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# Influence of the physicochemical properties of inorganic supports on the activity of immobilized bacteria for water denitrification

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ABSTRACT

The denitrification of polluted water was studied by using supported E-coli bacteria. The physicochemical characteristics of supports and the influence of these properties on the bacteria performance were analyzed. Inorganic supports oxides and zeolites were selected in order to cover a wide range of porosity and surface chemical properties and the denitrification process systematically studied. Consecutive denitrification cycles in batch experiments and the toxicity of supports were also analyzed. The acidity of supports provokes a slower reduction processes, favoring also a high concentration of intermediate nitrites in solution for longer periods. The $NO_3^-$ reduction is faster than the $NO_2^-$ one, being also less influenced by the support characteristics. Anyway, the total denitrification is reached in all cases. The best performance was obtained with bacteria supported on mesoporous and non-acid silica support.



## 1. Introduction

The extended use of fertilizers in intensive agriculture provokes very high nitrate contents in water used for irrigation. Nitrate is a typical pollutant from the environmental standpoint, causing eutrophication in lakes and water reservoirs, but is also dangerous from a human health point of view, taking into account that can induce matheomoglobinemia, disease or even cancer (Nuhoglu et al., 2002) and thus the limit concentration of nitrates-nitrites in drinking water supplies are severely set by the World Health Organization.

Different physicochemical technologies for nitrate removal including osmosis, ion exchange, heterogeneous catalysis, electrodialysis etc have been used (Karanasios et al., 2010). Biological processes are widely used in wastewater treatment plants due to its low cost and high-efficiency as well an ease implementation and environmental friendly performance. Biological denitrification is carried out by facultative bacteria that, under anoxic conditions, can use $NO_3^-$ as a terminal electron acceptor for respiration. Autotrophic or heterotrophic bacteria can be used, which show some advantages and disadvantages respectively. Heterotrophic bacteria used various carbon compounds ethanol, methanol as energy and electron sources while autotrophic denitrification bacteria typically use hydrogen and carbon dioxide or bicarbonate as energy source and carbon source, respectively (Karanasios et al., 2010).

Different types of reactors can also be used, from fluidized to fixed bed and membrane reactors. The correct colonization and activation of denitrifying bacteria communities on supports are critical factors to obtain high denitrification efficiency (Wallenstein et al., 2006). Environmental parameters such as C/N ratio, temperature or pH of polluted water but also the characteristics of the supports having different sites, influence the community structure and activity of denitrifying bacteria. The support media is considered to be one of the main parameters for the design of a packed-bed reactor (Karanasios et al., 2010) the selected materials and their characteristics such as shape and size have great influence on the system performance. The size and shape of supports determines interpaticle voids, the porosity and the specific surface area, influencing biofilm thickness and pore clogging (Nuhoglu et al., 2002). Constructed wetlands for water quality amelioration are generally created also in such a basis (Song et al., 2011).

Many different materials were previously used as bacteria supports, including metal oxides (Chen et al., 2012; Kurt et al.,

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1987), zeolites (Montalvo et al., 2012), biodegradable polymers (Chu and Wang, 2013), woods (Yamashita et al., 2011) or carbon materials (Moreno-Castilla et al., 2003). These studies are focused mainly to analyze the morphological characteristics of supports (Nuhoglu et al., 2002; Eldyasti et al., 2012), as previously commented. In this manuscript we study the performance of different inorganic materials including pure oxides as silica $SiO_2$, alumina $Al_2O_3$ and titania $TiO_2$ and some zeolites ZSM5, 13X and β-zeolite as supports of a heterotrophic bacteria *Escherichia coli*. Supports were characterized from a morphological, textural and chemical point of view and correlations between these parameters and the degree of denitrification observed have been established.

## 2. Materials and methods

Commercial oxides, including $SiO_2$ Silica Gel 100, Merck, $Al_2O_3$ Aluminum oxide acidic, Merck and $TiO_2$ anatase, Alfa Aesar and the zeolites 13X Aldrich and ZSM-5 Acros were used as received, without additional treatments. The acid β-zeolite EXXON was exchanged with alkali and alkali-earth cations according the procedure previously published (Maldonado-Hódar et al., 1997). Initially, β-zeolite was exchanged two times with a 0.5 M $NaNO_3$ solution. The process was developed under vigorous stirring at 80 °C, using a ratio volume of solution mL/zeolite weight g of 70. The dispersion was centrifuged and the solid washed and dried overnight at 110 °C obtaining the βNa-sample. Further, different portions of this sample were subsequently exchanged with $CsCH_3COO$, $Mg(NO_3)_2$ or $Ba(CH_3COO)_2$ following the same procedure. All the samples were finally calcined in air flow 3 L/h g at 500 °C for 2 h. The exchanged samples were referred as βNa, βCs, βMg and βBa respectively.

The morphology and size of supports was characterized by scanning electron microscopy SEM using a LEO Carl Zeiss GEMINI-1530. This technique was also used to study the colonization of supports by bacteria after the corresponding denitrification experiments. Information about the support acidity was obtained by measuring the $pH_{pzc}$. For that, 1 g of solid support was suspended in 20 $cm^3$ of $CO_2$-free distilled water and the slurry was maintained in a plastic bottle under shaken for 2 days until the pH had stabilized. The final pH of the slurry was recorded as the pH of the point zero charge $pH_{pzc}$ of the solid, according to the methodology previously described (Leon et al., 1992; Bailón-García et al., 2014).

Textural characterization was carried out by $N_2$ adsorption at −196 °C using a Quantachrome Autosorb-1 equipment. The total pore volume $V_{Total}$ was considered as the volume of $N_2$ adsorbed at $P/P_0 = 0.95$ (Bailón-García et al., 2014; Vivo-Vilches et al., 2013). The BET equation was applied to the $N_2$-adsorption isotherms to determine the apparent surface area $S_{BET}$ and the Dubinin–Radushkevich and Stoeckli equations applied to determine the micropore volume $V_{mic}$, the mean micropore width $L_0$ and the microporous surface $S_{mic}$, respectively (Brunauer et al., 1938; Dubinin, 1985; Bansal et al., 1988; Gregg and Sing, 1997). Furthermore, the Barrett–Joyner–Halenda BJH method was used to calculate the mesopore volume of the samples $V_{meso}$ and pore size distributions PSD (Barrett et al., 1951).

The bacteria used in the experiments was *E. coli*, ATCC 25922 strain. To immobilize these bacteria on the different supports it was first incubated at 310 K using a buffered media at pH 7 of Tryptic Soy Broth TSB, Difco Lab. Bacteria were supported on the different solids adding 1 mL of this suspension to 0.4 g of support suspended in 20 mL of TSB and the mixtures were shaken at 310 K for 3 days. After this time, the colonized supports were filtered and washed repeatedly with sterilized distilled water.

To study the denitrification process of water, the bacteria immobilized on the different supports the above 0.4 g was added to 100 mL of a solution containing 50 mg $L^{-1}$ of nitrate from $NaNO_3$ and 1.3 mL of ethanol. The suspension was buffered at pH 7 with an appropriate phosphate solution. The flasks used as bioreactors were flushed with argon gas to obtain anaerobic conditions and were placed in a thermostatised rotary shaker at 298 K. Periodically, the concentration of nitrate was measured directly in the bioreactors with a selective electrode supplied by Mettler, and simultaneously, a small volume of solution 1 mL was withdrawn for the determination of nitrite concentrations. This parameter was determined by UV-spectrometry at 543 nm after coupling diazotized sulphanilamide with N-1-naphthyl-ethylendiamine, using a Hitachi model U2000 spectrophotometer (Kesseru et al., 2002). Both equipment were previously calibrated. Consecutive denitrification cycles were performed following the same procedure described in order to analyze the bacteria activation/deactivation.

The toxicity of inorganic solids suspensions was measured based on inhibition of the luminosity intensity of marine bacteria Vibrio Fisheri, NRRL-B-11177, in accordance with the European guideline ISO 11348-2:2007. A suspension of bacteria is prepared according this procedure and the initial luminescence is determined. The solids supports are removed from their suspensions by centrifugation to avoid interferences in the determination of the light intensity, and water recovered used to dilute the bacteria suspension mixed in a 1:1 ratio. After a 15-min exposure bioluminescence was measured LUMISTOX system. In all measurements, the percentage inhibition % I was obtained by comparing the response of a control saline solution with that of the sample. Toxicity was expressed as the percentage inhibition of bacterial growth as a function of treatment time.

## 3. Results and discussion

The morphology of support particles and the structure of colonies of supported bacteria were studied by SEM. All supports studied are formed by aggregates of small microcrystallites. As an example, Fig. 1a shows that $Al_2O_3$ is composed by amorphous particles of several micrometer size, most of them in the range of the 100 μm, which are formed by small and polyhedral microcrystallites. Zeolites are also formed by aggregated microcrystallites, as showed in Fig. 1b for ZSM5 zeolite. In this case more or less cubic particles of around 1 μm size are observed. The particle size of supports observed by SEM are quite similar between them and are in good agreement with those values provided by suppliers 230 mesh for $SiO_2$ and $Al_2O_3$ or <325 meshes for $TiO_2$.

After denitrification processes, the surface of all these materials is coated by bacteria biofilms Fig. 2 and new structures, mainly filaments forming networks are observed together the corresponding bacteria. These filaments are biosynthesized polysaccharides that help to the microorganism to grow forming colonies attached to the supports. These structures can provide to the microorganism additional capacities, such as electron transfer processes (Reguera et al., 2005) which could be important in redox process like anaerobic denitrification and in some cases these polysaccharides have been extracted and purified because they can present some interesting properties and applications (Ganesh et al., 2004).

Both the bacteria concentration and the bacteria size are strongly influenced by the support nature. In general bacteria are smaller when supported on $Al_2O_3$ Fig. 2a than in the case of $SiO_2$ Fig. 2b or $TiO_2$ Fig. 2c where long bacteria, up to 8 μm, were observed. Bacteria are however more uniformly distributed on $Al_2O_3$ than on $TiO_2$ particles Fig. 2. The inter-particle spaces play an important role, because colonies can be placed between microcrystallites. Zeolites are also highly covered by bacteria biofilms after the corresponding denitrification cycles Fig. 2d–f. The higher