Journal of Environmental Management 232 (2019) 943–951

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Research article

Microbial characteristics of nitrifiers, denitrifiers and anammox bacteria on different support media to treat space mission wastewater

Fei Dai[a], Marina Celant De Prá[b], Matias B. Vanotti[c], Kevin R. Gilmore[d], William E. Cumbie[a,*]

[a] Pancopia, Inc., 1100 Exploration Way, Suite 302Q, Hampton, VA 23666, USA
[b] Department of Bioprocess Engineering and Biotechnology, Federal Technological University of Paraná - UTFPR, Dois Vizinhos, 85660-000, PR, Brazil
[c] United States Department of Agriculture, Agricultural Research Service, Coastal Plains Research Center, 2611 W. Lucas St., Florence, SC 29501, USA
[d] Department of Civil and Environmental Engineering, Bucknell University, 701 Moore Ave., Lewisburg, PA 17837, USA

ARTICLE INFO

Keywords:
Fluorescence in-situ hybridization
Biomass growth
Early planetary base wastewater
Biofilm support media

ABSTRACT

Biomass attachment and growth are important factors for the startup and stability of fixed-film biological reactors being proposed to recycle wastewater for potable water use in manned space activity. Eight different biofilm support media commonly used in wastewater treatment plants, aquaculture, and aquariums were compared for their relative ability to support attachment and growth of nitrifiers, denitrifiers, and anaerobic ammonia oxidizing (anammox) bacteria biomass. Accumulated total biomass was determined by comparing dry weight of each media before and after culturing of biomass. Fluorescence In-Situ Hybridization (FISH) analysis was used to quantify the proportion and relative activity of each organism group on each media. Measurements of dry biomass normalized to several media properties showed polyether polyurethane foam to have the highest extent of specific biomass attachment and colonization. Six of the eight media were able to sustain a population of anammox bacteria that was more abundant than the other cohorts.

1. Introduction

Wastewater reuse and recycling on the International Space Station (ISS) is an area of growing importance as the National Aeronautics and Space Administration (NASA) begins planning long-duration space missions to the moon and Mars that will accommodate large crews (Kanas, 1990; Kanas et al., 2009). Systems for treating space mission wastewater should be reliable, capable, economical, and lightweight (Cath et al., 2005). Currently, a physical/chemical wastewater treatment system is used in the ISS, consisting of a urine processor assembly and a water processor assembly (Carter et al., 2013). Biological wastewater treatment could have a high economic significance in space exploration due to its lower energy and consumables cost when compared with physical/chemical systems (Garland et al., 2005).

Conventional biological methods that remove organic carbon and nitrogen in wastewater have been widely applied for decades in municipal wastewater plants, most commonly through a combination of activated sludge followed by nitrification and denitrification unit processes (Paredes et al., 2007). However, low levels of organic carbon remaining after treatment with activated sludge are insufficient for complete denitrification (Ahn, 2006). Addition of external organic carbon for denitrification increases costs of consumables and of management of the surplus biosolids that must be disposed (Zhou et al., 2009). More recently attention has shifted towards emerging, cost-effective and environmental-friendly biological wastewater treatment technologies, such as deammonification. Deammonification makes use of aerobic ammonia oxidizing bacteria (AOB) and anaerobic ammonia oxidizing (anammox) bacteria (Mulder et al., 1995) to convert nitrogenous compounds (ammonia and nitrite) to nitrogen gas without the need for an organic carbon substrate (Paredes et al., 2007). Significantly less oxygen is required and smaller amounts of biosolids are produced compared to the conventional nitrification/denitrification (O'Shaughnessy et al., 2011; Regmi et al., 2012). However, deammonification offers some operational challenges since efficient operation requires precisely balanced flows of nitrite and ammonia into the anammox reactor, which can only be achieved through sophisticated process monitoring and control by highly trained operators (Dold et al., 2007). It was found that combination of AOB and anammox biomass in a single system displayed better bacterial activity and could be more economical than separate AOB and anammox process. (De Pra et al., 2016).

A process that addresses the operational drawbacks of

* Corresponding author.
E-mail addresses: fei@pancopia.com (F. Dai), marinapra@utfpr.edu.br (M.C. De Prá), matias.vanotti@ars.usda.gov (M.B. Vanotti), kg021@bucknell.edu (K.R. Gilmore), bill@pancopia.com (W.E. Cumbie).

https://doi.org/10.1016/j.jenvman.2018.12.008
Received 26 June 2018; Received in revised form 1 November 2018; Accepted 3 December 2018
Available online 10 December 2018

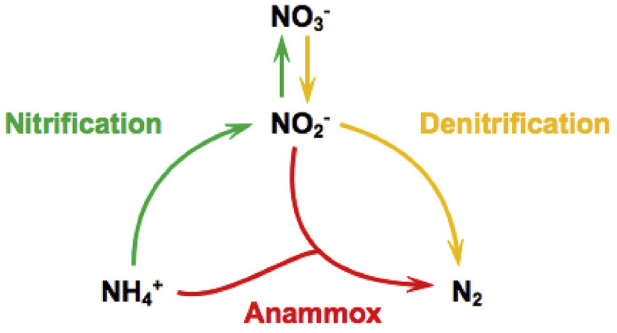


Fig. 1. NDX process pathways.

deammonification by synergistically combining deammonification with nitrification/denitrification in a process termed Nitrification-Denitrification-anammoX (NDX) (Cumbie et al., 2017) is being studied. The NDX bacterial consortium is comprised of three sets of microorganisms that include aerobic nitrifiers (a blend of AOB and NOB (nitrite oxidizing bacteria)), anaerobic denitrifiers, and anaerobic anammox bacteria. The NDX process consists of three pathways (Fig. 1). First, the partial oxidation of ammonia to nitrite by AOB under limited oxygen conditions, and a subsequent reduction of this nitrite to nitrogen gas with the remaining ammonium, by anammox bacteria under anaerobic conditions (also producing a small amount of nitrate). Secondly, the residual nitrate is reduced to nitrogen gas by denitrifiers using organic carbon as an energy source (Gao et al., 2012; Lan et al., 2011; Wang et al., 2010; Zhou et al., 2009). Thirdly, nitrate converted from nitrite by NOB can be reduced to nitrogen gas by denitrifiers to the extent that organic carbon is available for this process pathway. For the NDX process to be effective, it is necessary to keep the nitrifiers (both AOB and NOB), denitrifiers and anammox populations in balance.

Suitable support media along with biofilm composition and activity are critical factors in the successful operation and control of integrated fixed film processes of biological wastewater treatment (Kim et al., 2003; Lazarova and Manem, 1995). Microorganisms used in wastewater treatment have growth rates that span more than two orders of magnitude, and achieving the proper balance of organisms can be problematic. Cultivation and retention of anammox which has a doubling time of approximately two weeks (Grismer and Collison, 2017) is especially important. Support media are commonly used to provide a base for sessile microorganisms to colonize, sustain attached bacterial growth, and minimize washout of microorganisms from treatment systems. The exact microbial community composition in biological systems depends on wastewater composition, process design, and operation (Nielsen et al., 2009). This study investigated abundance and spatial distribution of an NDX bacterial consortium on eight different support media to determine the media's relative suitability for ersatz space wastewater treatment. All the support media were used in the treatment of the same wastewater under the same operational conditions. The wastewater was ersatz Early Planetary Base (EPB) wastewater, a synthetic wastewater containing high nitrogen and organic carbon similar to that generated on the ISS and commonly used as bioreactor influent in space wastewater research (Verostko et al., 2004). FISH analysis makes it possible to directly retrieve information on phylogenetic identities of the cells in a sample, to maintain the morphology of the cells, and to supply information on the distribution and population of identified organisms. The equivalent biovolume, the morphology, and architecture of bacteria were observed by the FISH analysis, and total dry weights of biofilm were quantified to determine the best-performing support media for attached growth of the NDX bacterial consortium treating EPB wastewater.

2. Materials and methods

2.1. Nitrifiers, denitrifiers and anammox

The NDX biomass to inoculate the batch reactor was a mix of three sets of microorganisms, including high performance nitrifying sludge (HPNS), denitrifiers, and anammox. The initial ratio of HPNS (including AOB and NOB), denitrifiers, and anammox settled biomass by volume was 1:1:2 (approximately 1.5% inocula mass fraction for each). The HPNS was obtained from United States Department of Agriculture-Agricultural Research Service (USDA-ARS). The HPNS is deposited under the provisions of the Budapest Treaty of the United Nations (WIPO) in the ARS Culture Collection in Peoria, IL, with deposit accession number NRRL B-50298. It is a patented composition (U.S. Patent No. 8,445,253 B2) of bacteria comprised of 35 strains or populations of isolated bacteria (Vanotti et al., 2013a,b). Based on 16S rRNA analysis, 26 of the bacteria are affiliated with Proteobacteria, 7 with Bacteroidetes, and 2 with Actinobacteria. The denitrifying biomass was collected from the Hampton Roads Sanitation District (HRSD) York River Treatment Plant. The plant has dedicated denitrification filters that permitted collection of a consistent set of organisms with good treatment capabilities. The anammox biomass was Candidatus *Brocadia caroliniensis*, a strain isolated and patented (U.S. Patent 8,574,885 B2.) by USDA-ARS (Vanotti et al., 2013a,b). It was deposited under the provisions of the Budapest Treaty in the ARS Culture Collection (NRRL) at Peoria, IL with accession number NRRL B-50286 (Vanotti et al., 2013a,b).

2.2. Space synthetic wastewater

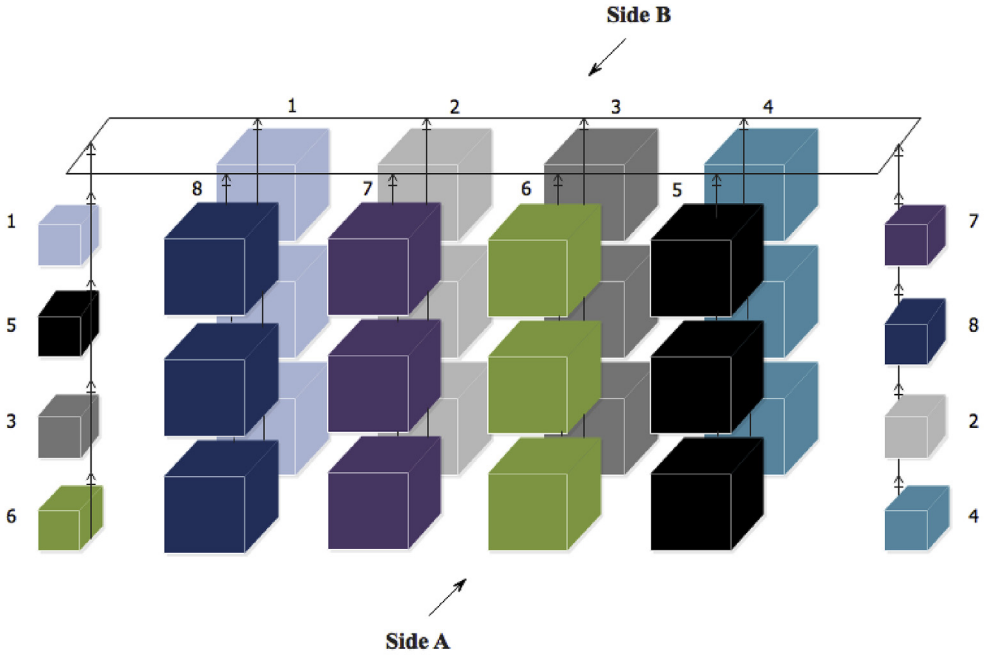
EPB wastewater was used as reactor feed, and prepared in accordance with instructions contained in the literature (Verostko et al., 2004), as noted in Table 1.

2.3. Reactor setup

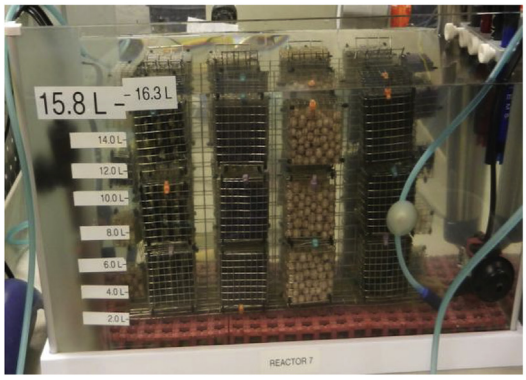
A bioreactor (Fig. 2) was initially operated with 16.3 L of half strength EPB wastewater. Two circulation pumps (Hydor, PICO 180, 4W) were placed in the reactor to provide uniform mixing at approximately 1.9 L/min. These also facilitated transport of inoculum to media surfaces during start-up and minimizing liquid-phase boundary layer thickness at the liquid-media interface. Oxygen was supplied by two air pumps (Tetra Whisper, 1.5 W) delivering lab air (flow rate = 0.3 L/min) to two ceramic air diffusers, one positioned just downstream of each circulation pump. The dissolved oxygen concentration in the tank varied between 0 and 2 mg/L. A quartz glass submersible heater (ViaAqua, 50 W) set to 30 °C was used to maintain the reactor

Table 1
Average solution properties of full strength ersatz EPB wastewater (Verostko et al., 2004).

Property	Formula	Units	Ave. Value	Std. Dev.
Potential of hydrogen	pH		8.9	± 0.2
Conductivity	EC	µS	6.869	± 1030
Total organic carbon	TOC	mg/L	631	± 63
Total inorganic carbon	TIC	mg/L	391	± 59
Chloride	Cl^-	mg/L	514	± 77
Nitrite	NO_2^-	mg-N/L	0	0
Nitrate	NO_3^-	mg-N/L	0	0
Phosphate	PO_4^{-3}	mg-P/L	116	± 17
Sulfate	SO_4^{-2}	mg/L	88	± 13
Sodium	Na^+	mg/L	331	± 50
Ammonium	NH_4^+	mg-N/L	852	± 128
Potassium	K^+	mg/L	240	± 36
Calcium	Ca^{+2}	mg/L	0	0
Magnesium	Mg^{+2}	mg/L	0	0



(a)



(b) (c)

Fig. 2. Scaffolds in reactor: (a) 3D schematic with corresponding media number in Table 2; (b) side A in reactor; (c) side B in reactor.

Table 2
Information of media used in reactor.

Number	Name	Type	Applications
1	Polyethylene	100% premium quality virgin polyethylene	Rusten et al., (2006)
2	Porous SiO_2	A complex pore system (65%–75% SiO_2) composed of 85% micro-tunnels and 15% micro-cavities	McCallum et al., (2017)
3	Porous ceramic	Ceramic bio-filter media with vast surface area and open-flow porosity	Sharma et al., (2018)
4	Activated carbon	Activated carbon that is shaped as a spherical bead of about 1.4 mm in diameter with a very uniform particle size distribution	Çeçen and Aktas, (2011)
5	Polyurethane foam with activated carbon	Polyurethane foam impregnated with a mixture of activated carbon and a copolymer RK dispersion as binder	Moe and Irvine, (2001)
6	Porous sintered glass	Highly porous artificial sintered glass	Perez et al., (1997)
7	Polyether polyurethane	Polyether polyurethane foam	(Pascik, 1990; Zhou et al., 2009)
8	PVC	Rigid and non-flammable PVC sheets	Ye et al., (2009)

Table 3
rRNA-targeted oligonucleotide probes used to detect nitrifying, anammox and denitrifying microorganisms in the biofilm samples.

Probe name	Target	Sequence (5'- 3')	Reference
FLC-labeled Nso190 oligonucleotide probe	Betaproteobacterial (AOB)	CGA TCC CCT GCT TTT CTC C	Mobarry et al., (1996)
FLC-labeled NIT3 oligonucleotide probe	Genus *Nitrobacter* (NOB)	CCT GTG CTC CAT GCT CCG	Wagner et al., (1996)
Cy3-labeled Flo1 oligonucleotide probe	*C. Brocadia carolinensis (anammox)*	TCG GTA GCC CCA ATT GCT TGG	Rothrock et al., (2011)
FLC-labeled Pae997 oligonucleotide probe	Most true *Pseudomonas spp. (denitrifier)*	GCT GGC CTA GCC TTC	Amann et al., (1996)

temperature. Bacteria were cultured simultaneously on the eight different media listed below (Table 2) in a single reactor inoculated with 250 mL NDX biomass (62.5, 62.5 and 125 mL of HPNS, denitrifiers and anammox, respectively), for 45 days. Each type of media was evaluated in triplicate using sets of 5 cm × 5 cm × 2.5 cm scaffolds to determine biomass accumulation over the culturing period.

Photos of the media before culturing are shown in the appendix (Fig. A.1). One small scaffold cube (2.5 cm × 2.5 cm × 2.5 cm) of each media was also cultured in the reactor over the same period for subsequent FISH analysis. The positions of each media in the reactor are indicated in Fig. 2.

Halfway through the incubation time (Day 23), the culturing solution (half strength EPB) in the reactor had to be replaced due to shedding of carbon particulates by 5 cm × 5 cm × 2.5 cm scaffold media samples of activated carbon (#4) and polyurethane foam with activated carbon (#5), and full strength EPB was selected so as to begin the feeding regimen. The washout of the carbon particulates resulted in extremely cloudy reactor water so both the activated carbon and the polyurethane foam with activated carbon were deemed unsuitable for application in wastewater treatment and were removed from the reactor for the remainder of the experiment. The 2.5 cm × 2.5 cm x 2.5 cm smaller scaffold cubes intended for FISH analysis were left in the reactor for the entire test duration. Some organisms in the carbon-contaminated water were lost during the water replacement. To address this issue, 500 mL anammox and 250 mL HPNS were added after the reactor was filled with full strength EPB. The reactor was operated in batch mode and not fed again until half of the ammonia in the full strength EPB was depleted (Day 33), at which point feed was initiated at a rate of 700 mL full strength EPB daily. This was done using peristaltic pumps to evenly feed 700 mL EPB wastewater and remove 700 mL final effluent per 24-hr period until the 45th day. The average NH_4-N concentration over the last ten days declined from 694 mg/L in influent to 181 mg/L in effluent while the effluent NO_3-N and NO_2-N increased from 0 mg/L each to 3 mg/L and 9 mg/L, respectively.

2.4. Biomass growth analysis

The 5 cm × 5 cm × 2.5 cm scaffolds were weighed before and after the 45-day testing period. Before weighing, media were oven-dried for 48 h at 60 °C and transferred to a desiccator. The scaffolds were then cooled and dried in the desiccator for 2 h, and then weighed. The amount of dry biomass grown on each scaffold was determined by the difference of its dry weight before and after the testing.

2.5. Fluorescence In situ hybridization (FISH)

The treated 2.5 cm cubes were collected from the reactor after the 45-day testing period and tested by FISH analysis. Media samples were taken from the 2.5 cm media scaffolds, washed three to four times using half strength EPB wastewater, and sonicated with an ultrasonic disrupter (42 kHz, 70 W; Bransonic Ultrasonic Cleaner, 151OR-MTH, Danbury, USA) for 1 min to release all cells off the support media. Flexible media (e.g., polyether polyurethane foam, #7) were also manipulated by hand to squeeze out biomass attached to interior surfaces. All samples were fixed with 4% paraformaldehyde for 6 h as described by Amann (Rudolf, 1995). The samples were then stored at −20 °C in a 1:1 (v/v) mixture of Ethanol and PBS (8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4{\cdot}12H_2O$, and 0.2 g KH_2PO_4 per liter of distilled water at pH 7.4). Prior to hybridization, each sample was homogenized by sonication for 1–2 min in order to disperse large aggregates. In situ hybridization was performed according to the standard hybridization protocol described by Amann (Rudolf, 1995). In brief, 10 µL of the fixed and homogenized sample was applied on a gelatin-coated microscope slide containing three wells (Tekdon Incorporated, Myakka City, Florida) and dried at 46 °C for 15 min, followed by a successive dehydration step with 50%, 80% and 99% ethanol for 3 min each. For hybridization, 9 µL of hybridization buffer with the optimal formamide concentration was mixed with 1 µL of labeled probe (50 ng/µL) and then pipetted on the microscope slide for a 3-h hybridization at 46 °C. After the hybridization, the microscope slide was rinsed with preheated (48 °C) washing solution and incubated for 15 min. Simultaneous hybridization with probes requiring different sequence (5'-3') was performed by the successive hybridization procedure described by Nielsen (Nielsen et al., 2009). Following the last hybridization, wells were stained with 4', 6-diamidino-2-phenylindole (DAPI) as an intercalating agent to quantify all bacteria, including both probe-targeted and non-targeted organisms. Table 3 describes the name, target, and sequence of specific probes employed to identify the microbial characterization of the samples. Negative controls without organisms were used to eliminate false positive results due to autofluorescence. The images used for quantification of bacterial groups were acquired through an epifluorescence microscope (Nikon Eclipse 50i) and were resolved using standard image controls (Nikon DS-5M digital camera and DS-L1 camera control unit). FISH images from epifluorescence microscope were examined by a digital image analyzer (Image J software) with a curated collection of Fiji's plugins pre-installed for quantification. Also, to see the AMX and AOB biomass architectures and morphology, additional images were acquired using a confocal microscope (Nikon Eclipse 80i) and were resolved using control and standard software (Nikon ACT-1, version 2.63).

2.6. Statistical analysis

Based on the dry biomass weight as well as the bacterial attachment of AOB, NOB, AMX and DN on different media, a statistical analysis to compare the differences of each media on the results was carried out using one-way analysis of variance (ANOVA) at $P < 0.05$ in Excel of Microsoft Office™ Software.

3. Results and discussion

3.1. Biomass attachment

Activated carbon (#4) and polyurethane foam with activated carbon (#5) were deemed not suitable for this application. Both media, made of activated carbon, released large amounts of inert carbon particles in the first half of the 45-day testing, resulting in contamination of the reactor water. After the reactor water was replaced by full strength EPB in the middle of the testing, the reactor was much clearer. Although trace amounts of carbon remained on the other scaffolds after water replacement, the amounts of the leaked carbon on each scaffold should have been similar due to the continuous mixing pattern in the

Table 4
Average dry biomass weight on each set of cubes at different positions.

Number-Material	Type of porosity	Average mass of biomass (mg)	Standard deviation	Rank	Internal surface area (m^2/m^3)	Mass of biomass per scaffold total internal surface area (mg/cm^2)	Rank	Scaffold average mass (g)	Mass of biomass per scaffold mass (mg/g)	Rank	Scaffold volume (cm^3)	Mass of biomass per scaffold volume (mg/cm^3)	Rank
1-Polyethylene	non-microporous	150.5	32.7	3	800	4.8555	2	16.5	9.1	2	25.81	5.8	3
2-Porous SiO_2	non-microporous	207.6	29.1	2	N/A	N/A	N/A	66.2	3.1	5		8.0	2
3-Porous ceramic	microporous	110.8	64.5	4	458661	0.0062	5	29.8	3.7	4		4.3	4
6-Porous sintered glass	microporous	82.6	44.4	6	44176	0.0483	4	52.4	1.6	6		3.2	6
7-Polyether polyurethane foam	non-microporous	255.5	25.1	1	1600	4.1215	3	9.1	28.1	1		9.9	1
8-PVC	non-microporous	83.5	9.2	5	226	9.5360	1	10.9	7.7	3		3.2	5

reactor and therefore have had similar effects on the results when comparing the dry weights of different media.

Average mass of dry biomass on each scaffold media was determined at the end of the study (45 days) in the remaining 6 media (Table 4). The mass of biomass in the various media was calculated in three ways: 1) per scaffold total internal surface area, 2) per scaffold mass, and 3) per scaffold volume (Table 4). Polyether polyurethane foam (#7) scaffolds had the greatest average dry biomass weight. With an internal surface area of approximately $1600\ m^2/m^3$, polyether polyurethane foam is at least twice as space efficient as the other non-microporous media, such as polyethylene (#1) and PVC (#8). The weight of the materials sent to space station should be also considered in reference to cost and energy, as materials with less weight are preferred. The polyether polyurethane foam maintained the highest biofilm growth per unit weight (mass of biomass per scaffold mass in Table 4). Although porous SiO_2 media (#2) had the second-highest biomass growth, it is the heaviest media among the eight tested media (scaffold average mass, Table 4), and is thus not suitable as material to be used on the space station. It is worth noting that the polyether polyurethane foam, polyethylene filter, and PVC are much lighter than the other three media. Polyethylene and PVC had the second and the third greatest biomass growth per unit weight, respectively. Media volume should also be considered for saving space when designing a system, and the polyether polyurethane foam achieved the highest mass of biomass per scaffold volume (Table 4). In conclusion, the ideal support media in terms of biomass attachment is the polyether polyurethane foam.

3.2. Molecular quantification of abundance

The relative abundances of nitrifying (AOB and NOB), denitrifying (DN), and anammox (AMX) bacterial populations on the eight support media were compared using FISH after the 45-day testing, as shown in Fig. 3. For comparison and controls, the last three microbial compositions (Fig. 3) present the analysis of anammox inocula (InAMX), nitrifying inocula (InNIT), and denitrifying inocula (InDN), respectively.

Anammox have a slower growth rate (9–11 days) compared to AOB, NOB, and DN, and possess a range of potential inhibitors (Bettazzi et al., 2010; Dapena-Mora et al., 2007; Strous et al., 1999). Thus it is favorable to create conditions to keep the anammox bacteria in the reactor as much as possible. Data in Fig. 3 show that 75% of the eight support media (6/8) were able to maintain anammox as the bacterial cohort with the greatest abundance of all attached organisms, with a relative abundance higher than AOB, NOB, or DN. Especially polyether polyurethane foam (#7) with 32.1% relative abundance, activated carbon (#4) with 31.8%, and porous ceramic media (#3) with 31.1%. However, #3 was made of a material that was found to be very fragile during reactor operation.

In the NDX process, AOB utilize ammonia and oxygen as substrates, while AMX require ammonia and nitrite. On the other hand, NOB make use of nitrite and oxygen, and can outcompete the AOB-AMX process due to oxygen competition with AOB and nitrite competition with AMX. Therefore, it is necessary to keep the amount of NOB relatively low. Additionally, from an energy standpoint a low NOB population reduces the required oxygen. Moderate NOB is helpful to generate nitrate as substrates used by denitrifiers to remove carbon. Fig. 3 indicates that the support media #7 had the least amount of NOB (0.9%), which is very advantageous for NDX application. While the mechanisms for preferential attachment of each bacterial group onto one media or another is not studied here, the apparent selectivity of polyether polyurethane foam for higher AMX and AOB with lower NOB and DN, combined with its high specific biomass accumulation and light weight, make the polyether polyurethane foam a preferred support substrate for a fixed-film process.

Although the presence of heterotrophic denitrifying bacteria is important for carbon and nitrate removal, some research found anammox

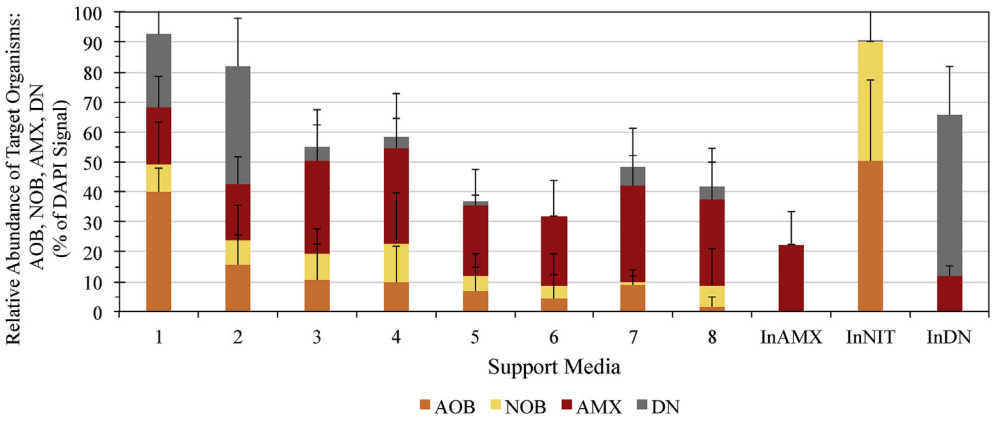


Fig. 3. Relative abundances of AOB, NOB, AMX and DN measured by probe-targeted FISH over the total bacterial population (targeted-DAPI), as well as abundance of all the bacteria in the field of view (FOV) during the analysis. One-sided error bars represent plus one standard deviation. The x-axis number 1 to 8 refers to media number in Table 2.

communities could be inactivated or eradicated under concurrent operation with denitrification when COD exceeds 300 mg/L so COD would be critical for process selection between anammox reaction and denitrification (Chamchoi et al., 2008). In addition, autotrophic AOB and anammox bacteria cannot incorporate exogenous organic compounds because they obtain energy from oxidation of inorganic compounds (De Pra et al., 2012; Yamamoto et al., 2011). The maximum growth rate of autotrophic AOB ($\mu_A$max) is much lower than the growth rate of aerobic heterotrophic bacteria ($\mu_H$max) as Wiesmann (1994) reported $\mu_H$max = 7.2 d^{-1} and $\mu_A$max = 0.77 d^{-1}. This significant difference in growth rates—coupled with the rapid growth of heterotrophic denitrifying bacteria fed by the carbon available in EPB wastewater—can promote competition by heterotrophic denitrifying bacteria for living space in a bioreactor, suppressing the growth of AOB and anammox. Therefore, control of heterotrophic denitrifying bacteria is important to successful operation of the NDX process. Polyethylene (#1) and porous SiO_2 media (#2) are not recommended because they were found to host a very large proportion of denitrifying bacteria (Fig. 3).

Fig. 4 shows the representative images from FISH using a confocal microscope, and illustrates the AMX and AOB populations' composition and architecture on eight support media. The biomass architectures were different, alternating from big aggregates dominated by AMX, to smaller and dispersed AOB flocs (Figs. 4 and 1 to 8). It is possible to see large aggregates of both bacteria with a nice consociated mix of AOB and AMX in image 1, 2, and 7 of Fig. 4. In the other images (3–6, and 8), the AOB were mainly present in small and dispersed flocs in the biomass, possibly explaining their apparently much smaller amount relative to AMX in most of the support media.

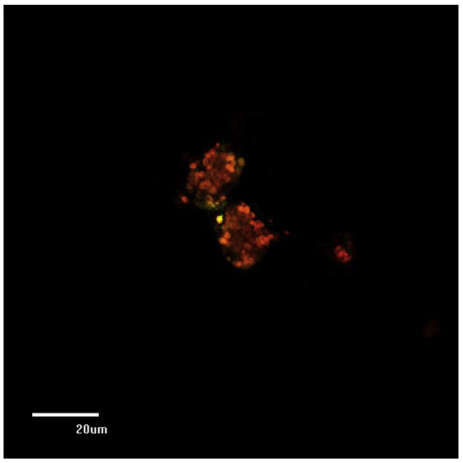


Fig. 5. Representative FISH image of polyether polyurethane foam under confocal microscope (anammox populations are displayed with red color allocation). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

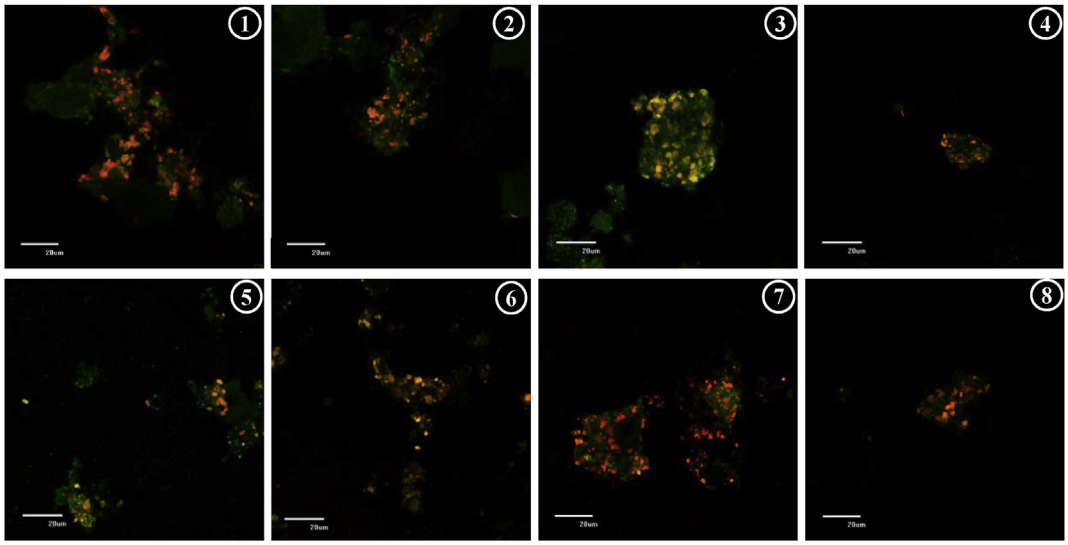


Fig. 4. Representative FISH images under confocal microscope illustrating the differences of the biomass compositions and architectures on eight different support media. Anammox populations are displayed with red color allocation and AOB in green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Results of one-way ANOVA statistics (*P* values).

	Dry biomass weight (mg)	Relative abundance				
		All bacteria	AOB	NOB	AMX	DN
P	0.0101	2.106E-05	5.325E-09	0.7264	0.0079	0.0003

In summary, the best support media in terms of microbial composition in the NDX process for space exploration is the polyether polyurethane foam media #7. It had better quantitative and qualitative measurements of AOB, AMX and DN than the other media, and thus showed significant capacity for attachment and growth of NDX bacteria. In addition, it is lightweight, which is important for space systems. From FISH image (Fig. 5) captured from the polyether polyurethane foam (#7), it is possible to visualize an intrinsic characteristic of anammox bacteria. As it is common for anammox bacteria to occur as small to large cell aggregates (Nielsen et al., 2009), anammox bacteria have a coccoid morphology and a "donut-like" shape after FISH analysis, with an apparent small hole in the center of the cells. The intracellular "hole" is anammoxosome that is ribosome-free (van Niftrik and Jetten, 2012) (and thus absence of target molecules for FISH probes) in this region.

3.3. Statistical analysis

One-way ANOVA performed on the results, at a significance level of 0.05, indicated that dry biomass weight as well as the bacterial attachment of AOB, AMX and DN were significantly affected by the media (Table 5). The significant bacterial attachment difference may be related to different oxygen level across the depth of the different media as well as contrasting media internal surface area (Table 4). Different media had no significant effect on NOB population. This may be because of the effect of low dissolved oxygen level (< 1 mg/L) in the NDX reactor on the growth of NOB. Relatively lower population of NOB compared to the other bacteria resulted in the relatively equal group variance. The overall (sum of AOB, NOB, AMX and DN) bacterial relative abundance were significantly different on the media, with a *P* value of 2.106E-05.

4. Conclusions

The extent of NDX bacteria growth on 8 different media carriers over a 45-day testing period was investigated. The results indicated that polyether polyurethane foam had the greatest biomass growth as well as the highest biofilm growth per unit mass and per unit volume, making it an optimal candidate for biological wastewater treatment in space. In addition, the functional microbial composition of polyether polyurethane foam, with the greatest AMX bacterial attachment (32.1% relative abundance), better quantitative and qualitative measurements of AOB, AMX and DN than the other media and relatively low NOB, would improve the treatment efficiency. The polyether polyurethane foam will be further studied on the effects of foam porosity and thickness variables on substrate transport and wastewater treatment performance as well as the risk analysis of its reliability in NDX-based bioreactors.

Acknowledgements

The authors thank NASA for its financial, technical, and administrative assistance in funding and managing this work (NASA SBIR contract number NNX16CJ12C). The comments and views detailed herein may not necessarily reflect the views of NASA. This research was funded in part by a grant from the Center for Innovative Technology. The authors also thank John Whitelaw at Pancopia and William Brigman at USDA-ARS for analysis assistances. At the time of this research, Marina Celant De Prá was USDA Visiting Scientist. Cooperation between Pancopia, Inc. and ARS through CRADA 58-6082-6-006 is acknowledged. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

Appendix

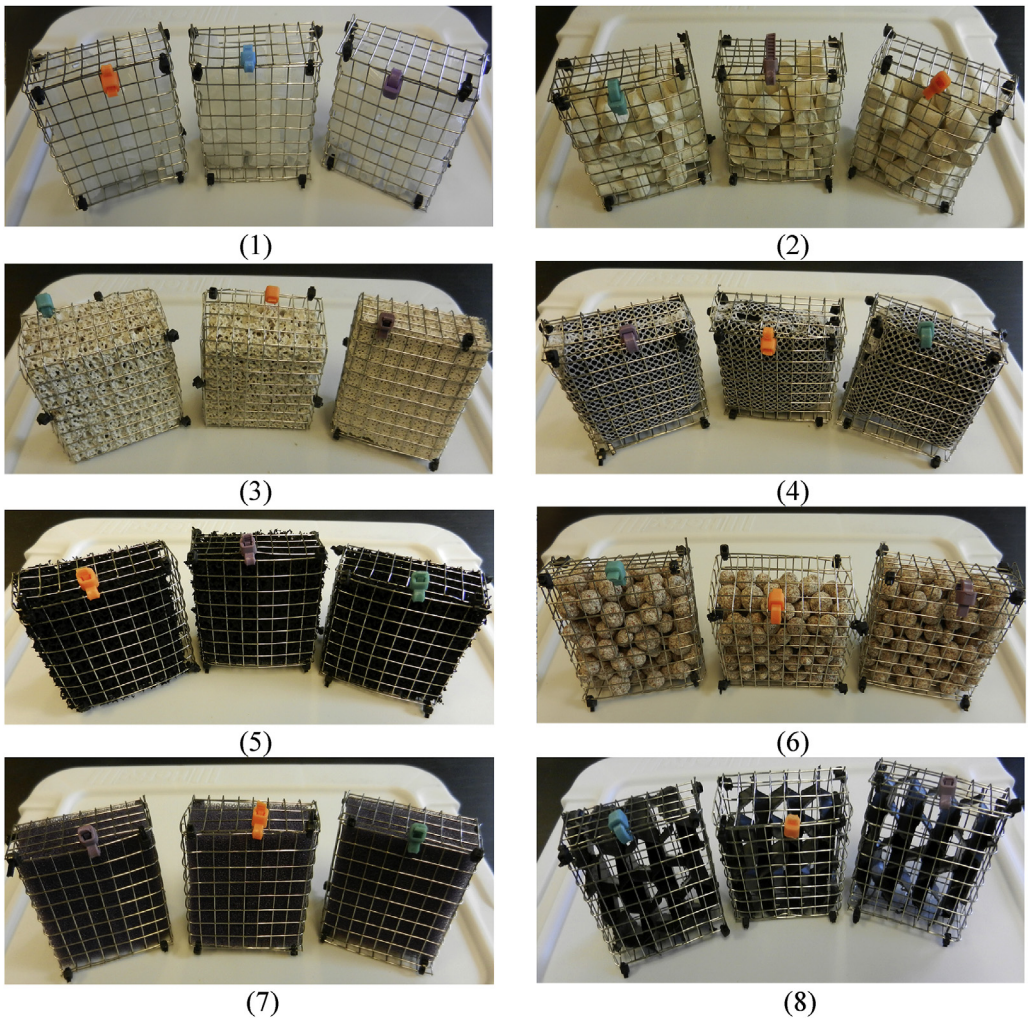


Fig. A.1. Triplicate scaffolds made of different media: (1) Polyethylene (2) Porous SiO_2 (3) Porous ceramic (4) Activated carbon (5) Polyurethane foam with activated carbon (6) Porous sintered glass (7) Polyether polyurethane (8) PVC.

References

Ahn, Y.-H., 2006. Sustainable nitrogen elimination biotechnologies: a review. Process Biochem. 41 (8), 1709–1721.

Amann, R., Snaidr, J., Wagner, M., Ludwig, W., Schleifer, K.-H., 1996. In situ visualization of high genetic diversity in a natural microbial community. J. Bacteriol. 178 (12), 3496–3500.

Bettazzi, E., Caffaz, S., Vannini, C., Lubello, C., 2010. Nitrite inhibition and intermediates effects on Anammox bacteria: a batch-scale experimental study. Process Biochem. 45 (4), 573–580.

Carter, D.L., Tobias, B., Orozco, N.Y., 2013. Status of ISS water management and recovery. In: 43rd International Conference on Environmental Systems, pp. 3509.

Cath, T.Y., Gormly, S., Beaudry, E.G., Flynn, M.T., Adams, V.D., Childress, A.E., 2005. Membrane contactor processes for wastewater reclamation in space: Part I. Direct osmotic concentration as pretreatment for reverse osmosis. J. Membr. Sci. 257 (1), 85–98.

Çeçen, F., Aktas, Ö., 2011. Activated Carbon for Water and Wastewater Treatment: Integration of Adsorption and Biological Treatment. John Wiley & Sons.

Chamchoi, N., Nitisoravut, S., Schmidt, J.E., 2008. Inactivation of ANAMMOX communities under concurrent operation of anaerobic ammonium oxidation (ANAMMOX) and denitrification. Bioresour. Technol. 99 (9), 3331–3336.

Cumbie, W., Whitelaw, J., Dai, F., Schafran, G., Gilmore, K., Vanotti, M., Bott, C., 2017. Biological Water Recycling System for the International Space Station: Phase II Update. WaterJam September 11-14, Hampton, vol. A. .

Dapena-Mora, A., Fernandez, I., Campos, J., Mosquera-Corral, A., Mendez, R., Jetten, M., 2007. Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. Enzym. Microb. Technol. 40 (4), 859–865.

De Pra, M.C., Kunz, A., Bortoli, M., Perondi, T., Chini, A., 2012. Simultaneous removal of TOC and TSS in swine wastewater using the partial nitritation process. J. Chem. Technol. Biotechnol. 87 (12), 1641–1647.

De Pra, M.C., Kunz, A., Bortoli, M., Scussiato, L.A., Coldebella, A., Vanotti, M., Soares, H.M., 2016. Kinetic models for nitrogen inhibition in ANAMMOX and nitrification process on deammonification system at room temperature. Bioresour. Technol. 202, 33–41.

Dold, P., Walker, R., Houweling, D., Fairlamb, M., Takacs, I., Jones, R., 2007. Treatment plant/control system simulation for design and operational benefits. Proceed. Water Environ. Federat. 2007 (10), 7385–7395.

Gao, F., Zhang, H., Yang, F., Qiang, H., Zhang, G., 2012. The contrast study of anammox-denitrifying system in two non-woven fixed-bed bioreactors (NFBR) treating different low C/N ratio sewage. Bioresour. Technol. 114, 54–61.

Garland, J., Rector, T., Anderson, M., 2005. The Effects of Alternative Biological Primary Processing Approaches on the Efficiency of an Integrated Water Processing System. SAE Technical Paper. 0148-7191. .

Grismer, M.E., Collison, R.S., 2017. The zeolite-anammox treatment process for nitrogen removal from wastewater—a review. Water 9 (11), 901.

Kanas, N., 1990. Psychological, psychiatric, and interpersonal aspects of long-duration space missions. J. Spacecraft Rockets 27, 457–463.

Kanas, N., Sandal, G., Boyd, J., Gushin, V., Manzey, D., North, R., Leon, G., Suedfeld, P., Bishop, S., Fiedler, E., 2009. Psychology and culture during long-duration space missions. Acta Astronaut. 64 (7), 659–677.

Kim, S., Park, C., Kim, T.-H., Lee, J., Kim, S.-W., 2003. COD reduction and decolorization of textile effluent using a combined process. J. Biosci. Bioeng. 95 (1), 102–105.

Lan, C.-J., Kumar, M., Wang, C.-C., Lin, J.-G., 2011. Development of simultaneous partial nitrification, anammox and denitrification (SNAD) process in a sequential batch reactor. Bioresour. Technol. 102 (9), 5514–5519.

Lazarova, V., Manem, J., 1995. Biofilm characterization and activity analysis in water and wastewater treatment. Water Res. 29 (10), 2227–2245.

McCallum, E.S., Krutzelmann, E., Brodin, T., Fick, J., Sundelin, A., Balshine, S., 2017. Exposure to wastewater effluent affects fish behaviour and tissue-specific uptake of pharmaceuticals. Sci. Total Environ. 605, 578–588.
Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E., Stahl, D.A., 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 62 (6), 2156–2162.
Moe, W.M., Irvine, R., 2001. Polyurethane foam based biofilter media for toluene removal. Water Sci. Technol. 43 (11), 35–42.
Mulder, A., Van de Graaf, A.A., Robertson, L., Kuenen, J., 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. 16 (3), 177–183.
Nielsen, P.H., Daims, H., Lemmer, H., Arslan-Alaton, I., Olmez-Hanci, T., 2009. FISH Handbook for Biological Wastewater Treatment. Iwa publishing.
O'Shaughnessy, M., Wett, B., Bott, C., Murthy, S., deBarbadillo, C., Kinnear, D., Chandran, K., Neethling, J., Shaw, A., Stinson, B., 2011. Full-plant deammonification for low-energy, low-carbon nitrogen removal. Proceed. Water Environ. Federat. 2011 (13), 3715–3720.
Paredes, D., Kuschk, P., Mbwette, T., Stange, F., Müller, R., Köser, H., 2007. New aspects of microbial nitrogen transformations in the context of wastewater treatment–a review. Eng. Life Sci. 7 (1), 13–25.
Pascik, I., 1990. Modified polyurethane carriers for biochemical waste water treatment. Water Sci. Technol. 22 (1–2), 33–42.
Perez, M., Romero, L.I., Nebot, E., Sales, D., 1997. Colonisation of a porous sintered-glass support in anaerobic thermophilic bioreactors. Bioresour. Technol. 59 (2–3), 177–183.
Regmi, P., Miller, M.W., Bunce, R., Hingley, D.M., Kinnear, D., Deammonification, B., 2012. Nitrogen Removal 3.0: a pilot study to evaluate the feasibility of mainstream deammonification. Proceed. Water Environ. Federat. 2012 (15), 1994–2017.
Rothrock, M.J., Vanotti, M.B., Szögi, A.A., Gonzalez, M.C.G., Fujii, T., 2011. Long-term preservation of anammox bacteria. Appl. Microbiol. Biotechnol. 92 (1), 147–157.
Rudolf, A., 1995. In Situ Identification of Microorganisms by Whole Cell Hybridization with rRNA-targeted Nucleic Acid Probes, Molecular Microbial Ecology Manual. Springer, Netherlands.
Rusten, B., Eikebrokk, B., Ulgenes, Y., Lygren, E., 2006. Design and operations of the Kaldnes moving bed biofilm reactors. Aquacult. Eng. 34 (3), 322–331.
Sharma, D., Taylor-Edmonds, L., Andrews, R.C., 2018. Comparative assessment of ceramic media for drinking water biofiltration. Water Res. 128, 1–9.
Strous, M., Kuenen, J.G., Jetten, M.S., 1999. Key physiology of anaerobic ammonium oxidation. Appl. Environ. Microbiol. 65 (7), 3248–3250.
van Niftrik, L., Jetten, M.S., 2012. Anaerobic ammonium-oxidizing bacteria: unique microorganisms with exceptional properties. Microbiol. Mol. Biol. Rev. 76 (3), 585–596.
Vanotti, M.B., Szogi, A.A., Ducey, T.F., High performance nitrifying sludge for high ammonium concentration and low temperature wastewater treatment, Patent and Trademark Office, 2013a. U.S. Patent No. 8,445,253 B2.
Vanotti, M.B., Szogi, A.A., Rothrock Jr., M.J., Anammox bacterium isolate. in: U.S. Patent and Trademark Office, 2013b. U.S. Patent 8,574,885 B2.
Verostko, C.E., Carrier, C., Finger, B.W., 2004. Ersatz Wastewater Formulations for Testing Water Recovery Systems. SAE Technical Paper. 0148-7191. .
Wagner, M., Rath, G., Koops, H.-P., Flood, J., Amann, R., 1996. In situ analysis of nitrifying bacteria in sewage treatment plants. Water Sci. Technol. 34 (1–2), 237–244.
Wang, C.-C., Lee, P.-H., Kumar, M., Huang, Y.-T., Sung, S., Lin, J.-G., 2010. Simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) in a full-scale landfill-leachate treatment plant. J. Hazard Mater. 175 (1–3), 622–628.
Wiesmann, U., 1994. Biological nitrogen removal from wastewater. In: Biotechnics/wastewater. Springer, pp. 113–154.
Yamamoto, T., Wakamatsu, S., Qiao, S., Hira, D., Fujii, T., Furukawa, K., 2011. Partial nitritation and anammox of a livestock manure digester liquor and analysis of its microbial community. Bioresour. Technol. 102 (3), 2342–2347.
Ye, J., McDowell, C.S., Koch, K., Kulick, F.M., Rothermel, B.C., 2009. Pilot testing of structured sheet media IFAS for wastewater biological nutrient removal (BNR). Proceed. Water Environ. Federat. 2009 (12), 4427–4442.
Zhou, L.-C., Li, Y.-F., Bai, X., Zhao, G.-H., 2009. Use of microorganisms immobilized on composite polyurethane foam to remove Cu (II) from aqueous solution. J. Hazard Mater. 167 (1–3), 1106–1113.