**Evaluation of natural zeolite as microorganism support medium in nitrifying batch
reactors: influence of zeolite particle size**

C. MERY ¹, L. GUERRERO ¹, J. ALONSO-GUTIÉRREZ ², M. FIGUEROA ², J.M. LEMA ²,
S. MONTALVO ³, R. BORJA ^{4*}

¹ *Departamento de Ingeniería Química y Ambiental, Universidad Técnica Federico Santa
María. Valparaíso, Chile.*

² *Departamento de Ingeniería Química, Universidad Santiago de Compostela. Santiago de
Compostela, Spain.*

³ *Departamento de Ingeniería Química, Universidad Santiago de Chile.
Santiago de Chile, Chile.*

⁴ *Instituto de la Grasa (CSIC). Sevilla, Spain*

ABSTRACT

An evaluation of natural zeolite as a microorganism carrier in nitrifying reactors operated in batch mode was carried out. Specifically, the influence of zeolite particle sizes of 0.5, 1.0 and 2.0 mm in diameter on microorganism adherence to zeolite, ammonium adsorption capacity and the identification of microbial populations were assessed. The greatest amount of total biomass adhered was observed for a zeolite particle size of 1 mm (0.289 g) which was achieved on the 12th day of operation. The highest ammonium adsorption capacity was

* Address correspondence to R. Borja, Instituto de la Grasa (C.S.I.C.), Avda. Padre García Tejero, 4. 41012-Sevilla, Spain (Phone: +34 95 4689654, Fax: +34 95 4691262, E-mail: rborja@cica.es).

observed for a zeolite particle size of 0.5 mm, which was 64% and 31% higher than that observed for particle sizes of 1.0 and 2.0 mm, respectively. The maximum de-sorption values were also found for a zeolite particle size of 0.5 mm, although when equilibrium was reached the ammonium concentrations were similar to those observed for a zeolite particle size of 1.0 mm. It was also found that the experimental data on ammonium adsorption fitted very well to the Freundlich isotherm for the three particle sizes studied. Finally, the nitrifying reactors showed similar microbial populations independently of the particle size used as microorganism carrier. The dominant bacterial community was *Gammaproteobacteria* making up 80% of the total population found. *Betaproteobacteria* were also identified and made up 12% approx. of the total population. *Ammonium Oxidant Betaproteobacteria* and *Nitrobacter* were also detected.

Keywords: Natural zeolite, nitrification, support medium, microorganisms, particle size.

INTRODUCTION

When nitrogen oxidized compounds (nitrite, nitrate) and ammonium nitrogen are poured directly into hydric bodies, they provoke toxicity in aquatic fauna and a reduction in dissolved oxygen (DO), which creates a more acidic environment and accelerates the eutrophication process.^[1-10] The toxic non-ionized form of ammonium (NH_3) has a negative effect on aquatic fauna in riverbed receptors. Ammonium and ammonia are in equilibrium, a few mg/L of free ammonia are toxic, and a slight increment in pH or temperature may also increase toxicity.^[11-14] Nowadays, nitrogen should be completely eradicated, and two methods can be used to this end: physicochemical and nitrification-denitrification biological elimination.^[15-17]

The biological removal of nitrogen consists of a treatment where two sequential processes are applied: nitrification and denitrification. The classical solution to the problem of ammonia removal is nitrification, which is the first step in biological wastewater treatment. ^[18] Typically, ammonia nitrogen can be reduced by up to 90% during secondary treatment processes which promote nitrification by using fixed film biological filters. In each case, the maintenance of high DO concentrations is crucial to sustaining effective ammonia removal. ^[18] These are “attached growth systems” which feature increased surface area available for bacterial growth. There are some limitations to these systems, including sensitivity to toxic shock, pH sensitivity, the high oxygen levels and low temperatures required. ^[18] With respect to shock loading it has been recently reported that an increase in total ammonia concentration by up to 80% in the range of 0.15-1.8 mg/L reduced the rate of nitrification in a trickling filter by 60%. ^[18] Nitrification can also be limited if the DO concentration is limited for example within the sub-saturation range of 0.5-2.5 mg/L. ^[18] When the DO concentration drops below 1 mg/L, denitrification can occur due to the anoxic conditions. In addition, the nitrification rate is invariant with respect to DO above 6 mg/L. Below this value the reduction in rate is significant and reaches only 33% maximum effectiveness when the DO concentration is reduced to 2 mg/L. ^[18]

On the other hand, biological aerated filters have been identified as fixed film systems on which biofilm support media are stationary during normal operation with aeration. The primary purposes of biological aerated filter technology were to simultaneously accomplish the biological oxidation of organics and the physical filtration of suspended solids. ^[19] This process is commonly known to have lots of advantages as compared with conventional activated sludge processes. The biofilm formation on an inert granular media in these aerated filters allows for a much higher concentration of active biomass than a suspended growth activated sludge system and therefore the size of the reactor can be reduced. Furthermore,

suspended solids can be captured physically by the media, thus eliminating the secondary clarifier. The aerated filter is also known as a powerful system for treating a stream as it has variations of pH and a high organic content. ^[19] When the aerated filter system is applied, the selection of packing media plays an important role in maintaining a high amount of active biomass and a variety of microbial populations. Natural materials, such as sand, shale or certain clays have frequently been used. ^[19] In addition, synthetic materials such as polystyrene, polyethylene have also been used. However, the production of these materials is usually costly and they are not significantly more effective than natural materials. ^[19]

Natural zeolite is a non-metallic mineral which characteristically has high porosity and a large specific surface area. In addition, it is an ion exchanger with a high affinity for the ammonium ion, which is reported to have a classical aluminosilicate cage-like structure and therefore exhibits significant macroporosity. ^[20] Earlier studies have shown that clinoptilolite, and certain other natural zeolites can be effective in removing ammonia from wastewaters, ^[21-23] and has been used to enhance the nitrification of biofilters. ^[20, 24]

Taking these points into account, the aim of the present work was to assess the performance of the nitrification process using natural zeolite as support medium in batch reactors. The influence of the zeolite particle size on the adherence of microorganisms, ammonia adsorption capacity and microbial populations immobilized on this support were also evaluated.

MATERIALS AND METHODS

With the aim of studying the influence of zeolite particle diameter on the nitrification process, three different groups or sets of assays were carried out in batch mode:

- I) Assays for the determination of microorganism adherence to natural zeolite by using particle diameters of 0.5 mm, 1.0 mm and 2.0 mm.

II) Experiments for the determination of the ammonium adsorption capacity of natural zeolite by using particle diameters of 0.5 mm, 1.0 mm and 2.0 mm.

III) Identification of microbial populations found in the above-mentioned zeolite particle sizes used during the nitrification process.

Characteristics of natural zeolite

The characteristics and chemical composition (% w/w) of the natural zeolite used in the three sets of assays was: SiO₂, 66.62%; Al₂O₃, 12.17%; Fe₂O₃, 2.08%; CaO, 3.19%; MgO, 0.77%; Na₂O, 1.53%; K₂O, 1.20%; and 11.02% ignition wastes. Its phase composition was 35% Clinoptilolite, 15% Mordenite, 30% Montmorillonite, and 20% others (calcite, feldespate and quartz). The Chilean natural zeolite used was obtained from Minera Formas, Chile (ZeoClean_R).

Three different zeolite particle diameters were selected for this study: 0.5 mm, 1.0 mm and 2.0 mm.

Chemical analyses

Chemical oxygen demand (COD), volatile suspended solids (VSS), volatile solids (VS), total phosphorus and pH analyses were carried out according to Standard Methods for the Examination of Waters and Wastewaters, 1998.^[25] Nitrate and ammonium nitrogen were determined by selective electrodes.

Experimental procedure

Assays for the determination of microorganism adherence to natural zeolite

To study microorganism adherence to the above-mentioned support material, three different zeolite particle sizes were used: 0.5 mm, 1.0 mm and 2.0 mm in diameter. Small batch reactors with an operating volume of 0.25 L working at a temperature of 20 °C were used for this set of experiments.

The characteristics of the inoculum used are summarized in Table 1. The concentration of the inoculum used in each reactor was 1.5 g VSS/L.

The composition and characteristics of the synthetic wastewater used for these assays are shown in Table 2. The total duration of these experiments was 25 days.

The quantification of the biomass attached to any zeolite diameter selected was evaluated by the difference between the estimated dried solids at 105°C and those estimated by burning at 550°C, which can be related to the concentration of volatile solids (VS).

Assays for the determination of ammonium adsorption capacity of natural zeolite

This second set of assays was performed in Jar Test reactors with a volume of 500 mL operating in batch mode. The reactors were placed in a thermostatic chamber at a constant temperature of 30 °C. Zeolite particle sizes with the above-mentioned diameters were used in these experiments.

The characteristics of the synthetic wastewater used in this group of experiments are detailed in Table 3. This wastewater was diluted to obtain four different initial ammonium concentrations (C) which were used in the experiments carried out with the three zeolite particle sizes assayed. Table 4 shows the initial concentrations of ammonium used for each experiment. The zeolite concentration in each reactor was 8 g/L. The content of the reactors was maintained homogeneous through stirring at 120 rpm.

Every 24 hours agitation was stopped for 5 minutes to take a sample of the supernatant liquid and to measure the concentration of N-NH_4^+ in these previously filtered samples.

Assays for identification of the microbial populations adhered to natural zeolite in the batch nitrification process

This third set of assays was performed in the small scale batch reactors previously described in the first set of assays. Zeolite particle sizes of 0.5 mm, 1 mm and 2 mm in diameter were also used in this group of experiments. The operating conditions of the reactors are shown in Table 5.

The inoculum used in this set of assays was derived from an activated sludge reactor with extended aeration which treats urban wastewaters.

Samples of biomass adhered to zeolite were taken from the reactors at the beginning and end of the experiment (25 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, to analyze 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.^[26] To be specific, samples were fixed in 4% para-formaldehyde, washed in phosphate buffer saline (PBS) and preserved in PBS–ethanol at -20 °C until used. For the hybridization procedure, the samples were fixed on a multi-dish slide at 46 °C for 20 minutes and dried in ethanol. All the probes carried out were summarized and specified in Table 6. DAPI staining (4', 6-diamidino-2-phenylindol) was used to corroborate that the observed fluorescence with the FISH technique corresponded to bacteria cells in order to quantify the existing microorganisms.^[26]

RESULTS AND DISCUSSION

Assays for the determination of microorganism adherence to natural zeolite

During the first set of assays (I) where the objective was to study microorganism adherence to zeolite, the pH values remained between 7.5 and 8.5 with DO concentrations of between 4 and 6 mg/L. These values can be considered as adequate for the optimum performance of the process.^[27, 28] Qiu et al. ^[29] also found optimum pH values of between 6.5 and 7.5 in the nitrification process carried out in biological aerated filters with zeolite and ceramic supports. Figure 1 shows the variation of the amount of total biomass (g) adhered to zeolite with time for the three particle sizes studied (0.5, 1.0 and 2.0 mm). As can be seen, the greatest amount of total biomass adhered was observed for a particle size of 1 mm, achieving its maximum total value (0.289 g) on the 12th day of operation time. For particle sizes of 0.5 and 2 mm the maximum values of total biomass adhered were 0.233 and 0.272 g respectively, which were achieved after 14 days of operation.

Assays for the determination of ammonium adsorption capacity of natural zeolite

Figures 2-4 show the main results obtained in the experiments of ammonium adsorption carried out during the second set of assays (II). The reactors were stirred for 6 days, during which time it was checked that equilibrium had been achieved. The pH ranged between 6.3 and 6.7 throughout the experimental runs made.

As can be seen in Figures 2-4, maximum ammonium adsorption was observed for a zeolite particle size of 0.5 mm, which was 64% and 31% higher than the adsorption rate for zeolite particle sizes of 1 mm and 2 mm, respectively. However, these maximum adsorption values decreased when the ammonium was de-adsorbed. Maximum de-sorption values were found for a zeolite particle size of 0.5 mm, although when equilibrium was reached, the ammonium concentrations were similar to those observed for a zeolite particle size of 1.0 mm. Therefore, the equilibrium concentrations were similar in the two cases studied, although the values were reached in different ways.

Table 7 shows the percentages of adsorption and de-sorption obtained in each experiment performed for the three zeolite particle sizes. As can be seen for particle sizes of 1 mm and 2 mm, the percentages of maximum adsorption achieved were relatively similar for the different initial ammonium concentrations (C1-C4) studied in each case.

Therefore, the results obtained in the present work demonstrate that a zeolite particle size of 0.5 mm allows for maximum adsorption values, which in turn would allow for a higher amount of available ammonium to be raised and degraded by the ammonium oxidant microorganisms adhered to its surface.

Other recent research works have demonstrated that natural zeolites such as clinoptilolite were also used to recover wastewater ammonium, decrease the nitrogen content in the effluent from on-site sanitation systems and in wastewater treatment plants when this nitrification-denitrification efficiency is low.^[30] Specifically, these results showed that the ammonium adsorption capacity increased with decreasing clinoptilolite particle size, and the highest experimental adsorption capacity was 2.7 mg NH₄⁺/g.^[30] In the same way, it was demonstrated that the addition of zeolite powder to sequencing batch reactor could also improve its performance, increasing significantly the NH₄⁺, COD and PO₄⁻³ removals compared to a control reactor without the addition of zeolite.^[31]

Yang^[32] also studied the influence of lower initial ammonium concentrations (50, 70 and 100 mg N/L) on the nitrification process in batchwise fluidized bed with zeolite as microorganism carrier. It was observed in this case that both ion exchange and nitrification effects were dominant over the effect of air-stripping.^[32]

Similar behaviour was reported in nitrification processes carried out in continuous mode. Specifically, around 85% COD and 70% NH₄⁺-N were removed constantly in an up-flow zeolite media biological aerated filter for treatment of aquaculture wastewater.^[33] It was observed that the nitrifying population occupied the top of the filter column.^[33] The effect of

the addition of zeolite on submerged membrane bioreactor (SMBR) operating in continuous mode was also studied.^[34] It was also found that a mean 25% higher total nitrogen (TN) removal took place in the test system compared to a control consisting of a SMBR without added zeolite. Ion exchange and simultaneous nitrification and denitrification were considered to be the main reasons for this high TN removal.^[34] The same effect was observed in a pilot-scale biofilter with biomass immobilised on zeolite containing expanded clay aggregate filtermedia.^[35]

Furthermore, it was found that the experimental data of ammonium adsorption obtained in the present work fitted well to both the Langmuir and Freundlich isotherms for the three zeolite particle sizes studied. To be specific, the highest determination coefficients (R^2) were achieved with the Freundlich isotherm with values of 0.96, 0.98 and 0.93 for zeolite particle sizes of 0.5, 1 and 2 mm, respectively. Sarioglu had also previously observed that both isotherms can be applied to fit ammonium adsorption data from municipal wastewaters using Turkish natural zeolite (Dogantepe).^[36] In addition, Du et al.^[37] demonstrated that the Freundlich model was more appropriate for representing data of ammonium adsorption on Chinese Clinoptilolite with wide ranges of ammonium concentration.

In the present work, the experimental data of ammonium adsorption on zeolite particle sizes of 0.5, 1 and 2 mm were fitted to a Freundlich isotherm ($Q_e = k \cdot C_e^{1/n}$), which is represented by equations (1), (2) and (3), respectively:

$$Q_e = 0.036 \cdot C_e^{1/0.93} \quad (1)$$

$$Q_e = 0.00033 \cdot C_e^{1/0.56} \quad (2)$$

$$Q_e = 0.056 \cdot C_e^{1/1.19} \quad (3)$$

where: Q_e is the mass of substance adsorbed per unit of mass of zeolite in the equilibrium (mg N/g zeolite); C_e is the concentration of substance in the liquid in the equilibrium (mg N/L); and k and n are constant characteristics of each pair adsorbed substance – zeolite. The values of k and n obtained in this work were lower than those obtained in the nitrification of small polluted urban river waters in areas without centralized sewage works using zeolite-coated nonwovens, for which k and n values of 0.28 and 1.17 were found, respectively.^[38] By contrast, the k values calculated in our case for particle sizes of 0.5 and 2.0 mm were much higher than those obtained in a biofilter with activated carbon as biomass carrier treating wastewater with high concentrations of TKN ($k = 0.0006$).^[39]

Assays for identification of the microbial populations adhered to natural zeolite in the batch nitrification process

The nitrifying reactors showed similar microbial populations independently of the particle size used as microorganism carrier. For all reactors used with the above-mentioned nitrifying culture (Table 5) the dominant population was *Gammaproteobacteria* representing 80% of the total communities. *Betaproteobacteria* were also identified and made up 12% approx. of the total population. Table 8 shows the percentages of the microorganisms found for each particle size studied, while Figure 5 illustrates the dispersion of these classes of bacteria (*Gammaproteobacteria* and *Betaproteobacteria*) over the total cells for a zeolite particle size of 0.5 mm. Among the *Betaproteobacteria*, the presence of *Ammonium Oxidant Betaproteobacteria* was also determined, a group that was also identified in other nitrifying related studies.^[40] *Nitrobacter* (responsible for the oxidation of nitrite to nitrate) was also detected, as occurred in similar studies.^[39, 41] It was recently reported that the number of nitrifiers on natural zeolite was greater than on sand media in the case of biological aerated filters with the above-mentioned microorganism carriers.^[19] To be specific, in the biofilm

grown on zeolite, *Nitrosomas* and *Nitrobacter* were counted at 3.0×10^9 CFU/mL and 2.2×10^9 CFU/mL, respectively, while those on sand were counted at 4.5×10^8 CFU/mL and 6.5×10^8 CFU/mL, respectively.^[19]

On the other hand, the high percentage of *Gammaproteobacteria* found and the scarce identification of *Ammonium Oxidant Betaproteobacteria* seems to indicate that both *Ammonium Oxidant* and *Nitrite Oxidant bacteria* belong to the *Gammaproteobacteria* class.

In addition, due to the natural fluorescence of zeolite it was not possible to accurately quantify the bacteria groups hybridated with probes marked with chromophore Fluors. As a consequence, the percentage of *Alphaproteobacteria* was not reported.

Nogueira et al.^[42] reported that *Betaproteobacteria* was the predominant group in a study on nitrifying populations immobilised on expanded polyethylene, while the *Gammaproteobacteria* were almost undetected. In addition, when granulated polyethylene was used as biomass carrier, 80% of the total bacteria corresponded to *Alphaproteobacteria* and *Betaproteobacteria*.^[42] By contrast, our research demonstrated that using Chilean natural zeolite as biomass carrier, 80% of the total bacterial population found corresponded to the *Gammaproteobacteria* class. Therefore, the type of support material used to immobilize the biomass influences the population diversity found colonizing it, despite the fact that its size has no influence on the type of microbial communities detected.

CONCLUSIONS

The highest adherence of microorganisms to zeolite was found for a particle size of 1 mm. Lower amounts of total biomass adhered, although with similar values, were found for zeolite particle sizes of 0.5 and 2.0 mm. Similar ammonium adsorption equilibrium values

were found for particle sizes of 0.5 and 1.0 mm in diameter, the de-sorption capacity being higher for a zeolite size of 0.5 mm. The microbial populations of the nitrifying reactors were very similar, independently of the particle size used. The dominant microbial population observed was *Gammaproteobacteria*, making up 80% of the total communities found. *Betaproteobacteria*, making up 12% approx. of the total, were also identified. Finally, taking into account the overall results derived from bacterial adherence, ammonium adsorption and identification of the microorganisms involved in the nitrification process as well as other practical aspects such as operational costs for obtaining different particle sizes, the contact microorganism-support and possible clogging of the reactor, it can be concluded that a particle size of 1.0 mm is the most suitable for use in fixed or fluidized beds.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to FONDECYT project no. 1090414 (Chile) and the University of Santiago de Chile for providing financial support.

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FIGURE CAPTIONS

Figure 1. Variation of the amount of total biomass (g) adhered to zeolite with operation time for the three particle sizes studied (0.5, 1.0 and 2.0 mm).

Figure 2. Variation of the ammonium concentration in the supernatant liquid with operation time for the zeolite particle size of 0.5 mm.

Figure 3. Variation of the ammonium concentration in the supernatant liquid with operation time for the zeolite particle size of 1.0 mm.

Figure 4. Variation of the ammonium concentration in the supernatant liquid with operation time for the zeolite particle size of 2.0 mm.

Figure 5. FISH of the nitrifying reactors with a particle size of 0.5 mm using the chromophore CY3 (red) to determine a specific class of bacteria over the total cells dyed with DAPI (blue). Left: *Gammaproteobacteria*; Right: *Betaproteobacteria*. Bar length indicates 10 μm .

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476 **Figure 1**

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Figure 2

Figure 3

Figure 4

Figure 5

Table 1. Characteristics of the inoculum used in the first set of assays (I).

Parameter	Unit	Concentration
COD	mg/L	18,480
pH		7.25
TSS*	mg/L	4,904
VSS	mg/L	3,903

* TSS: total suspended solids

Table 2. Characteristics of the synthetic wastewater used in the first set of assays (I).

Compound	Unit	Concentration
COD	mg/L	360
NH ₄ Cl	g/L	0.764
Urea	g/L	0.450
Yeast extract	g/L	0.28
FeSO ₄ ·7H ₂ O	g/L	0.0992
NaHCO ₃	g/L	3.12
KH ₂ PO ₄	g/L	1.5
Saline Solution*	mL/L	1

* EDTA: 0.15 g/L; HCl: 1 mL/L; FeSO₄: 2 g/L; HBr: 0.05 g/L; ZnCl₂: 0.05 g/L; MgCl₂: 0.05 g/L.

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594 **Table 3.** Composition of the synthetic wastewater used in the second set of assays (II).

Compound	Unit	Concentration
COD	mg/L	360
(NH ₄) ₂ SO ₄	mg N/L	707.1
MgSO ₄ ·7H ₂ O	mg Mg/L	3.6
K ₂ HPO ₄	mg P/L	43.9
KH ₂ PO ₄	mg P/L	43.9

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Table 4. Initial concentrations of N-NH_4^+ in each reactor in the second set of assays (II).

Initial concentrations (mg N-NH_4^+ /L)	Zeolite particle size (mm)		
	0.5	1	2
C1	400	408	412
C2	495	495	500
C3	889	867	852
C4	1153	1070	1153

Table 5. Characteristics of the batch reactors used in the third set of experiments (III).

Parameter	Value
Culture medium (mL)*	100
Zeolite(g)	40
Inoculum (mL)	12
Temperature (°C)	20
pH	8
Dissolved Oxygen (mg/L)	7
Operation time (days)	25

*NH₄Cl, 0.764 g/L; Urea, 0.45 g/L; Yeast extract, 0.28 g/L; FeSO₄·7H₂O, 0.0992 g/L; NaHCO₃, 3.12 g/L; KH₂PO₄, 0.181 and 1 ml/L of saline solution (EDTA, 0.15 g/L; HCl, 1 ml/L; FeSO₄, 2 g/L; HBr, 0.05 g/L; ZnCl₂, 0.05 g/L; MgCl₂, 0.05 g/L). COD, 360 mg/L.

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649 **Table 6.** Sequences, target organisms and percentage of formamide (%FA) used.

Probe	Chromo*	Sequence (5'→3')	FA (%) / NaCl (mM)	Target Organisms
EUB338I	Fluos	GCT GCC TCC CGT AGG AGT	20 / 225	<i>Bacteria</i>
ALF1B	Fluos	CGT TCG YTC TGA GCC AG	20 / 225	<i>Alphaproteobacteria</i>
NIT3*	Cy3	CCT GTG CTC CAT GCT CCG	40 / 56	<i>Nitrobacter</i> spp.
NITComp		CCT GTG CTC CAT GCT CCG		
BET42a*	Cy3	GCC TTC CCA CTT CGT TT	35 / 80	<i>Betaproteobacteria</i>
BETComp-		GCC TTC CCA CAT CGT TT		
NSO190	Cy3	CGA TCC CCT GCT TTT CTC C	55 / 20	<i>Ammonia-Oxidizing</i> <i>Betaproteobacteria</i>
GAM42a*	Cy3	GCC TTC CCA CAT CGT TT	35 / 80	<i>Gammaproteobacteria</i>

650 * Chromophore

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Table 7. Percentages of adsorption and de-sorption obtained in each case studied.

%	zeolite [mm]			
	0.5			
	C1	C2	C3	C4
Maximum adsorption	44	47	58	64
Equilibrium adsorption	28	34	27	35
De-sorption				
(subtraction)	16	13	31	29
1				
Maximum adsorption	33	26	32	31
Equilibrium adsorption	17	26	30	30
De-sorption				
(subtraction)	16	0	2	1
2				
Maximum adsorption	20	23	13	17
Equilibrium adsorption	13	18	12	14
De-sorption				
(subtraction)	7	5	1	3

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671 **Table 8.** Results derived from the FISH. Values expressed in percentages of the specific
672 microorganism class in relation to the total cells present in the sample.

Microorganisms (%)	Zeolite particle size		
	(mm)		
	0.5	1	2
<i>Betaproteobacteria</i>	15	10	10
<i>Gammaproteobacteria</i>	80	80	80

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