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Effect of bacteria biomass detachment on the ammonium oxidation yield

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Nitrification yield can be affected by fixed biomass or biofilm density. Infact, biofilm detachment may influence the nitrification. The present experiment investigated the effect of detachment biomass cells on nitrifying bacteria expressed via nitrification rate. We monitored nitrification rates before and after biomass detachment from gravel sampled in a small-scale model of wastewater treatment using macrophytes in vertical and horizontal filtersystems. The ammonia-oxidizing bacteria (AOB) number decreased after cell detachment whereas the number of nitrite-oxidizing bacteria (NOB) was lower and saving a constant value of 3.0 MPN/100 ml. Despite this detachment, the yield of ammonium oxidation in the vertical filter remains constant but the reaction required more time. After washing, the NO_3^--N concentration at the bottom of horizontal filter with fine gravel is more important (1.24 mg/l) than that observed at the medium (1.1 mg/l) and the top (0.8 mg/l) of basin; whereas, at the horizontal filter with coarse gravel, the nitrification performance is more important at the medium of basin with NO_3^--N concentration value of 1.14 mg/l than those obtained at the top (0.7 mg/l) and the bottom (0.98 mg/l).

Key words: Autotrophic bacteria, detachment, nitrification, turbidity.

INTRODUCTION

Nitrogen is present in the environment in a wide variety of chemical forms including organic nitrogen, ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxide (N_2O), nitric oxide (NO) or inorganic nitrogen gas (N_2). The ammonia, nitrite and nitrate form are toxic to living (WHO, 2006). Exposure to high levels of nitrates or nitrites has been associated with increased incidence of cancer in adults and brain tumors, leukemia and nasopharyngeal (nose and throat) tumors in children (Sanchez-Echaniz et al., 2001; Pogoda and Preston, 2001; USEPA, 2006).

In biological wastewater treatment processes, the nitrification is achieved by two types of bacteria, that is, ammonia-oxidizing bacteria (AOB) responsible for nitrite formation, and nitrite-oxidizing bacteria (NOB) for

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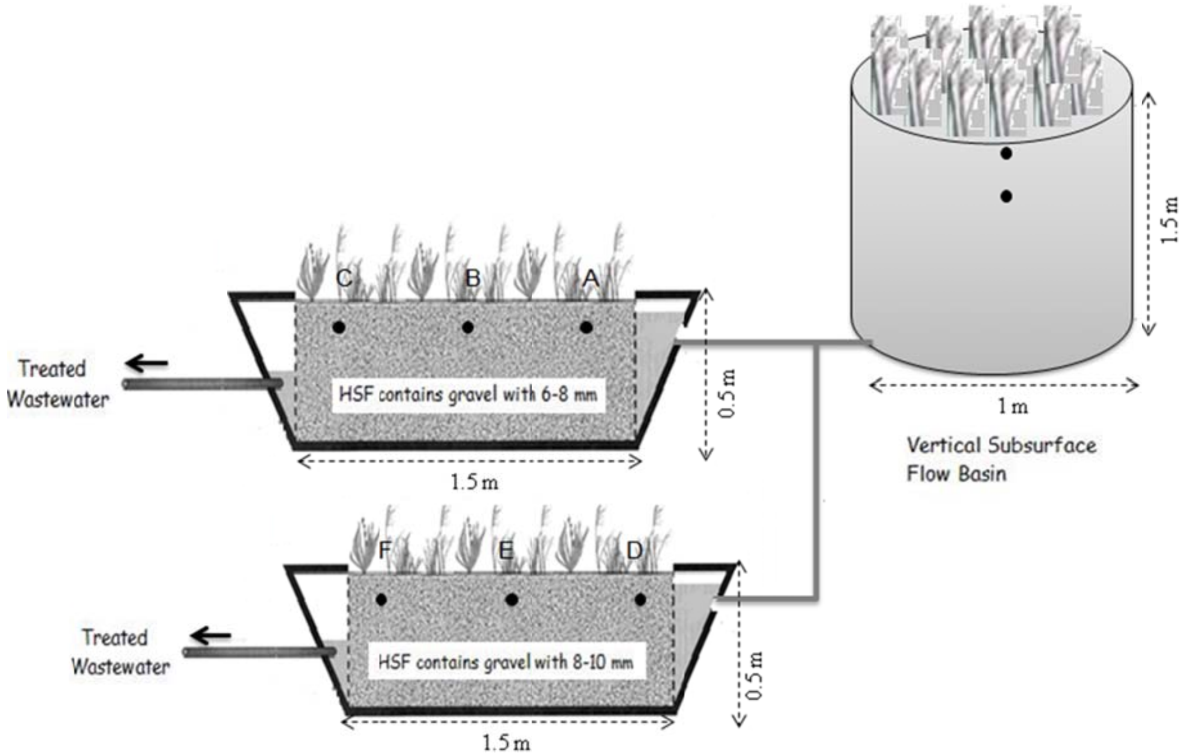

Figure 1. Schematic representation of the constructed wetlands system (small-scale model) placed under greenhouses. HSF: horizontal subsurface flow. The samples were taken from the top (A), middle (B) and the bottom (C) of the HSF with fine gravel. In addition others coarse gravel samples were taken at the top of the HSF (D), middle (E) and the bottom (F) gravel sampling point.

conversion of nitrite to nitrate (Ruiz et al., 2003; Tay et al., 2002). At temperatures higher than 25°C, the growth rate of ammonia-oxidizing microbes is higher than nitrite-oxidizing (Hellinga et al., 1998). Microbial biomass detachment can directly reduce the fixed biofilm (Stoodley et al., 1999; Tijhuis et al., 1996). Approximately 60 to 80% of nitrogen in domestic and municipal wastewater is in the form of ammonium (Gerardi, 2010). Strong ammonium concentrations can contribute to biofilm detachment from the filter of constructed wetlands. The biofilm detachment appears to be the major cause of no constructed wetlands efficiency in terms of nitrates reduction. Nevertheless, the biofilm is detached from the filterand washed out. Wash-out of solids proportional to the flow rate is foreseen only at higher flow rates. It is assumed that detached parts of the biofilm are retained within the pores and metabolized until washed out by a peak flow (Langergraber, 2008).

The biofilm detachment may reduce autotrophic bacterial populations despite the heterotrophic layer could have protecting nitrifiers from detachment (Michaud et al., 2006). The decrease of nitrifying bacteria number can induce a relatively low potential nitrification. Continuous detachment from a constant-thickness biofilm results invariability in nitrification rates. Detachment frequency can influence the competition between heterotrophic and autotrophic bacteria within the biofilm (Morgenroth and Wilderer, 2000; Rittmann et al., 2002).

These experiments investigated the effect of detachment of nitrifying bacteria from gravel on nitrification rate.

MATERIALS AND METHODS

Pilot-scale wastewater treatment

A pilot-scale model of wastewater treatment using macrophytes was installed in greenhouses in Gembloux, Belgium. Macrophytes were planted in vertical flow beds with a support medium composed of peagravel and non-limestone gravel from 6 to 8 mm in diameter (Figure 1). Two horizontal flow beds contained two different sizes of gravel. The first flow basin contained coarse gravel of 8-10 mm in diameter and the second flow basin included fine gravel of 6-8 mm. Macrophytes were planted in these beds. Sixty liters of bovine liquid manure diluted with clear water to reach 150 mg BOD_5/l was added daily to each system.

Sampling (gravel-biofilm)

The gravel was sampled to follow the biofilm development. Two samples were taken from the vertical filter at 5 and 20 cm depth. Six other samples were also considered at different positions from the horizontal flow basins at 5 cm depth (Figure 1).

Table 1. Effect of washing on the numbers of nitrifying and denitrifying bacteria

Nitrifying and denitrifying bacteria	Number of bacteria (MPN/100 ml)	
	First washing	Fourth washing
Ammonia-oxidizing bacteria (AOB)	23.0 ± 0.0	3.0 ± 0.0
Nitrite-oxidizing bacteria (NOB)	3.3 ± 0.4	3.0 ± 0.0
Denitrifying bacteria	161.5 ± 96.9	5.5 ± 2.7

Ammonium analysis

Ammonium was assayed using the indophenol blue-ISO 7150/1 (Merck-Spectroquant) method. Merck reagents Spectoquant ammonium was used. The optical density (OD) was determined at 692 nm by a spectrophotometer (Spectronic® 20 Genesys^M). The OD_{692} value was converted to NH_4^+mg/l, using the Excel/Fiexcel/Calcdos.

Nitrate analysis

For two milliliters of the sample were two milliliters of sodium salicylate solution (5 g/l) added. After mixing, the solution was evaporated at 60°C for 2 h and cooled in desiccators. Two ml of H_2SO_4 concentrated was added. After ten minutes, 15 ml of distilled H_2O and 15 ml of NaOH/sodium potassium tartrate (40/6%, vol/vol) were added. The mixture was brought to a final volume of 50 ml and OD was measured at 420 nm using a spectrophotometer UNICAM.

Turbidity measurements

Turbidity is the measure of relative clarity of a liquid. It is an optical characteristic of water and is an expression of the amount of light that is scattered by material in the water when a light is shined through the water sample. The turbidity of the solutions was measured by ISO 2100P turbidimeter HACH® and expressed in nephelometric turbidity units (NTU).

Nitrifying and denitrifying bacteria enumeration

The autotrophic nitrifying bacteria (AOB and NOB) and the heterotrophic denitrifying populations were enumerated using the most probable number (MPN) method (Lorch et al., 1995). Preparation and composition of the AOB, NOB and denitrifying medium were as described by Alef (1995). An incubation period of four weeks was used. After incubation, ammonia-oxidizing bacteria were counted with the MPN-Griess method, while NOB was counted with both the MPN-diphylamine (Both et al., 1990). Denitrifying bacteria presence was expressed by gas that can be observed after three weeks of incubation at room temperature in an anaerobic jar containing nitrogen gas. MPN values were calculated according to the statistical tables of De Man (1983).

Experimental protocol of biofilm detachment

Bacterial enumeration after successive washing

In this experiment, a single piece of gravel was removed from the top (F) of the horizontal filter and placed in a sterile tube. Three milliliters of sterile distilled water were added followed by agitation at 2 rpm during 4 s. The bacterial suspension in the tube was enumerated for nitrifying and denitrifying bacteria. The gravel was washed three more times following the same way and the number of bacteria was determined after each washing. This experience was released in triplicate.

Nitrification after washing

The kinetics of nitrification was followed after washing. Gravel was sampled from vertical and horizontal filter. For this, for a weight of 100 g of gravel was added a volume of 200 ml of sterile distilled water. After agitation at 2 rpm during 10 s the suspension was separated from the gravel and this fraction is considered as the first wash fraction. Four successive washes were applied and each obtained fraction was collected separately. The turbidity of the different fraction was determined. After the fourth wash, a volume of 200 ml of solution A[$(NH_4)SO_4$ 20 mg/l with pH 7,6 and 500 mg/l of $CaCO_3$ as carbon source] was added to the sample gravel included in bottles and the bottles were incubated at 25°C during increasing times (5 min, 4, 8, 24 and 48 h). Then, a volume of 10 ml was sampled and analyzed for the various nitrogen forms.

The kinetics of nitrification was followed before washing (as control samples). Each gravel sample (100 g), undergoing a successive washing, was placed into 500 ml bottle. After addition of 200 ml of solution A, bottles were incubated at 25°C. Then, a volume of 10 ml was taken at different time (5 min and 4, 8, 24 and 48 h) and analyzed for the various nitrogen forms.

Statistical procedures

Pearson's correlation coefficient (r) was used to show correlation between the analyzed parameters data using Statistical Package for the Social Sciences (SPSS) software (SPSS for Windows, SPSS Inc., Chicago, Il, USA).

RESULTS AND DISCUSSION

Detachment of nitrifying bacteria on gravel

The nitrifying and denitrifying bacteria decreased with subsequent washings (Table 1). The population of ammonia-oxidizing bacteria (AOB) in suspension was higher than 23 MPN/100 ml. After the fourth wash, this number decreased to attain 3.3 MPN/100 ml. In the same way, the number of denitrifying bacteria was affected by washing, as these populations decreased from 161.5 to 5.5 MPN/100 ml before and after fourth washing, respectively. The nitrite-oxidizing bacteria (NOB) populations present in suspension of sample (single piece of

Table 2. Biomass cell suspension expressed by the turbidity values obtained after the first and fourth sample washing at vertical filter (at 5 and 20 cm of depth), horizontal filter characterized by fine gravel (in the top, medium and bottom of filter) and horizontal filter characterized by coarse gravel (in the top, medium and bottom of filter).

Washing	Vertical filter (cm)		HF with fine gravel			HF with coarse gravel		
	5	20	Top	Medium	Bottom	Top	Medium	Bottom
First washing	57 ± 2[d]	97 ± 2[h]	63.5 ± 1.3[e]	76.4 ± 2[f]	88.5 ± 1.5[g]	35.3 ± 2[c]	29 ± 1.3[b]	23 ± 2.2[a]
Fourth washing	4.4 ± 2[b]	7 ± 1[bc]	6.9 ± 1.9[bc]	0.7 ± 1.6[a]	8.3 ± 1.4[c]	9.5 ± 2[c]	3.2 ± 0.16[ab]	3.8 ± 0.3[ab]

HF: Horizontal filter; (a, b, c, etc.): In each line for each sample, mean values followed by a different symbol are significantly different according to Student–Newman–Keuls test at $P < 0.05$.

gravel) were lower, saving a constant value of 3.3 MPN/100 ml. For this reason after washing, the number of NOB populations remained almost constant (3.0 MPN/100 ml). This may be due to the low number of NOB that failed to form a thick biofilm on the gravel. Thus, after washing, the detachment will be too minor.

In this study, the autotrophic bacteria (AOB) are detached and their concentration decreased. Similar study by Derlon (2008) showed that detachment causes a decrease in the number of autotrophic bacteria. The heterotrophic bacteria with fast growth in the outer layers of biofilm (substrate concentration and detachment rate are high) may cover nitrifying bacteria with slow growth in internals layers of the biofilm (Nogueiro et al., 2002). Thus, the heterotrophic bacteria affect positively the nitrifying bacteria by protecting them against detachment, when the oxygen levels were sufficient for their maintenance under the biofilm matrix (Furumai and Rittmann, 1994). In this study, the insufficient oxygen level in the horizontal filter (where the sample was taken) prevents the heterotrophic bacteria from protecting the autotrophic bacteria against the detachment.

Biomass density in suspension

Results showed that the turbidity was inversely proportional to the number of gravel washings (Table 2). In the vertical filter, the turbidity after the first washing was much higher (97 NTU at 20 cm) than the turbidity after thes ubsequent washings (7NTU after 4th wash). The statistical analysis indicated significant differences according to the Student–Newman–Keuls test at P<0.05 obtained between turbidity after the first and fourth washing of the gravel sample taken at 20 cm of depth from vertical filter (Table 2). In the vertical filter, we found that the turbidity of the first washing of gravel taken from 20 cm was higher (97 NTU) than that for gravel sampled at 5c m of depth (57 NTU). In addition, the statistical analysis indicated that turbidity marks significant differences according to Student–Newman–Keuls test at P<0.05 after the first washing of the gravel sample taken at 5 and 20 cm of depth from vertical filter (Table 2).

Generally, the turbidity was greater at the bottom of the horizontal beds (88.5 NTU during the first washing than that obtained at the top and medium of horizontal filter (63.5, 76.4 NTU, respectively). The bottom of horizontal filter consisted of more fine gravel and it may be that the microbial loading is greater in the bottom of filter. The statistical study indicated significant differences according to the Student–Newman–Keuls test at P<0.05 obtained between turbidity at the bottom of horizontal filter characterized by fine gravel and the turbidity obtained at the top and medium of horizontal filter (Table 2). Also, it is probable that the microbial biomass at the bottom is less fixed than that at top and medium of horizontal filter. At the top of horizontal filter characterized by coarse gravel the turbidity value is higher than the turbidity value obtained at the medium and the bottom of this basin with values of 35.3, 29 and 23 NTU, respectively (Table 2). The fine gravel loaded per unit mass of microorganisms indicated less fixed microbial biomass than those obtained with coarse gravel. The microorganisms loading are more important in the filter with fine gravel than the filter with coarse gravel. The turbidity values after the first wash of fine gravel saved a value ranging between 60 and 100 NTU. The statistical study indicated significant differences according to the Student–Newman–Keuls test at P<0.05 obtained between turbidity of sample characterized by fine and coarse gravel from horizontal filter after the first washing (Table 2).

The nature of the carrier media used requires development of a very thin, evenly distributed and smooth biofilm to enable transport of substrate and oxygen to the biofilm surface. The turbulence sloughs off excess biomass and maintains adequate thickness of biofilm. Biofilm thickness less than 100 µm for full substrate penetration is usually preferred (Odegaard et al., 1994). Nevertheless, extremely high turbulence detaches biomass from the carrier and therefore is not recommended.

Ammonium oxidation in vertical and horizontal filters

The gravel from 5 cm incubated for 8 h at 25°C did not show a reduction of NH_4^+-N amount expressed by constant value of ammonium saved at the level of samples from before and after washing 13.84 ± 3.6 and

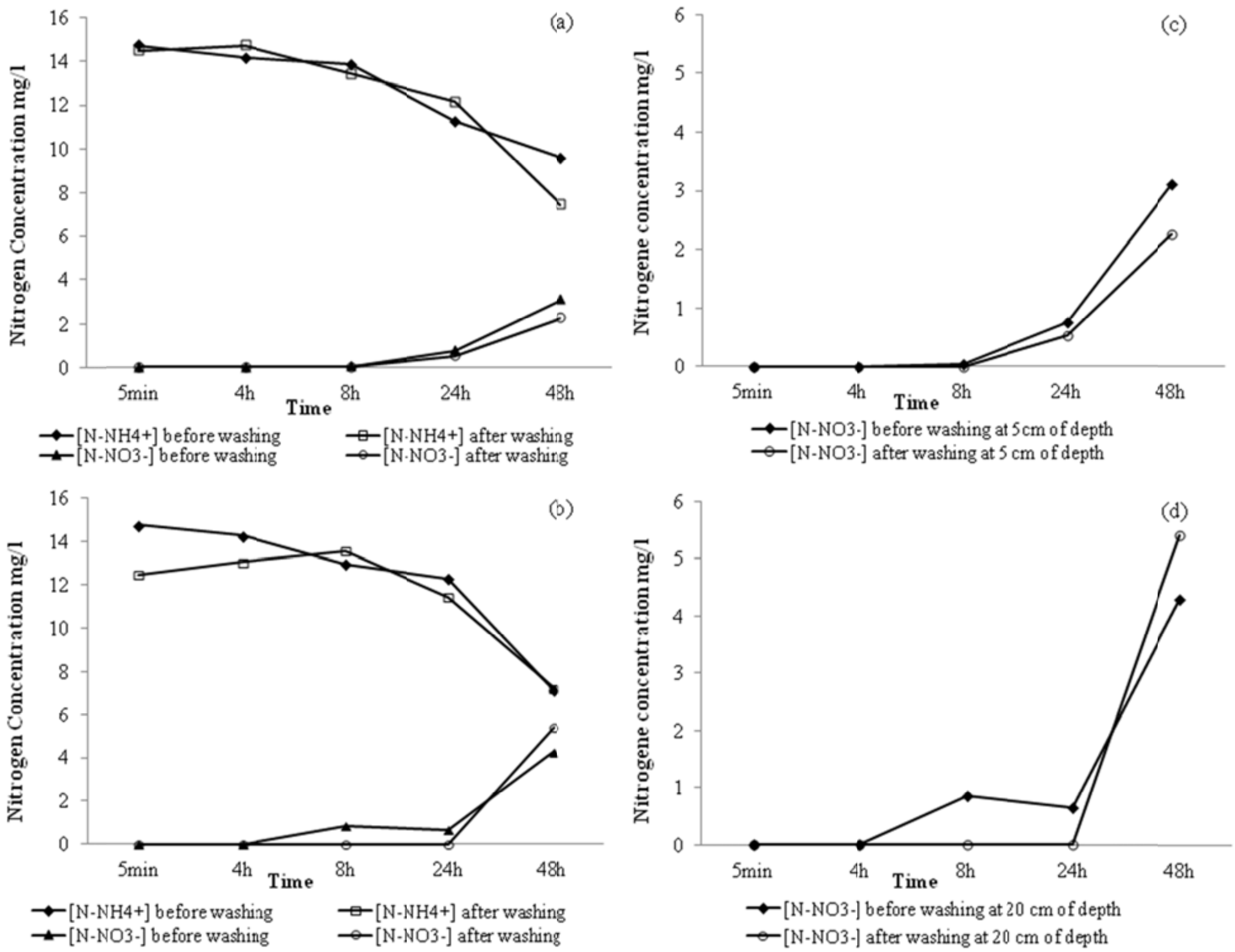

Figure 2. Evolution of nitrate oxidation on gravel sampled from vertical filter at 5 cm (a) and 20 cm of depth (b), NO_3^--N concentration at 5 (c) and 20 (d) cm of depth after first and fourth washing.

13.43 ± 2.5mg/l, respectively (Figure 2a). After incubation for 24 h, an increase of NO_3^--N concentration was observed, in samples before and after washing 0.8 and 0.5 mg/l, respectively. For samples taken from 20cm of depth and before washing the ammonium oxidation started after 8 h of incubation (Figure 2b), therefore, after washing, ammonium oxidation started after 24 h of incubation.

This result shows that washing sample gravel delayed ammonium oxidation. Washing or detachment seems to affect the nitrification performance by delaying nitrification. Using a bench-scale aerated biofilter, Ohashi et al. (1995) established that is no nitrification due to the biofilm detachment by daily backwash. However, their subsequent trials with lower substrate loading and backwash rates allowed stable nitrification.

However, after washing gravel sampled from vertical filter and following incubation during 48 h ammonium oxidation is more important at 20 cm (5.41 mg/l) than that obtained at 5 cm of depth (2.26 mg/l) (Figure 2c and d). The obtained result supposes that at 20 cm of depth microorganisms loading is more important than that at 5 cm of depth. Also, a significant, positively correlation between NO_3^--N concentration at 5 and 20 cm of depth before (r = 0.97) and after (r = 0.973) washing was obtained. In addition, a highly significant positively correlation between NO_3^--N concentration at 20 cm of depth before and after washing was obtained, with r = 0.977.

Nitrification performance of horizontal filter characterized by fine and coarse gravel after washing was grouped in Figure 3. NO_3^--N concentration at the bottom of horizontal filter with fine gravel is more important (1.24 mg/l at 48 h of incubation) than that observed at the medium and the top of basin saving values of 1.1 and 0.8 mg/l, respectively after 48 h of incubation (Figure 3a).

We supposed that nitrifying microorganisms are more important at the bottom of filter even after washing. Whereas, after washing the horizontal filter with coarse gravel, the nitrification performance is more important at the medium of basin with NO_3^--N concentration value of 1.14 mg/l than those obtained at the top and the bottom

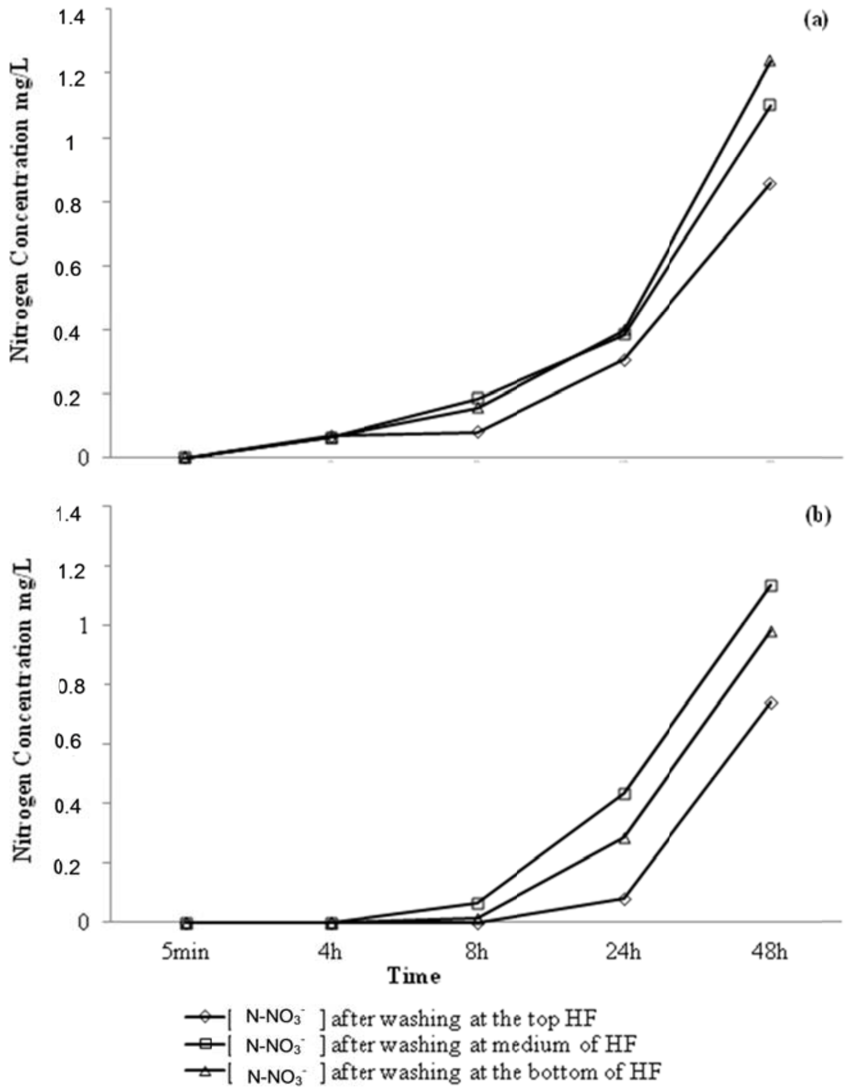

Figure 3. Evolution of nitrate oxidation at the top, medium and bottom of horizontal filter characterized by fine (a) and coarse (b) gravel after washing. HF: Horizontal filter.

of basin with values of 0.7 and 0.98 mg/l, respectively.

A statistical significance was showed at P<0.05 for NO_3^--N concentration before and after washing at horizontal filter. A positively correlation between NO_3^--N concentration at the top, medium and the bottom of horizontal filter characterized by fine gravel after and before washing (respectively, r=0.933, 0.93 and 0.966) was observed. After washing, a significant positively correlation (P<0.01) between NO_3^--N concentration at the top and the medium (r = 0.975), between the top and the bottom (r =0.966) and between the medium and bottom (r=0.999) of horizontal filter characterized by fine gravel were obtained. However, no significant correlation was noted at the top, medium and the bottom of horizontal filter characterized by coarse gravel after and before washing.

In this study, the detachment from a constant thickness biofilm resulted in nitrification reduction. However, a stable nitrification was obtained by an extension of the treatment period. Other studies showed that the washing induce reduction of the average of nitrification rates and reduced number of autotrophic bacteria (Elenter et al., 2007).

When dynamic of biofilm detachment is imposed on the system, a drop in the efficiency of nitrification is observed. In addition, some studies indicated that the effectiveness of nitrification drop 98 to 25% when the events of detachment is imposed, meaning that autotrophic bacteria are within biofilm (Derlon, 2008). In this investigation and unlike other studies, a stable and effective nitrification was obtained after washing. This difference may be due to the growth of nitrifying bacteria after washing or presence of substrate even in lower loading, thereby allowing a stable nitrification (Ohashi et al., 1995).

Conclusion

The decrease in AOB and denitrifying bacteria populations was affected by the number of washings, while the number of NOB present in suspension was lower and had a constant value of 3.0 MPN/100 ml. Washing seems to affect the nitrification performance by delaying nitrification.

Despite this detachment for gravel sampled from vertical filter, ammonium oxidation yield has been obtained by an extension of the treatment period. After washing, the ammonium oxidation was more important at 20 cm (5.41 mg/l) than at 5 cm of depth (2.26 mg/l). This supposes that at 20 cm of depth microorganisms loading was more important than at 5 cm depth.

In horizontal filter with fine gravel, the nitrification performance was more important at the bottom of filter even after washing. Whereas, at the horizontal filter (coarse gravel), the nitrification performance was more important at the medium of the basin.

Since the yield of nitrification is unaffected by detachment, enhancing detachment by acting in some physico-chemical parameters may lead to clogging prevention in fixed-biofilm wastewater treatment processes.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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