraction variations inside different sized carriers (a), R_5 , (b) R_{15} , (c) R_{30} , (d) zone and bacteria distribution inside R_{15} carrier (e-i) spatial arrangement patterns observed in different sized carriers. RU, relative unit (Daims et al., 2011).

Fig. 4. Nitrogen concentration variations in influent and effluent, (a) NH_4 -N reduction, (b) NO_2 -N reduction, (c) NH_4 -N removal efficiency, (d) NO_2 -N removal efficiency, (e) effect of different sized carriers on balancing shear and cohesive forces, (f) variation in SMP and NO_3 production

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Table 1

Variations in biofilms topography and bacterial size inside smaller, middle and larger sized carriers.

Carrier	Surface area (m ² /m ³)	Pore size (μm)		Bacteria size (μm)	Thickness (μm)
		Outer	Inner		
R ₅	500	>550	>350	0.5-0.7	488
R ₁₅	1200	20-80	5-30	1-1.2	2250
R ₂₀	866	100-150	40-80	0.75-0.85	1270
R ₅₀	510	>400	150-250	0.6-0.7	1048

Table 2

Effects of mixing at different speeds on biofilm detachment and subsequent removal performance.

Carriers	Low rpm (80–100)		Med rpm (120–150)		High rpm (180–200)	
	DT*(%)	DRE**(%)	DT (%)	DRE*(%)	DT (%)	DRE*(%)
R ₅	Nil	0	15	10	50	45
R ₁₀	Nil	4	10	15	40	40
R ₁₅	Nil	0	Nil	0	20	10
R ₂₀	Nil	8	N/A	16	40	25
R ₃₀	N/A	18	N/A	20	4	15
R ₅₀	N/A	25	N/A	15	Nil	23

Med: medium, ***DT:** detachment, ****DRE:** dropped removal efficacy, **Nil:** no detachment occurred **N/A:** neither adhesion nor mixing were optimal (but no detachment occurred); so, in this case, removal efficiency decreased only due to improper mixing.