

# Nitrifying, Denitrifying and Heterotrophic Biomass Present in Moving Bed-Reactor

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**Abstract:** This study was carried out to evaluate the main bacterial communities related to the removal of nitrogen in a moving bed-reactor treating landfill leachate, relating the physico-chemical parameters with the existence of these organisms in the mixed liquor (non-attached microorganisms) and Support Material (SM). The system was operated in two phases: Phase I (without effluent recirculation) and II (with recirculation, at a flow rate of 3 times the inlet flow). To monitor the system, physico-chemical analyzes were determined in the influent and effluent: pH, alkalinity, temperature, nitrogen species, Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD). To determine the concentration of nitrifying bacteria (Ammonia Oxidizing Bacteria-AOB and Nitrite Oxidizing Bacteria-NOB) and denitrifiers, the most probable number was estimated per 100 mL (MPN.100 mL<sup>-1</sup>). The concentration of heterotrophic bacteria was estimated by determination of colony forming unit per mL (CFU.mL<sup>-1</sup>). The reactor showed a high percentage of NH<sub>4</sub><sup>+</sup>-N removal in both phases of operation, reaching 80% removal efficiency in Phase I and 83% in II. At pH close to 5.4 NOB activity was practically ceased, with nitrite accumulation in the system. Although the oxygen concentration in the mixed liquor was above 2.0 mg.L<sup>-1</sup> the concentration of denitrifying bacteria was not affected. The concentration of heterotrophic bacteria was above 10<sup>9</sup> CFU.mL<sup>-1</sup>, but COD removal in the system was low due to low BOD/COD ratio in the mixed liquor. Analyzing the physico-chemical results and correlating them with the microbiological, it is verified that the MPN.100 mL<sup>-1</sup> of the nitrifying organisms were strongly affected by the effluent conditions, being necessary for an effective nitrification process the control of these parameters, mainly pH.

**Keywords:** Leachate, Support Material, Biofilm

## Introduction

The final disposal of solid waste in landfills is a widely used waste treatment technique. This technique minimizes the environmental impacts and allows the decomposition of the residues under controlled conditions until their transformation into inert and stabilized material (Renou *et al.*, 2008). The resulting liquid of wastes decomposition, added to moisture and precipitation, percolates through residues mass, forming leachate.

The leachate is a highly polluting effluent, which may contain organic substances, ammonia, heavy metals,

inorganic salts and other contaminants (Renou *et al.*, 2008; Naveen *et al.*, 2017). Leachate composition varies with the age of the landfill, climatic factors and characteristics of the waste (Kjeldsen *et al.*, 2002; Bhalla *et al.*, 2012).

Among the main contaminant compounds present in the leachate nitrogen has its significant share (Butkovskiy, 2009). In contrast to organic matter, the concentration of nitrogen present in the leachate does not depend on the decomposition phase of the residues, because during the life of the landfill what changes is the way in which it presents itself (Kjeldsen *et al.*, 2002).

In conventional biological removal of nitrogen, the removal of this compound occurs through two sequencing processes, nitrification and denitrification. In nitrification, ammonia ( $\text{NH}_4^+\text{-N}$ ) is converted to nitrite ( $\text{NO}_2^-\text{-N}$ ) by the action of autotrophic Ammonia Oxidizing Bacteria (AOB) and later this nitrite is transformed by autotrophic Nitrite-Oxidizing Bacteria (NOB) to nitrate ( $\text{NO}_3^-\text{-N}$ ). Both these may be used by denitrifying bacteria as substrate, generating as final product the gaseous nitrogen ( $\text{N}_2$ ) (Kampschreur *et al.*, 2009; Kin *et al.*, 2015).

The nitrification is the limiting step, because the nitrifying bacteria have slow growth and depend directly on the physical and chemical conditions of the medium, such as pH, alkalinity, temperature, Dissolved Oxygen (DO),  $\text{NO}_2^-\text{-N}$ ,  $\text{NO}_3^-\text{-N}$  and organic matter to develop and not be carried away from the system (Wang *et al.*, 2010; Cortés-Lorenzo *et al.*, 2015).

One of the techniques used to help the development and permanence of nitrifying organisms in treatment plants is the use of support material in reactors, the use of which favors the adhesion of microorganisms to their surface, keeping them in the system for longer (Odegaard *et al.*, 1994; Kermani *et al.*, 2008).

In the foregoing, the moving bed-reactor has been used successfully to favoring the development of the nitrifying biomass, since this reactor configuration uses suspension support material, providing a suitable surface for the binding of slow growing microorganisms (Cao *et al.*, 2015).

This reactor configuration, in addition to maintaining high concentrations of organisms in the

system, have high volumetric rates of removal of pollutants, relatively low installation area and do not present problems with bulking formation when compared to systems that only operate with suspended biomass (Chen *et al.*, 2008; Leyva-Díaz *et al.*, 2013).

In the literature few searches are found that evaluate the set of physico-chemical parameters and its relation with the density of nitrifying and denitrifying bacteria in the treatment of leachate in moving bed-reactor. However, evaluating these parameters, relating them to the characteristics of the system for the development of these organisms, can be useful to optimize the system as well as present itself as a tool to solve operational problems (Oliveira *et al.*, 2013).

From the above, the main objective of this paper was to quantify the nitrifying, denitrifying and heterotrophic bacteria present in a mobile bed reactor treating leachate, evaluating the operational conditions of the system for the development of these organisms.

## Materials and Methods

### Experimental Setup and Operational Period

An experimental installation was setup on bench scale, composed by a moving bed-reactor with continuous flow, with 3.4 L volume, measuring 77.0 cm and 8.0 cm in diameter. As Support Materials (SM) for microorganisms' immobilization were used low-density polythene carriers, undefined mark, occupying about 25% of reactor useful volume (Fig. 1).

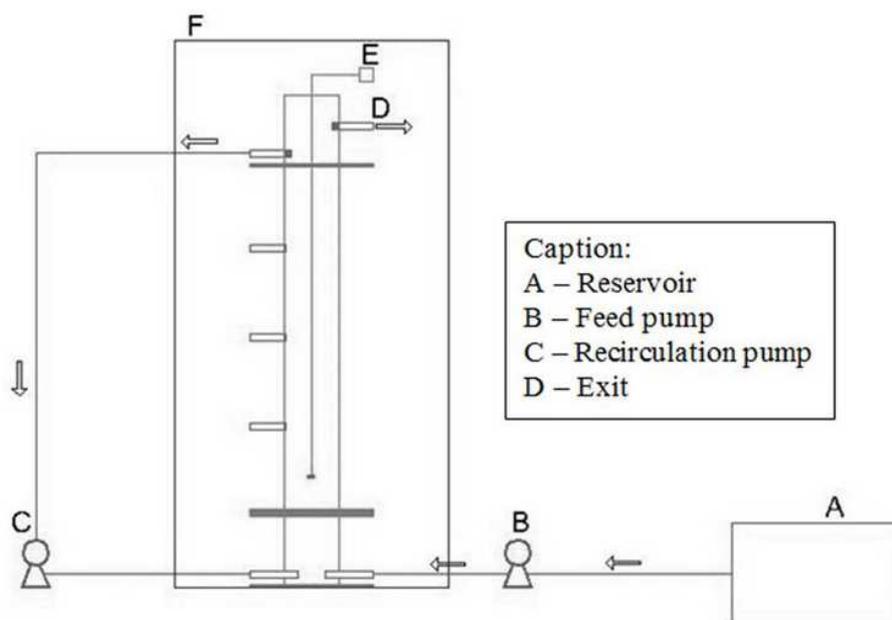


Fig. 1. Reactor installation in BOD chamber, feed pump and recirculation, entry points and effluent exit

The landfill leachate was collected from a landfill in the municipality of Rolândia. This landfill is about 13 years of activity and receives about 30 tons of urban solid waste daily. It is noteworthy that leachate collection (three collections) was made in low rainfall periods. After collecting, the leachate was stored under refrigeration. Table 1 shows the physico-chemical characteristics of the leachate collected and used in this research.

The reactor was fed with the leachate using a magnetic pump (ProMinent GALA model). For the leachate aeration an aquarium pump was installed (Brand BIG ALFA A230), with approximately 90L.h<sup>-1</sup> air flow, keeping dissolved Oxygen Concentrations (DO) above 2.0 mg.L<sup>-1</sup>. Throughout the system operation period, the reactor was maintained at a BOD chamber, with temperature controlled at 25±2°C.

The system was operated by 157 days, divided into two phases (Table 2), both operated with Hydraulic Retention Time (HRT) average of 13 days. In Phase I, lasting 66 days, system was operated without effluent recirculation, while in Phase II, lasting 91 days, the nitrified effluent recirculation was introduced (SRF = 3Q). A pump of the same trademark and model mentioned above carried out recirculation.

To start the biological treatment system adopted in this study, it was necessary the use of an inoculum. This was obtained by aeration of landfill leachate in a sequencing batch reactor with 3L capacity, for 70 days. After finding that there was nitrification in the batch reactor, the inoculum was placed to the moving bed-reactor and staying in contact with supports material for 3 days, in batch and under aeration. Then, it was initiated continuous leachate feeding in the reactor, operating under conditions specified in Table 2.

#### Physicochemical Parameters Monitoring

For determination of pH (4500-H B), total alkalinity (2320 B), temperature, Total Kjeldahl Nitrogen-TKN (4500-Norg/4500-NH3 C-Kjeldhl), NH<sub>4</sub><sup>+</sup>-N (4500-NH<sub>3</sub> B/C), Chemical Oxygen Demand-COD (5220 D) and Biochemical Oxygen Demand-BOD (5210 B/4500-OG) in influent (raw leachate) and effluent (treated leachate), were used methodologies described in APHA (2012). In addition, for determining nitrite (NO<sub>2</sub><sup>-</sup>-N) and nitrate (NO<sub>3</sub><sup>-</sup>-N) concentration the methodology proposed by Cataldo *et al.* (1975) was adopted.

Free ammonia (mgNH<sub>3</sub>.L<sup>-1</sup>) and free nitrous acid concentration were determined based on the equation showed in Anthonisen *et al.* (1976).

#### Microbiological Monitoring

In this search, bacteria present in the mixed liquor (non-attached microorganisms) were quantified in nitrifying, denitrifying and heterotrophic (Phases I and II) and SM (Phase II). They were held SM microscopic examination, using scanning electronic microscope at the end of reactor operational period.

Five carriers were took in each analysis to determine nitrifying, denitrifying and heterotrophic organisms' quantity. This material was transferred to a 100 mL flask, with 20 mL of sterile distilled water, then, glass beads were added in an amount four times SM mass. The flask was stirred manually for 20 min to provide the detachment of adherent organisms and, subsequently, a serial dilution procedure was followed, considering this preparation with the first dilution (10<sup>-1</sup>).

#### Nitrifying, Denitrifying and Heterotrophic Bacteria Quantification

Analyzes to quantify MPN.100 mL<sup>-1</sup> of nitrifying bacteria (AOB and NOB), was based on the methodology proposed by Schmidt and Belser (1984). For determination of MPN.100 mL<sup>-1</sup> of denitrifying organisms, the methodology proposed by Tiedje (1984) was used. MPN.100 mL<sup>-1</sup> calculation was carried out with combination of positive tubes, using Probability Standard Table retrieved in APHA (2012).

The quantification of nitrifying and denitrifying organisms present in mixed liquor was carried out in 5th, 38th, 74th, 125th and 153rd reactor operation day and SM in 74th, 125th and 153rd system operation days.

Table 1. Physico-chemical parameters of influent leachate used

Parameters (Units)	Medium values
pH	8.4
Alkalinity (mgCaCO <sub>3</sub> .L <sup>-1</sup> )	4209.0
NTK (mg.L <sup>-1</sup> )	1013.0
NH <sub>4</sub> <sup>+</sup> -N (mg.L <sup>-1</sup> )	926.0
COD (mg.L <sup>-1</sup> )	1120.0
BOD (mg.L <sup>-1</sup> )	135.0
BOD/COD	0.12

Table 2. System operating phases and their operational characteristics of Hydraulic Retention Time (HRT), external re-circulation (Re) and operating time

Stage	HRT (Days)	*Re	Operation time (Days)
I	~13	No	66
II	~13	Yes (SRF/Q = 3)	91
Total			157

\*Re = Recirculation ratio

To determine the number of Heterotrophic Bacteria (HB) present in mixed liquor and in SM, a standard plate count method was used to determining of Colony Forming Units (CFU.mL<sup>-1</sup>). HB quantification was performed in mixed liquor in 15th, 31st, 56th, 75th, 91st, 125th, 153rd operation days and in SM in 75th, 91st, 125th, 153rd days.

### Scanning Electronic Microscopy (SEM)

Microscopic examination of biofilm attached to SM, using scanning electronic microscopy, was performed at Electronic Microscopy and Microanalysis Laboratory of Londrina State University in a device of ZEISS brand, DSM-960 model in 10 to 20 keV and photographed with a video coprocessor (Mitsubishi), with photographic plate (Fuji) CK 100-S. This analysis was performed on a carrier (SM) taken from the reactor on the last day of operation (153rd) and prepared by using the HMSD (hexamethyldisitane) method of Nation (1983).

## Results and Discussion

### Nitrifying Organisms: MPN.100 mL<sup>-1</sup> of Ammonia Oxidizing Bacteria (AOB) and Nitrite Oxidizing Bacteria (NOB)

The results for MPN.100 mL<sup>-1</sup> of nitrifying organisms (AOB and NOB) are displayed in Fig. 2. It was not detected results to MPN.100 mL<sup>-1</sup> of AOB and NOB in 5th and 153rd operation days, respectively, in the mixed liquor.

In the mixed liquor, AOB ranged from 10<sup>6</sup> MPN.100 mL<sup>-1</sup> (38th operation day-Phase I) to 10<sup>8</sup> MPN.100 mL<sup>-1</sup> (125th day-Phase II) and NOB ranged from 10<sup>5</sup> MPN.100mL<sup>-1</sup> in the 5th day (Phase I) to 10<sup>7</sup> MPN.100 mL<sup>-1</sup> in 38th (Phase I) and 74th (Phase II) days.

In SM high numbers of these organisms were found, reaching AOB to 10<sup>8</sup> MPN.100 mL<sup>-1</sup> in 153rd operation day (Phase II) and NOB to 10<sup>6</sup> MPN.100 mL<sup>-1</sup> in 74th and 153rd days (Phase II).

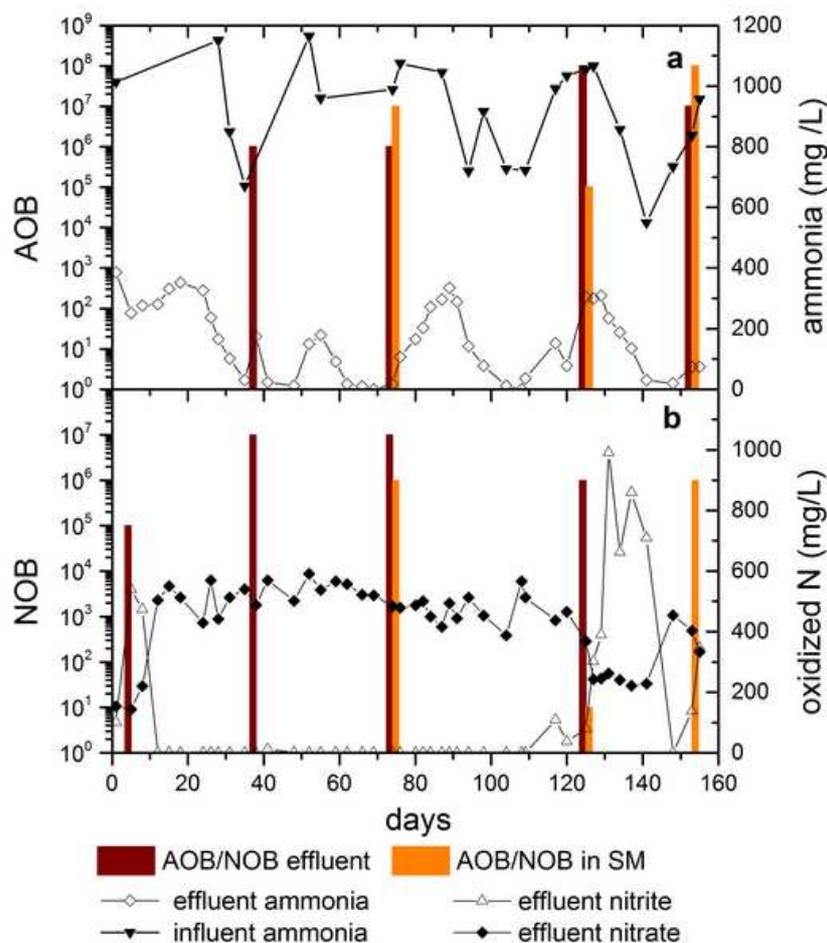


Fig. 2. (a) MPN.100 mL<sup>-1</sup> variation of Ammonia Oxidizing Bacteria (AOB) and NH<sub>4</sub><sup>+</sup>-N affluent and effluent values were shown (b) MPN.100 mL<sup>-1</sup> variation of nitrite-oxidizing bacteria and nitrite and nitrate values (Oxidized-N) effluent were shown

In 125th operation day, there was a decrease in AOB and NOB concentration in SM (Phase II). From 74th day to 125th, AOB decreased in the order of  $10^2$  MPN.100 mL<sup>-1</sup> and NOB in the order of  $10^5$  MPN.100 mL<sup>-1</sup>. This reduction in MPN.100 mL<sup>-1</sup> may have occurred because of a natural process of biomass detachment from biofilm.

NH<sub>4</sub><sup>+</sup>-N influent concentrations ranged along the reactor-monitoring period, which directly influenced the result of NH<sub>4</sub><sup>+</sup>-N effluent. In the influent the concentration of NH<sub>4</sub><sup>+</sup>-N was on average 893±162 mg.L<sup>-1</sup> and in the output, these values were 179±158 mg.L<sup>-1</sup> in Phase I and 111±152 mg.L<sup>-1</sup> in Phase II, reaching an average efficiency NH<sub>4</sub><sup>+</sup>-N removal of 80% and 83% in Phases I and II, respectively. When analyzing the data, it was possible to verify AOB activity efficiency in converting NH<sub>4</sub><sup>+</sup>-N into NO<sub>2</sub><sup>-</sup>-N.

The NO<sub>2</sub><sup>-</sup>-N concentrations in most of reactor operational time were below 1 mg.L<sup>-1</sup>, which also indicates good activity of NOB, showing that most NH<sub>4</sub><sup>+</sup>-N percentage converted into NO<sub>2</sub><sup>-</sup>-N was used by NOB in its metabolism and then, converted into NO<sub>3</sub><sup>-</sup>-N.

In initial period of reactor operation, NH<sub>4</sub><sup>+</sup>-N values and NO<sub>2</sub><sup>-</sup>-N in the effluent were high, reaching levels of 352 mg.L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>-N in 18th day and NO<sub>2</sub><sup>-</sup>-N to 539.6 mg.L<sup>-1</sup> in 5th day.

These NH<sub>4</sub><sup>+</sup>-N and NO<sub>2</sub><sup>-</sup>-N waste high concentrations at the beginning of reactor operating period may be related to the adaptation period of nitrifying biomass.

After the 116<sup>th</sup> operation day, the NO<sub>2</sub><sup>-</sup>-N concentration in treatment system increased again while the NO<sub>3</sub><sup>-</sup>-N concentration decreased, indicating the NO<sub>2</sub><sup>-</sup>-N accumulation in reactor. This may be due to pH decrease observed in this period, which reached values of 5.4. NOB activity is strongly influenced by pH, since in values below 6.5 these organisms activity virtually ceases. According to Jiménez *et al.* (2011), optimal values for these bacteria activity lie in a range of 7.5 to 9.95. Villaverde (2004) reports, that at pH below 6.5 the nitrification process practically stops by lack of free ammonia in the environment and nitrous acid high concentrations.

Silva Filho *et al.* (2007), studying nitrifying bacteria metabolic activity in Activated Sludge system, observed that in pH below 5.0, the two nitrifying bacteria groups (AOB and NOB) do not exhibit biological activity. However, at pH around 8.0, a metabolic capacity increase was noticed in both groups.

Due to the low pH values in the medium, 3 g.L<sup>-1</sup> of sodium bicarbonate was added to the influent on the 98<sup>th</sup> day of operation (Phase II) in order to increase the alkalinity of the medium and to control the pH.

Free ammonia average concentration in influent, during system operation, was of 119.8±29.4 mgNH<sub>3</sub>.L<sup>-1</sup> and at output, averaged 0.1 mgNH<sub>3</sub>.L<sup>-1</sup> in Phases I and II. According to Anthonisen *et al.* (1976), free ammonia concentrations from 10 mgNH<sub>3</sub>.L<sup>-1</sup> can inhibit AOB

activity and from 0.1 mgNH<sub>3</sub>.L<sup>-1</sup> may inhibition NOB activity. Apart from free ammonia, nitrous acid may also inhibit nitrifying bacteria activity, especially NOB. This acid concentration is dependent on the nitrite concentration, the pH and the environment temperature and in 0.2 to 2.8 mg.L<sup>-1</sup> range concentrations may impair NOB activity.

The estimated nitrous acid concentration during Phase I and II was on average 0.16 and 1.14, respectively, in the effluent. Based on these nitrous acid concentrations, it is believed that lowering pH favors nitrous acid formation, which consequently hampered significantly nitrification process in final stage reactor operation (Phase II).

The reactor-influent TKN was on average 910±141 mg.L<sup>-1</sup> and in output it was 273±133 mg.L<sup>-1</sup> and 157±87 mg.L<sup>-1</sup> in Phases I and II, respectively. TKN reactor removal percentage was in the range of 70.2% in Phase I and in Phase II, 82.5%. Average ratio NH<sub>4</sub><sup>+</sup>-N/NTK of leachate influent was 0.98 and 0.97.

Alkalinity in reactor inlet was set at a range of 5187±1179 mg CaCO<sub>3</sub>.L<sup>-1</sup> and in output, the value averaged 256±357 mgCaCO<sub>3</sub>.L<sup>-1</sup> and 132±93 mgCaCO<sub>3</sub>.L<sup>-1</sup> in Phases I and II, respectively. In these results high reactor alkalinity consumption was observed. In the nitrification process, high amounts of alkalinity are consumed due to H<sup>+</sup> ion release. Thus, for 1.0 g of N-NH<sub>4</sub><sup>+</sup> oxidation are consumed CaCO<sub>3</sub> or 8,64g 7,14g of HCO<sub>3</sub><sup>-</sup>. Therefore, in wastewater with high nitrogen concentration, if sufficient alkalinity is not provided, it is possible to occur nitrification process impairment (Sedlak, 1991).

Another factor that may interfere in nitrification process is C/N ratio present in influent. Using ratio BOD/TKN for evaluating C/N ratio in the leachate, it is registered that influent C/N ratio was 0.15 in Phase I (114±73 mg BOD<sub>total</sub>.L<sup>-1</sup> influent) and 0.11 in II (119±42 mg BDO<sub>filtered</sub>.L<sup>-1</sup> influent), these results disclose that the treated leachate had low biodegradable organic matter amount.

As C/N rate increases, heterotrophic organism growth is favored and these organisms compete with the nitrifying ones for oxygen and nutrients. For BOD/TKN relations around 0.5, nitrifying fraction, according to Metcalf and Eddy (2003) would be about 35% and as this ratio decreases, the nitrifying fraction increases.

The BOD/TKN ratio increase favors heterotrophic biomass dominance in biofilm, whose growth rate is usually higher than autotrophic ones. Therefore, substrates which have low organic fraction (soluble BOD) offer lesser competition possibility between heterotrophic and nitrifying bacteria. However, studies based on molecular biology techniques have pointed to lower percentages of nitrifying organisms in relation to heterotrophic biomass, even in low carbon systems

concentrations with efficient nitrification (Dionisi *et al.*, 2002; Li *et al.*, 2007).

### MPN.100 mL<sup>-1</sup> of Denitrifying Bacteria

The Denitrifying bacteria concentration ranged from 10<sup>11</sup> to 10<sup>7</sup> MPN.100 mL<sup>-1</sup> in mixed liquor and SM, respectively (Fig. 3). Although the reactor was aerated for 24 h, maintaining DO concentrations above 2 mg.L<sup>-1</sup>, denitrifying bacteria concentration in both mixed liquor and MS were higher than those of nitrifying bacteria.

High denitrifying bacteria concentration found in reactor indicate that possibly occurred nitrification and denitrification simultaneous process under the same overall operating conditions, which can offer significant advantages when compared to conventional systems in which the processes occur separately.

Because of the mutualistic interaction established between the nitrifying and denitrifying organisms, when co-immobilized, the system that has this kind of biological interaction takes advantage in terms of biological nitrogen removal compared to systems that have just nitrifying organisms (Kotlar *et al.*, 1996).

The nitrification and the denitrification process in a single reactor is possible due to physical and biological processes, which as result of oxygen concentration gradients formation within the *floc* or biofilm, different organisms can be established (Münch *et al.*, 1996) and as nitrification by-product is a substrate for denitrification, this biological interaction becomes interesting for these organisms.

From nitrogen balance, held with input and output data of TKN, nitrite and nitrate, we obtained a nitrogen deficit in the reactor outlet, where about 14.3 and 29.6% of influent nitrogen has not been quantified in effluent as TKN or oxidized-N, respectively in Phases I and II.

To achieve nitrogen balance it was considered all nitrogen (TKN, nitrite and nitrate) forms in influent. However, this stoichiometry is difficult to be perfect, because the same nitrogen can be found in other nitrogen compounds. These form that were not quantified, for example, but it intermediates the nitrification process and nitrogen used in cell synthesis.

It is important to highlight that nitrogen loss in ammonia form, by stripping, in this study can be considered negligible, in view of pH values found and considering reactor contact area with atmosphere, being small in relation to its volume.

### Heterotrophic Bacteria

CFU.mL<sup>-1</sup> result for heterotrophic bacteria present in mixed liquor and MS was high throughout reactor monitoring period (Fig. 4), accounting about 10<sup>13</sup> CFU.mL<sup>-1</sup> in the 15th operation day to 10<sup>14</sup> in 31st, both in Phase I. In SM, these organisms ranged from 10<sup>8</sup> CFU.ml<sup>-1</sup> in the 75th day to 10<sup>13</sup> CFU.ml<sup>-1</sup> in the 125th operation day (Phase II).

Even obtaining high concentrations of heterotrophic bacteria, average BOD removal efficiency was low yielding 23% in Phase I and Phase II there was virtually no removal.

COD<sub>total</sub> average influent value was 1228±122 mg.L<sup>-1</sup> and COD<sub>filtered</sub> of 1118±122 mg.L<sup>-1</sup>, reaching an average removal efficiency of 18% total COD in Phase I and 21% in Phase II. From BOD and COD averages removal efficiencies counted, it appears likely that used-leachate substrate contained recalcitrant compounds or slowly biodegradable matter.

Spagni and Marsili-Libelli (2009) obtained in a sequencing batch reactor, average COD removal efficiency in old landfill leachate of only 20% and attributed this value to low leachate biodegradability.

BOD/COD ratio to influent was in the range of 0.1±0.05, which indicates that treated leachate was in methanogenic phase of waste decomposition (Kjeldsen *et al.*, 2002), demonstrating an effluent of difficult biodegradation.

It is observed in Fig. 4 that there were no significant differences in CFU.mL<sup>-1</sup> found in SM in respect to mixed liquor, indicating that these organisms have adapted on immobilized and on suspended shape, although the substrate in environment presented low concentration and readily biodegradable organic matter (average of 114±73 mg BOD<sub>total</sub>.L<sup>-1</sup>, Phase I and 119±42 mg BDO<sub>filtered</sub>.L<sup>-1</sup>, Phase II).

Bassin *et al.* (2012) operating a moving bed reactor in order to examine different operational conditions effects in heterotrophic and nitrifying distribution, noticed that even when organic carbon source was not offered, heterotrophic bacteria were dominant. In this way, their growth was attributed to soluble microbial products release by autotrophic.

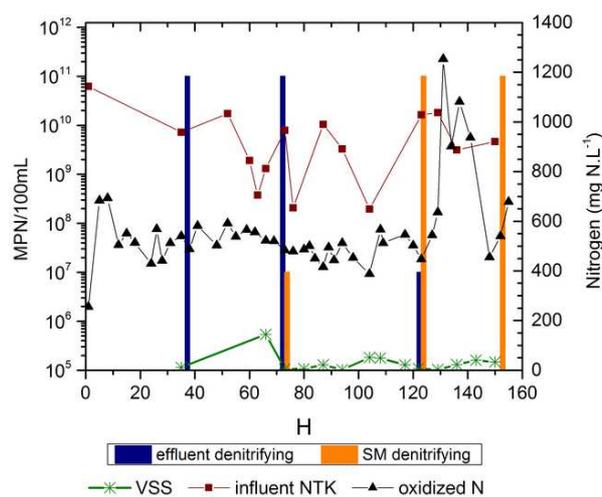


Fig. 3. Oxidized nitrogen values in effluent, influent TKN, effluent VSS and MPN. 100 mL<sup>-1</sup> of denitrifying bacteria present in mixed liquor and MS

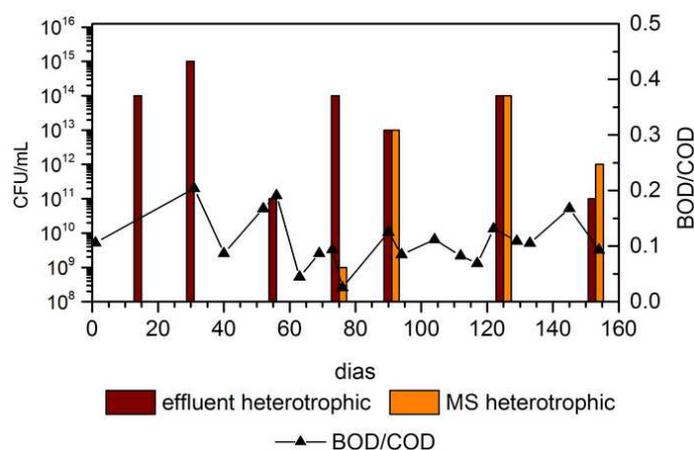


Fig. 4. CFU.mL<sup>-1</sup> of heterotrophic bacteria present in mixed liquor and SM and BOD/COD ratio in effluent

Heterotrophic bacteria have a growth rate around 4.8 d<sup>-1</sup> and NOB around 0.76 d<sup>-1</sup> and 0.84 d<sup>-1</sup>, respectively (Watanebe *et al.*, 1992; Rittmann and Snoeyink, 1984). From these values, it can be observed that heterotrophic bacteria present a high growth rate in relation to nitrifying autotrophic. This factor is often in treatment systems that primarily aim nitrogen compounds removal and can be a problem because heterotrophic bacteria compete strongly with nitrifying for oxygen and space (Wijeyekoon *et al.*, 2004), which can lead to biofilm structure stratification.

Due to the growth rate of heterotrophic bacteria in relation to nitrifying be greater, the first ones are usually located in biofilm outer layers, in places where the substrate concentration is higher. On the other hand, nitrifying bacteria are located internally. Thereby, heterotrophic bacteria may form a layer over nitrifying ones, which can be disadvantageous to these organisms, particularly when environment oxygen concentration is low. However, when oxygen concentration in the system is high enough to diffuse through biofilm layers, heterotrophic bacteria layer formation over nitrifying bacteria can be seen as advantageous, since this heterotrophic microbial layer may protect nitrifying from easily detach from biofilm (Furumai and Rittman, 1994), remaining much longer inside the reactor.

Taking into account the methodology used for heterotrophic organisms cultivation (nutrient broth and anaerobic conditions), it is assumed that cultivated organisms are mostly facultative anaerobic and heterotrophic, thus, much of the quantified heterotrophic organisms can be denitrifying bacteria responsible for environment nitrogen removal process.

### Scanning Electronic Microscopy (SEM)

In Fig. 5, captured images by scanning electronic microscopy in outer and inner surface of SM are displayed. It is observed that in SM external surface, in

smoother regions, there were few adhered microorganisms (Fig. 5A).

Few adherent microorganisms' presence can be attributed to recent adhesion and biomass formation, or recent natural detachment or even by the used technique in mounting material during SEM. According to Rusten *et al.* (2006), in moving bed reactors, little or no biofilm grows adhered to supports outside because of shear forces between SM and moving-bed reactor walls, growing only in protected parts of the inside.

Figure 5B shows in detail the inside of outer surface of SM roughness. In this Figure, a higher number of attached microorganisms than in smooth outer surface can be seen. Usually support materials with irregular contact surfaces, porous or provided with ridges (greater surface contact area) potentiate polysaccharides excretion of microorganisms, providing perfect conditions for biofilm establishment (Sutherland, 2001).

In Fig. 5C, it is visualized SM inner surface. This surface has a significant number of adhered microorganisms, plus a great diversity, with different morphologies, such as *cocos*, bacilli and rods. In addition to SM inner surface of observed bacteria, there were also some protozoa such as *thecamoebians* (Fig. 5D) and inorganic elements, such as sodium, evaluated by transmission microscopy. Finding organisms, such *thecamoebians*, in processing systems characterizes periods of nitrification good performance (Eikelboom, 2002).

Growth and biofilm thickness is usually influenced by hydrodynamic conditions and organic fillers applied. SM presence can favor bacteria distribution with faster growth rate in upper biofilm layers (where substrate passage and microorganisms shedding is higher), while slow growing nitrifying bacteria, grow inside the biofilm, preventing their drag out from the reactor, thus, biofilm formed of heterotrophic bacteria may present a greater thickness than biofilms composed of autotrophic bacteria (Okabe *et al.*, 1996a).

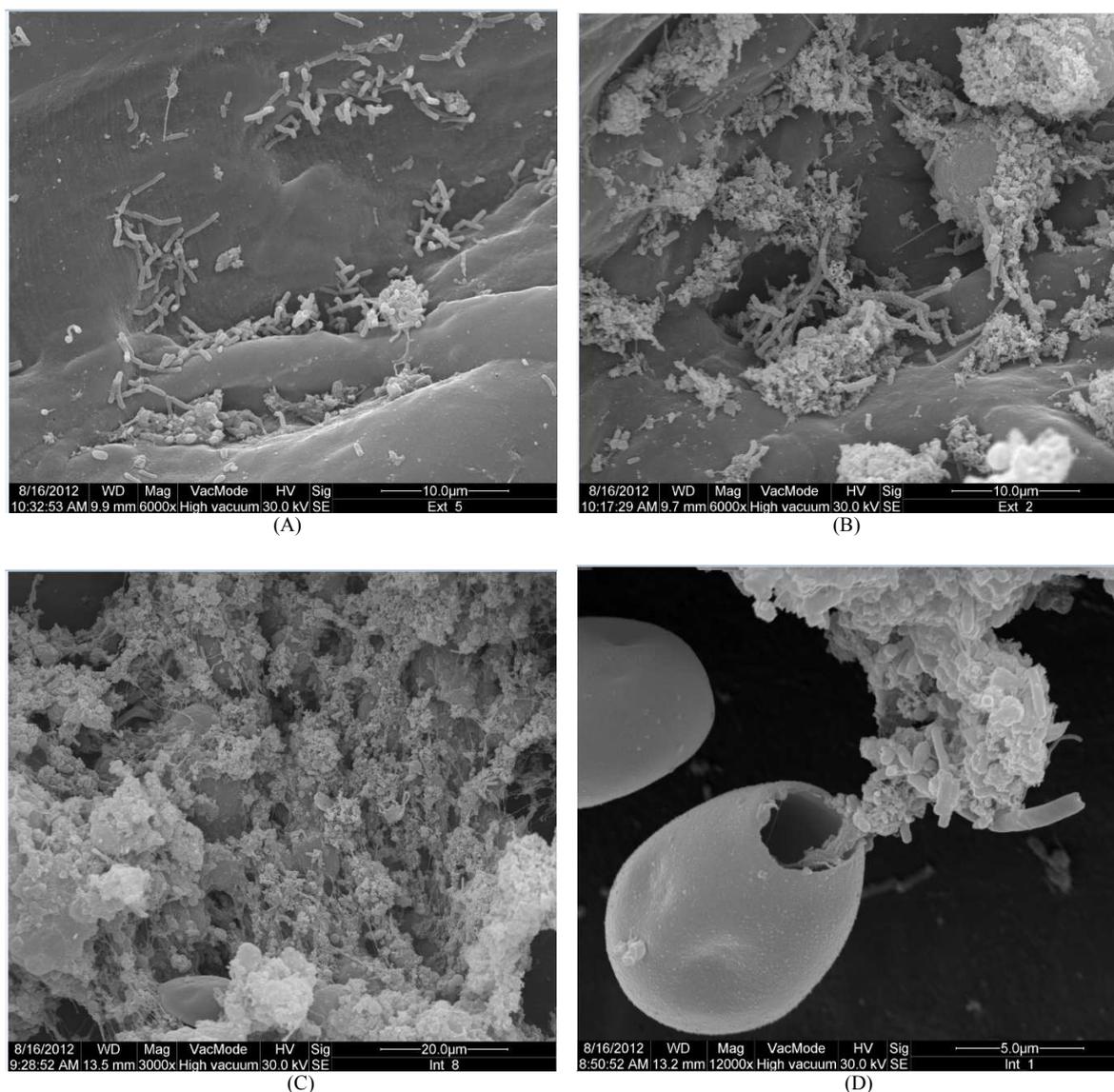


Fig. 5. Images obtained from scanning electronic microscopy performed in SM; (A) SM outer surface image; (B) SM outer surface roughness of biofilm colonized; (C) SM Inner surface colonized; (D) Adhered microorganism to SM internal roughness (similar to *Thecamoebian*)

Bassin *et al.* (2012) when analyzing biofilm on micrographs, observed that nitrifying biomass is significantly changed when effluent COD is reduced, it was also noticed that in some parts of its SM, biofilm detachment was registered when COD was diminished. With this detachment, the remaining biofilm was enriched by nitrifying bacteria, which consequently increased its specific rate of ammonia-N removal.

By studying biofilms dynamics of microorganisms, Okabe *et al.* (1996b) checked that spatial distribution of heterotrophic and nitrifying bacteria is dependent on C/N ratio and in carbon absence both groups may coexist in biofilm outer layers.

## Conclusion

The moving bed-reactor featured good performance in terms of  $\text{NH}_4^+$ -N removal in both reactor-operating phases (80% in Phase I and 83% in Phase II). It was observed that physicochemical conditions, which were verified in the effluent, possibly influenced significantly the number of nitrifying bacteria present in the reactor, being pH one of the parameters that may have impaired more their development, especially NOB.

It is emphasized that even the system being operated under aerobic conditions, due to the high concentration of nitrifying and denitrifying

microorganisms in the system, that the Simultaneous Nitrification and Denitrification (SND) process may have occurred.

Even the leachate not having high readily biodegradable organic matter values (BOD/COD:  $0.1 \pm 0.05$ ), the number of heterotrophic bacteria in both SM and mixed liquor was high.

About the concentration of organisms evaluated in this study, one can observe that it can be established even in systems operated at high concentrations of recalcitrant and non-biodegradable materials and that SM used in the system resulted in microorganisms' maintenance in the reactor.

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## Author's Contributions

All authors involved in the study, helped theoretical and practical development of research, since this article is a part of a dissertation and for the preparation there of, the involvement and direct contribution of the authors was necessary. The lead author (Andreliza D. G.O.) was a graduate student in question, the second author (Camila Z.C) contributed with the construction of part writing and structuring the article. Prof. Dr. Katia V.M.C.P, co-advisor of the dissertation and Prof. Dr. Deize D.L was the advisor of the dissertation studied and expressed in parts in this article.

## Ethics

All of the other authors have read and approved the manuscript and there are no ethical issues involved.

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