START–UP AND PERFORMANCE OF UASB REACTORS USING ZEOLITE FOR IMPROVEMENT OF NITRATE REMOVAL PROCESS

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ABSTRACT

A first study on the use of Chilean natural zeolite of different particle sizes (0.5, 1 and 2 mm in diameter) in laboratory-scale batch denitrificant reactors was carried out with the aim of assessing the microbial communities adhered to this material. Molecular techniques such as fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) fingerprints revealed a high microbial diversity with a strong presence of Gammaproteobacteria (70% of the total microorganisms) in reactors with zeolite 0.5 mm in diameter. Archaea were only detected in the reactors with zeolite 1 mm in diameter. In addition, the acclimatization and start-up of two UASB reactors

(one without zeolite and the other with added zeolite 1 mm in diameter) were conducted following three consecutive and progressive stages using upward velocities from 0.10 to 0.44 m/h in order to establish an experimental protocol suitable for the start-up of this type of reactors. Total (100%) nitrate reduction was achieved in the UASB reactors with and without zeolite on the 7^{th} and 11^{th} days, respectively, of the second stage of the start-up period, showing the suitability of the use of this material in this type of reactors. Finally, a third study carried out with both UASB reactors operating in continuous mode at a high organic loading rate (44 kg COD/m^3·d) and a very low HRT (2.5 h) revealed that the reactor with zeolite achieved a nitrate removal efficiency of 92.4% at a nitrogen load of 6.42 kg NO_3^-/(m^3·d). This last study also demonstrated the robustness of the UASB reactor with zeolite under nitrate load variations.

Keywords: denitrification, microbial communities, UASB reactors, start-up, zeolite.

1. INTRODUCTION

A continuous increase in the concentration of nitrate has been observed in groundwater reserves and surface waters (rivers, lakes, etc.) due to intensified agriculture, industrialization and urbanization (Benyoucef et al., 2013). To be specific, wastewaters with nitrogen compounds (nitrate, nitrite, ammonia, etc.) are generated by domestic services as well as many industries such as fertilizers, fisheries, metal finishing, agro-foods, slaughterhouses, etc. (Huiliñir et al., 2011; Cheikh et al., 2013). For instance, between 30%-70% of nitrogen fertilizers used in agriculture is lost into the environment and found in nitrate form in surface waters and ground waters, and used as drinking

water in some countries (Cheikh et al., 2013). At high concentrations, nitrate consumption causes methemoglobinemia in infants. Likewise, nitrate is reduced to nitrite in the intestine and is known to be linked to several cancers. Moreover, nitrosamines are carcinogenic compounds that can be formed from nitrite in the stomach (Cheikh et al., 2013). In addition, ammonia and increased reactive nitrogen discharged into aquatic environments can cause serious oxygen depletion in those environments and, in general, nitrogen compounds can cause eutrophication of lakes and rivers (Benyoucef et al., 2013).

There are physicochemical and biological methods for removing the nitrogen present in wastewaters (Zhang et al., 2009; Andalib et al., 2012; Malekian et al., 2011; Montalvo et al., 2011; Rezakazemi et al., 2012; Bravo et al., 2013; Jiang et al., 2013; Yu et al., 2013). Physicochemical methods do not completely solve the problem because they transfer the pollutants from one matrix to another and are not cost effective. By contrast, biological processes remove the pollutants and, operating under adequate conditions, their final products are innocuous for the environment and byproducts can even be obtained for use as fertilizers (Yetilmezsoy and Sapci-Zengin, 2009; Uysal et al., 2010). Therefore, among various nitrogen removal methods, nitrification-denitrification processes are the most frequently used from a practical point of view and seem to be the most promising (Morita et al., 2008; Rodríguez at al., 2011).

The nitrification-denitrification process is carried out in two steps. During the first step, ammonia is aerobically converted into nitrate through nitrite, and in the second anoxic step nitrate is reduced to nitrite, nitric oxid, nitrous oxide and nitrogen gas by heterotrophic bacteria, with organic materials usually used as the electron donor for this

reduction process. Finally, gaseous nitrogen (N_2) which escapes into the air causing no secondary contaminant effects is formed (Shen et al., 2013):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO\,(g) \rightarrow N_2O\,(g) \rightarrow N_2\,(g) \quad (1)$$

When using methanol as an extra carbon source while treating water with a low C/N ratio, the overall denitrification reaction can be expressed as follows:

$$6NO_3^- + 5CH_3OH \rightarrow 5CO_2 + 3N_2 + 7H_2O + 6OH^- \quad (2)$$

The following reaction represents a typical reaction for synthesis or generation of microorganisms:

$$3NO_3^- + 14CH_3OH + CO_2 + 3H^+ \rightarrow 3C_5H_7O_2N + 19H_2O \quad (3)$$

In practical terms, between 25% and 30% of the methanol needed as energy is used to generate microorganisms. From a laboratory test, the following equation has been developed to describe the global reaction of nitrate removal (Shen et al., 2013):

$$NO_3^- + 1.08CH_3OH + H^+ \rightarrow 0.065C_5H_7O_2N + 0.47N_2 + 0.76CO_2 + 2.44H_2O \quad (4)$$

The use of efficient biological reactors such as the upflow anaerobic sludge blanket (UASB) for organic matter degradation has been widely assessed and checked, leading to high COD removal efficiencies operating with low hydraulic retention times (HRTs) (Puyol et al., 2009; Basu and Gupta, 2010). The UASB reactor involves the anaerobic degradation of organic wastes using a biomass which is not attached to a support medium but which aggregates under favourable conditions to produce particles with good settlement characteristics. These particles are known as granules and their formation, commonly termed “granulation”, greatly enhances the efficiency of the process, producing high biomass retention times and enabling high organic loading rates to be achieved (Hulshoff Pol et al., 2004; Chong et al., 2012).

One problem or disadvantage of the anaerobic process is its slow start-up phase. With the aim of accelerating the start-up of the UASB reactor, different methods have been tested, among them the addition of certain materials that could help granule formation. The use of zeolite is an alternative that may be evaluated to promote granule formation taking into account the favourable influence of this material in other biological purification processes (Fernandez et al., 2007; Mery et al., 2012; Montalvo et al., 2012). On the other hand, an in-depth analysis of the microbial communities developing during organic matter decomposition in anaerobic reactors could represent an important step in better understanding and monitoring the performance of the process. Understanding the involvement of specific microorganisms would be useful for controlling performance and maximum substrate decomposition during the anaerobic degradation of wastewaters (Rincon et al., 2008).

In this light, the aim of this work was to study the use of natural zeolite (a mineral composed basically of alumina-silicates) to help to increase the anaerobic biomass in UASB reactors, assessing the performance of these reactors in removing nitrates in denitrification processes when operating with synthetic wastewater. The start-up period of the modified UASB reactors was closely evaluated in order to develop an adequate starting experimental procedure. Previously, the identification of the microbial communities adhered to zeolite was also carried out in denitrification experiments performed in batch mode.

2. MATERIALS AND METHODS

Two different experimental runs were carried out throughout this study. Firstly, a set of batch experiments was performed in small laboratory-scale reactors with the aim of assessing the microbial communities adhered to natural zeolites with different particle sizes. Molecular techniques such as fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) fingerprints were used to evaluate the microbial profiles. A second run of experiments was conducted in laboratory-scale UASB reactors with and without zeolite with the aim of setting up an appropriate experimental protocol for the start-up of this type of reactors, which were operated in continuous mode.

2.1. Batch denitrification experiments

A first run of experiments was carried out for the identification of the microbial populations adhered to natural zeolite in batch denitrification processes. These experiments were performed in small-scale batch reactors with an operating volume of 0.25 L working at a temperature of 37ºC. Zeolite particle sizes 0.5 mm, 1 mm and 2 mm in diameter were used in this group of experiments. The operating conditions of the reactors are shown in Table 1. The inocula used in this set of assays were derived from an anaerobic sludge digester reactor which treats urban wastewaters.

Samples of biomass adhered to zeolite were taken from the reactors at the beginning and end of the experiment (28 days). In order to separate the biomass from the zeolite, these samples were subjected to ultrasound for 30 seconds using the Branson Sonifier 150 ultrasound equipment with 40% amplitude. Subsequently, and with a view to analyzing the different microbial populations, samples were prepared using the molecular technique of fluorescence in situ hybridization (FISH) according to

the protocol described by Amann et al. (1990). Table 2 shows the sequences, target organisms and percentage of formamide used in each case. To be specific, samples were fixed in 4% paraformaldehyde, washed in phosphate buffer saline (PBS) and preserved in PBS–ethanol at −20ºC until used. For the hybridization procedure, the samples were fixed on a multi-dish slide at 46ºC for 20 minutes and dried in ethanol. All the probes carried out are summarized and specified in Table 2. DAPI staining (4', 6-diamidino-2-phenilindol) was used to corroborate that the observed fluorescence with the FISH technique corresponded to bacteria cells in order to quantify the existing microorganisms.

2.2. Characteristics of the zeolite

The Chilean natural zeolite used was obtained from the company *Minera Formas (ZeoClean^R)*. Its main chemical composition was: SiO_2, 67.00%; Al_2O_3, 13.01%; Fe_2O_3, 3.60%; CaO, 3.46%; Na_2O, 1.32%; TiO_2, 0.28%; MgO, 0.78%; K_2O, 0.53%. In addition, the mineralogical composition of the zeolite used in all assays was: Clinoptilolite, 35%; Mordenite, 15%; Montmorillonite, 30%; others (calcite, feldespate and quartz), 20%.

2.3. Characteristics of the inoculum

The anaerobic inoculum used in all experiments was obtained from a full-scale anaerobic digester installed at the Urban Wastewater Treatment Plant (UWTP) *La Farfana,* located in Santiago de Chile. The main characteristics of the inoculum were: volatile suspended solids (VSS), 14.9 g/L; percentage of VSS (dry basis), 65.5;

alkalinity (as $CaCO_3$), 3930 mg/L; volatile fatty acids (VFA, as acetic acid), 380 mg/L; pH, 7.2.

2.4. Start-up and operational conditions of the laboratory-scale UASB reactors

The second experimental run was carried out in two identical laboratory-scale UASB reactors, one operating with zeolite and the other without. The size and main characteristics of the UASB reactors were: length, 110 cm; internal diameter, 10.5 cm; thickness, 0.5 cm; surface, 86.6 cm^2; total volume, 9525 cm^3.

The composition of the synthetic wastewater used for feeding the UASB reactors was: methanol, 3.17 g/L; $NaNO_3$, 2.3 g/L; yeast extract, 0.1 g/L; peptone, 0.25 g/L; K_2HPO_4, 238 mg/L; KH_2PO_4, 183 mg/L; $NaHCO_3$, 4 g/L.

The start-up of the UASB reactors was carried out in 4 steps as follows:

1) Pre-acclimation step: initially 750 mL of anaerobic inoculum and 3 litres of synthetic wastewater were added to a UASB reactor, keeping this reactor at room temperature (18ºC-20ºC). The reactor operated in this way without feeding for one week.

2) Stage 1: after a pre-acclimation period of one week, 1750 mL of mixed liquor from one UASB reactor were added to the second UASB reactor, leaving the same volume of mixed liquor (1750 mL) in the first reactor. The remaining 250 mL were removed from the first reactor and subsequently used for chemical analysis. Both reactors operated in batch mode with total recirculation at an upward velocity of 0.1 m/h during the first day and 0.44 m/h during the second one. This stage lasted only 2 days.

3) Stage 2: upon completion of the first stage, 25 g of VSS were left in each reactor and 250 g zeolite were added to reactor 2 (at a ratio of 0.1 g VSS/g zeolite), while reactor 1 was operated without any added zeolite. Previous research works (Montalvo et al. 2005 and 2012) showed that anaerobic processes were favoured by the addition of natural zeolite at doses of between 0.05 and 0.3 g VSS/g zeolite, the optimum value being 0.1. Moreover, the increase of the zeolite doses may affect the mass transfer of organic matter, either nutrients and metabolites, in the vicinity of zeolite particles and the microorganisms associated. In addition, high amounts of zeolite may be toxic due to the accumulation of heavy metals (Montalvo et al., 2005). Both reactors operated in batch mode with total recirculation with an upward velocity of 0.1 m/h for 15 days.

4) Stage 3: both reactors (reactor 1 without zeolite and reactor 2 with added zeolite) were also operated in batch mode with total recirculation with an upward velocity of 0.44 m/h for 7 days.

2.5. Chemical analyses

Chemical oxygen demand (COD) and volatile suspended solids were analysed according to the closed digestion and colorimetric 5220D and 2540B methods, respectively, of the Standard Methods for the Examination of Waters and Wastewaters (APHA, 1998). pH was determined using a pH-meter model Crison 20 Basic. Nitrate and ammonium nitrogen were determined by selective electrodes.

3. RESULTS AND DISCUSSION

3.1. Identification of the microbial populations adhered to natural zeolite in the batch denitrification process

For the correct analysis of denitrifying reactors due to high microbial growth the samples had to be diluted. The dilution was carried out in Ethanol and PBS at a ratio of 1:1.5:1.5. Denitrifying reactors showed Gammaproteobacteria communities with ratios inversely proportional to the size of the zeolite, i.e. the highest percentage was found for the smallest diameter of 0.5 mm, for which 70% of these microorganisms was found, while for 1 mm and 2 mm diameters, the percentage of Gammaproteobacteria found was 40% and 30%, respectively. These bacteria were found in grouped form exclusively for the 0.5 mm diameter. For the other diameters they were dispersed within a dispersed total biomass. Within this bacterial class the presence of *Competibacter phosphatis* was identified in denitrifying reactors with a zeolite diameter of 0.5 mm and 2 mm with percentages close to 30%. Gammaproteobacteria include *Pseudomonas*, some of which use nitrate as an electron acceptor, and, therefore, play a key role in denitrification systems. Recent studies related to marine sediments (Mills et al., 2008) show a high abundance of Gammaproteobacteria in processes of nitrification and denitrification, attributable to a high level of metabolism of these microorganisms, which leads to rapid changes in oxygen and substrates as occurs in the sands of the sea. Figure 1 shows the images of FISH obtained for the reactors with zeolite diameters of 1.0 and 0.5 mm. As can be seen, a strong presence of Gammaproteobacteria was observed in the reactors with zeolite 0.5 mm in diameter.

Recent studies related to the biodegradation of PHAs under anoxic and anaerobic denitrifying batch conditions also revealed, based on 16S rDNA analysis, that the denitrifying enriched culture was mainly composed of Gammaproteobacteria (19 clones out of a total of 23 clones) (Lu et al., 2011), similar to what is observed in our study.

Denitrification is an anaerobic process carried out by distinct phylogenetic microorganic groups of bacteria and archaea (Cheneby et al., 2000), where archaea may have been involved in nitrogen removal. Only in the reactors with zeolite 1 mm in diameter, cells not belonging to Eubacteria were identified. For this reason, these reactors were analysed for the presence of archaea, finding an 80% presence (Figure 1). Within archaea, the presence of *Methanosaetaceae*, *Methanosarcina* and *Methanobacteria* was analysed because of the high organic content of the culture medium, finding no presence of these archaea groups. However, the non-presence of *Methanosarcina* is not categorical, since this was analysed with fluorochrome fluos. Previous works have clearly demonstrated that the total number of bacteria can be measured by DAPI while bacterial community structures were estimated with FISH using oligonucleotide probes specific to ribosomal RNA of the domain bacteria and the domain archaea (Abe et al., 2003).

Archaeal phylotypes very closely related to members of the orders *Methanosarcinales* and *Methanomicrobiales* were found in two production waters of a low-temperature and low-salinity petroleum reservoir in Canada, which were examined using cultural and molecular approaches (Grabowski et al., 2005). The authors found significant amounts of denitrifers in these reservoirs and the dominant members of the

culturable population were affiliated with the Firmicutes, the Deltaproteobacteria, the Epsilonproteobacteria, the Spirochaetes and the Euryarchaeota.

Since a greater microbial diversity in the samples of the anaerobic reactors whose diameter of zeolite was 1 mm was detected after applying the FISH technique, it was decided that a denaturing gradient gel electrophoresis (DGGE) analysis of bacteria from these reactors should be carried out by analysing samples of start-up (DNB 1) as well as samples of the end of the experiment (DNB 3). DGGE results show a pattern of bands of different intensity in different positions of the gel (Figure 2). In the course of the experiment, six communities of the phylum Firmicutes were developed, of which three were 99% similar to the genus *Clostridium*, which is considered a potential key player in denitrification processes.

The bacterial diversity in a sequencing batch biofilm reactor (SBBR) treating landfill leachate was also studied with a view to explaining the mechanism of nitrogen removal (Xiao et al., 2009). DGGE fingerprints based on total community 16S rRNA genes were analyzed with statistical methods, and excised DNA bands were sequenced. As in the present work, the results of phylogenetic analyses carried out by Xiao at al. (2009) also revealed high diversity within the SBBR biofilm community, and DGGE banding patterns showed that the community structure in the biofilm remained stable during the running period, observing also a dominant presence of the same microorganism groups than those detected in the present work. In the same way, denitrification processes in fixed bed reactors were simulated at laboratory-scale using anaerobic batch tests with immature compost (Trois et al., 2010a). This research showed that the original composition, nature, the carbon-to-nitrogen ratio (C/N) and the degree

of maturity and stability of the substrates clearly play a key role in the denitrification process, impacting directly on the development of the bacterial population and, therefore, on the long-term removal efficiency. Therefore, the above-mentioned factors clearly influence on the denitrification process being very important for an appropriate microbial diversity and ecosystem restoration (Benyoucef et al., 2013; Cheikh et al., 2013; Avila et al., 2013; Tait et al, 2013).

3.2. Start-up and acclimation of the UASB reactors with and without zeolite

On the basis of results achieved in experimental run 1 it was decided that zeolite with a diameter of 1 mm should be used in the second experimental run, for which two UASB reactors were tested, one without zeolite and the other with added zeolite. This zeolite particle size is within the diameter range considered to be adequate when other reactor configurations were used (Milan et al., 2001 and 2003; Montalvo et al., 2005).

Figures 3 shows the variation of the COD content with time during acclimation stages 2 and 3. As can be seen during the second step almost no reduction of the COD content was observed. This behaviour can be explained by the inoculum not fully acclimating to the synthetic substrate and by the origin of the inoculum, which was adapted to remove only organic matter but not to substrates with high nitrate concentrations. By contrast, Figure 3 also shows how a rapid COD degradation took place in the third stage, especially after the first 7 days of the operating period, which shows how this stage led to the development of a heterotrophic biomass such as denitrifier bacteria. During this stage the influence of the added zeolite was observed at

the end of the experimental run (lasting 5 days), with COD degradation higher than in the UASB reactor without zeolite. This can be attributed to the higher cell growth achieved in the reactor with zeolite, as can be seen in Figure 4 (a-d). Figure 4 (a and c) shows how the anaerobic sludge fills part of the internal wall of the reactor as well as the upper baffles in the UASB reactor with zeolite, while the reactor without zeolite shows low levels of biomass and a different light colour in its walls (Figure 4 (b-d)). It is worth mentioning that the UASB reactor with zeolite contained only 250 g of zeolite, which corresponds to only 2% of the total volume of the reactor. Moreover, and despite the high growth of biomass in this reactor, VSS in the process effluents (biomass losses) were not observed in any case.

Other digester configurations such as a hybrid anaerobic biofilm and sludge reactor (HABSR) were shown to be very effective in simultaneous methanogenesis and denitrification as was investigated in batch tests (Zhong et al., 2010). However, in this case as the nitrate concentration was increased from 75 to 600 mg/L, COD removal rates were reduced from 95 to 1.7 mg/(h·g).

Figure 5 shows the evolution of pH with time during the second stage of the start-up process. An increase in pH values with time was observed in both cases due to the use of nitrate as the oxidant agent and the formation of hydroxide ion according to equation (2). In the same way, a considerable increase in pH and alkalinity was also observed during the biological denitrification of anaerobic effluents carried out in batch reactors at a temperature of 22ºC with volatile fatty acid (VFA) concentration in the range of 20-200 mg/L (Elefsiniotis et al., 2004).

The variation of effluent nitrate concentration with time during the second stage of the start-up period is shown in Figure 6. As can be seen, during the first 4 days the nitrate concentration decreased by only 6.7% of its initial value despite the fact that a denitrifier population is apparently found in the reactors to remove the nitrate at higher rates. However, the nitrate was totally removed on the 7^{th} day in the reactor with zeolite, while it was not completely consumed until the 11^{th} day in the reactor without zeolite, which fits with the more accentuated growth of the dentrificant biomass in the reactor with zeolite.

Denitrification processes of landfill leachates in fixed bed reactors were simulated at laboratory scale using anaerobic batch tests and columns packed with immature compost and pine bark (Trois et al. 2010b). Leachates with nitrate concentrations of 350, 700 and 1100 mg N/L were used for these trials. These nitrate concentrations were lower than those used in the present study. Preliminary results suggested that after the acclimatization step (40 days for both substrates), full denitrification is achieved in 10-20 days for the pine bark and 30-40 days for the compost (Trois et al. 2010b).

Figure 6 also illustrates the variation of the ammonium concentration with time during the second stage of the acclimatization period. A marked decrease in ammonium concentration from the first days of the start-up period was observed, the reactor with zeolite showing the lowest values. This behaviour can be attributed to the cationic exchange and adsorption capacities of the zeolite and its selectivity by this compound. This hypothesis can be corroborated through different ammonium adsorption experiments using the same zeolite as used in the UASB reactor (Mery et al., 2012). These experiments revealed that natural zeolite 1 mm in diameter adsorbed the

ammonium ion following a Langmuir isotherm according to this expression: $Qe = 0.00033 \cdot Ce^{1/0.56}$, where Qe is the mass of substance adsorbed per unit of mass of zeolite in the equilibrium (mg NH_4^+/g zeolite), Ce is the concentration of substance in the liquid in the equilibrium (mg NH_4^+/L) (Mery et al., 2012). In addition, ammonium consumption by the microorganisms and its incorporation into their cell tissues also contributes to ammonium removal.

The simultaneous removal of carbon and nitrogen of the anaerobically pretreated distillery wastewater with nitrate, nitrite and added ammonium was studied in an expanded granular sludge bed (EGSB) reactor inoculated with methanogenic granular sludge (Li et al., 2011). At high C/N ratios of 10 and 8, little total Kjeldahl nitrogen (TKN) was removed. However, 30%-50% of TKN entering the reactor was removed at lower C/N ratios of 2, 3 and 4 suggesting the removal of ammonium (Li et al., 2011), in a similar way to what occurs in the present study.

3.3. Operation of the UASB reactors in continuous mode at pseudo-steady state

Once the acclimatization and start-up stages of the UASB were completed, both reactors operated in continuous mode at an organic loading rate of 44 kg COD/(m^3·day) and a hydraulic retention time of 2.5 hours for an operating period of 60 days. During this period the reactors operated with an upward velocity of 0.44 m/h, which is somewhat lower than that used in a conventional UASB (0.5-1 m/h).

Figure 7 shows the evolution of influent and effluent nitrate concentrations with time for the reactors with and without zeolite. Lower nitrate concentrations were always

observed in the reactor with zeolite, which led to nitrate concentrations as low as 17 mg NO_3^-/L.

With the aim of observing the robustness of the process against the variation of nitrate loads, the process performance was assessed for nitrate loads ranging between 4.18 and 7.52 kg NO_3^--N/(m^3·d). Under these conditions, nitrate removal efficiencies in the ranges of 43.5%-97.0% and 20.6%-54.5% were achieved for the reactors with and without zeolite, respectively. As can be observed in Figure 7, from day 43 onwards the influent nitrate concentration was kept constant, which was equivalent to a nitrate load of 6.42 kg NO_3^--N/(m^3·d), achieving nitrate removal efficiencies of 61.2% and 92.4% for the reactors without and with zeolite, respectively. The high nitrate removal efficiencies found in the UASB reactor with zeolite under continuous feeding can be explained by the lower mass transfer limitation for denitrifiers presumably located in the outer layer of biomass aggregates supported on zeolite (Kalyuzhni et al., 2007).

Figure 8 shows the variation of the pH with time in the reactors with and without zeolite, as well as the influent pH evolution. As can be seen, the effluent pH achieved slightly alkaline values ranging between 7.19 and 8.34 without detecting process inhibition. It could be observed that the pH values in the reactor with zeolite were always slightly higher than those obtained in the reactor without zeolite. In addition, the average COD removal efficiencies in the reactors with and without zeolite were 30% and 24%, respectively, during the operation period in continuous mode.

4. CONCLUSIONS

Laboratory-scale denitrificant reactors operating in batch mode with added zeolite showed high microbial diversity with the strong presence of Gammaproteobacteria. Archaea were only observed in the reactors with zeolite 1 mm in diameter. Bacteria belonging to the Gammaproteobacteria class were the best adapted to the denitrification conditions studied.

An experimental procedure or protocol following three stages was implemented for the start-up of UASB reactor with natural zeolite. This procedure and the adequate acclimatization of the inoculum led to higher nitrate removal efficiencies in this reactor than for the UASB without zeolite. A higher biomass growth was always observed in the reactor with zeolite.

The robustness of the reactor with zeolite achieving high nitrate removal efficiencies when operating under important variations of nitrate loads was shown. To be specific, nitrate removals higher than 92% were achieved when the reactor operated in continuous mode at high organic loading rates (44 kg COD/(m^3·d)) and low hydraulic retention times (2.5 h).

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Table 1. Characteristics of the batch reactors

	Unit	Amount
Culture medium*	mL	210
Zeolite**	g	20
Inoculum	mL	20
Temperature	°C	37
pH	-	7
C/N ratio	g/g	10.5
Operation time	days	28

*The culture medium of the denitrificant reactors was: 1.5 g/L of CH_3COOH; 0.08 g/L of $MgSO_4 \cdot 7H_2O$; 0.3 g/L of NaCl; 0.2 g/L of yeast extract; 1.56 g/L of KNO_3; 5.0 g/L of K_2HPO_4; 1.5 g/L of KH_2PO_4 and 1 ml/L of saline solution.

**For each culture medium, three zeolite diameters (0.5, 1.0 and 2.0 mm) were used separately. Triplicate reactors were used for all cases studied.

Table 2. Sequences, target organisms and percentage of formamide (% FA) used.

Probe	Chromo*	Sequence (5'→3')	% FA/ NaCl (mM)	Target organisms
GAM42a*	Cy3	GCC TTC CCA CAT CGT TT	35	Gammaproteobacteria
GAOQ431	Cy3	TCC CCG CCT AAA GGG CTT	35	Candidatus "Competibacter phosphatis"
ARC915	Cy3	GTG CTC CCC CGC CAA TTCCT	40	Archaea
MX825	Cy3	TCG CAC CGT GGC CGA CAC CTA GC	50	Some methanosaetaceae
MS821	Fluos	CGC CAT GCC TGA CAC CTA GCG AGC	40	Methanosarcina
MB1174	Cy3	TAC CGT CGT CCA CTC CTT CCT C	45	Methanobacteriales (minus Methanothermus)

*Chromosphero

FIGURE CAPTIONS

Figure 1. Images of FISH: **Left image** shows a sample of the denitrificant anaerobic reactor with zeolite 1 mm in diameter: *Archaea* (Cy3: red colour) put on all biomass (DAPI: blue colour). **Right image**: sample of the denitrificant reactor with zeolite 0.5 in mm diameter: *Gammaproteobacteria* (Cy3: red colour) put on all biomass (DAPI: blue colour). Bar indicates 10 μm.

Figure 2. **Left side**: denaturing gradient gel electrophoresis (DGGE) analysis showing the bands of the samples DNB 1 and DNB 3 with the different groups found. **Right side**: interest bands, access numbers and closest microorganisms phylogenetically found.

Figure 3. Variation of COD (mg/L) with time (days) during stages 2 and 3.

Figure 4. Photograph of the UASB with zeolite (Fig. 4a: lower part; Fig. 4c: entire reactor) and of the UASB without zeolite (Fig. 4b: lower part: Fig. 4d: entire reactor).

Figure 5. Variation of the pH with time (days) during stage 2 of the start-up period.

Figure 6. Variation of the nitrate and ammonium concentrations (mg/L) with time (days) during stage 2 of the start-up period.

Figure 7. Evolution of the nitrate concentration (mg/L) with time (days) during the operation of the reactors in continuous mode.

Figure 8. Evolution of the pH with time (days) during the operation of the reactors in continuous mode.

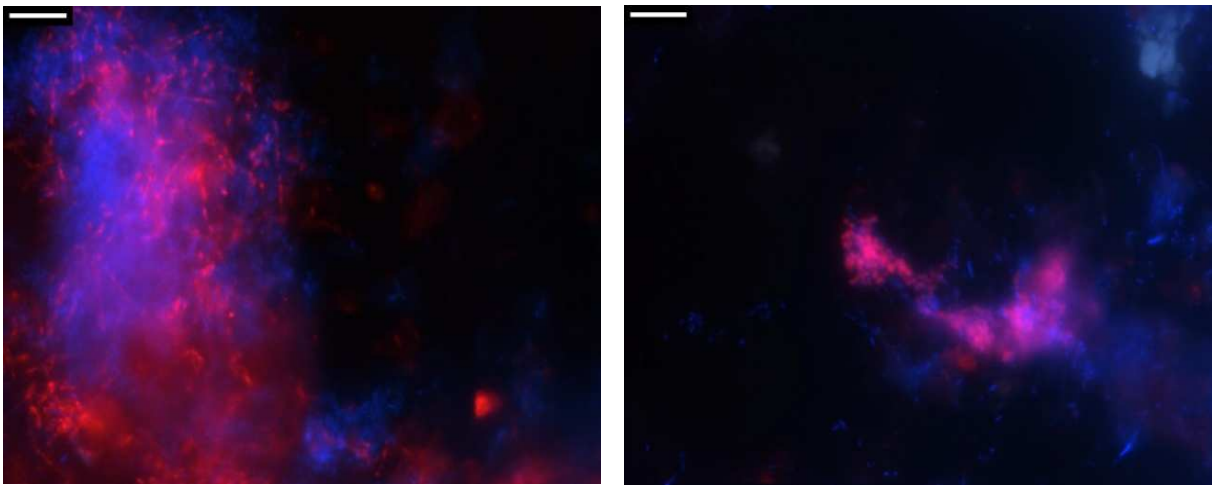
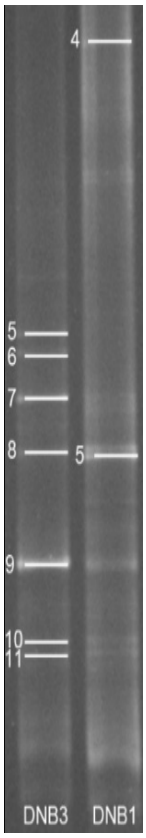


Figure 1



Sample	*Band*	*Best result (Access number)*	*Closest Microorganism (% similarity)*
DNB1	4	Uncultured bacterium nbw139f01c1 (GQ047959)	*Flavobacteria* (94%)
DNB1	5	Uncultured bacterium D242_27F_BAC_019 (AB447697)	*Bacteroidetes* (99%)
DNB3	5	Uncultured bacterium clone LaYa5b-55 (GU291589)	*Clostridium* (99%)
DNB3	6	Uncultured bacterium clone: TSNIR002_A18 (AB487194)	*Clostridium* (98%)
DNB3	7	Bacterium enrichment culture clone DPHE06 (GQ377119)	*Clostridium* (99)
DNB3	8	Uncultured bacterium clone HAW-RM37-2-B-877d-A14 (FN563219)	*Bacteroidetes* (99%)
DNB3	9	Uncultured Clostridiales bacterium clone DS166 (DQ234248)	*Frigovirgula* (100%)
DNB3	10	Uncultured Firmicutes QEDN2BH06 (CU925649)	*Firmicutes* (99%)
DNB3	11	Uncultured bacterium clone IA-23 (AJ488074)	*Firmicutes* (99%)

Figure 2.

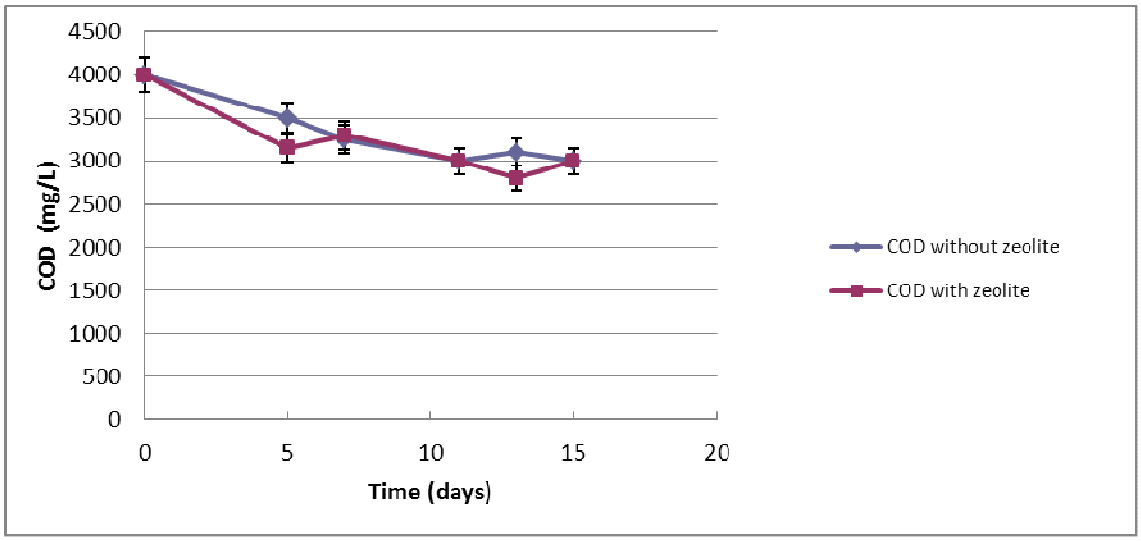

4500
4000
3500
3000
2500
2000
1500
1000
500
0
COD (mg/L)
0
5
10
15
20
Time (days)
COD without zeolite
COD with zeolite

Fig. 3a

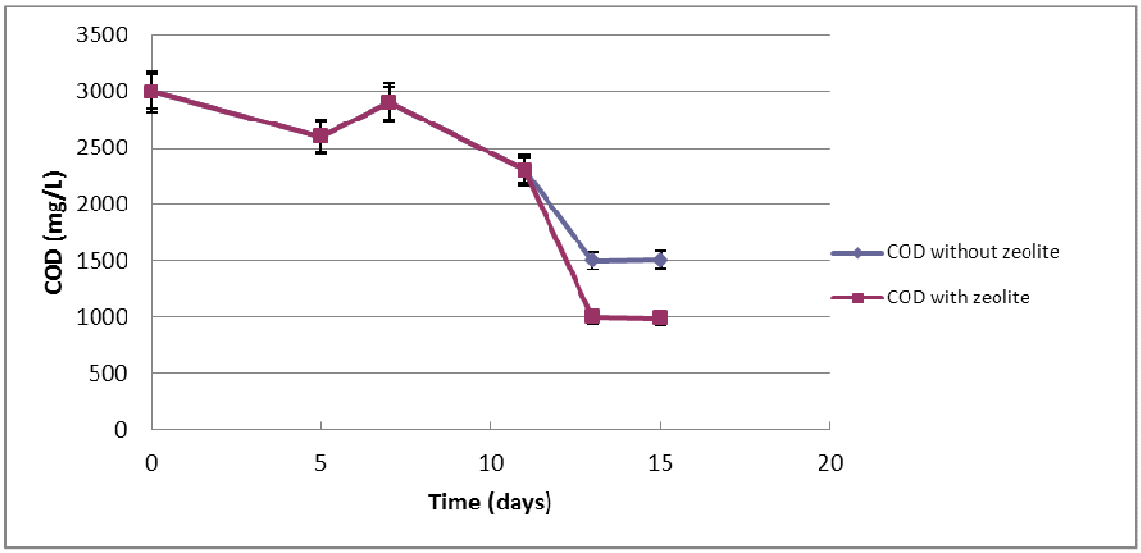

3500
3000
2500
2000
1500
1000
500
0
COD (mg/L)
0
5
10
15
20
Time (days)
COD without zeolite
COD with zeolite

Fig. 3b

Fig. 4a

Fig. 4b

Fig. 4c

Fig. 4d

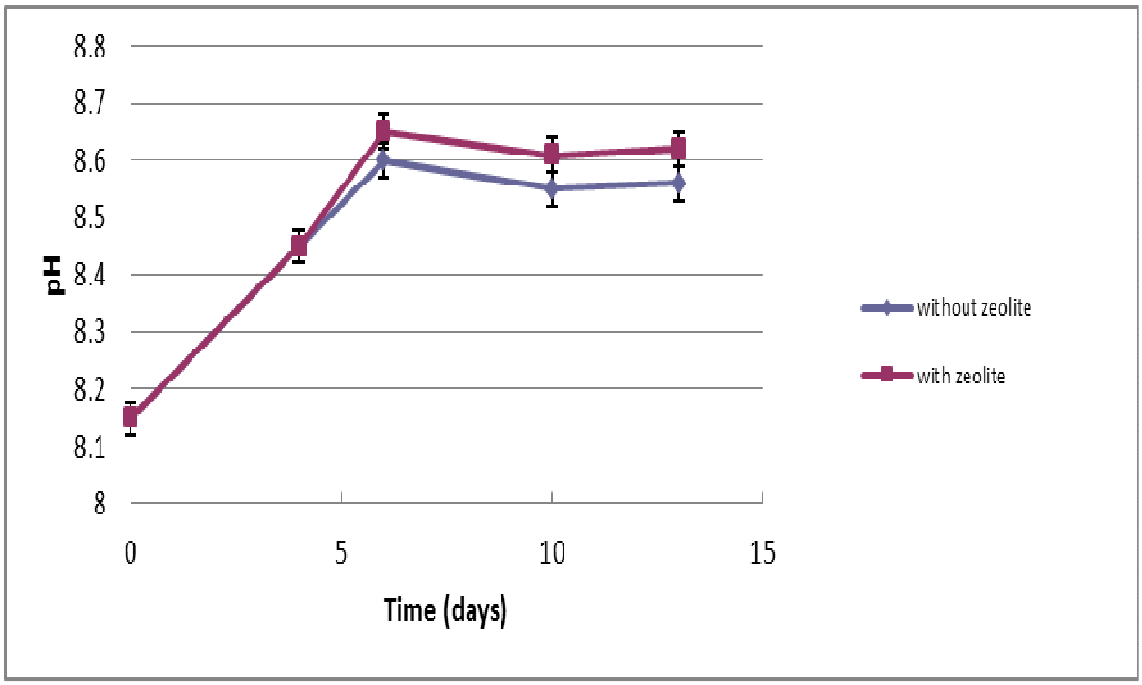

8.8
8.7
8.6
8.5
8.4
8.3
8.2
8.1
8
pH
0
5
10
15
Time (days)
without zeolite
with zeolite

Figure 5

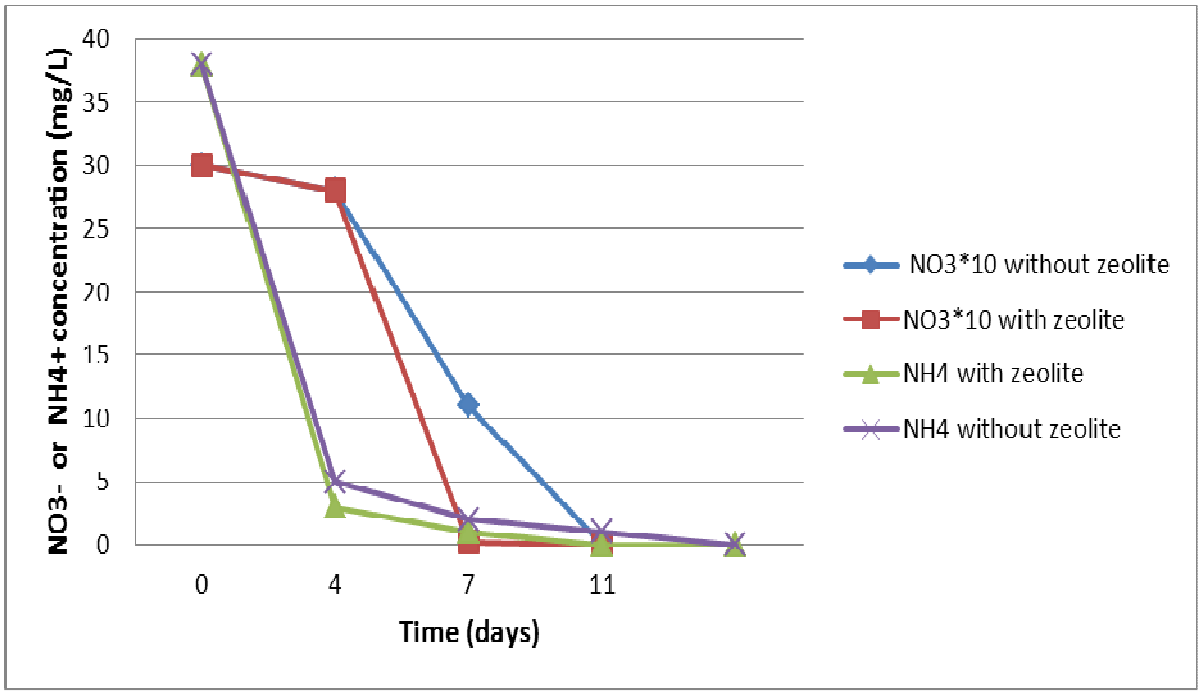

NO3- or NH4+ concentration (mg/L)
40
35
30
25
20
15
10
5
0
0
4
7
11
Time (days)
NO3*10 without zeolite
NO3*10 with zeolite
NH4 with zeolite
NH4 without zeolite

Figure 6

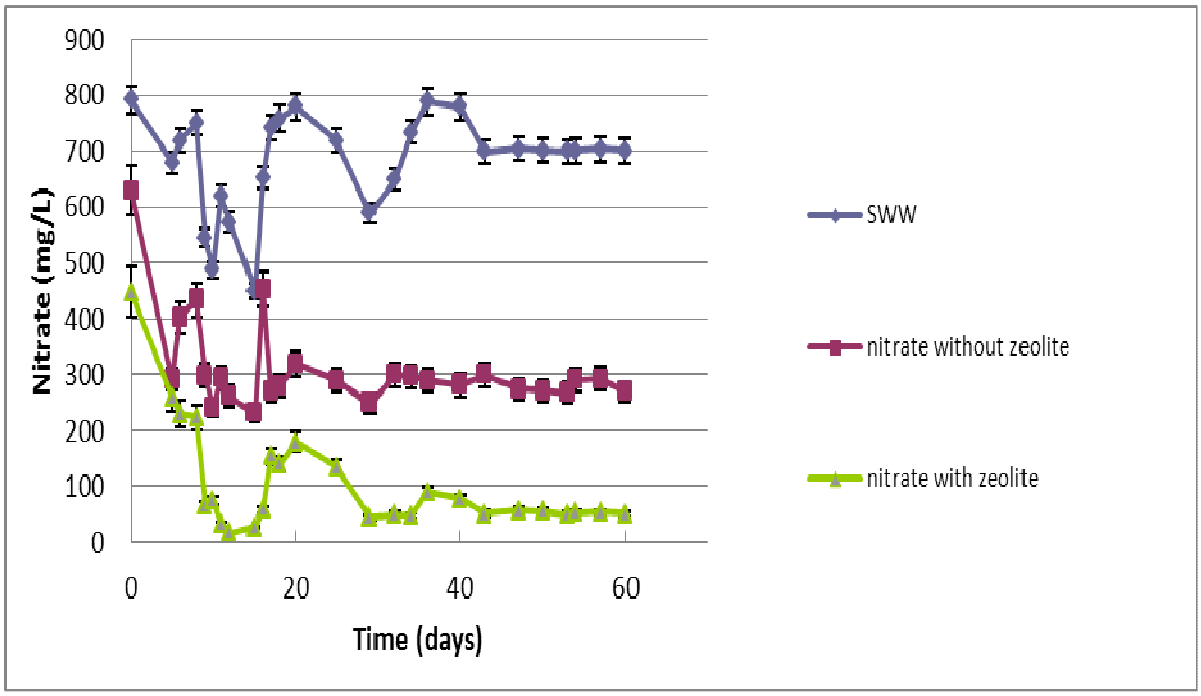

900
800
700
600
500
400
300
200
100
0
Nitrate (mg/L)
0
20
40
60
Time (days)
SWW
nitrate without zeolite
nitrate with zeolite

Figure 7

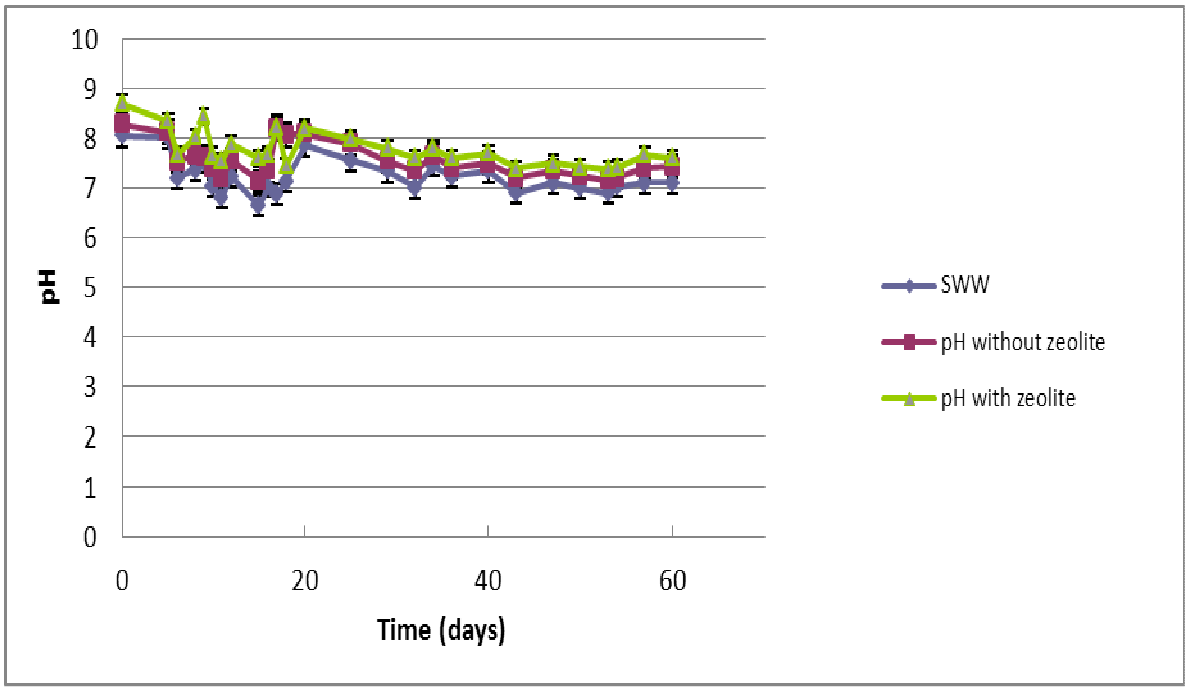

10
9
8
7
6
5
4
3
2
1
0
pH
0
20
40
60
Time (days)
SWW
pH without zeolite
pH with zeolite

Figure 8