Organic Matter Removal in a Simultaneous Nitrification-Denitrification Process Using Fixed-Film System.

Perla Gonzalez
Instituto Tecnológico de Sonora
Ana Aguilar Ruiz
Instituto Tecnológico de Sonora
Andrea Reynosa Varela
Instituto Tecnológico de Sonora
Ulises Durán Hinojosa
National Autonomous University of Mexico
Marco Garzón Zuñiga
Research Center for Integral Regional Development (CIIDIR)
Edna Meza Escalante
Instituto Tecnológico de Sonora
Luis Álvarez Valencia
Instituto Tecnológico de Sonora
Denisse Serrano Palacios (denisse.serrano@itson.edu.mx)
Instituto Tecnológico de Sonora

Research Article

Keywords: SND, support media, nitrogen removal, nitrifying activity, denitrifying activity

Posted Date: October 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-959611/v1

Abstract

This study focused on evaluating different support media for COD and nitrogen compound removal from an Upflow Anaerobic Sludge Blanket (UASB) reactor fed with swine wastewater. Maximum specific nitrification (MSNA) and denitrification (MSDA) activity tests were performed in two fixed-film systems with (1) polyurethane foam (R_1) and (2) polyethylene rings (R_2). The results showed that the R_2 system performed more efficiently than R_1, reaching organic matter removal of 77 ± 8% and nitrogen of 98 ± 4%, attributed to higher specific denitrifying activity recorded (5.3 ± 0.34 g NO_3^--N/g VTS •h). In this sense, MSDA tests indicated that the suspended biomass was responsible for at least 70% of nitrogen removal in the form of ammonium compared with 20% attributed to biomass in the form of biofilm. On the other hand, 40 ± 5% of initial nitrogen could not be quantified in the system effluents, but 10 ± 1% was attributed to loss by volatilization. According to the analyses, the previous information infers the development of simultaneous nitrification-denitrification (SND) routes. Respect to the analyses of microbial diversity and abundance in the biofilm of R_2 rings, the presence of the genus *Pseudomonas* dominated the prokaryotic community of the system in 54.4%.

Introduction

Nitrogen is an essential biological growth nutrient and one of the main constituents of all living organisms. However, its excessive presence should be avoided due to several reasons: (a) Nitrogen in reduced forms exerts oxygen demand in the receiving water body[1]. (b) Ammonia and nitrite are toxic for fish in concentrations higher than 0.045 and 120 mg/L, respectively[2]. (c) Wastewater with high nitrogen concentrations requires a great amount of chlorine for its disinfection[3]. and (d) Nitrite and nitrate in concentrations greater than 0.2 and 1.5 mg/L, respectively, jointly with phosphorus in concentrations greater than 0.10 mg/L may cause eutrophication of lakes and water bodies, which results in an uncontrolled growth of algae and other aquatic plants [4, 5].

Nitrogen appears in swine wastewater in different ionized forms, such as ammonia or ammonium (NH_3^+ and NH_4^+, respectively), nitrate (NO_3^-) and nitrite (NO_2^-) as well as organic compounds measured as total nitrogen[6]. Thus, the nitrogen found in swine wastewater must be removed, since it can reach a concentration of up to 2000 mg/L[7]. Nitrogen removal may be carried out by means of processes, such as biological nitrification and denitrification, so understanding the microbial structure that conforms the biological system is needed for its proper function and operation[1].

Nitrification is a biological aerobic process that oxidizes NH_4^+ to NO_2^- with the help of ammonia oxidizing bacteria (AOB) followed by the conversion of nitrite to nitrate by oxidizing bacteria (NOB), both bacterial groups are called chemoautotrophic nitrifying bacteria [8]. *Nitrosomonas, Nitrosococcus* and *Nitrosospira* are the main bacteria reported for ammonia oxidation whereas *Nitrobacter, Nitrocystis* and *Nitrospira* have been reported for nitrite oxidation [9].

On the other hand, denitrification is a non-assimilative reduction process of oxidized nitrogen forms (NO_2^- and NO_3^-) to molecular nitrogen under anoxic conditions. This process is carried out by heterotrophic bacteria using organic carbon source for their metabolism[9]. Denitrification may be carried out by diverse groups of bacteria, but they are generally heterotrophic microorganisms (*Phylum, Proteobacteria, Firmicutes, Thiobacillus versutus*, etc.) and less frequently by autotroph organisms (*Thiobacillus denitrificans* and *Mocrococus denitrificans*)[10] Additionally, other biological processes exist for nitrogen removal, for example, the (i) single reactor system for high ammonium removal over nitrite – best know as the SHARON process. This process is based on ammonium oxidation to nitrites of 50%, in

low oxygen conditions (< 0.7 mg of O_2/L) [11], (ii) Anaerobic ammonium oxidation (ANAMMOX), where ammonium functions as electron donor and nitrite oxygen as electron receptor to obtain gaseous nitrogen; and (iii) Simultaneous nitrification-denitrification (SND), which is given by forming anoxic microzones in the interior of the bacterial consortia found in the aerobic reactor[11]. Additionally, SND may be carried out by heterotrophic bacteria using an organic carbon source for metabolism[12, 13].

In the last years, some aerobic systems have been improved by installing support materials like polyurethane (PUR), polyvinylchloride (PVC) and polyethylene (PE) within the system for biomass growth in biofilm form that may favor SND. According to Ruiz et al.[14], this type of systems may be classified as hybrids. Hybrid support systems have a great diversity of microorganisms mainly due to the anoxic microzones that allow different types of bacterial growth, both aerobic and facultative[15]. Examples of the different bacterial population diversity, both in suspended biomass and biofilms, can be *Pseudomonas stutzeri*, *Enterobacter cloacae* HNR strain, *Vibrio diabolicus* and *Bacillus sp*. This bacteria have been reported to favor consumption of different contaminants that cannot be degraded totally in conventional systems[16].

In this context, the objective of this study is to assess nitrogen and organic matter removal from two aerobic fixed-film systems with polyurethane foam (R_1) and polyethylene rings (R_2). Additionally, a microbiological analysis of the most efficient pack-bed biofilm system is performed to determine the main microorganisms involved in nitrogen removal process.

Materials And Methods

Inoculum

The biomass used for the experiments was obtained from an aerated lagoon as part of the process of a wastewater treatment plant located southward from Cd. Obregón, Sonora, in northwest Mexico. The reactors were inoculated with the support media previously placed in contact for one week with 1 L of aerobic biomass (20.4 g/L of total solids (TS) and 7.66 g/L of Total Volatile Solids (TVS).

Operation of the aerobic systems

The two aerobic fixed-film reactors, R_1 (filled with polyethylene rings) and R_2 (filled with polyurethane foam) had a capacity of 0.9 L each one and operated continuously for 330 days with hydraulic retention time (HRT) from 0.4-0.5 days and an average dissolved oxygen (DO) concentration of 3.35 mg/L. Buttom up aeration was performed in the reactors through the mammoth pump. Figure 1 shows the schematic diagram of the systems. Table 1 shows the characteristics of the support materials.

Table 1
Packing media characteristics

Parameter	R_1	R_2
Material	Polyurethane	Polyethylene
Dimensions	Width: 0.5 cm; Height: 0.5 cm	Diameter: 3 cm; Height: 3 cm
Shape	Rectangular	Cylindrical
Percentage in system (%)	20	20
Support mass (unit)	1.2	6
Density (g/L)	7.3	77.8
Superficial Area (m^2/g)	23.5*	6.1**
*Sahariah et al.[17] **Silva et al.[18]		

Influent characteristics

The fixed-film reactors were fed with the effluent of an UASB with swine wastewater coming from a farm with a maternity production process. Table 2 shows the physicochemical characteristics of the two fixed-film system feed evaluated, which maintained an organic load rate (OLR) of 0.6 ± 0.3 kg of chemical oxygen demand (COD)/m^3day.

Table 2
Physicochemical characteristics of packed systems influent.

Parameter*	Concentration (mg/L)
Chemical oxygen demand (COD)	300 ± 100
Nitrate (NO_3^--N)	5 ± 4
Nitrite (NO_2^- -N)	5 ± 4
Ammonium (NH_4^+ -N)	100 ± 20
Total solids (TS)	650 ± 150
Total volatile solids (TVS)	200 ± 100
* The parameters were quantified according to the techniques described in the analytical methods section.	

Analytical methods

In the influent and effluent of the fixed-film reactors, the following analytical techniques were performed: organic matter chemical oxygen demand (COD), total solids (TS), Total Volatile Solids (TVS), nitrates, nitrites and ammonium according to that established in the American Public Health Association[19].

Nitrifying and denitrifying activities

To know the nitrifying and denitrifying activities of the systems evaluated, discontinuous assays were performed based on the proposed methodology by Bassin et al.[20]. In the nitrifying activity assay, R_1 and R_2 were evaluated (1) with suspended biomass + biofilm in the support material and (2) only with suspended biomass (without support

material). The nitrifying activity was performed discontinuously, stopping system feed, followed by injecting a stock solution of 100 mg NH_4^+-N /L. The samples were collected from the supernatant of each reactor every hour for 10 h and subsequently every two hours with a total of 36 h. To know the nitrifying activity, soluble nitrogen forms (NH_4^+-N, NO_2^--N and NO_3^- -N) were determined in each established time. After determining NH_4^+-N removal at the final sampling time, its volumetric removal rate (VRR) was calculated according to Equation 1:

$$\mathrm{VRR}(\mathrm{mg}NH_4^+ - \mathrm{N/L} \bullet \mathrm{h}) = \frac{NH_4^+ - \mathrm{N}_{\mathrm{initial}} - \mathrm{N} - NH_{4\mathrm{final}}^+ (\mathrm{mg/L})}{Time(h)} \quad \text{(Equation 1)}$$

The result of the maximum specific nitrifying activity (MSNA) in milligrams NH_4^+-N/g TVS•h was obtained from dividing the volumetric removal rate (VRR) of nitrogen in the form of ammonium by the TVS of suspended and fixed biomass concentration (Equation 2):

$$MSNA(mgNH_4^+ - \mathrm{N}/gVTS \bullet h) = \frac{\mathrm{VRR}(\mathrm{mg}NH_4^+ - \mathrm{N/L} \bullet \mathrm{h})}{(\mathrm{g/L})} \quad \text{(Equation 2)}$$

With respect to the denitrifying activity, it was developed similarly to the nitrifying one, also performed in the two aerobic systems discontinuously stopping their feed. During the maximum specific denitrifying activity (MSDA) the R_1 and R_2 systems were injected with a concentrated stock solution of $NaNO_3$, and $C_2H_3NaO_2$. As a result, concentrations at time zero 82 ± 0.17 and 77.71 ± 2.02 mg of NO_3^- -N /L and 261.42 ± 8.75 and 250±5.41 mg COD/L were obtained for R_1 and R_2, respectively, with the purpose of reaching a theoretical value of 80 mg of NO_3^- -N/L and 250 mg of COD/L, simulating the conditions of the influent in the continuous systems.

Times for sampling in this experiment were equal to those of the nitrifying activity. After determining nitrogen concentrations in nitrates in the final sampling time, the VRR of NO_3^- -N was calculated and obtained by dividing nitrogen concentration in the form of removed nitrate in mg/L by the time in hours (Equation 3):

$$\mathrm{VRR}(\mathrm{mg}NO_3^- - \mathrm{N/L} \bullet \mathrm{h}) = \frac{NO_3^- - \mathrm{N}_{initial} - NO_3^- - \mathrm{N}_{final} (\mathrm{mg/L})}{Time(h)} \quad \text{(Equation 3)}$$

Subsequently, the MSDA was obtained from dividing nitrogen VRR in nitrates by the TVS of biomass concentration in the systems (Equation 4):

$$MSDA(mgNO_3^- - \mathrm{N}/gVTS \bullet h) = \frac{\mathrm{VRR}(\mathrm{mg}NO_3^- - \mathrm{N/L} \bullet \mathrm{h})}{(\mathrm{g/L})} \quad \text{(Equation 4)}$$

Extraction, amplification and DNA analyses in supports

Biofilm volumes of 1.5 mL were taken in sterile conical microtubes and centrifuged at 5000 rpm for 15 min. The supernatant of each microtube was discarded, and the final pellet was used in the nucleic acid extraction. The total DNA extraction was performed starting from 0.1-0.12 g of pellets with the DNeasy PowerSoil Kit (Qiagen, Hiden, DE) according to the manufacturer's instructions. Subsequently to the extraction, DNA was quaintified in a fluorometer Qubit 3.0 (ThermoFisher Scientific, Waltham, MA, USA), preserving the samples at -20°C up to their subsequent processing. The diversity of bacteria and archaea was determined by amplifying the V4 16S rRNA region with the specific primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The endpoint polymerase chain reactions (PCR) were performed in a total volume of 25 µL, whose concentration for each reaction consisted of: ~10 ng DNA, 1X PCR buffer (free of Mg^{2+}), 0.4 µM of each oligonucleotide primer, 800 µM of deoxynucleoside triphosphate (dNTP) mix (dATP, dCTP, dTTP and dGTP), 5% of dimethylsulfoxide (DMSO), 1.5 mM of

$MgCl_2$ and 1 U of DNA Taq polymerase ExTaKaRa Taq (Takara Bio Inc. Kusatsu, Shiga, JP). The amplification protocole consisted of an initial DNA denaturalization at 95°C for 3 min, followed by 35 denaturalization cycles (95°C, 30 s), hybridation (52°C, 40 s) and extension (72°C, 90 s), with a final extension at 72°C for 10 min. The amplicons obtained were purified with magnetic pearls using the Agencourt AMPure XP PCR Purification System (Beckman Coulter, Brea, CA, USA) kit and shipped for sequencing in the plataform Illumina MiSeq (Yale University, USA). The sequences obtained were analyzed with the platform QIIME2 (https://qiime2.org)[21]. The amplicon sequence variant (ASV) were taxonomically classified using Silva (https://www.arb-silva.de/) database. The charts and relative abundance of the main microbial groups were performed with the program 'phyloseq'[22] in the RStudio (1.2.5042) environment of the R (The R Core Team 2012) platform.

Statistical analyses

The results are expressed as average standard deviation (SD) +/-. Data were analyzed using a two-way analysis of variance (ANOVA) with the Minitab (Version 17.0) software. When ANOVA identified the differences among groups, multiple comparison of means were performed using Tukey's honestly significant difference (HSD) test with a level of significance of $P < 0.05$.

Results And Discussion

Aerobic fixed-film system performance

Organic matter removal was evaluated by measuring organic load rate both in the influent and effluent of the systems (Fig. 2). The influent concentration was maintained in 300 ± 100 mg COD/L and both R_1 and R_2 indicated a similar COD removal of organic matter of 72 ± 7 and 77 ± 8%, respectively. It is worth to mention that the system feed was stopped to perform the nitrifying activity tests with suspended biomass + biofilm (day 150) only with suspended biomass (day 200) and denitrifying activity (day 250). Additionally, suspended biomass and biofilm samples were taken subsequent to each assay to quantify the volatile suspended solids (VSS). Thus, destabilization in R_1 and R_2 was observed in these days causing decreases in removal efficiency (Fig. 2). The statistical analyses showed that no significant difference was observed in organic matter removal in R_1 and R_2 ($P \leq 0.05$), reaching a stationary state after the first 50 days of operation.

Similar results have been reached by mobile packed-bed bioreactors coupled to membrane bioreactors (MBBR-MBR) fed with domestic wastewater with a COD concentration of 185.80 ± 45.8 mg/L and operating with a hydraulic retention time (HRT) of 0.5 days. These packed-bed systems with a commercial Kaldnes type "K_1", in a filling ratio of 35% of total volume, showed global COD removal efficiency of 83 ± 2.11%[23]. Mazioti et al.[24] reported COD removal efficiency of 86.6% when they operated a MBBR system of 4.5 L useful volume with domestic wastewater and influent concentration of 270 mg COD/L, operated with HRT of 1.1 day, packed with the commercial AnoxKaldnes type "k3" support material in a filling volume ratio of 30%. Boutet et al. [25] obtained COD removal efficiencies of 47%, operating packed-bed systems with inert material "BIONEST" using municipal wastewater with an average concentration of 457 mg/L COD and HRT of 0.5 day.

In general, the systems assessed in this study reached similar or higher efficiency to those reported by other authors, which not only depended on variables, such as filling material percentage, COD concentration in the influent or type of supporting material but also on biomass concentration developed in the system interior. The biomass developed on the material surface was 18 ± 5 and 21 ± 3 g/L of R_1 and R_2, respectively. This biofilm possibly gives rise to different oxygen concentration gradients towards the interior of these materials. The differences in oxygen concentration in the

system could favor the appearance of anoxic zones in the deepest areas of the biofilm where oxygen cannot easily penetrate. The previous allows heterotroph organisms to assimilate organic carbon for their metabolism and growth, giving rise to denitrification processes and favoring removal of organic matter in the form of COD [11, 16, 26].

Figure 3 shows the fixed-film system performance for NH_4^+-N removal. It is worth to point out that this performance represents the system behavior when fed with swine wastewater, while the nitrifying and denitrifying activities were performed with synthetic solutions as the methodology indicated. The influent concentration was maintained in 100 ± 35 mg NH_4^+-N /L for 330 days of operation of the systems R_1 and R_2 showing a complete removal efficiency of NH_4^+-N (99.9%).

Lo et al.[27] concluded that high efficiency is possible thanks to the nitrifying activity carried out in aerobic systems with support. The nitrifying activity is performed both because of the suspended biomass within the systems and fixed biomass adhered to the supports. However, Bassin et al.[20] demonstrated that suspended biomass plays the most important role in ammonium removal process, showing a nitrifying activity relatively greater compared to biofilm, which plays the main role in the denitrifying process. Additionally, total nitrogen removal may be achieved indirectly as a result of both SND process and nitrogen assimilation by the heterotroph organisms for new cell formation. Lo et al.[27] reported that approximately 34% of total initial nitrogen was used for biomass formation where a SND process was performed in a hybrid biofilm system with an HRT of eight hours. This result implies a process where the greatest part of nitrogen is removed by a SND process, but with a yield in biomass formation. Coupled to this process, bacteria involved in SND, such as *Pseudomonas*, have low replication times of up to 30 min[28] which is why nitrogen assimilation through these bacteria possibly plays an important role in R_1 and R_2 performance.

Matsumoto et al.[29] and Wu et al.[30] observed SND process in biofilm systems with inert materials, such as plastic and ceramic membranes, evidencing these processes by the presence of AOB and NOB bacteria in the internal zone of the biofilm and heterotroph bacteria in the same biofilm surface. In this sense, according to Bassin et al.[20], up to 20% of NH_4^+-N removal might be attributed to biofilm while the suspended biomass contributed up to 70% of this process. Lastly, Figure 3 also shows that NH_4^+-N removal efficiency reached by each system were stable and without significant differences between them, according to statistical analyses ($P < 0.05$).

Sahariah et al.[17] operated a sequential mobile packed-bed bioreactor with polymer foam support with a filling Volume of 15.7% and fed with a concentration of 125 mg NH_4^+-N /L. The reported systems showed removal efficiency of 68% of NH_4^+-N, a lower value than that obtained in this study. On the other hand, Bassin et al.[20] operarated two mobile packed-bed bioreactors of 1 L of useful Volume, one packed with the commercial synthetic support “Kaldnes K1” and the other one with “MutagBiochip” with the filling Volume of 50%, operated with HRT of 0.5 days and fed with a concentration of 100 mg NH_4^+-N/L. The authors achieved NH_4^+-N removal efficiency higher than 90%. It should be mentioned that the support materials evaluated in this study occupied a filling Volume with a range from 15-50%, reaching NH_4^+-N removal efficiency of >99%. In this sense, the systems evaluated in this study demonstrated much higher efficiency with respect to similar systems. As mentioned previously, this excellent performance could have been due to biofilm presence (18 ± 5 and 21 ± 3 g TVS/L for R_1 and R_2, respectively), which was measured at the end of the assays. Ødegaard et al. [31] and Bassin et al. [20] suggested that the quantity of adhered biomass to a support medium not onl1y depends on the superficial area but also its form or material configuration. These findings indicate that supports, as Mutag Biochip that has the form of a satellite dish, are frequently subjected to attrition forces due to the intense contact with the surrounding liquid, favoring biofilm detachment and the quantity of adhered solids. Whereas the types of support with cylindrical shape or rings favor biofilm accumulation.

Nitrifying and denitrifying activity

The nitrifying and denitrifying activity of the systems were assessed in a period of 36 h for R_1 and R_2. The systems operated continuously and for these assays they were set in batch mode, stopping feed flux. Figure 4 shows ammonium removal during the MSNA assays. The NH_4^+ removal was 20 ± 5% for both systems at hour 10 of the assay; five hours later the systems reached two-fold removal. Starting from hour 10, removal started to increase significantly until it reached 90 ± 6 and 98 ± 4% of NH_4^+-N removal for R_1 and R_2, respectively (Figure 4). This result could indicate an adaptation process of the microorganisms in the systems when they went from operating continuously to discontinously. Although it did not inhibit the nitrifying process, it made it slower.

Despite the behavior was similar for both assays (R_1 and R_2), the R_2 system reached greater NH_4^+-N removal, whereas R_1 showed a slightly lower removal though not significant according to the statistical analyses performed ($P > 0.05$). Additionally, at the end of the assay, loss of nitrogen was evident in both systems, but it was not found in any of the determined soluble forms, approximately 60% and 65% for R_1 and R_2, respectively. Presumably, these non-quantified nitrogen percentages have been converted to molecular nitrogen by means of SND. Garzón-Zuñiga et al. [11] explained that aeration systems with fixed biomass in support materials are capable of developing denitrifying processes starting from heterotroph bacteria that achieve growing in anoxic environments. On their part, Lo et al. [27] studied nitrogen transformation in the form of ammonium to nitrogen, gas in a hybrid biofilm system. The results showed that approximately 60% of soluble nitrogen was converted to nitrogen gas by a SND process. On the other hand, some *Pseudomonas* species have been reported capable of reducing nitrate to nitrite, among which *P. stutzeri* perform denitrification processes[28].

Table 3 shows the results of MSNA, as well as those reported by different authors, where the obtained values are found within the range bibliographically reported for systems operated under similar conditions. The results allow observing the importance of suspended biomass in MSNA: 3.13 and 2.05 mg NH_4^+-N /gTVS•h for R_1 and R_2 respectively, even higher including that reached by the systems with both biomass types (suspended and fixed): 0.352 and 0.253 mg NH_4^+-N /g TVS•h for R_1 and R_2, respectively. Lo et al. [27] observed that in a hybrid biofilm system, nitrification was produced mainly in suspended biomass while biofilm played the main role in denitrification. In this manner, biofilm and suspended sludge interaction in the same reactor gave as a result a better general yield in nitrogen removal by a SND. The previous information may be observed in the nitrifying assays (Table 3). On the other hand, Mašić and Eberl [32] found evidence through mathematical models that suspended biomass contributes in a more important manner to ammonium removal in biofilm systems. However, nitrifying activity is not considered frequently in the suspended biomass, assuming that nitrification only takes place in the biofilm [33].

Table 3
Specific Nitrifying Activity obtained in different laboratory-scale studies.

	Bassin et al. [20]	Salvetti et al. [34]	Bassin et al. [35]	Reif-Lopez [36]	Lu et al. [37]	This study	
Type system	MBBR	MBBR	SBR	MBR	SBBR	Packed-bed	
Material	Kaldnes K1 y MutagBiochip	KMT	NR	Membrane Zenon ZW-10	Polyurethane	Polyurethane	Polyethylene
Ammonium	0.2	1.96	0.2	0.04-0.08	NR	0.18	
OLR	0.82-3.2	1.28	0.9	0.45-0.9	NR	0.75	
HRT	12-3.1	0.3-0.6	5.2	24-12.1	12	12	
TVS	4.9-5.52	6.67	10.0-14.2	0.5-2	NR	15.79	20.44
MSNA	1.2-5.6[a]	15-44[a]	1.2-5.6[a] 9.5-18[b]	0.12-0.16[a]	7.0-22[a]	0.35[a] 3.13[b]	0.25[a] 2.05[b]

[a] Fixed biomass, [b] Suspended biomass. Where: Maximum specific nitrifying activity (MSNA) is expressed in mg NH_4^+-N/g TVS•h; Volumetric organic load rate (OLR): kg Chemical oxygen demand (COD) m^3/d; Ammonium load: kg NH_4^+-N /m^3; Hydraulic retention time (HRT): h; Total volatile solids (TVS): g/L. NR = No reported

On the one hand, MSDA was 4.64 ± 0.13 and 5.3 ± 0.34 mg of NO_3^- -N/g TVS•h, for R_1 and R_2, respectively, results that are found within the range reported for biological nitrogen removal (BNR) systems. On the other hand, the determined MSDA agreed with that reported for the SND processes (1.6-30 mg of NO_3^- -N/g TVS h) for BNR systems inoculated with aerobic biomass and fed with real wastewater. Whereas lower MSDA values were reported for conventional denitrification routes and ANAMMOX (0.5-1.56 mg of NO_3^- -N/g TVS h)[12]. In the case of R_1 and R_2, nitrification and denitrification metabolism activation is performed in the same system simultaneously SND and not in different or sequential reactors as conventionally reported[10]. The previous is due to the presence of anoxic microzones in the aerobic system, given as a result dissolved oxygen gradients that limit their diffusion through the systems[12].

In this sense, the main explanation for SND is because denitrification organisms may exist both in the biofilm and suspended biomass of the system. Additionally, the existence of facultative microorganisms has been proven that use NH_4^+-N as electron donor and NO_2^--N as electron receptor, producing N_2 and NO_X in SND[11].

In the case of MSDA, polyethylene rings showed superiority over polyurethane foam, which was directly related to the amount of developed biofilm. Thus, polyethylene rings were selected as the most efficient support material in NH_4^+-N removal. Additionally, the statistical analyses indicated a significant and higher difference ($P \leq 0.05$) in the denitrifying activity, where the rings could favor SDN alternate routes because of factors, such as configuration and

material type that could create better conditions to form anoxic zones where the denitrification process mainly takes place.

Figure 5 shows the results obtained from monitoring NO_3^- -N behavior, organic matter in the form of COD and removal efficiency for the MSDA assays. Differently from MSNA, R_1 and R_2 had a different behavior, of which system R_2 was the most efficient by removing 91 ± 2.24% of NO_3^- -N and 67.86 ± 0.4% of COD, whereas system R_1 removed 52.32 ± 0.6% of NO_3^--N and 57.42 ± 1.24% of COD. The results indicated that 2.54 g COD/g of NO_3^--N reduced were used, which correspond to the organic requirements reported by Chatterjee et al. [38] for heterotrophic denitrification (2.86 g COD/g of NO_3^- -N removed), and more specifically 2.08 g COD/g of NO_3^--N reduced when using $C_2H_3NaO_2$ as a carbon source[12].

Balance of nitrogen species

The result of the nitrogen forms measured in the effluents R_1 and R_2, evidenced a nitrogen concentration that could not be quantified (~ 40 ± 5%) with respect to NH_4^+-N measured in the influent. The material balance indicated that it was quantified in the effluents of R_1 and R_2: 55 ± 11% and 54 ± 10% in the form of NO_3^- -N; 2.58 ± 2 and 3.4 ± 2.5% in NO_2^--N and 3.03 ± 4.02% and 5.07 ± 6.84% as NH_4^+-N. Based on these results, the operated systems might have shown a SND process.

According to Matsumoto et al.[29] SND is associated to reactors that have suspended biomass and biofilm and show nitrogen loss in the effluent. Anoxia conditions activate denitrifying metabolism, which are given by the anoxic microzones in the interior of the biofilm bacterial consorptia. In these microzones, oxygen cannot penetrate, but the NOx generated by the nitrifying bacteria can. According to Garzón-Zúñiga, [11] the nitrates produced by the nitrifying bacteria in the superficial layers of the biofilm may penetrate toward the deepest layers by one concentration gradient. When they penetrate toward these deepest layers where oxygen concentration is very low or null, the denitrifying bacteria use nitrites and nitrates as receptor and transform them into molecular nitrogen (N_2), which escapes from the system with gaseous effluent, making it possible to be counted in soluble form.

The previous information agrees with the volatilization assays that were performed additionally where a loss of NH_4^+-N to the environment in gaseous form was determined 10 ± 1%. It is worth to mention that some authors also reported loss by Stripping of 8-15%[38, 11]. In this sense, Garzón-Zúñiga et al.[11] found that in a packed-bed biofilter with organic material, nitrogen loss was performed by biologic sorption, filtration and assimilation mechanisms. These authors reported that from a total NH_4^+-N found in the influent, 10% oxidized NO_2^--N and another 10% to NO_3^- N, 40% was lost during the SND processes, 10% volatilized, 6% was retained in the system and 3.5% was found as residual NH_4^+-N. Zhao et al. [39] also reported SND processes in packed-bed systems, examining the combination of different support media, such as grapefruit skin and several conventional plastics as polyurethane, SPR-1 suspension and elastic filling TA-II. The results showed that by combining these materials efficient SND processes could be achieved with total ammonium and nitrogen removal of 96.8 ± 4.0% and 78.9 ± 9.5%, respectively. Additionally, the microbial analysis evidenced dominant genera of *Thiothrix, Gemmata* and *Comanonadaceae*, which indicated a heterotroph nitrification –same which favored the SND process. Furthermore, Walters et al. [40] operarated a batch system with suspended biomass and biofilm adhered to a biodegradable support medium. The results and experiments of these authors clearly indicated that nitrification may be achieved in suspended biomass while denitrification is performed at the interior of the support structure pores.

Microbial community analyses

The microbial community found in the biofilms of polyethylene rings was analyzed. This system was selected for analysis to show better performace as to the capacity of nitrogen and organic matter removal besides a larger concentration with respect to polyurethane foam. This study was performed through the analysis of the 16S rRNA fragments. A taxonomic classification of the total microbial community diversity was performed, which highlighted the microorganisms obtained at the level of phylum and genus. The bacterial abundance obtained from the sample was 99%. Table 4 shows that *Proteobacteria* was the dominant phylum that biofilm rings conformed, followed by *Bacteroidetes* and *Firmicutes* that are common in swine wastewater [41]. This result agrees with that reported by Alzate [42], who mentioned that typical microbiology of aerobic systems with activated sludge are composed approximately of 95% bacteria. On the other hand, a certain abundance of archaea was observed, which was not significant (~1%).

Table 4
Relative abundance of different edges identified in biofilm of polyethylene rings

Dominium	Phylum	Relative abundance (%)
Bacteria	Proteobacteria	56.10
	Bacteroidetes	24.54
	Firmicutes	9.59
	Tenericutes	3.54
	Spirochaetes	2.43
	Fibrobacteres	0.73
	Kiritimatiellaeota	0.50
	Verrucomicrobia	0.36
	Epsilonbacteraeota	0.34
	Cloacimonetes	0.32
	Actinobacteria	0.20
	Otros	0.28
Archaea	Euryarchaeota	0.91
	Nanoarchaeaeota	0.17

Within these phyla, the presence of *Pseudomonas* was detected in the biofilm rings. This bacterial genus is associated to denitrification processes in the presence of aeration[43, 44]. Zhang et al. [45] identified *P. stutzeri* in swine wastewater. These authors concluded that this type of *Pseudomonas* may transform not only nitrate and nitrite but also ammonium with the capacity of a complete removal up to 200 mg/L of NO_3^--N and 170 mg/L of NO_2^--N in aerobic conditions. They also observed NH_4^+-N removal of approximately 95% through a denitrification process and from this one 39% of NH_4^+-N removed was oxidized completely to gaseous nitrogen in a total of 18 h. This result indicated that the strain has capacities for heterotrophic nitrification and aerobic denitrification with the notable capacity of removing nitrogen efficiently in the form of ammonium. This percentage agrees including with 40% ot the nitrogen not found in the effluent systems of this study in any of its soluble forms.

On the other hand, the presence of *Clostridium* (2.43%) in the biofilm rings would indicate nitrification processes[10]. Interestingly, bacteria of the type *Nitrosomonas* and *Nitrobacter* -responsible for nitrification in aerobic conditions were not found in the taxonomic analysis despite having obtained a removal efficiency of NH_4^+ greater than 95% in system R_2. It should be highlighted that according to the MSNA assays, only may 20% of NH_4^+-N removal be attributed to biofilm, whereas 80% of this process would have been performed by suspended biomass, which was not microbiologically analyzed.

Figure 6 shows a phylogenetic tree of the 50 most abundant bacteria found in biofilm polyethylene rings. The circles of different size correspond to abundance in readings of each microorganism, while the color indicates the order to which the genus represented in the tree belong. Finally, the bacteria not classified at the level of order and/or genus are indicated.

The following bacteria at the level of order and by abundance are: Pseudomonadales (54.81%), Bacteroidales (24.17%), Clostridiales (8.59%), Acholeplasmatales (3.01%), Spirochaetales (2.01%). The rest of the organisms that appear in Figure 6 were found in a percentage lower than 1%. In contrast with the results mentioned, Nascimento et al. [46] reported that bacteria of the order Clostridiales are usually the most abundant in aerobic biomass. However, Pseudomonales have the capacity of growing in limited media. In other words, this phylum which showed greater proportion at genus level (56.10%) could suppress the development of taxa as *Clostridium* including bacteria in charge of ammonium oxidation in nitrifying conditions as *Nitrosomonas* spp.

Conclusion

After 330 days of operation of the fixed-film systems with polyethylene rings (R_1) or polyurethane foam (R_2), removal efficiency of organic matter and nitrogen were greater than 70 and 95%, respectively. However, the results of the maximum specific nitrifying activity (MSNA) kinetics were higher in the polyethylene rings (5.3 ± 0.34 mg of NO_3^--N/g VTS •h) than in polyurethane foam (4.64 ± 0.13 mg of NO_3^--N/g VTS •h), associated to the depth of the developed biofilm. Furthermore, starting from the MSNA determination, the suspended biomass contribution was estimated at 70% compared with the biomass in the form of biofilm (20%).

Finally, evidence of SND process was observed estimating that (i) approximately 40 ± 5% of nitrogen in the effluents of R_1 and R_2 that could not be quantified in any of its oxidized forms may have been transformed to molecular nitrogen; (ii) the obtained MSDA values agree with processes where nitrogen is transformed to N_2; and (iii) the analyses of the 16S rRNA fragments of the biofilm rings showed an abundance of *Pseudomonas* higher than 50%, bacterial genus associated to the denitrifying process in the presence of oxygen.

Declarations

Acknowledgments

This work was supported by CONACYT fund Projects of Basic Science CB-2017–2018 [Grant number A1-S-43472] also by PROFAPI _2021_0047.

References

1. Waheed, H., Hashmi, I., Naveed, A. K. & Khan, S. J. Molecular detection of microbial community in a nitrifying-denitrifying activated sludge system. *International Biodeterioration and Biodegradation*, **85**, 527–532 https://doi.org/10.1016/j.ibiod.2013.05.009 (2013).
2. Tilak, K. S., Lakshmi, S. J. & Susan, T. A. The toxicity of ammonia, nitrite and nitrate to the fish, Catla catla (Hamilton). *Journal of environmental biology*, **23** (2), 147–149 (2002).
3. Dapena-Mora, A., Campos, J. L., Mosquera-Corral, A. & Méndez, R. Anammox process for nitrogen removal from anaerobically digested fish canning effluents. *Water Science and Technology*, **53** (12), 265–274 https://doi.org/10.2166/wst.2006.429 (2006).
4. Moreno-Ostos, E., Cruz-Pizarro, L., Basanta, A. & George, D. G. The influence of wind-induced mixing on the vertical distribution of buoyant and sinking phytoplankton species. *Aquat Ecol*, **43**, 271–284 (2009).
5. Dunck, B. *et al.* Responses of primary production, leaf litter decomposition and associated communities to stream eutrophication. *Environ. Pollut*, **202**, 32–40 https://doi.org/10.1016/j.envpol.2015.03.014 (2015).
6. Rodríguez-Hernández, L., Esteban-García, A. L. & Tejero, I. Comparison between a fixed bed hybrid membrane bioreactor and a conventional membrane bioreactor for municipal wastewater treatment: A pilot-scale study. *Bioresour. Technol*, **152**, 212–219 (2014).
7. Garzón-Zúñiga, M. A. & Buelna, G. Caracterización de aguas residuales porcinas y su tratamiento por diferentes procesos en México. *Revista Internacional de Contaminacion Ambiental*, **30** (1), 65–79 https://doi.org/10.1161/STROKEAHA.109.561472STROKEAHA.109.561472 (2014).
8. Siripong, S. & Rittmann, B. E. Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants. *Water Res*, **41** (5), 1110–1120 https://doi.org/10.1016/j.watres.2006.11.050 (2007).
9. Bothe, H., Jost, G., Schloter, M., Ward, B. B. & Witzel, K. P. Molecular analysis of ammonia oxidation and denitrification in natural environments. *FEMS Microbiology Reviews*, **24** (5), 673–690 https://doi.org/10.1111/j.1574-6976.2000.tb00566.x (2000).
10. Chen, H. *et al.* Iron robustly stimulates simultaneous nitrification and denitrification under aerobic conditions. *Environmental science & technology*, **52** (3), 1404–1412 (2018).
11. Garzón-zúñiga, M. A. (2005). *Mecanismos no convencionales de transformación y remoción del nitrógeno en sistemas de tratamiento de aguas residuales*. *XX*, 137–149.
12. Ahn, Y. H. Sustainable nitrogen elimination biotechnologies: A review. *Process Biochem*, **41** (8), 1709–1721 https://doi.org/10.1016/j.procbio.2006.03.033 (2006).
13. Holm-Nielsen, J. B., Al Seadi, T. & Oleskowicz-Popiel, P. The future of anaerobic digestion and biogas utilization. *Bioresour. Technol*, **100** (22), 5478–5484 https://doi.org/10.1016/j.biortech.2008.12.046 (2009).
14. Ruiz, G., Jeison, D. & Chamy, R. Nitrification with high nitrite accumulation for the treatment of wastewater with high ammonia concentration. *Water Res*, **37** (6), 1371–1377 https://doi.org/10.1016/S0043-1354(02)00475-X (2003).
15. Bassin, J. P. & Dezotti, M. (2018). Moving bed biofilm reactor (MBBR). In Advanced Biological Processes for Wastewater Treatment (pp. 37–74).Springer, Cham.
16. Zhang, S. *et al.* Responses of bacterial community structure and denitrifying bacteria in biofilm to submerged macrophytes and nitrate. *Sci. Rep*, **6** (October), 1–10 https://doi.org/10.1038/srep36178 (2016).
17. Sahariah, B. P., Anandkumar, J. & Chakraborty, S. Stability of continuous and fed batch sequential anaerobic–anoxic–aerobic moving bed bioreactor systems at phenol shock load application. *Environmental Technology (United Kingdom)*, **39** (15), 1898–1907 https://doi.org/10.1080/09593330.2017.1343388 (2018).

18. Silva, A. J., Hirasawa, J. S., Varesche, M. B., Foresti, E. & Zaiat, M. Evaluation of support materials for the immobilization of sulfate-reducing bacteria and methanogenic archaea., **12** (2), 93–98 https://doi.org/10.1016/j.anaerobe.2005.12.003 (2006).
19. APHA - American Public Health Association. *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association (APHA), Washington, DC, USA, 2015).
20. Bassin, J. P. *et al.* Effect of increasing organic loading rates on the performance of moving-bed biofilm reactors filled with different support media: Assessing the activity of suspended and attached biomass fractions. *Process Safety and Environmental Protection*, **100**, 131–141 https://doi.org/10.1016/j.psep.2016.01.007 (2016).
21. Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, **37**, 852–857 (2019).
22. McMurdie, P. J. & Holmes, S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one*, **8** (4), (2013). e61217
23. Leyva-Díaz, J. C., Muñío, M. M., González-López, J. & Poyatos, J. M. Anaerobic/anoxic/oxic configuration in hybrid moving bed biofilm reactor-membrane bioreactor for nutrient removal from municipal wastewater. *Ecol. Eng*, **91**, 449–458 (2016).
24. Mazioti, A. A., Stasinakis, A. S., Pantazi, Y. & Andersen, H. R. Biodegradation of benzotriazoles and hydroxy-benzothiazole in wastewater by activated sludge and moving bed biofilm reactor systems. *Bioresour. Technol*, **192**, 627–635 https://doi.org/10.1016/j.biortech.2015.06.035 (2015).
25. Boutet, E., Baillargeon, S., Patry, B. & Lessard, P. Simultaneous organic matter removal and nitrification of an inert self-supporting immersed media to upgrade aerated lagoons. *Water Science and Technology*, **77** (1), 51–59 https://doi.org/10.2166/wst.2017.511 (2018).
26. Liang, Y. *et al.* Microbial characteristics and nitrogen removal of simultaneous partial nitrification, anammox and denitrification (SNAD) process treating low C/N ratio sewage. *Bioresour. Technol*, **169**, 103–109 (2014).
27. Lo, I. W., Lo, K. V., Mavinic, D. S., Shiskowski, D. & Ramey, W. Contributions of biofilm and suspended sludge to nitrogen transformation and nitrous oxide emission in hybrid sequencing batch system. *Journal of Environmental Sciences*, **22** (7), 953–960 (2010).
28. Yang, X., Wang, S. & Zhou, L. Effect of carbon source, C/N ratio, nitrate and dissolved oxygen concentration on nitrite and ammonium production from denitrification process by Pseudomonas stutzeri D6. *Bioresour. Technol*, **104**, 65–72 https://doi.org/10.1016/j.biortech.2011.10.026 (2012).
29. Matsumoto, S. *et al.* Experimental and simulation analysis of community structure of nitrifying bacteria in a membrane-aerated biofilm. *Water Science and Technology*, **55** (8-9), 283–290 (2007).
30. Wu, W., Yang, L. & Wang, J. Denitrification performance and microbial diversity in a packed-bed bioreactor using PCL as carbon source and biofilm carrier. *Applied microbiology and biotechnology*, **97** (6), 2725–2733 (2013).
31. Ødegaard, H., Gisvold, B. & Strickland, J. The influence of carrier size and shape in the moving bed biofilm process. *Water Science and Technology*, **41** (4-5), 383–391 (2000).
32. Mašić, A. & Eberl, H. J. A modeling and simulation study of the role of suspended microbial populations in nitrification in a biofilm reactor. *Bulletin of mathematical biology*, **76** (1), 27–58 (2014).
33. Albiruzi, J., Van Loosdrecht, M. C. M. & Larrea, L. Extended mixed-culture biofilms (MCB) model to describe integrated fixed film/activated sludge (IFAS) process behavior. *Water Science and Technology*, **60** (12), 3233–3241 (2009).
34. Salvetti, R., Azzellino, A., Canziani, R. & Bonomo, L. Effects of temperature on tertiary nitrification in moving-bed biofilm reactors. *Water Res*, **40** (15), 2981–2993 https://doi.org/10.1016/j.watres.2006.05.013 (2006).

35. Bassin, J. P., Kleerebezem, R., Dezotti, M. & Van Loosdrecht, M. C. M. Simultaneous nitrogen and phosphate removal in aerobic granular sludge reactors operated at different temperatures. *Water research*, **46** (12), 3805–3816 (2012).
36. Reif, R., Suárez, S., Omil, F. & Lema, J. M. Fate of pharmaceuticals and cosmetic ingredients during the operation of a MBR treating sewage., **221** (1-3), 511–517 https://doi.org/10.1016/j.desal.2007.01.111 (2008).
37. Lu, Y. *et al.* Intensification and microbial pathways of simultaneous nitrification–denitrification in a sequencing batch biofilm reactor for seawater-based saline wastewater treatment. *Journal of Chemical Technology & Biotechnology*, **93** (9), 2766–2773 (2018).
38. Chatterjee, P., Ghangrekar, M. M. & Rao, S. Organic matter and nitrogen removal in a hybrid upflow anaerobic sludge blanket - Moving bed biofilm and rope bed biofilm reactor. *Journal of Environmental Chemical Engineering*, **4** (3), 3240–3245 https://doi.org/10.1016/j.jece.2016.07.002 (2016).
39. Zhao, J., Feng, L., Yang, G., Dai, J. & Mu, J. Development of simultaneous nitrification-denitrification (SND) in biofilm reactors with partially coupled a novel biodegradable carrier for nitrogen-rich water purification. *Bioresource technology*, **243**, 800–809 (2017).
40. Walters, E., Hille, A., He, M., Ochmann, C. & Horn, H. Simultaneous nitrification/denitrification in a biofilm airlift suspension (BAS) reactor with biodegradable carrier material. *Water research*, **43** (18), 4461–4468 (2009).
41. Liu, A., Chou, C., Chen, L. & Kuo, C. Bacterial community dynamics in a swine wastewater anaerobic reactor revealed by 16S rDNA sequence analysis. *Journal of Biotechnology*, **194**, 124–131 https://doi.org/10.1016/j.jbiotec.2014.11.026 (2015).
42. Alzate Marin, J. C. (2019). *Remoción biológica de nitrógeno por nitrificación y desnitrificación aeróbica en reactor de cargas secuenciales SBR*. 1–128.
43. Liang, S. C. *et al.* Isolation and characteristic of an aerobic denitrifier with high nitrogen removal efficiency. *African Journal of Biotechnology*, **10** (52), 10648–10656 (2011).
44. Ji, B. *et al.* 2015. Aerobic denitrification: A review of important advances of the last 30 years. Biotechnol. Bioproc. Eng. 20, 643-651
45. Zhang, J., Wu, P., Hao, B. & Yu, Z. Heterotrophic nitrification and aerobic denitrification by the bacterium Pseudomonas stutzeri YZN-001. *Bioresource technology*, **102** (21), 9866–9869 https://doi.org/10.1016/j.biortech.2011.07.118 (2011).
46. Nascimento, A. L. *et al.* Sewage sludge microbial structures and relations to their sources, treatments, and chemical attributes. *Frontiers in microbiology*, **9**, 1462 (2018).

Figures

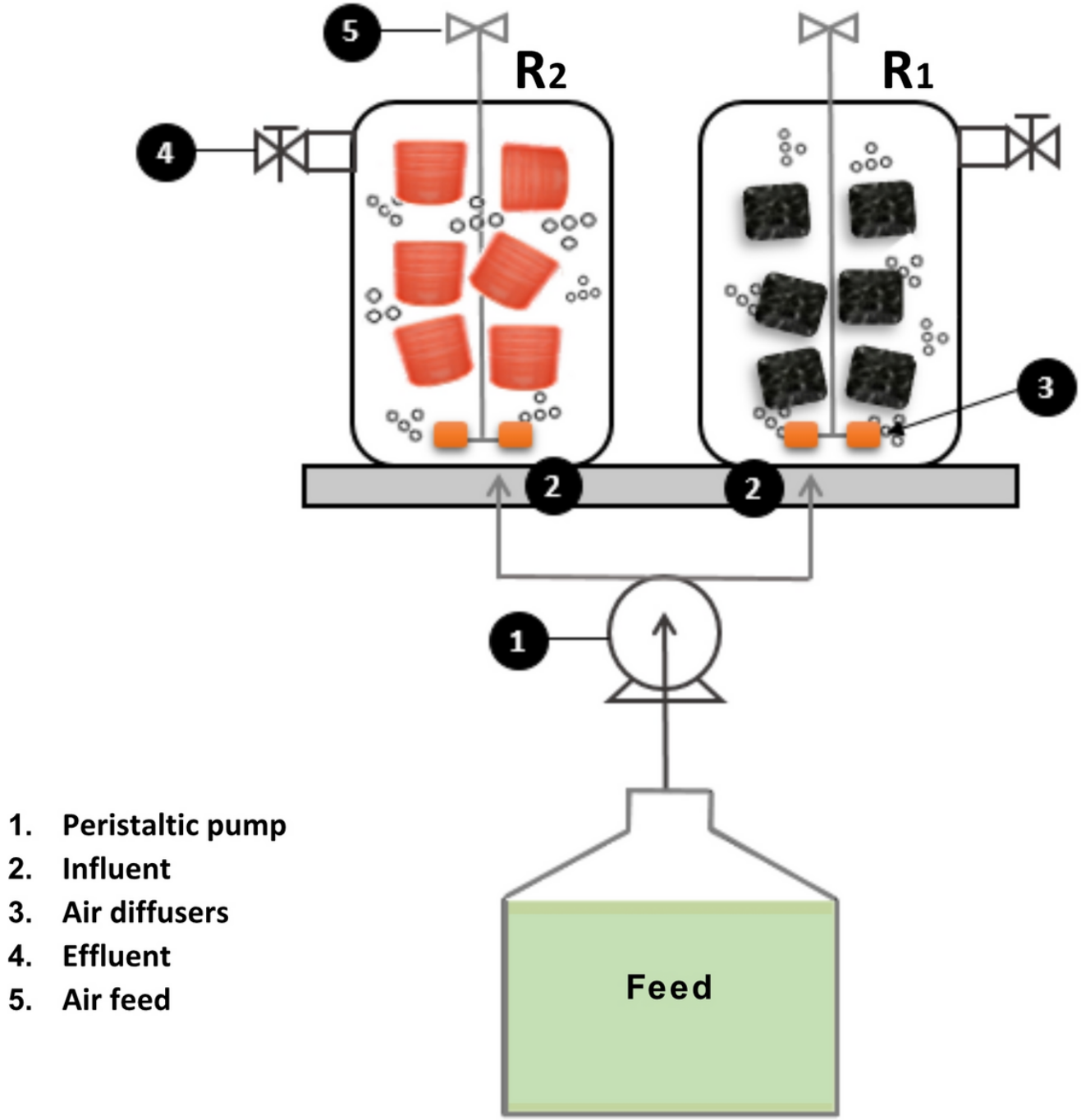

Figure 1

Schematic diagram of aerobic Fixed-film systems

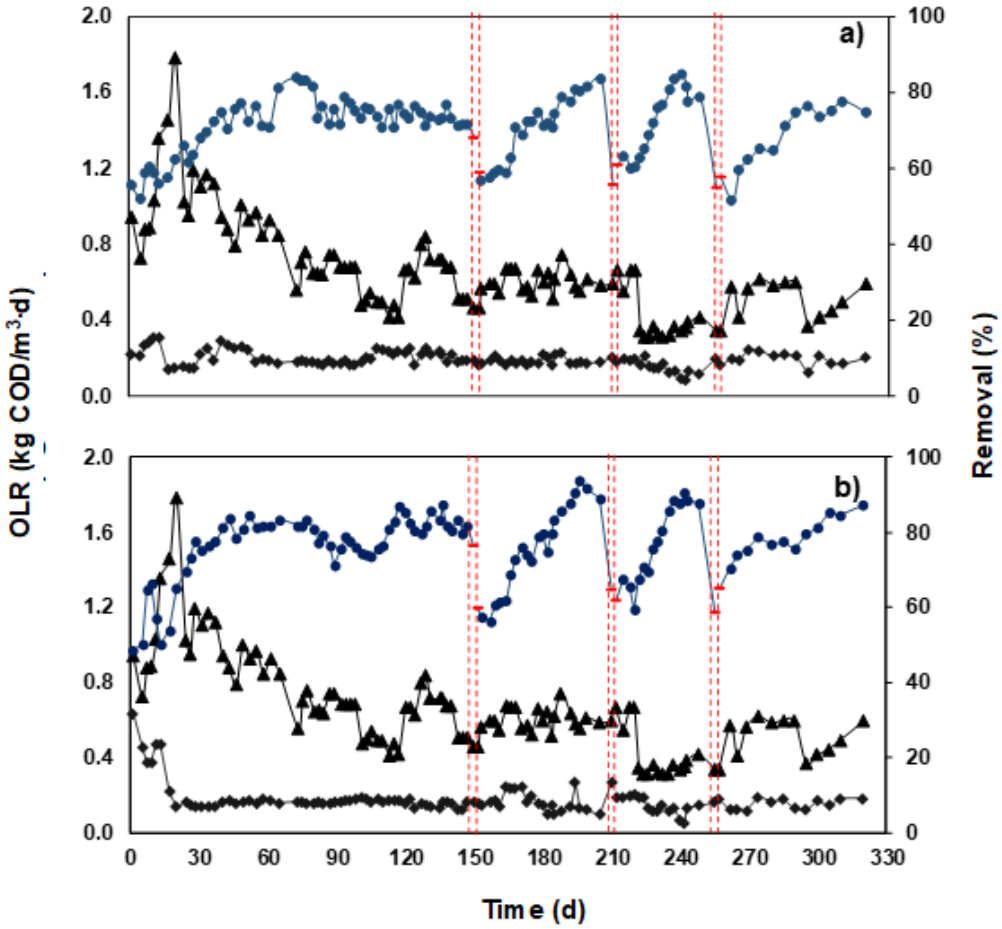

Figure 2

Removal efficiency of organic load rate (ORL) (kg COD/m3•d) with swine wastewater. Where: (a) is R1 with polyurethane foam and (b) is R2 with polyethylene rings. (●) Removal of chemical oxygen demand (COD) percentage; (▲) influent and (♦) effluent. Lines show days in which nitrifying and denitrifying activities were performed in both discontinuous reactors.

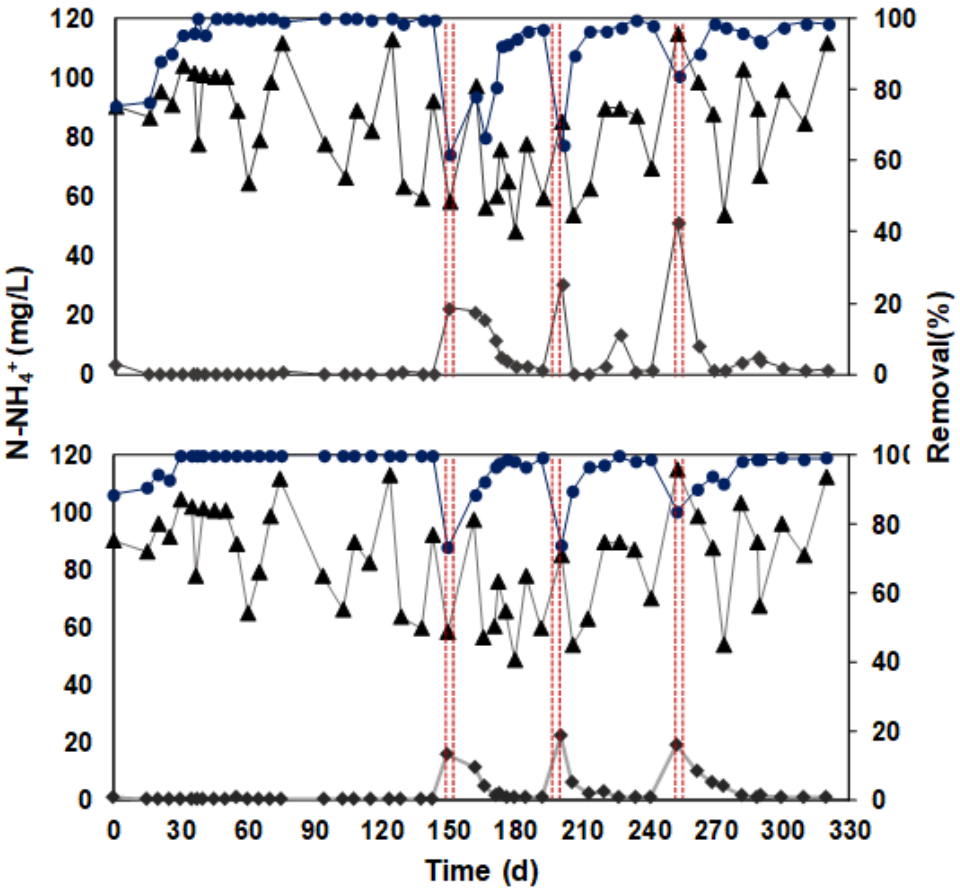

Figure 3

Removal efficiency of NH4+-N with swine wastewater. Where (a) is R1 polyurethane foam and (b) is R2 polyethylene rings. (●) Chemical Oxygen Demand (COD) removal percentage; (▲) influent and (♦) effluent. Lines show days in which the nitrifying and denitrifying activities were performed discontinuously in both reactors.

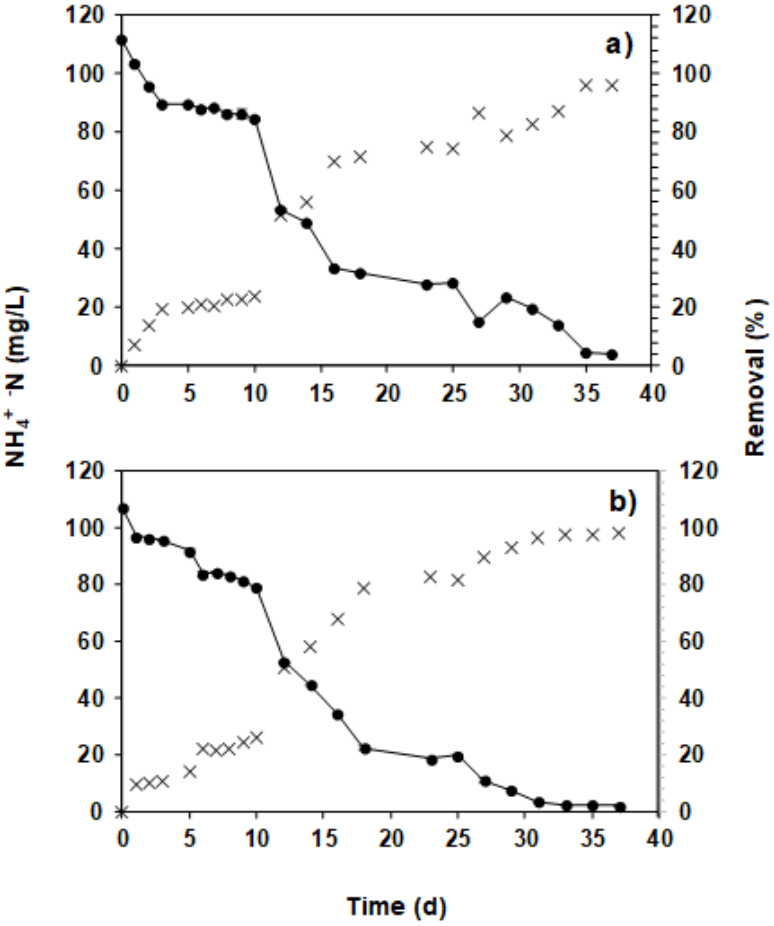

Figure 4

Behavior of NH4+-N concentration in suspended and fixed biomass assays (SB+FB), where: (a) R1 is polyurethane foam (b) R2 is polyethylene rings. (X) removal efficiency (●) follow-up of NH4+-N (mg/L).

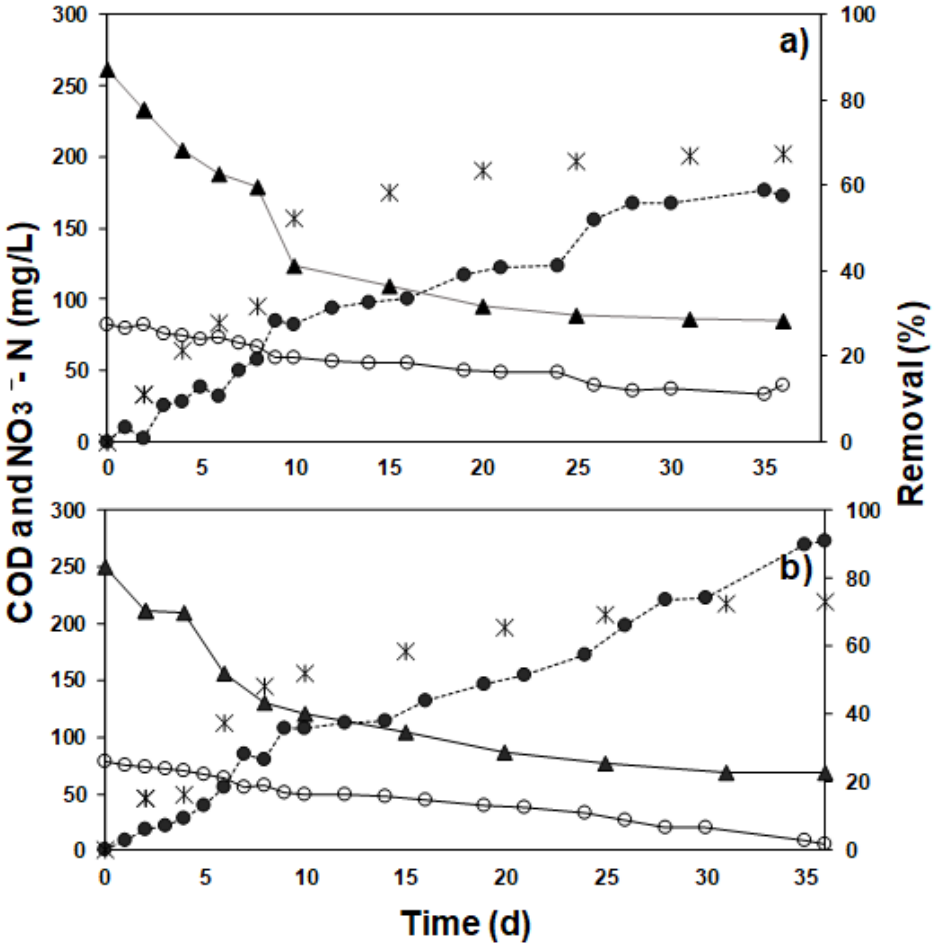

Figure 5

Time chart of consumption behavior NO3- -N and chemical oxygen demand (COD) during the maximum specific denitrification activity (MSDA) assays for (a) R1 and (b) R2. Where (●) is the concentration of NO3- -N ; (o) is NO3–N removal percentage; (▲) COD concentración (mg/L) and (x) is COD removal in the systems.

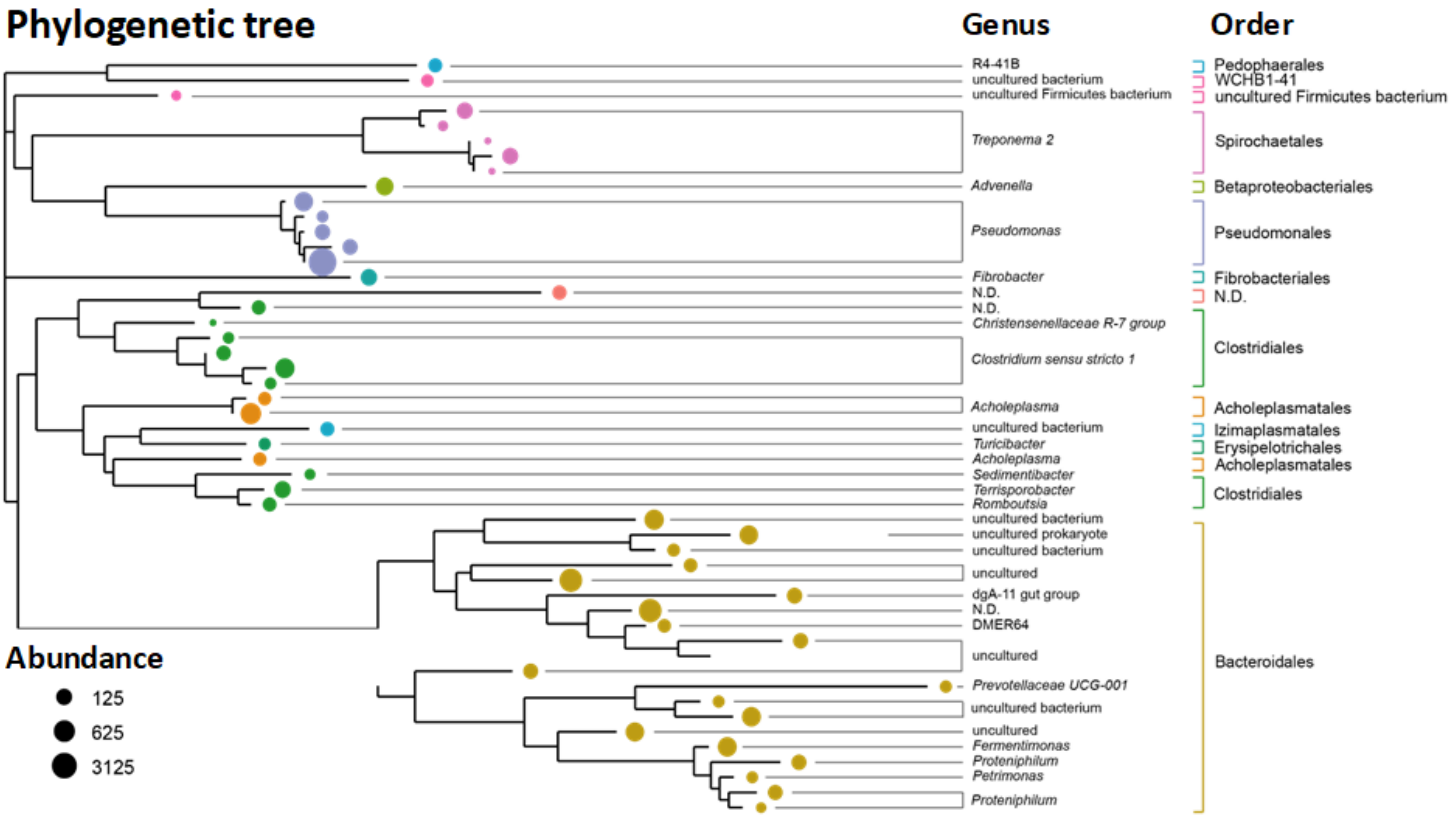

Figure 6

Phylogenetic tree of the most abundant bacteria present in the R2 biofilm.